2019

# Impacts of Trogocytosis-Mediated Intracellular Signaling on CD4+ T cell Effector Cytokine Production and Differentiation

Steven James Reed

# Impacts of Trogocytosis-Mediated Intracellular Signaling on $CD4^+$ T cell Effector Cytokine Production and Differentiation

By

STEVEN JAMES (JIM) REED

BS, University of Washington, Seattle, WA, 2010

Dissertation/Thesis

presented in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in Immunology

The University of Montana
Missoula, MT

Official Graduation Date (anticipated) August 2019

Submitted for approval by:

Scott Whittenburg,
Dean of the Graduate School

Dr. Scott Wetzel, Chair
Division of Biological Sciences

Dr. Jesse Hay
Division of Biological Sciences

Dr. Mike Minnick
Division of Biological Sciences

Dr. Kevan Roberts
Department of Biomedical and Pharmaceutical Sciences

Dr. Dave Shepherd
Department of Biomedical and Pharmaceutical Sciences

Reed, Steven (Jim), Doctor of Philosophy, Summer 2019 Immunology

Impacts of trogocytosis-mediated intracellular signaling on $CD4^+$ T cell effector cytokine production and differentiation

Chairperson: Scott Wetzel

Trogocytosis is the direct intercellular transfer of membrane and membrane associated molecules. Unlike other passive-membrane transfer events, trogocytosed molecules may remain fully functional and become re-expressed on the surface of the trogocytosis-positive ($trog^+$) recipient. This phenomenon commonly occurs between various cell types, including those of the immune system. $CD4^+$ T cell trogocytosis occurs during their activation by antigen presenting cells (APC). Consequently, the acquired molecules include ligands for signaling receptors on the T cell.

The impacts of $CD4^+$ trogocytosis on the immune response are largely unknown. While it has been demonstrated that $trog^+$ cells can present trogocytosed peptide:MHC (pMHC), and costimulatory molecules to activate other T cells, the consequences of trogocytosis on the individual $trog^+$ $CD4^+$ T cell have not been studied in-depth. We previously reported that $trog^+$ cells perform cell-autonomous signaling by trogocytosed ligands engaging surface receptors, referred here to as trogocytosis-mediated signaling. This signaling led to the enhanced survival of $trog^+$ cells *in vitro* compared to $trog^-$ cells after APC removal. Because the duration of T cell signaling influences the activation, lineage determination, and effector functionality of $CD4^+$ T cells; trogocytosis-mediated signaling has the potential to uniquely modulate the effector-cytokine production and differentiation of $trog^+$ $CD4^+$ T cell after separation from APC.

Examining this possibility is the foundation for this dissertation. Here, I will report my findings that: **1**. Between 0-72 hrs post-separation from APC, trogocytosis-mediated signaling drives IL-4 and GATA-3 expression, consistent with T helper type-2 ($T_H2$) differentiation; **3**. Extended trogocytosis-mediated signaling (>72 hrs) leads to the expression of Bcl-6, PD-1, CXCR5, and IL-21, consistent with T follicular helper ($T_{FH}$) differentiation; **4.** In absence of exogenous antigen (Ag), trogocytosis-mediated signaling is critical for the survival of the activated $CD4^+$ T cells with high memory-potential.

Despite the critical role for $CD4^+$ T cells in generating protective immunity in the host, much remains unknown about the differentiation of $CD4^+$ T cell effector subsets. The findings here present a novel mechanism for $CD4^+$ T cell activation and differentiation via trogocytosis-mediated signaling, demonstrating the broad implications for such signaling in matters of public health.

# Acknowledgments

I would like to first extend my gratitude to my graduate committee for dedicating their valuable time towards my progress throughout my graduate career and providing constructive criticism of my research

Special thanks to my mentor Dr. Scott Wetzel, who introduced me to trogocytosis, greatly expanded my knowledge of the immune system and $CD4^+$ T cells, and fluorescent microscopy. Scott also helped in improving my writing skills, through guidance, and re-writing half of what I wrote. The scientific insights, trust, and freedom to pursue my inclinations given to me by Scott has made me a better researcher, which I will be eternally grateful for.

Thanks to Dr. Mike Minnick who was a part of my graduate committee and has provided continued support throughout my time at the University of Montana. I would also like to thank Mike for: teaching microbial pathogenesis upon my request despite also teaching other undergraduate courses, his critical reviews of our manuscripts, prodiving letters of recommendation, and “letting” me TA medical microbiology for five consecutive years.

I would like to thank rest of my graduate committee; Dr. Dave Shepherd for his enthusiastic willingness to provide constructive criticism of my research, Dr. Kevan Roberts for his jovial presence and open attitude in reviewing my research, and Dr. Jesse Hay who taught me about cell biology and membrane dynamics.

In addition, I would like to thank others at the University of Montana -
Dr. Steve Lodmell for chairing my qualifying defense.
Dr. Jay Evans, Dr. Shannon Miller, and Dr. Allison Smith, for letting me use their LSRII. Without this access I would not have been able to gather the amount of data that I did.

Pam Shaw for helping me with flow cytometry and FACS sorting.

Lou Herritt for assisting me with confocal microscopy.

Sarah Bidwell for her hard work in preparing for the undergraduate labs I taught, and making TA-ing a more enjoyable experience.

Jill Burke, Zooey Zephyr, and Rochelle Krahn in the DBS office

Dr. Scott Samuels for keeping me alert by his violent sneezes which I could hear through the walls.

Other fellow CMMB members including faculty and graduate students

Thanks to my canine companion of the last 11 years, Marley.

Dr. Anthony Desbien, my primary mentor from the Infectious Disease Research Institute, for sharing his excitement of immunology with me and teaching me multiple lab techniques which let me hit the ground running upon starting graduate school.

Additional thanks to: My girlfriend Lily for always being supportive, her understanding for my staying in lab until midnight, or writing through the night, and her love and care for Marley and I; Her parents Susan and Roy who have given me a second family during my time in Missoula, and her grandmother Rebecca for being the most enthusiastic person to receive a copy of our manuscript on trogocytosis despite “not knowing what any of it means”.

I would like to thank Dr. Rhea Coler for her enthusiastic and continual support, letters of recommendation, and sincere interest and concern for my success and well-being for the past 20+ years.
To my family, I would like to thank: My siblings Matt and Sarah, and sister in-law Ashleigh, (and Roman), for their continual support and love.

My uncle Del for his compassion, wit, and trading me his camper van which we used to for multiple Montana adventures.

My aunt Karen, for ensuring that I follow my passions throughout my career

Finally, I would like to thank my mother Marianne Reed for teaching me patience and compassion, for being a friend, and providing unconditional love and support throughout my life; and my father Steve Reed for teaching me to develop a strong work ethic, for being an inspiring role model and introducing me to research, for taking me all over the world, and for always supporting me, and loving and caring for our family.

# Table of contents

# Figures

## Chapter 1

## Chapter 3

## Chapter 4

# Tables

# Chapter 1
# Introduction

## Part I
### Brief overview of the immune response

The immune system provides protection against infection from a multitude of pathogens in a series of events orchestrated by various leukocytes (white blood cells). The immune response is broadly broken down into three major stages, the first being the innate immune response, followed by adaptive immunity, and finally, long-lasting protective memory. As suggested by its title, the innate immune response occurs rapidly (minutes to hours), and independently of specific antigens (Ag). Cells of the innate immune system consist of those derived from common myeloid progenitors including: neutrophils, mast cells, basophils, and dendritic cells (DC); as well as natural killer (NK) cells, NK T cells, γδ T cells, and innate-like lymphocyte (ILC) derived from common lymphoid progenitors. A portion of innate cells reside in mucosal and epidermal layers to increase detection of pathogens by proximity, while others are found in circulation. Innate cells are activated by signaling through pattern recognition receptors (PRR) which include toll-like receptors (TLRs), rig-like receptors (RLR) and nod-like receptors (NLRs). These receptors detect conserved, pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), viral CpG DNA and RNAs, and flagellin. Signaling through PRRs triggers a rapid inflammatory response that recruits other immune cells to the site of infection [1-

3]. During this stage of the immune response, activated PAMP-stimulated antigen-presenting phagocytes, primarily DC, mature and migrate to secondary lymphoid organs, such as the lymph nodes. In these tissues they present peptide fragments of phagocytosed pathogens loaded into major histocompatibility complexes (MHC) to T cells, which are key mediators of the adaptive immune response [4].

## The Adaptive Immune Response

Unlike the innate immune response, cells of the adaptive response respond to specific antigenic epitopes. Adaptive immunity is established by two primary types of lymphocytes; T cells, named after the location of their development in the thymus, and B cells which develop in the bone marrow. T cells make up the largest proportion (40-75%) of the peripheral blood mononuclear cell population and are broken down further into two major subsets determined by CD4 ($CD4^+$) and CD8 ($CD8^+$) surface antigens. Cytotoxic $CD8^+$ T cells are associated with cell-mediated immunity and the killing of infected cells, whereas the primary role of $CD4^+$ “helper” T cells is to mediate the activity of other cells of the immune system through contact-dependent stimulation and cytokine secretion. B cells secrete protective antibodies (Abs) against Ags on pathogens leading to their neutralization, opsonization, or lysis through initiating the classical complement cascade and antibody-dependent cellular cytotoxicity (ADCC). An important hallmark of the adaptive immune response is the rapid clonal expansion of responding lymphocytes resulting in an increase of several orders of magnitude

in the number of Ag-specific lymphocytes within days after initiation. This expansion typically peaks 5-7 days post-infection and is followed by a rapid contraction phase, which coincides with clearance of Ag. Once Ag has been cleared, ~90-95% of activated lymphocytes die within a matter of days. The remaining cells survive as long-lived memory cells, which are maintained at relatively stable numbers for years after infection. In addition to increasing the pool of antigen-specific cells, memory cells respond more quickly, and cells are pre-differentiated to generate the appropriate type of response for the given pathogen. The difference in memory responses vs primary responses is quite significant, and the generation of stable memory populations is the basis of modern-day vaccines.

# Part II
# $CD4^+$ T cell Activation, Differentiation, and Functions in the Immune Response

## $CD4^+$ T cell Activation

$CD4^+$ T lymphocytes play a central role in generating protective immunity against infection. The importance of $CD4^+$ cells has been exemplified through the HIV epidemic of recent decades, in which viral-depletion of $CD4^+$ cells leads to AIDS progression. While low $CD4^+$ levels result in impaired immunity against infection, aberrant $CD4^+$ T cell responses can lead to, or exacerbate, autoimmune diseases such as asthma [5, 6], allergy [6], multiple sclerosis [7-9], [10-12], systemic lupus erythematosus (SLE) [13], psoriasis [14], irritable bowel disease

(IBD) [10, 15-20], and tumor persistence, amongst others [21]. *Despite the clear implications of T cells in modulating the immune response, the mechanisms behind T cell activation and subsequent differentiation are still being elucidated*. Thus, additional research is necessary to develop a comprehensive understanding of the mechanisms which govern these events and have far-reaching implications in human health.

## T cell Maturation and Central Tolerance

Due to the profound impact T cells have on the generation of immune responses, T cell development is a highly regulated process. The model for central tolerance involves two phases known as positive selection and negative selection. Both $CD4^+$ and $CD8^+$ T cells arise from common progenitors which express CD2 but are double-negative for CD4 and CD8. The majority of T cells contain $\alpha\beta$ TCR (while ~20% have $\gamma\delta$ TCR). In early development, diversity among the TCR repertoire is established through genetic recombination of DNA encoding segments for TCR by RAG1 and RAG2 recombinases. This leads to different variable (V) and common chain subunits to be expressed by each T cell through recombination of V(D)J regions of the of TCR $\beta$ chain, and VJ recombination in the $\alpha$ chain. For a developing $CD4^+$ T cell to progress through primary selection, the rearranged TCR must be able to recognize self-MHCII. This step, in positive selection is necessary due to the diversity of MHC proteins between individuals. In fact, the Human MHC is locus (referred to as HLA, for human leukocyte antigen) is the most polymorphic region in the human genome,

with >10,000 different alleles identified to-date [22, 23]. As a result, depending on the number of different inherited alleles, an individual may express up to 6 different MHC I molecules, or haplotypes. MHC II are made up of two subunits (A and B), and humans can express between 3 and 12 different MHC II molecules [23]. In mice, there are three classical MHC I subclasses, (H2-K, D, L) and two MHCII classes H-2A(I-A) and H-2E(I-E) with many haplotypes indicated by lower case superscripts (such as I-$E^k$) seen later in this dissertation [24]. Endogenous T cells in the periphery only recognize Ag presented by host MHC haplotypes, but foreign MHC molecules have the potential to be antigenic as evident through the large role these molecules have in transplant rejection [25, 26]. During positive selection, if the re-arranged TCR cannot recognize self-MHC, the T cell dies from lack of stimulation in the cortex of the thymus. Negative selection subsequently serves to eliminate strongly self-reactive T cells which could lead to autoimmunity. During negative selection, single-positive $CD4^+$ T cells which have survived the process of positive selection are exposed to a variety of self-Ag by medullary thymic epithelial cells (mTECs), along with DCs and macrophages, which present various self and non-self Ags. T cells that have high affinity TCR for self-Ags are eliminated through APC-induced apoptosis, while some lower-affinity self-reactive T cells are programmed to become natural T regulatory ($nT_{reg}$). T cells that recognize self-Ag weakly, or not at all are able to exit as mature, naïve T cells and circulate throughout the periphery. Central tolerance results in a population of mature T cells that display, each of which displays unique T cell receptor (TCR) specificity for a single peptide epitope (Ag). Thus,

the population of T cells can recognize a diverse array of Ags, while limiting the risk of unwanted immune responses by maintaining high Ag specificity.

The peptides presented to $CD4^+$T cells are typically 12-25 amino acids in length, and alterations in the sequence may alter the T cell response significantly. While each TCR has a precise sequence for optimal Ag-recognition, TCR may recognize similar peptide sequences (with ~1-3 amino-acid substitutions) with varying affinity. Lower affinity interactions typically lead to a weaker TCR ligation, and subsequent TCR signaling. Therefore, a single amino acid replacement could be the difference between a T cell becoming activated or not [27]. Due to the immense diversity among TCRs, and physiological limitations of an organism, the frequency of circulating naïve T cells which recognize a particular epitope is approximately 0.01%, or roughly 100 cells in a WT mouse [28]. Peripheral naïve $CD4^+$ T cells circulate throughout the lymphatic system where in lymph nodes (LN), they scan 160-200 DC per hour in search of cognate peptide:major histocompatibility complexes (p:MHC) complexes [29].

## Regulation of T cell Activation

T cell activation requires TCR recognition of antigenic peptide presented by MHC on the surface of an APC. However, recognition of cognate p:MHC and subsequent TCR signaling alone is insufficient to drive complete activation. In order to become fully activated, naïve $CD4^+$ T cells require concurrent costimulatory receptor engagement including CD28, ICOS, and CD27 [30, 31]. The critical role of costimulatory signaling in T cell activation is apparent, as in

models where costimulatory molecules were knocked out, ~8000 TCR:pMHC contacts were required to induce T cell activation and proliferation. In the presence of CD28 signaling, this number was reduced to only ~1500 contacts [32]. In typical immune settings, insufficient costimulatory signaling following Ag recognition results in the T cell becoming hyporesponsive, or “anergic” [33].

This checkpoint limits the activation of $CD4^+$ T cells in absence of infection, as T cell costimulatory ligands such as CD80/CD86 (ligands for CD28), ICOS-L, and OX40L are not highly expressed on resting APC, but become upregulated following PRR stimulation [34]. Initial priming and activation of naïve T cells primarily occurs through interaction with DC [35], however B cells are also potent APC later in the response after many Ag-bearing DC have died, and Ag-specific B cells have become abundant following clonal expansion [36].

Activated T cells, and memory cells have been observed to respond more quickly to stimuli compared to naïve T cells [37], although the exact mechanism for these differences is unclear. One study has proposed that it may be attributed to differential signaling through Erk pathways in naïve vs previously activated cells [38]. Other studies have found that memory cells have higher levels of ZAP-70, thus may more readily form TCR signaling complexes than naïve cells [39]. In addition, activated cells are significantly larger than naïve cells, so the number of initial TCR:pMHC contact points is likely greater on activated cells. These results suggest that $CD4^+$ T cells that have passed through the initial activation checkpoints and undergone clonal expansion require less stimuli compared to naïve T cells.

# T cell:APC Interactions

## The Immune Synapse

A single T cell expresses ~30,000 TCR, while an APC typically displays ~100 cognate ligands spread across the cell surface [40, 41]. In the case of naïve $CD4^+$ T cells, interaction between a single TCR:pMHC is insufficient for complete activation. In order to obtain sufficient levels of TCR and costimulatory signaling required for activation, engagement of TCR with cognate p:MHC complexes leads to the formation of the immunological synapse [42-49]. Initiation of the immunological synapse may be triggered by a single TCR:pMHC interaction, but an elegant study by Irvine *et al.*, visualized labeled peptide bound to MHC, and found 10 or more TCR:pMHC ligands are necessary to produce a functional immunological synapse [50]. The formation of the immunological synapse involves the spatio-temporal rearrangement of the TCR, costimulatory molecules, and adhesion molecules into distinct, spatially-segregated supra-molecular activation complexes (SMACs) [48, 51-54]. This rearrangement is actin-dependent [55, 56], and is initiated by a rapid calcium influx initiated upon TCR:pMHC ligation. In effector cells, TCR and costimulatory molecules such as CD28 and ICOS, as well as the regulatory molecule CTLA-4, form connections at multiple points of T cell:APC contact and rapidly migrate towards the center of the SMAC forming the central SMAC (c-SMAC) [45, 57, 58]. Exclusion of adhesion molecules from the c-SMAC leads to the formation of a proximal ring (p-SMAC) of adhesion molecules such as LFA1-ICAM1, talin, and CD4 around the c-SMAC, stabilizing the interaction. Surrounding the p-SMAC are larger

molecules not directly involved in the immune synapse including CD43, CD44, and CD45, which form a distal SMAC (d-SMAC) [42, 47].

This entire process of rearrangement typically occurs within 2-3 minutes of initial Ag recognition [31, 59]. As TCR reach the center of the c-SMAC, they lose signaling capacity and become internalized by the T cell and are either recycled to the surface or are ubiquitinated, leading to their degradation [60]. The loss of TCR signaling and surface expression have been proposed as a mechanism for dissociation from APC [61], though the exact events which initiate the termination of the immunological synapse remain unknown. The inhibitory molecule CTLA-4 which shares the APC ligands CD80 and CD86 with the T cell costimulatory molecule CD28 may also be involved in dissociation of the immune synapse by outcompeting CD28 for shared ligands on APC [62]. Dissociation from APC generally occurs within 15-60 minutes in activated $CD4^+$ cells [31]. Naive $CD4^+$ T cells tend to maintain longer synapses and have been commonly observed to maintain contact with APC for hours [63]. One study found that at the immunological synapse, as many as 20,000 TCRs could be triggered by only 100 p:MHC complexes [64], showing the great efficiency of the immunological synapse in T cell activation. Depictions of the formation of the immunological synapse, and molecules involved are seen in figure 1 below.

Figure 1.1

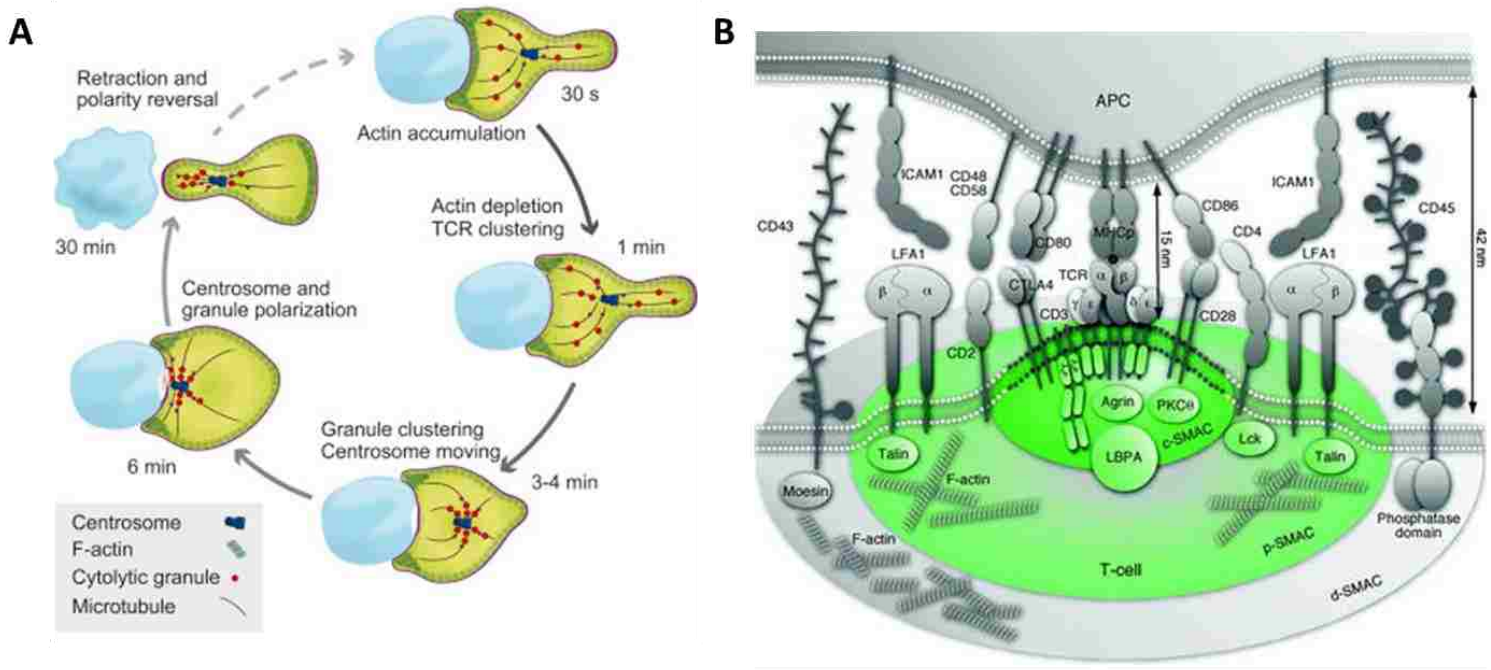



**Figure 1.1. The formation of, and the molecules involved in the immunological synapse.** **(A)** Cytoskeletal rearrangement by T cell during formation of the immunological synapse, *from: M. Davis. 2003. Nature reviews immunology* **(B)** Diagram of the molecules involved in the immune synapse between APC (top) and T cell (bottom). TCR:pMHCII ligation in the center of the cSMAC, & flanked by CD28:CD86, CTLA4:CD80. CD2:CD45/CD58 and LFA1:ICAM1 interactions are observed in the p-SMAC, and CD43 and CD45 are excluded to the d-SMAC. *Source: Yokosuka T. & Saito T. 2010. Current Topics in Microbiology and Immunology, vol 340.*

## Kinapses, and Microvessicles and the Distal Pole Complex

## Immune Kinapses

Immune synapses are not the only mechanism for T cells to receive stimulation. Shorter-lived interactions between highly motile T cells and APC also occur and have termed kinapses [46, 57]. Similar to the immunological synapse, kinapses typically have a focal point of contact between the T cell and APC. However, unlike synapses that are stable structures, with kinapses the T cell does not arrest upon TCR signaling thus, these are unstable structures and signaling is

intermittent [65]. From imaging experiments, signaling molecules that are found in the cSMAC of a classical synapse appear to be localized to the trailing edge (uropod) the migrating T cell (Fig. 1.2B).

**Figure 1.2**

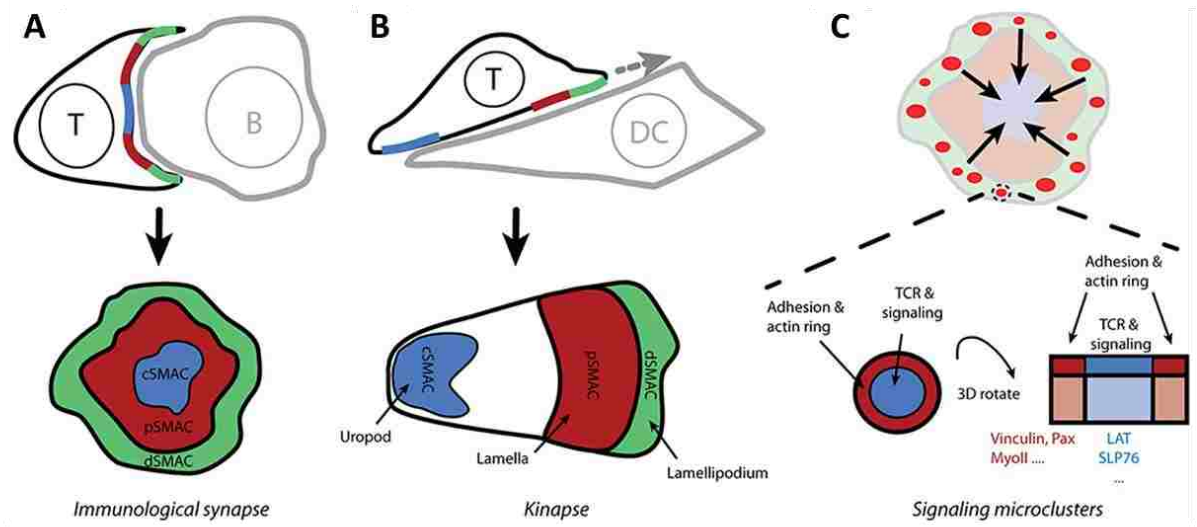



**Figure 1.2. Structural organization of T cell signaling events. (A)** Classical depiction of the immunological synapse between T cell (left) and B cell (right). **(B)** Kinapse formation between T cell (top) and DC (bottom). Arrow indicates direction of T cell migration. **(C)** TCR signaling microclusters formed in a ring around the cSMAC and migrate towards the cSMAC. *Source: Glatzova and Cebecauer, 2019. Frontiers in Immunology*

It has been proposed that kinapses may form as opposed to a classical synapse due to the high mobility of the T cell, or in cases where chemokine receptor signaling is greater than signaling triggered by APC molecules [57]. Recently, an elegant study by Akkaya *et al.*, proposed a model for kinapse morphology involving T cell microvilli, (small protrusions containing TCR/costimulatory molecules on the surface of T cells) that form the main points of contact during synapses [66].

Kinapses are not generally associated with the activation of naïve T cells, though it has been found multiple kinapses can result in the accumulation of sufficient signaling to drive T cell activation [67]. Kinapses also serve as a mechanism to maintain the activation state of already activated T cells [67]. These findings, combined with the proposed model for kinapse formation via microvilli [66], suggest that microvilli may play a larger role than previously thought in the context of active TCR signaling.

## Microclusters

A third physically distinct signaling mechanism that has been observed between T cell and APC involves small clusters of TCR and costimulatory molecules such as CD28 termed microclusters. The initiation of both the immune synapse and formation of microclusters involve filamentous like protrusions on the T cells known as microvilli [66, 68]. Microvilli have high concentrations of TCR, thus enabling T cells to efficiently scan the surface of APC for pMHC complexes. Microvilli also contain costimulatory molecules such as CD28. Microclusters form outside of the dSMAC upon microvilli binding pMHC and costimulatory molecules on APC. These microcluster signaling complexes provide a major source of TCR/costimulatory molecule signaling. The synaptic microcluster morphology commonly occurs in synapses formed with DC [45], while the classical “bulls-eye” centrally focused synapses are predominantly formed during Ag presentation by B cells [45, 69]. Because DC play a primary role in the activation of naïve T cells, this is a notable observation.

One particularly interesting aspect of TCR microclusters is their unique migration patterns. Signaling via microclusters precedes the formation of the immunological synapse, and rapidly initiates (0-30 seconds) following engagement of TCR and MHC and CD28:CD80/86. Microclusters generally migrate towards the cSMAC, but also have been observed to migrate away from the immunological synapse and localize at the distal pole of the T cell [70]. In contrast to some of the original models of the immune synapse, where maximal signaling was predicted to occur in the cSMAC, more recent models suggest that TCR signaling primarily occurs in microclusters outside of the SMAC [69, 71-73]. It has been proposed that while active signaling is occurring in the microclusters, the cSMAC mainly serves as the location where TCR that are no longer actively signaling are endocytosed and either recycled to the surface or ubiquinated, leading to their destruction [69, 74, 75].

Curiously, microclusters migrating away from the immune synapse have been shown to retain active TCR signaling, despite their apparent lack of APC contact [70, 76]. An interesting study by Hashimoto *et al.*, observed that TCR microclusters form a surrounding ring of adhesion molecules, and on a micro-scale are similar in structure to the immunological synapse [71] (Fig. 1.3). Microclusters outside of the cSMAC have also been observed to exclude the phosphatase protein CD45 [74]. CD45 can act to enhance TCR signaling through the dephosphorylation of autoinhibitory tyrosine in clusters of Lck [77]. On the other hand, CD45 also plays an important role in regulating TCR signaling by dephosphorylating ITAMs and TCR in the sMAC, leading to the termination of the

TCR signaling [78, 79]. The formation of the ring surrounding TCR microclusters has been thought to aid in their exclusion of CD45, permitting TCR signaling to be maintained in absence of APC contact.

**Figure 1.3**

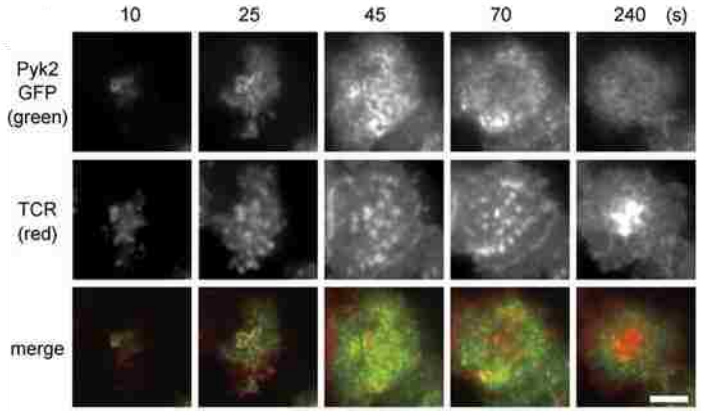



**Figure 1.3 TCR microclusters are bordered with focal adhesion molecules.** Time-course of a T cell forming a microcluster, with the focal adhesion molecules Pky2 (top, bottom green) and TCR (middle, bottom red). Scale bar = 1.5 µm *Source: Hashimoto-Tane et al, 2016. Journal of Experimental Medicine*

## The Distal Pole Complex

During the formation of the immunological synapse, cytoskeletal rearrangements and establishment of an active signaling location leads to distinct polarity within the T cell. This axis of polarity is established as TCR and costimulatory molecules migrate towards the T cell:APC interface to form the proximal pole, which is the location of the highest active signaling. Concurrent with the formation of the synapse, surface molecules not involved in the immune synapse such as CD43, SHP-1, PI3K, scribble (PKCζ), Numb, Par3/6, and Disks Large (hDLG) [80], migrate to the side of the T cell directly opposite the immune synapse. Together these molecules form the distal pole complex (DPC) [70].

While the DPC contains negative-regulators of T cell activation,

surprisingly, active signaling of molecules including ZAP-70, have also been observed to occur at the DPC [76, 81]. Although the mechanism is unknown, the presence of active TCR signaling at the DPC has been observed to precede the formation of the immune synapse in a TCR-independent, but contact-dependent manner [70]. Randriamampita *et al.* have termed active signaling at the DPC the "antisynapse". [76]. Upon formation of a stable immunolocigal synapse, actively-signaling TCR at the antisynapse are recruited to the proximal pole until eventually signaling localized to the distal pole is no longer detectable [70]. The mechanisms behind active TCR signaling at the distal pole have not yet been characterized [76]. However, it is likely that the antisynapse is formed by microclusters which retain active signaling outside of the cSMAC [74, 76].

## Additional Variables That Influence Synapse Morphology

While it has been suggested that T cell activation via microclusters vs classical synapse are dependent on APC cell type, factors such as Ag dose, or T cell subset also have been shown to influence the morphology of the T cell:APC interaction [31, 82]. For example, Thauland *et al.,* found that the majority of pre-polarized $T_H2$ cells formed multifocal synapses with low doses of Ag, while high doses lead to the majority of synapses forming the classical "bull's-eye" morphology [83]. Meanwhile, polarized $T_H1$ cells formed canonical synapses under both low and high Ag dose [83] (Fig. 1.4).

**Figure 1.4**

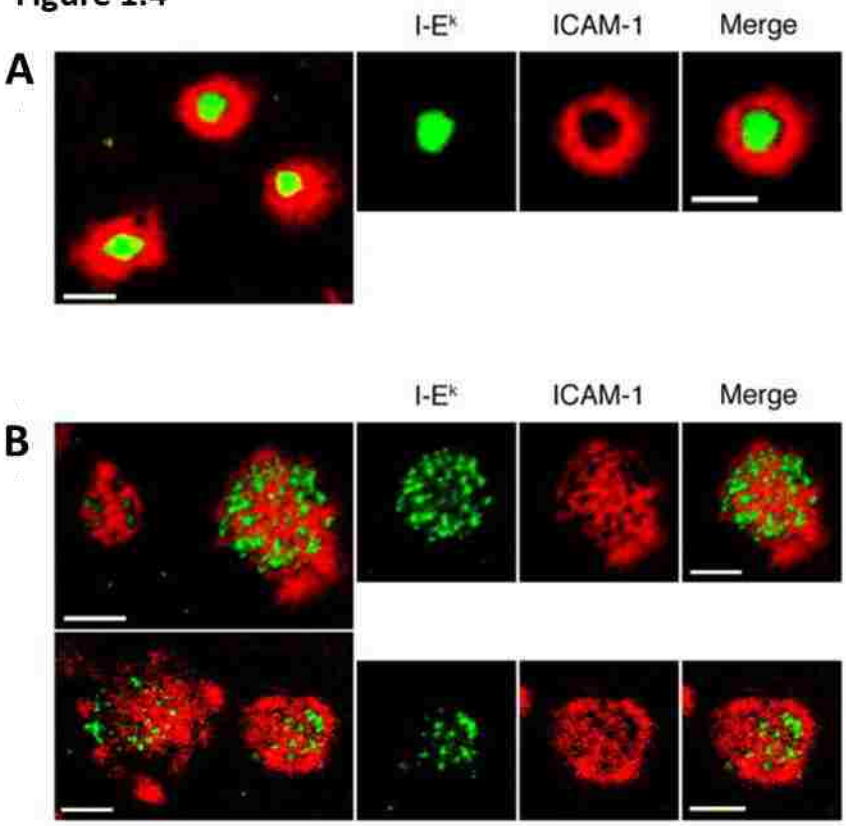



**Figure 1.4. $T_H1$ and $T_H2$ cells form morphologically distinct synapses.** $T_H1$ and $T_H2$ cells were incubated on supported planar bilayers containing ICAM-1-Cy5 (red) and peptide-loaded I-$E^k$-488 (green). Images were captured between 20 and 35 minutes. **(A)** $T_H1$ cells consistently formed IS with a compact, monofocal accumulation of MHC/TCR. **(B)** The majority of $T_H2$ cells formed multifocal IS. *Source: Thauland et al. 2008. Journal of Immunology*

The existing models of T cell activation, requires the ligation of many TCR by pMHC, CD28 ligation with CD80/CD80, and the interaction of adhesion molecules. Thus, regardless of the physical location of these interactions, synaptic or microclusters, a period of stable signaling molecule ligation is necessary to achieve full $CD4^+$ T cell activation.

# T cell Signaling: Pathways, Consequences, and Regulation

## T cell activation

Following TCR and costimulatory receptor engagement, downstream signaling leads to the activation of three canonical transcription factors which play the primary roles in T cell activation, proliferation, and differentiation. These transcription factors are; nuclear factor-κB (NF-κB) (required for proliferation and differentiation), activator protein 1 (AP-1) (involved in IL-2 production and activation), and nuclear factor of activated T cells (NFAT) (drives IL-2 and IL-2Rα expression, and important in effector functions and differentiation) [84]. An overview of T cell signaling events is shown in figure 1.5.

Following recognition of p:MHC by TCR formed by αβ heterodimers, the Src-family kinase $p56^{Lck}$ (Lck), which is non-covalently associated with CD4 molecules, is recruited to the TCR signaling complex [85]. Lck is recruited to the early synapse, upon CD4 binding to the non-polymorphic β2 domain of MHCII [86]. CD4 is therefore often categorized as part of the TCR signaling complex, and also acts as a coreceptor because it enhances the sensitivity of TCR by up to 100-fold [87-90]. Upon removal of an autoinhibitory phosphate by CD45, and phosphorylation of Tyr 505, activated Lck phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) on the CD3 and TCR ζ chains, which then become binding sites for the SH2 domains of the Syk-family kinase zeta associated protein 70 kDa (ZAP-70) [91]. Lck phosphorylates and activates recruits ZAP-70. Phosphorylated Ltk/ZAP-70 stabilize the TCR signal [92] and

phosphorylate four major tyrosine sites on the adaptor protein linker of activated T cells (LAT). The proximity of LAT to TCR-signaling complexes is maintained via its single transmembrane domain, and the multiple cytoplasmic binding domains of LAT serve as scaffold for multiple signaling molecules. The recruitment of these signaling molecules to activated LAT forms the proximal signaling complex which plays a central role in the initiation of the multiple signaling pathways downstream of TCR signaling [93]. The formation of this complex promotes interaction with recruited proteins including the adaptor Grb2-related adaptor protein-2 (GRAP2/Gads), SLP-76, IL-2 inducible T cell kinase (ITK), Grb2, p85 PI-3 kinase, and phosphoinositide-specific phospholipase C1 (PLCγ1) [94]. Here the signal diverges through signaling of GRB2/SOS and SLP-76, as well as PLCγ1, to act on different transcription factors involved in T cell activation.

Grb2 and Gads are adaptor proteins which bind Son of Sevenless (SOS) and SLP-76, leading to Ras and subsequent MAP kinase cascade. This signaling cascades leads to ERK1/2 activation, which acts in the nucleus to regulate c-Jun and c-Fos, which form the AP-1 transcription factor. AP-1 plays roles in IL-2 production by binding to the IL-2 promotor to induce gene expression and also stabilizes IL-2 mRNA [95]. Its activation also promotes survival by indusing expression of the anti-apoptotic gene Bcl-xL [96]. Downstream of AP-1, NF-κB activation occurs, which drives T cell proliferation and differentiation through IL-2 expression, enhancing translation of subset-specific transcription factors, increased effector functions, and upregulation of surface activation and costimulatory markers [93, 97-99].

PLCγ1 hydrolyzes $PIP_2$ to generate the secondary messengers inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG), which activate different downstream pathways. $IP_3$ diffuses in the cytoplasm and becomes bound by its receptor ($IP_3R$) on the endoplasmic reticulum (ER). This causes the release of $Ca^{2+}$ stores from the ER leading to the subsequent influx of extracellular calcium through channels in the plasma membrane. Elevated levels of cytosolic $Ca^{2+}$ activate various proteins, including the phosphatase calcineurin. Calcineurin dephosphorylates the transcription factor nuclear factor of activated T cells (**NFAT**), allowing it to traffic into the nucleus. In the nucleus, NFAT is involved in stabilization and induction of lineage-specific transcription factors T-bet ($T_H1$), Gata3 ($T_H2$), RORγt ($T_H17$), and Foxp3 ($iT_{reg}$) [100]. The specific transcription factor NFAT promotes is dependent on the convergence of other signaling pathways such as cytokine receptor signaling to activate respective STAT signaling pathways. NFAT also is critical for the expression of IL-2 and IL-2Rα (CD25), which enable T cell proliferation [101].

Activated DAG initiates signaling in two pathways. The first pathway involves the calcium independent activation of protein kinase Cθ (PKCθ). DAG binding to PKCθ leads to conformational changes in PKCθ to form an open, active state. Activated PKCθ can phosphorylate cytoplasmic IkB kinase (IKK), leads to **NF-κB** translocation to the nucleus where it acts as a transcription factor driving T cell proliferation and differentiation [102]. The second role for DAG is to initiate the RAS – MAPK cascade ending with the activation of kinases ERK1/2

which, as described above, leads to the nuclear translocation and activation of **AP-1.**

At the immunological synapse, costimulatory signaling primarily involves the costimulatory receptor CD28. CD28 ligation activates phosphoinositide 3-kinase (PI3k) which subsequently phosphorylates phosphatidylinositol 4,5-bisphosphate ($PIP_2$) to yield phosphatidylinositol (3,4,5)-trisphosphate ($PIP_3$). $PIP_3$ recruits proteins to the inner leaflet of the plasma membrane, such as ITK kinase [103]. ITK binds to $PIP_3$ and SLP-76, which localize it to the plasma membrane and cause its activation. Activated ITK can then phosphorylate PLCγ1, thus CD28 signaling pathways converge with TCR signaling to amplify RAS/MAPK and PCKθ signaling. CD28 signaling also increases expression of the costimulatory CD40L to aid in the maintenance of T cell activation [104]. Signaling through CD28 alone can enhance the survival of activated T cells.

Importantly, TCR + CD28 activation of PI3k also activates the protein kinase B (Akt) / mammalian target of rapamycin (mTOR) signaling pathway. mTOR includes two unique signaling complexes mTORC1 and mTORC2. Through inhibition of STAT signaling, mTORC1 has been found to play a role in $T_H1$, $T_H2$, and $T_H17$ differentiation, while a role for mTORC2 has only been found in the differentiation of $T_H1$, and $T_H2$ [105]. While early studies suggested the absence of mTOR signaling in the presence of TCR signaling led to anergy [106], more recent studies have found that absence of mTOR signaling leads to induction of $T_{reg}$ [107]. mTORC1 signaling leads to glycolysis, and trafficking of the glucose transporter Glut1 to the cell membrane [108].The induction of

mTORC glycolic metabolism is associated with $CD4^+$ effector function. It also is directly involved in protein synthesis through inhibition of eukaryotic initiation factor 4E binding protein (4EBP1) (which inhibits protein translation), and activation of p70 S6K ribosomal protein [109, 110]. In addition, mTORC1 increases lipid metabolism and synthesis, as well as mitochondrial biogenesis which aids in T cell effector functionality and clonal expansion [111]. mTORC2 acts by phosphorylating other proteins including Akt, which forms a positive feedback loop [112], leading to increased cell survival, cytoskeletal organization and migration [113]. mTOR signaling can lead to increased effector functionality by promoting the production of effector cytokines [114]. Collectively, the mTOR signaling complex integrates multiple cell signaling events to regulate cell proliferation, differentiation, effector functionality, and survival.

## Figure 1.5

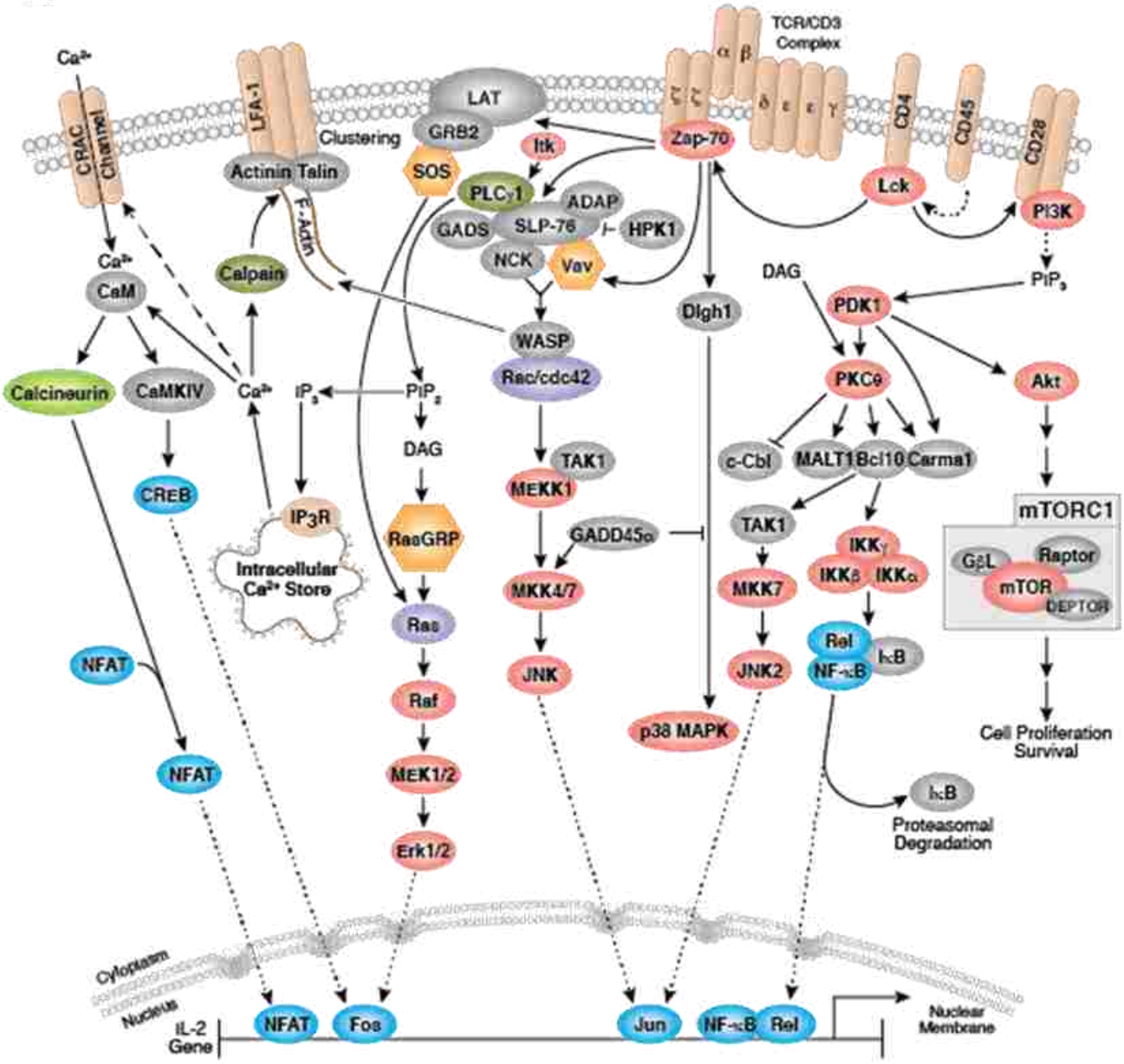



**Figure 1.5. Simplified model of T cell signaling events during the immune synapse, as described above.** *Source: Cell Technologies: T cell signaling pathways*

## Regulation of T cell signaling

Proximal signaling through ZAP-70 is negatively regulated by the binding of the E3-ubiquitin ligases Cbl c-Cbl and Cbl-b. Cbl ubiquinates lysine residues on ZAP-70 targeting it for proteasomal degradation [115]. Excessive Cbl-recruitment can lead to insufficient signaling for activation and is largely associated with T cell anergy. While anergic T cells are able to form mature immunological synapses, it was found by Doherty *et al.*, that anergic synapses showed higher recruitment of Cbl-b to the cSMAC compared to control cells, leading to decreased pERK signaling [116]. Cytoplasmic phosphatases have also been shown to reduce ZAP-70 activity through removal of activating tyrosine phosphate [117]. Dysregulation of ZAP-70 signaling can lead to cell exhaustion, or excessive responses implicated with autoimmune diseases. Along these lines, inhibitors of ZAP-70 signaling have been proposed for use in treatment of autoimmune diseases and organ transplant rejection [118, 119].

The Cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), which is expressed after T cell activation, binds to the same ligands as CD28 (CD80/CD86), but rather than providing positive costimulation, CTLA-4 signaling inhibits T cell activation [120]. CTLA-4 resides in intracellular vesicles and upon strong TCR ligation, these vesicles move to and fuse with the plasma membrane delivering CTLA-4 into the immunological synapse [121, 122]. Although expressed at much lower levels than CD28, CTLA-4 binds to CD80/86 with significantly higher affinity than CD28. CTLA-4 ligation provides competitive inhibition of costimulatory signaling, as well as inducing unique signals that inhibit

IL-2 production [120]. The immunoregulatory effects CTLA-4 comes from studies using antibody-mediated blockade of CTLA-4 signaling, which has become a promising component in anti-tumor therapies [123, 124]. However, the absence of CTLA-4 has also been shown to lead to CD28-mediated autoimmunity in mice and humans [62, 125]. In these particular cases, it is likely that CTLA-4 blockade not only increased costimulatory signaling through CD28, but also decreased the function of $T_{reg}$. The high expression levels of CTLA-4 by $T_{reg}$ plays an important role in peripheral tolerance, and are discussed in greater detail in later sections.

# Events Following T-cell Activation

## T cell Clonal Expansion

Following activation, naive $CD4^+$ T cells rapidly proliferate, resulting in clonal expansion of up to 100,000-fold within 3-4 days [126]. Despite early contradictory findings [127], continued Ag signaling is not required during the expansion phase [128, 129]. This allows for rapid T cell proliferation without the requirement for daughter cells to scan APC for Ag. Clonal expansion leads to a massive increase in Ag-specific lymphocytes which can then rapidly clear an infection. It also underscores the necessity for the regulation of T cell activation through activation checkpoints and $T_{reg}$, in order to conserve physiological resources and prevent unwanted inflammation and autoimmunity due to potential self-reactive T cells that have escaped negative selection.

Following expansion, activated $CD4^+$ effector cells provide “help” to other cells of the immune system through effector-cytokine secretion and contact

dependent mechanisms [130]. Effector T cells undergo a genetic reprogramming into specialized lineages of T helper $T_H$ subsets. Each $T_H$ subset exhibits effector functions associated with protection against a given type of pathogen, or interacts with a specific type of cell, such as $T_{FH}$ help for B cells. This reprogramming from undifferentiated precursors is known as T cell differentiation.

## $CD4^+$ T Helper Differentiation

The immune system must be highly adaptable in order to provide protection against a diverse array of pathogens with various modes of infection. For example, a cell-mediated response is optimal for combating intracellular pathogens, while a humoral response leading to the production of antibodies is more effective at neutralizing extracellular infections. Typically, a fine balance is required to provide optimal protection. $CD4^+$ T cells play a central role in directing the type of immune response generated. They are differentiated from naïve, undifferentiated precursors, into specialized subsets upon activation. While some T cell lineages such as natural T regulatory ($nT_{Reg}$) [131], and NK T cells [132] are predetermined during maturation in the thymus, the majority of naïve $CD4^+$ T cells in the periphery are undifferentiated.

The first insights into $T_{FH}$ differentiation came from the work of Mossmann and Coffman in the late 1980s when they observed two distinct subsets of $CD4^+$ T cells that produced either IFNγ ($T_H1$), or IL-4 ($T_H2$) [133, 134]. In the years since, new subsets with specialized functions have been discovered including $T_H17$, $T_H9$, $T_H22$, induced T regulatory ($iT_{reg}$/ $T_{reg}$), T follicular helper ($T_{FH}$), and

cytotoxic $CD4^+$ T cell ($CD4^+$ CTL) [135-137]. The primary model for naïve $T_H$ differentiation, known as the three-step model, proposes that TCR signaling primes the T cell (step 1), while costimulatory signaling is required for T cell activation (step 2). The effector subset differentiation is driven by cytokine signaling concomitant with, or shortly after, activation (step 3). This differentiation is ultimately controlled by PRR signaling in APCs, which leads to the expression of cytokines associated with protection against the encountered pathogen. In this way, $CD4^+$ T cells differentiate into lineages which promote the most effective immune response against the pathogen at hand [138] (Fig. 1.6).

**Figure 1.6**

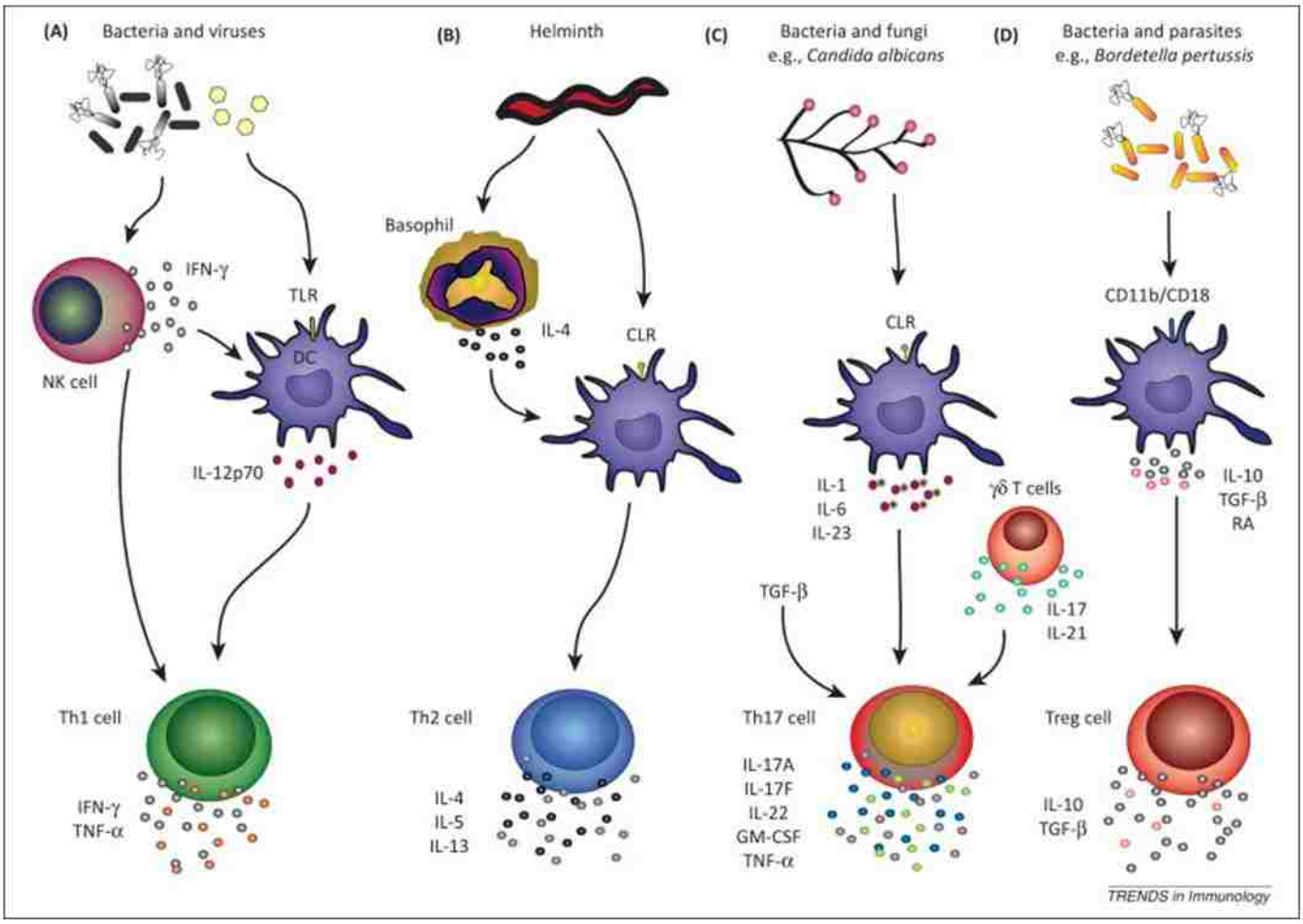



**Figure 1.6. Signals received through pattern recognition receptor signaling on APC are transferred from DC to T cells in the form secreted cytokines.** Examples of cytokine expression by APC or other immune cells induced through contact with various pathogens. Cytokines then impact the differentiation of $CD4^+$ T cells into effector subsets. *Source:* Walsh & Mills, *2013. Trends in Immunology*

While this model holds true for the majority of $T_H$ differentiation cases, recent evidence has found this process to be more complex than initially thought. One example comes from findings that the strength of TCR signaling may override cytokine signaling in the context of $T_H2$ differentiation. Weak TCR signaling has been found to drive cells to differentiate towards $T_H2$ even when cells are in a $T_{H1}$ -polarizing environment [139]. In contrast, strong TCR signaling has been shown to favor $T_H1$ differentiation [82, 140, 141]. The duration, or

persistence, of TCR and costimulatory signaling also impacts $T_H$ differentiation, with short-lived interactions favoring a $T_H2$ phenotype and longer synapses promoting $T_H1$ differentiation [82]. In the case of $T_{FH}$ cells, repeated cognate interactions with cognate APC are required for their differentiation [142, 143]. To add to the complexity, under opposing polarizing conditions, there is plasticity between differentiated $T_H$ subsets, and concurrent expression of two or more subset-characteristic cytokines and/or transcription factors in the same $T_H$ cell is possible [144-149] (Fig. 1.7B)

**Figure 1.7**

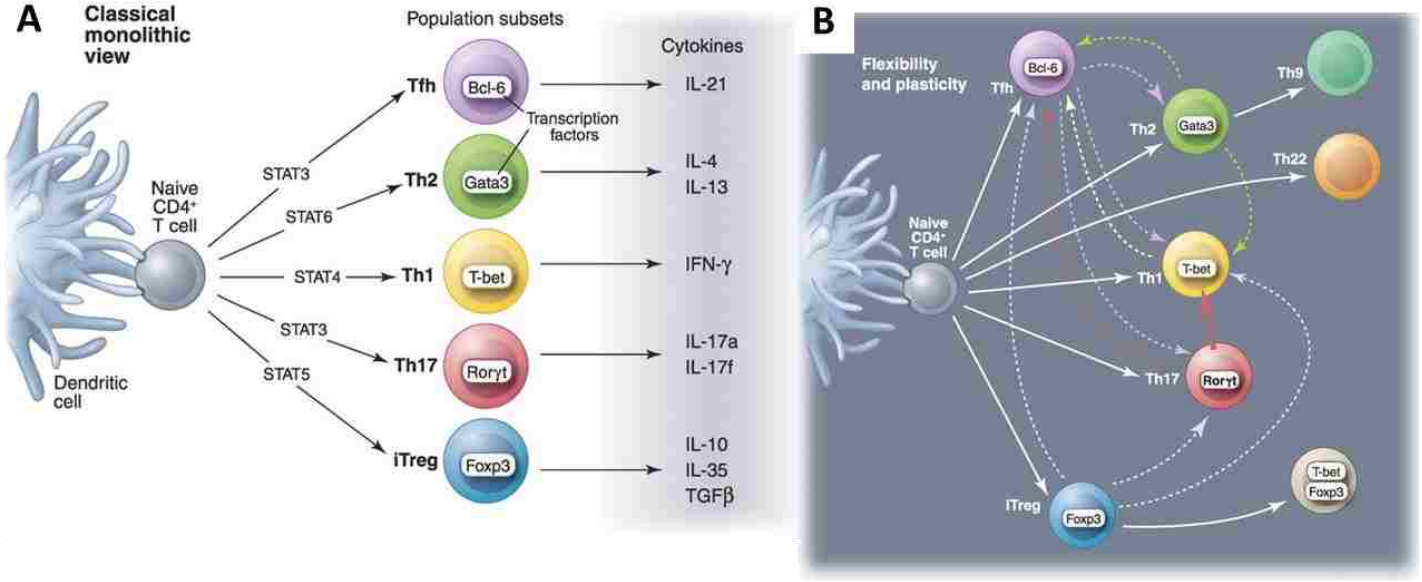



**Figure 1.7. Classical model of CD4⁺ T cell differentiation vs model of plasticity and flexibility between subsets. (A)** Classical monolithic view of the differentiation of naïve CD4⁺ T cells through APC-derived cytokine induction of STAT signaling pathways, leading to subset-characteristic transcription factor expression and respective differentiation. Effector cytokines associated with each subset are shown on the right. (B) Model showing flexibility between differentiated $T_H$ subsets. *Adapted from O'Shea and Paul, 2010. Science.*

## Functions of CD4$^+$ $T_H$ Subsets

As described above, while $T_H$ differentiation is a complex process, the cytokines present at the time of activation play a major role in $T_H$-subset differentiation. Through the activation of specific STAT proteins, cytokine signaling is able to activate transcription factors that act as master regulators of differentiation, and the expression of cytokines which further drive $T_H$ differentiation, or by inhibiting the expression of transcription factors associated with other $T_H$ subsets. The cytokines, transcription factors, and primary functions associated with known effector subsets is shown in table 1 below.

**Table 1**

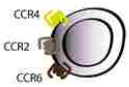
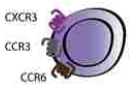
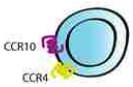
| | Th1 | Th2 | Th17 | Th9 | Th22 | iTreg | Tfh |
|---|---|---|---|---|---|---|---|
| |   |   |   |   |   |   |   |
| Major cytokines driving differentiation | IL-12 | IL-4 | TGFβ IL-6 | TGFβ IL-4 | IL-6 TNFα IL-1β?? | TGFβ IL-2 | IL-21 IL-6?? |
| Master transcriptional regulator | T-Bet | GATA-3 | RORγt | PU.1 | AhR? | FoxP3 | Bcl-6 |
| Major cytokines produced | IFNγ | IL-4 | IL-17 | IL-9 | IL-22 | IL-10 TGFβ | IL-21 |
| Major function | Intracellular infections | Extracellular Infections | Extracellular Infections | Extracellular Infections | Extracellular Infections | Regulation | Extracellular Infections |
| Major site of effector function | Peripheral tissues | Peripheral tissues | Peripheral tissues | Peripheral tissues | Peripheral tissues | Peripheral tissues & secondary lymphoid organs | Secondary lymphoid organs |

**Table 1. Driving factors and characteristics of known TH subsets.** This table shows the primary cytokines involved in the differentiation of respective T helper subsets shown on the top. Below are subset-characteristic transcription factors expressed by each subset, followed by major functions, and typical sites of function in the context of infection. *From Kara et al., 2014. PLoS Pathogens*

## $T_H1$: Cell-mediated immunity against intracellular pathogens

$T_H1$ differentiation is driven primarily by the polarizing cytokine IL-12, while IL-18, IL-23, and IL-27 also favor $T_H1$ differentiation [150]**.** Meanwhile opposing signals from IL-4 can inhibit $T_H1$ differentiation and effector functionality [151]. Although other APC are capable of driving $T_H1$ differentiation, $CD8\alpha^+$ dendritic cells (DC) localized in lymphoid organs [152] secrete IL-12, thus are effective promoters of promoting $T_H1$ differentiation [153]. The master transcriptional regulator T-bet inhibits GATA3 expression [154], and is a defining marker for $T_H1$ lineage [150]. Strong TCR and costimulatory signaling through high Ag dose [155-157], and extended T:DC interactions also favors $T_H1$ differentiation [157]. $T_H1$ cells aid in cellular-mediated immunity through secretion of IFNγ, IL-2, TNFα and TNFβ, which activate cytotoxic $CD8^+$ T cells (CTL) and NK cells to kill infected cells [158], or tumors [159, 160]. These cytokine also induce nitric oxide production in macrophages to enhance pathogen killing [161, 162]. IFNγ induces B cell class switching towards IgG2 Ab production which are highly effective in activating the classical complement pathway [163, 164]. Aberrant $T_H1$ responses contribute to pathogenicity of organ-specific autoimmune diseases including type 1 diabetes, psoriasis, Crohn's disease, and multiple sclerosis [7, 153, 165]

## $T_H2$: Humoral-Mediated Immunity and Protection Against Extracellular Pathogens

$T_H2$ differentiation cells are identified by the master regulator transcription factor GATA3 [166, 167], and produce effector cytokines IL-4, IL-5, IL-6, and IL-13. The presence of IL-4 following activation drives GATA3 expression via STAT6 signaling [168], while IL-12 negatively regulates $T_H2$ differentiation [169]. IL-4R signaling not only prevents $T_H1$ differentiation, but also negatively regulates IFNγ expression [170], following a clear pattern for $T_H1$/$T_H2$ negative regulation. In addition, IL-2-induced STAT5 has been found to be required for $T_H2$ differentiation [171]. Lower signal strength or short interactions with APC favor $T_H2$ differentiation [139, 156, 172], thought to be due to weaker signaling leading to early IL-4 production [173]. Some contrasting reports have found high signal strength to drive $T_H2$ differentiation [174, 175], and the consensus is that either low, or high signal strength can drive $T_H2$ differentiation, while medium to high signal strength drives $T_H1$ [155-157].

These cells specialize in providing B cell help through directed cytokine secretion upon recognition of cognate Ag displayed by the B cell. The cytokines IL-4 and IL-6 in particular are important in driving B cell class switching and proliferation in the germinal center response, with IL-4 favoring class switching towards IgE and IgG1 isotypes [6, 176]. $T_H2$ cells activate eosinophils [177] and play a role in airway inflammation. $T_H2$ responses are also associated with protection against large extracellular pathogens such as helminths [178]. Dysregulation in the activation of $T_H2$ cells can lead to increased allergic

inflammation, ulcerative colitis, atopic dermatitis, chronic rhinosinusitis, as well as airway inflammation and asthma [6, 165, 179].

## $T_H17$: Mucosal and Surface-Barrier Immunity

The third effector subset of $CD4^+$ T cell to be identified were $T_H17$ cells, induced by a combination of TGFβ + IL-6 or IL-23 signaling leading to the expression of transcription factors STAT3, RORγt, and RORα [180]. While TNFα, IL-21, and IL-23 can enhance $T_H17$ differentiation, IL-4, IFNγ, IL-12, and IL-27 inhibit their differentiation. $T_H17$ cells secrete IL-17A, IL-17F, IL-21, IL-22, and granulocyte-macrophage colony-stimulating factor (GM-CSF) [181, 182]. These cells are associated with inflammation and mucosal immunity, primarily act at barrier tissues, and provide protection against extracellular bacteria, fungi, and enteric parasites such as *Giardia* [183, 184]. IL-17 in combination with IL-22 has been found to induce expression of β-defensin 2 and other antimicrobial peptides, suggesting that $T_H17$ cells play a role in skin immunity [185]. Cytokines produced by $T_H17$ cells can induce neutrophil production, and lead to recruitment of other immune cells to sites of inflammation. $T_H17$ cells are also able to regulate the B cell *Pigr* gene, which is crucial for the expression of the poly Ig receptor for IgA, pIgR. The pIgR enables IgA molecules to cross luminal membranes [183], again, demonstrating the important role $T_H17$ cells play in mucosal immunity. As $T_H17$ cells are associated with inflammation, excessive $T_H17$ responses have been found to play a role in exacerbation of diseases in a manner similar to $T_H1$ cells. Excessive numbers of $T_H17$ likely increases pathology of psoriasis [14], as drugs which neutralize IL-17A have been effective in reducing the symptoms of

psoriasis [186]. IL-17A has also been associated with rheumatoid arthritis, multiple sclerosis, airway inflammation and COPD, asthma, atopic dermatitis, and irritable bowel disease [10, 15-20].

## $T_H22$: Surface barrier immunity

$T_H22$ cells are a more recently described subset of cells, which are related $T_H17$ cells. it has been found that $T_H22$ cells are identified by expression of the transcriptional regulator, the Aryl Hydrocarbon Receptor (AhR) and they produce TGFα and IL-22, but not IFNγ, IL-4, or IL-17 [187]. It has been proposed that IL-22 differentiation is driven by IL-6, IL-23, IL-1β, and FICZ [188]. While originally considered to be identical to $T_H$ 7 [185] these cells have roles in immune function distinct from $T_H17$ cells [189]. High numbers of $T_H22$ localize to the epidermis and $T_H22$-produced IL-22 has been found to play important roles in protection against enteropathogenic bacteria [190]. The impact of IL-22 differs between locations, as pathogens may enter through the skin, gut, and respiratory tract [191, 192]. However, the end result is production of antimicrobial peptides on surface barriers, and mucus-associated glycoproteins at mucosal barriers, both which provide defense against bacterial infection [193]. IL-22 also exhibits epithelial reparative and regenerative properties [194]. Interestingly, the depletion of $T_H22$ cells during HIV infection led to the epithelial barrier becoming compromised due to decreased IL-22 levels, suggesting IL-22 may play a role in protection from HIV, among other viral infections, at mucosal membranes [194]. Excessive $T_H22$

responses can exacerbate symptoms of rheumatoid arthritis [10, 19], skin allergy [195] and atopic dermatitis, psoriasis, irritable bowel syndrome, and potentially systemic lupus erythematosus (SLE) [13].

### $T_H9$: Type-2 Associated extracellular Pathogen, (and anti-tumor?) Immunity

The recently discovered $T_H9$ subset [196] is induced by IL-2 and IL-4 + TGFβ [197] and is typically associated with a type 2 Immune response. $T_H9$ cells require transcription factors STAT6, IRF4, GATA3, and are identified by expression of PU.1 [198]. While the redundancy of $T_H9$ cells with $T_H2$ has led to uncertainty whether they play an indispensable role in immune protection, $T_H9$ cells have been found to uniquely produce IL-10, IL-21, and IL-9 [137]. IL-9 stimulates growth, proliferation and survival of T cells and mast cells, increases secretion of mucus, and enhances IgE production by B cells [199-202]. suggesting a protective role for $T_H9$ cells in mucosal immunity. Results have found $T_H9$ cells drive immunity against helminth infection [203] through increasing IL-5 and IL-13 production in T cells, and basophil and eosinophil numbers at the site of infection [204]. IL-9-secreting cells have also recently been found to play a role in anti-tumor immunity in melanoma models [205, 206]. While the mechanism for IL-9 driven anti-tumor activity is unclear, it was proposed that IL-9 induced recruitment of mast cells [205], DC and cytotoxic $CD8^+$ cells to the site of the tumor through increasing expression of the chemoattractant CCL20 [206]. The impacts of $T_H9$ cells on autoimmunity are still undetermined, though transfer of $T_H9$ cells in a colitis model led to increased inflammation [197]. It was

unclear however, if this was a result of IL-9 production, as transferred cells were observed to increase lFNγ expression, thus the results may have been due to a $T_H9$-$T_H$ 1 effector transition [197].

## T follicular helper cells: Key Players in the Humoral Response

T follicular helper cells ($T_{FH}$) play an essential role in providing B cell help in the germinal center (GC) reaction. Their proximity to B cells, secretion of IL-21 and IL-6, maintained expression of the C-X-C chemokine receptor type 5 (CXCR5) along with inducible T-cell costimulatory (ICOS) and programmed cell death protein-1 (PD-1) [207-211], sets them apart from the $T_H$ 2 subset. Unlike other $CD4^+$ T cell effector subsets, $T_{FH}$ differentiation does not occur following initial priming of naïve $T_H0$ cells by antigen-bearing DC, *but requires subsequent interaction with APC for full differentiation* [142, 212-215]. The current model of $T_{FH}$ differentiation seen in Fig. 1.8, holds that pre-$T_{FH}$ are primed by DC through IL-6 and ICOS signaling which leads to the upregulation CXCR5. As activated T cells downregulate CCR7 (which keeps cells in T cell zones), the expression of CXCR5 enables them to chemotax in response to the CXCL13 chemokine gradient present in B cell zones [212, 216-218]. At the edge of the B cell follicle, pre-$T_{FH}$ interact with antigen-bearing, cognate B cells and these repeated interactions in combination with either IL-6 or IL-21 induce STAT3 signaling [219, 220]. STAT3 signaling leads to upregulation of the transcription factor Bcl-6 to drive full $T_{FH}$ differentiation (in the absence of the transcription factors Blimp-1

and KLF2) [209, 213, 215, 219, 221-225]. Bcl-6 acts as a transcriptional repressor for GATA3 and inhibits the activity of T-bet and RORγt, thus inhibiting $T_H2$, $T_H1$, and $T_H17$ differentiation, respectively [226]. The transcription factor Tcf1 has also been found to be necessary for full differentiation in the context of viral infection [227].

**Figure 1.8**

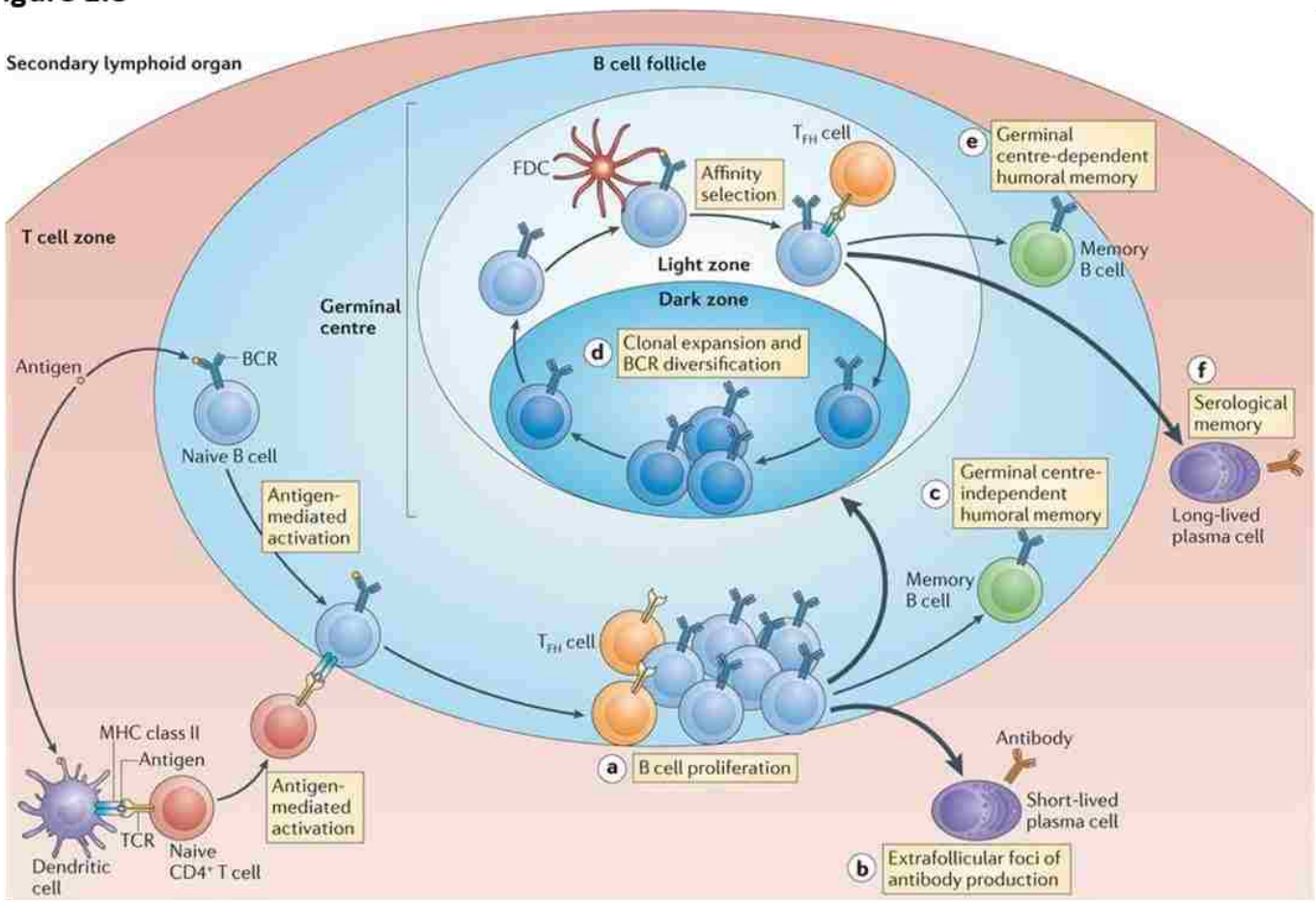



**Figure 1.8. Proposed model for T follicular-helper ($T_{FH}$) differentiation.** Naïve CD4+ T cells are primed by DC to move towards the B cell follicle. Cognate signaling with B cells in the B cell drives TFH differentiation and subsequent entry into the germinal centre. *Source: Kurosaki et al. 2015. Nature Reviews, Immunology*

The proposed requirement for B cells in $T_{FH}$ differentiation is potentially due to the fact that pre-$T_{FH}$ are much more likely to encounter antigen-bearing B cells than DC upon their CXCR5-driven migration to the B cell follicles. In

addition, about 3 days following the initiation of the immune response, the majority of APC are Ag-specific B cells. This possibility is supported by the fact that continual stimulation by repeated immunizations in the absence of B cells was found to produce $T_{FH}$ [213]. This, combined with results from other studies [228], suggests that B cells do not provide unique signals which drives $T_{FH}$ differentiation, but simply serve to prolong Ag stimulation that is required for $T_{FH}$ differentiation.

Upon full $T_{FH}$ differentiation, high Bcl-6 expression leads to loss of Epstein-Barr virus-induced G-protein coupled receptor 2 (EBI2). EBI2 is present in the B cell follicle, but not the germinal center (GC) where the majority of B cell class switching and somatic hypermutation occurs. The high expression of CXCR5 and CXCR4, in combination with low surface expression of CCR7 and sphingosine 1-phosphate 1 receptor (S1P1R) displayed by $T_{FH}$ allows them to migrate into the GC [229, 230]. Once in the GC, $T_{FH}$ provide B cell help through contact-dependent co-stimulation and secretion of high levels of IL-21 and IL-6, which induce B cell maturation, proliferation, and class switching [208, 231-233]. $T_{FH}$ cells in mice have also been found to produce intermediate amounts of IL-4 [234], and low amounts of IL-5, IFNγ, and IL-17 [235, 236]. The diverse cytokine profile of $T_{FH}$ is in part, due to the fact that differentiated $T_H1$ [237], $T_H2$ [238], $T_H17$ [148], and possibly $T_{reg}$ [239], cells have the ability to convert to $T_{FH}$. The differentiation of these cells is driven in the same manner as un-differentiated T cells, through repeat cognate interactions. These effector-$T_{FH}$ retain the expression of their lineage-associated cytokines in addition to $T_{FH}$-associated

markers and are classified by their co-localization with B cells in the GC. Less evidence exists for the differentiation of $T_{reg}$ to $T_{FH}$ $T_{FH}$-regulatory ($T_{FR}$), and it has been speculated that these cells may arise from naïve precursors, or transition from $T_{FH}$ to $T_{FR}$ [239-241]. $T_{FR}$ regulate the GC reaction and play an important role in preventing excessive humoral responses which could lead to Ab-mediated autoimmune disease. Interestingly, CXCR4-expressing cells that otherwise resemble $T_{FH}$ in surface phenotype and function, have been detected outside of B cell follicles and can induce class switching in extrafollicular B cells [242]. The exact mechanisms that drive this phenotype have yet to be elucidated.

Due to the heterogenicity of the $T_{FH}$ pool, the Ig subclasses produced generally correlate with the effector-like phenotype of the $T_{FH}$ themselves (i.e. $T_H$ 2-like $T_{FH}$) [243]. Since their identification as a distinct subset of $T_H$ cells, $T_{FH}$ cells have been found to be crucial in GC formation and effective antibody-mediated protection [244]. Dysregulation of $T_{FH}$ function is a major cause of systemic Ab-mediated autoimmunity such as SLE [245], and has been implicated in human diseases including myasthenia gravis (MG), autoimmune thyroiditis, Sjögren's syndrome (SS), rheumatoid arthritis (RA), multiple sclerosis (MS), ulcerative colitis, Crohn's disease, ankylosing spondylitis (spinal arthritis), and type 1 diabetes [245, 246].

## Cytotoxic $CD4^+$ ($CD4^+$ CTL): MHCII-Restricted Killers

$CD4^+$ CTL secrete granzyme B and perforin to kill infected cells in a similar fashion to $CD8^+$ T cells [247, 248]. IL-2 is required for their differentiation through upregulation of the transcription factor EOMES which leads to IFNγ expression and perforin [249, 250]. It also appears that there is an inverse correlation between $CD4^+$ CTL and $T_{FH}$ differentiation, as $CD4^+$ CTL express high levels of Blimp-1, which inhibits Bcl-6 expression [251]. These cells are thought to be related to $T_H1$ cells as they most often occur in viral infection models, express T-bet, and secrete IFNγ [252]. Although they have also been found to develop from $T_H0$ [253, 254], $T_H2$ [255], $T_H17$ [256], and $T_{reg}$ [257]. Class I-restricted T cell-associated molecule (CRTAM) has been identified as a likely early marker for identification of $CD4^+$ CTL [258]. The majority of $CD4^+$ CTL are found in peripheral tissues, such as in the lung, but this is likely due, at least in part, to their increased presence in viral infection models of the lung. $CD4^+$ CTL have been found to have a major role in late-onset EAE with implications for exacerbation of multiple sclerosis in humans [259]. It is also possible that these cells play a major role in the onset of colitis [257].

## Regulatory T cells ($T_{reg}$): Preventing Excessive, and Undesirable Immune Responses

Regulatory T cells ($T_{reg}$) function to suppress the activities of the other effector subsets and play a role in tolerance to non-self Ags [260, 261]. While $nT_{reg}$ exit the thymus pre-differentiated, inducible $T_{reg}$ ($iT_{reg}$) are differentiated by a combination of TGF-β and IL-10. $T_{reg}$ express the transcription factor Foxp3 [262, 263], and often display the surface marker CD25. Foxp3 acts as a transcriptional

regulator which suppresses the activation of T cells by inhibiting NFAT and NF-κB [264]. Foxp3+ $T_{reg}$ regulate peripheral tolerance through multiple inhibitory mechanisms and are a critical component in preventing the activation of cells reactive to self or allergy-associated Ags, as well as limiting infection-induced organ pathology, fetal and transplant rejection, and graft versus host disease (GvHD) [265-271]. The immunosuppressive roles by $T_{reg}$ are carried out both in a contact-dependent manner and through secretion of inhibitory cytokines and other glycoproteins. Unlike conventional $CD4^+$ effector cells, $T_{reg}$ constitutively express the inhibitory molecule CTLA-4 which binds costimulatory CD80 and CD86 on APC. CTLA-4 binds these ligands with a significantly higher affinity than CD28 and can inhibit the activation of conventional T cells via competitive ligation [272-274]. Interestingly, in addition to limiting conventional $T_H$ access to costimulatory molecules through the binding of CD80/CD86, $T_{reg}$ have been observed to physically remove CD80/CD86 from DC in a process which has been termed transendocytosis [275, 276]. While studies have proposed this acquisition resembles trogocytosis [277], transendocytosis by $T_{reg}$ differs slightly from trogocytosis [278, 279] in that acquired molecules are degraded within the $Tr_{reg}$ rather than expressed on the surface as they are after trogocytosis [275, 280-287]. In addition to the physical removal or engagement of CD80/86 from APC, ligation with CTLA-4 itself drives signaling through CD80/86 on the DC which lead to the nuclear translocation of Foxo3, which then inhibits the expression of IL-6 and TNFα [288]. It has also been observed that ligation with CTLA-4 induces production of the immunosuppressive tryptophan catabolizing

enzyme IDO by DC [289], which would further inhibit T cell activation. It has been speculated that the high expression of CD25 (IL-2Rα) by $T_{reg}$ may also act as a sink for IL-2, limiting the amount of IL-2 available to effector T cells and thus hampering their proliferation and activation

$T_{reg}$ can also directly influence the activation of conventional T cells by secreting the cytokines TGF-β, IL-10 and IL-35, as well as Granzyme-B, perforin, cAMP, Galectin, and Neutropilin. [290, 291]. In an immunosuppressive context, TGF-β inhibits T cell proliferation. While TGF-β + IL-6 can drive $T_H17$ differentiation [292]. $T_{reg}$ can limit $T_H17$ differentiation through CTLA-4:CD80/86-mediated downregulation of IL-6 by DC. IL-10 can inhibit $T_H1$ and $T_H2$ responses through activation of STAT3, while enhancing the differentiation of $T_{reg}$ in a positive-feedback loop [293]. The immunosuppresive cytokine IL-35 is produced almost exclusively by regulatory cells, and reduces the activation of naïve T cells, and inhibits proliferation in activated cells [290].

$T_{reg}$ have been observed to release cyclic-AMP (cAMP) onto effector cells through gap-junctions between $T_{reg}$ and effector T cells [294]. Granzyme B and perforin secreted by $T_{reg}$ has been shown to kill effector T cells and activated B cells *in vitro* [295-297]. $T_{reg}$ have been found to generate high levels of the β -galactoside–binding protein galectin-1 which is secreted as well as found on the surface membrane. Galectin-1 binds to surface glycoproteins on effector T cells, such as CD7, CD43, and CD45, leading to cell-cycle arrest, apoptosis, and the inhibition of inflammatory cytokines such as IL-2 and IFNγ [298-301].

Through the mechanisms mentioned above, $T_{reg}$ play a critical role in peripheral tolerance. Thus, excessive $T_{reg}$ responses may hamper an immune response leading to insufficient protection, and decrease anti-tumor immunity [266, 302, 303]. On the other hand, insufficient $T_{reg}$ presence can result in excessive immune responses associated with all $T_H$ subsets and therefore has been associated with numerous autoimmune diseases, allergy, and asthma [8, 304-307].

In addition to conventional $T_{reg}$, other regulatory T cell subsets that can conditionally express Foxp3 include Tr1 and $T_H3$ cells. Tr1 secrete IL-10 and TGFβ which inhibit proinflammatory $T_H1$ responses [308]. $T_H$ 3 cells share many similarities with conventional $T_{reg}$, and Tr1 cells, but can also secrete IL-4 [309-311]. Both Tr1 and $T_H3$ cells are important for oral tolerance, as secreted TGFβ increases IgA production by B cells plays a large role in mucosal immunity [309, 312]. Further contributing to $T_{reg}$ diversity is the ability for naive $nT_{reg}$ to differentiate into effector $T_{reg}$ ($eT_{reg}$) in the context of the current immune response [313]. $eT_{reg}$ occurs concurrently with effector T cell activation, and by receiving similar stimuli from APC, $eT_{reg}$ differentiate similarly to T effector cells. This allows the $eT_{reg}$ to display similar homing patterns to the effector T cells, enhancing their ability to regulate the activation of effector T cells in the periphery [313, 314]. The multiple pathways for $T_{reg}$ development, and high diversity of regulatory mechanisms show the high diversity of the $T_{reg}$ pool and emphasizes the broad implications in $T_{reg}$-mediated immunological tolerance and homeostasis.

## Memory CD4$^+$ T cells: Providing Long-Term Immunity

Following Ag clearance, the vast majority of activated T cells die within days while ~ 5 -10% persist as long-lived memory cells, which maintain stable populations for years. Due to the ability of multiple CD4$^+$ effector subsets to become memory cells, memory CD4$^+$ T cells are comprised of a heterogenous population [315]. Therefore, memory cells are largely defined by the ability to survive for extended periods of time in absence of Ag [316], and exhibit rapid recall responses upon re-encountering their cognate Ag [39].

The exact mechanisms which drive activated CD4$^+$ T cells to become memory remain unclear. Studies have found that T cells that receive weaker signals upon activation, along with late cognate interactions are more likely to become memory cells [317-320]. A proposed mechanism for the survival of previously activated CD4$^+$ T cells is through the permanent upregulation of the IL-7 receptor alpha chain (CD127) [321-323]. IL-7 is secreted throughout the body by multiple cell types, including epithelial cells [324], stromal cells [325], skeletal muscle cells [326], and follicular DCs [327]. CD127 ligation signals through Jak3/1 - STAT5 promotes cell survival by inducing expression of the anti-apoptotic genes Mcl-1 and Bcl-2 [328]. CD127 is highly expressed on naïve cells, but is downregulated on activated cells, facilitating their clearance during the contraction of the immune response. While the mechanisms which allow memory-fated cells to survive during the contraction phase are unknown, it has been suggested that autocrine IL-2/CD25 (IL-2Rα) signaling induced by TCR

signaling late in the immune response may be required, by driving constitutive CD127 (IL-7Rα) expression [317, 319].

A second path to becoming memory cells is through asymmetrical cell division, where one daughter cell becomes a memory-precursor or maintains a quiescent memory phenotype, and the other displays effector functionality [329-331] (Fig. 1.9). Existing memory T cells have also been observed to asymmetrically divide upon restimulation to produce effector cell progeny and a daughter cell which retains memory-like capacity [331].

**Figure 1.9**

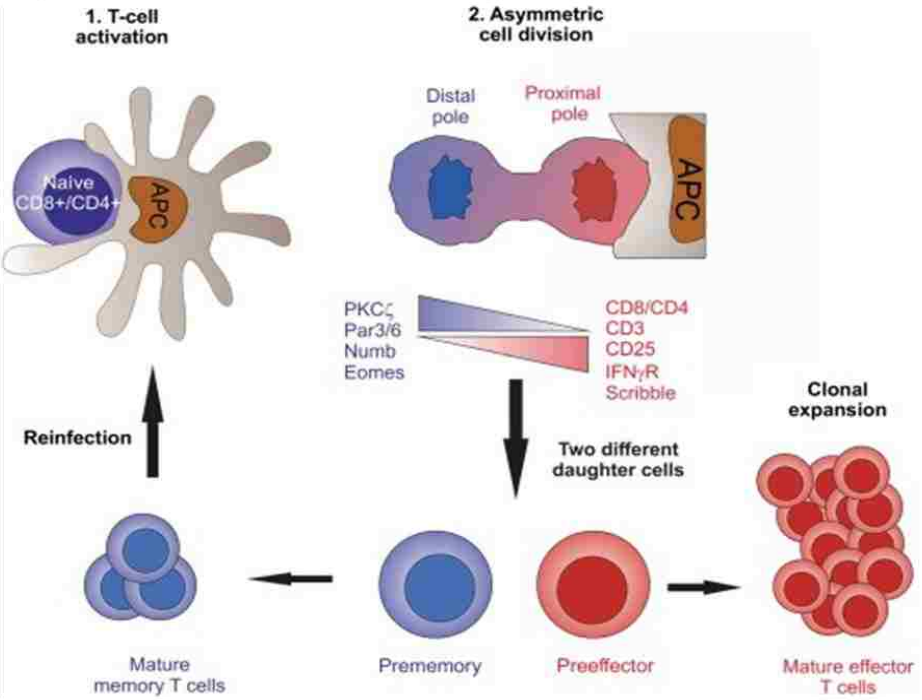



**Figure 1.9. Following activation, T cells asymmetrically divide to produce distinct progeny.** Model for asymmetric T cell division, where the daughter containing the distal pole becomes prememory and the daughter containing the proximal pole becomes a preeffector which undergoes clonal expansion. *Source: Bustos-Moran et al. 2016. International Review of Cell and Molecular Biology*

## $CD4^+$ Memory Subsets

Memory $CD4^+$ cells are categorized into various subsets based on their physiologic locations and recall responses. Variable expression of CD44 and CD62L was the basis for the initial characterization of two distinct $CD4^+$ memory populations. Cells expressing both CD62L and CD44 are classified as T central memory ($T_{CM}$), while CD44+ CD62L- are classified as T effector memory ($T_{EM}$) [332]. Additional subsets of $CD4^+$ memory cells have been identified in recent years, including Tissue-resident memory ($T_{RM}$) T cells, and stem cell-like memory T cells ($T_{SCM}$). These subsets are largely defined by their physiological location that is dictated by expression of the chemokine receptor CCR7, and CD62L (L selectin), (Fig. 1.9). The ligands for CCR7, CCL19 and CCL21, are highly expressed by stromal cells in T cell zones of the lymph node [333], mature DCs, and on the luminal side of HEVs [334, 335]. Thus, expression of CCR7 by naïve, and memory subsets allow these cells to exit the periphery and home to T cell/DC rich areas in the LNs. CD62L is a member of the selectin family of adhesion molecules. Expression of CD62L contributes to the tethering and rolling action of lymphocytes along luminal surfaces of venules through interactions with P-selectin glycoprotein ligand 1 (PSGL1) [336, 337]. Therefore, CD62L expression permits T cells to cross endothelial barriers and enter lymphoid organs from the periphery.

**Figure 1.10**

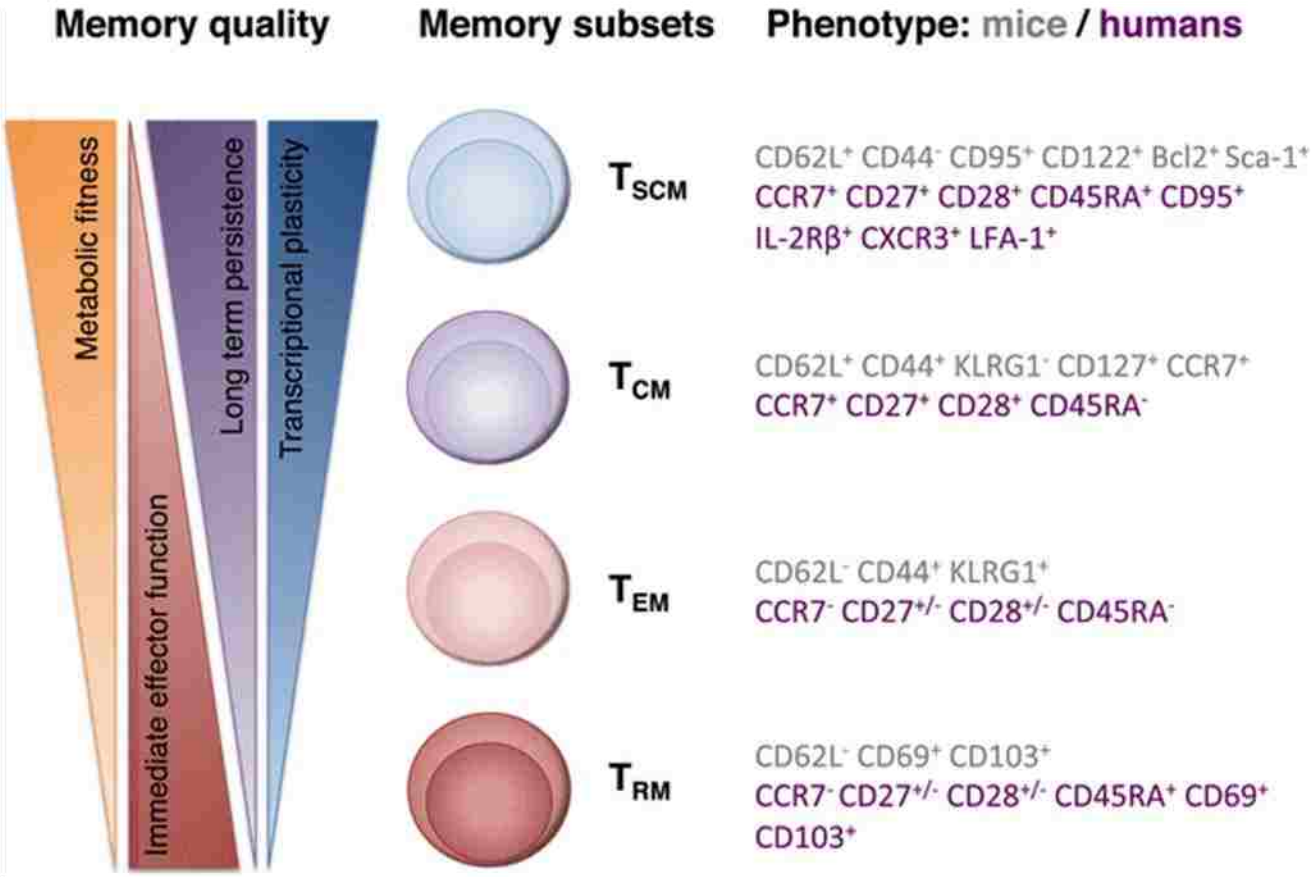



**Figure 1.10. Functions. memory quality, and defining phenotype of memory T cell subsets:** Stem Cell-like Memory ($T_{SCM}$), T effector memory ($T_{EM}$), T central memory ($T_{CM}$), Tissue resident memory ($T_{RM}$), in mice (grey) and humans (purple). Source: *Jandus et al. 2016. Methods in Molecular Biology*

Another marker commonly used to identify memory T cells is CD44. This surface protein is not expressed by naïve T cells but is upregulated following activation. Thus, expression of CD44 indicates that T cells have been previously activated. After contraction of cells during the effector phase, its presence can be used to identify Ag-experienced cells. The primary receptor for CD44 is hyaluronic acid (HA) which is a component of the extracellular matrix, and is also

expressed by some by endothelial cells [338-340]. It has been speculated that CD44 may aid in lymphocyte trafficking through the extracellular matrix, through HEV, and aid in the retention of T cells to luminal barriers. CD44 has been found to also act as costimulatory molecule [341-343].

## T central memory ($T_{CM}$)

$T_{CM}$ cells account for a large percentage of $CD4^+$ memory cells and may arise from undifferentiated precursors, or from differentiated effector cells [344, 345]. $T_{CM}$ are identified by surface expression of both $CD44^+$ and $CD62L^+$, while also commonly expressing CCR7 [346]. The expression of CD62L and CCR7 allows these cells to circulate in blood, cross through high endothelial venules (HEVs), and traffic through lymphoid organs [332]. As a result, $T_{CM}$ are the primary $CD4^+$ memory population found in lymphoid tissue and upon restimulation, rapidly produce IL-2 and proliferate to produce effector cell progeny, and can serve to stimulate Ag-presenting DCs to increase costimulatory molecule expression [332, 347].

## T effector memory ($T_{EM}$)

$T_{EM}$ cells share a similar phenotype to activated effector cells and are thought to arise primarily from effector cells which have transitioned to memory. This is supported through the observations that $T_{EM}$ show less plasticity between $T_H$ subset phenotypes compared to $T_{CM}$ [348]. As mentioned above, these cells express CD44, but not CD62L or CCR7. As a result, $T_{EM}$ continually scan

peripheral tissue and circulate in blood, and are able to rapidly migrate to sites of inflammation. Upon re-activation, $T_{EM}$ quickly exert effector functions, but do not rapidly proliferate compared to $T_{CM}$. [332, 349, 350] However, there has been some debate whether $T_{EM}$ are simply effector cells that arise from $T_{CM}$ upon reactivation and subsequent proliferation.

## Tissue resident memory ($T_{RM}$)

During infection, activated $CD4^+$ T cells migrate to peripheral tissue to induce local responses and clear pathogens. Among the memory cells that survive after Ag clearance include a subset of cells that take up residence in tissues such as the lung, small intestine, and skin [351-353]. Upon Ag-reencounter, localized $T_{RM}$ induce rapid localized inflammation to recruit other immune cells [354]. These cells are retained in peripheral tissue through constitutive expression of CD69 [355], and many maintain expression of CD103. While CD69 is commonly used as an early activation marker, one of its biological roles is to suppress the function of sphingosine 1-phosphate receptor-1 (S1P1) [356]. As high concentrations of S1P are found in blood and lymph, CD69 expression prevents the egress of CD69+ cells into these fluids [357]. Different migration patterns have been observed between $CD8^+$ and $CD4^+$ $T_{RM}$. While $CD8^+$ migrate and localize at sites of infection, $CD4^+$ cells were more mobile and continually scanned through skin [358]. One study using mice, as well as mice bearing human skin xenografts, found that human skin $T_{RM}$ were able to downregulate CD69 expression and enter circulation [353]. These cells then took up residency

in secondary skin sites where they resumed a $T_{RM}$ phenotype [353]. As such, $T_{RM}$ may not be as strictly compartmentalized as previously thought, and $T_{RM}$ that develop early may downregulate CD69 to become more evenly distributed throughout the body. Although the mechanism for $T_{RM}$ re-entry into circulation remains unknown, it is possible that this could also play a role in recruitment of $T_{RM}$ to sites of subsequent infection.

## T stem cell-like memory ($T_{SCM}$)

Human $T_{SCM}$ are identified by the surface expression pattern $CD45RO^{-}$, $CCR7^{+}$, $CD45RA^{+}$ $CD62L^{+}$, $CD27^{+}$, $CD28^{+}$ and $IL\text{-}7R\alpha^{+}$ [359]. In mice, the surface phenotype of $T_{SCM}$ largely resembles that of a naïve T cell ($CD62L^{+}$ $CD44^{-}$). Similar to $T_{CM}$, the expression of CD62L and CCR7 allows these cells to circulate in blood, cross through high endothelial venules (HEVs), and traffic through lymphoid organs. $T_{SCM}$ cells are Ag-specific, but retain $sT_{EM}$ cell-like properties in that they are able to undergo homeostatic proliferation and replenish the memory pool of Ag-specific cells of both $T_{EM}$ and $T_{CM}$ [359, 360]. Human $T_{SCM}$ have also been found to rapidly proliferate, to a greater extent than $T_{CM}$ or $T_{EM}$ [359]. $T_{SCM}$ were produced upon *Mycobacterium tuberculosis* infection [361], however the exact mechanisms that drive $T_{SCM}$ differentiation have yet to be elucidated.

In all, the rapid recall responses of the various populations of memory $CD4^{+}$ T cells provide long-lived Ag-specific immunity. This is accomplished by $T_{CM}$ in the LN rapidly proliferating to produce effector cells and stimulating the activation of other responding cells; $T_{EM}$ and $T_{RM}$ residing in surface and

peripheral organs to rapidly exert effector functions upon encountering Ag in the periphery; and $T_{SCM}$ performing homeostatic proliferation to replenish the memory pool.

# Part III
## Trogocytosis

Trogocytosis, stemming from the Greek words trogo, or “to gnaw” and cyto meaning cells, is the direct, intercellular transfer of membrane and membrane-associated molecules in a contact-dependent manner. Trogocytosis occurs rapidly and between live cells, and therefore is not a simply a result of phagocytosis of apoptotic vesicles. In addition, unlike endocytosis of CD80/CD86 by $T_{reg}$ where acquired molecules become degraded [275, 276], a defining feature of trogocytosis is that acquired molecules remain functional, and may become re-expressed on the surface of the trogocytosis-positive ($trog^{+}$) T cell. Images of a live-cell experiment showing trogocytosis by $CD4^{+}$ T cells is shown in Fig. 1.11

The first report of trogocytosis came from the research of Cone, Sprent, and colleagues in 1972, where they detected donor B cell MHCII molecules on adoptively transferred T cells in mice [362]. They concluded that the MHC molecules were integrated into the T cell surface, and similar in composition to the membrane-bound form on B cells. While the mechanism was unknown at the time, mouse T cells do not endogenously express MHCII [363], thus these molecules were acquired from the host B cells. One year later, Bona et al were the first to detect the transfer of Ag from APC to lymphocytes using B cells that

acquired LPS from macrophages in a B cell receptor-dependent manner [364]. Several years later, Hudson and Sprent became the first to report the transfer of surface molecules between APC and T cells when they found B cell-derived IgM on the surface of activated T cells that had been transferred into irradiated allogenic (MHC-mismatched) mice [365]. This discovery provided an explanation for the earlier findings by Cone and Sprent, as well as other instances where signaling molecules were detected on cells in which they are not endogenously expressed (as determined by the absence of mRNA for these molecules). Shortly after two, separate groups observed MHC transferred to T cells in donor thymocytes cells from host splenic cells [366], and in cultures of proliferating T cells [367]. In the years since, this phenomenon has been observed in $CD4^+$ [81, 285, 287, 368-372], $CD8^+$ [370, 373-376], and $\gamma\delta$ [377] T cells, B cells [378, 379], NK cells [380-382], basophils [283], macrophages [383, 384], neutrophils [385-387], dendritic cells [388, 389], and between tumor cells [390]. Despite the widespread occurrence and immunomodulatory potential of trogocytosis by immune cells, the biological impacts of trogocytosis remain largely unknown.

Figure 1.11

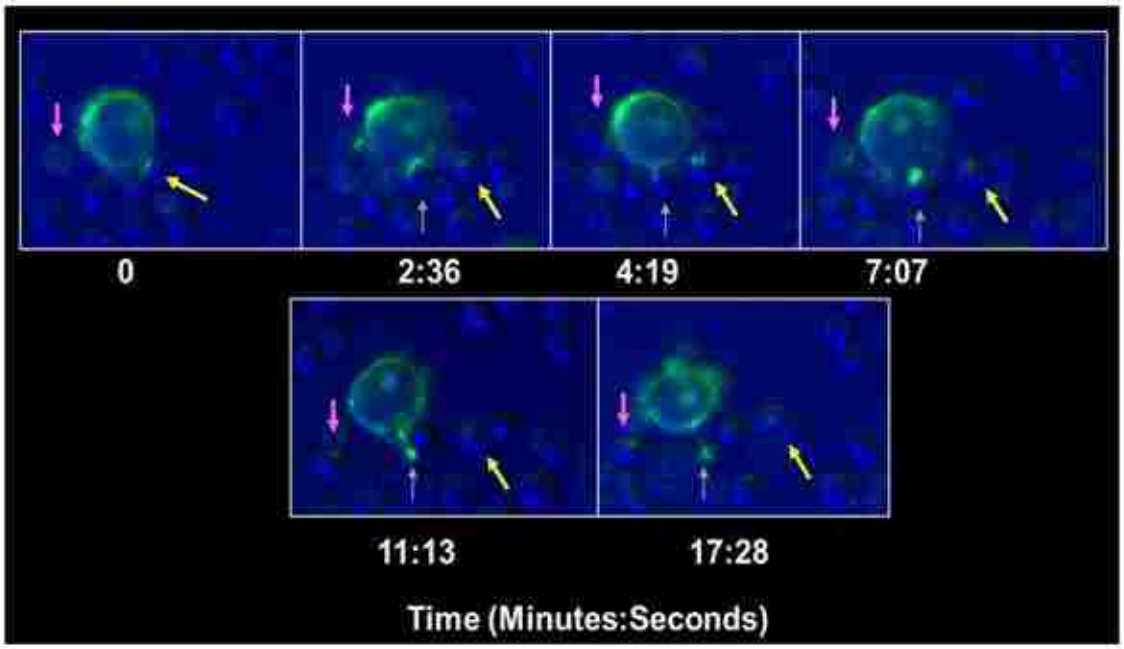



**Figure 1.11. CD4⁺ T cells acquire p:MHC complexes from APC during the immune synapse**. Live-cell imaging of MCC-specific TCR transgenic CD4⁺ T cells (small blue cells) interacting with APC expressing GFP-tagged MHCII bound with MCC peptide (large green cell). T cells rapidly recognize Ag and form synapses between time 0 and 7:07. By 11:13 T cells indicated by yellow arrows have dissociated from APC with trogocytosed p:MHCII complexes. *Source: Wetzel et al. 2001. Journal of Immunology*

# Mechanisms for Trogocytosis

## Contributions of T cell Signaling and Activation State on Trogocytosis

Although significant work over the past decade has been conducted in deciphering the mechanism of T cell trogocytosis, the exact details of this event remain unknown. This is in part, due to multiple factors which appear to influence trogocytosis such as the activation state of the T cell [391-396] and APC [383]; Ag-dose [287, 373, 397] and affinity [397, 398]; and costimulatory molecule signaling [394]. In contrast to B cells, Natural killer (NK) cells [399], and DCs that may passively perform trogocytosis [370, 400, 401], evidence suggests that active

signaling is required for T cell trogocytosis. We, and others, have previously shown that trogocytosis by $CD4^+$ T cells occurs at the immunological synapse formed between Ag-specific $CD4^+$ T cells and APC (Fig 1.11) [287, 392, 394, 397, 402]. This is consistent with the vast majority of reports which have found Ag-specificity to be a requirement for T cell trogocytosis [285, 287, 375, 399, 403]. In fact, the presence of trogocytosed molecules has been used as an identifying marker for tumor- [399, 403], and viral-, reactive cells [404]. In addition, this method has recently been described as a method to identify antigen-epitopes for specific T cells in vivo [399].

The strength of TCR signaling by Ag-dose positively correlates with the trogocytosis [287, 373, 397]. The activation state of T cells impacts the efficiency in which cells perform trogocytosis, as activated T cell blasts perform trogocytosis to a greater extent than naïve T cells [391-396]. This could be due, in part, to the larger size of activated T cells compared to naïve cells, which have increased surface area contact with APC. It has also been hypothesized that increased avidity of the activated T cells due to their increased expression of adhesion and costimulatory molecules, such as CD28 and LFA-1, and CD44, stabilizes the immune synapse to facilitate trogocytosis [394, 405]. Costimulatory molecule ligation such as CD28:CD80/CD86 can also lead to acquisition of CD80 and CD86 [394], thus increasing the amount of total material trogocytosed by the T cell.

Similar to the effect of adhesion molecules in stabilizing the immune synapse and promoting trogocytosis, T cell microvilli (as mentioned under

kinapses and microclusters) are also central factors in trogocytosis. These filamentous like protrusions concentrated with TCR form multiple contact points with APC and enhance signaling in both the T cell and APC [68]. In a recent study, Akkaya *et al.*, found that microvilli stabilize T:APC interactions and correlate with the acquisition of MHCII by the T cell [66]. $iT_{reg}$ had the highest level of microvilli, formed the most stable contacts, and showed the highest acquisition of MHCII, compared to activated effector T cells which had higher levels of microvilli compared to naïve t cells (Fig. 1.12). They also found that microvilli acquired MHCII with cognate, but not irrelevant peptide, suggesting that the acquisition of MHCII occurred in an Ag-specific manner.

**Figure 1.12**

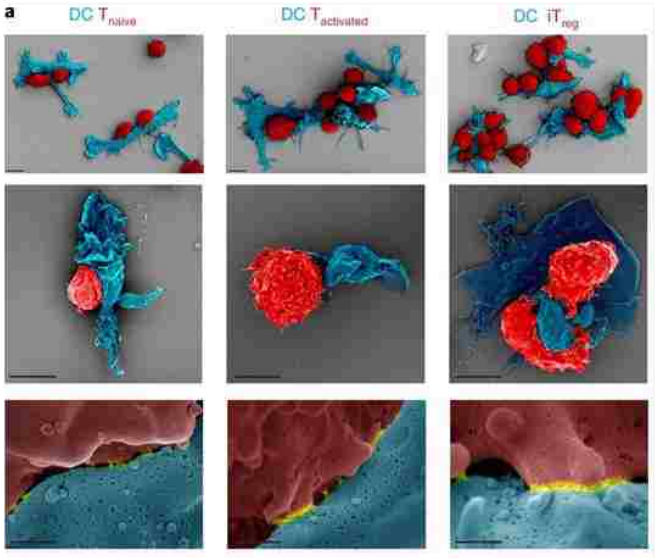



**Figure 1.12. Activated T cells and iTreg form large areas of contact with APC *via* microvilli, while naïve T cells do not**. SEM images of naïve (left) activated (middle) and iTreg (right) contacts with DC in clusters (top) and individually (middle). Scale bar, 5 µm. The bottom row shows membrane fusion domains (yellow) at the T cell:APC binding site, scale bar, 300nm. *Source: Akkaya et al., 2019. Nature Immunology*

Further insight into the mechanism of trogocytosis comes from studies showing that treatment of cells with latrunculin or cytochalasin D to inhibit actin polymerization severely limited trogocytosis [370, 405]. Blocking proximal TCR signaling through ZAP-70 has also been found to significantly reduce the rate of trogocytosis [373]. Intriguingly, trogocytosis of CD80 and CD86 may occur

independently of CTLA-4, or CD28-signaling in $T_{reg}$ [277]. Studies with conventional effector cells found that antibody blockade of CD80 had minimal on trogocytosis of GFP-tagged MHCII, while blocking MHCII or CD3 significantly reduced the amount of trogocytosed MHCII [287].

Collectively, the above data suggests that trogocytosis is an active process in T cells that occurs predominantly in an Ag-specific manner and requires stable-T cell:APC interactions.

## Proposed Physical Mechanisms of Trogocytosis

The possibility that more than one pathway exists for the acquisition of APC molecules by T cells has contributed to the elusiveness in defining the underlying mechanisms. While it is understood that trogocytosis requires stable, cognate interactions and trogocytosed molecules may be re-expressed on the T cell surface, the pathways leading to this end-result may differ. For example, $CD8^+$ CTL have been observed to acquire membrane and associated proteins from target cells through small regions of fused membrane termed membrane bridges (Fig. 1.13A) [406]. These membrane bridges are distinct from the significantly longer nanotubules formed between T cell and APC (Fig. 1.13B) that are thought to extend cell communication [407].

Figure 1.13

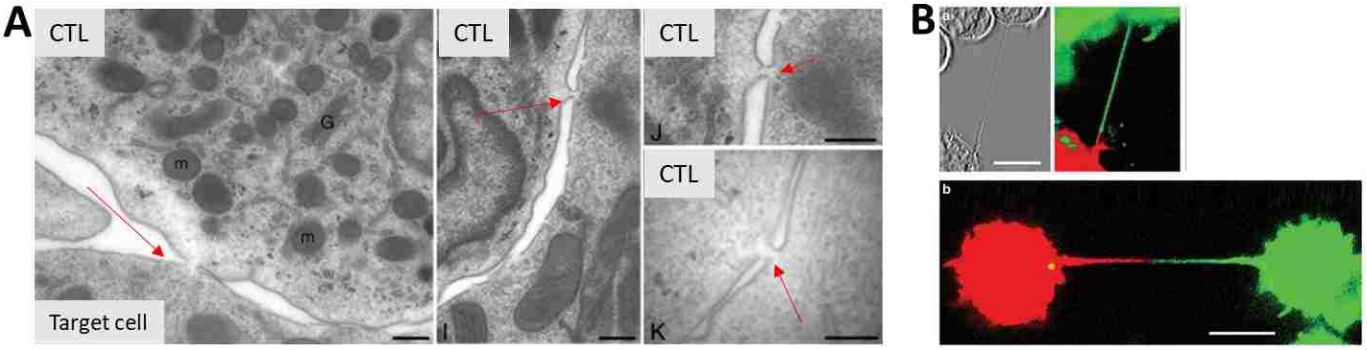



**Figure 1.13. Cytotoxic T Lymphocytes (CTL) form membrane bridges that facilitate transfer of APC-derived molecules, and are distinct from nanotubules**. **(A)** Membrane bridges between CTL and APC conjugates as indicated by red arrows. Scale bar, 250 nm. Source: Stinchcombe et al., 2001. Immunity **(B)** Nanotubules formed between Jurkat T cells (top) and human primary T cells (bottom) with DiD labeled membrane (red) and APC with GPI-anchored GFP (Green), scale bar, 10 µm. *Source: Sowinski et al., 2007. nature cell biology*

However, the presence of T cell:APC membrane bridges in $CD4^+$ T cells has not been reported. Live-cell imaging data from Wetzel *et al.*, showed that GFP-tagged MHCII was removed from APC during spontaneous dissociation from the immune synapse [287] (Fig. 1.11). As the immune synapse is comprised of a multitude of ligated molecules, the strength of these interactions is greater than the integrity of the APC membrane. Spontaneous dissociation during the immunological synapse results in the stripping of APC molecules by the migrating T cell.

Multiple proposed models for trogocytosis suggest that APC-derived molecules become internalized by the T cell through endocytosis. These models diverge from one another in how the APC molecules arrive on the surface of T cell. In one theory, APC-derived exosomes containing various molecules are taken up by the T cells and then re-expressed on the surface [408]. It has been observed that strong MHCII:TCR interactions leads to the migration of late

endosomal compartments containing MHCII towards the immunological synapse, thus TCR signaling may increase the release of APC exosomes directed towards the T cell [409]. In a separate study, electron microscopy revealed that MHCII was transferred to T cells in T cell:DC conjugates via exosomes which were concentrated at the immunological synapse [408]. Here, the authors proposed that the exosomes were endocytosed by the T cell during TCR recycling, then were re-expressed on the T cell. Similar results were observed by Huang *et al.,* in a model using *Drosophila* APC transfected with mouse MHC and costimulatory molecules, where they observed small packets containing MHCII localized between T:DC synapses [397]. The authors in this study observed that acquired MHCII were co-internalized with recycling TCR and were found to be present in both endosomes and lysosomes. Thus, they concluded that TCR-endocytosis was driving the internalization and subsequent acquisition of APC-derived signaling molecules [397]. However, results from numerous studies suggest that the acquisition of APC-derived molecules via exosomes is distinct from trogocytosis. For example, the transfer of PD-L1 and MHC is blocked when co-cultured T cells and APC are separated by a trans-well membrane [375, 410]. In addition, blocking exosome formation by DC with the ATPase inhibitor CMA was found to only slightly decrease the amount of trogocytosed material compared to untreated controls [375].

Figure 1.14

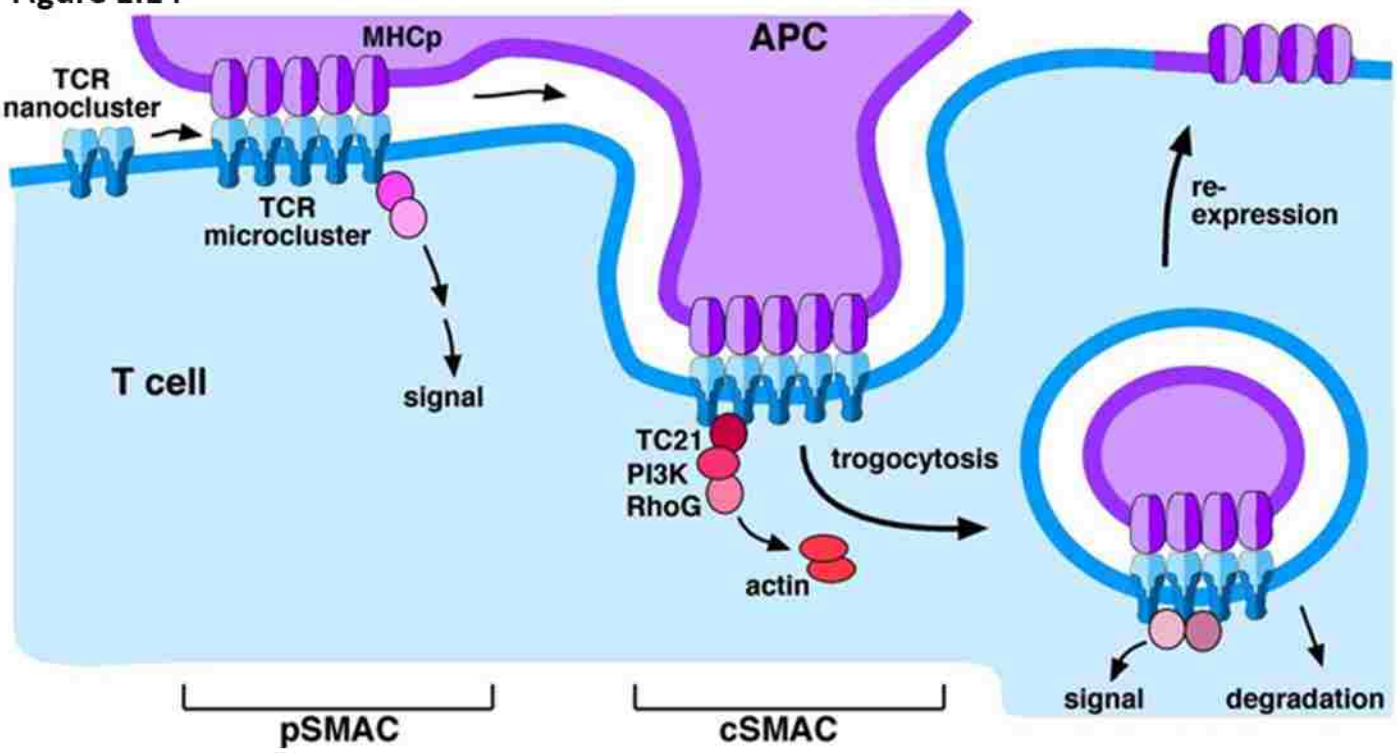



**Figure 1.14. Proposed TC21/RhoG-dependent model for trogocytosis**. APC peptide:MHC bound to TCR become endocytosed in a TC21/RhoG-dependent manner. Trogocytosed molecules become re-expressed on the surface of the T cell in the correct topological orientation. *Source: Dopfer et al. 2011. Immunity, based off of research by Martinez-martin et al. 2011. Immunity*

The most recent model for trogocytosis comes from studies by Martinez-Martin *et al.,* proposing that APC-derived membrane and membrane proteins are internalized in tandem during TCR downmodulation. Consistent with much of the published research, this model proposes that T cell trogocytosis is an active event occurring in a TC21/RhoG dependent manner. Once internalized by the T cell, recycling endosomes containing acquired APC fragments fuse with the T cell plasma membrane resulting in APC-derived molecules being displayed on the T cell surface in their native topological orientation (Fig. 1.14, *above*) [278, 411] This model is consistent with the majority of published data

including results of Sprent and colleagues that found trogocytosed GFP-tagged MHCI is internalized by $CD8^+$ T cells before surface re-expression [397]. Finally, pre-treatment of T cells with the vacuolar ATPase CMA to inhibit endosomal formation and trafficking led to a significant decrease in trogocytosis [375], strengthening a model for endocytosis-dependent trogocytosis.

Our live-cell imaging experiments also show that GFP-labeled p:MHC found on the surface of the T cell moves from the immunological synapse and localizes at the distal pole region (Fig. 1.15). We also observed that in fixed T:APC conjugates, >82% of T cells with detectable trogocytosed molecules had p:MHCII localized to the distal pole [81]. Similar distal pole localization of trogocytosed MHCI was reported by Huang *et al.,* using $CD8^+$ T cells and *Drosophila* APC [397]. Regardless of the mechanisms in which the molecules are acquired from APC, true trogocytosis results in the expression, or re-expression, of the acquired molecules on the T cell surface.

**Figure 1.15**

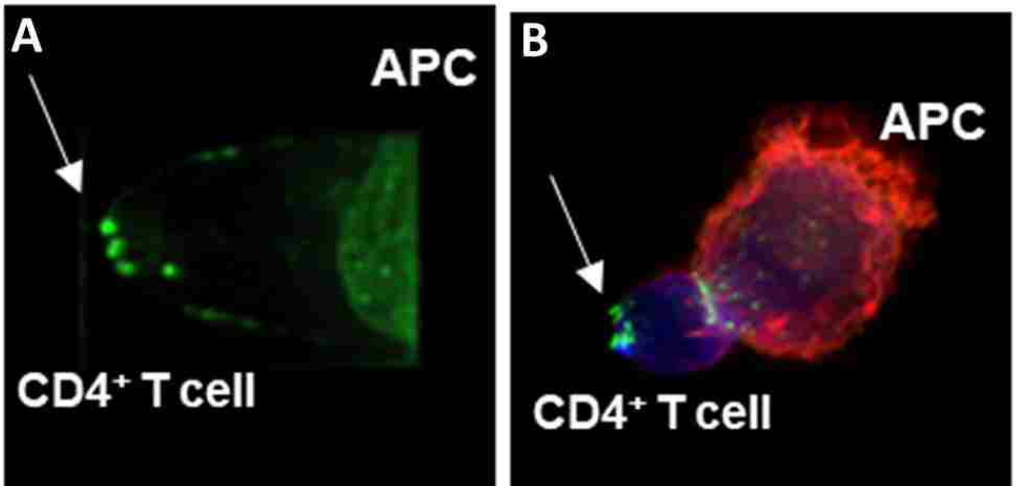



**Figure 1.15. Trogocytosed molecules localize to the distal pole during the immuneological synapse. (A)** GFP-tagged pMHCII transferred to the distal pole of T cells (clear) during the immune synapse. **(B)** CD4+ T cell (blue) forming an immune synapse with APC expressing with ICAM-1 (red) and GFP-tagged pMHCII (green). *Source: Scott Wetzel*

## Biological Consequences of T cell Trogocytosis

While the intracellular exchange of proteins is common among various cell types, the outcome of trogocytosis has distinct biological implications in that; 1. the molecules acquired may be involved in active signaling; and 2. these signaling molecules become re-expressed in the native topological orientation on the surface of recipient cell. As mentioned above, molecules trogocytosed from APC by T cells include molecules that can trigger active signaling within the T cells including pMHCI/MHCII complexes [66, 81, 282, 285, 287, 376, 399, 412], CD80/CD86 [277, 281, 282, 284], OX40-L [368], and PDL-1 [375], as well as adhesion molecules such as ICAM-1 [400]. Due to the nature, and accessibility of these trogocytosed molecules, this event has numerous biological implications. Elevated levels of trogocytosis have been documented in sites of autoimmune inflammation [391], viral and parasitic infections [282, 413, 414], rheumatoid arthritis [11], and in tumor environments [284, 403]. While the function of trogocytosis in these cases is not yet established, this widely-observed event has been proposed to play a role in the modulation of immune responses [277, 388, 415-419].

## Antigen Presentation by Trog$^+$ T cells

The potential for trog$^+$ cells to act as APC themselves has inspired significant research focused on the ability of the trogocytosis-positive (trog$^+$) T cells to present antigen, in the context of other acquired molecules, to responding T cells [369, 371, 372, 415, 420-426]. The outcome of such presentation appears to correlate with the nature of the acquired molecules and phenotype of the trog$^+$

cell. For example, trog$^+$ CD4$^+$ cells displaying acquired costimulatory molecules such as CD80, along with MHC:peptide can activate responding naïve T cells both *in vitro*, and in vivo [371, 410, 420, 424]. One such example comes from the work of Zhou *et al.*, who found that FACS-sorted hemagglutinin (HA)-specific trog$^+$ T cells induced proliferation of naïve HA-specific T cells *in vitro* [406]. Through bystander acquisition of MHCI from DC, OVA-specific trog$^+$ CD4$^+$ T cells have been found to activate naïve CD8$^+$ T cells both *in vitro*, and in vivo after adoptive transfer of trog$^+$ cells [421]. In a study using human T cells, Game *et al.* showed that in an allogenic response, trog$^+$ cells can stimulate both naïve and allogenic and autologous T cells to proliferate [410]. The antibody blockade of CD80/86 limited these responses, suggesting that, similar to Ag-specific responses, the presence of allogenic MHC alone on T cells is insufficient to induce a response in other allogenic T cells.

A separate study compared the effects of trogocytosed CD80 in both an allogenic and Ag-specific response. Similar to the results above, they found that the trog$^+$ cells could induce activation of other cells in both types of responses. However, the trog$^+$ cells only were shown to have significant effects on stimulating naive T cells in the first 48 hours of their activation [427]. In the case of allogenic responses they proposed that T cells expressing CD80 could act to as a mechanism to boost the costimulatory presence for responding naïve T cells. Interestingly they also noticed a difference in the chemokine receptor pattern between allogenic and Ag-specific responses. T cells that performed antigen-independent acquisition of CD80 retained expression of CCR7 and low

levels of CCR5, thus were more likely to remain in the T cell zones of the lymph node after activation. On the other hand, $CD80^+$ cells which were stimulated with cognate Ag-presenting DCs downregulated CCR7 and increased expression of CCR5, thus, they would be more likely to exit the lymph node and act on peripheral sites to modulate the response of peripheral T cells. Finally, it was observed in samples from human donors with either malignant ascites or autoimmune thyroiditis, circulating T cells had increased levels of CD86 compared to healthy controls, suggesting that these cells may play a role in augmenting these autoimmune disorders [428].

While T:T Ag-presentation has been shown to augment an immune response, numerous studies have also found it may play a role in suppressing the immune function of other cells. In an in vivo study, Helft *et al.*, found that Ag presentation by $trog^+$ cells to activated T cells induced responder cell death, but it stimulated naïve T cells to proliferate. The authors proposed that this was likely due to insufficient levels of costimulatory molecules upon p:MHC presentation by $trog^+$ cells [429]. The authors speculated that T cell Ag-presentation may play a role in preventing peripheral memory, but not naïve T cells, from entering activated lymph nodes by interacting with T cells in the periphery.

Attenuation of the immune response via presentation of trogocytosed molecules to T cells has also been observed in T cells from donors with multiple myeloma. In vitro incubation of patient cells with myeloma cell lines led to trogocytosis of CD86 and the immunosuppressive molecule HLA-G by T cells [284]. These $HLA\text{-}G^+$ $trog^+$ cells were functionally comparable to $nT_{reg}$ in

proliferation of trog- cells. In samples directly from patients, a significantly higher frequency of circulating T cells had detectable HLA-G and CD86 compared to healthy controls, and the increase in the frequency of these trog$^{+}$ cells was associated with poor prognosis [284].

In addition to conferring regulatory functions to non-$T_{reg}$ cells [277, 375, 415, 417, 430-432], $T_{reg}$ themselves display high-rates of Ag-specific trogocytosis [66]. CD80 and CD86 have been detected on the surface of both $nT_{reg}$ and $iT_{reg}$ [277], and has been proposed that these trog$^{+}$ $T_{reg}$ use trogocytosed molecules to form connections with activated T cells, thus enhancing their suppressive [431,432].

Trogocytosis itself also has been found to suppress the immune response in a manner similar to $T_{reg}$, where pMHC complexes and costimulatory molecules are removed via trogocytosis rendering APC inefficient stimulators of other responding T cells, or inhibiting the detection and killing of tumor cells [284, 433-436]

Another means of immune suppression is the killing of Ag-presenting T cells by CTL through fratricide. This phenomenon is most widely-associated with viral infection, but CD8$^{+}$ T cells with trogocytosed PD-L1 can induce fratricide of neighboring T cells expressing PD-1. In addition, trog$^{+}$ themselves may be targeted by cytotoxic cells through displaying trogocytosed pMHC [434].

Collectively, the studies described underscore the biological significance of trogocytosis and subsequent Ag presentation by trog$^{+}$ T cells

## Trogocytosis-Mediated Signaling

While the ability of trog$^+$ T cells to present trogocytosed molecules to other T cells has been fairly well-documented, much less is known about the biological consequences of trogocytosis on an individual trog$^+$ cell. Trogocytosed molecules are retained in punctate areas on the CD4$^+$ T cell surface and co-localize with their receptors and signaling molecules [81, 287]. This raises the possibility that trogocytosed molecules may drive intracellular signaling within the T cell. It has been observed that trog$^+$ cells are more activated compared to their trog- counterparts, as indicated by expression of CD44, CD69, and/or CD25 [277, 417, 420, 422, 432, 437, 438], and, in some cases, express higher levels of effector cytokines compared to similarly-stimulated trog- cells [81, 282, 412, 439]. One possible explanation for these observations is that highly activated cells are more efficient at performing trogocytosis. Another possibility is that the trogocytosed molecules induce intracellular signaling and are playing a role in augmenting the activation state and effector functions of the trog$^+$ cells.

In our previous studies, TCR signaling was detected in trog$^+$, but not trog$^-$, CD4$^+$ T cells up to 72 hours after separation from APC. This sustained signaling was mediated by trogocytosed molecules engaging their cognate receptors on the trog$^+$ T cell, resulting in autopresentation, referred to here as trogocytosis-mediated signaling. This signaling led to the enhanced survival of trog$^+$ cells compared to trog$^-$ cells, up to five days after APC removal [81]. This was not due to residual signaling from the T-APC interaction, as treatment with

the Src-family kinase inhibitor PP2 was used to halt signaling after APC removal. Signaling, as measured by ZAP-70 and ERK 1/2 phosphorylation resumed only in trog$^{+}$ cells. Consistent with these findings, Zhou *et al.* found that trog$^{+}$ CD4$^{+}$ T cells displayed sustained activation of NFκB and AP1, 24 hours after removal from APC. Interestingly, the trog$^{+}$ cells also developed a unique cytokine profile [439]. This suggests that a qualitative difference may exist between trogocytosis-mediated signaling and signals received from APC, raising the possibility that this non-canonical signaling has the potential to modulate the immune response.

Trogocytosis-mediated signaling, by altering the activation state, survival, and effector cytokine production has the potential to significantly impact the physiology and subset differentiation of the trog$^{+}$ cell, and by extension, an immune response. This paradigm-shifting event may play a significant role in the generation and control of protective immune responses, cancer immunotherapy, and vaccine efficacy. However, we have a very limited knowledge of trogocytosis-mediated signaling, and more work is necessary to improve our understanding of this underappreciated phenomenon and the role it plays in modulating the immune response.

## Rationale for the Current Study

While the ability of $CD4^+$ T cells to differentiate into specific subsets with specialized roles is a defining feature of the adaptive immune response, the mechanisms behind their differentiation are not fully understood. While canonical T cell activation is dependent on APC, $trog^+$ $CD4^+$ cells can sustain active TCR signaling through engaging trogocytosed molecules with their surface receptors (trogocytosis-meidated signaling) [81]. This signaling was observed up to 72 hrs after APC removal, and despite being similarly activated by APC, the $trog^+$ cells displayed enhanced survival compared to $trog^-$ cells in the absence of exogenous stimulation [81]. These findings strongly suggest that trogocytosis-mediated signaling in the $trog^+$ T cells can modulate their survival.

The overall objective of this study was to determine the biological effects of trogocytosis-mediated signaling on $CD4^+$ T cell activation, effector cytokine production, and differentiation. Due to the demonstrated ability for $trog^+$ cells to retain active signaling and maintain a heightened state of activation, ***I hypothesized that trogocytosis-mediated signaling would drive sustained effector cytokine production and influence the differentiation of the recipient $trog^+$ cell*.**

This was examined through the following three aims:

**Aim 1. Determine the activation state, effector cytokine production, and $T_H$ -subset differentiation of $trog^+$ and $trog^-$ cells after APC removal, and the contribution of trogocytosis-mediated signaling to the observed phenotype**

*Rationale and supporting data*: Sustained T cell signaling is not only required for

effector T cell and survival, but also mediates T cell activation state and effector cytokine production. In addition, trog$^+$ cells display higher levels of activation and increased effector functionality *in vitro* and *in vivo*. Whether trogocytosis is a result, plays a causative role in these phenotypes has yet to be determined.

Because variations in signaling events, including the frequency, and duration, of TCR signaling, can significantly impact the differentiation of CD4$^+$ cells [82, 139-143], it is plausible that sustained trogocytosis-mediated signaling may also impact these aspects in the trog$^+$ cell.

**Aim 2. Ascertain the impacts of trogocytosis-mediated signaling in the differentiation of trog$^+$ CD4$^+$ T cells towards $T_{FH}$**

*Rationale and supporting data.*

While events during initial T cell activation are sufficient to drive CD4$^+$ cells to differentiate into common effector subsets including $T_H1$, $T_H2$, and $T_H17$, the differentiation of T follicular helper ($T_{FH}$) requires prolonged, and/or repeated TCR and costimulatory signaling [142, 143]. Multiple studies have suggested that following T cell activation, subsequent interactions with B cells presenting cognate Ag are required for full $T_{FH}$-differentiation, [209, 221-224]. However, signaling from DC in absence of B cells is capable of driving $T_{FH}$ differentiation [213]. Thus, the key factor in $T_{FH}$ differentiation is likely sustained T cell signaling, and not a signal uniquely attained through B cell interactions. It is possible that sustained trogocytosis-mediated signaling may promote $T_{FH}$ differentiation after separation from APC.

**Aim 3. Determine the impact of trogocytosis-mediated signaling in the generation of memory-precursor CD4$^+$ T cells.**

*Rationale and supporting data:* Though the exact requirements for driving CD4$^+$ T cells to become long-lived memory cells are unknown, sustained and/or repeated TCR and costimulatory signaling from APC promote CD4$^+$ transition to memory. It is possible that by emulating T:APC interactions, trogocytosis-mediated signaling may satisfy signaling requirements to transition into memory cells. One of the defining criteria for memory cells is the ability to survive in the absence of Ag. We previously found that sustained trogocytosis-mediated signaling leads to enhanced survival of trog$^+$ cells compared to trog- cells after APC removal, suggesting that such signaling could play a role in the survival of memory/memory-precursor cells.

Results from aim 1 showed that trog$^+$, but not trog$^-$ cells produced IL-4 and IL-21, both of which have been shown to aid in CD4$^+$ survival. In addition, IL-21 has also been found to be critical for CD4$^+$ and CD8$^+$ memory cell generation [440]. Therefore, it is possible that autocrine signaling through IL-4 and IL-21 produced by the trog$^+$ cells would enhance their survival, as well as promote transition to memory.

In addition to effector-to-memory transition, T cells may be fated to become memory cells during initial T cell activation and subsequent asymmetric division. In our previous study, during T:APC synapses, it was found that over 83% of these cells had trogocytosed molecules at the distal pole, and these

molecules were driving TCR signaling. Thus, it is possible that the commonly observed TCR signaling at the distal pole is due to TCR engagement with trogocytosed pMHC complexes. If trog$^+$ cells undergo asymmetric division and trogocytosed molecules are retained on the distal daughter cell, this would implicate that trogocytosis-mediated signaling may play a role in the survival of memory cells that are generated through asymmetric division.

These aims were addressed primarily using our well-established standard *in-vitro* trogocytosis-assay using fibroblast cell line and peptide-loaded mouse BMDC cells as APC. Briefly, naïve or non-polarized resting CD4$^+$ T Cell blasts were incubated with fibroblasts which constitutively express covalently-attached MCC to the I-E$^k$ $\beta$-chain, or MCC-loaded BMDC, for 90' at 37 $^o$C. T cells are removed from APC from T cell cultures and are analyzed immediately, or cultured at low density to limit potential T:T interactions. In addition, *in vivo* experiments were conducted using MCC-specific TCR-transgenic mice, and wild-type (WT) mouse models. These experiments involved immunization of whole protein to examine the phenotype of cells which have performed *in vivo* trogocytosis. Studies on memory T cell survival included the adoptive transfer of CD4$^+$ T cells recovered from an *in vitro* trogocytosis assay into naïve animals. Full details of these experiments are described in the materials and methods section.

Results from this study provide insight into both, the biological consequences of trogocytosis, as well as the mechanisms behind CD4$^+$ activation and differentiation. Due to the common occurrence of trogocytosis by

$CD4^+$ T cells upon activation, and the central role of $CD4^+$ T cells in immune responses, these findings are of significance in advancing our current knowledge of basic cell biology while also providing insight into better understanding the pathology of infectious and autoimmune diseases, allergies, along with future drug or vaccine design.

# Chapter 2

# Materials and Methods

## Animals

B10.A and C57BL/6 CD45.1 mice were purchased from The Jackson Laboratory (Sacramento, CA). 5C.C7 TCR ($V\beta3^+$) transgenic Rag-$1^{-/-}$ mice specific for pigeon cytochrome c fragment 88–104 and reactive against moth cytochrome c (MCC) fragment 88–103 [441] were purchased from Taconic (Rensselaer, NY). For some experiments, 5C.C7 mice were crossed with B10.A mice and the splenocytes from F1 generation animals were used. Both male and female mice were used in this study, and no differences were observed between male and female T cell phenotypes. Mice were housed in the University of Montana Laboratory Animal Resources facility and were allowed food and water *ad libitum*. All procedures were supervised and in accordance with the University of Montana Institutional Animal Care and Use Committee.

## Antibodies and Reagents

The following conjugated or unconjugated antibodies were purchased from: Biolegend (San Diego, CA): anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD69 (H1.2F3), anti-CD80 (16-10A1), anti-Foxp3 (150D), anti-I-$E^k$ (17-3-3), anti-IA/IE (M5/114.15.2), anti-IFN$\gamma$ (XMG1.2), anti-IL-2 (JES6-5H4), anti-IL-4 (11B11), anti-IL-6 (MP5-20F3), anti-IL-9 (RM9A4), anti-IL-17A (TC11-18H10.1), anti-IL-21

(FFA21), anti-Bcl-6 (IG19E/A8), anti-T-bet (4B10), anti-PD-1 (29F.A12), anti-CCR7 (4B12), anti-Ki-67 (16A8), anti-CD25 (3C7) anti-CD62L (MEL-14), anti-CD127 (A7R34), anti-ICOS-L (HK.53), anti-CD44 (IM7), anti-GATA-3 (16E10A23), Streptavidin (PE-Dazzle594). Conjugated or unconjugated antibodies were also purchased from BD Biosciences (Franklin Lakes, NJ): anti-pZAP-70 (17A/P-ZAP-70), anti-Vβ3 (KJ25), anti-IL-12 (C15.6), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-CD90.2 (30-H12)], and eBiosciences (San Diego, CA): anti-IL-5 (TRFK5), anti-IL-10 (JES5-16E3), anti-RORγt (AFKJS-9), anti-Tcf1/Tcf7 (S33-966). AlexaFluor 488- and AlexaFluor 647-conjugated Streptavidin were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Whole pigeon cytochrome c (PCC) and Ovalbumin (Ova) proteins were obtained from Sigma-Aldrich (St. Louis, MO) and moth cytochrome C fragment 88-103 ($MCC_{88-103}$) peptide was obtained from Genscript (Piscataway, NJ). Peptides were dissolved in sterile nano-pure water at 500 µM and aliquots were stored at -80 °C until used**.**

**Culture Media**

Fibroblast APC were cultured in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA), 1 mM L-glutamine, 100 mg/ml sodium pyruvate, 50 mM 2-ME, essential and nonessential

amino acids (Life Technologies), 100 U/ml penicillin G, 100 U/ml streptomycin, and 50 mg/ml gentamicin (Sigma-Aldrich).

Primary splenocytes, T lymphocytes, and bone marrow-derived dendritic cells (BMDC) were maintained in complete RPMI which consisted of RPMI 1640 medium (Life Technologies) supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA), 1 mM L-glutamine, 100 mg/ml sodium pyruvate, 50 mM 2-ME, essential and nonessential amino acids (Life Technologies), 100 U/ml penicillin G, 100 U/ml streptomycin, and 50 mg/ml gentamicin (Sigma-Aldrich).

**Antigen Presenting Cells**

In *in vitro* trogocytosis experiments, peptide-pulsed B10.A BMDC or the previously described MCC:FKBP cell line [287] were used as APC. The MCC:FKBP is a $CD80^{high}$ $Ltk^{-}$ fibroblast cell line that has been transfected with ICAM-1, I-$E^{k}$ α-chain, and an I-$E^{k}$ β-chain with covalently attached antigenic $MCC_{88–103}$ peptide via a flexible linker, and a cytoplasmic tail containing 3 copies of FK506-binding protein (Ariad Pharmaceuticals, Cambridge, MA). This cell line expresses levels of CD80 and ICAM-1 comparable to B10.BR splenocyte APC [287].

**Surface-Labeling of APC**

MCC:FKBP APC or BMDC APC cells were surface biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Scientific, Waltham, MA), or in some cases surface

labeled with BODIPY™ FL NHS Ester (Succinimidyl Ester) conjugated to AlexaFluor 647 (ThermoFisher), in PBS pH 8.0 for 20 min at RT. Following surface labeling, excess biotin or BODIPY dye was quenched with 15x volume of PBS pH 7.4 containing 100mM glycine, followed by an additional 3 washes with PBS + 100 mM glycine. Cells were then resuspended in cRPMI prior to counting and plating for use in the trogocytosis assay.

**Generation of BMDCs**

Bone marrow cells were isolated from femurs and tibiae of B10.A mice and cultured for 6 days in sterile non-tissue culture grade petri dishes at $10^5$ cells/ml in complete RPMI medium containing 30 ng/ml recombinant murine granulocyte macrophage-colony stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ) at 37 °C and 5% $CO_2$. Fresh culture media and GM-CSF were added on day 3, and non-adherent cells were harvested on day 6. Cells were activated by plating on tissue culture-coated 6-well plates with addition of Sigma adjuvant sys$T_{EM}$ at 125 ng/ml 18 hours prior to use. Adherent cells were surface biotinylated and exogenously loaded with $MCC_{88–103}$ peptide at a final concentration of 2.5 µM for 2 hours prior to use. Purity was verified via flow cytometry to be >90% $CD11c^+$.

***In vitro* T cell priming**

Non-polarized TCR-transgenic $CD4^+$ T cell blasts were generated *in vitro* to increase the potential for trogocytosis relative to naïve $CD4^+$ T cells [392]. Single-cell suspensions of splenocytes from 6- to 12-week-old 5C.C7 or 5C.C7 x B10.A

F1 transgenic mice were depleted of erythrocytes by hypotonic lysis using Gey's balanced salt solution (155 mM $NH_4Cl$, 10mM $KHCO_3$) and resuspended in complete RPMI 1640. Splenocytes from 5C.C7 x B10.A F1 mice were activated *in vitro* for 6 days by addition of 2.5 µM $MCC_{88–103}$ peptide to splenic cell suspensions. 5C.C7 splenocytes were stimulated for 48 hours on pre-coated anti-CD3 (1 µg/ml) and anti-CD28 (1 µg/ml) plates followed by a 2-day incubation after removal from antibody-coated plates prior to use. Viable lymphocytes were isolated from priming cultures by density centrifugation using Lympholyte M (Cedarlane Labs, Burlington, NC). When culturing B10.A x 5C.C7 F1 or C57BL/6 cells, B cells were depleted from cultures by incubating cells for 30 min with biotin-labeled anti-B220 (BioLegend), followed by 3 washes in PBS. Cells were then incubated for 20 min with 3.75 µm streptavidin-coated magnetic particles (Spherotech) followed by removal of $B220^+$ cells by magnetic separation. Cultures of cells from peptide T cell blast cultures were 90-95% $CD4^+$ immediately prior to use in the trogocytosis-assay.

### *In vitro* $T_H1$ and $T_H2$ polarization to induce $T_H1$ or $T_H2$ effector subset differentiation

Primary T cells were stimulated directly *ex vivo* on anti-CD3 (1 µg/ml) and anti-CD28 (1 µg/ml) coated plates, as described above, with either $T_H1$ (5 ng/ml IL-12 and 20 µg/ml anti-IL-4 (11B11), or $T_H2$ (10 ng/ml rmIL-4 and 20 µg/ml anti-IFN$\gamma$) differentiation cocktails [83]. On day 2, 5 U/ml rmIL-2 was added to all cultures. The exogenous cytokines were obtained from Peprotech, (Rocky Hill, NJ) and

neutralizing antibodies were purchased from Biolegend. Cells were removed from Ab-coated plates on day 4. Polarization was confirmed through flow cytometry analysis of transcription factor and cytokine staining for GATA-3, T-Bet, IL-4 and IFNγ. Immediately prior to use in the trogocytosis assay, >95% of $CD4^+$ blasts generated under $T_H1$-polarizing conditions were T-$bet^+$, and >60% of $CD4^+$ blasts generated under $T_H2$-polarizing conditions were GATA-$3^+$.

**Standard *in vitro* trogocytosis assay**

To assess trogocytosis by the $CD4^+$ 5C.C7 T cells, we used our previously described standard *in vitro* trogocytosis assay ([81] Fig. S3.1A). Briefly, 0.5 x $10^6$ surface biotinylated or BODIPY labeled MCC:FKBP fibroblast or 1 x $10^6$ similarly labeled BMDC APC were plated into individual wells of a 6-well tissue culture plate (Greiner, Monroe, NC) and incubated overnight at 37 °C. The MCC:FKBP cell line doubling time is approximately 12 hours, so following the overnight incubation wells contained $10^6$ APC at the time T cells were added. To facilitate magnetic depletion of APC from recovered T cells, iron-containing polystyrene 2.22 µm beads (Spherotech, Green Oaks, IL) were added to the overnight cultures at a final concentration of 0.01% w/v. Twelve to 18 hours later, free magnetic beads were removed from cultures by rinsing, and 2.5 x $10^6$ *in vitro*-primed T cells, (for a final 2.5:1 T:APC ratio), were added to each well and subsequently incubated for 90 min at 37 °C. For experiments using naïve $CD4^+$ T cells, B220 negative selection was performed as described in *in vitro* T cell priming, and the same 2.5:1 T:APC ratio was used, but T cells were incubated

with APC for 18 h at 37 °C. After the co-incubation, T cells were recovered from the cultures by rinsing with cold PBS pH 7.4. Greater than 95% of residual APC were removed by magnetic separation and greater than 70% of the input T cells were routinely recovered from the culture at 90 min after the PBS wash. After two additional PBS washes, recovered T cells (containing both $trog^+$ and $trog^-$ cells) were analyzed immediately by flow cytometry, or were resuspended in complete RPMI 1640 medium at low density ($10^4$ /ml) to minimize T:T contact, and cultured for 60 min in 6-well plates or petri dishes to allow for residual APC to settle and bind to the surface of the 6-well plate. Cells were then gently aspirated and placed into unused 6-well plates with 2 mL/well at $10^4$ cells/ml additional time periods. These cultures of recovered cells contained >95% $CD4^+$ cells, and <0.1% residual APC (Fig. S3.1B).

**Inhibition of TCR Signaling**

To confirm the role of trogocytosis-mediated signaling in effector function, TCR signaling was extinguished using the reversible Src family tyrosine kinase inhibitor PP2 (Life Technologies), as previously reported [81, 442]. Immediately post-trogocytosis assay, recovered T cells were incubated for 20 min in 20 μM PP2, followed by three washes in PBS to remove the PP2. Cells were then assessed immediately to confirm the treatment halted all TCR-signaling, or were cultured as described above and assessed for TCR-downmodulation, transcription factor, and intracellular cytokine expression via flow cytometry.

**CFSE / CellTrace Violet Labeling of T cells**

Primary mouse splenocytes were washed twice with CFSE loading buffer ( 0.1% FBS in PBS pH 7.4). Cells were then resuspended at $10^7$ cells/ml in pre-warmed CFSE loading buffer containing 5 µM CFSE (Sigma-Aldrich), or 5 µM CellTrace Violet (ThermoFisher) and incubated at 37 °C for 10 minutes. The reaction was stopped by addition of an equal volume of complete RPMI 1640 medium containing 10% FBS, followed by two additional washes in complete RPMI.

***In vivo* Trogocytosis Experiments**

In adoptive transfer experiments, B10.A mice were immunized via subcutaneous injection (s.c.) (base of tail) with either 300 µg pigeon cytochrome c (PCC) protein (Sigma-Aldrich) in 100 µl of Sigma Adjuvant System (SAS) or with 100 µl of PBS as a negative control. 24 hours later, 2 x $10^6$ CFSE labeled 5C.C7 primary splenocytes in PBS were injected i.v. Draining inguinal lymph nodes were collected five days post-adoptive transfer and analyzed using flow cytometry. Donor 5C.C7 cells were identified as being V$\beta$3$^+$ and CFSE$^+$. Of these transferred donor cells, trog$^+$ cells were identified as CD3$^+$ CD4$^+$ CD80$^+$ I-E$^{k+}$ (Fig. S.31D).

To generate *in vivo* trog$^+$ cells in a non-transgenic setting, C57Bl/6 CD45.1 mice were subcutaneously immunized at the base of tail with 300 µg of chicken egg white albumin (OVA) (Sigma-Aldrich) in 100 µl SAS, or 100 µl SAS alone as a control. 14 days later, OVA-immunized mice were boosted s.c. with 300 µg OVA

in 100 µl of PBS, control mice were injected with 100 µl PBS alone. Five days post-boost, draining inguinal lymph nodes and spleens were harvested and analyzed via flow cytometry. $Trog^+$ cells were identified as $CD3^+$ $CD4^+$ $CD80/86^+$ $I\text{-}A/E^+$ (Fig. S3.5). To confine analysis of cytokine and transcription factor expression to cells with similar activation states, $CD4^+$ $CD69^{High}$ cells were gated on prior to identification of $trog^+$ and $trog^-$ populations.

**Flow cytometry**

Cells were recovered from cultures and resuspended at $10^6$/ml in FACS buffer (PBS pH 7.4 containing 2% BSA Fraction V (Sigma-Aldrich), 2.5 mM EDTA, and 0.1% $NaN_3$). To assess viability of cells, cells were stained for 10 min at RT with Zombie NIR fixable viability dye (Biolegend) diluted in PBS then washed 3x with PBS. Cells were then stained for surface markers with the indicated reagents for 30 min on ice in FACS buffer. After three additional washes in FACS buffer, cells were stained for 20 min with secondary reagents in FACS buffer, when necessary. Following a final set of three washes in FACS buffer, cells were resuspended in 400 µl of FACS buffer and stored on ice until being analyzed within 2 hours of staining. Alternatively, cells were fixed in ice-cold fixative (4% paraformaldehyde and 0.5% glutaraldehyde), or Biolegend Fixation Buffer, for 30 min at 4 °C followed by 3 washes in FACS buffer. Fixed cells were resuspended in 400 µl of FACS buffer and stored in the dark at 4 °C until being analyzed the following day.

For the detection of antigen-specific cells, an MCC:I-$E^{k\ (88–103)}$ MHC class-II tetramer was obtained through the NIH tetramer core facility (Emory University, Atlanta, GA). Prior to surface staining, tetramer was diluted 1:100 in FACS buffer, and spun down at 1000 x g for 10 minutes to remove aggregates. Cells were blocked with FC receptor-block, then stained with recovered tetramer in suspension, for 30 min at 37 °C, or with an irrelevant isotype control tetramer. For detection of previously-activated cells gating included selection of $CD44^{+}$ cells (Fig. S4.3)

**Intracellular Cytokine Staining**

To enhance detection of intracellular cytokines, in Figs. 3.2, 3.4A-C, 3.6, 3.7, 3.8, 4.4, 4.8, 4.12, 4.16-18, 4.29, and 4.35B, cells were re-stimulated at for 5 hours 37 °C with 500 ng/ml PMA (Phorbol 12-myristate 13-acetate) (Sigma-Aldrich), 1 µg/ml Ionomycin (Sigma-Aldrich), in the presence of 5 µg/ml brefeldin A (BioLegend). After staining surface molecules on live cells, as described above, cells were fixed for 30 min in BD Bioscience cytofix/cytoperm followed by three washes in 1x Biolegend permeabilization/wash buffer (PWB). Cells were then stained for intracellular targets for 60 min on ice or overnight at 4 °C with staining reagents diluted in 1x PWB. Following intracellular staining, cells were washed three times in PWB and were either analyzed immediately, or were stored in FACS buffer in the dark at 4 °C and analyzed the following day.

**Transcription Factor Staining**

For transcription factor staining, live cells were stained for surface molecules, as described above, then fixed in Biolegend True-Nuclear transcription factor fixation buffer for 60 min at room temperature. Following 3 washes in True-Nuclear Perm Buffer, intercellular targets cells were stained with antibodies diluted in True-Nuclear Perm Buffer for 60 minutes on ice, or overnight at 4 °C. After a final set of 3 washes in Perm Buffer, cells were resuspended in 300 µl FACS buffer and analyzed immediately via flow cytometry.

Cells were analyzed on a FACSAria (BD Bio-sciences) in the University of Montana Fluorescence Cytometry Core, or an LSRII (BD Bio-sciences) in the University of Montana Center for Translational Medicine. Data were analyzed with FlowJo 8.8.7 and FlowJo 10 software (Tree Star, Ashland, OR). Geometric mean flourescence intensity (MFI) values were determined, where indicated.

**Gating Strategies**

Identification of trog$^+$ cells was done by gating on lymphocyte population (SSC-A vs. FSC-A) followed by rigorous doublet exclusion (FSC-W vs. FSC-H, and SSC-W vs. SSC-A). Live CD3$^+$ CD4$^+$ cells were identified by the absence of fixable live/dead staining, and trog$^+$ cells were identified by the presence of trogocytosed biotinylated-APC membrane protein (greater than stained, anti-CD3/CD28-stimulated, or unstimulated controls), or the presence of CD80/CD86 + I-E$^k$ for experiments using 5C.C7 TCR transgenic mice, or presence of CD80/CD86 + MHCII I-A/E for experiments using C57BL6/J mice (Fig. S3.5). Ag-specific

staining was confirmed by comparison to respective isotype controls of matched populations confirmed by background levels on unstimulated $CD4^+$ T cells (Fig. S3.5).

Intracellular cytokines and transcription factor gating was established using matched-isotype control Ab staining for respective populations. $trog^+$ and $trog^-$ isotype control intensities were nearly identical, and vertical lines in histogram data depict fluorescence intensity greater than 99% of cells stained with isotype controls.

**Removal of Peptide from Trogocytosed-MHCII**

Recovered T cells were washed in PBS at RT and 5 x $10^6$ cells/ml were incubated at RT for 2.5 min with citric acid buffer (0.1M sodium citrate (Sigma), 0.1M citric acid (Sigma) in PBS pH 4.5 to induce reversible-conformational changes in MHCII [443]. The reaction was neutralized by adding 15x volume of cRPMI, followed by two additional washes in cRPMI. Following washing of unbound peptide, aliquots of cells were supplemented with 10 mM hemoglobin (Hb) peptide, or MCC peptide. When MCC-loaded BMDC were exposed to the same treatment, then used in a trogocytosis-assay, in cultures with supplemented Hb the rate of trogocytosis, and activation (as measured by CD69 expression) of $CD4^+$ T cells recovered from the trogocytosis-assay were reduced by >80% relative to cells recovered from BMDC cultures supplemented with MCC-peptide. $Trog^+$ cells recovered from cultures where peptide was stripped

and replenished with Hb showed a substantial reduction in pZAP-70 signaling compared to untreated, or peptide-stripped cultures where MCC was replenished (Fig. S4.2). Acid treatment did not have a significant effect on activation state of cells as determined by CD69 expression (data not shown).

**Antibody-Neutralization of Trogocytosed MHCII/CD80**

Following recovery from the trogocytosis-assay and removal of contaminating APC, 20 µg/ml of purified anti-I-$E^k$ (17-3-3) (BioLegend) and anti-CD80 (Clone) (BioLegend) were added to an aliquot of recovered cells, and replenished 3d post-recovery. In experiments involving neutralizing antibodies, trogocytosis was measured by the presence of trogocytosed biotin-labeled APC membrane proteins. $trog^+$ cells recovered from cultures containing neutralizing antibodies showed a substantial decrease in pZAP-70 compared to $trog^+$ cells from untreated cultures (Fig. S4.2)

***In vitro* Cytokine Supplementation**

Immediately following recovery from the trogocytosis assay, an aliquot of recovered T cells was supplemented with recombinant mouse IL-2 to a final concentration of 100 iU/mL. Cultures were re-supplemented with IL-2 at 24, and 48 hrs post-recovery.

**Isolating Lymphocytes from Blood**

Blood was collected into tubes containing 0.2 mM EDTA followed by RBC lysis with Gey's solution then stained as described above.

**Isolation of lymphocytes from skin**

Prior to immunization mouse hair was trimmed in a ~2 $cm^2$ patch around the injection site. At the time of harvest, ~1.5 cm 2 skin was collected from the injection site and minced into 3-5mm pieces. Tissue was incubated with 2.5 mM HBSS for 30 min at 37 °C degrees followed by an additional 60 min digestion at 37 °C after the addition of collagenase D (Sigma) to a final concentration of 100 U/ml. Skin was washed in cRPMI and tissue was dissociated with glass slides, and filtered through a 70 µm filter prior to staining for flow cytometric analysis.

**Treatment of T cells with Cytochalasin D**

Prior to imaging of proliferating naïve cells, cytochalasin D (Sigma), was added to cultures for a final concentration of 10 µM, followed by a 4 hr incubation at 37 °C before collection and staining.

**Edu Incorporation Assays**

Twenty-four hours prior to collection and analysis, 2 mM 5-Ethynyl-2´-deoxyuridine (EdU) was added aliquots of recovered cells. After collection, cells were stained for surface molecules, with the exception of PE-conjugated Abs.

Cells were fixed with 4% PFA for 20 min at RT and permeabilized with 0.2% Triton-x 100. Edu staining was conducted according to manufacturer's protocol, followed by 3 washes and when applicable, staining with PE-conjugated Abs.

**Detection of Extracellular Cytokines**

The detection of secreted cytokines in culture medium was performed using BioLegend LEGENDplex beads according to the manufacturer's directions. Briefly, culture medium from cells was centrifuged to remove contaminating cells and debris, and immediately stored at -80 °C until used. Samples and known standards for each cytokine analyzed were subsequently incubated with cytokine-capture beads and biotinylated-detection antibodies in polypropylene microfuge tubes shaking at 1000 rpm at room temperature for 2 hours. Following this incubation, SA-PE was added, followed by a 30 min incubation while shaking at 1000 rpm. Beads were washed and immediately analyzed via flow cytometry.

LEGENDplex beads were analyzed on a LSRII (BD Bio-sciences). Standard curves were generated, and data were analyzed with LEGENDplex software (Biolegend/ VigeneTech). Cytokine levels in samples were confirmed using BioLegend LEGEND MAX sandwich cytokine-capture ELISA kits according to manufacturer's protocol.

## Cell Staining and Image Collection for Microscopic Analysis

T cells recovered and isolated from the standard trogocytosis assay were cultured at low density ($10^4$ cells/ml) for three days in complete RPMI at 37 °C. Live cells were isolated using Lympholyte M density centrifugation prior to detection of trogocytosed molecules with fluorochrome-conjugated streptavidin and anti-I-$E^k$ antibodies diluted in FACS buffer for 30 min on ice. Cells were then washed 3x in PBS and ~$10^6$ recovered T cells were placed in 0.01% poly-L-lysine (Sigma) precoated #1.5 LabTek II eight-chambered coverslips (Nunc) for 10 min at 37 °C in PBS. Cells were fixed with ice-cold fixative (4% paraformaldehyde and 0.5% glutaraldehyde in PBS) in a dark, a humidified chamber for 20 min at room temperature, followed by permeabilization with 0.2% Triton X-100 in PBS for 10 min. Intracellular cytokine and phospho-ZAP70 staining was performed for 1 hour at room temperature in a dark humidified chamber followed by washing with PBS and addition of SlowFade Gold anti-fade reagent (Thermo Fisher, Eugene, OR). 0.3 µm optical sections were collected, on an Olympus Fluoview FV1000 laser scanning confocal mounted on an inverted IX81 microscope using a Nikon 60x objective with 1.4 N.A, housed in the UM Molecular Histology and Fluorescent Imaging core facility.

## Image analysis

Constrained, iterative deconvolution was performed using the Applied Precision SoftWorx software package (GE Healthcare, Issaquah, WA). The integrated

intensity of streptavidin, which is a measure of the amount of fluorescently labeled molecules trogocytosed, was obtained for areas ≥ six-times above background fluorescence. For analysis of phosphorylated ZAP-70, the integrated intensity and mean fluorescent intensity was obtained for areas 6-fold above background. Between 25 and 55 $trog^+$ cells were imaged in each of five independent experiments. Cells were selected for analysis by the presence of streptavidin or I-$E^k$ (6-fold above background intensity). Unstimulated cells were also stained and examined to establish levels of background and non-specific staining. To determine IL-4 or IL-21 polarization towards trogocytosed molecules, cells were divided into 4 quadrants using the ImageJ quadrant picker plugin, with trogocytosed molecules in the center of one radian along the circumference of the cell. The presence of IL-4 staining in the same quadrant as trogocytosed molecules was defined as specific accumulation. Images presented are maximum intensity projections of three consecutive 0.3 µm-thick Z-axis optical sections centered around the highest intensity staining of trogocytosed molecules on the cells surface were created by stacking images in ImageJ. IL-21 and Tcf-1 images in chapter 4, and the additional images presented in Fig. S3.4, are single 0.3 µm-thick optical sections

**Statistical Analysis and Graphing**

Statistical analysis was determined by student's t test, and one-way ANOVA followed by Tukey's multiple comparison test when more than two samples were

compared, using Prism 4 (GraphPad Software, La Jolla, CA). Significance was defined as * p≤ 0.05, ** p≤ 0.01, *** p≤ 0.001, and **** p≤ 0.0001.

# Chapter 3

## Trogocytosis-mediated Intracellular Signaling in CD4$^+$ T cells Drives $T_H$2-associated Effector Cytokine Production and Differentiation

The following are results as presented in the Journal of Immunology, 2019, minus the materials and methods section which has been consolidated into chapter II, and references which have been consolidated in the bibliography of this dissertation.

**Trogocytosis-mediated Intracellular Signaling in CD4$^+$ T cells Drives $T_H$2-associated Effector Cytokine Production and Differentiation[1,2]**

Jim Reed[†] and Scott Wetzel*[†,§]

† Program in Cellular, Molecular and Microbial Biology, Division of Biological Sciences, University of Montana, Missoula, MT 59812, USA
§Center for Environmental Health Sciences, University of Montana, Missoula, MT 59812, USA

**1.** Work funded by R03AI122167 (to S.A.W). Fluorescence Cytometry and Molecular Histology and Florescence imaging core facilities used to perform studies are supported by P30RR033379.

**2. Abbreviations used:**

BMDC, bone marrow-derived dendritic cells; FKBP, FK506-binding protein; ICS, Intracellular cytokine staining; Lck, P56$^{Lck}$; MFI, mean fluorescence intensity; MCC, moth cytochrome-c; OVA, ovalbumin; pZAP-70, phosphotylated ZAP-70; Trog $^{(+)}$, trogocytosis-(positive); Trog $^{(-)}$, trogocytosis-(negative)

**Keywords:** Trogocytosis, $CD4^+$ T cell, $T_H$ **2**, IL-4, effector cytokine, intracellular signaling, cell differentiation, mouse.

## Abstract

$CD4^+$ T cells have been observed to acquire APC-derived membrane and membrane-associated molecules through trogocytosis in diverse immune settings. Despite this, the consequences of trogocytosis on the recipient T cell remain largely unknown. We previously reported that trogocytosed molecules on $CD4^+$ T cells engage their respective surface receptors, leading to sustained TCR signaling and survival after APC removal. Using peptide-pulsed BMDC and transfected murine fibroblasts expressing antigenic MHC:peptide complexes as APC, we show that trogocytosis-positive $CD4^+$ T cells display effector cytokines and transcription factor expression consistent with a $T_H2$ phenotype. *In vitro* polarized $T_H2$ cells were found to be more efficient at performing trogocytosis than $T_H1$ or non-polarized $CD4^+$ cells, while subsequent trogocytosis-mediated signaling induced $T_H2$ differentiation in polarized $T_H1$ and non-polarized cells. Trogocytosis-positive $CD4^+$ T cells generated in vivo also display a $T_H2$ phenotype, in both TCR-transgenic and wild type models. These novel findings suggest that trogocytosis-mediated signaling impacts $CD4^+$ T cell differentiation and effector cytokine production, and may play a role in augmenting or shaping a $T_H2$-dominant immune response.

## Introduction

T lymphocytes acquire lipids and membrane-bound molecules directly from the surface of antigen presenting cells (APC) in a phenomenon termed trogocytosis [279, 373]. This event has been frequently observed in $CD4^+$ T-helper ($T_H$) [81, 285, 287, 368-372], $CD8^+$ [370, 373-376], and $\gamma\delta$ [377] T cells. Though the consequences of trogocytosis on recipient cells are not completely understood, it has been described as “an inherent feature in $CD4^+$ T cell activation” [420]. This phenomenon is not exclusive to T cells, as B cells [378, 379], NK cells [380-382], basophils [283], macrophages [383, 384], neutrophils [385-387], and dendritic cells [388, 389], have all been reported to perform trogocytosis. With elevated levels of trogocytosis documented in sites of autoimmune inflammation [391], viral and parasitic infections [282, 413, 414], rheumatoid arthritis [11], and in tumor environments [284, 403], this widely observed event has been proposed to play a role in the modulation of immune responses [277, 388, 415-419].

Work over the past decade has begun to decipher the mechanism of T cell trogocytosis. We, and others, have previously shown that trogocytosis by $CD4^+$ T cells occurs at the immunological synapse formed between Ag-specific $CD4^+$ T cells and APC [287, 392, 394, 397, 402]. The formation of the immunological synapse involves the spatio-temporal rearrangement of the TCR, costimulatory molecules, and adhesion molecules, into distinct, spatially-segregated supramolecular activation complexes (SMACs) [51-54]. Upon binding MHC:peptide complexes, TCRs migrate towards the center of the SMAC where they become internalized by the T cell and are either recycled to the surface, or

ubiquitinated leading to their degradation [60]. Martinez-Martin *et al.,* have proposed a model of trogocytosis in which APC-derived membrane and membrane proteins are internalized in tandem with TCR via T-cell phagocytosis. Recycling endosomes containing acquired APC fragments then fuse with the T cell plasma membrane resulting in APC-derived molecules being displayed on the T cell surface in their native topological orientation [278, 411]. Trogocytosed molecules are retained in a focused spot on the $CD4^+$ T cell surface [81, 287] and remain fully functional, as demonstrated by the ability for trogocytosis-positive ($trog^+$) T cells to present antigen, in the context of other acquired molecules, to responding T cells [369, 371, 372, 415, 420-426]. The consequences of such presentation appear to correlate with the nature of the acquired molecules and phenotype of the $trog^+$ cell. While $trog^+$ $CD4^+$ cells displaying acquired CD80 and MHC:peptide have been shown to activate responding naïve T cells [371, 410, 420, 424], presentation of Ag to activated cells has been reported to induce responder cell death [429]. In a regulatory context, $trog^+$ T regulatory ($T_{reg}$), or $T_H$ cells displaying trogocytosed molecules associated with immune-suppression, such as HLA-G, show enhanced suppressive capabilities [417, 420, 422, 432, 437, 438]. Collectively, the above findings underscore that $CD4^+$ T cell trogocytosis, and the subsequent presentation of acquired molecules, are biologically significant events in the context of regulating immune responses.

While the ability of trog$^+$ T cells to act as APC has been fairly well documented, much less is known about the biological consequences of trogocytosis on an individual trog$^+$ cell. In our previous studies, we detected TCR signaling in trog$^+$, but not trog$^-$, CD4$^+$ T cells up to 72 hours after separation from APC. This sustained signaling was mediated by trogocytosed molecules engaging their cognate receptors on the trog$^+$ T cell, resulting in “autopresentation”, referred to here as “trogocytosis-mediated signaling”. This signaling led to the enhanced survival of trog$^+$ cells compared to trog$^-$ cells, up to five days after APC removal [81]. Consistent with these findings, Zhou *et al.* found that trog$^+$ CD4$^+$ T cells displayed sustained activation of NFκB and AP1, 24 hours after removal from APC. Interestingly, the trog$^+$ cells also developed a unique cytokine profile [439], raising the possibility that a difference exists between trogocytosis-mediated signaling and signals received from APC. Taken together, the above results suggest that trogocytosis-mediated signaling has the potential to uniquely impact the survival, activation state, and effector cytokine production and/or maintenance of the trog$^+$ CD4$^+$ T cell after separation from APC.

In this study, we examined whether trogocytosis-mediated signaling impacted T cell effector cytokine production and differentiation in the context of the individual trog$^+$ cell. Using non-polarized *in vitro* CD4$^+$ T cell blasts, we found that shortly after APC removal, a high frequency of trog$^-$ cells expressed IFNγ, consistent with a T helper 1 ($T_H1$) phenotype. The trog$^-$ cells did not maintain IFNγ expression over a subsequent 72-hour incubation, and by 72 hours only minimal levels of any effector cytokine examined were detected. In contrast, 5

hours after APC removal the frequency of IFN$\gamma^+$ trog$^+$ cells was significantly lower than IFN$\gamma^+$ trog$^-$ cells. Interestingly, over the subsequent 72-hour incubation, IL-4 expression was significantly increased in the trog$^+$ cells, consistent with a $T_H2$ phenotype. Because trogocytosis-mediated signaling induces preferential survival of trog$^+$ cells [81], the appearance of the $T_H2$ phenotype by trog$^+$ cells could be due to differences in the ability of $T_H1$ and $T_H2$ cells to perform trogocytosis. Consistent with this possibility, *in vitro* polarized $T_H2$ cells were more efficient at performing trogocytosis than $T_H1$ or non-polarized CD4$^+$ cells. However, further investigation revealed that trogocytosis-mediated signaling was directly contributing to the $T_H2$-associated effector cytokine and transcription factor expression in non-polarized trog$^+$ CD4$^+$ cells. In addition, *in vitro* $T_H$ 1-polarized trog$^+$ cells lost expression of IFN$\gamma$ and T-bet, and began expressing IL-4 and GATA-3, suggesting that trogocytosis-mediated signaling was inducing $T_H$ 1 to $T_H2$ conversion. Finally, using both TCR-transgenic and non-transgenic models, and distinct Ag systems, we show that in vivo, a significantly higher frequency of trog$^+$ CD4$^+$ T cells display a $T_H2$ phenotype when compared to trog$^-$ cells from the same animal. Cumulatively, these findings suggest that trogocytosis-mediated signaling has the potential to significantly impact CD4$^+$ T cell effector cytokine production and differentiation, and subsequently may play a role in sustaining, augmenting, or shaping, $T_H2$-dominant immune responses.

# Results

### Intracellular TCR signaling and elevated activation is maintained in trog$^+$, but not trog$^-$ CD4$^+$ T cells after APC removal.

At sites of inflammation and immune activation, there is a correlation between CD4$^+$ trogocytosis and a heightened activation state [397]. This is consistent with data showing that highly activated cells display enhanced trogocytic activity [287, 391]. An additional explanation for the heightened activation observed in trogocytosis-positive (trog$^+$) cells is that that trogocytosed molecules could engage their receptors on the trog$^+$ CD4$^+$ T cell and sustain intracellular signaling. Such trogocytosis-mediated signaling may be playing an important and unappreciated role in driving and/or maintaining T cell activation, effector functions, and differentiation. We have previously shown that that after APC removal, there is selective survival of trog$^+$ cells over 5 days *in vitro* compared to trog$^-$ cells [81]. In addition, both TCR-proximal (ZAP-70 phosphorylation) and distal signaling (ERK 1/2 phosphorylation) was detectable in trog$^+$, but not trog$^-$ cells 72 hours after APC removal. This sustained-signaling occurred independently of APC, and was driven by the engagement of trogocytosed molecules and their receptors on T cells (i.e. trogocytosis-mediated signaling) [81].

To extend on our previous study, we began by comparing the activation state and TCR-proximal signaling in trog$^+$ and trog$^-$ cells up to 72 hours after recovery from a 90-minute *in vitro* trogocytosis assay. Recovered T cells were analyzed immediately, or cultured for 3 days at low density ($10^4$ cells/ml) to minimize T:T

interactions, and samples were collected daily. At indicated time-points (Fig. 3.1), cells were analyzed via flow cytometry, and trog$^{+}$ cells were identified by the presence of trogocytosed, biotin-labeled APC-derived molecules (Fig. S3.1C). Consistent with our previous findings, both trog$^{+}$ and trog$^{-}$ cells showed similar levels of TCR downmodulation immediately after the trogocytosis-assay, indicating that both populations had recognized antigen (Fig. 3.11A, B). In agreement with the TCR downmodulation data, both trog$^{+}$ and trog$^{-}$ cells also displayed elevated levels of the activation marker CD69, compared to unstimulated cells (Figs. 3.11C-E). However, after APC were removed from the system, only the trog$^{+}$ cells maintained TCR downmodulation and an activated (CD69$^{High}$) phenotype throughout the 72-hour incubation. In contrast, at the 48 and 72-hour time points, the trog$^{-}$ cells displayed significantly lower TCR downmodulation levels compared to the trog$^{+}$ cells (Fig. 3.1B), and surface TCR levels were similar to unstimulated T cell blasts (Figs. 3.11A, B). The loss of TCR downmodulation coincided with a decrease in CD69 expression by the trog$^{-}$ cells to levels similar to unstimulated T cell blasts (Figs. 3.1C, D), and at the 48 and 72-hour time points, the trog$^{+}$ cells displayed significantly increased levels of CD69 compared to trog$^{-}$ cells (Fig. 3.1D). This marked difference was not only due to the loss of CD69 expression by trog$^{-}$ cells, but also a result of trog$^{+}$ cells enhancing CD69 expression, and the loss of CD69$^{Low}$ trog$^{+}$ cells (Figs. 3.1C-E). In agreement with our previous study [81], the trog$^{+}$ cells displayed enhanced survival compared to the trog$^{-}$ cells, as seen by the increase in trog$^{+}$ frequencies, and decrease in trog$^{-}$ frequencies after APC removal (Fig. 3.1C). While the

phenotype of the trog$^+$ cells is consistent with active-TCR signaling, the phenotype displayed by the trog$^-$ cells is consistent with published data showing that CD69 expression peaks between 18-48 hours after removal of *in vitro* TCR stimulation [444].

**Figure 3.1 A-D**

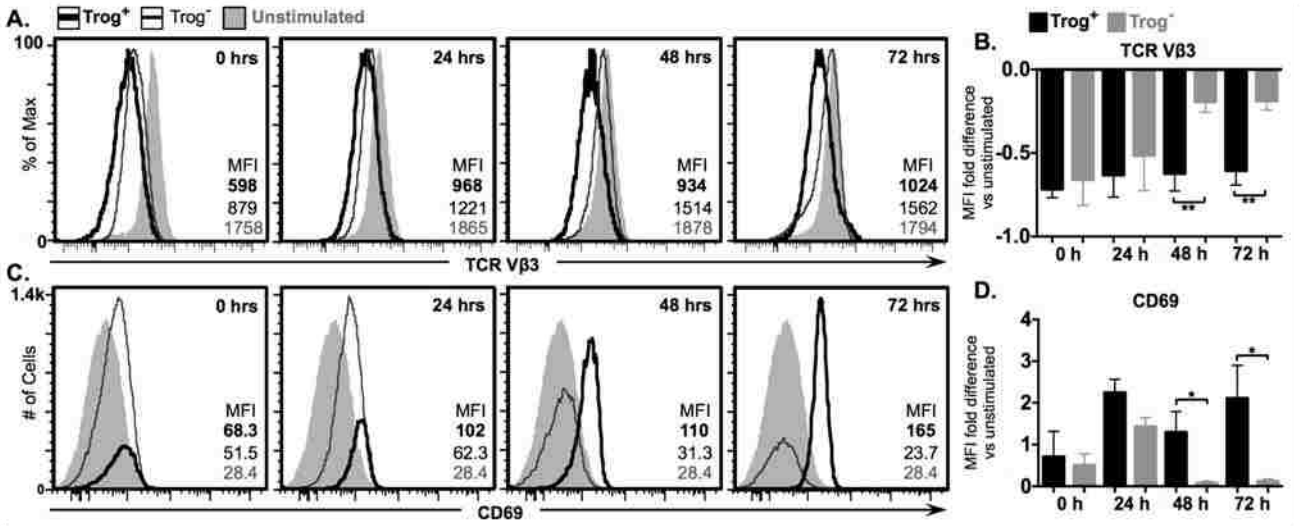



**Figure 3.1 A-D. Sustained survival, and activation in trog$^+$, but not trog$^-$, CD4$^+$ T cells after APC removal.** CD4$^+$ T cells recovered from standard *in vitro* trogocytosis assay were cultured at low density ($10^4$ cells/ml) for 72 hours. At indicated time-points, expression of TCR Vβ3, CD69 and phosphorylated ZAP-70 were determined via flow cytometry. **(A)** Representative histograms showing TCR Vβ3 expression in trog$^+$ *(thick black line),* trog$^-$ *(thin black line),* and unstimulated *(shaded grey)* cells. **(B)** Mean fold-difference in TCR Vβ3 expression between trog$^+$ *(black)* and trog$^-$ *(grey)* cells compared to unstimulated T cell blasts. **(C)** CD69 expression in in trog$^+$ *(thick black line),* trog$^-$ *(thin black line),* and unstimulated *(shaded grey)* cells. Numbers represent geometric mean fluorescence intensity (MFI) of respective populations. **(D)** Mean fold-difference in CD69 expression between trog$^+$ *(black)* and trog$^-$ *(grey)* cells compared to unstimulated T cell blasts. In Figs. B and D, error bars represent ±SEM from three independent experiments with * = p≤ 0.05 and ** = p≤ 0.01. Unstimulated samples represent CD4$^+$ T cell blasts prior to the trogocytosis assay. All data is representative of thee independent experiments.

To confirm that the TCR engagement displayed by trog$^+$ cells resulted in TCR-proximal signaling, the levels of phosphorylated ZAP-70 (pZAP-70) were

assessed [445, 446]. In support of the data in Figs. 3.1A and B, and consistent with our previous studies [81], the trog$^{+}$, but not trog$^{-}$ cells, maintained pZAP-70 levels after APC removal (Fig. 3.1E). At each observed time-point, the trog$^{+}$ cells had significantly higher levels of pZAP-70, as determined by MFI, compared to the trog$^{-}$ cells (Fig. 3.1F). Because nearly no APC were present in the cultures of CD4$^{+}$ T cells following the trogocytosis-assay (Fig. S3.1B), the increased CD69 expression, TCR downmodulation, and elevated pZAP-70 maintained in trog$^{+}$, but not trog$^{-}$, cells over the 72-hour incubation suggests that cell-autonomous, trogocytosis-mediated signaling sustained the T cell activation.

**Figure 3.1 E-F**

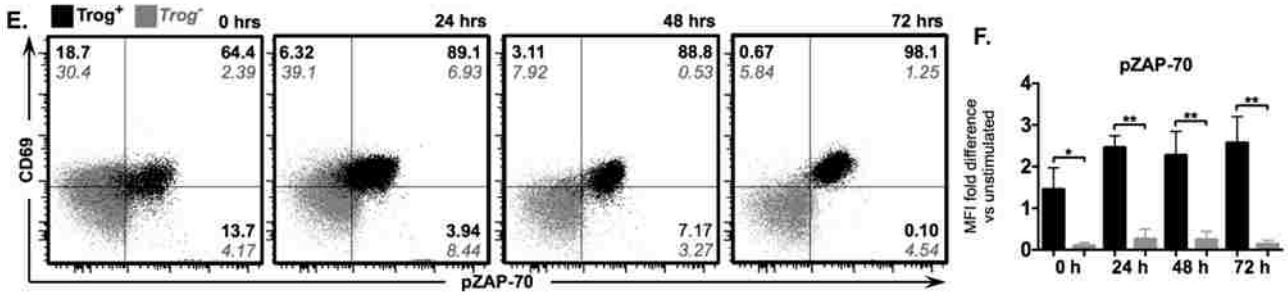



**Figure 3.1 E-F. Sustained TCR signaling, in trog$^{+}$, but not trog$^{-}$, CD4$^{+}$ T cells after APC removal.** CD4$^{+}$ T cells recovered from standard *in vitro* trogocytosis assay were cultured at low density ($10^{4}$ cells/ml) for 72 hours. **(E)** 2D plots showing phosphorylated ZAP-70 vs CD69 in trog$^{+}$ *(black)* and trog$^{-}$ *(grey)* CD4$^{+}$ T cells. Quadrant gates were determined by unstimulated CD4$^{+}$ cells, where >99% of cells were contained in the bottom left quadrant. **(F)** Mean fold-difference in the MFI of phosphorylated ZAP-70 in trog$^{+}$ *(black)* and trog$^{-}$ *(grey)* cells compared to unstimulated CD4$^{+}$ cells. Error bars represent ±SEM from three independent experiments with * = p≤ 0.05 and ** = p≤ 0.01. Unstimulated samples represent CD4$^{+}$ T cell blasts prior to the trogocytosis assay. All data is representative of thee independent experiments.

### Trog$^{+}$ CD4$^{+}$ T cells express elevated levels of IL-4 and IL-5, whereas trog$^{-}$ cells express high levels of IFN$\gamma^{+}$ and little IL-4 or IL-5.

The sustained activation and TCR proximal signaling in trog$^{+}$ cells after removal from APC raised the possibility that trogocytosis-mediated signaling may impact

effector cytokine production within these cells. To examine this, culture supernatants from T cells recovered from the trogocytosis assay were analyzed for cytokines characteristic of various $T_H$ subsets using cytokine-capture beads. Five hours after recovery from the *in vitro* trogocytosis-assay, T cell supernatants contained high levels of IFNγ and IL-2, but negligible amounts of IL-4, IL-6, IL-21 or IL-13 (Fig. 3.2A). Interestingly, by 72 hours IFNγ levels were significantly decreased, while IL-4 levels had increased significantly (Fig. 3.2A). To examine a potential correlation between trogocytosis and the observed cytokine production, intracellular cytokine staining (ICS) was performed on recovered cells at 24-hour intervals over a 72-hour incubation after APC removal. The fold-difference in MFI for $T_H$ subset-characteristic cytokine expression of $trog^+$ and $trog^-$ cells compared to T cell blasts which did not undergo the trogocytosis-assay (unstimulated cells) is shown in Fig. 3.2B. Five hours post-recovery, neither the $trog^+$ nor $trog^-$ cells showed increased levels of IL-4, IL-5, IL-12, IL-6, IL-9, or IL-17, and the only cytokine showing substantially increased expression compared to unstimulated controls was IFNγ (Fig. 3.2B). Interestingly, at 5 hours IFNγ levels were increased in the $trog^-$ cells significantly more than in the $trog^+$ cells (Fig. 3.2D). By 72 hours, there were still minimal expression of IL-12, IL-6, IL-9, or IL-17 by both populations. The robust IFNγ expression seen in $trog^-$ cells at 5 hours was no longer apparent and had decreased to levels only marginally higher than that of unstimulated cells, while IFNγ levels remained minimal in $trog^+$ cells. However, the $trog^+$ cells had developed a striking 2.2-fold increase for IL-4 and 1.85-fold increase for IL-5, and 0.89-fold increase for IL-2, all of which were found to be

significantly higher in the trog$^+$ cells compared to the trog$^-$ cells (Fig. 3.2B). Representative histograms for IFNγ and IL-4 expression by trog$^+$, trog$^-$, and unstimulated cells are presented in Fig. 3.2C. Enhanced IL-4 expression was also observed in trog$^+$ cells from parallel experiments using MCC peptide-pulsed BMDCs as APC (Fig. S3.2). Because the only significant differences in subset-characteristic cytokines detected between trog$^+$ and trog$^-$ cells were $T_H1$ and $T_H2$-associated, the subsequent experiments focused on these subsets.

**Figure 3.2 A-B**

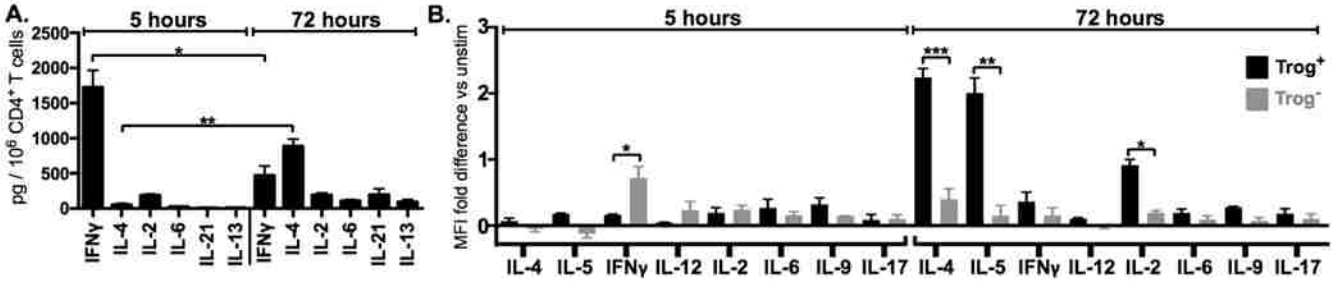



**Figure 3.2 A-B. After APC removal, trog$^+$ cells increase expression of $T_H2$-associated effector cytokines, while trog$^-$ cells express decreasing levels of $T_H1$-associated cytokines.** Expression of cytokines in CD4$^+$ T cells recovered from a standard *in vitro* trogocytosis assay was assessed at indicated time points by flow cytometry. **(A)** Mean levels of subset-characteristic T-helper cytokines in culture supernatant of total recovered T cells at 5 hours *(grey)* and 72 hours *(black)* post trogocytosis-assay. **(B)** Mean fold-difference in the expression of subset characteristic T-helper cytokines between trog$^+$ *(black)* and trog$^-$ *(grey)* cells, compared to unstimulated CD4$^+$ T cells, at 5 and 72 hours post-recovery, as measured by intracellular cytokine staining (ICS) Error bars represent ±SEM from three independent experiments, where * = p≤ 0.05, ** = p≤ 0.01, and *** = p≤ 0.001. All ICS data is representative of at least three independent experiments.

Further assessment of the IFNγ and IL-4 production on a per-cell basis showed that the frequency of IL-4$^+$ cells increased exclusively in the trog$^+$ cells, from an average of 12.5% at 5 hours to an average of 73% by 72 hours (Fig. 3.2D). By comparison, the frequency of trog$^-$ IL-4$^+$ cells remained below 5% at all time-points. This resulted in significantly more trog$^+$ IL-4$^+$ cells than trog$^-$ IL-4$^+$ cells at 24, 48, and 72 hours (Fig. 3.2D *left*). In contrast, on average, 34% of the trog$^-$

cells, but only 10% of trog$^+$ cells were IFN$\gamma^+$ at 5 hours. Unlike IL-4, IFN$\gamma$ expression was not maintained in trog$^+$ cells. Despite a dramatic loss of trog$^-$ IFN$\gamma^+$ cells, there was still a significantly lower frequency of trog$^+$ IFN$\gamma^+$ cells compared to the trog$^-$ IFN$\gamma^+$ cells at each observed time-point (Fig. 3.2D *right*). The decrease in trog$^-$ IFN$\gamma^+$ cells over the 72-hour incubation correlates with the loss of TCR signaling and activation seen in Fig. 3.1, and the massive cell death in this population as observed in Fig. 3.1C and our previous study [81].

**Figure 3.2 C-D**

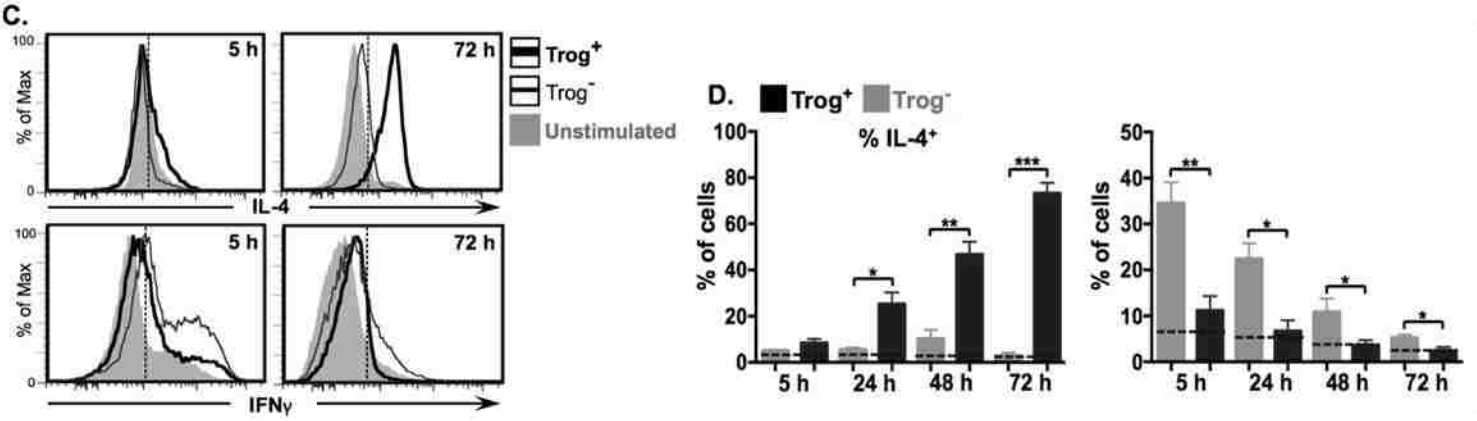



**Figure 3.2 C-D. After APC removal, trog$^+$ cells increase expression of $T_H$2-associated effector cytokines, while trog$^-$ cells express decreasing levels of $T_H$1-associated cytokines.** Expression of cytokines in CD4$^+$ T cells recovered from a standard *in vitro* trogocytosis assay was assessed at indicated time points by flow cytometry. **(C)** Representative histogram plots of ICS data showing fluorescence intensity of IL-4 *(top)* and IFNγ *(bottom)* in trog$^+$ *(thick black line)* and trog$^-$ *(thin black line)* CD4$^+$ T cells. Unstimulated controls are shown in shaded grey for comparison. Dashed vertical line represents the maximum MFI for >99% relevant isotype control. **(D)** Frequency of IL-4$^+$*(left)* or IFNγ$^+$*(right)* CD4$^+$, trog$^+$ *(black),* and trog$^-$ cells over 72 hours after APC removal. Dashed horizontal line represents mean percentage of unstimulated CD4$^+$ T cells for respective cytokines. Error bars represent ±SEM from three independent experiments, where * = p≤ 0.05, ** = p≤ 0.01, and *** = p≤ 0.001. All ICS data is representative of at least three independent experiments.

The strong IFN$\gamma^+$ expression in trog$^-$ cells at 5 hours further demonstrates that both trog$^+$ and trog$^-$ cells are fully activated by the initial APC encounter. Thus, differences in the phenotype between trog$^+$ and trog$^-$ cells do not simply reflect whether T cells have recognized Ag. In addition, although trog$^+$ cells displayed

sustained signaling and activation (Fig. 3.1), IFNγ expression in these cells was not maintained. Combined with the increase in IL-4 expression by trog$^+$ cells after removal from APC, these results are consistent with the possibility that trogocytosis-mediated signaling was impacting the effector cytokine production of the trog$^+$ cells, in a manner consistent with a $T_H2$ phenotype.

**$T_H2$ cells are more efficient than $T_H1$ or non-polarized CD4$^+$ T cells at performing trogocytosis.**

One potential explanation for the increased IL-4 expression and frequency observed in trog$^+$ cells is that pre-$T_H2$ or $T_H2$-like cells had performed trogocytosis more efficiently than pre-$T_H1$ / $T_H1$-like cells. Thus, the increase in IL-4$^+$ trog$^+$ cells may simply reflect the superior survival displayed by trog$^+$ cells after removal from APC [81]. To compare the trogocytic potential of differentiated $T_H1$ and $T_H2$ cells, *in vitro*-polarized $T_H1$ and $T_H2$ blasts were used in an *in vitro* trogocytosis assay (Figs. 3.3A, B). Representative histograms in Fig. 3C and D show that GATA-3$^+$ $T_H2$ blasts were highly efficient at performing trogocytosis, with an average of 71.5% displaying biotin-labeled APC-membrane and 58.1% having I-E$^k$ on their surface. In contrast, T-bet$^+$ $T_H1$ cells were weakly trogocytic, averaging only 14.2% of cells with detectable APC-membrane and 10.4% being I-E$^{k+}$. For comparison, non-polarized blasts, which showed a predominant $T_H1$ phenotype (Figs. 3.3A, B), averaged 26.8% of cells with detectable APC-membrane, and 16.8% with trogocytosed I-E$^k$. Thus, while non-polarized cells had a significantly higher frequency of trog$^+$ cells compared to polarized $T_H1$ cells, the $T_H2$ cells performed trogocytosis at significantly higher rates than both

non-polarized, and $T_H1$ cells (Fig. 3.3E). These results support the hypothesis that the predominant $T_H2$-associated cytokine production in the trog$^+$ cells at 72 hours (Fig. 3.2) was, at least in part, due to the increased ability of $T_H2$ cells to perform trogocytosis and their subsequent enhanced survival, which was driven by trogocytosis-mediated signaling.

**Figure 3.3**

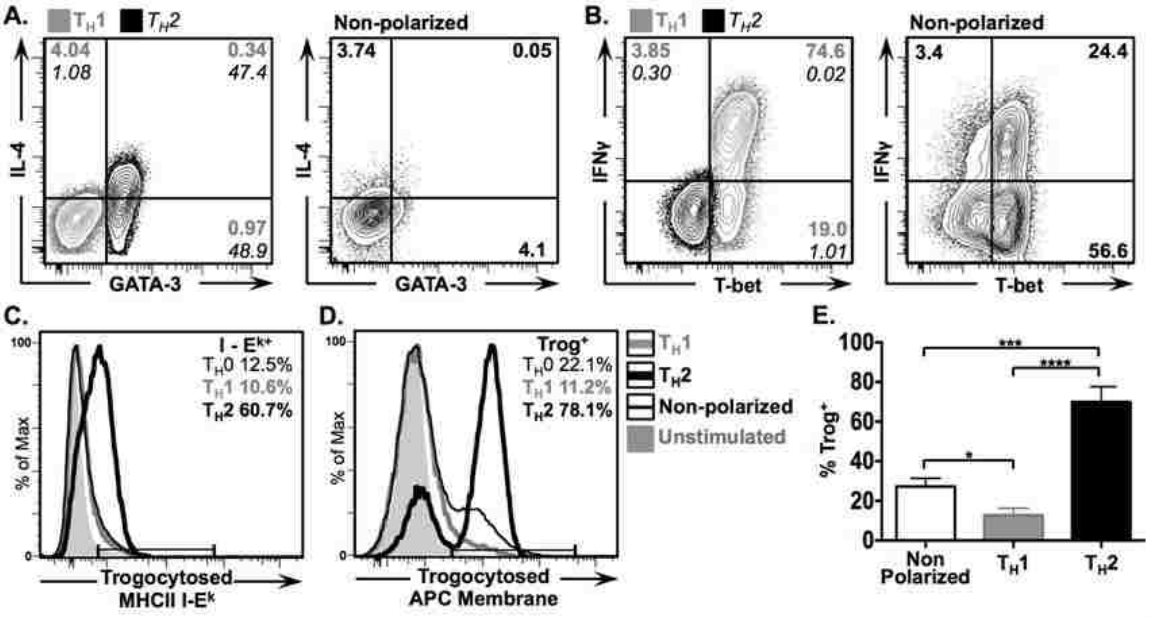



**Figure 3.3. *In vitro* polarized $T_H2$ CD4$^+$ T cells are more efficient at performing trogocytosis than $T_H1$ and non-polarized cells.** Non-polarized and *in vitro* polarized $T_H1$ and $T_H2$ T cell blasts were used in standard *in vitro* trogocytosis assay and expression of IL-4, GATA-3, IFNγ and T-bet in recovered T cells was assessed via flow cytometry. **(A & B)** 2D plots showing fluorescence intensity of (A) IL-4 vs. GATA-3, and (B) IFNγ vs. T-bet in polarized $T_H2$ *(grey)* and $T_H1$ *(black),* and non-polarized cells, 1 hour post recovery. Numbers in corners represent frequencies of cells in respective quadrants. **(C & D)** Histogram overlays showing the frequency of trogocytosed *(C)* MHCII I-E$^k$ and *(D)* biotinylated APC membrane by polarized $T_H1$ *(grey line)* $T_H2$ *(thick black line),* and non-polarized cells *(thin black line),* with unstimulated controls in shaded grey for comparison. **(E)** Mean frequency of trog$^+$ cells from non-polarized *(left),* $T_H1$ *(middle),* and $T_H2$ *(right)* CD4$^+$ cells 1 hour post-recovery. Error bars represent ±SEM from three independent experiments, * = p≤ 0.05, *** = p≤ 0.001, and **** = p≤ 0.0001. All data shown is representative of three independent experiments.

**Trogocytosis-mediated signaling is sufficient, and effective, in driving IL-4 expression.**

Based on the data in Fig. 3.3 showing that $T_H2$ cells possess higher trogocytic potential than $T_H1$ or non-polarized cells, the increase in trog$^+$ IL-4$^+$ cells may simply be due to trogocytosis-mediated signaling enhancing the survival of predestined $T_H2$ cells. If this were the case, then sustained-canonical TCR and costimulatory signaling would be expected to augment the $T_H2$ phenotype of pre-$T_H2$ or $T_H2$ cells. To examine this possibility, an aliquot of the same T cell blasts to be used in a trogocytosis-assay were stimulated with plate-bound anti-CD3 + anti-CD28 in parallel with T cells recovered from a standard *in vitro* trogocytosis-assay. At 72 hours, on average, 77.4% of the trog$^+$ cells were IL-4$^+$, but only 3.5% of trog$^-$ cells and 1.8% of Ab-stimulated blasts were IL-4$^+$ (Figs. 3.4A-C). In contrast, Ab-stimulated blasts maintained a similar frequency of IFN$\gamma^+$ cells over the 72 hours (Fig. 3.4A), while the frequency of IFN$\gamma^+$ trog$^+$ cells dropped from an average of 13.4% at 5 hours to 1.5% by 72 hours (Fig. 3.4C). The striking difference between the IFN$\gamma$ and IL-4 expression patterns between the trog$^+$ and Ab-stimulated blasts suggests that trogocytosis-mediated signaling provides additional, potentially unique, signals that favor $T_H2$-associated cytokine expression, beyond the signaling induced by repeated CD3 + CD28 signaling. These results further strengthen the association between trogocytosis-mediated signaling, and a $T_H2$ phenotype.

**Figure 3.4**

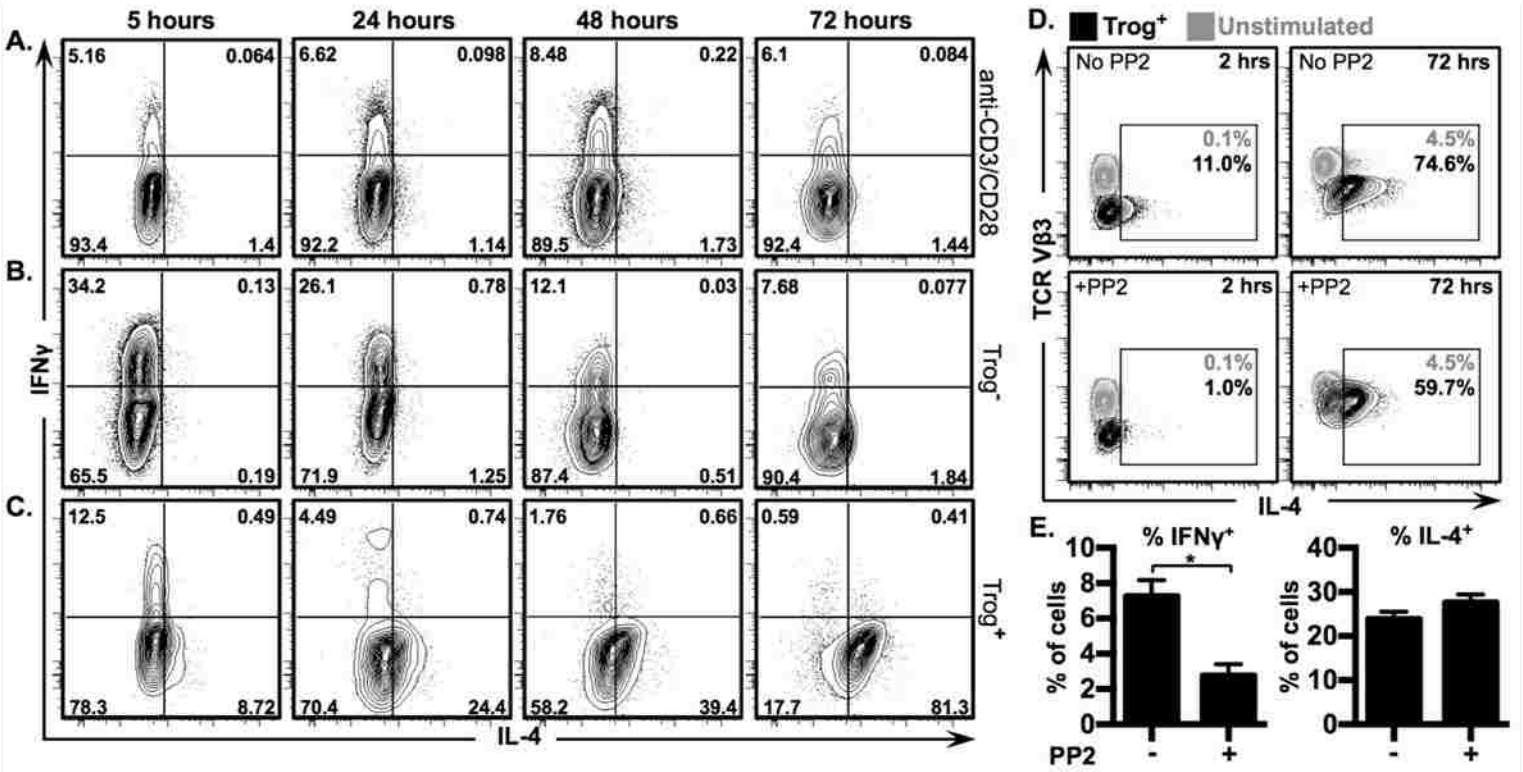



**Figure 3.4. Trogocytosis-mediated signaling drives IL-4, but not IFNγ, expression in trog⁺ cells.** Non-polarized CD4⁺ T cells were used in a standard *in* vitro trogocytosis assay, or were stimulated with plate-bound anti-CD3 + anti-CD28. Levels of IL-4 and IFNγ were measured by ICS and analyzed via flow cytometry. Representative 2D flow plots show fluorescence intensity of IL-4 vs IFNγ in **(A)** Ab-stimulated blasts, **(B)** trog⁻ cells, and **(C)** trog⁺ cells, at indicted time points post-recovery. Gates were established using respective isotype-control staining where >99% of CD4⁺ cells stained with isotype controls fell in the lower left quadrant. Numbers in corners represent frequency of cells in respective quadrants. Data are representative of at least 3 independent experiments. **(D)** Immediately after recovery from an *in vitro* trogocytosis assay, cells were left untreated *(top row)* or were treated for 20 minutes with 20 μM PP2 *(bottom row)* to halt TCR signaling. PP2 was removed from cultures and cells were analyzed at 2 *(left column),* and 72 hours *(right column),* after PP2 removal. Trog⁺ cells are shown in black, compared to unstimulated cells in grey. Numbers represent frequency of cells included in respective gates. **(E)** Mean frequency of IFNγ⁺ and IL-4⁺ trog⁺ cells, 24 hours after PP2 removal, compared to untreated trog⁺ cells. Error bars represent ±SEM from three independent experiments, * = p≤ 0.05. All data is representative of at least three independent experiments.

As seen in Fig. 3.3, the majority of non-polarized T cells did not displayed a $T_H1$ phenotype prior to the trogocytosis assay, yet the vast majority of trog⁺ cells developed a $T_H2$ phenotype after APC removal. While the data thus far is suggestive that trogocytosis-mediated signaling was driving this phenotype, the

possibility that residual signaling from the T:APC interaction was driving the observed IL-4 expression in trog$^+$ cells remained. To confirm that trogocytosis-mediated signaling alone was capable of driving IL-4 production in trog$^+$ cells, Lck signaling was interrupted in cells recovered from the trogocytosis assay with the reversible Src family kinase inhibitor PP2. Using this technique, we previously demonstrated that PP2 treatment terminated TCR signaling, however, after removal of PP2, signaling was re-initiated in trog$^+$, but not trog$^-$ cells [81]. Here, we hypothesized that if trogocytosis-mediated signaling was driving IL-4 expression, then cytokine production would resume in trog$^+$, but not trog$^-$ cells after removal of PP2. If neither trog$^+$ nor trog$^-$ cells re-initiated IL-4 expression after PP2 was removed, it would indicate that trogocytosis-mediated signaling alone was insufficient to drive the expression of IL-4. Immediately following the trogocytosis assay, recovered cells were treated for 20 min with PP2 to halt TCR signaling, and then incubated for an additional 2 hours after PP2 removal. Results in Fig. 3.4D show that PP2 treatment did not alter the engagement of TCR by trogocytosed MHC:peptide complexes, as nearly identical levels of TCR downmodulation were observed in PP2-treated trog$^+$ and untreated trog$^+$ cells, at 2 (MFI reduced 2.9 fold or 2.81 fold vs. unstimulated controls, respectively) and 72 hours (1.7 fold vs. 1.3 fold reduction, respectively) (Fig. 3.4D). Treatment with PP2 did however, reduce the frequencies of trog$^+$ IL-4$^+$ cells at 2 hours by an average of 83.5% (from 11% to 1%). However, 72 hours after PP2 removal, the frequency of IL-4$^+$ trog$^+$ cells had rebounded from 1% at 2 hours, to nearly 60% at 72 hours (Fig. 3.4D*, right*). PP2 treatment also decreased the modest IL-4

expression of trog$^-$ cells at 2 hours, and, as expected, neither the PP2-treated or untreated populations of trog$^-$ cells expressed IL-4 at 72 hours (Fig. S3.3C). The ability of trog$^+$ cells to resume IL-4 production in absence of APC provides compelling evidence that trogocytosis-mediated signaling was sufficient to drive IL-4 expression in trog$^+$ cells.

Treatment with PP2 reduced the frequency of trog$^+$ IFN$\gamma^+$ cells by 72%, and trog$^-$ IFN$\gamma^+$ cells by 80% at 2 hours (Fig. S3). However, consistent with the hypothesis that trogocytosis-mediated signaling was driving IL-4, but not IFN$\gamma$ expression, the expression of IL-4, had rebounded in trog$^+$ cells 24 hours after PP2 treatment, while IFN$\gamma$ expression was significantly lower in PP2-treated cells compared to untreated cells (Fig. 4E). This suggests that IFN$\gamma$ expression was due to signaling occurring at the immunological synapse, and that trogocytosis-mediated signaling was insufficient to maintain IFN$\gamma$ production in these cells.

**Intracellular IL-4 is polarized towards trogocytosed molecules**

The sustained expression of IL-4 by trog$^+$ cells up to 72 hours after removal from APC (Fig. 3.2) suggested that trogocytosis-mediated signaling may be contributing to the maintenance of $T_H2$-associated cytokine production in these cells. As a major function of $T_H2$ cells is to provide contact-dependent help to cognate B cells upon Ag recognition through B cell directed cytokine secretion, we hypothesized that trogocytosis-mediated signaling may mimic these T:B interactions. If so, intracellular IL-4 would likely be polarized towards areas of

trogocytosis-mediated TCR-signaling. Three days after recovery from a standard *in vitro* trogocytosis assay, recovered cells were stained for trogocytosed molecules, intracellular IL-4, and pZAP-70, and analyzed by confocal microscopy. Consistent with the flow-cytometry data (Fig. 3.2), IL-4 was only detected in 5% of trog$^-$ cells in which polarization of IL-4 towards any distinct region was only observed in 8% of trog$^-$ IL-4$^+$ cells (Figs. 3.5C, S4). In contrast, the trog$^+$ IL-4$^+$ cells had significantly higher frequencies of polarized IL-4 compared to the trog$^-$ IL-4$^+$ cells, and 86% of these cells had IL-4 polarized towards trogocytosed molecules on the T cell membrane (Fig. 3.5C). Representative images illustrate that IL-4 was polarized towards trogocytosed molecules (Fig. 3.5A), and that proximal TCR-signaling was occurring at the trogocytosed molecules, as indicated by pZAP-70 staining (Fig. 3.5B). Additional images are presented in Fig. S3.4. These images are reminiscent of observations made by Kupfer *et al.*, who revealed that during $T_H2$ help for B cells, IL-4 delivery was localized to the $T_H2$:B cell interface [447]. Thus, data in Fig. 3.5 are supportive of a model where trogocytosis-mediated signaling was stimulating polarized IL-4 secretion, and also further reinforce the $T_H2$ phenotype displayed by trog$^+$ cells.

Figure 3.5

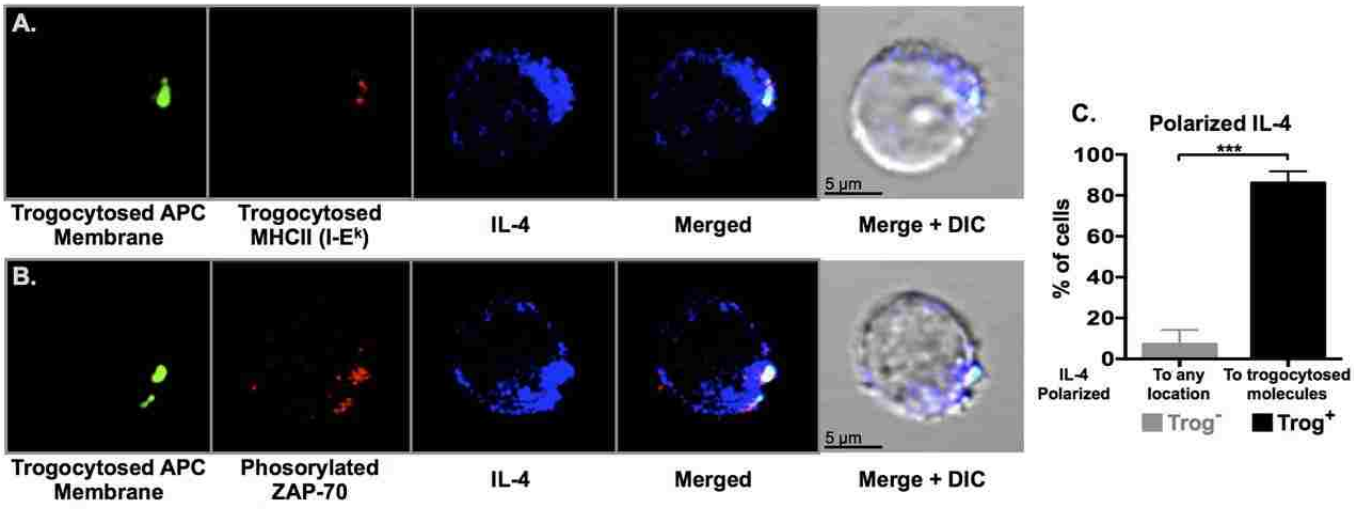



**Figure 3.5. IL-4 is polarized towards trogocytosed molecules and the location of active TCR signaling.** CD4$^+$ T cells were analyzed by confocal microscopy 72-hours after recovery from a standard *in vitro* trogocytosis assay. Representative optical sections of trog$^+$ cells are shown with **(A)** trogocytosed APC membrane proteins *(green),* trogocytosed I-E$^k$ *(red)*, and intracellular IL-4 *(blue)*. **(B)** Trogocytosed APC membrane proteins *(green),* phosphorylated-Zap-70 *(red)* and intracellular IL-4 *(blue)*. Data is representative of five independent experiments, with over 120 individual trog$^+$ CD4$^+$ cells analyzed in total. **(C)** IL-4$^+$ trog$^-$ *(grey)* and trog$^+$ *(black)* cells were analyzed for IL-4 polarization, as defined by the localization of intracellular IL-4 to any constrained area for the trog$^-$ cells, and polarization towards trogocytosed molecules in the trog$^+$ cells. Error bars represent ±SEM from three independent experiments, *** = p≤ 0.001.

### Trog$^+$ cells develop a $T_H2$ phenotype while trog$^-$ cells maintain a $T_H1$ phenotype after separation from APC.

Although IL-4 is widely accepted as the representative $T_H2$ effector-cytokine, other T cell subsets, such as $T_{FH}$, also express IL-4 [448]. Therefore, expression of IL-4 alone does not indicate $T_H2$-differentiation. To determine whether trog$^+$ cells were differentiated to $T_H2$, the expression of subset-characteristic transcription factors was examined. The data in Fig. 3.6A show no detectable differences in expression of GATA-3 ($T_H2$), T-bet ($T_H1$), Foxp3 ($T_{reg}$), Bcl-6 ($T_{FH}$) or RORγt ($T_H17$) between trog$^+$ and trog$^-$ cells, 5 hours post-recovery from an *in vitro* trogocytosis-assay. This further supported that the unstimulated blasts consisted of a homogenous population prior to use in the trogocytosis-assay. By

72 hours however, the trog$^{+}$ cells displayed a 1.7-fold increase for GATA-3, and 90% lower levels of T-bet compared to trog$^{-}$ cells, while only minimal differences were detected for Foxp3, Bcl-6, or ROR$\gamma$t (Fig. 3.6A). In agreement with the cytokine profiles observed in Fig. 3.2, over the 72-hour incubation, T-bet expression in trog$^{+}$ cells decreased to a level below unstimulated bulk T cell blasts, while trog$^{-}$ cells maintained elevated T-bet expression (Fig. 3.6B). In contrast, at 72 hours, the majority of the trog$^{+}$ cells were GATA-3$^{+}$, and GATA-3 expression was negligible in the trog$^{-}$ cell population (Fig. 3.6B). While trog$^{+}$ and trog$^{-}$ displayed similar levels of T-bet and GATA-3 five hours after recovery, by 72 hours the trog$^{+}$ cells displayed significantly increased GATA-3 expression, and significantly decreased T-bet expression compared to trog$^{-}$ cells (Fig. 3.6C). These results confirmed that the IL-4$^{+}$ trog$^{+}$ cells were consistent with a $T_H2$ phenotype, and support the hypothesis that trogocytosis-mediated signaling was driving $T_H2$ differentiation rather than simply enhancing the survival of $T_H2$-commited cells.

Figure 3.6 (A-C)

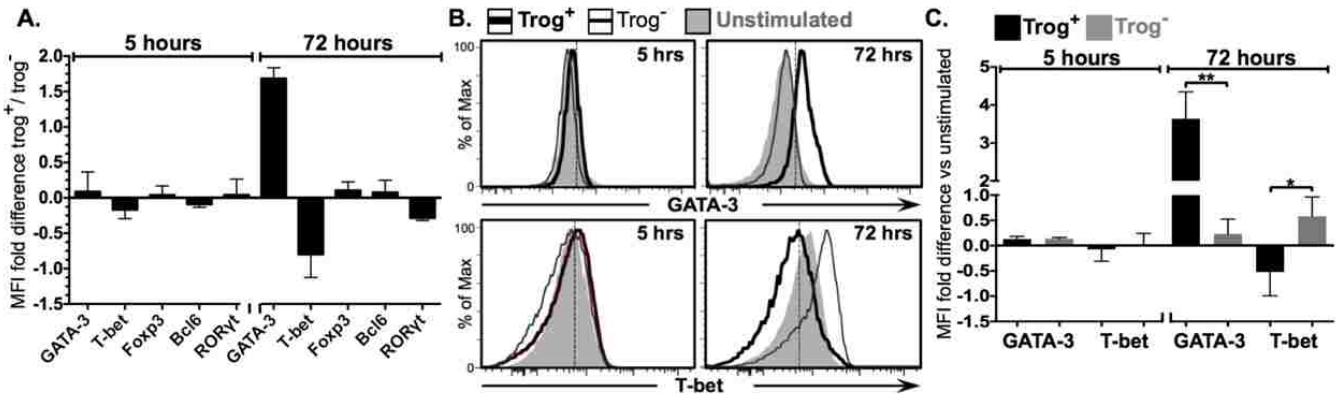



**Figure 3.6 (A-C). Expression of GATA-3 increases in trog⁺ cells after APC removal.** CD4⁺ T cells were recovered from a standard *in vitro* trogocytosis assay and characteristic T-helper subset transcription factor expression was examined by flow cytometry. **(A)** Mean fold-difference in expression of indicated transcription factors between trog⁺ cells and trog⁻ cells at five, and 72, hours post-recovery as determined by MFI. **(B)** Representative histograms of samples in 6A showing GATA-3 *(top)* and T-bet *(bottom)* levels in trog⁺ *(thick black line)* and trog⁻ *(thin black line)* cells, with unstimulated control cells shown in shaded grey for comparison. Dotted vertical lines represent the fluorescence intensity >99% of recovered CD4⁺ cells stained with respective isotype controls. **(C)** Mean fold-difference in GATA-3 and T-bet expression between trog⁺ *(black)* and trog⁻ *(grey)* cells compared to unstimulated cells, at 5, and 72 hours post recovery. In A and C, error bars represent ±SEM from three independent experiments, * = p≤ 0.05, ** = p≤ 0.01. All data is representative of three independent experiments.

## Trogocytosis-mediated signaling, rather than IL-4 availability, is required for GATA-3 upregulation after APC removal.

While IL-4 is a major product of $T_H2$ cells, it is also an inducer of this subset, as IL-4R-signaling significantly contributes to $T_H2$ differentiation [449]. Because the trog⁺ cells produced significantly higher amounts of IL-4 than trog⁻ cells (Fig. 3.2), it was possible that the $T_H2$ phenotype displayed by the trog⁺ cells was simply the result of IL-4 availability. To examine whether IL-4R signaling was playing the central role in the $T_H2$-phenotype observed in trog⁺ cells, exogenous IL-4 (20 µg/ml) was added to cultures of cells immediately after recovery from the trogocytosis assay, and replenished at 24 hours. As seen in Fig. 3.6D, at 72 hours, the addition of IL-4 had minimal effects on GATA-3 expression in unstimulated cells. Similarly, the trog⁻ cells from cultures containing supplemented IL-4, showed only an 8.6% average increase in GATA-3

expression, while trog$^+$ cells from the same cultures had increased GATA-3 expression by an average of 25.5%, as determined by MFI (Fig. 3.6E). However, the addition of IL-4 had little impact on the frequency of GATA-3$^+$ trog$^-$ or trog$^+$ cells, as no significant differences between cells from untreated cultures, and cultures with supplemented IL-4, were detected in either population (Fig. 3.6F). Thus, IL-4R signaling was not playing the central role in the increase in GATA-3$^+$ expression by the trog$^+$ cells. These results confirm that trogocytosis-mediated signaling, and not simply the availability of IL-4, was essential for the observed $T_H2$ phenotype developed in trog$^+$ cells, consistent with the finding that GATA-3 translation is dependent on TCR signaling [450].

**Figure 3.6 (D-F)**

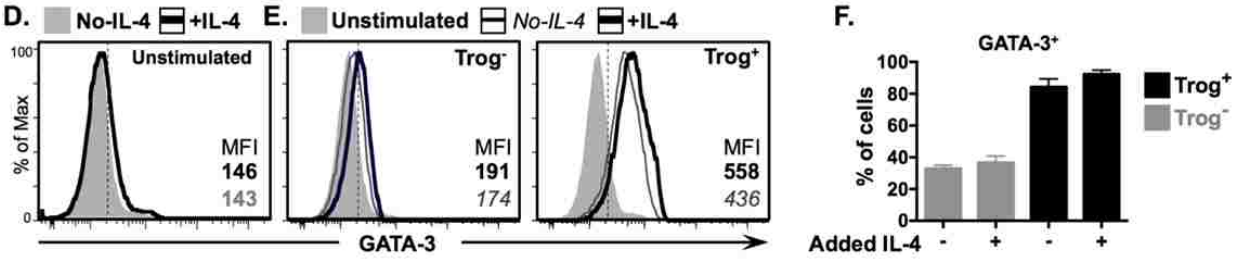



**Figure 3.6 (D-F). Trogocytosis-mediated signaling is required for GATA-3 expression in trog$^+$ cells.** CD4$^+$ T cells were recovered from a standard *in vitro* trogocytosis assay and characteristic T-helper subset transcription factor expression was examined by flow cytometry. **(D-E)** Exogenous IL-4 (20 μg/ml) was added to recovered cell cultures immediately after the trogocytosis assay, and supplemented 24 hours later. (D) GATA-3 levels 72 hours post-recovery are shown for (left) unstimulated cells with (*black line*) and without *(shaded grey)* supplemental IL-4 **(E)** GATA-3 levels 72 hours post-recovery trog$^-$ cells *(left),* and trog$^+$ cells *(right),* with *(black line)* or without *(thin grey line)* supplemental IL-4. Unstimulated cells cultured without supplemented IL-4 in shaded grey are included for comparison. Dotted vertical lines represent the fluorescence intensity >99% of recovered CD4$^+$ cells stained with respective isotype controls **(F).** Mean frequency of GATA-3$^+$ trog$^-$ *(grey)* and trog$^+$ *(black)* cells from cultures with, and without, supplemented IL-4. Error bars represent ±SEM from three independent experiments, * = p≤ 0.05, ** = p≤ 0.01. All data is representative of three independent experiments.

### Trogocytosis-mediated signaling drives $T_H1$ cells to express IL-4 and GATA-3

The results in Figs. 3.4 and 3.6 are consistent with the hypothesis that trogocytosis-mediated signaling drives $T_H2$ differentiation in trog$^+$ cells. To examine this hypothesis, we tested whether trogocytosis-mediated signaling

could induce *in vitro* $T_H1$ polarized trog$^+$ cells to start producing $T_H2$ characteristic proteins. If such conversion was observed in trog$^+$, but not trog$^-$, polarized $T_H1$ cells it would strongly support the hypothesis that sustained, trogocytosis-mediated signaling was inducing a $T_H2$ phenotype. *In vitro* $T_H1$ and $T_H2$ polarized blasts were generated and used in a standard *in vitro* trogocytosis assay. In parallel, polarized $T_H1$ and $T_H2$ blasts were stimulated on anti-CD3 + anti-CD28 coated plates to provide sustained signaling throughout the 72-hour incubation. Because plasticity between $T_H1$ and $T_H2$ subsets occurs only after days of exposure to alternate polarizing conditions [144, 169, 451, 452], the 90 minutes of exposure to APC during the trogocytosis-assay alone would not likely be sufficient to induce $T_H1$ to $T_H2$ conversion. Cells were examined at 5 hours to examine baseline conditions, and at 72 hours after APC removal, to assess potential phenotypic changes.

The results in Fig. 3.7A show that sustained Ab-stimulation resulted in stable IFN$\gamma$ expression in polarized $T_H1$ cells, with an average of 61% being IFN$\gamma^+$ at 5 hours, and 57% being IFN$\gamma^+$ at 72 hours. As anticipated, these $T_H1$ polarized cells did not express IL-4 at any time-point. The $T_H1$ polarized trog$^+$ cells showed a phenotype similar to Ab-stimulated $T_H1$ cells at 5 hours, with an average of 79% being IFN$\gamma^+$, and only 8% expressing IL-4$^+$ (Fig. 3.7B). However, unlike the Ab-stimulated $T_H1$ cells, the frequency of trog$^+$ IFN$\gamma^+$ cells decreased at each successive time-point, and by 72 hours only ~5% remained IFN$\gamma^+$. IFN$\gamma$

expression was not detected in the trog$^+$ $T_H2$, or the Ab-stimulated $T_H2$ blasts at any time-point (Figs. 3.7 C, D).

**Figure 3.7**

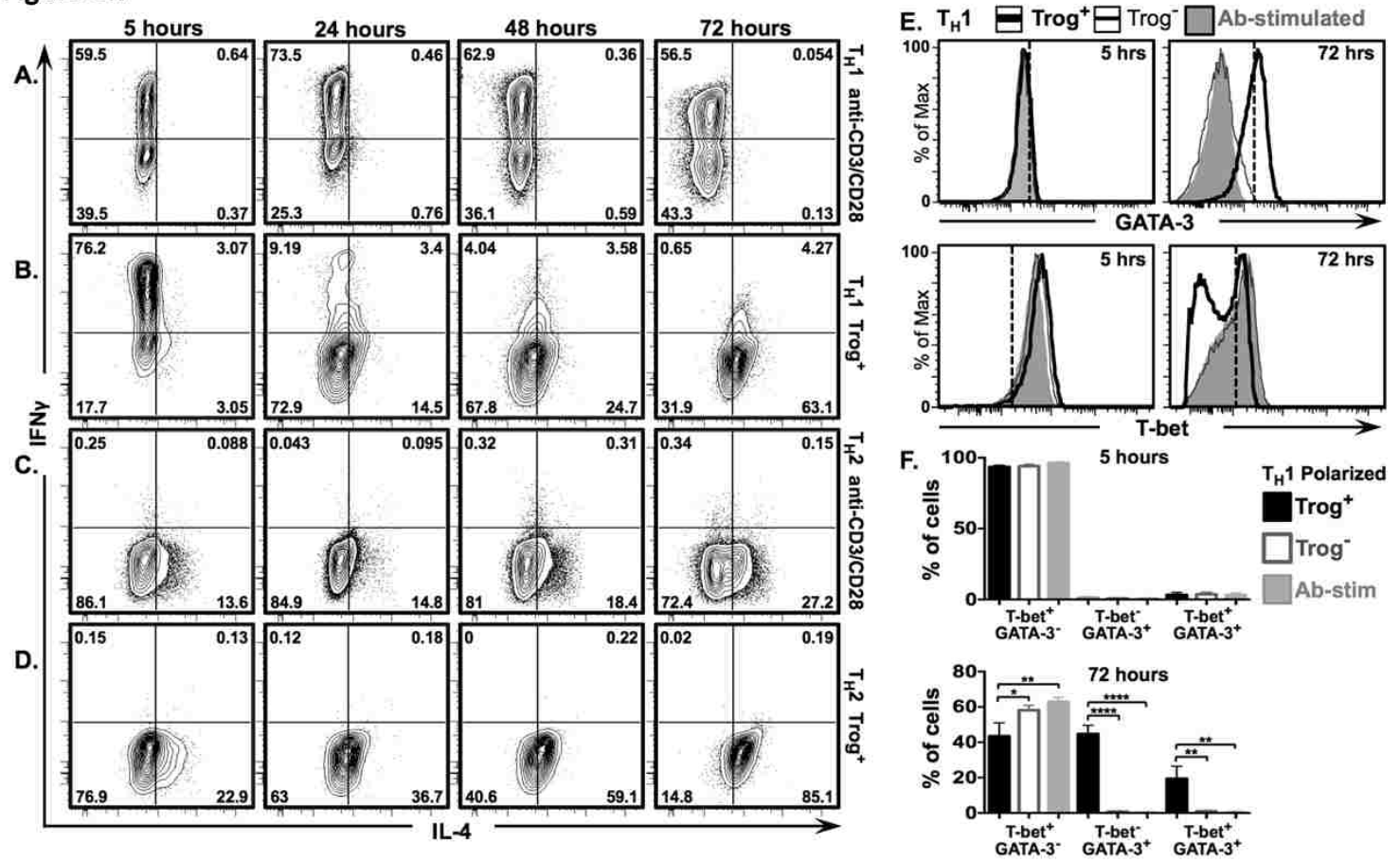



**Figure 3.7. IL-4 expression increases and IFNγ expression decreases in polarized $T_H1$ trog$^+$ cells after removal of APC.** *In vitro* polarized $T_H1$ and $T_H2$ CD4$^+$ T cells were used in a standard *in vitro* trogocytosis assay. In parallel, aliquots of polarized cells were stimulated with plate-bound anti-CD3 + anti-CD28 Abs. Fluorescence intensity of IL-4 vs. IFNγ is displayed in representative 2D scatter plots at 5 hrs. *(left),* 24 hrs., *(2$^{nd}$ from left),* 48 hrs. *(2$^{nd}$ from right),* and 72 hrs. *(right)*for **(A)** anti-CD3 + anti-CD28 stimulated $T_H1$ polarized cells, **(B)** $T_H1$ polarized trog$^+$ cells **(C)** anti-CD3 + anti-CD28 stimulated $T_H2$ polarized, and **(D)** $T_H2$ polarized trog$^+$ cells. Numbers represent frequency of cells in each quadrant. **(E)** Representative histograms showing intensity of GATA-3 *(top)* and T-bet *(bottom)* expression in $T_H1$ polarized trog$^+$*(thick black line)* and trog$^-$ *(thin black line)* cells, compared to anti-CD3/CD28 stimulated blasts *(shaded grey)*, at 5 *(left),* and 72 *(right),* hours post-recovery. **(F)** Mean frequency of T-bet$^+$ GATA-3$^-$ *(left),* T-bet$^-$ GATA-3$^+$ *(middle),* and T-bet$^+$ GATA-3$^+$ *(right)*, *in vitro* polarized $T_H1$ trog$^+$ *(black)*, trog$^-$ *(grey outline),* and anti-CD3/CD28 stimulated cells *(shaded grey)* at 5 *(top)*, and 72 *(bottom)* hours post-recovery. Error bars represent ±SEM from three independent experiments, * = p≤ 0.05, ** = p≤ 0.01, *** = p≤ 0.001, and **** = p≤ 0.0001. All data is representative of three independent experiments.

In contrast to the significant decrease in IFNγ expression, the frequency of trog$^+$ $T_H$1 polarized cells expressing IL-4 increased from 8% at 5 hours, to approximately 70% at 72 hours (Fig. 3.7B). The presence of a unique population (averaging 4.7%) of $T_H$1 polarized trog$^+$ cells which were IFNγ$^+$ IL-4$^+$ double-positive cells at 72 hours (Fig. 3.7B) supported the idea that these cells converted from $T_H$1 towards a $T_H$2 phenotype [178]. For comparison, while Ab-stimulation of the polarized $T_H$1 maintained IFNγ expression, on average, less than 0.2% of these cells were for IFNγ$^+$ IL-4$^+$ double-positive at 72 hours (Fig. 37A). Within the polarized $T_H$2 cells, on average only 0.22% of trog$^+$, and 0.31% of Ab-stimulated cells were IFNγ$^+$ IL-4$^+$ (Fig. 3.7C, D).

While the $T_H$2 polarized trog$^+$ population displayed an average of 84.2% of cells being IL-4+, somewhat unexpectedly, only 28% of Ab-stimulated cells were IL-4$^+$ at 72 hours (Fig. 3.7C, D). These are similar to the results in Fig. 4, and further support that trogocytosis-mediated signaling is favorable for driving and/or augmenting a $T_H$2 phenotype, while sustained anti-CD3 + anti-CD28 Ab-stimulation is not.

In addition to shutting down IFNγ expression and upregulating IL-4, the trog$^+$ $T_H$1 polarized cells also displayed a shift in transcription factor expression. These cells were GATA-3 negative and expressed high levels of T-bet at 5 hours, but by 72 hours, the trog$^+$ $T_H$1 cells had upregulated GATA-3 expression, and approximately half of the population lost T-bet expression (Fig. 3.7E). In contrast,

the Ab-stimulated and trog$^-$ $T_H1$ cells remained GATA-3$^-$ and maintained expression of T-bet at 72 hours. While there were no significant differences in T-bet or GATA-3 expression between $T_H1$ polarized trog$^+$, trog$^-$, and Ab-stimulated cells 5 hours after recovery, by 72 hours the frequency of T-bet$^+$ cells was significantly lower in the trog$^+$ population compared to trog$^-$ population. This was concomitant with significantly more trog$^+$ cells expressing GATA-3 compared to trog$^-$ and Ab-stimulated cells (Fig. 3.7F). Consistent with the cytokine expression data where the $T_H1$ polarized trog$^+$ cells expressed both IL-4 and IFN$\gamma$ (Figs. 37A, B), significantly more trog$^+$ cells also expressed both T-bet and GATA-3 compared to trog$^-$ and Ab-stimulated cells (Fig. 3.7F). Taken together, the data in figure 7 strongly suggest that trogocytosis-mediated signaling induced $T_H1$ to $T_H2$ conversion, strengthening the conclusion that trogocytosis-mediated signaling drove the observed $T_H2$ phenotype in trog$^+$ cells.

**Trogocytosis$^+$ CD4$^+$ T cells generated *in vivo* display a $T_H2$ phenotype.**

The results so far strongly support the hypothesis that trogocytosed molecules engage cognate receptors on T cells to sustain intracellular signaling, leading to $T_H2$ biasing. To examine whether this *in vitro* phenotype was consistent with in vivo immune responses, a protein immunization model in an adoptive transfer system, using TCR-transgenic T cells transferred into wild type animals was used, as well as the direct protein immunization of wild type animals. In the adoptive transfer model, B10.A mice were immunized subcutaneously with whole pigeon cytochrome-c (PCC) protein. Twenty-four hours later, naïve 5C.C7 TCR-

transgenic T cells were adoptively transferred into the immunized animals (Fig. 3.8). Five days after the adoptive transfer, cells were harvested from draining lymph nodes and analyzed. Of the recovered, adoptively transferred $CD4^+$ T cells, there was significantly higher rates of trogocytosis in the PCC-immunized animals (averaging 19.4% $trog^+$) compared to PBS-injected controls (averaging 0.28% $trog^+$) (Fig. 3.8A). Based on CD69 upregulation and TCR downmodulation, both $trog^+$ and $trog^-$ adoptively transferred $CD4^+$ T cells had recognized Ag and were activated (Fig. 3.8B). Consistent with the *in vitro* results in Fig. 3.1, the $trog^+$ $CD4^+$ T cells showed trends of higher activation and TCR downmodulation compared to $trog^-$ $CD4^+$ T cells from the same animal (Fig. 3.8B). Within the activated ($CD69^{High}$) $CD4^+$ T cells, there was a significantly higher frequency of $trog^+$ IL-$4^+$ cells than $trog^-$ IL-$4^+$ cells (Fig. 3.8C). On average, 15% of $trog^-$ cells and 11% of $trog^+$ cells were IFN$\gamma^+$ (Fig. 3.8C), resembling the phenotypes observed at 48 hours following recovery from the *in vitro* trogocytosis assay (Fig. 3.2D). Also, consistent with the phenotype of cells recovered from the *in vitro* trogocytosis assays (Fig. 3.2C), the expression of IL-4 on a per-cell basis, was higher in $trog^+$ cells than similarly activated $trog^-$ cells (Fig. 3.8D).

**Figure 3.8 A-D**

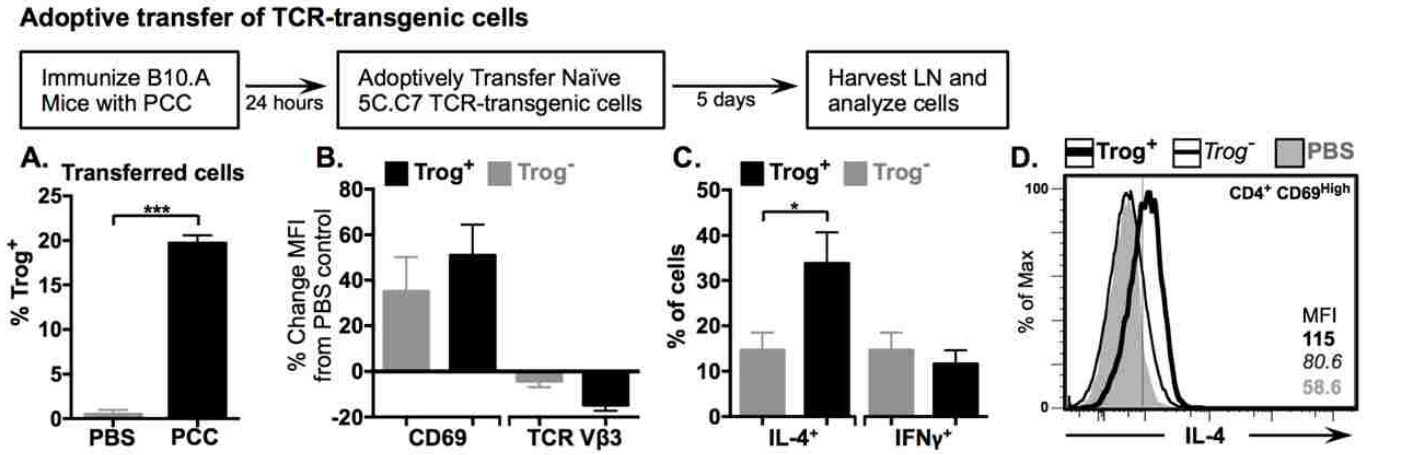



**Figure 3.8 (A-D). Adoptively-transferred *in vivo*-generated trog⁺ CD4⁺ T cells display a $T_H2$ phenotype.** B10.A mice (n=3) were immunized s.c. with 300 µg of PCC protein, or PBS as a control. Twenty-four hours after immunization, 2 x $10^6$ naïve CFSE-labeled 5C.C7 T cells were adoptively transferred into immunized B10.A recipients. Cells were harvested and analyzed via flow cytometry 5 days following adoptive transfer. **(A)** Mean frequency of trog⁺ cells from recovered adoptively transferred cells in PBS injected *(grey)* and PCC immunized *(black),* mice. **(B)** Mean percent change in CD69 *(left)* and TCR Vβ3 *(right)* expression in trog⁺ *(black)* and trog⁻ *(grey)* recovered, adoptively transferred CD4⁺ cells, compared to recovered, adoptively transferred CD4⁺ cells, from PBS control animals as determined by in MFI. **(B)** Frequency of IL-4⁺ *(left)* and IFNγ⁺ *(right)* trog⁺ *(black)* and trog⁻ *(grey)* CD4⁺ $CD69^{High}$ adoptively transferred cells recovered from PCC immunized animals **(D)** Representative histogram of intracellular IL-4 expression of trog⁺ *(thick black line)* and trog⁻ *(thin black line)* CD4⁺ $CD69^{High}$ adoptively transferred cells recovered from PCC immunized animals, compared to adoptively transferred CD4⁺ T cells recovered from PBS control animals *(shaded grey)*. Dotted vertical lines represent the maximum fluorescence intensity of 99% of CD4⁺ cells stained with respective isotype controls In Figs. A-C, Error bars represent ±SEM from three biological replicates, * = p≤ 0.05 and *** = p≤ 0.001. Data is representative of at least two separate experiments.

In a parallel set of experiments, non-transgenic C57BL/6 mice were immunized with chicken ovalbumin (OVA), followed by a booster immunization 14 days later. $CD4^+$ T cells were recovered from draining lymph nodes five days after the second immunization. These time-points were chosen to reintroduce antigen at the end of the effector stage of the immune response, but before establishment of a stable memory population. OVA-immunized animals had significantly higher frequencies of $trog^+$ $CD4^+$ T cells compared to PBS-injected control mice (Fig. 3.8E). Consistent with our previous study [81], the $trog^+$ cells displayed sustained survival *ex vivo*, as the frequency of isolated $trog^+$ $CD4^+$ T cells from OVA-immunized mice increased from 8% on the day of harvest to nearly 60% after a five-day *in vitro* incubation (Fig. 3.8D). Similar to the results with the TCR-

transgenic model, the trog$^{+}$ CD4$^{+}$ T cells were more activated than trog$^{-}$ CD4$^{+}$ T cells as determined by CD69 staining (Fig. 3.8F). Within the activated (CD69$^{High}$) CD4$^{+}$ cell populations, the trog$^{+}$ cells displayed increased expression of GATA-3 and IL-4, whereas their expression in trog$^{-}$ cells from the same animal was nearly identical to that of CD4$^{+}$ T cells recovered from PBS-control animals (Fig. 3.8F). Similar to results from *in vitro* and in vivo TCR-transgenic experiments (Figs. 3.2, 3.8C), the frequency of GATA-3$^{+}$ CD4$^{+}$ cells was significantly higher in the trog$^{+}$ cells compared to the trog$^{-}$ cells (Fig. 3.8G). Consistent with these results, of cells recovered from OVA-immunized mice, the frequency of IL-4$^{+}$ cells was significantly higher in CD69$^{High}$ trog$^{+}$ cells, at nearly 50%, compared to the CD69$^{High}$ trog$^{-}$ cells, of which 22.3% were IL-4$^{+}$. (Fig. 3.8H). Collectively, the results in Fig. 3.8 provide strong corroboration of the results obtained from the *in vitro* experiments, as trog$^{+}$ CD4$^{+}$ T cells generated in vivo displayed enhanced survival *in vitro,* and displayed greater activation, as well as increased GATA-3 and IL-4 expression, compared to trog$^{-}$ cells from the same animal. These results support the hypothesis that trogocytosis-mediated signaling may play a role in $T_H2$ differentiation *in vivo.*

**Figure 3.8 (E-H)**

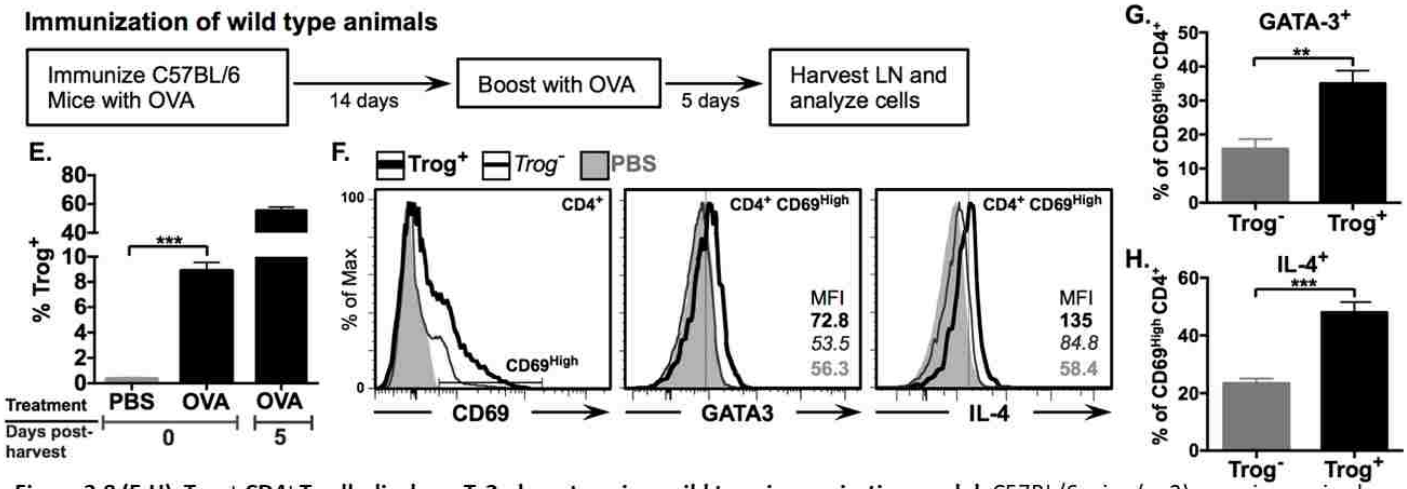



**Figure 3.8 (E-H). Trog$^+$ CD4$^+$ T cells display a $T_H2$ phenotype in a wild type immunization model.** C57BL/6 mice (n=3) were immunized s.c. with 300 µg of OVA protein or PBS as a control and boosted with OVA 14 days later. Draining lymph nodes were collected five days following booster immunization (day 19) and analyzed immediately. An aliquot of recovered cells was cultured post-recovery and analyzed on day five. **(E)** Mean frequency of trog$^+$ CD4$^+$ T cells from PBS injected *(left)* and OVA-immunized *(center)* mice immediately following recovery. *(Right)* The frequency of trog$^+$ CD4$^+$ T cells from OVA-immunized mice after a day five culture *in vitro*. **(F)** Representative histogram overlays showing levels of CD69 in CD4$^+$ T cells *(left)* and the expression of GATA-3 *(middle)* and IL-4 *(right)* in trog$^+$ *(thick black line)* and trog$^-$ *(thin black line)* CD69$^{High}$ CD4$^+$ T cells. CD4$^+$ T cells from PBS control mice are shown in shaded grey. **(G-H)** Frequency of (G) GATA-3 and (H) IL-4$^+$, trog$^+$ *(black)* and trog$^-$ *(grey)* CD69$^{High}$ CD4$^+$ T cells recovered from OVA immunized mice. For histograms, dotted vertical lines represent the maximum fluorescence intensity of 99% of CD4$^+$ cells stained with respective isotype controls. In Figs. E, G and H, error bars represent ±SEM from three biological replicates, * = p≤ 0.05, ** = p≤ 0.01 and *** = p≤ 0.001. Data is representative of at least two separate experiments.

## Discussion

Trogocytosis by CD4$^+$ T cells results in the presence of functional, APC-derived molecules, including MHC:peptide complexes, on the surface of the trog$^+$ T cell. Many of these acquired molecules are not expressed endogenously by the T cell, but they clearly have an impact on T cell biology. This has been demonstrated by the ability of trog$^+$ cells to impact the activation of other T cells through the presentation of trogocytosed molecules [372, 420]. We have found that trogocytosed molecules are also engaged by cognate receptors on the trog$^+$ T cell [81], however the biological implications of this phenomenon are largely unknown. Because trogocytosis commonly occurs during the activation of CD4$^+$

T cells, it is important to develop a comprehensive understanding of the biological consequences of this event.

In this study, we examined the impact of sustained, trogocytosis-mediated signaling on the activation, effector cytokine production, and differentiation of the trog$^+$ T cell. trog$^+$ cells have sustained TCR proximal signaling for at least 72 hours after APC removal, consistent with cell-autonomous signaling resulting from engagement of the receptors on the T cell by trogocytosed molecules (Fig. 3.1). This sustained signaling was not due to T:T presentation or the presence of contaminating APC, as only the trog$^+$ cells maintained a phenotype consistent with active TCR signaling and sustained activation, despite the cultures containing both trog$^+$ and trog$^-$ cells throughout the incubation period. This conclusion is further supported by images showing that active TCR signaling occurred proximal to trogocytosed molecules on the surface of trog$^+$ cells, 72 hours after removal from APC (Fig. 3.5). Similar to our previous studies, the sustained signaling led to preferential survival of the trog$^+$ cells, as the frequency of CD4$^+$ cells that were trog$^+$ increased from roughly 25% immediately after recovery from the trogocytosis-assay, to nearly 80% 72 hours later (Fig. 3.1C, [81].

Because the trog$^+$ cells had sustained TCR signaling and remained activated 72 hours after APC removal, we investigated whether trogocytosis-mediated signaling might impact the effector cytokine production of these cells. Intracellular

cytokine staining of cells 5 hours after recovery from the trogocytosis-assay showed that a significantly higher frequency of trog$^-$ cells were IFN$\gamma^+$ compared to trog$^+$ cells. However, IFN$\gamma$ levels decreased to resting levels in both trog$^-$ and trog$^+$ cells over a subsequent 72-hour incubation (Fig. 3.2B,C). While the frequency of trog$^-$ IL-4$^+$ cells remained at approximately 5% over the 72-hour incubation, the average frequency of trog$^+$ IL-4$^+$ cells increased from 10% at 5 hours, to over 70% at 72 hours (Fig. 3.2D). Because the trog$^+$ cells also displayed enhanced survival after APC removal (Fig. 3.1C), the trog$^+$ cells accounted for over 98% of the total IL-4$^+$ CD4$^+$ cells at 72 hours. The increase in the frequency of trog$^+$ IL-4$^+$ cells over the 72-hour incubation was likely not due to increased proliferation of the trog$^+$ cells, as the amount of trogocytosed molecules on the trog$^+$ cells remained constant and was not diluted, as would be expected for dividing cells. In addition, our previous study showed no discernable proliferation of trog$^+$ cells, up to 5 days after removal from APC [81]. These results are consistent with the observations that trogocytosed molecules are retained in a punctate spot on the membrane of the trog$^+$ cell (Fig. 4, [81, 287]. Thus, the data suggest that trogocytosis-mediated signaling led to sustained survival of IL-4$^+$ cells, and/or directly impacted the IL-4 expression in the trog$^+$ cells.

If the trogocytosis-mediated signaling was simply sustaining the survival of IL-4-expressing cells, the apparent increase in IL-4$^+$ trog$^+$ cells after APC removal might be due to a difference in the ability of $T_H1$ and $T_H2$ cells to perform

trogocytosis. We found that when *in vitro* polarized $T_H1$ and $T_H2$ cells and non-polarized T cells were compared, the $T_H2$ polarized cells were indeed more efficient at performing trogocytosis (Fig. 3). However, the difference in the efficiency of trogocytosis alone isn't sufficient to account for the observed phenotypes. While the frequency of trog$^+$ IL-4$^+$ cells increased, the frequency of trog$^+$ IFN$\gamma$ producing cells decreased from 10% at 5 hours after recovery to 0.5% at 72 hours, suggesting that trogocytosis-mediated signaling was not simply boosting global intracellular signaling and enhancing the survival of all trog$^+$ cells. If that were the case, the frequency of IFN$\gamma$-expressing and IL-4-expressing cells would be expected to remain relatively stable. Rather, our results suggested that trog$^+$ cells were differentiating into $T_H2$ (GATA-3$^+$ IL-4$^+$) cells after the trogocytosis-assay. While robust GATA-3 expression was detected in trog$^+$ cells by 72 hours (Fig. 6), anti-CD3 + anti-CD28 stimulation of an aliquot of the unstimulated T cell blasts used in the trogocytosis-assay did not result in a similar $T_H2$ phenotype (Fig. 4A). Furthermore, T cell blasts immediately prior to the trogocytosis assay displayed a relatively homogeneous $T_H$ 0/$T_H1$ phenotype (Figs. 3.2, 3.3, 3.6). This is consistent with the inherent bias towards a $T_H1$ phenotype possessed by the 5C.C7 TCR transgenic mice used in our experiments [453, 454], and may explain the rapid IFN$\gamma$ production, and delay in IL-4 expression, observed in the cells after recovery from the trogocytosis-assay.

To eliminate the possibility that the observed IL-4 production in the trog$^+$ cells was induced by residual signaling received from the T:APC interaction and

directly examine the role of trogocytosis-mediated signaling in the observed $T_H2$ phenotype of trog$^+$ cells, the reversible Lck inhibitor PP2 was used to halt TCR signaling after APC removal, then washed out to allow trogocytosis-mediated signaling to resume. We found that after PP2 removal, IFN$\gamma$ expression did not resume in either trog$^+$ or trog$^-$ cells (Figs. 3.4D, 3.4E, S3.3), consistent with IFN$\gamma$ production being induced by interactions with APC, and not induced further by trogocytosis-mediated signaling. In contrast to IFN$\gamma$, the frequency of trog$^+$ IL-4$^+$ cells from PP2-treated cultures rebounded to levels equal to untreated cells by 24 hours after PP2 removal (Fig. 3.4E), and robust IL-4 production was observed in trog$^+$ cells 72 hours after PP2 treatment (Figs. 3.4B, C). These significant findings show that trogocytosis-mediated signaling was sufficient to drive IL-4 expression in trog$^+$ cells. The results showing that *in vitro* $T_H1$ polarized trog$^+$ cells began expressing IL-4 and GATA-3, while at the same time decreasing expression of IFN$\gamma$ and T-bet (Fig. 3.7), further support that, at least in absence of external stimuli, trogocytosis-mediated signaling promotes $T_H2$ differentiation. Although it is possible that a portion of the trog$^+$ blasts generated under $T_H1$-polarizing conditions were not fully differentiated to $T_H1$ prior to use in the trogocytosis assay, the unique population of double-positive cells expressing both IFN$\gamma$ and IL-4 (Fig. 3.7B), and the transcription factors T-bet and GATA-3 (Fig. 3.7F), was only apparent within the trog$^+$ cells generated under $T_H1$-polarizing conditions. Thus, the data in Fig. 3.7 supports the possibility that trogocytosis-mediated signaling is capable of inducing $T_H1$ to $T_H2$ conversion. That a greater frequency of $T_H2$ polarized trog$^+$ cells produced IL-4 than Ab-

stimulated $T_H2$ polarized cells at 72 hours (Fig. 3.7C, D), further supports the hypothesis that trogocytosis-mediated signaling is potent in driving IL-4 expression. Taken together, the data presented here are consistent with the concept that trogocytosis-mediated signaling can drive the differentiation of $CD4^+$ T cells towards a $T_H2$ phenotype.

The $T_H2$ phenotype observed *in vitro* with $trog^+$ cells was also apparent in in vivo immune responses. Using TCR-transgenic or wild type cells, and with different antigen sys$T_{EM}$s, we observed that in vivo derived $CD4^+$ $trog^+$ cells expressed IL-4 and GATA-3 at greater levels, and higher cell frequencies, compared to $trog^-$ cells from the same animal (Fig. 3.8B, C). The observed $T_H2$ phenotype of in vivo $trog^+$ cells was less robust than the phenotype developed in *in vitro* assays, however, this is consistent with findings that in some cases, GATA-3 expression in $T_H2$ $CD4^+$ T cells is less pronounced *in vivo* than *in vitro* [143]. The observed phenotype may also be attributed to the inherent nature of the mice used in our study towards $T_H1$, which, consequently, further underscores the significance of the $T_H2$ phenotype developed by $trog^+$ cells in this study. Further studies are underway to characterize the $T_H2$ phenotype of in vivo-generated $trog^+$ cells at additional time-points, and after immunization with $T_H1$-inducing components.

It is interesting to speculate on the mechanisms leading to the observed association between $CD4^+$ $trog^+$ cells and a $T_H2$ phenotype. It has been found that the strength, duration, and “summation” of TCR and costimulatory molecule

signaling can substantially impact T helper differentiation [143, 157, 455, 456]. In non-differentiated cells, it is possible that immune synapses that result in trogocytosis may be of shorter duration and/or generate weaker TCR signaling. This would be consistent with observations that weaker TCR signaling drives early IL-4 production by T cells [156, 157, 173, 457]. Because only a fraction of the APC molecules involved in the immunological synapse are transferred to the T cell, trogocytosis-mediated signaling is likely weaker than signaling at the synapse. This signaling could further promote IL-4 production, consistent with the increased levels of IL-4 observed in trog$^+$ cells over a 72-hour incubation (Figs. 3.2, 3.4, 3.7). The $T_H1$ to $T_H2$ conversion observed with $T_H1$ polarized trog$^+$ cells (Fig. 3.7) further supports this model, as weak TCR-signaling drives $T_H2$ differentiation, even under $T_H1$- polarizing conditions [139]. In contrast, IFN$\gamma$ production and $T_H1$ differentiation have been shown to require strong TCR-signaling [82, 140, 141].

The differences in trogocytosis efficiency between polarized $T_H1$ and $T_H2$ cells may also be attributed to morphological differences in the immunological synapse formed. We have previously shown that at low Ag concentrations, $T_H1$ synapses form the classical “bull’s-eye” shape, while $T_H2$ cells form multi-focal synapses [83]. In separate live-cell imaging experiments, we have observed that small “packets” of MHC:peptide are transferred from APC to non-polarized T cells from the immunological synapse, before becoming localized to a punctate spot at the distal pole of the T cell membrane [31]. It is inviting to speculate that

the multi-focal synapses formed by $T_H2$ cells facilitate trogocytosis much more efficiently than the synapses formed by $T_H1$ cells, although that was not directly tested here.

There are many biological implications of trogocytosis-mediated signaling driving and/or augmenting a $T_H2$ phenotype, while also antagonizing a $T_H1$ phenotype. Such implications are amplified when considering that trog$^+$ cells display sustained survival, along with enhanced activation and effector cytokine production (Figs. 3.1, 3.2, [81]). The $T_H2$ phenotype itself may contribute to the enhanced survival displayed by trog$^+$ cells (Fig. 3.2C, [81]), as IL-4 has been found to enhance CD4$^+$ survival both *in vitro* and in vivo [458]. Additionally, a $T_H2$ phenotype may aid in the heightened activation commonly observed in trog$^+$ cells, as $T_H2$, but not $T_H1$ cells, have been shown to be able to revert from an anergic state to resume effector functionality [459]. It is possible that trog$^+$ $T_H2$ cells may significantly aid in the generation of B cell germinal centers, and/or increase the quality and duration of protective antibody generation when Ag is limited. On the other hand, the low trogocytic potential of $T_H1$ cells, and the $T_H2$ phenotype induced by trogocytosis-mediated signaling, could act as checkpoint to limit unwanted $T_H1$-associated inflammation after Ag clearance.

In cases where a $T_H2$ response is undesirable, excessive CD4$^+$ trogocytosis may play a role in exacerbating $T_H2$-mediated autoimmune diseases such as SLE and rheumatoid arthritis, heighten allergic reactions, or negatively impact protective

cell-mediated responses. In a study by Brown *et al.,* $CD4^+$ T cells from patients with multiple myeloma showed increased rates of trogocytosis and the $trog^+$ cells displayed inhibitory effects on proliferation of stimulated T cells [284]. The authors proposed that trogocytosis might play a role in tumor-induced immune suppression through T-cell fratricide and deletion in patients with multiple myeloma. It is possible that in tumor environments where antigen is presumably abundant, a high frequency of $CD4^+$ trogocytosis and subsequent $T_H2$-differentiation/conversion could significantly suppress an anti-tumor response. Such suppression may be attributed to the inhibition of anti-tumor promoting $T_H1$ cell differentiation by trogocytosis-mediated signaling, and the high IL-4 production by $trog^+$ cells, as IL-4 has been shown to both inhibit IFN$\gamma$ production and prevent activation of naïve T cells [460].

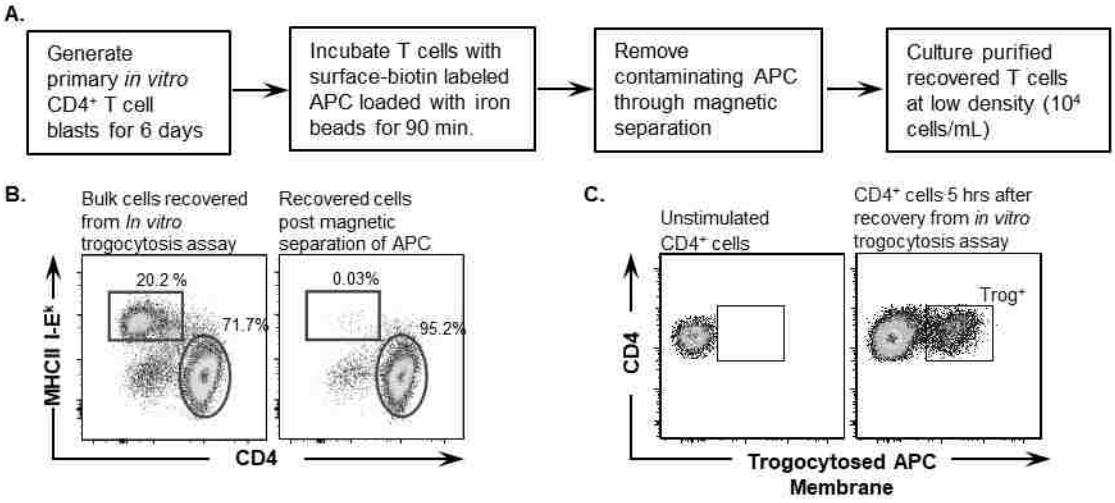
Beyond driving a $T_H2$ phenotype, continual trogocytosis-mediated signaling may aid in the generation of $CD4^+$ memory and/or $T_{FH}$ cells, as both subsets require sustained-TCR signaling through repeated Ag-encounter for their differentiation [142, 143]. As $T_H2$ to $T_{FH}$ conversion has been found to take between 5 to 7 days to occur in vivo [238], examining the phenotype of $trog^+$ cells at extended time-points is likely necessary to determine this possibility. Studies are currently underway in our lab to examine the potential role of trogocytosis-mediated signaling in the generation of both $T_{FH}$ and memory $CD4^+$ T cells.

In conclusion, the results from this study provide further insight into the role of trogocytosis and trogocytosis-mediated signaling in the activation, effector cytokine production and differentiation of $CD4^+$ T cells. We report a strong association between $CD4^+$ trogocytosis and a $T_H2$ phenotype, which is twofold, as $T_H2$ cells are highly efficient at performing trogocytosis, while trogocytosis-mediated signaling induced $T_H2$ differentiation in both non-polarized, and polarized $T_H1$ cells. We propose a model for trogocytosis-mediated $CD4^+$ differentiation in which trogocytosed MHC:peptide complexes and costimulatory molecules sustain intracellular signaling by engaging their cognate receptors on the $trog^+$ T cell. The relatively weak intensity of this signaling leads to early IL-4 production, which is sustained by trogocytosis-mediated signaling. In the presence of IL-4, the sustained trogocytosis-mediated TCR-signaling drives GATA-3 expression, and thus, $T_H2$ differentiation [450]. Because $trog^+$ $CD4^+$ possess the unique ability to remain activated independently of further APC encounter via trogocytosis-mediated signaling, results from this study raise the possibility that $CD4^+$ trogocytosis may play a role in augmenting, or inducing a $T_H2$-dominant immune response.

**Acknowledgments**

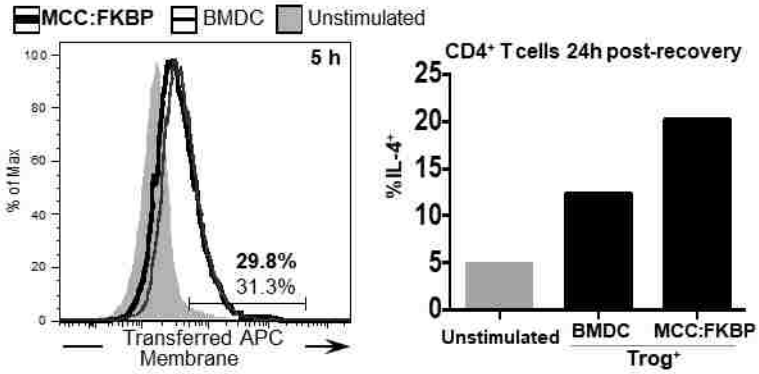
We thank the University of Montana Fluorescence Cytometry core and the core staff scientist Pam Shaw, for expert technical assistance with flow cytometry. We also thank Dr. Jay Evans, and Dr. Alyson Smith and Center for Translational Medicine for the use of their LSRII and for helpful discussions. In addition, we thank the University of Montana Molecular Histology and Fluorescence Imaging Core and the core staff scientist Lou Herritt for expertise and technical assistance with imaging experiments. Finally, we would like to thank Dawit Mengistu, Morgan Stark, Dr. Shannon Miller, and Dr. Mike Minnick for critical review of the manuscript.

## Figure S3.1



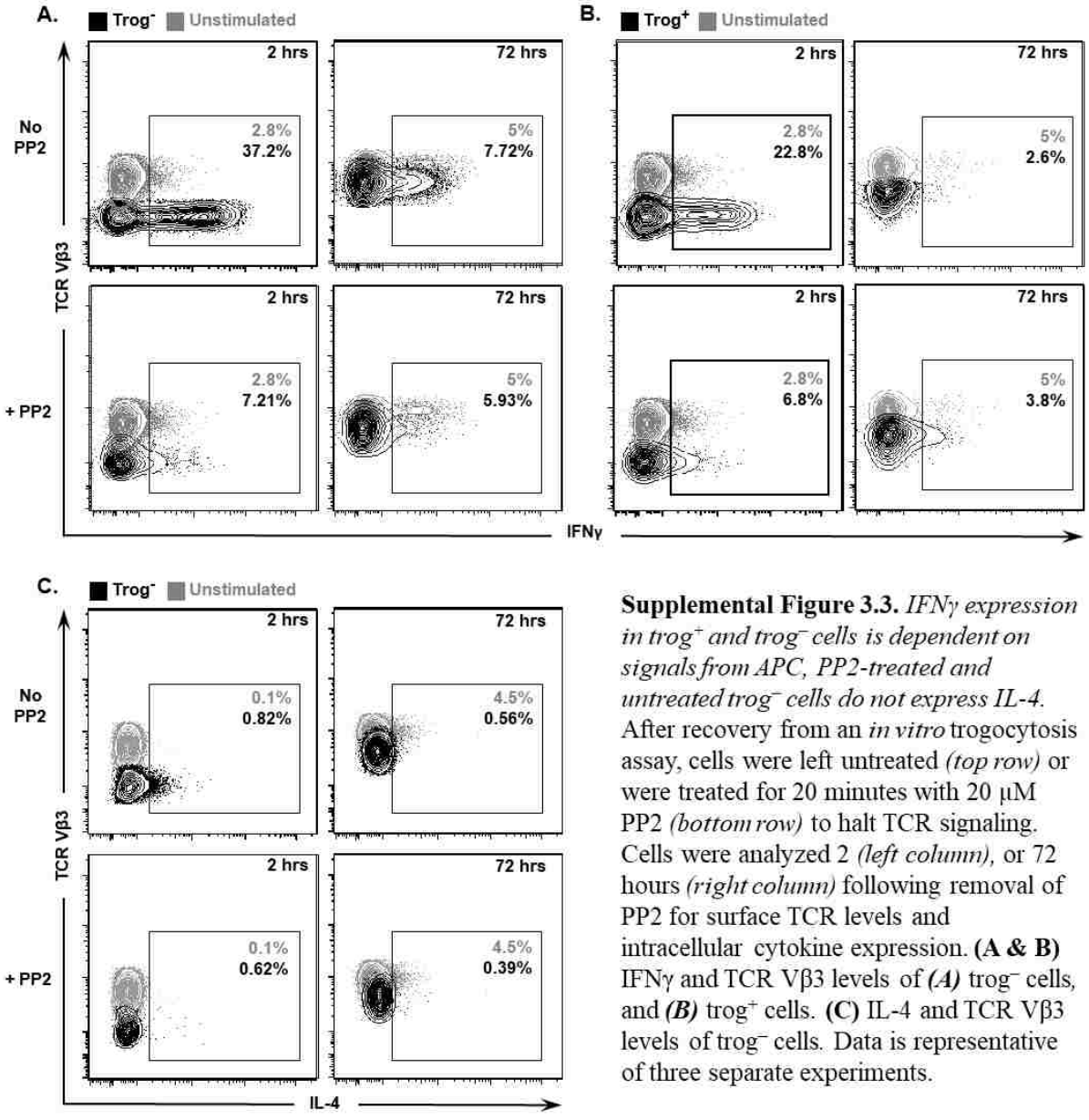

**Supplemental Figure 3.1.** *Cultures of recovered cells do not contain APC, and trog⁺ cells are identified by presence of APC membrane* **(A)** Schematic timeline of a standard *in vitro* trogocytosis assay. **(B)** Purity of CD4⁺ T cells following recovery from an *in vitro* trogocytosis assay. APC shown in square box and CD4⁺ cells are shown in the oval gate. Plots show recovered samples pre-magnetic separation *(left)* and post-magnetic separation of APC *(right)*. **(C)** Identification of trog⁺ cells as determined by the presence of trogocytosed APC membrane.

## Figure S3.2



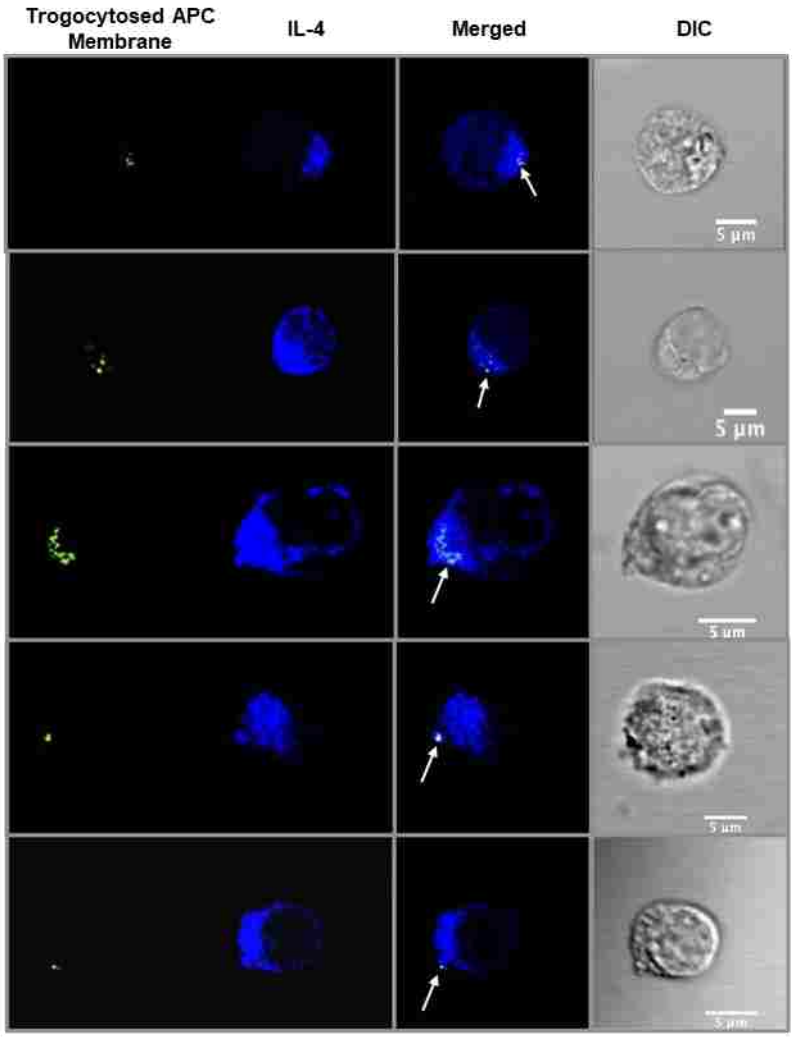

**Supplemental Figure 3.2.** *CD4⁺ T cells perform trogocytosis at similar frequencies and trog⁺ cells express IL-4 using BMDC and MCC:FKBP fibroblasts.* BMDC and MCC:FKBP fibroblasts were used as APC in parallel standard *in vitro* trogocytosis-assays. **(Left)** Histograms showing frequency, and levels of trogocytosed APC membrane from BMDC *(thin grey line) and* MCC:FKBP fibroblasts *(thick black line)*, with unstimulated cells *(shaded grey)* for comparison. **(Right)** Frequency of unstimulated IL-4⁺ cells *(grey),* IL-4⁺ trog⁺ cells recovered from a standard trogocytosis-assay using MCC-pulsed BMDC (*middle)* and MCC: FKBP fibroblasts *(right),* 24 hours post-recovery.

**Figure S3.3**



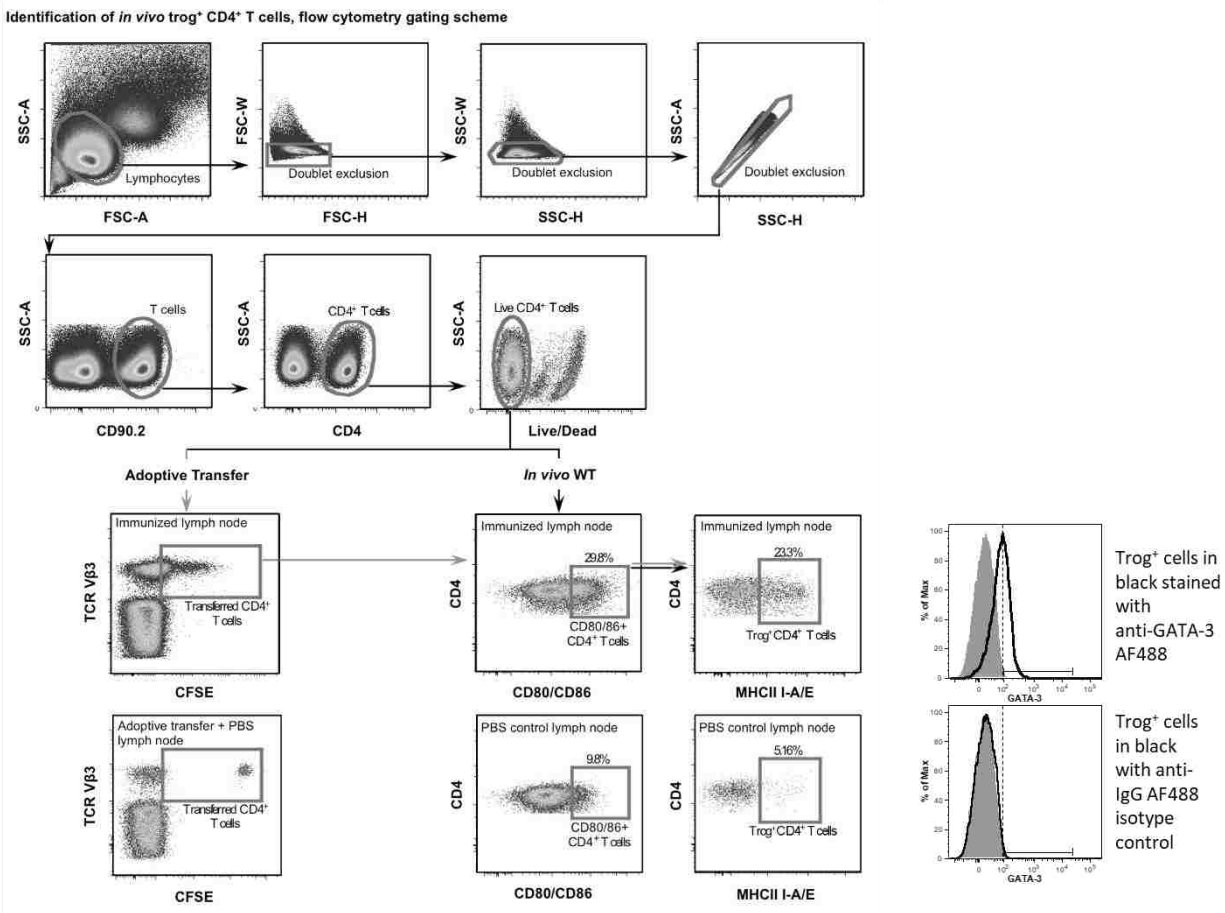

**Supplemental Figure 3.3.** *IFNγ expression in trog⁺ and trog⁻ cells is dependent on signals from APC, PP2-treated and untreated trog⁻ cells do not express IL-4.* After recovery from an *in vitro* trogocytosis assay, cells were left untreated *(top row)* or were treated for 20 minutes with 20 μM PP2 *(bottom row)* to halt TCR signaling. Cells were analyzed 2 *(left column)*, or 72 hours *(right column)* following removal of PP2 for surface TCR levels and intracellular cytokine expression. **(A & B)** IFNγ and TCR Vβ3 levels of ***(A)*** trog⁻ cells, and ***(B)*** trog⁺ cells. **(C)** IL-4 and TCR Vβ3 levels of trog⁻ cells. Data is representative of three separate experiments.

## Figure S3.4




**Supplemental Figure 3.4.** *Intracellular IL-4 is polarized towards trogocytosed molecules.* Images show trog$^{+}$ cells 72 hours post recovery, with trogocytosed APC molecules (green) and IL-4 (blue)

**Figure S3.5**




**Supplemental Figure 3.5** *Trog+ CD4+ cells are identified in vivo by presence of CD80/86 and MHCII.* Representative gating scheme for the identification of trog+ cells *in vivo* from data acquired by flow cytometry. CD80/86+ and MHCII+ cells were identified using respective isotype controls from identical populations. Light grey arrow shows identification of adoptively transferred cells prior to identifying trog+ cells, when applicable, representative isotype control gating and placement of vertical lines on histograms is shown on the right.

# Chapter 4.
## Trogocytosis-Mediated Signaling Drives a $T_{FH}$ Phenotype with High Memory-Potential

Work funded by R03AI122167 (to S.A.W). Fluorescence Cytometry and Molecular Histology and Florescence imaging core facilities used to perform studies are supported by P30RR033379.

## Introduction

The adaptability of the immune response is largely defined by the differentiation of $CD4^+$ T cells into specialized subsets. Despite extensive research efforts, the mechanisms which govern $CD4^+$ T cell differentiation are not fully understood. This is largely due to variability in events at the immunological synapse which have been found to impact $CD4^+$ differentiation such as TCR affinity [456, 461-465], antigen density and dose [155, 157, 456, 462, 463], TCR dwell time [82, 466], and costimulatory molecule signaling [155, 156, 217, 467-475]. In addition, $T_{FH}$ differentiation and effector-to-memory transition have been shown to require multiple signaling events through subsequent cognate interactions [145, 213, 476, 477]. Further convoluting the process of deciphering pathways behind $CD4^+$ differentiation is the growing evidence for plasticity between various $CD4^+$ subsets [144-148, 178, 451, 452]. Due to the wide spread implications for $CD4^+$ T cell differentiation in infectious and autoimmune disease, allergy and asthma, as well as vaccine and immunotherapy design, understanding the mechanisms behind $CD4^+$ differentiation is critical for public health.

One notable but unappreciated deviation from the canonical model for T cell activation has been the discovery that T cells may act as APC themselves. This is accomplished through the acquisition, and subsequent presentation of APC-derived signaling molecules including peptide:MHC (p:MHC) complexes, and costimulatory molecules such as CD80 and CD86, via trogocytosis [81, 285, 287, 368-372]. Trogocytosed molecules may be fully functional and become properly re-expressed on the surface of trogocytosis-positive ($trog^+$) T cells [278, 411]. Numerous studies have found that $trog^+$ cells are able to impact the activation of responding T cells through the presentation of trogocytosed molecules [369, 371, 372, 415, 420-426]. In addition, we previously reported that $trog^+$ $CD4^+$ T cells are able to perform cell-autonomous signaling through engagement of their receptors with trogocytosed ligands, which we have termed trogocytosis-mediated signaling [81, 412]. In T cell blasts recovered from APC after a 90 min incubation, and cultured without additional stimuli, TCR signaling and a heightened state of activation was maintained for >72 hours in $trog^+$, but not $trog^-$, cells [81, 412]. The $trog^+$ cells subsequently developed a $T_H2$-phenotype and increased expression of IL-4, while the $trog^-$ cells produced IFNγ shortly after removal of APC, but dramatically decreased IFNγ expression until reaching levels comparable to unstimulated cells [412]. These results are consistent with models of TCR signal strength in $T_H2$ differentiation, in which the presumably weaker signal from trogocytosis-mediated signaling had induced IL-4 expression [139, 449, 457, 478]. It was also observed that IL-4 was directed towards trogocytosed molecules in a manner reminiscent of $T_H2$ help of B cells [48]. As

both $T_H2$ and $T_{FH}$ specialize in providing B cell help [21, 179, 479-481], $T_H2$ readily differentiate to $T_{FH}$ [238], and $T_{FH}$ differentiation requires multiple TCR signaling events [213, 214, 231, 479, 482]; it was possible that the observed sustained trogocytosis-mediated signaling could further promote the differentiation of trog$^+$ cells towards $T_{FH}$. Here, we researched this possibility using naïve, and activated TCR-transgenic MCC-specific 5C.C7 CD4$^+$ T cell blasts and APC expressing CD80, and I-E$^k$ with covalently attached MCC, or peptide-pulsed BMDC expressing high levels of CD80, ICOS, CD86, and I-E$^k$. We also examined the phenotype of trog$^+$ cells *in vivo* using adoptively transferred 5C.C7 cells, and pigeon cytochrome c (PCC) or (ovalbumin) OVA in wild type animals.

Our results suggest that trogocytosis-mediated signaling drives a phenotype consistent with $T_{FH}$ (CD69$^+$ PD-1$^+$ CXCR5$^+$ ICOS$^+$ IL-21$^+$) *in vitro*, between 3-5 days post removal from APC. Inhibiting signaling through trogocytosed I-E$^k$ and CD80 with neutralizing antibodies, or removal of antigenic peptide from trogocytosed MHCII, significantly reduced the $T_{FH}$ phenotype. *In vivo*-generated trog$^+$ cells developed a $T_{FH}$ phenotype more rapidly than trog$^-$ cells following immunization with PCC in Sigma Adjuvant System (SAS). The trog$^+$ cells also showed increased expression of IL-6, IL-21, and Bcl-6 compared to similarly activated trog$^-$ cells 5 days post-immunization of WT mice with OVA.

At later time-points the trog$^+$ cells in immunized mice contained a major population that resembled T central memory cells ($T_{CM}$) [483, 484]. Interestingly, early $T_{CM}$-precursors and $T_{FH}$ often share a similar phenotype including increased levels of CXCR5 and Bcl-6 [483, 485], and IL-21 has been found to be critical for T cell memory formation [440, 486-489]. In addition, repeat cognate-Ag interactions that are required for $T_{FH}$ also promote effector-to-memory transition [490]. These similarities raise the possibility that trogocytosis-mediated signaling may promote the formation of memory CD4$^+$ cells, and is supported by the enhanced survival of trog$^+$ cells in absence of APC [81, 412].

Our results suggested that trogocytosis-mediated signaling enhances IL-2 production, proliferation, and survival of trog$^+$ cells after APC removal. The trog$^+$ cells also showed higher expression of the anti-apoptotic protein Bcl-2, and CD127. Interestingly, proliferating trog$^+$ cells appeared to unequally distribute trogocytosed molecules amongst progeny to produce a trog$^+$ and a trog$^-$ daughter cell. The daughter cell that retained the trogocytosed molecules also expressed levels of Tcf1, reminiscent of memory-precursors formed through asymmetric division [491].

Results from this study show that extended trogocytosis-mediated signaling is capable of driving CD4$^+$ T cells to develop a phenotype consistent with $T_{FH}$ after separation from APC. We also report a strong correlation between trogocytosis and memory precursor cells and propose a model in which trogocytosis-mediated signaling maintains the survival of CD4$^+$ T cells which have received weaker

signals from APC. These findings provide additional insights into both $T_{FH,}$ and memory generation in the absence of APC, through the non-conventional mechanism of trogocytosis-mediated signaling.

## Results

### Trog$^{+}$, but not trog-, cells maintain a heightened state of activation after APC removal.

While multiple studies have suggested that repeated cognate interactions with B cells are required for full $T_{FH}$-differentiation, [209, 221-224], it been demonstrated that sustained TCR signaling can drive $T_{FH}$ differentiation independently of B cells [213]. As a critical component in $T_{FH}$ differentiation is sustained TCR/costimulatory signaling, we hypothesized that sustained trogocytosis-mediated signaling may drive and/or augment $T_{FH}$ differentiation after separation from APC. To examine this possibility, *in vitro* trogocytosis assays were performed using T cell blasts from 5C.C7 TCR-Tg mice. Following the trogocytosis-assay, APC were removed resulting in cultures >95% CD4$^{+}$ T cells and <0.1% APC [412]. Recovered T cells were analyzed immediately (day 0), or cultured at low density $10^4$ cells/ml to minimize cell:cell interactions and analyzed 3, and 7 days post-recovery, at 24 h intervals. We previously have shown that both trog$^{+}$ and trog$^{-}$ cells downmodulate TCR levels and increase CD69 levels, suggesting that both populations were similarly activated by APC during the trogocytosis assay [412]. Consistent with those findings; Figure 4.1 shows that the trog$^{-}$ and trog$^{+}$ cells have similar CD69 levels immediately after recovery from the trogocytosis assay with either MCC:FKPB APC or BMDC (Fig. 4.1).

**Figure 4.1**

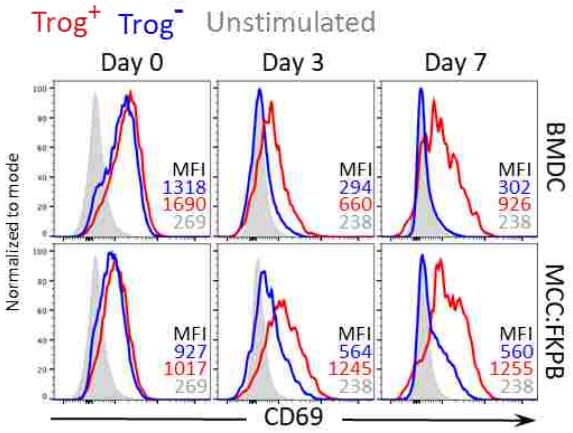



**Figure 4.1. After separation from APC, trog$^+$ cells maintain a heightened state of activation while trog$^-$ cells do not.** 5C.C7 CD4$^+$ T cell blasts were incubated for 90 min with MCC-loaded BMDC or MCC:FKPB APC in a standard *in vitro* trogocytosis assay. Following separation from, and removal of APC, cells were analyzed immediately (Day 0), or incubated at low density ($10^4$ cells/mL) *in vitro* until analysis via flow cytometry at indicated time-points of 24 h intervals. Plots show CD69 expression levels of trog$^+$ (red), trog$^-$ (blue), or unstimulated cells (shaded gray), at indicated time-points post-recovery from the trogocytosis assay with MCC-loaded BMDC (top), or MCC:FKPB APC (bottom). Data is representative of three individual experiments.

As with previous results, the trog$^+$, but not trog$^-$, cells retained high CD69 expression at 3 days and 7 days post recovery (Fig. 4.1). This supports the model of trog$^+$ cells retaining active TCR-signaling and subsequent activation in the absence of APC. A notable difference in the activation state of cells recovered from BMDC or MCC:FKPB APC was observed at day 0. Both the trog$^+$ and trog$^-$ cells recovered from MCC:FKPB fibroblasts had significantly lower levels of CD69 compared to respective populations recovered from BMDC. This is likely due to substantially higher expression of both I-E$^k$ and CD86, and slightly higher levels of CD80 and ICOS-L on BMDC compared to MCC:FKBP (Fig. S4.1). The trog$^+$ cells recovered from MCC:FKPB APC increased CD69 expression between Day 0 and Day 7, while the median CD69 expression of the trog$^-$ cells decreased, by 43% on Day 3, and was only 30% higher than resting cells 5 days after removal of APC. In contrast, trog$^+$ and trog$^-$ cells recovered from BMDC decreased in CD69 expression between Day 0 and Day 3, by 61% or 81% respectively. However, at 7 days post-recovery, the CD69 expression in

trog⁻ cells was unchanged, while on the remaining trog⁺ cells CD69 expression increased by 40% (Fig. 4.1).

### *In vitro*-generated trog⁺, but not trog⁻ cells, develop a $T_{FH}$-like phenotype after separation from APC.

Signaling from the inducible T cell costimulator (ICOS) has been found to play a major role in $T_{FH}$ differentiation, [209, 217, 242, 492, 493]. Immediately after recovery from APC, the trog⁺ cells showed moderately higher levels of ICOS compared to the trog⁻ cells (65% in cells recovered from BMDC, and 34% in cells recovered from MCC:FKBP) (Fig. 4.2, top). At 7 days post-recovery, there was a clear difference in expression in ICOS expression with the trog⁺ cells recovered from BMDC showing a 3.2-fold increase in ICOS levels over trog⁻ cells, and a 2.2-fold increase in cells recovered from MCC:FKBP APC (Fig. 4.2, bottom).

**Figure 4.2**

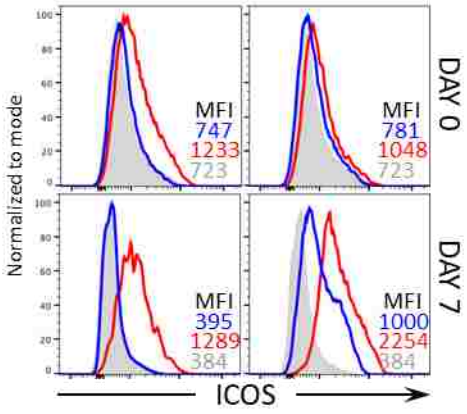



**Figure 4.2 Trog⁺, but not trog⁻ cells express high levels of ICOS 7days post-APC removal.** Cells were stimulated as in Fig. 4.1. Plots show Expression levels of ICOS of trog⁺(red), trog⁻ (blue), or unstimulated cells (shaded gray), at 0 (top) and 7 (bottom) days post-recovery. Data is representative of at three individual experiments.

$T_{FH}$ cells are largely identified by high expression of CXCR5 and PD-1 [494-496], as well as CD69 [497], which was maintained in trog⁺, but not trog⁻ cells (Fig 4.1). Consistent with the ICOS expression levels (Fig. 4.2), and the kinetics of $T_{FH}$-differentiation [498], figure 4.3 shows that immediately post-recovery (Day 0),

a $T_{FH}$-like phenotype, (PD-1+ CXCR5+), was apparent in 9.2% of trog$^{+}$ cells, but only 3% of trog$^{-}$ cells recovered from BMDC (Fig. 4.3, left). Similar levels were observed in cells recovered from MCC:FKBP (7.04% of trog$^{+}$ and 3.54% of trog$^{-}$ cells) (Fig. 4.3, right), suggesting that the higher activation induced by BMDC did not immediately impact CXCR5 and PD-1 surface expression levels. In cells recovered from BMDC, the trog$^{+}$ cells maintained CXCR5 expression at both 3 days and 7 days post recovery, while the frequency of the CXCR5$^{+}$ PD-1$^{+}$ population increased to 15%, and 19% of trog$^{+}$ cells, respectively (Fig. 4.3, left, *red*). In contrast, the trog$^{-}$ cells initially had lower CXCR5 levels at 3 days compared to trog$^{+}$ cells, and by 7 days, CXCR5 expression was similar to unstimulated cells (Fig. 4.3 left, *blue*). Accordingly, only 2.23%, and 0.3% of the trog$^{-}$ cells were PD-1$^{+}$ CXCR5$^{+}$ at 3 and 7 days post-recovery.

The trog$^{+}$ cells recovered from MCC:FKBP APC showed markedly different expression patterns compared to those recovered from BMDC. High CXCR5 expression was maintained in the trog$^{+}$ cells at 3 and 7 days post-recovery, and the frequency of PD-1$^{+}$ CXCR5$^{+}$ cells increased to 26.2% by Day 3 and had reached 57.5% on day 7 (Fig. 4.3, right, *red*). The trog$^{-}$ cells showed a very slight increase from 3.5% at Day 0 to 5.0% by Day 3. By Day 7 the CXCRS$^{+}$ PD-1$^{+}$ cells were only 4.4% of trog$^{-}$ cells (Fig. 4.3, bottom, *blue*).

**Figure 4.3**

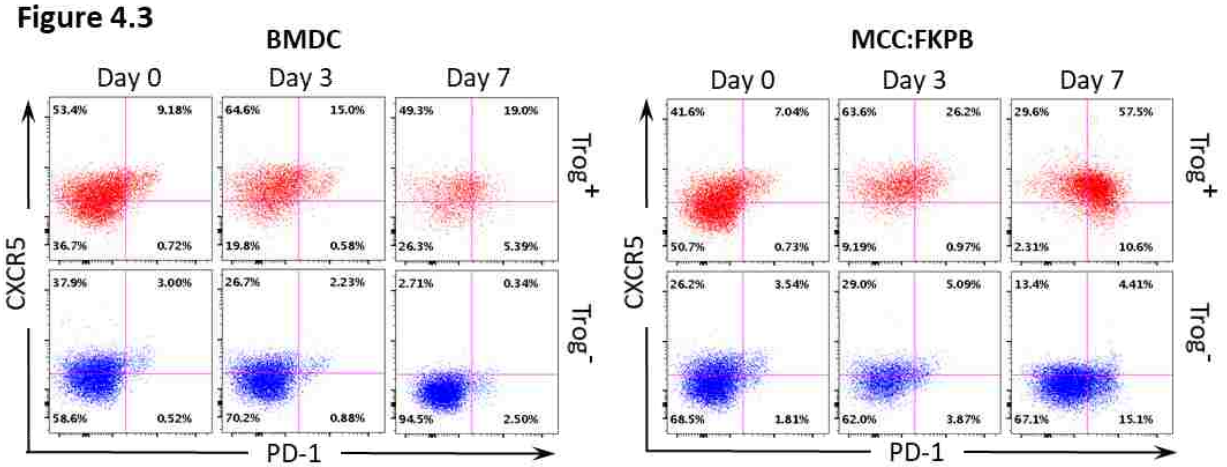



**Figure 4.3 Trog$^+$, but not trog$^-$ cells develop a TFH-phenotype 7 days post- APC removal.** Cells were stimulated as in Fig. 4.1. 2D plots showing PD-1 vs CXCR5 expression levels in trog$^+$ and trog$^-$ cells at indicated time-points post-recovery from BMDC (top) and MCC:FKPB APC (bottom). Numbers in corners represent frequency of cells in respective quadrants. Quadrants were established off of isotype staining of bulk CD4$^+$ cells recovered from the trogocytosis assay, and >97% of unstimulated cells fell in the lower left quadrant at each observed time-point (not shown). Data is representative of at three individual experiments.

Consistent with a $T_{FH}$ phenotype, the trog$^+$ cells from either APC cultures expressed higher levels of Bcl-6 and IL-21 compared to trog$^-$ cells 7 days post-recovery (Fig. 4.4). Consistent with the surface phenotype data (Fig 4.1-3), when trog$^+$ cells from the two different APC cultures were compared, the cells recovered from MCC:FKPB APC expressed higher levels of Bcl-6 and IL-21 (Fig. 4.4A, *right*), compared to trog$^+$ cells recovered from BMDC (Fig. 4.4A, *left*). When BMDC-stimulated populations were compared, a significantly higher frequency of trog$^+$ cells were IL-21$^+$ at days 2, 3, 5, and 7. IL-21 expression by trog$^+$ cells peaked on Day 5, when 29% of the cells were IL-21$^+$ (Fig. 4.4B, *top*). Similarly, when cells recovered from MCC:FKPB APC were compared, a significantly higher percentage of trog$^+$ cells were IL-21$^+$ at each time-point observed from 1 to 7 days post-recovery (Fig. 4.4B, *bottom*).

**Figure 4.4**

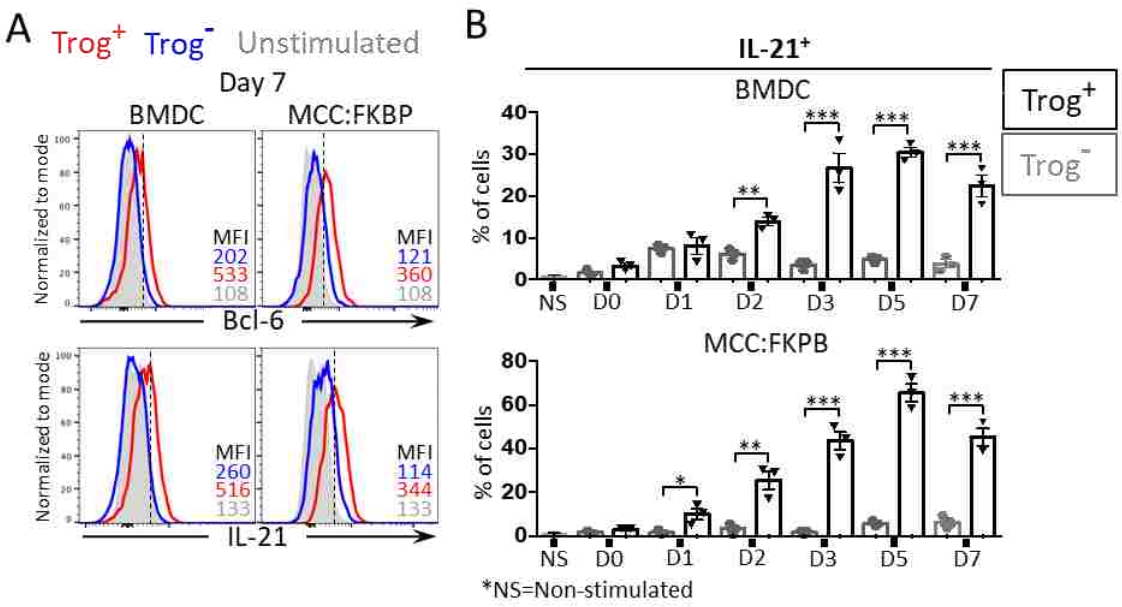



**Figure 4.4 Trog⁺ express higher levels Bcl-6, and IL-21 compared to trog⁻ after APC removal.** Cells were stimulated as in Fig. 4.1. **(A)** Expression of Bcl-6 (top) and IL-21 (bottom), in trog+, trog⁻, or unstimulated cells at day 0 (left) and day 5 (right) post-recovery. **(B)** Frequency of IL-21+ trog+ (black) and trog⁻ (gray), at indicated time points post-recovery from BMDC (top), or MCC:FKBP (bottom). Error bars represent ±SEM where * = P<0.5, ** = P<0.01, *** = P<0.001. Data is representative of at three individual experiments.

Collectively, the data in Figs. 4.1 to 4.4 show that trog⁺, but not trog⁻, cells are highly efficient at developing a CXCR5⁺ PD-1⁺ BCl-6⁺, IL-21⁺ phenotype consistent with $T_{FH,}$ after APC removal. When trog⁺ cells recovered from both BMDC and MCC:FKPB APC were compared, cells recovered from MCC:FKPB showed a more robust $T_{FH}$-like phenotype. Regardless of the APC type, these data strongly suggest that trogocytosis-mediated signaling promotes $T_{FH}$ differentiation in trog⁺ cells.

**Trogocytosis-mediated signaling drives a $T_{FH}$ phenotype**

The data in Figs. 4.2-4.2 show that after removal of APC, a substantial percentage of trog$^{+}$ cells developed a phenotype consistent with $T_{FH}$, while the trog$^{-}$ cells do not. However, initial priming of CD4$^{+}$ T cells by DC may result in a pre-$T_{FH}$ phenotype including the upregulation of CXCR5 to promote T cell migration towards B cell follicles/GC [218]. Therefore, the observed $T_{FH}$-like phenotype in the trog$^{+}$ cells may be due to signals from APC promoting $T_{FH}$ differentiation, and not a direct result of trogocytosis-mediated signaling. To address the impact of trogocytosis-mediated signaling on development of the observed $T_{FH}$ phenotype, CD4$^{+}$ T cell blasts were used in an *in vitro* trogocytosis assay with MCC-loaded BMDC or MCC:FKPB APC. Immediately following recovery from BMDC, peptide was removed from trogocytosed MHC by mild acid elution. Aliquots of acid-stripped cells were then cultured with 10 µM cognate MCC peptide or 10 µM irrelevant hemoglobin (Hb) peptide as a negative control. With cells recovered from MCC: FKPB APC, trogocytosed TCR and CD28 signaling was neutralized using 20 µM anti-I-E$^{k}$ and/or 20 µM anti-CD80 purified antibodies. Because the MCC: FKPB APC do not express CD86 (Fig. S4.1A), antibodies against CD86 were not included.

After recovery on Day 0, cells recovered from BMDC and MCC:FKPB APC exhibited minimal difference in PD-1 and CXCR5 expression on trog$^{+}$ and trog$^{-}$ cells, similar to the Day 0 in figure 4.1 (data not shown). As the $T_{FH}$-phenotype peaked at 5 days post-recovery (Fig. 4.4B) cells were examined at this time-

point. The acid-stripped cells cultured with Hb peptide had substantially decreased TCR signaling as determined by pZAP-70 signaling, compared to non-treated or acid-stripped cells cultured in the presence of MCC (Fig. S4.1B). Similarly, neutralization of I-E$^k$ and CD80 in MCC:I-E$^k$-recovered cultures greatly decreased TCR signaling (Fig. S4.1B).

**Figure 4.5**

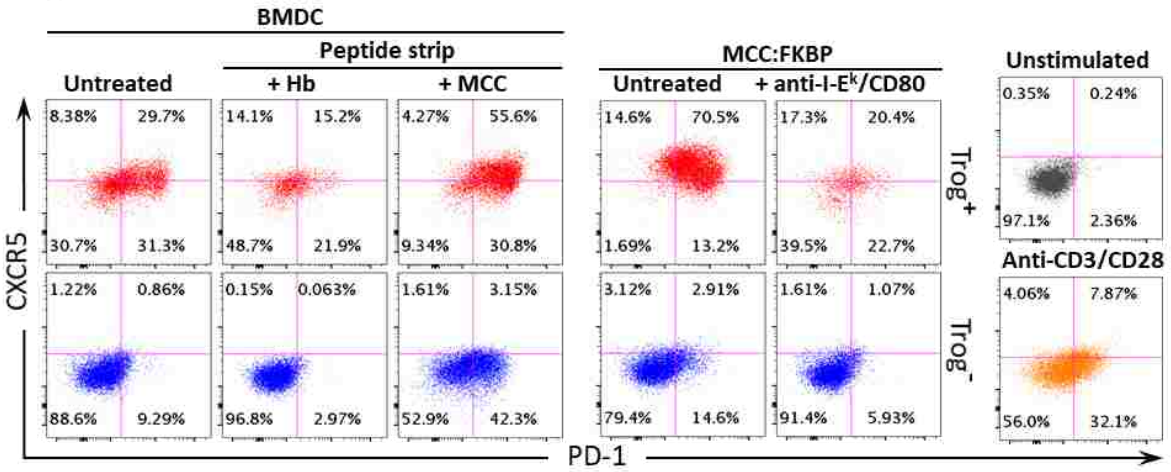



**Figure 4.5. Trogocytosis-mediated signaling drives a $T_{FH}$ phenotype in activated T cells after separation from APC. (A)** 5C.C7 CD4$^+$ T cell blasts were incubated for 90 min with MCC-loaded BMDC (left) or FKPB MCC:I-E$^k$ APC (right), and cultured in vitro after separation from APC until analysis on day 5 via flow cytometry. Plots show PD-1 vs CXCR5 expression in trog$^+$ (top row), and trog$^-$ cells (bottom row) in (BMDC) untreated (far left), peptide-stripped + hemoglobin (Hb) (middle), peptide-stripped + MCC (right), and (MCC:FKPB APC) untreated (left), antibody-neutralized (right) cultures. Unstimulated (top far right) and plate-bound anti-CD3+/anti-CD28 stimulated cells (far right bottom) are shown for comparison. Numbers in corners represent frequencies in respective quadrants. Data is representative of three separate experiments

Figure 4.5 shows that removal of antigenic-peptide had a drastic impact on the phenotype of the trog$^+$ cells, on Day 5, reducing the frequency of PD-1$^+$ CXCR5$^+$ trog$^+$ cells by nearly 50% (15.2% PD-1$^+$ CXCR5$^+$) compared to untreated controls (29.7% PD-1$^+$ CXCR5$^+$) (Fig. 4.5, *left*). On the other hand, addition of MCC to peptide-stripped cells increased the frequency of PD-1$^+$ CXCR5$^+$ cells by 87%, with 55.6% of these cells displaying the $T_{FH}$-like phenotype (Fig. 4.5, *left*). Similar

results were obtained with antibody neutralization as anti-I-$E^k$ and anti-CD80 reduced the frequency of $trog^+$ PD-$1^+$ $CXCR5^+$ cells by 71% to 20.4% PD-$1^+$ $CXCR5^+$ compared to the untreated $trog^+$ cells (70.5% PD-$1^+$ $CXCR5^+$) (Fig. 4.5, *right*). The frequency of PD-$1^+$ $CXCR5^+$ was very low in $trog^-$ cells across the board, ranging from 2.91% in untreated BMDC cultures to 1.07% in Hb-supplemented cultures, to 3.15% in MCC-supplemented cultures. Similarly, of cells stimulated with MCC:FKPB, the frequency of $CXCR5^+$ PD-$1^+$ $trog^-$ cells was 2.91% in untreated cultures and 1.07% in Ab-neutralized cultures (Fig. 4.5, *middle*).

In parallel cultures, T cell blasts were stimulated for 6 hours each day during a 5 day incubation as a control. These cells did not develop a robust PD-$1^+$ $CXCR5^+$ population, consistent with the requirement for IL-21 or IL-6, and costimulatory molecules such as ICOS for $T_{FH}$ differentiation. It also suggests that the $T_{FH}$-like phenotype displayed by the $trog^+$ cells was not simply a result of sustained-signaling TCR and CD28 signaling.

**Trogocytosis-mediated signaling drives a $T_{FH}$ phenotype in naïve T cells in absence of APC**

We next examined whether trogocytosis-mediated signaling could drive a $T_{FH}$-phenotype in $trog^+$ naïve $CD4^+$ cells. Like the activated blasts, $trog^+$ and $trog^-$ naïve cells displayed comparable levels of activation immediately after recovery from the trogocytosis assay (as determined by CD69 expression) (Fig.

4.6). As anticipated, naïve cells were more highly activated by MCC-loaded BMDC compared to MCC:FKBP APC (Fig. 4.6).

**Figure 4.6**

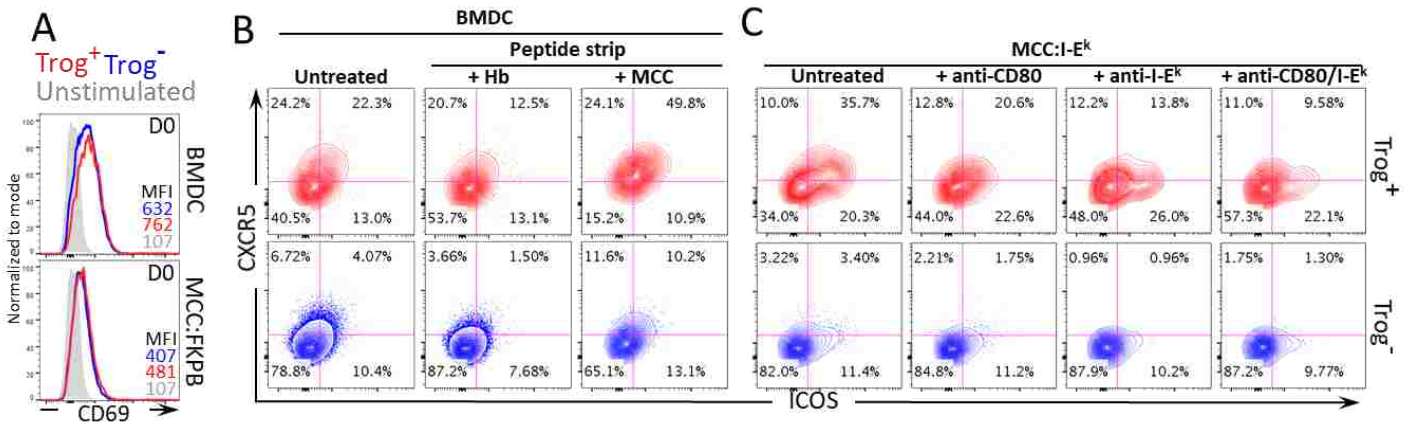



**Figure 4.6. Trogocytosis-mediated signaling drives a $T_{FH}$ phenotype in naïve T cells in absence of APC. (A)** Naïve 5C.C7 CD4$^+$ T cells were incubated for 18 hrs with MCC-loaded BMDC (top) or MCC:FKPB APC (bottom). Plots show CD69 expression immediately after recovery from the trogocytosis assay in trog$^+$ (red), trog$^-$ (blue) and unstimulated (shaded gray) cells. **(B)** Naïve 5C.C7 CD4$^+$ T cells were incubated for 18 hrs with MCC-loaded BMDC (left) or MCC:FKPB APC (right) and cultured in vitro until analysis on day 5 by flow cytometry. Plots show PD-1 vs ICOS expression in trog$^+$ (red), and trog$^-$ cells (blue) in (BMDC) untreated (far left), peptide-stripped +hemoglobin (Hb) (second from left), peptide-stripped + MCC (third from left) and - **(C)** (FKBP MCC:MHCII APC) in untreated (left) anti-CD80 (second from left), anti-I-E$^k$ (second from right), and anti-CD80 + anti-I-E$^k$ (right). Data are representative of at least two separate experiments.

Figure 4.6 shows that approximately 22.3% of the trog$^+$ naïve T cells cultured for 5 days after removal from an 18 hour trogocytosis assay using MCC-loaded BMDC were CXCR5$^+$ ICOS$^+$. In comparison, acid-stripped cells cultured in Hb peptide had a 4.5% decrease in the frequency of trog$^+$ CXCR5$^+$ ICOS$^+$ cells to 12.5% (Fig 4.6, left). Addition of MCC to acid-stripped cultures led to a 2.2-fold increase to 49.8% of the trog$^+$ CXCR5$^+$ ICOS$^+$ cells (Fig. 4.6, left). The trog$^-$ cells in Hb-supplemented peptide-stripped cultures showed a 78% decrease in the frequency of CXCR5$^+$ ICOS$^+$ cells compared to untreated cultures, to 4.07% CXCR5$^+$ ICOS$^+$. MCC-supplementation increased this frequency 2.5-fold to 10.2%, which, while substantially higher compared to trog$^-$ cells from untreated

cultures, was well below the frequency of trog$^+$ cells displaying this phenotype (Fig. 4.6, left)

To assess the individual roles of TCR and costimulatory signaling, neutralizing Abs anti-CD3 and anti-CD28 or anti-CD3 alone or anti-CD28 alone, were added to naïve cells recovered from MCC:FKBP APC as in Fig. 4.3. The data in Fig. 4.6 (right) suggests that TCR signaling through trogocytosed-p:MHC complexes and CD80 signaling both played a role in development of the apparent $T_{FH}$ phenotype. Compared to trog$^+$ cells from untreated cultures, the trog$^+$ cells from cultures with only neutralizing CD80 Abs had a 42% decrease in CXCR5$^+$ ICOS$^+$ cells (Fig. 4.6, *second from left*). The trog$^+$ cells from cultures containing neutralizing anti-I-E$^k$ Abs showed a 61.5% decrease in this population (Fig. 4.6, *second from right*), while the trog$^+$ cells from cultures containing both anti-I-E$^k$ and anti-CD80 showed a further 83% reduction in the phenotype to 9.58% (Fig. 4.6, *far right*), compared to trog$^+$ cells from untreated cultures (Fig. 4.6, *far left*).

**Trog$^-$ cells can develop a $T_{FH}$ phenotype in the presence of APC**

It was apparent that trogocytosis-mediated signaling was playing a role in driving a $T_{FH}$-phenotype in the trog$^+$ cells, however it was also possible that the cells that performed trogocytosis were predisposed to developing the $T_{FH}$ phenotype, and trogocytosis-mediated signaling was simply sustaining their survival. To examine whether the trog$^+$ cells inherently developed a $T_{FH}$-like phenotype more readily than trog$^-$ cells in cases where Ag receptor signaling was not limited to

trogocytosis-mediated signaling, unlabeled MCC:FKBP APC were added back at a 1:10 APC:T cell ratio to stimulate cultures of $CD4^+$ T cell blasts immediately after recovery from an 90 min trogocytosis-assay with biotin-labeled MCC:I-$E^k$ APC. Figure 4.7 shows that addition of MCC:FKBP to cultures after recovery from the trogocytosis assay, increased the frequency of PD-$1^+$ $CXCR5^+$ $trog^–$ cells increased from 5.56% to 22.5%. Interestingly, in these same cultures the frequency of PD-$1^+$ $CXCR5^+$ $trog^+$ cells decreased from 63.7% in untreated cultures, to 38.4% (Fig. 4.7, right). Consistent with previous data, neutralizing antibodies against trogocytosed I-$E^k$ and CD80 led to a 67.2% decrease in the frequency of PD-$1^+$ $CXCR5^+$ $trog^+$ cells compared to cells recovered from untreated cultures (Fig. 4.7, middle).

**Figure 4.7**

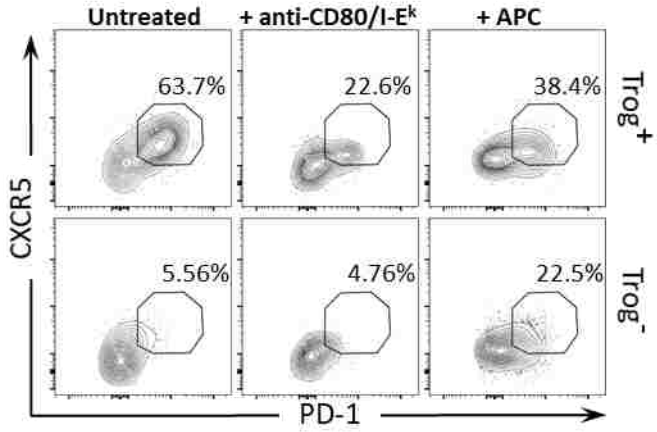



**Figure 4.7. $Trog^-$ cells can develop a $T_{FH}$ phenotype in the presence, but not absence of APC.** Naïve 5C.C7 $trog^+$ and $trog^–$ (bottom) cells 5d post-recovery from APC as in 4.5. Cultures were, untreated (left), supplemented with anti-I-$E^k$ + anti-CD80 neutralizing Abs (middle), or supplemented with APC 1:10 APC. Data is representative of two separate experiments.

### IL-21 expression in $trog^+$ cells is enhanced by trogocytosis-medited signaling *in vitro*

In line with the observed decrease in PD$1^+$ $CXCR5^+$ $trog^+$ cells, after acid-stripping or Ab blockade of trog-mediated signaling (Fig. 4.5), the frequency of IL-$21^+$ $trog^+$ cells at 5 days was significantly reduced by the same treatments (Fig.

4.8). Significantly fewer trog$^+$ cells from peptide-stripped cultures supplemented with Hb were IL-21$^+$ (22.8%) compared to untreated cultures (32.5%). In contrast, the frequency of IL-21$^+$ trog$^+$ cells in acid-stripped and MCC-supplemented cultures increased to 40.3% (Fig. 4.8, *left*). Similarly, neutralizing trogocytosis-mediated signaling with anti-I-E$^k$ and anti-CD80 decreased the frequency of IL-21$^+$ trog$^+$ by 55% (Fig. 4.8, *right*).

**Figure 4.8**

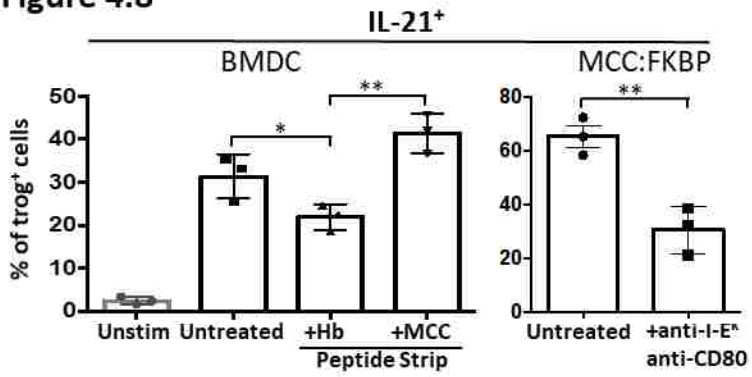



**Figure 4.8. IL-21 expression in trog$^+$ cells is enhanced by trogocytosis-medited signaling *in vitro*.** Frequency of IL-21$^+$ trog$^+$ CD4$^+$ T cells 5 days post-recovery from a 90 min trogocytosis assay as described in Fig. 4.5. Error bars represent ±SEM from three separate experiments. *=p<0.05, **=p<0.01.

### In absence of APC, the development of a $T_{FH}$ phenotype is dependent on trogocytosis-mediated signaling

To confirm that the trog$^+$ cells were developing a $T_{FH}$-like phenotype in absence of APC, naïve 5C.C7 cells recovered from a standard 18 h *in vitro* trogyctosis-assay were FACS-sorted into trog$^+$ and trog$^-$ populations. These purified trog$^+$ and trog- cells were cultured individually *in vitro* and analyzed 72 hours later. At this early time-point, 11.2% of the trog$^+$, but only 1.53% of the trog$^-$ cells recovered from MCC:APC were CXCR5$^+$ PD-1$^+$ (Fig. 4.9, top). Similarly 11.8% of trog$^+$ and 3.36% of trog$^-$ cells acid-stripped cells cultured in MCC peptide were CXCR5$^+$ PD-1$^+$ (Fig. 4.9, middle). Culturing with irrelevant Hb peptide after acid-stripping led to a massive decrease in the frequency of CXCR5$^+$ PD-1$^+$ cells to only 2.72% of the trog$^+$ and 1.75% of the trog$^-$ cells (Fig. 4.9, bottom). Due to low

recovery following the FACS sort, later time-points were not examined in these experiments.

**Figure 4.9**

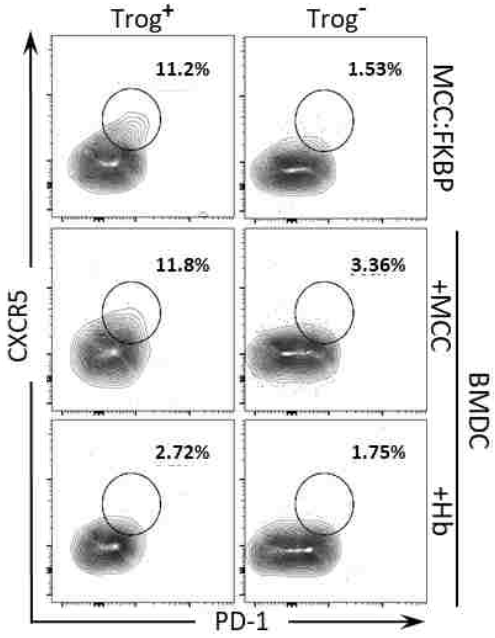



**Figure 4.9. In absence of APC, the development of a $T_{FH}$ phenotype is dependent on trogocytosis-mediated signaling.** Naïve 5C.C7 cells were used in a standard in vitro 18 trogocytosis assay with MCC:FKPB APC or MCC-loaded BMDC. Immediately post-recovery cells were FACS sorted into trog$^+$ and trog$^-$ populations and cultured individually in vitro. The post-sort purity of trog$^+$ cells was, on average 92% trog$^+$, and the trog$^-$ cultures were >98% trog$^-$. Plots show PD-1 vs CXCR5 expression in cells recovered from FACS sorted trog$^+$ (left) and trog$^-$ (right) cultures 96 hrs post-recovery from MCC:FKBP APC (top), and BMDC (middle, bottom). Cells recovered from BMDC were peptide-stripped as in 4.5, and separate aliquots were supplemented with MCC (middle) or Hb (bottom). Data is representative of two separate experiments

### $T_{FH}$-associated cytokines IL-21, IL-4 and IL-6 are polarized towards trogocytosed molecules

We previously reported that IL-4$^+$ trog$^-$ cells, >80% of trog$^+$ cells had IL-4 directed towards trogocytosed molecules, while there was no IL-4 polarization in trog$^-$ cells. To expand those previous studies, here intracellular polarization of IL-6 and IL-21 was examined. As seen in figure 4.9, 3 days post-recovery, IL-21 and IL-6 were directed towards trogocytosed molecules. As $T_{FH}$ cells help cognate B cells through directed secretion of these cytokines, this data strengthens the case for development of a $T_{FH}$-phenotype observed in the trog$^+$ cells.

**Figure 4.10**

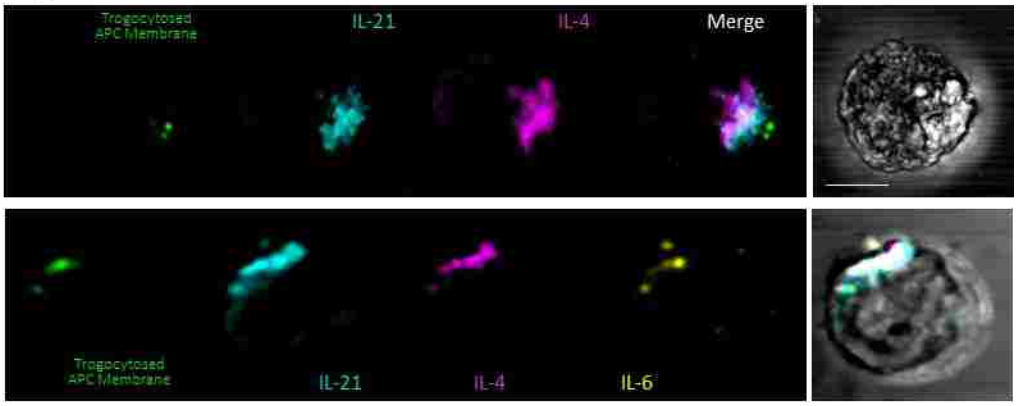



**Figure 4.10. $T_{FH}$-associated cytokines IL-21, IL-4 and IL-6 are polarized towards trogocytosed molecules.** Confocal images of 5C.C7 T cell blasts 72 hrs after a 90 min in vitro trogocytosis assay with MCC:MHC APC. Trogocytosed molecules are shown in green, IL-4 in cyan, IL-21 in magenta, (top), and IL-6 in yellow (bottom). IL-21 data is representative of three separate experiments with 18-30 cells analyzed per experiment. Il-6 data is representative of two separate experiments with 12-14 cells analyzed per experiment. Error bars represent 5 µm. IL-21 data is representative of three separate experiments, and IL-6 is representative of two separate experiments.

### *In vivo*-generated trog$^+$ cells can rapidly display a $T_{FH}$-like phenotype

The *in-vitro* data presented above suggests that trogocytosis-mediated signaling drives development of a $T_{FH}$ phenotype in trog$^+$ cells after separation from APC. To examine whether a similar phenotype occurs in vivo, $10^5$ naïve 5C.C7 CD4$^+$ T cells were adoptively transferred into naïve WT B10.A mice. Six hours later, recipients were immunized S.C. with PCC protein emulsified in Sigma Adjuvant System (SAS). Mice with adoptively transferred cells immunized with SAS alone served as negative controls. Spleens were harvested 3, and 7 days post-immunization and Ag-specific CD4$^+$ T cells were identified by MCC:pMHCII tetramer staining (Fig. S4.1C). As murine CD4$^+$ cells do not endogenously express MHCII, trog$^+$ cells were identified by the presence of trogocytosed

MHCII. Three days post-immunization, 2.3% of MCC:MHCII$^+$ CD44$^+$ cells were PD-1$^+$ CXCR5$^+$ (Fig. 4.11A, *left*) This number increased to 7.4% by 7 days post-immunization (Fig. 4.11A, middle). Transferred cells from mice immunized with SAS alone did not develop a $T_{FH}$-phenotype, as expected in absence of Ag (Fig. 4.11A, *right*). The data in Fig. 4.11B suggests that the transferred trog$^+$ cells were able to more rapidly express PD-1 and CXCR5 compared to transferred trog- cells. Of the PD-1$^+$ CXCR5$^+$ cells recovered 3 days post-immunization, 78.4% were trog$^+$ (Fig. 4.11B, *middle*). However, by 7 days post-immunization, the frequency of CXCR5$^+$ PD-1$^+$ cells that were trog$^+$ had decreased to an average of 37.2% (Fig. 4.11B, *right*). These findings suggest that even in the presence of APC, the trog$^+$ cells were more proficient at rapidly developing a phenotype consistent with $T_{FH}$.

**Figure 4.11**

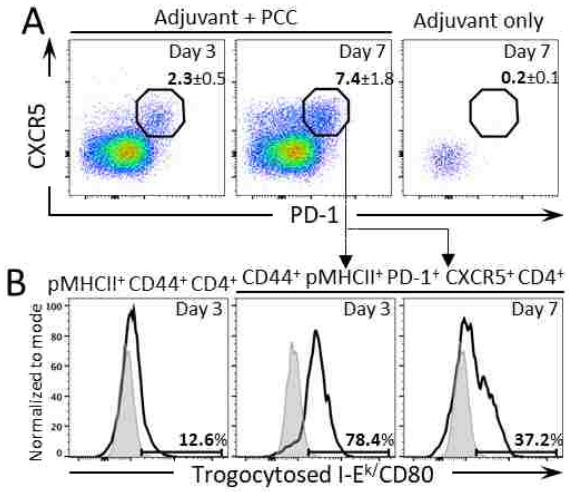



**Figure 4.11. *In vivo*-generated early $T_{FH}$ cells are primarily trog$^+$.** 5 x $10^4$ naïve 5C.C7 CD4$^+$ T cells were adoptively transferred I.V. into naïve WT B10.A mice. Six hours later, adoptive transfer recipients were immunized S.C. with Pigeon cytochrome C (PCC) emulsified in Sigma-adjuvant system (SAS), or SAS alone. Spleens were harvested at indicated time-points and analyzed via flow cytometry. **(A)** Plots show PD-1 vs CXCR5 in CD44+ MCC:MHCII-tetramer+ cells recovered at 3 (left) and 7 (middle) days post immunization with PCC+ (SAS). MCC:MHCII-tetramer+ CD4$^+$ cells 7 d after injection with with SAS alone is shown on the far right. Numbers represent mean frequency ±SEM of three separate animals. **(B)** Histograms showing fluorescence intensity of trogocytosed I-E$^k$/CD80 in CD44$^+$ MCC:pMHCII$^+$ CD4$^+$ cells (left, black line) and CD44$^+$ MCC:pMHCII$^+$ CXCR5$^+$ PD-1$^+$ CD4$^+$ T cells at 3 (middle, black line), and 7, days post-immunization (right, black line). MCC:MHCII+ CD4$^+$ T cells from SAS only-injected animals on day 7 are shown in shaded grey. Numbers show frequency of I-E$^k$/CD80$^+$ cells in respective populations from PCC+SAS-immunized mice as determined by isotype control staining. SAS-only controls were 1.4% I-E$^k$/CD80$^+$. Data is representative of two separate experiments with n=3 mice per experiment

### Trog$^{+}$ cells express higher levels of IL-21, IL-6, and Bcl-6 compared to trog$^{-}$ cells *in vivo*

To further examine the *in vivo* relationship between trog$^{+}$ cells and a $T_{FH}$ phenotype protein, WT B10.a mice were immunized S.C. with OVA protein in SAS, or SAS alone. Proximal draining lymph nodes were collected 5 days post-immunization and recovered cells were analyzed via flow cytometry. Figure 4.12A shows that of CD44$^{+}$ similarly-activated (CD69$^{High}$) CD4$^{+}$ T cells, the trog$^{+}$ cells (red) had increased expression levels of IL-21 (left), IL-6 (middle), and Bcl-6 (right), compared to trog$^{-}$ cells (blue). Meanwhile, the trog$^{-}$ cells had only slightly higher expression levels of IL-21, IL-6, and BCL-6 than CD4$^{+}$ T cells from SAS-only controls (shaded grey). A significantly greater frequency of trog$^{+}$ cells were IL-21$^{+}$ (10.2%), IL-6+ (17.1%), and BCL-6+ (6.8%) compared to CD4$^{+}$ cells from SAS-control mice. The trog$^{+}$ cells had significantly higher frequencies of IL-21$^{+}$ (38.4%), IL-6+ (39.2%) and BCL-6+ (22.1%), compared to CD4$^{+}$ cells recovered from SAS-control mice (Fig. 4.12B).

**Figure 4.12**

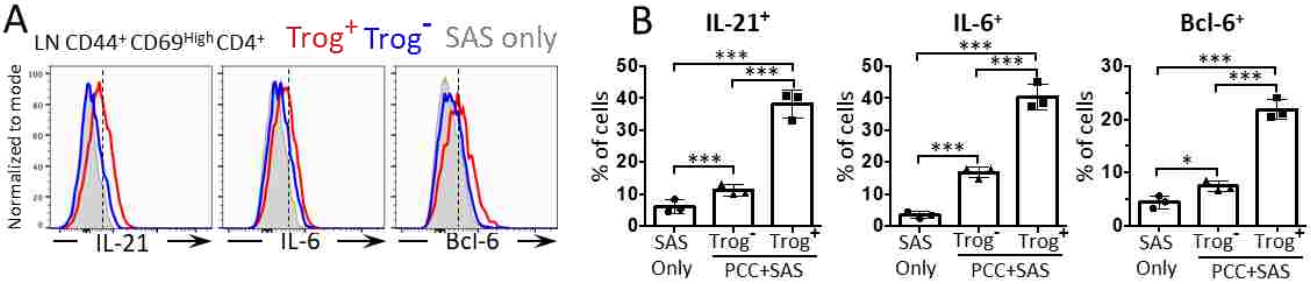



**Figure 4.12. Trog⁺ cells express higher levels of IL-21, IL-6, and Bcl-6, compared to trog⁻ cells *in vivo*.** **(A)** WT B10.A mice were immunized S.C. with 200 µg PCC in SAS, or SAS alone, proximal draining lymph nodes were collected 5 days post-immunization. Trog⁺ cells were identified by presence of trogocytosed I-E$^k$ and/or CD80. Histogram plots show expression levels of IL-21 (left), IL-6 (middle), and Bcl-6 (right) in trog⁺ (red) and trog⁻ (blue) CD44⁺ CD69⁺ CD4⁺ T cells, with CD4⁺ T cells from SAS-only controls in shaded grey. Dotted vertical lines represent intensity value >99% of MCC:MHCII ⁺ CD4⁺ cells recovered from immunized animals stained with respective isotype controls. Data is representative of two separate experiments with n=3 animals per group. **(B)** Frequency of IL-21⁺ (left), IL-6⁺ (middle), and Bcl-6⁺ (right) in CD4⁺ T cells from SAS-only immunized controls (left), and CD44⁺ CD69⁺ trog⁻ (middle) and trog⁺ (right) CD4⁺ T cells. Error bars represent ±SEM from n=3 replicate animals, with *=P<0.05 and ***=p<0.001.

### *In vitro*-generated trog⁺, but not trog⁻, cells develop a $T_{FH}$ *phenotype in vivo*, in absence of exogenous antigen

While the phenotype was consistent between *in vivo*-generated and *in vitro*-generated trog⁺ cells, the role of trogocytosis-mediated signaling in the phenotype in vivo could not be determined above. To more directly examine the role of trogocytosis-mediated signaling in vivo, an 18 hr trogocytosis assay using naïve 5C.C7 T cells and MCC:FKBP APC was performed. Recovered T cells were purified from APC and adoptively transferred into naïve WT B10.A mice. trog⁺ cells were identified by the presence of biotinylated APC membrane-derived proteins. Similar to the *in vitro* data (Fig. 4.2), neither the trog⁺ or trog⁻ cells contained a substantial population of PD-1⁺ CXCR5⁺ cells 24 hrs post-adoptive transfer (Fig. 4.13A, *left*). However, by 72 hrs post-transfer, 17% of the trog⁺ cells were PD-1⁺ CXCR5⁺, while only 1.4% of the trog⁻ cells shared this phenotype

(Fig. 4.13, *right*). Since the only source of stimulation in these animals was trogocytosed pMHCII and other APC-derived proteins, the appearance of the CXCR5$^+$ PD-1$^+$ population only in the trog$^+$ cells strongly supports the hypothesis that trogocytosis-mediated signaling can drive $T_{FH}$ differentiation both *in vitro* and *in vivo.*

**_In vivo_-generated trog$^+$ cells display a $T_{FH}$ phenotype during an active immune response, but resemble T central memory cells after contraction of the immune response**.

The data thus far suggested that trogocytosis-mediated signaling was playing a critical role in the apparent $T_{FH}$-like phenotype, however, the role of trogocytosis-mediated signaling in maintaining this phenotype was unknown. We observed that 7 days following the adoptive transfer of naïve 5C.C7 cells into WT B10.A mice, and immunization with MCC+SAS as in Fig. 4.11, a significantly greater frequency of trog$^+$ cells resembled PD-1$^+$ CXCR5$^+$ $T_{FH}$-like cells at 25%, compared to only 2.9% of trog$^-$ cells. Although the total numbers of PD-1$^+$ CXCR5$^+$ cells from each population was similar, due to only 10% of responding cells being trog$^+$, and thus consistent with the data in Fig. 4.11. The $T_{FH}$ phenotype was not maintained beyond the expected time for Ag-persistence in trog$^+$ or trog- cells, and by 28 days post-immunization the $T_{FH}$-like PD-1$^+$ CXCR5$^+$ population had decreased by 95% and 68% in the trog$^+$, and trog$^-$ populations, respectively. (Fig. 4.13A, *left*).

**Figure 4.13**

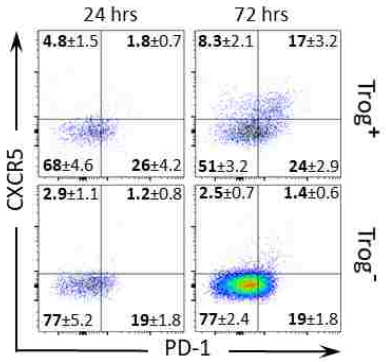



**Figure 4.13. *In vitro*-generated trog⁺ but not trog⁻ cells develop a $T_{FH}$ phenotype after adoptive transfer into naïve WT mice.** Naïve 5C.C7 cells were used in 18 h trogocytosis-assay with surface biotin-labeled MCC-I-$E^k$ APC. After APC removal, recovered T cells were labeled with CelltraceViolet (CTV) and transferred into naïve B10.A WT mice. Trog⁺ cells were identified by presence of trogocytosed biotin-labeled protein. Plots show PD-1 vs CXCR5 expression in trog⁺ (top) and trog⁻ (bottom) CTV⁺ cells recovered from spleens at 24 (left) and 72 (right) hours post-transfer. Numbers in corners represent mean frequency ±SEM of three separate animals. Quadrants in were established by respective isotype staining in MCC:MHCII⁺ CD4⁺ recovered cells, where >99% of cells stained with isotype controls fell in the lower left quadrant. Data is representative of three separate experiments.

We did however, observe a noticeable difference in the phenotype of the trog⁺ and trog- cell at this time-point, as a substantial percentage (57% on average) of the trog⁺ cells were expressing CXCR5, but not PD-1, compared to only 6.8% of trog⁻ cells. Interestingly, these results are consistent with a study by Pepper *et al.*, which showed that early in the immune response, CXCR5⁺ PD-1- cells contain a high frequency of CCR7+ T central memory ($T_{CM}$)-precursors which displayed sustained-survival after Ag-clearance [484]. In contrast, CXCR5⁺ PD-1⁺ $T_{FH}$ population decreased in frequency after Ag-clearance until disappearing altogether [484]. Looking back, a significantly higher frequency of the the trog⁺ cells were CXCR5+ PD-1- compared to trog- cells at 7 days post-immunization. In addition the trog⁺ cells also expressed higher levels of CCR7 compared to trog⁻ cells at D28 (Fig. 4.14B), consistent with a $T_{CM}$-phenotype. Recent studies have found many similarities between $T_{FH}$ and CD4⁺ memory precursors, and $T_{FH}$ themselves are able to become memory cells. Thus the apparent $T_{FH}$-like phenotype displayed early by the trog⁺ cells did not exclude the possibility that these cells were memory/memory-$T_{FH}$ precursors

**Figure 4.14**

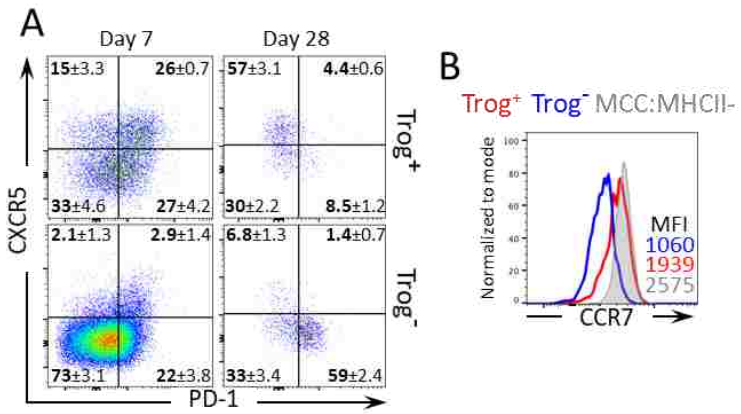



**Figure 4.14. *In vivo*-generated trog⁺ cells display a $T_{FH}$ phenotype during an active immune response, but resemble T central memory cells after contraction of the immune response (A)** Naïve 5C.C7 cells were adoptively transferred into naïve B10.A WT mice and immunized S.C. with PCC+SAS as in 4.11. Spleens were harvested at 7 (left), and 28 (right), days post-immunization. Plots show PD-1 vs CXCR5 expression in MCC:MHCII⁺ CD44⁺ CD4⁺ trog⁺ (top) and trog⁻ (bottom) cells. Numbers in corners represent mean frequency ±SEM of three separate animals. Data is representative of two separate experiments. **(B)** Representative histogram of CCR7 expression in trog⁺ (red), trog⁻ (blue) and MCC:MHCII⁻ CD4⁺ cells from immunized mice on day 28. Quadrants in A were established by respective isotype staining in MCC:MHCII+ CD4⁺ recovered cells, where >99% of cells stained with isotype controls fell in the lower left quadrant.

## Trogocytosis-mediated signaling increases memory-potential in CD4⁺ T cells

The results in Fig. 4.14 indicated that the while the trog⁺ cells displayed a phenotype consistent with $T_{FH}$, this phenotype was not observed at late time-points. Rather, the phenotype of the trog⁺ cells 28 days post-immunization resembled that of memory-precursors. Interestingly, a recent study by Kaji *et al.*, found that while GC B cells were critical for full $T_{FH}$ differentiation, a population of memory precursors that resembled $T_{FH}$ cells in CXCR5, PD-1, and BCL-6 expression developed in absence of GC B cells [499]. In addition, $T_{FH}$ cells themselves are able to readily become memory $T_{FH}$ [222, 500, 501], and in human studies, circulating CXCR5⁺ memory cells represent a distinct subset of memory cells which are able to rapidly migrate to B cell follicles and promote secondary memory responses [483]. Thus, the collective results from Fig. 4.2-4.14 substantiates the *in vitro* $T_{FH}$ data, while also raising the possibility that trog⁺ cells may also contain a high frequency of $T_{CM}$ precursors.

**Trogocytosis-mediated signaling enhances CD4$^+$ T cell survival.**

A defining hallmark of memory T cells is their ability to survive for extended durations after Ag clearance. [502-504]. While the exact mechanisms which permit the survival of CD4$^+$ memory cells in absence of Ag is currently unknown, prolonged Ag exposure and sequential cognate T cells:APC interactions promote the transition to memory [318, 499]. Consistent with this, our previous reports show that after separation from APC, trog$^+$, but not trog$^-$ cells retain active signaling and that trog$^+$ enhanced survival compared to trog$^-$ cells up to 5 days *in vitro* [81, 412].

To investigate whether the frequency of trog$^+$ cells also increased in vivo as Ag became depleted, WT B10.A mice were immunized with PCC in SAS as in Fig. 412. Spleens were harvested and MCC:MHC tetramer$^+$ (Fig. S4.1D) CD44$^+$ CD4$^+$ cells were analyzed for the presence of trogocytosed MHCII and CD80/CD86 at 5, 7, 15, 21, and 28 days post immunization. A sharp increase in the percentage of trog$^+$ cells was observed between 7 and 14 days post-immunizationn from an average of 6.8% during the peak of the immune response (Day 7), to 22% 14 days post-immunization. The frequency of trog$^+$ cells further increased on Day 21 to an average of 52% of cells being trog$^+$, then decreased slightly to an average of 42.2% on 28 days post-immunization (Fig. 4.15A).

**Figure 4.15**

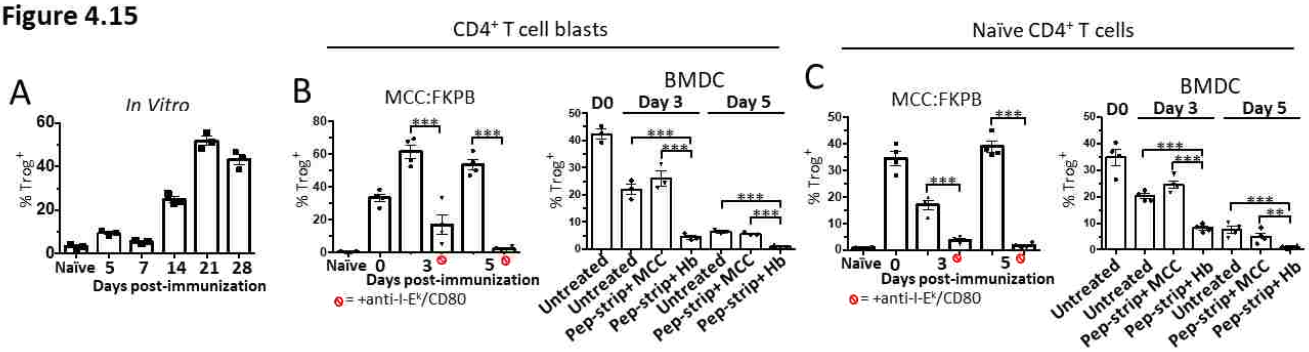



**Figure 4.15 Trogocytosis-mediated signaling is critical for the survival of trog+ cells (A)** WT B10.A mice were immunized S.C. with 200 µg PCC in SAS and spleens were collected at indicated time-points. CD44+ MCC:MHCII+ CD4+ cells were analyzed for the presence of trogocytosed I-Ek and CD80. The mean frequency of trog+ cells at indicated times post-immunization are shown with error bars representing ±SEM of three separate animals. Data is representative of two separate experiments. **(B)** 5C.C7 T cell blasts were used in a 90 min trogocytosis assay with surface biotin-labeled APC and trog+ cells were identified by the presence of biotinylated APC membrane protein. (Left) Mean frequency of trog+ cells at indicated time-points post-recovery from MCC:FKBP APC in untreated cultures, or cultures containing 10 µg/ml anti-I-Ek + 20 µg/ml anti-CD80. (Right) frequency of trog+ cells recovered from MCC-loaded BMDC in either untreated cultures, or peptide-stripped cultures supplemented with either Hb or MCC. **(C)** Naïve 5C.C7 cells used in 18 h in vitro trogocytosis assay with MCC:I-Ek APC (left) or MCC-loaded BMDC (right). Respective treatments were given as in Fig. 4.3. Graphs show frequency of trog+ cells at indicated time-points post-recovery. Data in B and C represent mean frequency ±SEM of four separate experiments.

However, in this in vivo system, it was unclear when these cells performed trogocytosis, and it was possible that cells present in later time-points had recently performed trogocytosis from APC expressing residual Ag. To directly assess the role of trogocytosis-mediated signaling in the enhanced survival displayed by the trog$^+$ cells, the frequency of trog$^+$ cells was measured in cells recovered from *in vitro* trogocytosis assays in the presence of neutralizing antibodies or with MCC peptide removed as in Figs. 4.4 and 4.5. Figure 4.15B shows that blocking trogocytosis-mediated signaling with neutralizing antibodies clearly impacted the survival of T cell blasts at 3, and 5 days post-recovery from a 90 min *in vitro* trogocytosis-assay with MCC:FKBP. While on average, 33.5% of recovered CD4$^+$ T cells were trog$^+$ immediately after recovery from the trogocytosis assay, by day 3, untreated cultures were on average, 61.4% trog$^+$. Antibody blockade of trogocytosis-mediated signaling resulted in a significantly lower frequency of trog$^+$ cells, and only 16.3% of cells from cultures containing

anti-I-Ek + anti-CD80 Abs were trog$^+$ (Fig. 4.15B, *left*). At 5 days post recovery, the frequency of trog$^+$ cells in cultures containing neutralizing Abs had dropped further to 1.8%, while 54% of the cells from untreated cultures were trog$^+$(Fig. 4.15B, *left*). Similar trends in survival were observed in cultures of T cell blasts recovered from a 90 min trogocytosis assay with MCC-loaded BMDC. While the frequencies of trog$^+$ cells were nearly equal in untreated and peptide-stripped + supplemented MCC, the cultures where peptide was removed and supplemented with Hb showed a 79% reduction in trog$^+$ cell frequencies to only 4.8% trog$^+$, compared to the 22.8% trog$^+$ from cultures where MCC was replenished (Fig. 4.15B, *left*). By 5 days post-recovery, only 1.2% of CD4$^+$ cells from peptide-stripped cultures supplemented with Hb were trog$^+$, which was significantly lower than the 4.8%, and 5.5% of trog$^+$ cells in untreated, or MCC-supplemented cultures, respectively (Fig. 4.15B, *right*).

Trogocytosis-mediated signaling also appeared to be critical for the survival of naïve T cells following activation. In cultures where naïve cells were used in an 18-hr trogocytosis assay with MCC:FKBP APC, 33.6% of recovered cells immediately after the trogocytosis assay were trog$^+$ (Fig. 4.14C, *left*). Blocking trogocytosed I-E$^k$ led to a significant decrease in the frequency of trog$^+$ cells at 3 days post-recovery as only 4.7% of cells from cultures containing neutralizing Abs were trog$^+$, compared to 16.5% of untreated cells (Fig. 4C, *left*). By 5 days post-recovery only 0.9% of cells from cultures containing neutralizing Abs were trog$^+$, which was significantly lower than the 4.2% of cells from untreated cultures (Fig. 4.15C, *left*). Finally, in cultures of naïve cells recovered

from an 18-hr trogocytosis assay with MCC-loaded BMDC, 34.4% of $CD4^+$ cells were $trog^+$ immediately following recovery from the trogocytosis assay. At 5 days post-recovery, on average, 6.8% of cells from peptide-stripped cultures supplemented with MCC were $trog^+$, compared to 4.7% of $CD4^+$ cells from untreated cultures being $trog^+$ (Fig. 4.15C, *right*). The removal of Ag-peptide however, led to a significant decrease in the frequency of $trog^+$ cells to only 0.8% of cells from these cultures being $trog^+$ cells (Fig. 4.15C, *right*). These results suggest that trogocytosis-mediating is critical for the survival of $trog^+$ cells in the absence of exogenous Ag.

**Trogocytosis-mediated signaling drives IL-2 expression in $trog^+$ cells**

To examine downstream effects of sustained trogocytosis-mediated signaling that could impact the survival of $trog^+$ cells, expression levels of IL-2 and CD25 (IL-2Rα) were examined, as early IL-2 signaling is critical for T cell activation, survival, and proliferation [505-507]. Naïve 5C.C7 $CD4^+$ T cells were used in an *in vitro* 18 hr trogocytosis assay using either MCC-loaded BMDC, or MCC:FKPB APC. Five days post-recovery, cells recovered from BMDC were, on average, 42.5% of the $trog^+$ but only 18.2% of the $trog^-$ cells were expressing both IL-2 and CD25 (Fig. 4D, left). In cultures where peptide was stripped following the trogocytosis assay, the addition of irrelevant Hb peptide resulted in a significantly lower frequency (15.2% average) of $IL\text{-}2^+$ $CD25^+$ $trog^+$ cells, compared to the 59.6% average of $trog^+$ cells recovered from MCC-supplemented cultures. While the $trog^-$ cells from cultures supplemented with Hb showed a 41% decrease in

IL-2$^{+}$ CD25$^{+}$ cells compared to untreated cultures, from 24.6% to 14.7% (Fig. 4.16, *third from left*). The addition of MCC appeared to have little impact on IL-2 expression in trog$^{+}$ cells, leading to an increase from 24.6% in untreated cultures, to 25.6% in MCC-supplemented cultures. The addition of MCC had substantial impacts on the trog$^{+}$ cells however, increasing the frequency of IL-2-expressing CD25$^{+}$ cells by 36% to 68.4% of trog$^{+}$ cells (Fig. 4.16, *third from left)*. Correlating with the massive cell death observed in Fig. 4.15, neutralizing trogocytosis-mediated signaling with anti-I-E$^{k}$ + anti-CD80 significantly resulted in an 80% decrease in IL-2$^{+}$ CD25$^{+}$ trog$^{+}$ cells. From 62.6% of trog$^{+}$ cells from untreated cultures, to only 12.5% of trog$^{+}$ cells (Fig. 4.16, *right)*. In addition to a decrease in frequency, there were clearly lower numbers of trog$^{+}$ cells in peptide-stripped+ Hb (Fig. 4.16D, *second from left*), and cultures containing neutralizing Abs (Fig. 4.16, *far right*), compared to untreated (Fig. 4.16, *left*), or peptide-stripped cultures supplemented with MCC (Fig. 4.16, *third from left*).

**Figure 4.16**

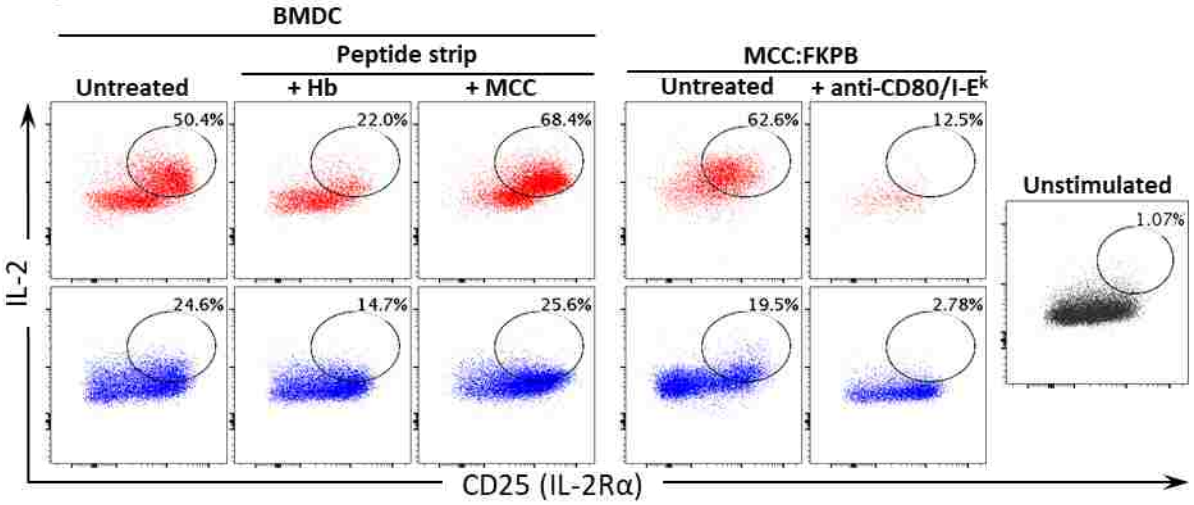



**Figure 4.16. Trogocytosis-mediated signaling drives IL-2 expression.** Naïve 5C.C7 were used in standard in vitro trogocytosis assays and treated as in 4.15C Plots show expression levels of CD25 vs IL-2 in trog$^+$ (red) and trog$^-$ (blue), and unstimulated (far right, black) cells. Numbers in plots represent frequency of cells within gate, which indicates CD25$^+$ IL-2$^+$ cells. Data is representative of three separate experiments..

Consistent with the decrease in IL-2 expression, and survival, inhibiting trogocytosis-mediated signaling by removing antigenic peptide or blocking the signaling with neutralizing Abs led to decreased activation (as indicated by CD69) (Fig. 4.17, *top*). The clear reduction in IL-2 expression resulting from the inhibition of trogocytosis-mediated signaling is further made apparent in histogram overlays (Fig. 4.17, *bottom)*

**Figure 4.17**

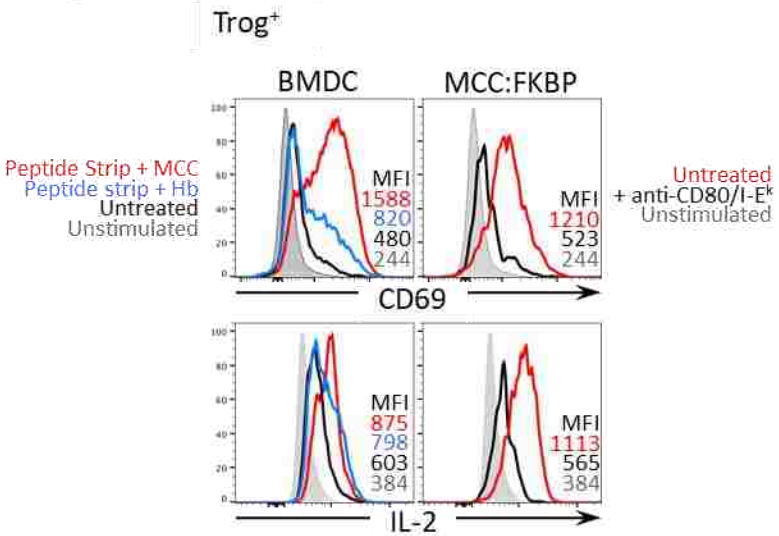



**Figure 4.17. IL-2 expression in trog⁺ cells correlates with the activation state and is dependent on trogocytosis-mediated signaling.** Naïve 5C.C7 recovered from a standard *in vitro* trogocytosis assay as in 4.16. Plots show expression of CD69 (top) and IL-2 (bottom) in trog⁺ cells 5 days post-recovery from **(left)** BMDC: Peptide-stripped + MCC (red), untreated (black), or peptide-stripped +Hb (black) cultures; and **(right)** MCC:FKBP: Untreated (red) and anti-I-E$^k$ + anti-CD80-containing cultures (black). Unstimulated cells are shown in shaded grey. Numbers in plots correspond to respective MFI.

*In vivo*-generated trog⁺ cells showed a similar phenotype. Ninety-six hours post-immunization with PCC+SAS, the trog⁺ adoptively transferred naïve 5C.C7 cells expressed higher levels of IL-2 and CD25 compared to trog⁻ cells from the same animals (Fig. 4.18).

**Figure 4.18**

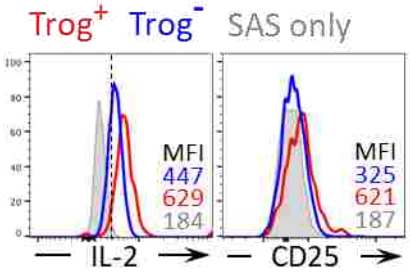



**Figure 4.18. *In* vitro-generated trog⁺ cells express higher levels of IL-2 and CD25 compared to trog⁻ cells, *in vivo*.** Naïve 5C.C7 cells were used in 18 hr *in vitro* trogocytosis-assay with surface biotin-labeled MCC-I-E$^k$ APC. After APC removal, recovered T cells were labeled with CelltraceViolet (CTV) and transferred into naïve B10.A WT mice. Spleens were recovered 96 hours after adoptive transfer and trog⁺ cells were identified by presence of trogocytosed biotin-labeled protein. Plots show expression levels of IL-2 (left) and CD25 (right) in trog⁺ (red) and trog⁻ (blue), and transferred unstimulated (shaded gray). Data is representative of two separate experiments with n=3 mice per-experiment

### Trogocytosis-mediated signaling sustains the survival of trog$^{+}$ cells in an IL-2-independent manner

The inhibition of trogocytosis-mediated signaling led to a reduced expression of pro-survival cytokines including IL-2 (Fig. 4.16) and IL-21 (Fig. 4.8) by the trog$^{+}$ cells. While trogocytosis-mediated signaling appeared to clearly play a role in the survival of the trog$^{+}$ cells in the absence of APC, the direct impact of trogocytosis-mediated signaling vs the presence of survival-associated cytokines was unclear. To address this, exogenous recombinant IL-2 (100 U/mL) was added to an aliquot of T cell blasts in cultures with, or without, anti-I-E$^{k}$/anti-CD80 neutralizing antibodies. In cultures supplemented with IL-2 alone, there was a modest, but significant increase in the number of trog$^{+}$ cells relative to input compared to untreated cultures from 26.5%, to 32%, respectively. The trog$^{-}$ cells showed a greater response to IL-2, showing a survival rate of 23% compared to only 9.5% of cells from untreated cultures (Fig. 4.19, *left*). In cultures where anti-I-E$^{k}$/anti-CD80 neutralizing antibodies were added, addition of exogenous IL-2 led to significantly increased survival of both the trog- and trog$^{+}$ cells. However, the increased survival was most apparent in the trog- cells, which increased from 6.2% to 22% relative to input, which was similar to the trog- cell numbers in the absence of neutralizing Abs + IL-2. On the other hand, the trog$^{+}$ cell numbers were only partially rescued by supplemented IL-2 in the presence of neutralizing Abs, increasing the survival rate from 12% to 18.2%, which was significantly lower than the survival rate of trog$^{+}$ cells in cultures with IL-2 alone.

**Figure 4.19**

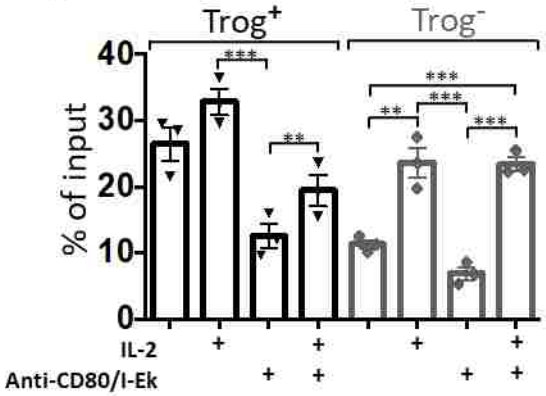



**Figure 4.19. Trogocytosis-mediated signaling sustains the survival of trog⁺ cells in an IL-2 independent manner.** 5C.C7 T cell blasts were used in a standard, 90 min *in vitro* trogocytosis assay with MCC:FKPB APC. Aliquots of cells were either supplemented with recombinant IL-2 (100 U/ml), anti-I-E$^k$ + anti-CD80 neutralizing Abs, or both. Graph shows mean frequency of viable trog⁺ (left) and trog⁻ (right) cells relative to input at the time of plating, prior to the in vitro culture ±SEM from three separate experiments. **=p<0.01, ***=p<0.001.

The data in Fig. 4.15 show that trogocytosis-mediated signaling plays a major role in the survival of both trog⁺ and trog⁻ cells after separation from APC, but through different mechanisms. While it appeared that the survival of recovered trog⁻ cells blasts was largely dependent upon exogenous IL-2, trogocytosis-mediated signaling appeared to be playing a distinct role in the survival of the trog⁺ cells independently of IL-2 (Fig. 4.19).

Consistent with previous studies, the trog⁺ blasts recovered from MCC:FKPB APC showed enhanced survival compared to the trog⁻ cells (Fig. 4.15, left). In contrast to this pattern, the frequency of naive trog⁺ cells recovered from either MCC:FKBP, or BMDC decreased between 0 and 3 days post-recovery (Fig. 4.15C). Interestingly, at day 5 the frequency of trog⁺ cells recovered from MCC:FKBP APC had rebounded, while the cells recovered from BMDC continued to decrease (Fig. 4.15C). One explanation for these observed differences is that the cells more highly activated by BMDC were proliferating at a greater rate than the cells that were comparably less-activated by MCC:FKBP. As the trogocytosed material was limited to what was acquired during the trogocytosis assay, either trogocytosed molecules were being diluted or

internalized by the trog$^{+}$ cells resulting in undetectable levels, or unequal distribution of trogocytosed molecules was occurring during the division of trog$^{+}$ cells. It was also possible that the trog$^{-}$ cells were showing greater rates of proliferation.

**Trog$^{+}$ cells rapidly proliferate, and produce trog$^{+}$ and trog$^{-}$ progeny**

To ascertain whether the discrepancies in the frequency of trog$^{+}$ cells in cultures where naïve cells were used in the trogocytosis-assay, or BMDC were used as APC, a series of experiments was conducted to assess the proliferation, and retaining of trogocytosed molecules on proliferating trog$^{+}$ cells. First, we examined the proliferation in trog$^{+}$ and trog$^{-}$ cells using CFSE-labeled naïve 5C.C7 T in an 18 h *in vitro* trogocytosis assay with MCC:I-E$^{k}$ APC. For comparison, an aliquot of the CFSE-labeled naïve cells was added to anti-CD3 + anti-CD28 coated plates at the same time cells were added to the APC during the trogocytosis-assay (t= -18 h). Figure 4.20A shows that at 24 hrs post-recovery, of CD44$^{+}$ CD4$^{+}$ cells, a small portion of the trog$^{+}$ cells and nearly none of the trog$^{-}$ or Ab-stimulated cells had performed a single cells division (Fig. 4.20A, top). By 48 hrs the majority of the trog$^{+}$ cells had performed at lest one cell division. Meanwhile a noticeably lower frequency of the trog$^{-}$ cells had divided, and Ab-stimulated cells showed a similar proliferation pattern as the trog$^{-}$ cells had undergone one or two cell-divisions (Fig. 4.20A, *middle gray*). Interestingly, at 72 hrs the majority of the trog$^{+}$ cells remained at 1- 2 divisions,

while it appeared that the trog⁻ and Ab-stimulated cells had continued to divide (Fig. 4.20A, bottom).

**Figure 4.20**

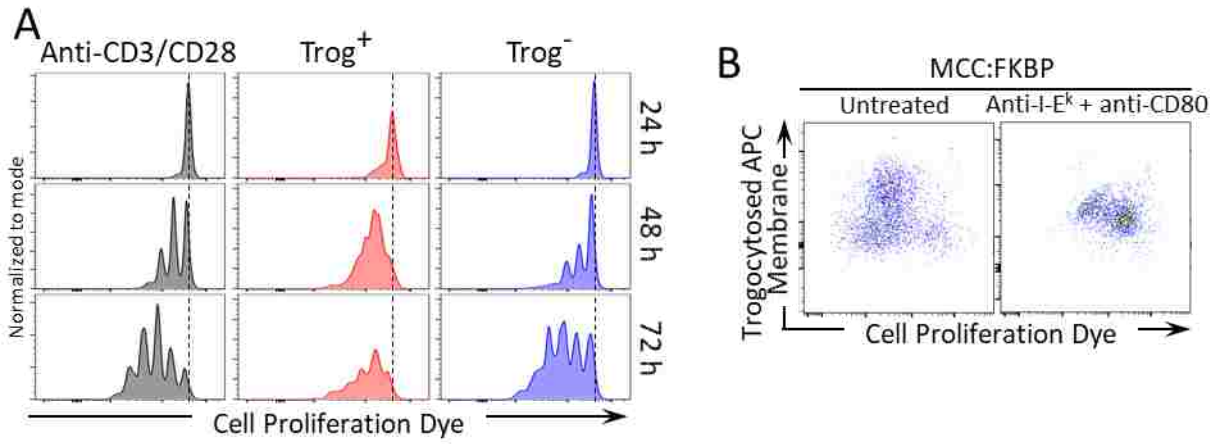


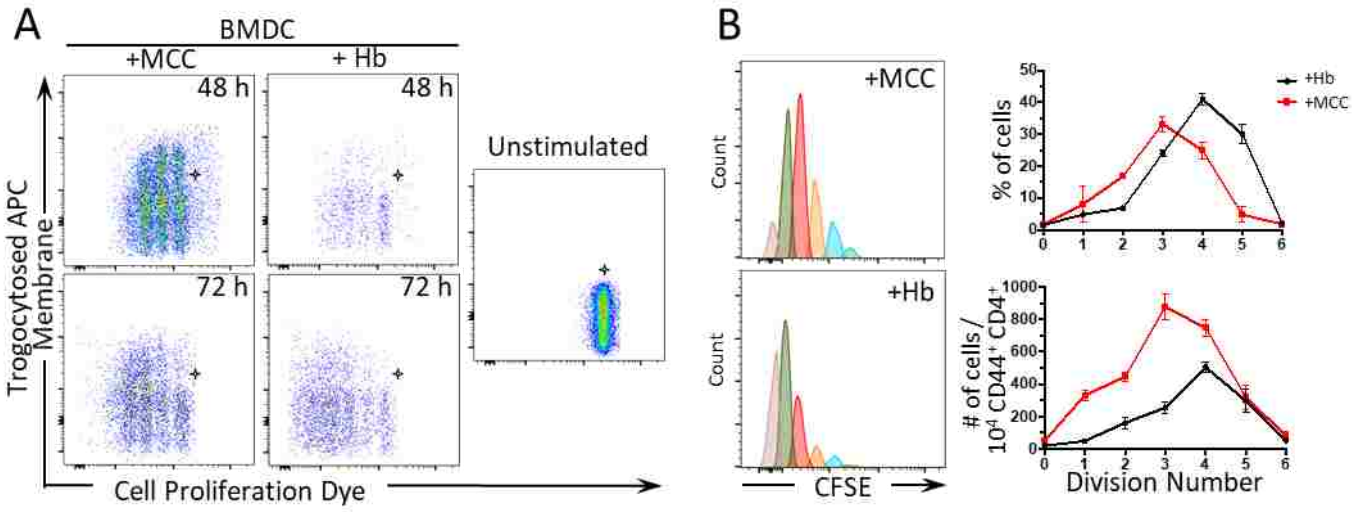

**Figure 4.20. Trogocytosis-mediated signaling is critical for proliferation, and survival in weakly activated cells. (A)** Carboxyfluorescein succinimidyl ester (CFSE)-labeled naïve 5C.C7 cells used in 18 hr *in vitro* trogocytosis assay with MCC:FKPB APC, or added to pre-coated anti-CD3+anti-CD28 6-well plates at the time cells were added to the trogocytosis assay. Plots show CFSE intensity in anti-CD3/C28-stimualted (left), trog⁺ (red, middle) and trog⁻ (blue, right) cells at 24 (top), 48 (middle), and 72 hrs (bottom) post-recovery. Vertical line represents MFI of undivided cells at 24 hrs. Data is representative of three separate experiments. **(B)** Cell proliferation dye (CFSE) vs trogocytosed biotin-labeled APC membrane in naïve T cells 5 days post-recovery from an *in vitro* trogocytosis assay as in 4.10A, either untreated (left), or cultured in the presence anti-I-Ek + anti-CD80 as in Fig. 4.5. Data is representative of three separate experiments.

Antibody-blockade against I-Ek and CD80 dramatically reduced the proliferation, and survival of trog⁺ and trog- cells (Fig. 4.20B), suggesting that trogocytosis-mediated signaling was necessary for the proliferation of the trog⁺ cells after APC removal.

Compared to naïve cells recovered from MCC:FKPB APC, cells recovered from BMDC showed markedly different proliferation patterns. The trog⁻ cells appeared to undergo comparable, if not more rounds of cell division compared to trog⁺ cells at 48 hrs, and similar levels to the trog⁺ cells by 72 hrs (Fig. 4.21, left). Removal

of peptide and supplementation with Hb led to decreased survival of both trog$^{+}$ and trog$^{-}$ cells, consistent with the findings in Fig. 4.20 (Fig. 4.21A, *left*), however, there was no substantial difference in the extent of proliferation in the trog$^{+}$ cells from Hb-supplemented cultures compared to MCC-supplemented cultures (Fig. 4.21 *left*). Somewhat unexpectedly, at 72 hrs, a higher frequency of the trog$^{+}$ cells from Hb-supplemented cultures had undergone 4-5 rounds of cell division compared to trog$^{+}$ cells from MCC-supplemented cultures (Fig. 4.21B, *top right*). However, in terms of cell numbers, the removal of Ag and supplementation with Hb led to a decrease in cell numbers within all cellular divisions (Fig. 4.21B, *right bottom*).

**Figure 4.21**




**Trog$^{+}$ cells show increased proliferative responses compared to trog- cells**

The seemingly disproportionate increase in the number of trog$^{-}$ cells that had undergone 3-4 divisions between 48 and 72 hrs (Fig. 4.20) could be due to a delay in the proliferation of these cells. Another possibility for this observation could be that the proliferating trog$^{+}$ cells were producing trog$^{-}$ progeny. To test

this, 5-ethynyl-2′-deoxyuridine (EdU)-incorporation was examined in proliferating trog$^{+}$ and trog$^{-}$ cells. Using a combination of EdU incorporation and Ki-67 Gossel *et al.* have reported a strategy to determine whether a cell is currently undergoing cell division or has recently divided but it not actively proliferating. If a cell is actively dividing, it will express both Ki-67 and have incorporated Edu. If a cell is not expressing Ki-67 but has incorporated Edu, this suggests that the cell has recently divided, but is no longer actively proliferating, or a progeny of an actively dividing cell [508]. Therefore, if unequal distribution of the trogocytosed molecules was occurring during cell division, it would be expected that the trog$^{-}$ cells would contain a high proportion of EdU$^{+}$ Ki-67$^{-}$ cells, while a higher frequency of the trog$^{+}$ cells would be both EdU$^{+}$ and Ki-67$^{+}$. The data in Fig. 4.22A show that 48 hrs after naïve 5C.C7 cells were recovered form a standard *in vitro* trogocytosis assay, 25.6% of the trog$^{+}$, but only 7.4% of the trog$^{-}$ cells were actively dividing, as determined by Ki-67 expression (Fig. 4.21A). At 72 hrs post-recovery, 16.5% of the trog$^{+}$ and 13.1% of the trog$^{-}$ were expressing Ki-67. In comparison, 21% and 12.2% of anti-CD3 + anti-CD28-stimulated cells were Ki-67$^{+}$ at 48 and 72 hours, respectively. Between 48 and 72 hrs, the frequency of recently-divided (EdU$^{+}$), Ki-67$^{-}$ cells increased 3.13-fold in the Ab-stimulated cells, and 3.13-fold in the trog$^{+}$ cells (Fig. 4.22, right, left). The trog$^{-}$ cells however, showed a 5.79-fold increase in this population, which did not fit well with the frequency of actively proliferating cells detected at 48 hrs (Fig. 4.22, middle).

**Figure 4.22**

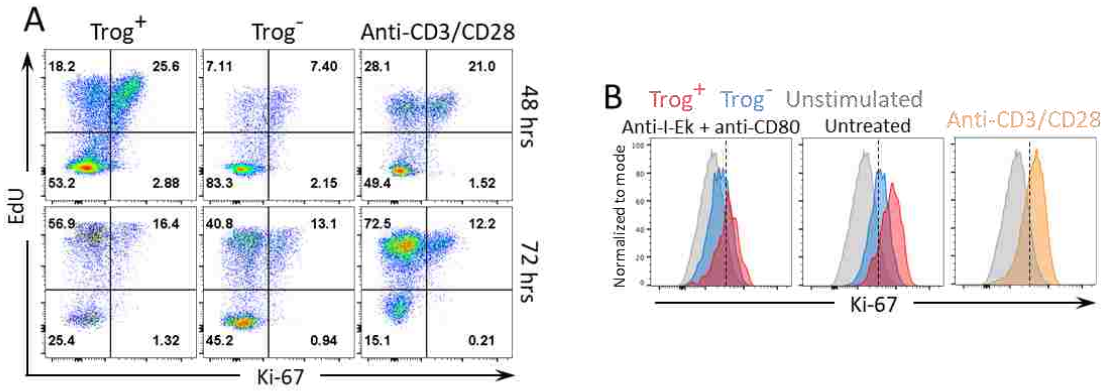



**Figure 4.22. Trog⁺ cells show increased proliferative responses compared to trog- cells, mediated by trogocytosis-sisnaling (A)** Naive 5C.C7 cells were used in a standard *in vitro* trogocytosis assay or stimulated with Ab-coated plates as in Fig. 4.5. Twenty-four hrs prior to collection and analysis, EdU was added to cultures to be incorporated into dividing cells. Plots show the proliferation marker Ki-67 vs incorporated Edu in CD44⁺ CD4⁺ trog⁺ (left), trog⁻ (middle) and anti-CD3+anti-CD28 stimulated (right) cells at 48 (top) and 72 (bottom) hrs post-recovery. Data is representative of three separate experiments. **(B)** 5C.C7 CD4⁺ blasts were used in a 90 min *in vitro* trogocytosis assay with MCC:FKBP APC. Cultures of cells were either untreated, or anti-I-E$^k$ + anti-CD80 neutralizing Abs were added as in Fig 4.5. Plots show Ki-67 expression in CD44⁺ CD4⁺ trog⁺ (red), trog⁻ (blue), or untreated (gray) cells, 72 h post-recovery. Anti-CD3 + anti-CD28 stimulated CD4⁺ cells are shown in orange (right) for reference. Data is representative of three separate experiments. Dashed vertical lines represent intensity value >99% of CD44⁺ CD4⁺ cells recovered from the trogocytosis assay stained with respective isotype control.

## Trogocytosis-mediated signaling enhances the cellular division of trog⁺ cells

As Ag-experienced CD4⁺ T cells require less stimulation compared to naïve cells to induce proliferation [37, 509], trogocytosis-mediated signaling may differently impact the proliferation of previously-activated and naïve cells. Due to the variability in the size of activated blasts, it is difficult to assess proliferation confidently with cell proliferation dyes. Ki-67 was instead used to identify actively-proliferating cells. It appeared that trogocytosis-mediated signaling was impacting the proliferation of previously-activated trog⁺ cells, as antibody blockade against trogocytosed I-E$^k$ and CD80 led to a decrease in Ki-67 in the trog⁺ cells (Fig. 4.22B).

**Trogocytosed molecules are retained through early cell divisions**

While trogocytosis-mediated signaling appeared to be playing a role in driving proliferation of lesser activated cells *in vitro* (Fig. 4.21), it was unclear whether in vivo-generated trog$^{+}$ and trog$^{-}$ cells would show similar patterns. To examine this, naïve CellTrace-labeled 5C.C7 cells were adoptively transferred into naïve WT B10.A mice. Six hours later, adoptive-transfer recipients were immunized with SAS alone, or PCC+SAS. Spleens were harvested 96 hrs later and cell proliferation was assessed by dilution of CellTrace dye. As expected, adoptively transferred cells in SAS-only immunized controls did not proliferate in the absence of Ag (Fig. 4.23A). In contrast, the transferred cells in SAS+PCC-immunized mice underwent massive expansion. Interestingly, trogocytosed I-E$^{k}$ and CD80 were clearly present on cells which had undergone fewer (1-5) rounds of cell division, but was scarce on cells which had undergone >6 rounds of cell division (Fig. 4.23A). Consistent with the phenotype of memory-precursors early in the immune response [510, 511], of adoptively transferred CD44$^{+}$ CD4$^{+}$ T cells that had performed 1-4 cell divisions, expression levels of the anti-apoptotic protein Bcl-2 were higher in trog$^{+}$ cells compared to trog$^{-}$ cells (Fig. 4.23B, *left*). However, this was not the case in the few trog$^{+}$ cells that had performed 5$^{+}$ cell divisions, as they displayed similar expression patterns to the trog- cells of similar cell divisions (Fig. 4.23B, *right*).

**Figure 4.23**

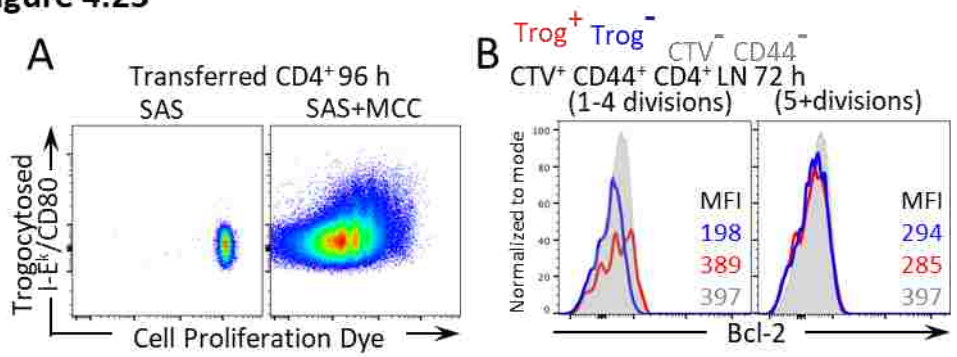



**Figure 4.23. Trogocytosed molecules are retained through early cell divisions, and of cells which have undergone 1-4 cell divisions the trog$^+$ cells express higher levels of Bcl-2 compared to trog$^-$ cells *in vivo*.** Naïve CellTrace Violet (CTV)-labeled 5C.C7 CD4$^+$ T cells were adoptively transferred into naïve WT B10.A mice. Six hours later recipients from the adoptive transfer were immunized S.C. with PCC+SAS, or SAS alone as in Fig. 4.14. Spleens were harvested 96 hrs post immunization. **(A)** Plots show CTV vs trogocytosed MHCII + CD80 in transferred (CTV$^+$) cells from animals immunized with SAS alone (left), or SAS+PCC (right). **(B)** Histograms showing Ki-67 staining in trog$^+$ (red) and trog$^-$ (blue) CTV$^+$ CD44$^+$ CD4$^+$ T cells, with CTV$^+$ CD4$^+$ T cells from SAS-only controls are shown in shaded gray. Data is representative of two separate experiments with n=3 mice per group.

The results in Figs. 4.20-4.23 suggest that trogocytosis-mediated signaling was critical for the proliferation of weakly-activated cells, but the extent of proliferation in highly activated cells was occurring independently of trogocytosis-mediated signaling (Fig. 4.21). Although, it was apparent that trogocytosis-mediated signaling was playing a role in the survival of the trog$^+$ cells, particularly those that had undergone fewer rounds of cell division (Fig. 4.21B). The disproportionate frequency of trog$^-$ cells that had divided between 48 and 72 hours relative to the frequency of cells actively-dividing at 48 hrs (Fig. 4.22A) could be due to the increased survival of recently-divided cells. When also considering the maintained levels of trogocytosed material (as determined by staining intensity), through multiple rounds of cell division, these results raised the possibility that these molecules may be unequally distributed to daughter

cells during the division of the trog$^{+}$ cells. Thus, potentially accounting for the increase of trog- cells which appeared to have divided multiple times at 72 hrs.

**During asymmetric division of trog$^{+}$ cells, daughter cells that retain trogocytosed molecules resemble memory precursors.**

In addition to the effector-to-memory transition model, it has been found that naïve T cell progeny may become predestined for memory fate during the immune synapse through subsequent asymmetric division [329, 491, 512, 513]. In this model, the signaling molecules converged at the immune synapse, along with high levels of localized TCR and costimulatory signaling, form a region of polarity known as the proximal pole. Concurrent with the formation of the immune synapse, surface molecules including CD43 and CD45 migrate, and localize at a focal point opposite of the immune synapse to form the distal pole complex [70, 514]. As this axis of polarity is established during the immune synapse, it has been proposed that asymmetric division occurs in the first few rounds of cell division following activation [329]. In this model, the daughter cell containing the proximal pole which received strong TCR and costimulatory signaling from the APC becomes an effector cell which continues to proliferate at high rates. Meanwhile, the cell containing the distal pole complex are more likely to be memory-precursors, which are less terminally differentiated, and do not expand as rapidly as the effector cell population [329, 330, 515]. Asymmetric division is not limited to naïve T cells, as memory T cells have also been observed to asymmetrically divide upon re-challenge [331]. Interestingly, we have previously

observed that during the immune synapse, >82% of trog$^{+}$ cells had trogocytosed molecules localized to the distal pole [81] (Fig. 4.24).

**Figure 4.24**

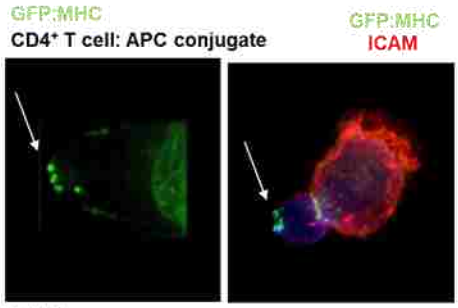



**Figure 4.24. Trogocytosed molecules localize to the distal pole during the immunological synapse.** Images of MCC-specific TCR Tg CD4$^{+}$ T cell forming synapses with APC expressing GFP-tagged MCC:FKBP. White arrows indicate transferred molecules on the distal pole of the T cell. (Left) GFP expressed on the APC (right) with GFP-labeled MHCII transferred to the distal pole of the T cell (clear). (Right) Large APC on right expressing high levels of ICAM-1 (red)

To examine whether the cells with retained trogocytosed molecules resembled memory precursors, we examined Tcf1 expression in trog$^{+}$ and trog$^{-}$ cells. In asymmetrically dividing CD4$^{+}$ T cells, Tcf1 has been observed to be retained at high levels on the daughter cell resembling $T_{CM}$ precursors [516]. In an influenza model, of CD4$^{+}$ T cells which had undergone only several rounds of divisions, Tcf1$^{High}$ cells retained the capacity to self-renew, producing Tcf1-silenced daughter cells which resembled terminally-differentiated effector cells, and a memory-like sibling cell which maintained high Tcf1 expression [491]. Consistent with these findings and the possibility that trog$^{+}$ cells resemble memory-precursor cells, 48 hrs post-recovery from a trogocytosis assay using naïve 5C.C7 CD4$^{+}$ cells, the trog$^{+}$ cells recovered from MCC:FKBP APC largely maintained high Tcf1 expression through 1-3 cell divisions, while the trog$^{-}$ cells within equal divisions expressed low levels of Tcf1 (Fig. 4.25A, *top*). This pattern was not observed in the cells recovered from BMDC, and both trog$^{+}$ and trog$^{-}$ cells showed lower levels of Tcf1 compared to trog$^{+}$ cells recovered from MCC:FKBP

APC (Fig. 4.25B). Intriguingly, the trog$^+$ cells recovered from BMDC which were peptide-stripped and supplemented with Hb, showed higher levels of Tcf1 compared to the trog$^+$ cells from peptide-stripped cultures supplemented with MCC (Fig. 4.25B).

**Figure 4.25**

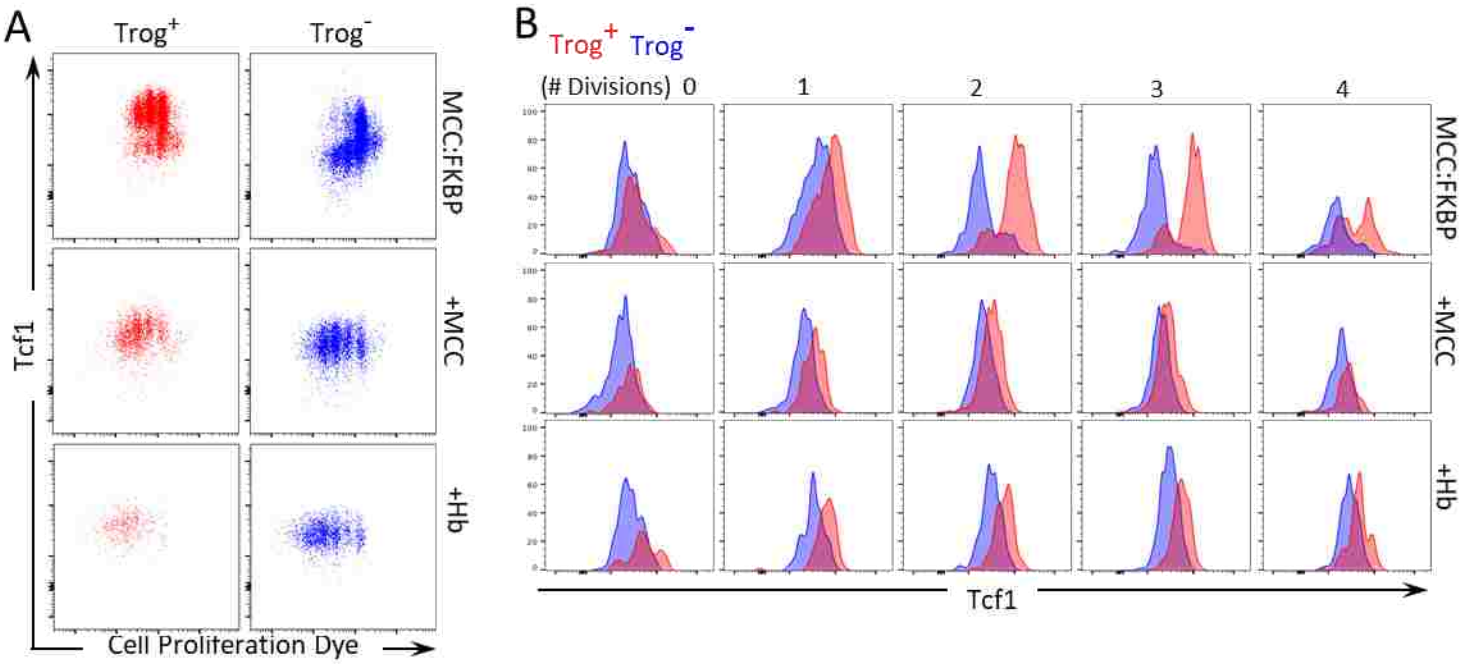



**Figure 4.25. In lesser-activated cells, Tcf1 is retained in early-dividing trog$^+$ but not trog$^-$ cells.** Naïve CFSE-labeled 5C.C7 cells were used in an 18 hr *in vitro* trogocytosis assay with surface biotin labeled - MCC:I-E$^k$ APC, or MCC-loaded BMDC. Cells recovered from BMDC were peptide-stripped and supplemented with Hb or MCC peptide as in Fig. 4.5. Cells were analyzed 48 hrs post-recovery. **(A)** Plots show cell proliferation (CFSE) vs Tcf1 staining in trog$^+$ (red) and trog$^-$ (blue) in cells recovered from MCC:FKBP APC (top), BMDC peptide stripped +MCC (middle) or +Hb (bottom). **(B)** Tcf1 expression in trog$^+$ (red), and trog$^-$ (blue), cells recovered from MCC:FKPB APC (top), BMDC with supplemented MCC (middle), or Hb (bottom). Plots show cells within indicated cell divisions (0-4), increasing from left to right. Data is representative of three separate experiments.

A similar phenotype was observed in in vivo-generated trog$^+$ cells. Of adoptively transferred cells as in Fig. 4.23., in cells that had undergone 1-4 cell divisions following immunization with PCC+SAS, the trog$^+$ cells showed higher levels of

Tcf1 compared to the trog⁻ cells (Fig. 4.26). Similar to the trends for Bcl-2 expression (Fig. 4.23), of adoptively transferred CD44⁺ cells which had undergone >5 rounds of cell division, the difference between Tcf1 expression in trog⁺ vs trog⁻ cells was less apparent and did not resemble asymmetric distribution of Tcf1. However, the trog⁺ cells still showed 62% higher expression of Tcf1 as determined by MFI, compared to the trog⁻ cells (Fig. 4.26, *right*).

**Figure 4.26**

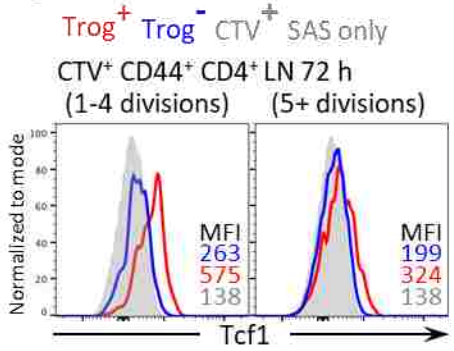



**Figure 4.26. *In vivo*-generated trog⁺ cells which have performed 1-4 cell divisions express high levels of Tcf1, while trog⁻ cells and highly-divided trog⁺ and do not.** Plots showing Tcf1 expression in trog⁺ (red), trog⁻ (blue), CD44⁺ CTV+ CD4⁺ T cells within 1-4 cell divisions (left) or 5+ divisions (right) in cells recovered 96 hrs post-immunization as in Fig. 4.23. CellTrace Violet-positive CD4⁺ T cells from SAS-only controls are shown in shaded gray. Data is representative of two separate experiments with n=3 mice per group.

To test the possibility that asymmetric distribution of trogocytosed molecules was occurring, naïve 5C.C7 cells were used in an 18 h *in vitro* trogocytosis assay with fluorescent NHS-Sulfo BODIPY-labeled MCC-loaded BMDC. Recovered cells were FACS sorted into trog⁺ and trog⁻ populations, CFSE labeled, then cultured separately *in vitro* for 72 h. While >95% of the trog⁺ cells were displaying trogocytosed molecules immediately following the FACS sort, by the end of the 3-day incubation, the frequency of cells displaying trogocytosed molecules decreased within each successive cell division (Fig. 4.27, *middle*). The trog⁻ sorted as expected, did not display trogocytosed molecules within cells of any cell division. (Fig. 4.2, *right*),

**Figure 4.27**

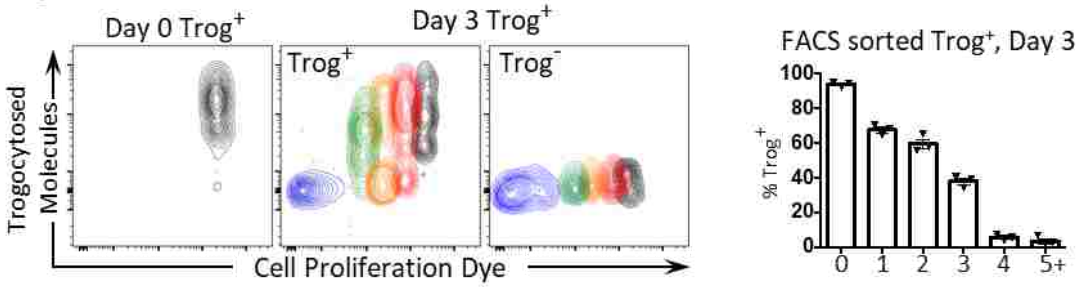



**Figure 4.27. Trogocytosed molecules are unequally distributed during cell division.** Naïve 5C.C7 CD4$^+$ T cells were used in a standard 18-hr *in vitro* trogocytosis assay with NHS-sulfo BODIPY surface-labeled APC. Cells were FACS sorted into trog$^+$ and trog$^-$ populations immediately after recovery, CFSE labeled, and cultured individually. **(A)** Plots show cell proliferation (CFSE) vs trogocytosed BODIPY-labeled APC membrane proteins in CD4$^+$ cells from sorted-trog$^+$ cells at 0 (left) and 3 (middle) days post-recovery. Cells recovered from sorted-trog$^-$ cultures 3 days post-recovery are shown on the far right. Each color corresponds to a separate cell division. **(B)** Frequency of cells recovered from trog$^+$-sorted cultures containing BODIPY-labeled APC membrane proteins with subsequent cell-divisions, 3 days post-recovery. Data is representative of two separate experiments with primary cells from n=3 individual mice.

The above data suggest that the trog$^+$ cells had undergone asymmetric division, where one cell retained both the trogocytosed molecules and Tcf1. To confirm this, immunofluorescent imaging was performed on naïve 5C.C7 cells 32 hrs post-recovery from an *in vitro* trogocytosis assay with MCC:FKBP APC. Four hours prior to fixation and staining, cells were treated with the actin polymerization inhibitor cytochalasin D to inhibit cytokinesis. Consistent with the data in Fig. 4.27, in >70% of the dividing cell conjugates, the cells with trogocytosed molecules also had higher levels of Tcf1. Representative images are shown in Fig. 4.28. Interestingly, it also appeared that unlike observations in trog$^+$ T cell blasts [412], the majority of naïve trog$^+$ cells had multiple punctate spots of trogocytosed molecules. Suggesting that it is possible for a trog$^+$ cell to produce more than one trog$^+$ progeny throughout it's proliferation cycle.

**Figure 4.28**

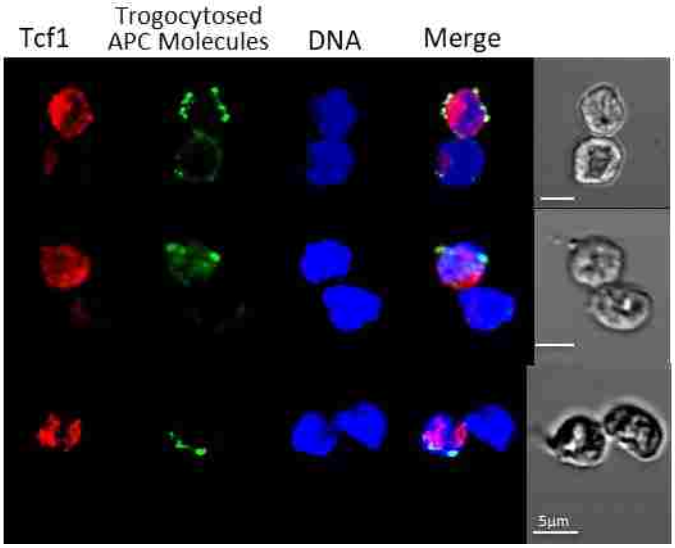



**Figure 4.28. In early-dividing cells, trogocytosed molecules and Tcf1 are retained on the same daughter cell.** Naïve 5C.C7 cells were used in an 18-hr standard *in vitro* trogocytosis assay with MCC:FKBP APC. Thirty-two hours post-recovery, cells were treated for 4 hrs with cytochalasin D to prevent cell cycle from G1 to S phase. Fixed cells were stained for Tcf1 (red), trogocytosed APC membrane (green), and DNA (Hoerscht 33342) (blue). Scale bars represent 5 μM. Data is representative of two separate experiments, with 6-12 conjugates images in each experiment.

Collectively, the data in above strongly suggest that during cell division, at least in lesser activated naïve $CD4^+$ T cells, trogocytosed molecules are unequally distributed to daughter cells in a manner reminiscent of asymmetric division. In this case, the daughter cells which retained the trogocytosed molecules resembled memory precursors, while the $trog^-$ progeny did not.

### $Trog^+$ cells rapidly increase expression of $T_{FH}$-associated molecules PD-1, CXCR5, and IL-21 upon *in vitro* re-stimulation

In addition to extended periods of survival, another important function of memory cells is to display rapid recall responses. To test the recall responses of $trog^+$ and trog- cells, naïve 5C.C7 cells were used in an 18-hr *in vitro* trogocytosis assay with MCC:FKBP APC. Recovered cells were cultured *in vitro* for 7 days and then re-stimulated for 3 hrs at a 1:1 ratio with MCC:FKBP APC, followed by a 3-hr incubation. Following this brief period of re-stimulation, the $trog^+$ cells increased in their frequency of PD-$1^+$ $CXCR5^+$ cells by 50%, (from 39.5% to 59.3%), while

the trog$^-$ cells showed a 32% increase in the frequency of this population. Although, at significantly lower overall frequencies compared to the trog$^+$ cells, the frequency of PD-1$^+$ CXCR5$^+$ trog$^-$ cells increased from 5.36% to 7.06% following *in vitro* re-stimulation (Fig. 4.29). To examine IL-21 expression in the context of a recall response, the trog$^+$ cells generated *in vivo* from the experiment in Fig. 4.14 were isolated from spleens of PCC+SAS-immunized mice 28 days post-immunization, and were re-stimulated *in vitro* as in Fig. 4.29A. Here, the trog$^+$ cells also displayed rapid expression of IL-21 upon re-stimulation while the trog- cells did not. Although, this may be an unfair comparison due to the overall lack of a robust $T_{FH}$-like phenotype displayed by the trog$^-$ cells. Regardless, these results show that the trog$^+$ cells were able to rapidly upregulate $T_{FH}$-associated markers upon re-stimulation, consistent with a memory-phenotype.

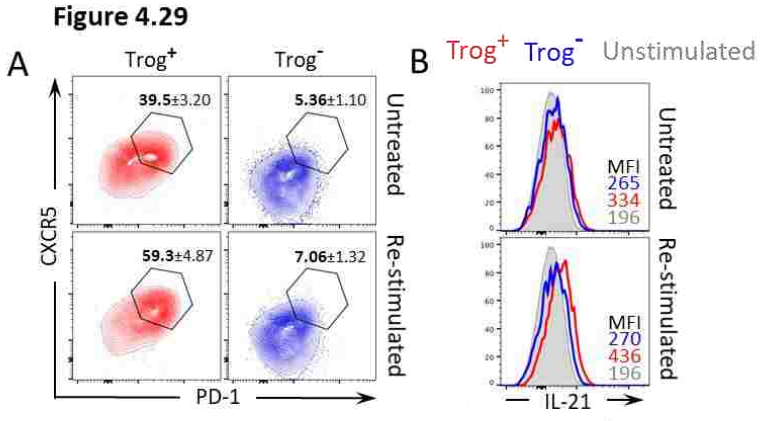



**Figure 4.29. Trog$^+$ cells rapidly increase expression of $T_{FH}$-associated molecules PD-1, CXCR5, and IL-21 upon re-stimulation. (A)** Naïve 5C.C7 cells were used in an 18-hr *in vitro* trogocytosis assay with MCC:FKBP APC. Recovered cells were cultured in vitro for 7 days and then re-stimulated for 3 hrs at a 1:1 ratio with MCC:FKBP APC, followed by a 3-hr incubation. Plots show PD-1 vs CXCR5 expression in trog$^+$ (red, left) and trog$^-$ (blue, right) in untreated (top) and re-stimulated cells (bottom) Numbers represent mean frequency of PD-1$^+$ CXCR5$^+$ cells ±SEM of cells recovered from three separate animals. **(B)** MHC:MHCII tetramer $^+$ cells recovered from the spleens of animals 28 days post-immunization with PCC+SAS and adoptive transfer of naïve 5C.C7 CD4+ T cells as in 4.14. Cells were re-stimulated as in 4.28A. Data is representative of two separate experiments with n=3 animals.

### *In vitro*-generated trog$^{+}$ cells display prolonged survival *in vivo*, in absence of antigen

The results thus far suggested that the trog$^{+}$ cells displayed characteristics consistent with memory-precursor cells. However, the results showing that the trog$^{+}$ cells can produce trog- progeny through asymmetric division, and the uncertainty of when trogocytosis occurred in the trog$^{+}$ cells in vivo made it difficult to ascertain the long-term phenotype of the initial trog$^{+}$ cell. To isolate the time frame for trogocytosis to occur to a set duration, and limit Ag to trogocytosed molecules, naïve 5C.C7 cells were used in an 18-hr *in vitro* trogocytosis assay with MCC:FKPB.

**Figure 4.30**

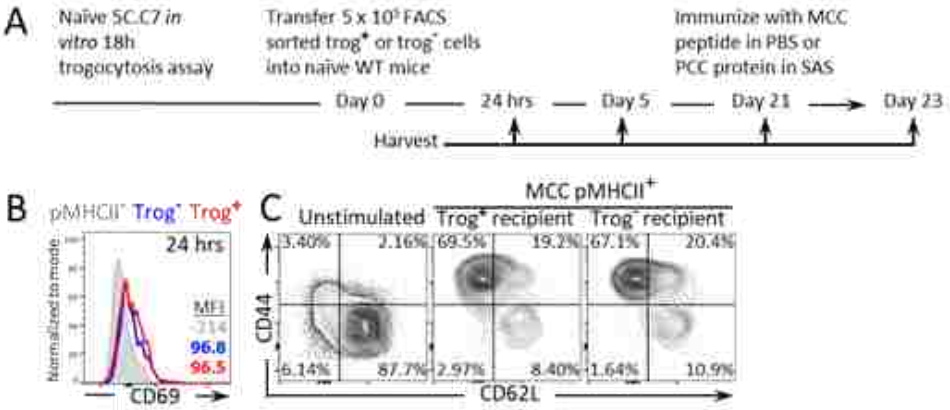



**Figure 4.30. *In vitro*-generated trog$^{+}$ and trog$^{-}$ cells are similarly activated, 24 hours post-adoptive transfer.**
Naïve 5C.C7 cells were used in an 18 h *in vitro* trogocytosis assay with MCC:I-E$^{k}$ APC. Recovered cells were FACS sorted into trog$^{+}$ and trog$^{-}$ populations and 2 x 10^5 trog$^{+}$ or trog$^{-}$ cells were transferred into separate naive WT B10.A mice (n=3 per time-point). Unstimulated 5C.C7 cells were transferred into separate animals for controls **(A)** Graphic timeline of experimental procedures **(B)** CD69 expression in MCC:MHCII$^{+}$ cells recovered from spleens of trog$^{+}$ (red) and trog$^{-}$ (blue) recipients 24h post-adoptive transfer. MCC:MHCII$^{-}$ cells are shown in shaded gray for comparison. (B) in a trog$^{+}$ recipient are shown in gray for reference. **(C)** 2D plots showing CD62L vs CD44 expression in MCC:MHCII$^{+}$ cells recovered from unstimulated (left), trog$^{+}$ (middle), and trog$^{-}$ (right) recipients 24 hrs post-adoptive transfer. All data in Figs. 4.30-4.35 are representative of two separate experiments

Immediately following recovery, cells were FACS sorted into $CD4^+$ $trog^+$ and $trog^-$ populations. Immediately following the FACS sort, 2 x $10^5$ FACS-sorted $trog^+$, $trog^-$, or unstimulated 5C.C7 cells were transferred into separate naïve WT B10.A recipient mice. An experimental timeline is shown in Fig 4.30A. In line with the *in vitro* data (Figs. 4.1, 4.5), 24 hrs after adoptive transfer, cells recovered from the spleens of $trog^+$ and $trog^-$ adoptive transfer recipients showed a similarly activated phenotype as indicated by CD69 levels (Fig. 4.30B), and high expression of CD44 and low CD62L (Fig. 4.30C).

Five days post-adoptive transfer, the transferred (MCC:MHCII tetramer-positive $CD44^+$) cells that had migrated to the LN of $trog^-$ recipients contained a higher frequency of actively-proliferating cells (46.7% on average) as determined by Ki-67 staining (Fig. 4.31), and had slightly increased numbers (Fig. 4.32) compared to the cells from $trog^+$ recipients.

**Figure 4.31**

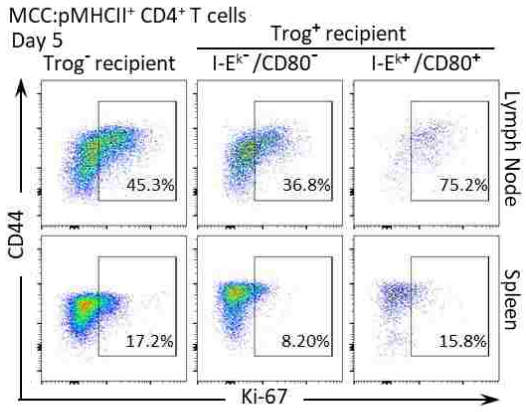



**Figure 4.31. *In vitro*-generated $trog^+$ and $trog^-$ cells proliferate at similar frequencies after adoptive-transfer into naïve WT mice. Cells with retained trogocytosed I-Ek/CD80 contain higher frequencies of $Ki\text{-}67^+$ cells 5 days post-transfer.** Recovered MCC:$MHCII^+$ $CD4^+$ cells adoptively as in 4.30. Ki-67 vs CD44 expression in MCC:$MHCII^+$ cells recovered from pooled axillary, and inguinal lymph nodes (top) and spleen (bottom row) from $trog^-$ (left) and $trog^+$ (middle, right) recipients. Middle column shown $I\text{-}E^k$- cells and right column shows $I\text{-}E^{k+}/CD80^+$ cells from $trog^+$ recipients.

Consistent with the *in vitro* data, on average 73.4% of the transferred cells which retained trogocytosed I-E$^k$ and CD80 (which accounted for on average 11% of CD44+ MC:MHCII$^+$ cells in the spleen, and 8.5% in the lymph nodes) were actively proliferating, compared to 35.4% of the cells without detectable I-E$^k$ / CD80 (Fig. 4.31). It also appeared that the trog$^+$ had noticeably higher levels of CD44 in the cells recovered from spleens compared to the transferred cells from the trog$^-$ recipients (based off of fluorescence intensity) (Fig. 4.31), suggesting that the cells from the trog$^+$ recipients had remained highly activated, and that those from the trog- recipients were becoming less activated.

**Figure 4.32**

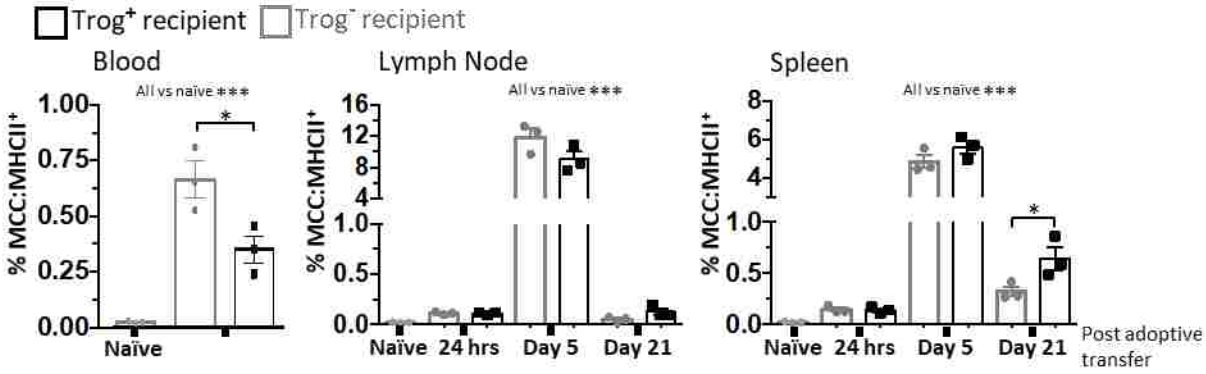



**Figure 4.32. *In vitro*-generated trog$^+$ cells display enhanced survival compared to trog$^-$ cells *in vivo*, in absence of exogenous Ag.** MCC:MHCII$^+$ CD4$^+$ cells adoptively transferred as in 4.30. Frequency of MCC:pMHCII$^+$ cells in blood (left), lymph nodes (middle) and spleen (right) recovered from trog$^+$ (black) and trog$^-$ (gray) recipients at indicated time-points post adoptive transfer. Cells recovered from naïve WT B10.A recipients are shown on the far left of each plot. Data represents mean frequency ±SEM of replicate animals (n=3), with *=P>0.05.

It appeared that the trog$^+$ cells more rapidly exited circulation compared to trog$^+$ cells, as 24 hours post-transfer, there was a significantly lower frequency of transferred cells in trog$^+$ recipients in circulation compared to trog$^-$ recipients (Fig. 4.32, *left*), while similar frequencies of transferred trog$^+$ and trog$^-$ cells were

observed in cells recovered from the lymph nodes and spleen (Fig. 4.32, *middle, right*). In support of the *in vitro* data, the transferred cells in the trog$^{+}$ recipients showed enhanced survival compared to the cells from trog$^{-}$ recipients, and at 21 days post-adoptive transfer, there was a significantly higher frequency of transferred cells in the spleens of trog$^{+}$ recipients compared to trog$^{-}$ recipients (Fig. 4.32, *right*). In support of the *in vitro* data, the transferred cells from trog$^{+}$ recipients which had retained trogocytosed I-E$^{k}$/CD80 also expressed high levels of CXCR5, (Fig. 4.33, *left*) while neither the I-E$^{k}$/CD80$^{-}$ cells in these animals, or the transferred cells recovered from trog$^{-}$ recipients expressed CXCR5 (Fig. 4.33, *left*). Similar to the transferred cells in immunized mice (Fig. 4.13), 21 days post-transfer, the transferred cells from trog$^{+}$ and trog$^{-}$ recipients did express high levels of PD-1, although the cells from trog$^{+}$ recipients showed lower levels of PD-1 expression compared to those from trog$^{-}$ recipients (Fig. 4.33, *middle*). Compared to transferred cells recovered from the trog$^{-}$ recipients, the cells from trog$^{+}$ recipients expressed higher levels of CCR7, consistent with CD4$^{+}$ $T_{CM}$ cells (Fig. 4.33, *right*).

**Figure 4.33**

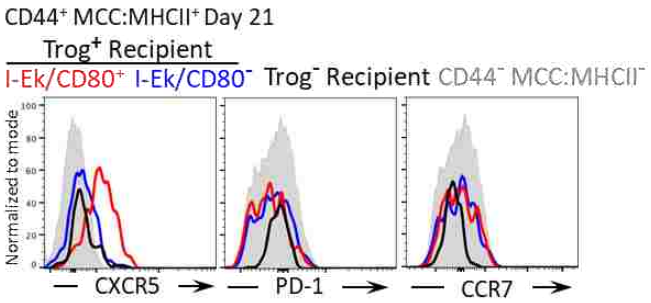



**Figure 4.33. Twenty-one days post-adoptive transfer, *In vitro*-generated trog$^+$ cells display high levels of CXCR5, decreased PD-1, and increased CCR7 compared to trog$^-$ cells.** Plots showing expression levels of CXCR5 (left), PD-1 (middle), and CCR7 (right) in cells recovered from spleens of adoptive transfer recipients from 4.30, 21 days post-adoptive transfer. Of cells recovered from trog$^+$ recipients, transferred cells displaying trogocytosed I-E$^k$/CD80 are shown in red, and I-E$^k$/CD80- cells are shown in blue. Transferred CD44$^+$ cells from trog$^-$ recipients are shown in black, and CD44- MCC:MHCII- cells from trog$^+$ recipients are shown in shaded gray for reference.

Consistent with the frequency observed in the immunized WT mice, an average of 61.5% of transferred cells in trog$^+$ recipients had retained I-E$^k$ and CD80 expression on day 21, compared to only 6.95% of the cells from trog$^-$ recipients (Fig. 4.34, left). To test the recall ability of the transferred cells, mice were immunized with MCC peptide alone or PCC emulsified in SAS on day 21. Forty-eight hours later, spleens, proximal draining lymph nodes, and skin from the injection site were collected. While immunization with peptide alone in the absence of high costimulation typically leads to anergy in naïve CD4$^+$ cells [33], memory CD4$^+$ cells have been found to respond to injection with a single peptide epitope [517]. Interestingly, 47% of the MCC:MHCII$^+$ cells recovered from the spleens of trog$^+$ recipients displaying trogocytosed I-E$^k$/CD80, expressed Ki-67 after immunization of peptide alone, while only 18% of the cells without trogocytosed molecules were Ki-67$^+$. In transferred cells recovered from trog$^-$ recipients, only 8.7% of all transferred CD44$^+$ CD4$^+$ cells were Ki-67$^+$. It appeared

that proliferation in these cells was induced by peptide, as <1% of the cells from either trog$^{+}$ or trog$^{-}$ recipients were Ki-67$^{+}$ on day 21 prior to the immunization. In the presence of adjuvant, the transferred cells from trog$^{+}$ recipients showed only a slight increase in the frequency of Ki-67$^{+}$ cells (49.6% on average) compared to MCC peptide-immunization (40.5% average Ki-67$^{+}$). The I-E$^{k}$/CD80-negative cells showed a substantial increase in the frequency of actively-dividing cells however, from 18% in MCC-immunized animals, to an average of 44.3% in PCC+SAS-immunized animals (Fig. 4.34A, middle). Meanwhile, the cells from the trog$^{-}$ recipients responded more robustly to immunization with MCC+SAS compared to MCC alone, increasing the total frequency of Ki-67$^{+}$ transferred cells to an average of 15.5%.

**Figure 4.34**

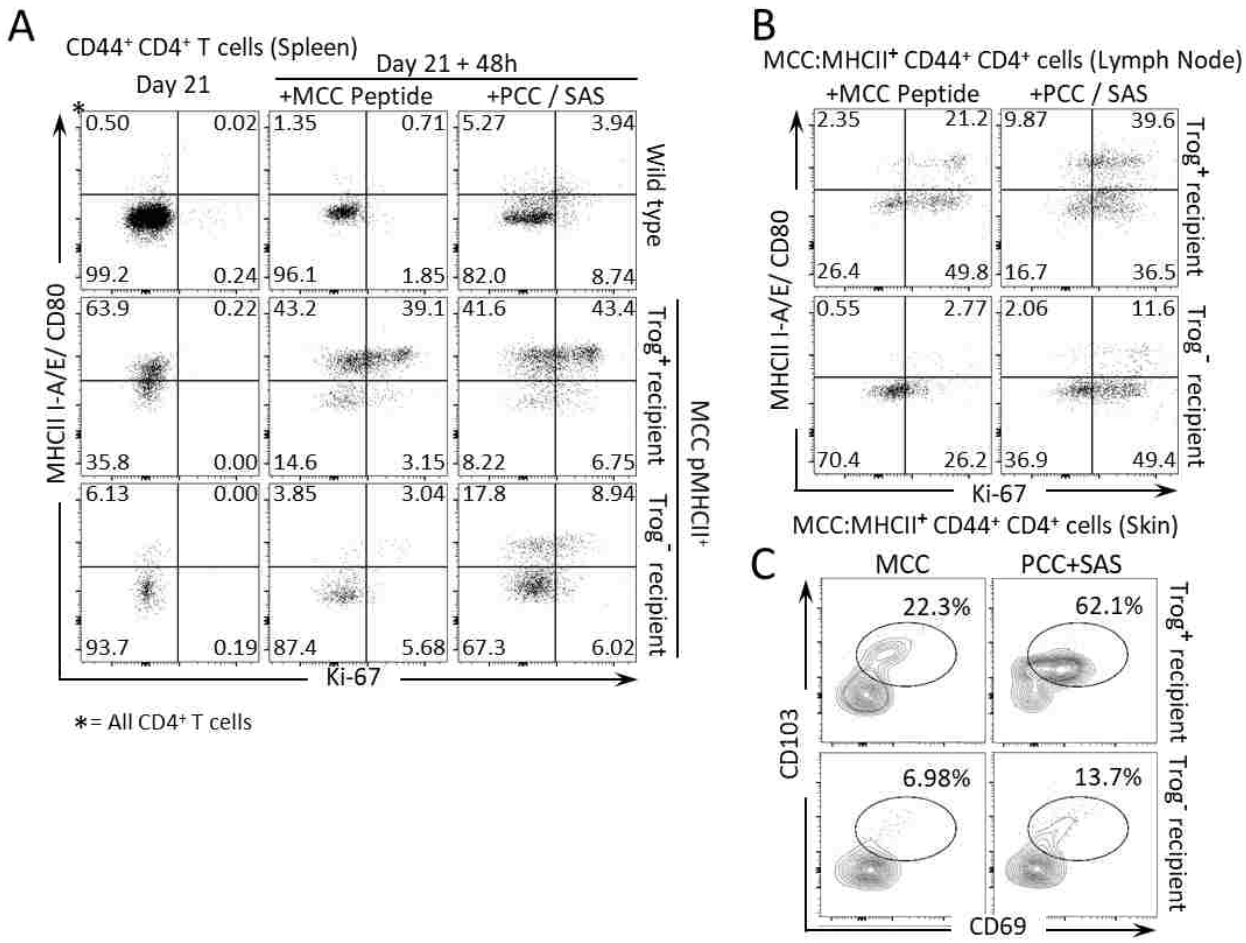



**Figure 4.34. *In vitro*-generated trog⁺ cells display rapid recall responses *in vitro*, 21 days post-adoptive transfer.** Twenty-one days post-adoptive transfer, spleens from WT B10.A mice and adoptive-transfer recipients were harvested (left column). A set of animals from trog⁺ and trog⁻ recipients were immunized S.C with either 500 µg MCC peptide alone (middle column) or 200 µg PCC protein in SAS (right column). Forty-eight hours later, spleens and draining lymph nodes were harvested. **(A)** Plots show Ki-67 vs MHCII/CD80 levels of CD4⁺ (top left) and CD44⁺ CD4⁺ T cells recovered from MCC (middle) and PCC+SAS-immunized (right) naïve WT B10.A Mice. Below are MCC:MHCII+ CD4⁺ T cells recovered from trog⁻ (middle row) and trog⁺ (bottom row) recipients. Numbers in corners represent frequency of cells in respective quadrants, which were determined by respective isotype control staining. **(B)** Plots show cells recovered from lymph nodes of mice immunized as in Fig. 4.31. **(C)** CD44⁺ CD4⁺ MCC:MHCII⁺ T cells recovered from skin at the site of injection 48 hrs post-immunization with MCC (left) or PCC+SAS (right) in trog⁺ (top) and trog⁻ (bottom) recipients.

Notably, 59% of the Ki-67⁺ cells in trog⁻ recipients on day 23 had acquired I-E$^k$ + CD80, suggesting that these cells performed trogocytosis from APC *in vivo* and more readily proliferated than cells which did not perform trogocytosis (Fig.

4.34A, *bottom right*). This is supported by the fact that cells from trog$^{-}$ recipients on day 21, and those recovered from mice 48 hrs post-immunization with MCC alone showed similar frequencies of I-E$^{k}$/CD80$^{+}$ cells (6% and 6.9%, respectively), however after immunization with PCC + SAS, 26.7% of cells in trog$^{-}$ recipients had become trog$^{+}$ (Fig. 4.34A, bottom).

Interestingly, the cells in the lymph nodes did not display the same pattern as those in the spleen, as both I-E$^{k}$/CD80 positive, and negative cells from the trog$^{+}$ recipients response to peptide alone, and a lower frequency of the cells in the draining LN were I-E$^{k}$/CD80$^{+}$ compared to those in the spleen (Fig. 4.34B). However, a noticeable difference was still observed between the cells recovered from the trog$^{+}$ (71% Ki-67$^{+}$) and trog$^{-}$ (29% Ki-67$^{+}$) recipients after immunization with MCC (Fig. 4.34B, *right*). The cells from the trog$^{-}$ recipients responded to immunization with PCC+SAS, and 61% of MCC:MHCII$^{+}$ cells in these animals were Ki-67$^{+}$ 48 hours post-immunization (day 23), compared to 76% of cells from trog$^{+}$ recipients (Fig. 4.34, *right*). To address potential $T_{RM}$ cells, skin was collected from the immunization site, as well as a portion of skin from the distal side of the mouse. Very few MCC:MHCII$^{+}$ cells were detected in a similar-sized portion of the skin distal to the injection site (not shown). However, there was a striking difference between the cells in the trog$^{+}$ and trog$^{-}$ recipients at the site of the immunization (Fig. 4.34C). Using the markers CD69 and CD103 to identify $T_{RM}$ cells, of the transferred CD44$^{+}$ cells in the trog$^{+}$ recipients, 22.3% of cells in MCC-immunized mice, and 62.1% of cells in MCC+SAS-immunized mice showed a $T_{RM}$-phenotype (Fig. 4.34C, *top*). In contrast, only 6.98% of transferred

CD44$^{+}$ cells in trog$^{-}$ recipients immunized with MCC, and 13.7% of SAS+PCC-immunized mice shared this phenotype (Fig. 4.34C, *bottom*).

To further assess the recall responses of transferred cells, the frequency of MCC:MHC$^{+}$ cells relative to all CD4$^{+}$ T cells in the spleens of mice at day 21 and 48 hrs after immunization on day 21, was determined. Immunization with PCC+SAS led to a significantly higher frequency of MCC:MHCII-tetramer$^{+}$ cells in naïve WT, trog$^{+}$ and trog$^{-}$ recipients compared to the frequencies on day 21. In naïve WT animals however, this frequency remained below 0.15% (Fig. 4.35A, *left*). Immunization with PCC+SAS also led to significantly higher frequencies of MCC:MHCII$^{+}$ cells compared to MCC-immunized mice in naïve WT, and trog$^{-}$ recipients, while the difference between MCC:MHCII+ cell numbers in PCC+SAS immunized trog$^{+}$ recipients was found to be significantly greater compared to day 21, but was not significantly different compared to MCC-immunized mice (Fig. 4.35A). This suggests that the response of the Ag-specific cells in the trog$^{+}$ recipients was consistent with memory cells.

Finally, IL-21 expression was examined in cells recovered from the spleens of trog$^{+}$ and trog$^{-}$ recipients 48 hrs after immunization with MCC or PCC+SAS. Despite the apparent increase in proliferation displayed by cells in the trog$^{+}$ recipients after immunization with MCC alone, the IL-21 expression was similar in cells from MCC-immunized trog$^{+}$ and trog$^{-}$ recipients (Fig. 4.35B, *left*). The transferred cells from the trog$^{+}$ recipients however displayed a 28% increase in

IL-21 expression compared to transferred CD44$^{+}$ cells from trog$^{-}$ recipients after immunization with PCC+SAS (Fig. 4.35B, *right*).

**Figure 4.35**

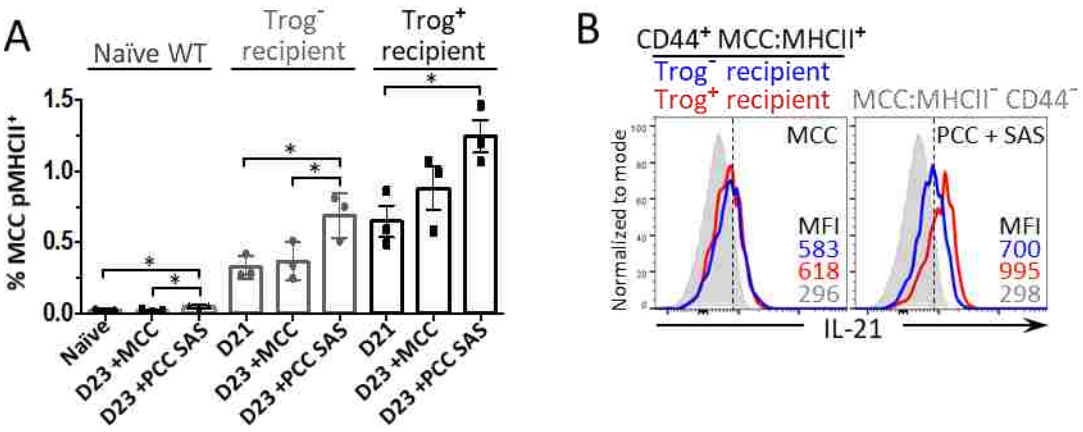



**Figure 4.35. *In vitro*-generated trog$^{+}$ cells produce a population which displays enhanced *in vivo* recall responses. (A)** Frequency of MCC:MHCII$^{+}$ CD4$^{+}$ T cells recovered from spleens of untreated (D21), followed by MCC and PCC+SAS-immunized mice (D23). Naïve WT B10.A mice are shown on the left, trog$^{-}$ recipients in gray (middle), and trog$^{+}$ recipients in black (far right). Data represents mean frequency ±SEM of replicate animals (n=3), with *=P>0.05. **(B)** Plots showing IL-21 expression in MCC:MHCII+ CD44$^{+}$ CD4$^{+}$ T cells recovered from spleens of MCC (left) and PCC+SAS-immunized (right) trog$^{+}$ (red) and trog$^{-}$ (blue) recipients, following a 5 h PMA+Ionomycin stimulation in the presence of BfA. CD4$^{+}$ cells from mice that did not receive adoptively transferred cells are shown in shaded gray. Data in Fig 7 is representative of two separate experiments.

As the early-memory checkpoint in mouse models typically falls between 21-28 days after the start of the immune response [518], at 21 days it is difficult to confirm that these cells were true-memory cells, which can persist for months to years after infection. However, in typical models the early checkpoint has been based off of immunization models, where Ag-reserves may persist for weeks, and thus making it difficult to determine if the cells were true memory or were activated by Ag-reserves. In this system, Ag was limited to the APC during the trogocytosis assay, and trogocytosed molecules. Therefore, these results

suggest that trog$^+$ cells contain a population of initial cells, or produce progeny, that show enhanced survival, and recall responses consistent with a memory-phenotype. Because in the absence of exogenous Ag the rate of trogocytosis is expected to be low, that a substantial percentage of surviving cells in the trog$^+$ recipients still displayed trogocytosed I-E$^k$ / CD80 21 days post-transfer (Fig. 4.34), suggests that the original cells which performed trogocytosis made up a major portion of the persisting early memory-like population.

## Discussion

The ability of CD4$^+$ T cells to differentiate into specialized $T_H$ subsets is a critical component in generating protective immunity against a multitude of pathogens. Despite the central role these cells play in generating adaptive immunity, the mechanisms behind CD4$^+$ differentiation are not completely understood. In particular, the multi-step signaling process required for $T_{FH}$ differentiation, [519], and the mechanisms governing memory T cell formation are largely unknown. Here, we set out to investigate the potential role for trogocytosis-mediated signaling in $T_{FH}$ differentiation. We hypothesized that, because $T_{FH}$ differentiation requires repeat-cognate TCR+costimulatory signaling events, that sustained trogocytosis-mediated signaling may promote $T_{FH}$ differentiation after APC removal.

While it has been agreed on that $T_{FH}$ development requires repeat cognate interactions, the required source of these interactions has not been solidified. The proposed requirement for B cells in the development of $T_{FH}$ is most

likely due to the fact that the B cells become the most abundant APC for activated T cells in the lymph node following clonal expansion. This is supported by the fact that repeated interactions with DC can also drive $T_{FH}$ differentiation [213]. Irrespective of the APC type, $T_{FH}$ differentiation requires repeated TCR signaling after initial activation [213, 228, 231, 244]. In this system, T cells were cultured in absence of APC, but still developed a $T_{FH}$-like phenotype. This strongly suggests that trogocytosis-mediated signaling is capable of driving $T_{FH}$ differentiation in the absence of APC. If there were contaminating residual APC in the cultures, then it would be expected that a higher frequency of the $trog^{-}$ cells would also be able to develop a $T_{FH}$ phenotype. This is supported by the results that the addition of APC to cultures of recovered cells led to a significant increase in the amount of $CXCR5^{+}$ $PD\text{-}1^{+}$ $trog^{-}$ cells (Fig. 4.7). Somewhat unexpectedly, the addition decreased the frequency of $CXCR5^{+}$ $PD\text{-}1^{+}$ $trog^{-}$ cells compared to cultures containing only recovered T cells. This could possibly be due to hyporesponsiveness developed by the T cells, which can occur after repeat exposure to APC [520].

The T cells recovered from the MCC:FKBP APC were highly efficient at developing a $T_{FH}$ phenotype, consistent with the positive correlation between TCR binding strength and $T_{FH}$ differentiation [521]. That trogocytosis-mediated signaling was contributing to the apparent $T_{FH}$ phenotype developed in the $trog^{+}$ cells was further supported by the findings that removal of MCC from trogocytosed pMHCII complexes led to a dramatic decrease in CXCR5, PD-1, and IL-21 expression.

Following adoptive transfer of naïve 5C.C7 cells into naïve animals, and immunization with PCC, 3 days -post immunization we found that nearly 80% of the PD-1+ CXCR5+ cells were also trog$^{+}$ (Fig. 4.11). However, the frequency of trog$^{+}$ cells decreased to 40% by 7 days post-immunization. This strongly suggests that the trog$^{+}$ cells are able to more rapidly become $T_{FH}$ compared to trog$^{-}$ cells, while trog$^{-}$ cells are able to efficiently become $T_{FH}$ given extra time. One explanation for these results could be that trogocytosis-mediated signaling contributed to the signaling required for $T_{FH}$ differentiation after the trog$^{+}$ cells dissociated from APC. On the other hand, the trog$^{-}$ cells would require re-engaging APC to receive sequential TCR signaling, reflected by a delay in the differentiation to $T_{FH}$. The kinetics of the immune response and $T_{FH}$ differentiation further support this theory. Because purified populations of CD4$^{+}$ TCR-Tg T cells were transferred into the naïve animal, at early time-points, the number of responding B cells would be expected to be low. Therefore, due to the time it takes for $T_{FH}$ differentiation to occur (at least 2-3 days *in vivo*), the trog$^{+}$ cells likely started developing a pre-$T_{FH}$ phenotype shortly after activation by DC outside of the B cell follicles. Seven days post-immunization, the host B cells would have had time to undergo clonal expansion, and thus effectively drive $T_{FH}$ differentiation in the trog$^{-}$ cells through increased B:T cell interactions. These findings raise the possibility that trogocytosis-mediated signaling may have significant impacts on the kinetics of GC formation and B cell activation.

Trogocytosis-mediated signaling appeared to enhance IL-21 expression in the trog$^{+}$ cells, as blocking signaling or removal of Ag from MHCII led to a

significant decrease in the frequency of IL-21$^+$ trog$^+$ cells. These results, combined with the observation that IL-21 was polarized towards trogocytosed molecules, suggest that trogocytosis-mediated signaling may further promote $T_{FH}$ differentiation by driving IL-21 expression. The IL-21 produced by the trog$^+$ cells may play a separate role in vivo, as IL-21 can impact multiple aspects of T cell immunity including maintaining cell survival, proliferation, effector function, and memory formation.

The observed proliferation of I-Ek/CD80- cells in the LN in response to immunization with MCC alone could be due to residual activation in the LN compared to the spleen, as a higher frequency of the LN cells were performing active cell division, and displayed higher levels of CD44 compared to those in the spleen 5 days post-transfer (Fig. 4.34B).

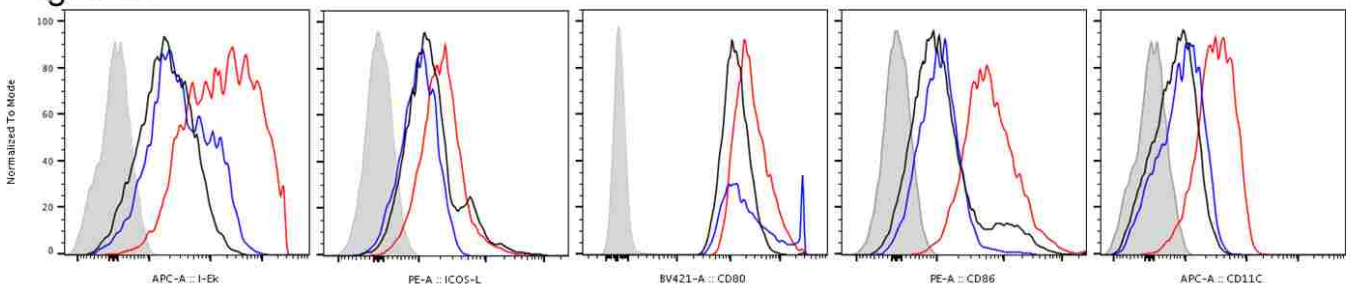
It also appeared that the cells from the trog$^+$ recipients were able to respond to immunization with PCC+SAS in the periphery more readily than the cells in the trog$^-$ recipients. This was indicated by a massive recruitment of these cells to the site of injection in trog$^+$ recipients compared to the trog$^-$ recipients (Fig. 4.34C). Interestingly, these cells resembled $T_{RM}$, however they were not largely present in the injection site of mice immunized with MCC peptide alone, suggesting that the presence of these cells may be an artifact of the adjuvant. However, it has been shown that human $T_{RM}$ are able to leave the tissue to enter circulation and take up residency in other sites. Therefore, it is possible that the trog$^+$ cells also produce $T_{RM}$. Without an initial site of Ag-delivery to induce a primary response in this model, it was difficult to assess $T_{RM}$ responses. In the

study shown in Fig. 4.14, skin from the injection site was collected at the time of harvest (7 and 28 days post-immunization), and no substantial differences in MCC:MHCII$^{+}$ CD69$^{+}$ CD103$^{+}$ $T_{RM}$ cells were observed between cells isolated from the skin proximal, or distal, to the injection site (data not shown).

From these experiments, the possibility for T:T presentation between the trog$^{+}$ cells after adoptive transfer into animals cannot be ruled out. However, even with this possibility, the results suggest that the trog$^{+}$ cells produce a more robust memory-like population compared to the trog$^{-}$ cells in absence of exogenous Ag and is a novel finding.

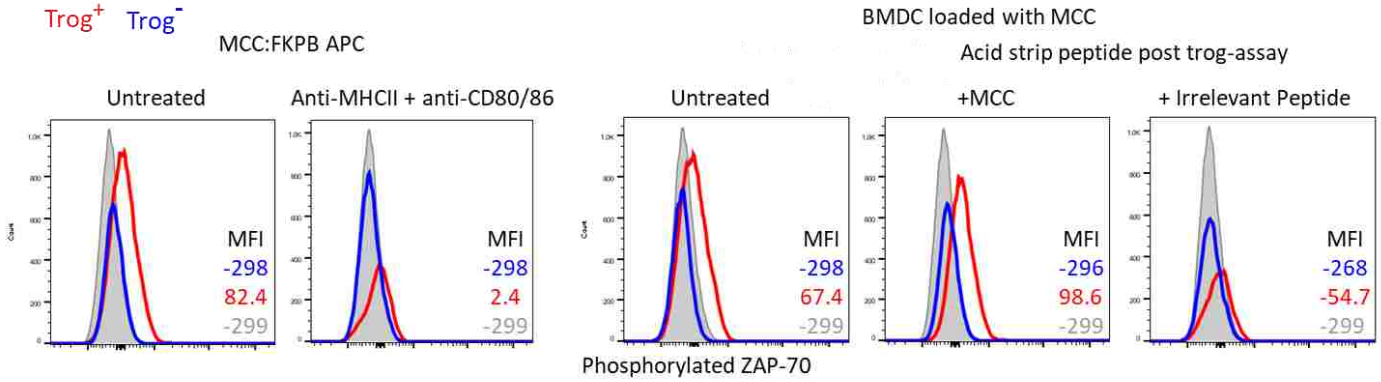
Collectively the results from chapter 4 suggest that trogocytosis-mediated signaling plays a role in the generation of cells resembling both $T_{EM}$ and $T_{CM}$. Importantly, the results suggest that trogocytosis-mediated signaling plays a critical role early in the response by enhancing the survival and proliferation of cells weakly-activated by APC. Such activation may promote Tcf1 expression and enhance the differentiation to $T_{FH}$, or transition to memory [522].

Fig. S4.1



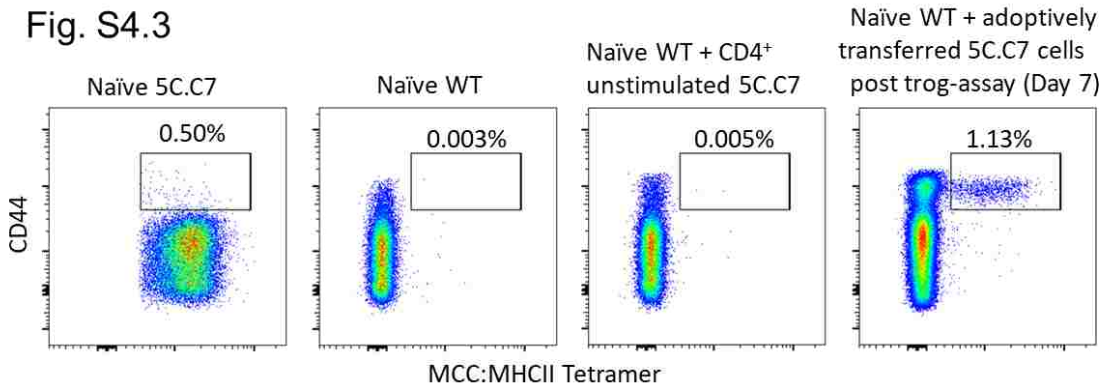

**Supplemental figure 4.1. Surface expression of signaling molecules on APC used in this study**: BMDC (red), MCC:FKBP APC (black), and APC transfected with empty MHC class-II I-$E^k$ (blue), compared to unstimulated $CD4^+$ T cells (shaded gray)

Fig. S4.2




Supplemental figure 4.2. Blocking I-$E^k$ or removal of Antigenic peptide lead to decreased TCR signaling in $trog^+$ cells. $CD4^+$ T cell blasts were used in a standard *in vitro* trogocytosis assay with indicated APC (top). Recovered cells were either untreated, or supplemented with neutralizing antibodies against I-Ek + CD80 (second from left), or when recovered from BMDC, stripped of peptide followed by supplementation with antigenic MCC, or irrelevant Hb peptide, as indicated. Plots show intensity of phosphorylated ZAP-70 in trog+ (red), trog- (blue), 72 hours post-recovery, in the context of respective treatments. With unstimulated T cells shown in gray.

Fig. S4.3




Supplemental Figure 4.3. Identification of Ag-experienced MCC:$MHCII^+$ cells. Gating scheme for identification of Ag-specific cells *in vivo* using a MCC:MHCII tetramer

# Chapter 5

# Discussion

Although trogocytosis was first observed nearly a half-century ago [362], the biological implications of this event were not appreciated until recent decades with the discovery that trog$^+$ cells can act as functional APCs through the presentation of trogocytosed molecules. T-T Ag presentation after trogocytosis can significantly impact the activation state of the responding cell. The various effector phenotypes and distinct migratory patterns of trog$^+$ cells add additional layers of complexity to models of canonical T cell activation and differentiation. The biological implications of trogocytosis-mediated signaling are underscored by the fact that trogocytosis occurs at significant frequencies (~10-20%) in responding T cells during an active immune response. Collectively, the published literature, and the results from this dissertation, demonstrate the clear immunomodulatory potential of trogocytosis, and subsequent signaling events.

A largely-understudied component of trogocytosis is how this event impacts the trog$^+$ cell itself. Studies have shown that irrespective of the context, the trog$^+$ cells show enhanced effector ability compared to their trog$^-$ counterparts [406][282, 374, 391, 400]. The authors in these studies suggested that trogocytosis was the result of a highly activated state in the trog$^+$ cells. While the heightened state of activation likely contributed to trogocytosis, the findings here suggest that trogocytosis-mediated signaling itself could have been

responsible for the observed phenotype in trog$^+$ cells. While activated cells perform trogocytosis more efficiently than naïve cells [391], the activation kinetics of trog$^+$ cells are not consistent with those of trog$^-$ cells after removal of TCR stimulation [81,406,412]. This suggests that the trogocytosed molecules impact the activation and, potentially effector functions of the trog$^+$ cell. We previously reported that T cells can engage their surface receptors with trogocytosed signaling molecules to achieve sustained TCR signaling [81]. This signaling correlated with a heightened activation state, and enhanced survival of the trog$^+$ cells. Because the duration and frequency of TCR signaling can significantly impact T cell activation, effector function, and differentiation, it is possible that trogocytosis-mediated signaling impacts the trog$^+$ cells beyond allowing for sustained survival. The objective of this dissertation was to examine how trogocytosis-mediated signaling impacted CD4$^+$ effector function and subset differentiation in the context of the trog$^+$ T cell.

# Part I. The Mechanisms of CD4$^+$ Trogocytosis

### On the Requirement for Active Signaling in T cell Trogocytosis

T cell trogocytosis is largely dependent on active signaling, and primarily involves the acquisition of molecules that are bound to ligands on APC. However, the acquisition of “bystander” molecules such as MHC class-I have been observed on trog$^+$ CD4$^+$ T cells [523]. It has been proposed that such acquisition occurs due the proximity of the non-engaged molecules to recycling TCR bound to ligands on the APC. Support for this come from the findings by Gu *et al.*, who

demonstrated that CTLA-4 knockout $nT_{reg}$, or $iT_{reg}$ with CTLA-4 neutralized with anti-CTLA-4 Abs, acquired their CD80 and CD86 from DC [277]. This trogocytosis event was not due to CD28 engagement with CD80/CD86 as neutralizing antibody blockade against CD28 did not affect the acquisition of CD80 or CD86. Curiously, they also reported that the addition of an endocytosis inhibitor (unnamed) to T cell:DC co-cultures did not impact CD80/CD86 acquisition by the $T_{reg}$. Thus, they speculated that $T_{reg}$ acquire CD80/86 via an unknown mechanism independently of CTLA-4, CD28, or PD-L1 binding, or endocytosis. Why they did not examine MHC in this study was unclear. One possible explanation for these results could be that CD80/CD86 were transferred via APC-derived exosomes that were bound to T cell surface receptors.

**APC-Derived Exosome Acquisition as a Mechanism for Trogocytosis**

The acquisition of pMHC complexes by T cells through APC or tumor-derived exosomes has been found to confer T cells with antigen-presenting ability, and has been proposed as one mechanism of intercellular membrane-protein transfer [524]. APC-derived exosomes include signaling, and non-signaling molecules, including lysosomal membrane protein-1 (Lamp-1), CD86, CD37, MHC1, MHCII, and transferrin receptor (TfR) [525-528]. Thus, the acquisition of these exosomes is consistent with the presence of non-engaged molecules detected on $trog^{+}$ T cells. In support of this theory, cognate T cell interactions with DC or B cells increase the rate of MHCII-rich exosomal formation by APC [529]. Furthermore, DC and B cell-derived exosomes can

stimulate activated, but not naïve $CD4^+$ T cells both *in vitro*, and *in vivo* [529]. However, evidence suggests that exosomal acquisition of APC-derived molecules is distinct from trogocytosis. For example, pre-treatment of DC with the ATPase inhibitor CMA (which also inhibits exosome formation) prior to co-incubation with $CD8^+$ T cells, led to only a minimal decrease in PD-L1 transfer to T cells [375]. However, separation of co-incubated T and DC by a trans-well membrane prevented transfer of PD-L1 to T cells [375], suggesting that the PD-L1 trogocytosis was contact-dependent. In a similar study using human cells, while co-cultured DC and T cells resulted in high levels of HLA-DR acquisition, separation of T cells and DC by a trans-well membrane inhibited virtually all transfer of HLA-DR to T cells [410]. These studies did not account, for the fact that contact with T cells induces increased exosomal release by APC [529]. Interestingly, when exosomes containing p:MHCII complexes and costimulatory molecules were added to cultures of naïve $CD4^+$ T cells, Ag-specific activation occurred only in the presence of DC CD80+/CD86+, but was independent of MHCII expression by the DC [530]. These results suggest that MHC acquired via APC-derived exosomes is only functional upon T cell:APC contact.

Additional evidence discounting the role of exosomes as the mechanism for trogocytosis come from findings with DC whose membranes were labeled with the extremely stable lipophilic dye PHK26. Levels of PHK26 on the T cell decreased between 6 and 24 h of $iT_{reg}$ co-incubation with DC while, in contrast, levels of CD80 and CD86 increased [277]. This suggests that receptor-mediated transfer was occurring, rather than absorption of membrane-bound exosomes

which would be expected to increase the levels of PHK26 similarly to CD80/86 [277]. Our previous data using APC-conditioned media also observed minimal acquisition of APC-derived exosomes by T cells [287]. In addtion, unpublished data from our lab using mild acid treatment of antibodies against trogocytosed molecules did not alter the amount of troocytosed molecules associated with a trog$^{+}$ T cell. These data show that the trogoctosed molecules are integrated into the T cell membrane, even at early time points, and were not on APC-derived exosomes adsorbed to the T cell surface.

While exosomes may contribute to the amount of transferred material to T cells, exosomal transfer alone is unlikely to result in intracellular trogoctosis mediated signaling. We, and others have observed trogocytosed molecules in distinct, punctate spots on the surface of the T cell. These spots are orders of magnitude larger than exosomes (~30-100 nm). Thus, dramatic re-arrangment of TCR/costimulatory molecules as well as APC-acquired molecules via exosomes would have to occur to form clusters equivelant to those which have been visualized. If the observed spots of trogocytosed molecule/TCR complexes were formed by T cell signaling events, then abolishing T cell signaling should lead to the dispersion of molecules and loss of these spots. Such dispersion was not observed in experiments where ZAP-70 signaling was inhibited with the Srk kinase inhibitor PP2 [81]. Taken together, the published data suggests that intracellular trogocytosis-mediated signaling is not dependent on exosomal transfer.

**Microclusters as a Mechanism for Trogocytosis**

Another potential mechanism for trogocytosis is the transfer of APC molecules to the T cell via microclusters. We, and others have observed small clusters of MHC being transferred from APC to T cells during the immune synapse [397] [81]. In live-cell imaging experiments, it has been observed that clusters containing pMHC move away from the immunological synapse and migrate to the distal pole upon T cell:APC contact (Fig. 1.15). This transfer event is strikingly similar to the formation of the distal pole complex or, the "antisynapse" [70, 76]. A perplexing characteristic of the antisynapse is that, despite its location at the distal pole, it contains active TCR signaling molecules ZAP-70, LAT, PCLy1, P-Tyr, and Lck [76]. It has been proposed that the formation of the antisynapse likely involves TCR microclusters which were activated by pMHC that move to the distal pole [70]. That microclusters may be involved in the active TCR signaling at the anti-synapse is strengthened from the fact that TCR microclusters form an adhesion ring composed of LFA-1, focal adhesion molecules paxillin and Pyk2, and myosin II (MyoII) [71]. It has been proposed that this structure allows microclusters to exclude the suppressive molecule CD45 and thus are able to retain active TCR signaling [69, 72, 74]. However, even by exclusion of CD45, there is little evidence that the TCR proximal signaling complex would be sustained for the duration that has been observed at the antisynapse, in the absence of MHC binding and subsequent TCR signaling. Coincidentally, we have observed that the clusters of MHC which

transfer to the T cell and localize to the distal pole also co-localize with TCR and active proximal TCR signaling molecules ZAP-70, Lck, P-Tyr [81]. In fact, the images we previously reported are strikingly similar to those of antisynapses (Fig. 5.1).

**Figure 5.1**

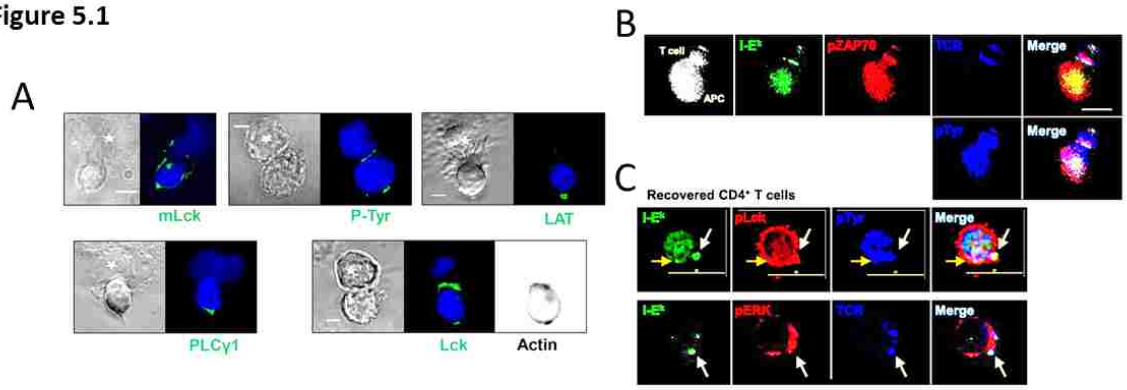



**Figure 5.1. Upon T cell:APC contact, TCR-signaling occurs distal to the immunological synapse (and is co-localized with trogocytosed MHC) (A)** Active P-Tyr, LAT, PLCγ1, and LCK signaling detected at the antisynapse following contact between Ag-loaded Raji B (indicated by white star) and Jurkat T cells. Images show formation between 3-5 min of T:APC contact. Green indicates activated respective signaling molecule. Nuclei were labeled with Hoechst 33342 (blue). Scale bars = 5 μm *Source: Guedj et al. 2016. Journal of Cell Science* **(B) Top:** $CD4^+$ T cells (top cell) conjugated with APC expressing GFP-tagged I-Ek (bottom cell). Images show pZAP-70 (red) and pTyr (blue, bottom row) levels colocalize with GFP-tagged pMHC (green) and the TCR (blue, top row) at the immune synapse and at the distal pole. **(C)** pLck (top red),total pTyr (top blue), pERK (bottom red) colocalize with trogocytosed pMHC (green) and TCR (bottom blue) regions (indicated by yellow and orange arrows) on T cells 30 min post-recovery from in vitro trogocytosis assay. *Source: Osborne and Wetzel, 2012. Journal of Immunology*

Further support for microclusters as a mechanism for trogocytosis, is that microcluster formation is found to be resistant to treatment with PP2 [73]. This potentially explains how punctate signaling complexes, or what I will refer to as "trogosomes", were observed after treatment of $trog^+$ cells with PP2 [81]. The uncanny resemblance between the antisynapse and our studies on trogocytosis in the context of location and active signaling events, is consistent with the hypothesis that active-TCR signaling at the antisynapse is driven by pMHC complexes bound to the TCR.

The location of APC-signaling molecules on the distal pole of the T cell could result in trogocytosis when the T cell dissociates from the APC. In this model, the transfer of APC-derived molecules is highly dependent on active signaling, consistent with current models for trogocytosis. This would also provide an explanation for the observed punctate spots on trog$^+$ cells, which would be derived from pre-existing microclusters rather than having to independently form from re-expressed trogocytosed molecules. Consistent with this possibility is the data from Fig. 3.3 which shows that pre-polarized $T_H2$ cells perform trogocytosis at significantly higher frequencies compared to polarized $T_H1$, or non-polarized cells. As $T_H2$ cells have been observed to form multi-focal synapses which resemble microclusters [83] this morphology could facilitate trogocytosis of pMHCII and costimulatory molecules.

In this proposed microcluster model, it is possible that the acquired molecules are endocytosed with recycling TCR. However, if a focal point of signaling is already pre-established by the signaling complex formed at the distal pole this would promote the migration of newly-bound TCR:pMHC to the site of active signaling. This could be an explanation for the observed punctate trogosomes detected on trog$^+$ cells days after APC removal (Fig. 3.5).

It is possible that highly activated cells are more efficient at tearing off membrane from the immunological synapse, as they have higher migration patterns and form shorter synapses. Perhaps trogocytosis through this mechanism would be more likely to enhance activation and effector functions of the trog$^+$ cell. The live-cell imaging should also be further analyzed, as the data

show that as the T cell dissociates, the T:APC molecules bound near the dSMAC appear to get “pulled in” towards the central point of T:APC contact and torn away from the APC by the T cell. This is suggestive that the acquisition of microclusters may also occur upon T cell dissociation.

The current literature has referred to trogocytosis in the context of all of the aforementioned mechanisms, however this broad categorization has led to contrasting results in not only the mechanisms of trogocytosis, but its biological outcomes as well. The microcluster model fits well with our previous results, and those in this study, showing that trogocytosis-mediated signaling is sustained for up to 72 hrs after APC removal, and leads to increased activation and effector cytokine production. In contrast, the acquisition of APC molecules not engaged with T cell receptors through bystander endocytosis or exosomes may lead to a different phenotype in the context of the trog$^{+}$ cell. Whether there is a difference in the overall result between the different mechanisms of APC-derived signaling molecules remains to be determined.

# Part II

# T:T Antigen Presentation and Trogocytosis-Mediated Signaling in T cell Activation, and Immuno-Regulation

The results in this study suggest that trogocytosis-mediated signaling enhances the activation state of the $trog^+$ cell independently from activation by APC. However, other studies have reported T:T presentation of trogocytosed molecules signaling to inhibit the activation of responding cells. The most likely explanation for such conclusions is simply, that the $trog^+$ cells which induce repressive modes of action are actually $T_{reg}$, which are highly efficient at performing trogocytosis. In such a case, trogocytosis-mediated interactions would enhance the $trog^+$ $T_{reg}$ suppressive capabilities rather than provide insufficient signaling to induce anergy in activated cells. While some studies claim that the $trog^+$ cells which showed repressive functions were "not $T_{reg}$", the argument for this is questionable. For example, Helft *et al.* reported that when $trog^+$ $CD4^+$ cells were mixed with previously-activated T cells, T:T presentation of Ag to activated cells induced responder cell death, but stimulated naïve T cells to proliferate. The authors proposed that this was likely due to insufficient levels of costimulatory molecules upon p:MHC presentation by $trog^+$ cells. Interestingly, when DC were added to cultures containing a mixture of $trog^+$ and either activated or naïve T cells, the presence of $trog^+$ cells inhibited the activation of activated, but not naïve cells, compared to controls which did not contain $trog^+$ cells [429]. Firstly, activated cells require less stimulation than naïve T cells to maintain an activated state, thus the argument for insufficient stimulation for

naïve vs activated cells is not sound. Second, if insufficient stimulation by trogocytosis-mediated signaling was the reason for activated cells to become inactive, the addition of APC to mixed trog$^{+}$/trog$^{-}$ cultures should have rescued at least some of the phenotype. Rather than determining the phenotype of the trog$^{+}$ cells, it was suggested that because the suppression of activated cells occurred in an Ag-specific manner, the trog$^{+}$ cells were not $T_{reg}$, which function in an Ag-independent manner. In complete contradiction to this reasoning, a study by Bacheli et al found that $T_{reg}$ efficiently perform trogocytosis and formed contacts with responding T cells leading to a contact-dependent mechanism for immunosuppression by trog$^{+}$ $T_{reg}$. [432]. In addition, three years prior to the publication by Helft *et al.* it was published that $T_{reg}$ perform contact-dependent immunosuppression through granzyme B release onto ligated cells [296]. While insufficient levels of trogocytosed costimulatory molecules are likely to induce anergy, inadequate phenotyping of the trog$^{+}$ cells in previous studies has resulted in a convoluted consensus of the impacts of non-$T_{reg}$ T:T presentation.

Our results suggest that in effector CD4$^{+}$ cells, trogocytosis-mediated signaling enhances the activation of the trog$^{+}$ cells (Figs. 3.1, 4.1). This is consistent with a role for trogocytosis signaling in the heightened state of activation observed in trog$^{+}$ cells from other studies. The sustained activation observed here occurred in the context of peptide bound to MHCII. This timing is the average $t_{1/2}$ half-life for peptide binding to MHC is ~72-96 hours, which is consistent with the kinetics of trogocytosis-mediated signaling we have observed in the activation state of trog$^{+}$ cells recovered from BMDC, or other APC with

non-covalent peptide. At later time-points, as peptide dissociates from MHC, engagement with a combination of empty MHC and pMHC complexes would be insufficient to trigger T cell responses.

Trogocytosis-mediated signaling also involves costimulatory receptor signaling, which could also contextually lead to differences in $T_H$ subset differentiation, for example, the acquisition of ICOS-L, and OX40L and subsequent trogocytosis-mediated signaling promoting a $T_{FH}$ phenotype. Costimulatory signaling through CD28 in the absence of TCR signaling has been found to be sufficient to induce the nuclear translocation of NF-κB [531]. Thus, as peptide dissociates from the MHC and TCR signaling dcreases, co-stimulatory signaling may be playing a larger role in the apparent phenotype at later time points.

# Part III
## Biological Implications of Intracellular Trogocytosis-Mediated Signaling

### Implications of Trogocytosis-Mediated Signaling and a $T_H2$ Phenotype

The strong correlation between trogocytosis and the subsequent $T_H2$ phenotype observed in chapter 3 supports the hypothesis that trogocytosis-mediated signaling plays a role in T cell effector function and subset differentiation. In cells that are weakly activated by APC (which also promotes $T_H2$ differentiation), trogocytosis-mediated signaling could help overcome the threshold of signaling required for T cell survival and activation. In recently activated cells, or $T_H2$ effector cells, the production of IL-4 driven by trogocytosis-mediated signaling

could enhance cell survival [458]. By driving $T_H2$ differentiation, trogocytosis-mediated signaling could further promote the survival of the trog$^+$ cells by increasing their resistance to $T_{reg}$ suppression. It has been found that $T_H2$ cells are resistant to galectin-mediated apoptosis by $T_{reg}$, while $T_H1$ and $T_H17$ are susceptible [299]. The $T_H2$ phenotype could also aid in preventing an exhausted phenotype from sustained trogocytosis-mediated signaling as $T_H2$ cells suppress the expression of the apoptosis-inducing antigen FAS Ligand, making them less susceptible to activation induced cell death (AICD) [532]. On the other hand, $T_H1$ cells are highly susceptible to AICD [533]. Interestingly, $T_H2$, but not $T_H1$, cells have been shown to be able to revert from an anergic state to resume effector functionality [459], which may promote the transition of $T_H2$ cells to memory. These characteristics may be a reason for the observed bias for trogocytosis-mediated signaling to drive a $T_H2$ phenotype, over a $T_H1$ or $T_H17$ phenotype. The high susceptibility of $T_H1$ cells to $T_{reg}$ suppression and AICD may also help explain the extremely low frequency of $T_H1$ trog$^+$ cells at later time-points.

The implications of trogocytosis-mediated signaling and development of a $T_H2$ phenotype were discussed in detail at the end of chapter 3. One additional point of emphasis should be made regarding this phenotype in the context of solid tumors. Increasing evidence has been found from both animal studies and samples from human patients that trogocytosis occurs at high rates in solid tumor environments and by multiple cell types [388, 389, 399, 435, 534, 535]. In these cases of a localized tumor environment, a $T_H2$ phenotype driven by trogocytosis-mediated signaling could inhibit $T_H1$ effector functions, and the activation of naïve

CD4 T cells. $T_H1$ responses are generally considered protective in tumor models. As Ag is removed from tumor cells the responding T cells would receive weaker TCR signals from tumor cells or APC, leading to imbalanced $T_H2$ differentiation and augmenting a localized $T_H2$ environment. This could lead to generation of a non-protective humoral response. Effective immunotherapy against solid tumors has not yet been developed. The results from this study suggest that the differentiation/effector cytokine production by $CD4^+$ $trog^+$ T cells in the tumor microenvironment is worthy of consideration in future strategies to combat solid tumors.

**Trogocytosis-Mediated Signaling in the Apparent $T_{FH}$ Phenotype**

The phenotype observed in the $trog^+$ cells in chapters 3 ($T_H2$) and 4 ($T_{FH}$) overlap in that both of these subsets specialize in providing B cell help. The differentiation towards $T_H2$ could ultimately promote $T_{FH}$ differentiation by increasing the proximity of the T cell to the B cell and increasing the likelihood of interactions with cognate B cells. In addition, $T_H2$ cells have been shown to readily become $T_{FH}$ themselves [148, 231, 238]. In these studies, trogocytosis-mediated signaling drives cells towards a $T_{FH}$-like phenotype, while still retaining qualities of $T_H2$. This possibility is supported by observations that $T_{FH}$-like cells can concurrently express Bcl-6 and GATA3 [148], and both $T_H2$ and $T_{FH}$ cells commonly express IL-4 [234, 238, 448, 481, 536]. It is temping to conclude that the early $T_H2$ phenotype developed by the $trog^+$ cells may act as a favorable intermediate for the $T_{FH}$ phenotype observed at later time-points.

An interesting observation from the results in chapter 4 was, in vivo, the trog$^{+}$ cells developed a $T_{FH}$-phenotype more readily than trog$^{-}$ cells (Fig. 4.11). In addition, when cultures of cells recovered from the trogocytosis assay were supplemented with APC, a greater frequency of the trog$^{+}$ cells displayed a $T_{FH}$-like phenotype compared to the trog$^{-}$ cells (Fig. 4.7). These results suggest that the trog$^{+}$ cells were more efficient at becoming $T_{FH}$ upon initial priming by APC compared to trog$^{-}$ cells. This also raises the possibility that trogocytosis-mediated signaling may have significant impacts on the kinetics of GC formation and B cell activation. By rapidly upregulating high levels of CXCR5 and producing high levels of IL-21 (Figs. 4.2-5), the trog$^{+}$ cells could rapidly provide help to the B cells to increase Ab production, affinity maturation, class switching and proliferation to further increase the chances of B:T cell interactions early during the effector phase of the immune response. This is an area of active inquiry in the Wetzel lab.

It is also likely that trogocytosis-mediated signaling would have significant impacts on $T_{FH}$ differentiation in cases of low Ag. If a low number of APC displaying cognate-Ag were present, Ag-specific T cell:APC interactions would be less likely to occur. The current models of $T_{FH}$ differentiation require 2 or more distinct T-APC interactions. However, the necessity for additional APC could be circumvented by sustained trogocytosis-mediated signaling after the first interaction, thus making the trog$^{+}$ cells more likely to differentiate to $T_{FH}$ than the trog$^{-}$ cells.

Finally, trogocytosis-mediated signaling also resulted in the sustained expression of IL-21, and IL-4 by the trog$^+$ cells (Figs. 3.3, 4.5). This is important because the initial T-DC interactions that result in pre-$T_{FH}$ cells do not induce IL-21 expression, which has been proposed to occur only after subsequent interactions with B cells. This implies the Trog-mediated signaling is contributing to full $T_{FH}$ differentiation. It is possible that the trog$^+$ cells would be more efficient $T_{FH}$ early in response by secreting high amounts of these cytokines in the GC without requiring cognate B cell interactions. Furthermore, if trogocytosis-mediated signaling can drive $T_{FH}$ differentiation in the absence of B cells, then the trog$^+$ cells could act also as extra-follicular $T_{FH}$-like cells, that readily provide help to B cells outside of B cell follicles/GCs.

The sustained activation and high production of IL-4 and IL-21 by trog$^+$ cells could also play a major role in humoral autoimmunity. Hyperreactive $T_H2$ and $T_{FH}$ responses play a major role in Ab-mediated autoimmune diseases such as myasthenia gravis, autoimmune thyroiditis, Sjögren's syndrome, rheumatoid arthritis (RA), multiple sclerosis, SLE, ulcerative colitis, Crohn's disease, ankylosing spondylitis (spinal arthritis), and type 1 diabetes [245, 246]. Interestingly, extrafollicular cells, which resemble $T_{FH}$, have been found to be key mediators in such autoimmune diseases by forming ectopic lymphoid structures with B cells to induce class switching, affinity maturation, and high antibody production [537]. These structures have been observed in patients with RA [538], and have shown to play a major role in progression of SLE [539]. It is possible

that trogocytosis-mediated signaling could be contributing to the $T_{FH}$-like phenotype of these cells outside of the GC.

### IL-21 promotes T cell Activation, Survival, Effector Function, and Memory Generation

The high production of IL-21 by trog$^+$ cells has biological implications beyond driving a $T_{FH}$ phenotype and providing B cell help. IL-21 may contribute to the sustained survival displayed by the trog$^+$ cells, as IL-21 has been shown to be critical for the survival of activated CD4$^+$ and CD8$^+$ T cells in cases where IL-2 is limited [540]. Is it also possible that IL-21 production by trog$^+$ cells aids in their ability to sustain effector functionality, as addition of IL-21 has been shown to protect T cells from suppression by $T_{reg}$ [541]. In CD8$^+$ T cells, IL-21 has been shown to preserve effector function in exhausted cells, and presence of IL-21-producing CD4$^+$ T cells has been shown to enhance Ag-presentation and the generation functional anti-tumor CTL [499]. This raises the possibility that IL-21-producing trog$^+$ cells may also play an important role in anti-tumor immunity. Consistent with the results in chapter 4, IL-21 production driven by trogocytosis-mediated signaling may aid in the transition of trog$^+$ cells to memory, as IL-21 is critical for the formation of CD4$^+$ and CD8$^+$ memory T cells [440, 489].

### Tcf1 is a Common Feature in $T_H2$, $T_{FH}$, and Memory Cells

A shared feature between the $T_H2$, $T_{FH}$, and memory-precursor phenotypes observed in the trog$^+$ cells here is expression of the repressive transcription factor T cell factor 1 (Tcf1). The results in chapter 4 show that the

trog$^{+}$ cells displayed higher levels of Tcf1 compared to trog$^{-}$ counterparts, and it is possible that Tcf1 was playing a central role in the observed differentiation patterns.

Tcf1 likely contributed to the $T_H2$ phenotype observed in chapter 3, as Tcf1 suppresses IFNγ and IL-17 expression, and it enhances the expression of GATA-3 [9, 244]. Consistent with the results from chapter 4, Tcf1 also is critical in $T_{FH}$ differentiation by regulating Blimp1 expression and repressing IL-2 signaling to promote the expression of Bcl-6 [227]. The high levels of Tcf1 expression in the trog$^{+}$ cells may explain their ability to develop a $T_{FH}$-like phenotype in the presence of high levels of IL-2. In addition, in a viral model Tcf1 had a critical role in $T_{FH}$ development, and was required for memory T cell formation [542]. Human lymph nodes have also been found to contain memory T cells which maintain high expression of TCF1, and these cells show superior functional potential compared to TCF1$^{low}$ memory cells [543]. Similar to the phenotype of the trog$^{+}$ cells in chapter 4, during T cell asymmetric division Tcf1 is inherited by the distal daughter cell and correlates with self-renewal and the transition to memory [491].

The regulation of Tcf1 is also consistent with a model in which trogocytosis and trogocytosis-mediated signaling are correlated with weaker TCR signaling. Strong TCR signaling has been shown to repress Tcf1 expression through high levels of PKCθ activation [522]. In addition, inflammatory cytokines such as IL-12 can suppress Tcf1 activation and drive effector differentiation [544]. It is possible that the IL-4 expression driven by trogocytosis-mediated signaling acts to shield the trog$^{+}$ cells from inflammatory cytokines through a

negative-feedback loop and aids in their progression to become a memory cell. The compelling correlation between Tcf1 function in each of the phenotypes observed in trog$^{+}$ cells in this study suggest that Tcf1 plays a central role in T cell differentiation via trogocytosis-mediated signaling.

## Trogocytosis-Mediated Signaling in CD4$^{+}$ Memory Generation

### Trogocytosis-Mediated Signaling in Effector-to-Memory Transition

Our previous results [81], and results from this study, show that despite similar activation by APC, the trog$^{+}$ cells display superior survival compared to trog$^{-}$ cells after APC removal (Fig. 2.1). Results in chapter 4 show that trogocytosis-mediated signaling was necessary for the high levels of IL-2 expression by the trog$^{+}$ cells, which was a major contributor the survival of both trog$^{+}$ and trog$^{-}$ cells (Fig. 4.19). These results suggest that trogocytosis-mediated signaling can promote effector-to-memory transition, as it has been found that autocrine IL-2/IL-2R signaling induced by late-cognate interactions with APC are required for memory transition [317]. As mentioned above, it is also likely that the IL-21 produced by the trog$^{+}$ cells would aid in the transition to memory, as IL-21 has been found to be critical in CD4$^{+}$ memory formation [440].

The sustained TCR downmodulation by the trog$^{+}$ cells also has implications for memory transition. While trogocytosis-mediated signaling may be sustained at a level which promotes survival and licensing to become memory, subsequent interactions with APC may drive terminal effector differentiation of the trog$^{+}$ cell. By maintaining low levels of surface TCR, the chances of

subsequent interactions between the trog$^+$ cells and APC would be decreased, thus favoring memory formation over terminal-effector differentiation.

Currently, the mechanisms which allow memory cells to persist after Ag clearance are not well defined. The data presented here raise the possibility that trogocytosis-mediated signaling plays a role in the sustained survival of the trog$^+$ cells and subsequent effector-to-memory transition.

### Trogocytosis-Mediated Signaling in T cell Memory Generation via Asymmetric Division

The results in chapter 4 show a strong correlation for trogocytosis and asymmetric division. The presence of trogocytosed molecules on the cell resembling the distal daughter cell during asymmetric division could be established during the immunological synapse. However, our results showed that it was apparent that trogocytosis-mediated signaling enhanced the survival of trog$^+$ cells and was critical for the proliferation, and survival of the lesser activated cells. The initial description of asymmetric division and memory development suggested differential signaling at the synapse and the distal region of the cell set up the asymmetry. Our imaging experiments show reduced, but present, signaling at the distal pole associated with trogocytosed molecules. This may have major biological implications in the context of memory formation, as cells which receive stronger signaling develop terminally-differentiated effector cells, while the cells which receive less intense signaling upon activation retain high memory-potential.

In addition to promoting the survival of cells which received weaker signals from APC, it is possible that trogocytosis-mediated signaling plays a role in the asymmetric division of cells. An unknown variable in the model for asymmetric division is how a T cell which has been separated from an APC for several rounds of cell division is able to maintain its axis of polarity established at the immune synapse. One proposed mechanism is strong PI3K signaling and the formation of the proximal TCR signaling complex established this polarity [513]. However, this still does not explain how the polarity is maintained long after the T cell separates from the APC and has undergone multiple rounds of cell division. It is possible that the sustained trogocytosis-mediated signaling at the distal pole complex/antisynapse is responsible for maintaining this polarity.

## Proposed Model for the Presented Results

The phenotypes displayed by the trog$^+$ cells in this study can be centered around a few common mechanisms, all of which can enhance the survival of the trog$^+$ cell. In the context of trogocytosis-mediated signaling, the most obvious factor is the signaling itself. The results in this dissertation show that trogocytosis-mediated signaling can influence the activation, proliferation, and survival of the trog$^+$ cell after APC removal. In addition, the primary cytokines we found trogocytosis-mediated signaling to promote were IL-2, IL-4, and IL-21. These cytokines are all able to promote CD4$^+$ T cell survival, and depending on the context of TCR/costimulatory signaling, could differently impact the effector functions and differentiation of the trog$^+$ cell. For example, strong trogocytosis-

mediated signaling in the presence of IL-21 could lead to $T_{FH}$ differentiation, while weaker signaling would further promote IL-4 production. IL-4 in the context of trogocytosis-mediated signaling could then drive GATA-3 expression and $T_H2$ differentiation. Trogocytosis-mediated signaling could also enhance the T cell memory pool through the survival of memory-precursors formed through asymmetric division. By rapidly, and sustainably, producing high levels of IL-2 the $trog^+$ cells could aid in the proliferation of not only the $trog^+$ cell, but the surrounding $trog^-$ cells as well. Later in the immune response IL-2 and IL-21 have been found to be critical in generating a stable memory T cell population from effector cells, again, in the context of TCR signaling. The common theme in these potential outcomes, is these are all mechanisms that could enhance the survival of the $trog^+$ cell through potentially self-sustainable mechanisms.

Throughout this dissertation I have also proposed that trogocytosis-mediated signaling of relatively weak strength, at least when compared to signaling from interaction with APC. This hypothesis is supported in that, weak TCR signaling can drive $T_H2$-differentiation, and promote memory formation, which is consistent with the phenotypes observed in $trog^+$ cells in this study. $T_H2$ cells display superior survival mechanisms compared to inflammatory subsets such as $T_H1$ and $T_H17$. It is possible that the survival of the individual cell would be enhanced through phenotypic changes towards a less inflammatory-associated subset such as the $T_H1$ to $T_H2$ conversion seen in Fig. 3.7 or become a memory T cell as proposed in chapter 4. Weak signaling also promotes Tcf1 expression, which as mentioned above, can drive $T_{FH}$, $T_H2$, and memory T cell

formation. In addition, it is likely that strong trogocytosis-mediated signaling would lead to T cell exhaustion. Thus, the surviving trog$^{+}$ cells are primarily those receiving a sustainable level of continual signaling.

This raises the question of whether the weak signaling by trogocytosis-mediated signaling drives phenotypes, or simply maintains the survival of cells programmed towards a particular subset by APC. Both of these possibilities are likely scenarios.

In an *in vitro* system where transgenic cells display uniform TCR and APC which display the same peptide antigen, if all T cells encounter Ag and are similarly activated, why do some cells perform trogocytosis while others do not? In this scenario, a major variable is the duration of contact between T cell and APC. If this is the case, then why did the trog+ cells display a phenotype associated with weaker T cell signaling ($T_H2$), while the trog$^{-}$ cells displayed a phenotype associated with stronger signaling ($T_H1$)? These data are seemingly contradictory to the published data showing that T cell trogocytosis is associated with a heightened state of activation, which is also what was observed here, as indicated by CD69 levels in the trog$^{+}$ and trog$^{-}$ cells (Figs. 3.1, 4.1).

One possible example is that the acquisition of pMHC/costimulatory molecules occurs via microclusters. Microclusters can localize to the distal pole rapidly upon T:APC contact, but become recruited to the immunological synapse within minutes. Therefore, if a T cell were to form a short-lived synapse and spontaneously dissociate before the microclusters integrate into the SMAC, this would result in the transfer of pMHC/costimulatory molecules to the T cell.

Consequently, in the *in vitro* system used here, it is likely that a shorter synapse would also be associated with weaker TCR signaling events. The weaker signaling would allow the trog$^{+}$ cells to retain high Tcf1 levels which could promote a $T_H2$ and/or $T_{FH}$ phenotype, effector-to-memory transition, or memory-precursor formation through asymmetric division. In contrast, longer-lived synapses would allow extra time for pMHC complexes in these microclusters to migrate back towards the immune synapse and thus would be less likely to be acquired by the T cell. The extended duration of the synapse would likely also subsequently lead to stronger signaling, providing an explanation for the predominant $T_H1$ phenotype and nearly exclusive IFNγ expression by the trog$^{-}$ cells observed in chapter 3.

In addition, the morphology of microclusters granting them ability to exclude regulatory molecules allows them to sustain active signaling for longer durations compared to TCR in the sMAC. This could also play a significant role in the sustained trogocytosis-mediated signaling observed in this study.

If a weaker signal promotes $T_H2$ and memory generation, then why do the trog$^{+}$ display equal, if not greater levels of CD69 compared to the trog$^{-}$ cells immediately after recovery from APC? Figures 3.1 and 4.1 show that the trog$^{+}$ cells not only maintained high CD69 expression after APC removal, but levels of CD69 increased over subsequent incubation. This strongly suggests that trogocytosis-mediated signaling was driving CD69 expression, as in absence of stimulation, CD69 levels peak within 24 hours of stimulation. Therefore, it is possible that compared to the trog$^{-}$ cells, the trog$^{+}$ cells were not as activated by

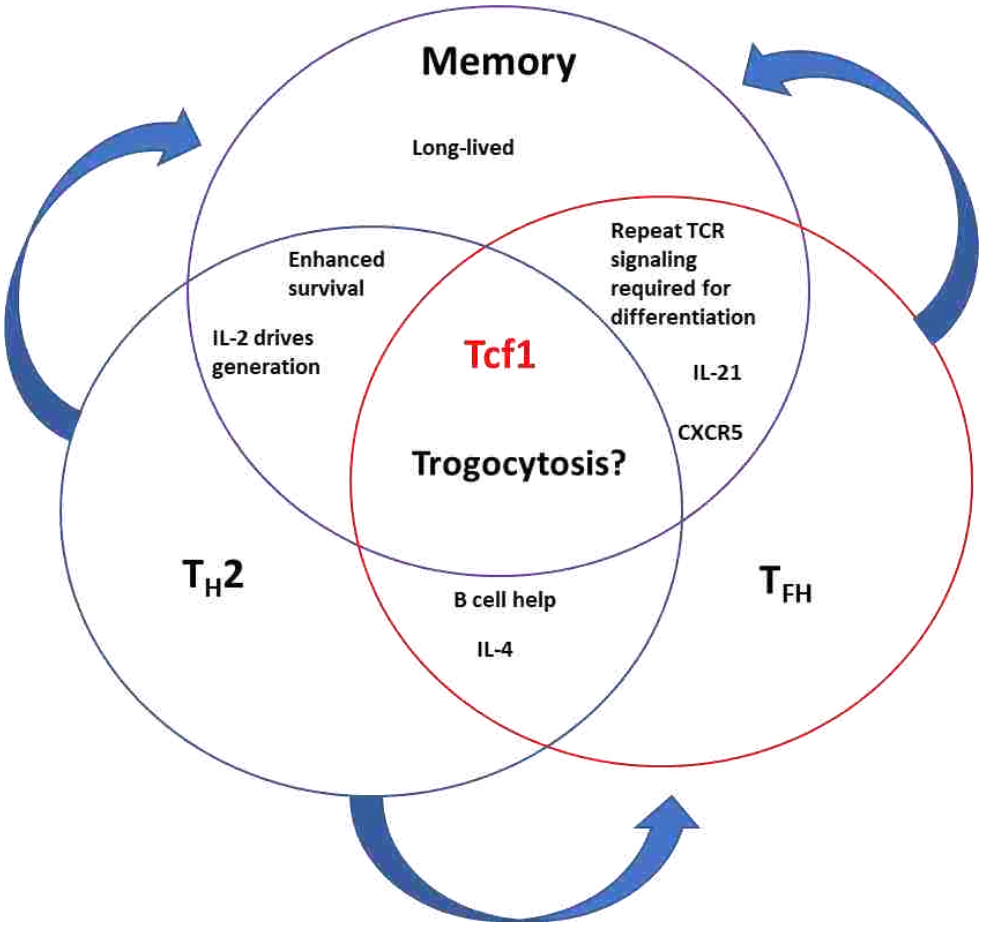
the APC, but this was not reflected in their activation state due to trogocytosis-mediated signaling augmenting CD69 expression in the trog$^{+}$ cells.

This proposed models is intended to serve as an explanation for the results obtained in this study, however as described in earlier sections, the broad consequences of trogocytosis-mediated signaling are likely context dependent.

## Outro

As the number of identified $T_H$ subsets continues to grow, and plasticity between differentiated subsets is becoming more apparent, determining the exact mechanisms governing $T_H$ differentiation has become a challenging topic of research. In particular, the multi-step processes involved in the generation of $T_{FH}$ comes with extra layers of complexity. In addition to $T_H$ differentiation, the mechanisms which drive memory formation are largely unknown. This combined lack of knowledge has been a major roadblock in vaccine design and efficacy, and anti-tumor immunotherapy. Therefore, improving our understanding of the mechanisms governing the differentiation of effector subsets, and memory cells are of paramount importance in human health. The results here provide insight into a novel mechanism for CD4$^{+}$ T cells to differentiate towards $T_H2$ and/or $T_{FH}$ in the absence of further interaction with APC. They also provide compelling evidence for trogocytosis-mediated signaling to play a role in the generation of CD4$^{+}$ memory cells through effector-to-memory transition, and asymmetric division.

I will end with a few questions for speculation. Could there be a distinct phenotype of T cell which displays all of these characteristics? Is trogocytosis simply a common, but understudied mechanism in the differentiation of these subsets? And finally, does weak TCR signaling itself promote a $T_H2$ phenotype and memory formation, or does weak TCR signaling actually promote trogocytosis?




## Future Directions

To address whether a shortened T cell:APC contact leads to increased trogocytosis via microclusters, additional live-imaging experiments should be conducted over a wider time-course to observe the rate of dissociation in comparison to the MHC that remains on the T cell microclusters. Additionally, collecting the cells which spontaneously dissociate from APC at different time points for immediate analysis, and assessment of the downstream phenotype will help address the hypothesis that shorter T cell:APC contact duration favors trogocytosis and memory cell generation.

While the differentiation of previously-activated trog$^{+}$ cells in chapter 3 was towards a $T_H2$ phenotype, this could be an artifact of the APC used in the system. While a greater frequency of the trog$^{+}$ cells from BMDC produced IL-4 compared to the trog$^{-}$ cells, the frequency of IL-4+ trog$^{+}$ cells was substantially lower in cultures from BMDC vs MCC:FKPC APC. This is likely due to the weaker activation state induced by the FKPB:APC compared to BMDC which has been shown to promote $T_H2$ differentiation. In addition, other model systems should be utilized with pathogen-associated antigenic proteins in *in vivo* studies, or the addition of different agonists which trigger separate TLR/RLR (PRR receptors) to observe whether the $T_H2$ phenotype of trog$^{+}$ cells holds true in inflammatory settings which promote a $T_H1$ phenotype.

While we observed a phenotype in the trog$^{+}$ cells consistent with $T_{FH}$, it was not actually demonstrated that the trog$^{+}$ cells were indeed $T_{FH}$, as defined by their

ability to help B cells. Further studies to examine the functional role of trog$^{+}$ cells in the help of B cells should therefore be conducted to confirm this phenotype. Assays should include B cell help assays containing mixtures of trog$^{+}$ and trog$^{-}$ FACS sorted cells to assess B cell activation, class switching, and proliferation. To assess the migratory location, FACS-sorted trog$^{+}$ and trog$^{-}$ cells may be labeled with different dyes and adoptively transferred in to mice 3-5 days following immunization with either a cognate or irrelevant Ag. Histology on the lymph nodes to observe the proximity of the trog$^{+}$ cells and trog$^{-}$ cells to B cell follicles can be assessed 12-24 hours post-transfer, and 48-72h post-transfer to also stain for Ig class switching proximal to the trog$^{+}$ or trog$^{-}$ cells. If the trog$^{+}$, but not trog$^{-}$ cells migrate to the B cell follicle/GC in animals immunized with irrelevant Ag, this would suggest that the trog$^{+}$ cells are able to home towards B cell follicles independently of cognate B cell interactions. The role of trogocytosis-mediated signaling through costimulatory molecules shown to be involved in $T_{FH}$ differentiation such as ICOS:ICOS-L and OX40:OX40-L interactions, should also be assessed. The acquisition of these molecules is likely to impact IL-21 and Bcl-6 expression through trogocytosis-mediated signaling.

It is also possible that trogocytosis-mediated signaling could lead to the generation of extrafollicular cells displaying a $T_{FH}$-like phenotype, including IL-21 secretion, which have been observed in other studies [242]. This is a possibility that should be examined further, as this would be an area where trogocytosis-mediated signaling would be most influential on the immune response, as

opposed to GCs where the all of the $CD4^+$ T cells in proximity would have access to APC contact.

In the context of asymmetric division, it is possible that the axis of polarity are maintained by trogocytosis-mediated signaling at the distal pole. This is an intriguing hypothesis that should be explored further. While it was difficult to ascertain whether this possibility was true due to the massive cell death and decreased proliferation of the $trog^+$ cells when trogocytosis-mediated signaling was neutralized with anti-I-Ek/CD80 antibodies, it is possible that the supplementation of sufficient survival cytokines would allow the T cells to survive and proliferate long enough to examine this possibility. I did find some evidence for reduced asymmetric distribution in the few cells which divided in cases where trogocytosis-mediated signaling was neutralized (data not shown). However, due to extremely low cell numbers, I cannot conclude with confidence that trogocytosis-mediated signaling promotes asymmetric division.

Following up with the asymmetric division model, another interesting possibility is the potential role for the molecule CD43. Thought to be due to its repressive activity and physically large size, CD43 has been observed to become excluded from the immunological synapse, and travel to the distal pole complex during the immune synapse. Interestingly, anti-CD43 Ab-induced CD43 signaling alone in $T_H2$ cells led to IL-4 production, increased proliferation, and CD69 and CD25 upregulation [545]. CD43 signaling has also been found to act as a costimulatory

molecule, acting independently of CD28 to enhance T cell activation, particularly in low levels of TCR signaling. High levels of CD43 signaling have been shown to inhibit TCR/CD3-mediated apoptosis, which may aid in the survival of trog$^{+}$ cells expressing pMHC. Due to its physical size, CD43 has also been proposed to inhibit cell:cell interactions, and thus could prevent MHC-recognition and killing of trog$^{+}$ by CTL. The role of CD43 in the activation, differentiation, and survival in the context of trog$^{+}$ cells is worth examining further.

Signaling through Notch proteins may also play a role in trogocytosis. These previously overlooked molecules have been shown to play significant roles in T, and other cell development and activation. Interestingly Notch signaling has been found to be important in activating the long isoforms of Tcf1, which promote $T_{FH}$ differentiation and are critical for memory formation, at least in the context of a viral model [542]. Therefore, the presence of these molecules should be assessed on trog$^{+}$ cells, as well as determining whether they play a role in trogocytosis, and/or trogocytosis-mediated signaling.

In the context of Tcf1, the Wnt/$\beta$-catenin pathway is an important regulator of Tcf1 and should be further examined in the context of trogocytosis-mediated signaling.

# Bibliography


1. Honda, K. and T. Taniguchi, *IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors.* Nature Reviews Immunology, 2006. **6**(9): p. 644.
2. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-820.
3. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nature immunology, 2010. **11**(5): p. 373.
4. Benvenuti, F., et al., *Dendritic cell maturation controls adhesion, synapse formation, and the duration of the interactions with naive T lymphocytes.* The Journal of Immunology, 2004. **172**(1): p. 292-301.
5. McGee, H.S. and D.K. Agrawal, *Naturally occurring and inducible T-regulatory cells modulating immune response in allergic asthma.* Am J Respir Crit Care Med, 2009. **180**(3): p. 211-25.
6. Deo, S.S., et al., *Role played by Th2 type cytokines in IgE mediated allergy and asthma.* Lung India: Official Organ of Indian Chest Society, 2010. **27**(2): p. 66.
7. Sospedra, M. and R. Martin, *Immunology of multiple sclerosis.* Annu. Rev. Immunol., 2005. **23**: p. 683-747.
8. Venken, K., et al., *Natural naive CD4+ CD25+ CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression.* The Journal of Immunology, 2008. **180**(9): p. 6411-6420.
9. Yu, Q., et al., *T cell factor-1 negatively regulates expression of IL-17 family of cytokines and protects mice from experimental autoimmune encephalomyelitis.* J Immunol, 2011. **186**(7): p. 3946-52.
10. Zhang, L., et al., *Elevated Th22 cells correlated with Th17 cells in patients with rheumatoid arthritis.* Journal of clinical immunology, 2011. **31**(4): p. 606-614.
11. Verwilghen, J., et al., *Expression of functional B7 and CTLA4 on rheumatoid synovial T cells.* J Immunol, 1994. **153**(3): p. 1378-85.
12. Gu-Trantien, C. and K. Willard-Gallo, *PD-1(hi)CXCR5(-)CD4(+) TFH Cells Play Defense in Cancer and Offense in Arthritis.* Trends Immunol, 2017. **38**(12): p. 875-878.
13. Tian, T., S. Yu, and D. Ma, *Th22 and related cytokines in inflammatory and autoimmune diseases.* Expert opinion on therapeutic targets, 2013. **17**(2): p. 113-125.
14. Belge, K., J. Brück, and K. Ghoreschi, *Advances in treating psoriasis.* F1000prime reports, 2014. **6**.
15. Chen, K. and J.K. Kolls, *Interluekin-17a (il17a).* Gene, 2017. **614**: p. 8-14.
16. Miossec, P. and J.K. Kolls, *Targeting IL-17 and T H 17 cells in chronic inflammation.* Nature reviews Drug discovery, 2012. **11**(10): p. 763.
17. Wakashin, H., et al., *Role of IL-23-Th17 cell axis in allergic airway inflammation.* International archives of allergy and immunology, 2009. **149**(Suppl. 1): p. 108-112.
18. Dardalhon, V., et al., *Role of Th1 and Th17 cells in organ-specific autoimmunity.* Journal of autoimmunity, 2008. **31**(3): p. 252-256.

19. Zhang, L., et al., *Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis.* PloS one, 2012. **7**(4): p. e31000.
20. Fujita, H., *The role of IL-22 and Th22 cells in human skin diseases.* Journal of dermatological science, 2013. **72**(1): p. 3-8.
21. Crotty, S., *T follicular helper cell differentiation, function, and roles in disease.* Immunity, 2014. **41**(4): p. 529-42.
22. Mungall, A., et al., *The DNA sequence and analysis of human chromosome 6.* Nature, 2003. **425**(6960): p. 805.
23. Rock, K.L., E. Reits, and J. Neefjes, *Present yourself! By MHC class I and MHC class II molecules.* Trends in immunology, 2016. **37**(11): p. 724-737.
24. Hedrick, P.W., *Balancing selection and MHC.* Genetica, 1998. **104**(3): p. 207-214.
25. Loveland, B. and E. Simpson, *The non-MHC transplantation antigens: neither weak nor minor.* Immunology Today, 1986. **7**(7-8): p. 223-229.
26. Le Moine, A., M. Goldman, and D. Abramowicz, *Multiple pathways to allograft rejection.* Transplantation, 2002. **73**(9): p. 1373-1381.
27. Sewell, A.K., *Why must T cells be cross-reactive?* Nature Reviews Immunology, 2012. **12**(9): p. 669.
28. Whitmire, J.K., N. Benning, and J.L. Whitton, *Precursor frequency, nonlinear proliferation, and functional maturation of virus-specific CD4+ T cells.* J Immunol, 2006. **176**(5): p. 3028-36.
29. Mandl, J.N., et al., *Quantification of lymph node transit times reveals differences in antigen surveillance strategies of naive CD4+ and CD8+ T cells.* Proceedings of the National Academy of Sciences, 2012. **109**(44): p. 18036-18041.
30. Yong, P.F., U. Salzer, and B. Grimbacher, *The role of costimulation in antibody deficiencies: ICOS and common variable immunodeficiency.* Immunological reviews, 2009. **229**(1): p. 101-113.
31. Wetzel, S.A., T.W. McKeithan, and D.C. Parker, *Live-cell dynamics and the role of costimulation in immunological synapse formation.* J Immunol, 2002. **169**(11): p. 6092-101.
32. Viola, A. and A. Lanzavecchia, *T cell activation determined by T cell receptor number and tunable thresholds.* Science, 1996. **273**(5271): p. 104-106.
33. Fathman, C.G. and N.B. Lineberry, *Molecular mechanisms of CD4+ T-cell anergy.* Nat Rev Immunol, 2007. **7**(8): p. 599-609.
34. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses.* Clinical microbiology reviews, 2009. **22**(2): p. 240-273.
35. Ni, K. and H. O'neill, *The role of dendritic cells in T cell activation.* Immunology and cell biology, 1997. **75**(3): p. 223-230.
36. Chen, X. and P.E. Jensen, *The role of B lymphocytes as antigen-presenting cells.* Archivum immunologiae et therapiae experimentalis, 2008. **56**(2): p. 77.
37. Croft, M., L.M. Bradley, and S.L. Swain, *Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells.* The Journal of Immunology, 1994. **152**(6): p. 2675-2685.
38. Adachi, K. and M.M. Davis, *T-cell receptor ligation induces distinct signaling pathways in naive vs. antigen-experienced T cells.* Proceedings of the National Academy of Sciences, 2011. **108**(4): p. 1549-1554.

39. Chandok, M.R., et al., *A biochemical signature for rapid recall of memory CD4 T cells.* J Immunol, 2007. **179**(6): p. 3689-98.
40. Harding, C.V. and E.R. Unanue, *Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation.* Nature, 1990. **346**(6284): p. 574.
41. Demotz, S., H.M. Grey, and A. Sette, *The minimal number of class II MHC-antigen complexes needed for T cell activation.* Science, 1990. **249**(4972): p. 1028-1030.
42. Cemerski, S. and A. Shaw, *Immune synapses in T-cell activation.* Curr Opin Immunol, 2006. **18**(3): p. 298-304.
43. Depoil, D., et al., *Immunological synapses are versatile structures enabling selective T cell polarization.* Immunity, 2005. **22**(2): p. 185-94.
44. DeMond, A.L. and J.T. Groves, *Interrogating the T cell synapse with patterned surfaces and photoactivated proteins.* Curr Opin Immunol, 2007. **19**(6): p. 722-7.
45. Dustin, M.L., et al., *T cell-dendritic cell immunological synapses.* Curr Opin Immunol, 2006. **18**(4): p. 512-6.
46. Dustin, M.L., *T-cell activation through immunological synapses and kinapses.* Immunological reviews, 2008. **221**(1): p. 77-89.
47. Fooksman, D.R., et al., *Functional anatomy of T cell activation and synapse formation.* Annu Rev Immunol, 2010. **28**: p. 79-105.
48. Grakoui, A., et al., *The immunological synapse: a molecular machine controlling T cell activation.* Science, 1999. **285**(5425): p. 221-227.
49. Lee, K.-H., et al., *T cell receptor signaling precedes immunological synapse formation.* Science, 2002. **295**(5559): p. 1539-1542.
50. Irvine, D.J., et al., *Direct observation of ligand recognition by T cells.* Nature, 2002. **419**(6909): p. 845.
51. Monks, C.R., et al., *Three-dimensional segregation of supramolecular activation clusters in T cells.* Nature, 1998. **395**(6697): p. 82-6.
52. Qi, S.Y., J.T. Groves, and A.K. Chakraborty, *Synaptic pattern formation during cellular recognition.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6548-53.
53. Grakoui, A., et al., *The immunological synapse: a molecular machine controlling T cell activation.* Science, 1999. **285**(5425): p. 221-7.
54. van der Merwe, P.A., et al., *Cytoskeletal polarization and redistribution of cell-surface molecules during T cell antigen recognition.* Semin Immunol, 2000. **12**(1): p. 5-21.
55. Valitutti, S., et al., *Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton.* Journal of Experimental Medicine, 1995. **181**(2): p. 577-584.
56. Billadeau, D.D., J.C. Nolz, and T.S. Gomez, *Regulation of T-cell activation by the cytoskeleton.* Nat Rev Immunol, 2007. **7**(2): p. 131-43.
57. Azar, G.A., et al., *Subcellular dynamics of T cell immunological synapses and kinapses in lymph nodes.* Proceedings of the National Academy of Sciences, 2010. **107**(8): p. 3675-3680.
58. Wang, J.-h. and M.J. Eck, *Assembling atomic resolution views of the immunological synapse.* Current Opinion in Immunology, 2003. **15**(3): p. 286-293.
59. Gascoigne, N.R.J. and T. Zal, *Molecular interactions at the T cell–antigen-presenting cell interface.* Current Opinion in Immunology, 2004. **16**(1): p. 114-119.
60. Liu, H., et al., *On the dynamics of TCR:CD3 complex cell surface expression and downmodulation.* Immunity, 2000. **13**(5): p. 665-75.
61. Lasserre, R., et al., *Release of serine/threonine-phosphorylated adaptors from signaling microclusters down-regulates T cell activation.* J Cell Biol, 2011. **195**(5): p. 839-853.

62. Chikuma, S., *CTLA-4, an essential immune-checkpoint for T-cell activation*, in *Emerging Concepts Targeting Immune Checkpoints in Cancer and Autoimmunity*. 2017, Springer. p. 99-126.
63. Stoll, S., et al., *Dynamic imaging of T cell-dendritic cell interactions in lymph nodes.* Science, 2002. **296**(5574): p. 1873-1876.
64. Valitutti, S., et al., *Serial triggering of many T-cell receptors by a few peptide–MHC complexes.* Nature, 1995. **375**(6527): p. 148.
65. Glatzová, D. and M. Cebecauer, *Dual Role of CD4 in Peripheral T Lymphocytes.* Frontiers in Immunology, 2019. **10**(618).
66. Akkaya, B., et al., *Regulatory T cells mediate specific suppression by depleting peptide–MHC class II from dendritic cells.* Nature immunology, 2019: p. 1.
67. Mayya, V., et al., *Durable interactions of T cells with T cell receptor stimuli in the absence of a stable immunological synapse.* Cell reports, 2018. **22**(2): p. 340-349.
68. Kim, H.R., et al., *T cell microvilli constitute immunological synaptosomes that carry messages to antigen-presenting cells.* Nat Commun, 2018. **9**(1): p. 3630.
69. Alarcon, B., D. Mestre, and N. Martinez-Martin, *The immunological synapse: a cause or consequence of T-cell receptor triggering?* Immunology, 2011. **133**(4): p. 420-5.
70. Guedj, C., et al., *T cell adhesion triggers an early signaling pole distal to the immune synapse.* J Cell Sci, 2016. **129**(13): p. 2526-37.
71. Hashimoto-Tane, A., et al., *Micro–adhesion rings surrounding TCR microclusters are essential for T cell activation.* Journal of Experimental Medicine, 2016. **213**(8): p. 1609-1625.
72. Saito, T. and T. Yokosuka, *Immunological synapse and microclusters: the site for recognition and activation of T cells.* Curr Opin Immunol, 2006. **18**(3): p. 305-13.
73. Campi, G., R. Varma, and M.L. Dustin, *Actin and agonist MHC-peptide complex-dependent T cell receptor microclusters as scaffolds for signaling.* J Exp Med, 2005. **202**(8): p. 1031-6.
74. Varma, R., et al., *T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster.* Immunity, 2006. **25**(1): p. 117-27.
75. Balagopalan, L., V.A. Barr, and L.E. Samelson, *Endocytic events in TCR signaling: focus on adapters in microclusters.* Immunological reviews, 2009. **232**(1): p. 84-98.
76. Randriamampita, C., et al., *A novel ZAP-70 dependent FRET based biosensor reveals kinase activity at both the immunological synapse and the antisynapse.* PLoS One, 2008. **3**(1): p. e1521.
77. Furlan, G., et al., *Phosphatase CD45 both positively and negatively regulates T cell receptor phosphorylation in reconstituted membrane protein clusters.* Journal of Biological Chemistry, 2014. **289**(41): p. 28514-28525.
78. Dustin, M.L., et al., *Antigen receptor engagement delivers a stop signal to migrating T lymphocytes.* Proceedings of the National Academy of Sciences, 1997. **94**(8): p. 3909-3913.
79. Freiberg, B.A., et al., *Staging and resetting T cell activation in SMACs.* Nature immunology, 2002. **3**(10): p. 911.
80. Cannon, J., et al., *CD43 interaction with ezrin-radixin-moesin (ERM) proteins regulates T-cell trafficking and CD43 phosphorylation.* Molecular biology of the cell, 2011. **22**(7): p. 954-963.
81. Osborne, D.G. and S.A. Wetzel, *Trogocytosis results in sustained intracellular signaling in CD4(+) T cells.* J Immunol, 2012. **189**(10): p. 4728-39.

82. van Panhuys, N., *TCR Signal Strength Alters T-DC Activation and Interaction Times and Directs the Outcome of Differentiation.* Front Immunol, 2016. **7**: p. 6.
83. Thauland, T.J., et al., *Th1 and Th2 cells form morphologically distinct immunological synapses.* J Immunol, 2008. **181**(1): p. 393-9.
84. MacIver, N.J., R.D. Michalek, and J.C. Rathmell, *Metabolic regulation of T lymphocytes.* Annual review of immunology, 2013. **31**: p. 259-283.
85. Artyomov, M.N., et al., *CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery.* Proceedings of the National Academy of Sciences, 2010. **107**(39): p. 16916-16921.
86. König, R., L.-Y. Huang, and R.N. Germain, *MHC class II interaction with CD4 mediated by a region analogous to the MHC class I binding site for CD8.* Nature, 1992. **356**(6372): p. 796.
87. Marrack, P., et al., *The major histocompatibility complex-restricted antigen receptor on T cells. II. Role of the L3T4 product.* Journal of Experimental Medicine, 1983. **158**(4): p. 1077-1091.
88. Hampl, J., Y.-h. Chien, and M.M. Davis, *CD4 augments the response of a T cell to agonist but not to antagonist ligands.* Immunity, 1997. **7**(3): p. 379-385.
89. Madrenas, J., et al., *The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide–MHC molecule ligands.* Journal of Experimental Medicine, 1997. **185**(2): p. 219-230.
90. Li, Q.-J., et al., *CD4 enhances T cell sensitivity to antigen by coordinating Lck accumulation at the immunological synapse.* Nature immunology, 2004. **5**(8): p. 791.
91. Cemerski, S., et al., *The stimulatory potency of T cell antigens is influenced by the formation of the immunological synapse.* Immunity, 2007. **26**(3): p. 345-55.
92. Bartolo, V.D., et al., *Tyrosine 315 determines optimal recruitment of ZAP-70 to the T cell antigen receptor.* European journal of immunology, 2002. **32**(2): p. 568-575.
93. Balagopalan, L., et al., *The LAT story: a tale of cooperativity, coordination, and choreography.* Cold Spring Harbor perspectives in biology, 2010. **2**(8): p. a005512.
94. Braiman, A., et al., *Recruitment and activation of PLCγ1 in T cells: a new insight into old domains.* The EMBO journal, 2006. **25**(4): p. 774-784.
95. Rincon, M. and R. Flavell, *AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes.* The EMBO journal, 1994. **13**(18): p. 4370-4381.
96. Mak, T.W. and M.E. Saunders, *The immune response: basic and clinical principles*. 2005: Academic Press.
97. Zhang, W., et al., *Association of Grb2, Gads, and Phospholipase C-γ1 with Phosphorylated LAT Tyrosine Residues EFFECT OF LAT TYROSINE MUTATIONS ON T CELL ANTIGEN RECEPTOR-MEDIATED SIGNALING.* Journal of Biological Chemistry, 2000. **275**(30): p. 23355-23361.
98. Liu, S.K., et al., *The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors.* Current Biology, 1999. **9**(2): p. 67-75.
99. Yablonski, D., et al., *Uncoupling of nonreceptor tyrosine kinases from PLC-γ1 in an SLP-76-deficient T cell.* Science, 1998. **281**(5375): p. 413-416.
100. Hermann-Kleiter, N. and G. Baier, *NFAT pulls the strings during CD4+ T helper cell effector functions.* Blood, 2010. **115**(15): p. 2989-2997.

101. Kim, H.P. and W.J. Leonard, *The basis for TCR-mediated regulation of the IL-2 receptor α chain gene: role of widely separated regulatory elements.* The EMBO journal, 2002. **21**(12): p. 3051-3059.
102. Paul, S. and B.C. Schaefer, *A new look at T cell receptor signaling to nuclear factor-κB.* Trends in immunology, 2013. **34**(6): p. 269-281.
103. Andreotti, A.H., et al., *T-cell signaling regulated by the Tec family kinase, Itk.* Cold Spring Harbor perspectives in biology, 2010. **2**(7): p. a002287.
104. Harding, F.A., et al., *CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones.* Nature, 1992. **356**(6370): p. 607.
105. Chi, H., *Regulation and function of mTOR signalling in T cell fate decisions.* Nature reviews immunology, 2012. **12**(5): p. 325.
106. Powell, J.D., C.G. Lerner, and R.H. Schwartz, *Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation.* The Journal of Immunology, 1999. **162**(5): p. 2775-2784.
107. Delgoffe, G.M., et al., *The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment.* Immunity, 2009. **30**(6): p. 832-844.
108. Gerriets, V.A. and J.C. Rathmell, *Metabolic pathways in T cell fate and function.* Trends in immunology, 2012. **33**(4): p. 168-173.
109. Volarević, S. and G. Thomas, *Role of S6 phosphorylation and S6 kinase in cell growth.* Progress in nucleic acid research and molecular biology, 2001. **65**: p. 101-127.
110. Sabatini, D.M., *mTOR and cancer: insights into a complex relationship.* Nature Reviews Cancer, 2006. **6**(9): p. 729.
111. Laplante, M. and D.M. Sabatini, *mTOR signaling at a glance.* Journal of cell science, 2009. **122**(20): p. 3589-3594.
112. Yuan, H.-X. and K.-L. Guan, *The SIN1-PH domain connects mTORC2 to PI3K.* Cancer discovery, 2015. **5**(11): p. 1127-1129.
113. Jacinto, E., et al., *Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive.* Nature cell biology, 2004. **6**(11): p. 1122.
114. Weichhart, T., M. Hengstschläger, and M. Linke, *Regulation of innate immune cell function by mTOR.* Nature Reviews Immunology, 2015. **15**(10): p. 599.
115. Lupher, M.L., et al., *The Cbl Phosphotyrosine-binding Domain Selects a D (N/D) XpY Motif and Binds to the Tyr292Negative Regulatory Phosphorylation Site of ZAP-70.* Journal of Biological Chemistry, 1997. **272**(52): p. 33140-33144.
116. Doherty, M., et al., *Anergic CD4+ T cells form mature immunological synapses with enhanced accumulation of c-Cbl and Cbl-b.* J Immunol, 2010. **184**(7): p. 3598-608.
117. Vang, T., et al., *Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant.* Nature genetics, 2005. **37**(12): p. 1317.
118. Visperas, P.R., et al., *Identification of inhibitors of the association of ZAP-70 with the T cell receptor by high-throughput screen.* SLAS DISCOVERY: Advancing Life Sciences R&D, 2017. **22**(3): p. 324-331.
119. Wang, H., et al., *ZAP-70: an essential kinase in T-cell signaling.* Cold Spring Harbor perspectives in biology, 2010. **2**(5): p. a002279.
120. McCoy, K.D. and G. Le Gros, *The role of CTLA-4 in the regulation of T cell immune responses.* Immunology and cell biology, 1999. **77**(1): p. 1-10.
121. Valk, E., C.E. Rudd, and H. Schneider, *CTLA-4 trafficking and surface expression.* Trends in immunology, 2008. **29**(6): p. 272-279.

122. Egen, J.G. and J.P. Allison, *Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength.* Immunity, 2002. **16**(1): p. 23-35.
123. Larkin, J., et al., *Combined nivolumab and ipilimumab or monotherapy in untreated melanoma.* New England journal of medicine, 2015. **373**(1): p. 23-34.
124. Seidel, J.A., A. Otsuka, and K. Kabashima, *Anti-PD-1 and anti-CTLA-4 therapies in cancer: mechanisms of action, efficacy, and limitations.* Frontiers in oncology, 2018. **8**: p. 86.
125. Sanderson, K., et al., *Autoimmunity in a phase I trial of a fully human anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibody with multiple melanoma peptides and Montanide ISA 51 for patients with resected stages III and IV melanoma.* Journal of Clinical Oncology, 2005. **23**(4): p. 741-750.
126. Swain, S.L., et al., *From naive to memory T cells.* Immunological reviews, 1996. **150**(1): p. 143-167.
127. Obst, R., et al., *Antigen persistence is required throughout the expansion phase of a CD4+ T cell response.* Journal of Experimental Medicine, 2005. **201**(10): p. 1555-1565.
128. Lee, W.T., et al., *Continued antigen stimulation is not required during CD4(+) T cell clonal expansion.* J Immunol, 2002. **168**(4): p. 1682-9.
129. Jelley-Gibbs, D.M., et al., *Two distinct stages in the transition from naive CD4 T cells to effectors, early antigen-dependent and late cytokine-driven expansion and differentiation.* J Immunol, 2000. **165**(9): p. 5017-26.
130. Brenchley, J., et al., *Expansion of activated human naïve T-cells precedes effector function.* Clinical & Experimental Immunology, 2002. **130**(3): p. 432-440.
131. Shevach, E.M., *From vanilla to 28 flavors: multiple varieties of T regulatory cells.* Immunity, 2006. **25**(2): p. 195-201.
132. Bendelac, A., P.B. Savage, and L. Teyton, *The biology of NKT cells.* Annu Rev Immunol, 2007. **25**: p. 297-336.
133. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties.* Annu Rev Immunol, 1989. **7**: p. 145-73.
134. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* J Immunol, 1986. **136**(7): p. 2348-57.
135. Luckheeram, R.V., et al., *CD4(+)T cells: differentiation and functions.* Clin Dev Immunol, 2012. **2012**: p. 925135.
136. Takeuchi, A. and T. Saito, *CD4 CTL, a cytotoxic subset of CD4+ T cells, their differentiation and function.* Frontiers in immunology, 2017. **8**: p. 194.
137. Kaplan, M.H., *Th9 cells: differentiation and disease.* Immunol Rev, 2013. **252**(1): p. 104-15.
138. Jin, B., et al., *The effects of TLR activation on T-cell development and differentiation.* Clin Dev Immunol, 2012. **2012**: p. 836485.
139. Turner, M.S., et al., *Low TCR signal strength induces combined expansion of Th2 and regulatory T cell populations that protect mice from the development of type 1 diabetes.* Diabetologia, 2014. **57**(7): p. 1428-36.
140. Leitenberg, D., et al., *CD4 regulation of TCR signaling and T cell differentiation following stimulation with peptides of different affinities for the TCR.* J Immunol, 1998. **161**(3): p. 1194-203.
141. Leitenberg, D. and K. Bottomly, *Regulation of naive T cell differentiation by varying the potency of TCR signal transduction.* Semin Immunol, 1999. **11**(4): p. 283-92.

142. Linterman, M.A. and C.G. Vinuesa, *Signals that influence T follicular helper cell differentiation and function.* Semin Immunopathol, 2010. **32**(2): p. 183-96.
143. Zhu, J., H. Yamane, and W.E. Paul, *Differentiation of effector CD4 T cell populations (*).* Annu Rev Immunol, 2010. **28**: p. 445-89.
144. Zhu, J. and W.E. Paul, *CD4+ T cell plasticity-Th2 cells join the crowd.* Immunity, 2010. **32**(1): p. 11-3.
145. Lu, K.T., et al., *Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells.* Immunity, 2011. **35**(4): p. 622-32.
146. Geginat, J., et al., *Plasticity of human CD4 T cell subsets.* Front Immunol, 2014. **5**: p. 630.
147. DuPage, M. and J.A. Bluestone, *Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease.* Nat Rev Immunol, 2016. **16**(3): p. 149-63.
148. Cannons, J.L., K.T. Lu, and P.L. Schwartzberg, *T follicular helper cell diversity and plasticity.* Trends Immunol, 2013. **34**(5): p. 200-7.
149. Peine, M., et al., *Stable T-bet(+)GATA-3(+) Th1/Th2 hybrid cells arise in vivo, can develop directly from naive precursors, and limit immunopathologic inflammation.* PLoS Biol, 2013. **11**(8): p. e1001633.
150. Szabo, S.J., et al., *A novel transcription factor, T-bet, directs Th1 lineage commitment.* Cell, 2000. **100**(6): p. 655-69.
151. Rengarajan, J., S.J. Szabo, and L.H. Glimcher, *Transcriptional regulation of Th1/Th2 polarization.* Immunology today, 2000. **21**(10): p. 479-483.
152. Shortman, K. and W.R. Heath, *The CD8+ dendritic cell subset.* Immunological reviews, 2010. **234**(1): p. 18-31.
153. Szabo, S.J., et al., *Molecular mechanisms regulating Th1 immune responses.* Annual review of immunology, 2003. **21**(1): p. 713-758.
154. Usui, T., et al., *T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription.* J Exp Med, 2006. **203**(3): p. 755-66.
155. Rogers, P.R. and M. Croft, *CD28, Ox-40, LFA-1, and CD4 modulation of Th1/Th2 differentiation is directly dependent on the dose of antigen.* The Journal of Immunology, 2000. **164**(6): p. 2955-2963.
156. Tao, X., et al., *Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation.* J Immunol, 1997. **159**(12): p. 5956-63.
157. van Panhuys, N., F. Klauschen, and R.N. Germain, *T-cell-receptor-dependent signal intensity dominantly controls CD4(+) T cell polarization In Vivo.* Immunity, 2014. **41**(1): p. 63-74.
158. Boehm, U., et al., *Cellular responses to interferon-γ.* Annual review of immunology, 1997. **15**(1): p. 749-795.
159. Brunda, M.J., et al., *Role of interferon-gamma in mediating the antitumor efficacy of interleukin-12.* Journal of immunotherapy with emphasis on tumor immunology: official journal of the Society for Biological Therapy, 1995. **17**(2): p. 71-77.
160. Vivier, E., et al., *Functions of natural killer cells.* Nature immunology, 2008. **9**(5): p. 503.
161. Nathan, C.F., et al., *Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity.* Journal of Experimental Medicine, 1983. **158**(3): p. 670-689.
162. Lowenstein, C.J., et al., *Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide.* Proceedings of the National Academy of Sciences, 1993. **90**(20): p. 9730-9734.

163. Severinson, E., C. Fernandez, and J. Stavnezer, *Induction of germ-line immunoglobulin heavy chain transcripts by mitogens and interleukins prior to switch recombination.* European journal of immunology, 1990. **20**(5): p. 1079-1084.
164. Bindon, C.I., et al., *Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q.* Journal of Experimental Medicine, 1988. **168**(1): p. 127-142.
165. Neurath, M.F., S. Finotto, and L.H. Glimcher, *The role of Th1/Th2 polarization in mucosal immunity.* Nature medicine, 2002. **8**(6): p. 567.
166. Zhu, J., *Transcriptional regulation of Th2 cell differentiation.* Immunol Cell Biol, 2010. **88**(3): p. 244-9.
167. Zheng, W. and R.A. Flavell, *The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells.* Cell, 1997. **89**(4): p. 587-96.
168. Ansel, K.M., et al., *Regulation of Th2 differentiation and Il4 locus accessibility.* Annu. Rev. Immunol., 2006. **24**: p. 607-656.
169. Szabo, S.J., et al., *Developmental commitment to the Th2 lineage by extinction of IL-12 signaling.* Immunity, 1995. **2**(6): p. 665-75.
170. Maggi, E., et al., *Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones.* The Journal of Immunology, 1992. **148**(7): p. 2142-2147.
171. Le Gros, G., et al., *Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells.* Journal of Experimental Medicine, 1990. **172**(3): p. 921-929.
172. Paul, W.E., *What determines Th2 differentiation, in vitro and in vivo?* Immunol Cell Biol, 2010. **88**(3): p. 236-9.
173. Constant, S., et al., *Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells.* J Exp Med, 1995. **182**(5): p. 1591-6.
174. Yamashita, M., et al., *T cell antigen receptor-mediated activation of the Ras/mitogen-activated protein kinase pathway controls interleukin 4 receptor function and type-2 helper T cell differentiation.* Proceedings of the National Academy of Sciences, 1999. **96**(3): p. 1024-1029.
175. Balamuth, F., et al., *Distinct patterns of membrane microdomain partitioning in Th1 and Th2 cells.* Immunity, 2001. **15**(5): p. 729-738.
176. Stevens, T.L., et al., *Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells.* Nature, 1988. **334**(6179): p. 255.
177. Lambrecht, B.N., et al., *Myeloid dendritic cells induce Th2 responses to inhaled antigen, leading to eosinophilic airway inflammation.* The Journal of clinical investigation, 2000. **106**(4): p. 551-559.
178. Panzer, M., et al., *Rapid in vivo conversion of effector T cells into Th2 cells during helminth infection.* J Immunol, 2012. **188**(2): p. 615-23.
179. Nakayama, T., et al., *Th2 cells in health and disease.* Annual review of immunology, 2017. **35**: p. 53-84.
180. Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.
181. !!! INVALID CITATION !!! {Aggarwal, 2003 #1229;Weaver, 2006 #1221;Cua, 2003 #3477;Veldhoen, 2006 #3530}.
182. Bettelli, E., et al., *Ouk-ka M., Weiner HL, Kuchroo V. K, 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* 2006.

183. Dann, S.M., et al., *IL-17A promotes protective IgA responses and expression of other potential effectors against the lumen-dwelling enteric parasite Giardia.* Experimental parasitology, 2015. **156**: p. 68-78.
184. Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* J Exp Med, 2006. **203**(10): p. 2271-9.
185. Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* Journal of Experimental Medicine, 2006. **203**(10): p. 2271-2279.
186. Griffiths, C.E., et al., *Comparison of ixekizumab with etanercept or placebo in moderate-to-severe psoriasis (UNCOVER-2 and UNCOVER-3): results from two phase 3 randomised trials.* The Lancet, 2015. **386**(9993): p. 541-551.
187. Trifari, S., et al., *Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells.* Nat Immunol, 2009. **10**(8): p. 864-71.
188. Plank, M.W., et al., *Th22 Cells Form a Distinct Th Lineage from Th17 Cells In Vitro with Unique Transcriptional Properties and Tbet-Dependent Th1 Plasticity.* J Immunol, 2017. **198**(5): p. 2182-2190.
189. Eyerich, S., et al., *Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling.* The Journal of clinical investigation, 2009. **119**(12): p. 3573-3585.
190. Basu, R., et al., *Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria.* Immunity, 2012. **37**(6): p. 1061-1075.
191. Aujla, S.J., et al., *IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia.* Nat Med, 2008. **14**(3): p. 275-81.
192. Wolk, K., et al., *IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis.* Eur J Immunol, 2006. **36**(5): p. 1309-23.
193. Jia, L. and C. Wu, *The biology and functions of Th22 cells.* Adv Exp Med Biol, 2014. **841**: p. 209-30.
194. Kim, C., et al., *A role for mucosal IL-22 production and Th22 cells in HIV-associated mucosal immunopathogenesis.* Mucosal immunology, 2012. **5**(6): p. 670.
195. Eyerich, K. and S. Eyerich, *Th22 cells in allergic disease.* Allergo journal international, 2015. **24**(1): p. 1-7.
196. Dardalhon, V., et al., *IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells.* Nat Immunol, 2008. **9**(12): p. 1347-55.
197. Dardalhon, V., et al., *IL-4 inhibits TGF-β-induced Foxp3+ T cells and, together with TGF-β, generates IL-9+ IL-10+ Foxp3− effector T cells.* Nature immunology, 2008. **9**(12): p. 1347.
198. Chang, H.C., et al., *The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation.* Nat Immunol, 2010. **11**(6): p. 527-34.
199. Van Snick, J., et al., *Cloning and characterization of a cDNA for a new mouse T cell growth factor (P40).* Journal of Experimental Medicine, 1989. **169**(1): p. 363-368.
200. Gounni, A.S., et al., *IL-9-mediated induction of eotaxin1/CCL11 in human airway smooth muscle cells.* The Journal of Immunology, 2004. **173**(4): p. 2771-2779.
201. Dong, Q., et al., *IL-9 induces chemokine expression in lung epithelial cells and baseline airway eosinophilia in transgenic mice.* European journal of immunology, 1999. **29**(7): p. 2130-2139.

202. Uyttenhove, C., R.J. Simpson, and J. Van Snick, *Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity.* Proceedings of the National Academy of Sciences, 1988. **85**(18): p. 6934-6938.
203. Licona-Limón, P., et al., *Th9 cells drive host immunity against gastrointestinal worm infection.* Immunity, 2013. **39**(4): p. 744-757.
204. Schmitt, E., M. Klein, and T. Bopp, *Th9 cells, new players in adaptive immunity.* Trends Immunol, 2014. **35**(2): p. 61-8.
205. Purwar, R., et al., *Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells.* Nat Med, 2012. **18**(8): p. 1248-53.
206. Lu, Y., et al., *Th9 cells promote antitumor immune responses in vivo.* The Journal of clinical investigation, 2012. **122**(11): p. 4160-4171.
207. Yang, J., et al., *Critical roles of mTOR Complex 1 and 2 for T follicular helper cell differentiation and germinal center responses.* Elife, 2016. **5**.
208. Suan, D., et al., *T follicular helper cells have distinct modes of migration and molecular signatures in naive and memory immune responses.* Immunity, 2015. **42**(4): p. 704-18.
209. Choi, Y.S., et al., *ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6.* Immunity, 2011. **34**(6): p. 932-46.
210. Nurieva, R.I., et al., *Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages.* Immunity, 2008. **29**(1): p. 138-49.
211. Kolenbrander, A., et al., *Generation of T follicular helper cells in vitro: requirement for B-cell receptor cross-linking and cognate B- and T-cell interaction.* Immunology, 2018. **153**(2): p. 214-224.
212. Cyster, J.G., et al., *Follicular stromal cells and lymphocyte homing to follicles.* Immunol Rev, 2000. **176**: p. 181-93.
213. Deenick, E.K., et al., *Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling.* Immunity, 2010. **33**(2): p. 241-53.
214. Ise, W., *Development and function of follicular helper T cells.* Biosci Biotechnol Biochem, 2016. **80**(1): p. 1-6.
215. Weber, J.P., et al., *ICOS maintains the T follicular helper cell phenotype by down-regulating Krüppel-like factor 2.* The Journal of Experimental Medicine, 2015. **212**(2): p. 217-233.
216. Breitfeld, D., et al., *Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production.* J Exp Med, 2000. **192**(11): p. 1545-52.
217. Stone, E.L., et al., *ICOS coreceptor signaling inactivates the transcription factor FOXO1 to promote Tfh cell differentiation.* Immunity, 2015. **42**(2): p. 239-251.
218. Ballesteros-Tato, A. and T.D. Randall, *Priming of T follicular helper cells by dendritic cells.* Immunol Cell Biol, 2014. **92**(1): p. 22-7.
219. Poholek, A.C., et al., *In vivo regulation of Bcl6 and T follicular helper cell development.* J Immunol, 2010. **185**(1): p. 313-26.
220. Vogelzang, A., et al., *A fundamental role for interleukin-21 in the generation of T follicular helper cells.* Immunity, 2008. **29**(1): p. 127-37.
221. Johnston, R.J., et al., *Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation.* Science, 2009. **325**(5943): p. 1006-10.
222. Luthje, K., et al., *The development and fate of follicular helper T cells defined by an IL-21 reporter mouse.* Nat Immunol, 2012. **13**(5): p. 491-8.

223. Baumjohann, D., et al., *Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype.* Immunity, 2013. **38**(3): p. 596-605.
224. Goenka, R., et al., *Cutting edge: dendritic cell-restricted antigen presentation initiates the follicular helper T cell program but cannot complete ultimate effector differentiation.* J Immunol, 2011. **187**(3): p. 1091-5.
225. Lee, J.Y., et al., *The transcription factor KLF2 restrains CD4(+) T follicular helper cell differentiation.* Immunity, 2015. **42**(2): p. 252-264.
226. Nurieva, R.I., et al., *Bcl6 mediates the development of T follicular helper cells.* Science, 2009. **325**(5943): p. 1001-1005.
227. Wu, T., et al., *TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection.* Cell Rep, 2015. **12**(12): p. 2099-110.
228. Benson, R.A., et al., *Antigen presentation kinetics control T cell/dendritic cell interactions and follicular helper T cell generation in vivo.* Elife, 2015. **4**.
229. Kerfoot, S.M., et al., *Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone.* Immunity, 2011. **34**(6): p. 947-60.
230. Moriyama, S., et al., *Sphingosine-1-phosphate receptor 2 is critical for follicular helper T cell retention in germinal centers.* J Exp Med, 2014. **211**(7): p. 1297-305.
231. Vinuesa, C.G. and J.G. Cyster, *How T cells earn the follicular rite of passage.* Immunity, 2011. **35**(5): p. 671-80.
232. Xie, M.M., et al., *AMP kinase promotes Bcl6 expression in both mouse and human T cells.* Mol Immunol, 2017. **81**: p. 67-75.
233. Kitano, M., et al., *Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity.* Immunity, 2011. **34**(6): p. 961-72.
234. Sahoo, A., et al., *Batf is important for IL-4 expression in T follicular helper cells.* Nat Commun, 2015. **6**: p. 7997.
235. Hsu, H.-C., et al., *Interleukin 17–producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice.* Nature immunology, 2008. **9**(2): p. 166.
236. Nurieva, R.I., et al., *Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages.* Immunity, 2008. **29**(1): p. 138-149.
237. Fahey, L.M., et al., *Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells.* Journal of Experimental Medicine, 2011. **208**(5): p. 987-999.
238. Glatman Zaretsky, A., et al., *T follicular helper cells differentiate from Th2 cells in response to helminth antigens.* J Exp Med, 2009. **206**(5): p. 991-9.
239. Maceiras, A.R., et al., *T follicular helper and T follicular regulatory cells have different TCR specificity.* Nat Commun, 2017. **8**: p. 15067.
240. Aloulou, M., et al., *Follicular regulatory T cells can be specific for the immunizing antigen and derive from naive T cells.* Nat Commun, 2016. **7**: p. 10579.
241. Maceiras, A.R., et al., *T follicular regulatory cells in mice and men.* Immunology, 2017. **152**(1): p. 25-35.
242. Odegard, J.M., et al., *ICOS-dependent extrafollicular helper T cells elicit IgG production via IL-21 in systemic autoimmunity.* J Exp Med, 2008. **205**(12): p. 2873-86.
243. Reinhardt, R.L., H.E. Liang, and R.M. Locksley, *Cytokine-secreting follicular T cells shape the antibody repertoire.* Nat Immunol, 2009. **10**(4): p. 385-93.
244. Vinuesa, C.G., et al., *Follicular Helper T Cells.* Annu Rev Immunol, 2016. **34**: p. 335-68.
245. Linterman, M.A., et al., *Follicular helper T cells are required for systemic autoimmunity.* J Exp Med, 2009. **206**(3): p. 561-76.

246. Mesquita, D., Jr., et al., *Follicular helper T cell in immunity and autoimmunity.* Braz J Med Biol Res, 2016. **49**(5): p. e5209.
247. Marshall, N.B. and S.L. Swain, *Cytotoxic CD4 T cells in antiviral immunity.* BioMed Research International, 2011. **2011**.
248. Mucida, D., et al., *Transcriptional reprogramming of mature CD4+ helper T cells generates distinct MHC class II–restricted cytotoxic T lymphocytes.* Nature immunology, 2013. **14**(3): p. 281.
249. Pipkin, M.E., et al., *Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells.* Immunity, 2010. **32**(1): p. 79-90.
250. Pearce, E.L., et al., *Control of effector CD8+ T cell function by the transcription factor Eomesodermin.* Science, 2003. **302**(5647): p. 1041-1043.
251. Donnarumma, T., et al., *Opposing development of cytotoxic and follicular helper CD4 T cells controlled by the TCF-1-Bcl6 nexus.* Cell reports, 2016. **17**(6): p. 1571-1583.
252. Brown, D.M., et al., *Multifunctional CD4 cells expressing gamma interferon and perforin mediate protection against lethal influenza virus infection.* Journal of virology, 2012. **86**(12): p. 6792-6803.
253. Yasukawa, M., et al., *Granule exocytosis, and not the Fas/Fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4+ as well as CD8+ cytotoxic T lymphocytes in humans.* Blood, 2000. **95**(7): p. 2352-2355.
254. Brown, D.M., et al., *IL-2 and antigen dose differentially regulate perforin-and FasL-mediated cytolytic activity in antigen specific CD4+ T cells.* Cellular immunology, 2009. **257**(1-2): p. 69-79.
255. Lancki, D.W., C.-S. Hsieh, and F.W. Fitch, *Mechanisms of lysis by cytotoxic T lymphocyte clones. Lytic activity and gene expression in cloned antigen-specific CD4+ and CD8+ T lymphocytes.* The Journal of Immunology, 1991. **146**(9): p. 3242-3249.
256. Brown, D.M., *Cytolytic CD4 cells: Direct mediators in infectious disease and malignancy.* Cellular immunology, 2010. **262**(2): p. 89-95.
257. Sujino, T., et al., *Tissue adaptation of regulatory and intraepithelial CD4+ T cells controls gut inflammation.* Science, 2016. **352**(6293): p. 1581-1586.
258. Yeh, J.-H., S.S. Sidhu, and A.C. Chan, *Regulation of a late phase of T cell polarity and effector functions by Crtam.* Cell, 2008. **132**(5): p. 846-859.
259. Raveney, B.J., et al., *Eomesodermin-expressing T-helper cells are essential for chronic neuroinflammation.* Nature communications, 2015. **6**: p. 8437.
260. Asano, M., et al., *Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation.* J Exp Med, 1996. **184**(2): p. 387-96.
261. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
262. Fontenot, J.D., et al., *Regulatory T cell lineage specification by the forkhead transcription factor foxp3.* Immunity, 2005. **22**(3): p. 329-41.
263. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
264. Kim, C.H., *FOXP3 and its role in the immune system*, in *Forkhead Transcription Factors*. 2009, Springer. p. 17-29.
265. Sakaguchi, S., *Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses.* Annu. Rev. Immunol., 2004. **22**: p. 531-562.

266. Schmidt, A., N. Oberle, and P.H. Krammer, *Molecular mechanisms of treg-mediated T cell suppression.* Frontiers in immunology, 2012. **3**: p. 51.
267. Somerset, D.A., et al., *Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset.* Immunology, 2004. **112**(1): p. 38-43.
268. Aluvihare, V.R., M. Kallikourdis, and A.G. Betz, *Regulatory T cells mediate maternal tolerance to the fetus.* Nature immunology, 2004. **5**(3): p. 266.
269. Samstein, R.M., et al., *Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict.* Cell, 2012. **150**(1): p. 29-38.
270. Sakaguchi, S., et al., *Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance.* Immunological reviews, 2001. **182**(1): p. 18-32.
271. Coombes, J.L., et al., *Regulatory T cells and intestinal homeostasis.* Immunological reviews, 2005. **204**(1): p. 184-194.
272. Cederbom, L., H. Hall, and F. Ivars, *CD4+ CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells.* European journal of immunology, 2000. **30**(6): p. 1538-1543.
273. Oderup, C., et al., *Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression.* Immunology, 2006. **118**(2): p. 240-249.
274. Misra, N., et al., *Cutting edge: human CD4+ CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells.* The Journal of Immunology, 2004. **172**(8): p. 4676-4680.
275. Qureshi, O.S., et al., *Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4.* Science, 2011. **332**(6029): p. 600-603.
276. Hou, T.Z., et al., *A transendocytosis model of CTLA-4 function predicts its suppressive behavior on regulatory T cells.* The Journal of Immunology, 2015. **194**(5): p. 2148-2159.
277. Gu, P., et al., *Trogocytosis of CD80 and CD86 by induced regulatory T cells.* Cell Mol Immunol, 2012. **9**(2): p. 136-46.
278. Dopfer, E.P., S. Minguet, and W.W. Schamel, *A new vampire saga: the molecular mechanism of T cell trogocytosis.* Immunity, 2011. **35**(2): p. 151-3.
279. Joly, E. and D. Hudrisier, *What is trogocytosis and what is its purpose?* Nat Immunol, 2003. **4**(9): p. 815.
280. LeMaoult, J., J. Caumartin, and E.D. Carosella, *Exchanges of membrane patches (trogocytosis) split theoretical and actual functions of immune cells.* Hum Immunol, 2007. **68**(4): p. 240-3.
281. Caumartin, J., J. Lemaoult, and E.D. Carosella, *Intercellular exchanges of membrane patches (trogocytosis) highlight the next level of immune plasticity.* Transpl Immunol, 2006. **17**(1): p. 20-2.
282. Rosenits, K., et al., *T cells acquire cell surface determinants of APC via in vivo trogocytosis during viral infections.* Eur J Immunol, 2010. **40**(12): p. 3450-7.
283. Miyake, K., et al., *Trogocytosis of peptide-MHC class II complexes from dendritic cells confers antigen-presenting ability on basophils.* Proc Natl Acad Sci U S A, 2017. **114**(5): p. 1111-1116.
284. Brown, R., et al., *CD86+ or HLA-G+ can be transferred via trogocytosis from myeloma cells to T cells and are associated with poor prognosis.* Blood, 2012. **120**(10): p. 2055-63.
285. Wetzel, S.A. and D.C. Parker, *MHC transfer from APC to T cells following antigen recognition.* Crit Rev Immunol, 2006. **26**(1): p. 1-21.

286. Wetzel, S.A. and J. Reed, *Trogocytosis-Mediated Signaling Drives GATA-3 and IL-4 Expression in CD4<sup>+</sup> T cells.* The Journal of Immunology, 2016. **196**(1 Supplement): p. 55.11-55.11.
287. Wetzel, S.A., T.W. McKeithan, and D.C. Parker, *Peptide-specific intercellular transfer of MHC class II to CD4+ T cells directly from the immunological synapse upon cellular dissociation.* J Immunol, 2005. **174**(1): p. 80-9.
288. Dejean, A.S., et al., *Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells.* Nature immunology, 2009. **10**(5): p. 504.
289. Grohmann, U., et al., *CTLA-4–Ig regulates tryptophan catabolism in vivo.* Nature immunology, 2002. **3**(11): p. 1097.
290. Collison, L.W., et al., *The inhibitory cytokine IL-35 contributes to regulatory T-cell function.* Nature, 2007. **450**(7169): p. 566.
291. Shevach, E.M., *Mechanisms of foxp3+ T regulatory cell-mediated suppression.* Immunity, 2009. **30**(5): p. 636-45.
292. Yoshimura, A. and G. Muto, *TGF-β function in immune suppression*, in *Negative Co-Receptors and Ligands*. 2010, Springer. p. 127-147.
293. Saraiva, M. and A. O'garra, *The regulation of IL-10 production by immune cells.* Nature reviews immunology, 2010. **10**(3): p. 170.
294. Bopp, T., et al., *Cyclic adenosine monophosphate is a key component of regulatory T cell–mediated suppression.* Journal of Experimental Medicine, 2007. **204**(6): p. 1303-1310.
295. Grossman, W.J., et al., *Human T regulatory cells can use the perforin pathway to cause autologous target cell death.* Immunity, 2004. **21**(4): p. 589-601.
296. Gondek, D.C., et al., *Cutting edge: contact-mediated suppression by CD4+ CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism.* The Journal of Immunology, 2005. **174**(4): p. 1783-1786.
297. Zhao, D.-M., et al., *Activated CD4+ CD25+ T cells selectively kill B lymphocytes.* Blood, 2006. **107**(10): p. 3925-3932.
298. Rabinovich, G.A., et al., *Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response?* Trends in immunology, 2002. **23**(6): p. 313-320.
299. Toscano, M.A., et al., *Differential glycosylation of T H 1, T H 2 and T H-17 effector cells selectively regulates susceptibility to cell death.* Nature immunology, 2007. **8**(8): p. 825.
300. Garín, M.I., et al., *Galectin-1: a key effector of regulation mediated by CD4+ CD25+ T cells.* Blood, 2007. **109**(5): p. 2058-2065.
301. Hernandez, J.D. and L.G. Baum, *Ah, sweet mystery of death! Galectins and control of cell fate.* Glycobiology, 2002. **12**(10): p. 127R-136R.
302. Sakaguchi, S., K. Wing, and T. Yamaguchi, *Dynamics of peripheral tolerance and immune regulation mediated by Treg.* Eur J Immunol, 2009. **39**(9): p. 2331-6.
303. Cao, W., et al., *Regulatory T cell expansion and immune activation during untreated HIV type 1 infection are associated with disease progression.* AIDS research and human retroviruses, 2009. **25**(2): p. 183-191.
304. Robinson, K., et al., *Helicobacter pylori-induced peptic ulcer disease is associated with inadequate regulatory T cell responses.* Gut, 2008. **57**(10): p. 1375-1385.
305. Robinson, D.S., M. Larché, and S.R. Durham, *Tregs and allergic disease.* The Journal of clinical investigation, 2004. **114**(10): p. 1389-1397.
306. Zorn, E., et al., *Reduced frequency of FOXP3+ CD4+ CD25+ regulatory T cells in patients with chronic graft-versus-host disease.* Blood, 2005. **106**(8): p. 2903-2911.

307. Yamano, Y., et al., *Virus-induced dysfunction of CD4+ CD25+ T cells in patients with HTLV-I–associated neuroimmunological disease.* The Journal of clinical investigation, 2005. **115**(5): p. 1361-1368.
308. Roncarolo, M.G., S. Gregori, and M. Levings. *Type 1 T regulatory cells and their relationship with CD4^+ CD25^+ T regulatory cells*. in *Novartis Foundation symposium*. 2003. Wiley Online Library.
309. Weiner, H.L., *Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells.* Immunological reviews, 2001. **182**(1): p. 207-214.
310. Peterson, R.A., *Regulatory T-cells: diverse phenotypes integral to immune homeostasis and suppression.* Toxicologic pathology, 2012. **40**(2): p. 186-204.
311. Akdis, C., et al., *Immunological mechanisms of sublingual immunotherapy.* Allergy, 2006. **61**: p. 11-14.
312. Iijima, H., I. Takahashi, and H. Kiyono, *Mucosal immune network in the gut for the control of infectious diseases.* Reviews in medical virology, 2001. **11**(2): p. 117-133.
313. Cretney, E., A. Kallies, and S.L. Nutt, *Differentiation and function of Foxp3+ effector regulatory T cells.* Trends in immunology, 2013. **34**(2): p. 74-80.
314. Duhen, T., et al., *Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Th cells.* Blood, 2012. **119**(19): p. 4430-4440.
315. Ahmadzadeh, M., S.F. Hussain, and D.L. Farber, *Heterogeneity of the memory CD4 T cell response: persisting effectors and resting memory T cells.* J Immunol, 2001. **166**(2): p. 926-35.
316. Boyman, O., et al., *Homeostatic proliferation and survival of naive and memory T cells.* Eur J Immunol, 2009. **39**(8): p. 2088-94.
317. McKinstry, K.K., et al., *Effector CD4 T-cell transition to memory requires late cognate interactions that induce autocrine IL-2.* Nat Commun, 2014. **5**: p. 5377.
318. Daniels, M.A. and E. Teixeiro, *TCR Signaling in T Cell Memory.* Front Immunol, 2015. **6**: p. 617.
319. Devarajan, P., et al., *New Insights into the Generation of CD4 Memory May Shape Future Vaccine Strategies for Influenza.* Front Immunol, 2016. **7**: p. 136.
320. Snook, J.P., C. Kim, and M.A. Williams, *TCR signal strength controls the differentiation of CD4(+) effector and memory T cells.* Sci Immunol, 2018. **3**(25).
321. Kondrack, R.M., et al., *Interleukin 7 regulates the survival and generation of memory CD4 cells.* J Exp Med, 2003. **198**(12): p. 1797-806.
322. Chetoui, N., et al., *Interleukin-7 promotes the survival of human CD4+ effector/memory T cells by up-regulating Bcl-2 proteins and activating the JAK/STAT signalling pathway.* Immunology, 2010. **130**(3): p. 418-26.
323. Read, K.A., et al., *IL-2, IL-7, and IL-15: Multistage regulators of CD4(+) T helper cell differentiation.* Exp Hematol, 2016. **44**(9): p. 799-808.
324. Watanabe, M., et al., *Interleukin 7 is produced by human intestinal epithelial cells and regulates the proliferation of intestinal mucosal lymphocytes.* The Journal of clinical investigation, 1995. **95**(6): p. 2945-2953.
325. Sudo, T., et al., *Interleukin 7 production and function in stromal cell-dependent B cell development.* Journal of Experimental Medicine, 1989. **170**(1): p. 333-338.
326. Haugen, F., et al., *IL-7 is expressed and secreted by human skeletal muscle cells.* American Journal of Physiology-Cell Physiology, 2010. **298**(4): p. C807-C816.
327. Kröncke, R., et al., *Human follicular dendritic cells and vascular cells produce interleukin-7: a potential role for interleukin-7 in the germinal center reaction.* European journal of immunology, 1996. **26**(10): p. 2541-2544.

328. Carrette, F. and C.D. Surh. *IL-7 signaling and CD127 receptor regulation in the control of T cell homeostasis*. in *Seminars in immunology*. 2012. Elsevier.
329. Arsenio, J., P.J. Metz, and J.T. Chang, *Asymmetric Cell Division in T Lymphocyte Fate Diversification.* Trends Immunol, 2015. **36**(11): p. 670-683.
330. Chang, J.T., et al., *Asymmetric T lymphocyte division in the initiation of adaptive immune responses.* Science, 2007. **315**(5819): p. 1687-91.
331. Ciocca, M.L., et al., *Cutting edge: Asymmetric memory T cell division in response to rechallenge.* J Immunol, 2012. **188**(9): p. 4145-8.
332. Sallusto, F., et al., *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708.
333. Randolph, G.J., V. Angeli, and M.A. Swartz, *Dendritic-cell trafficking to lymph nodes through lymphatic vessels.* Nature Reviews Immunology, 2005. **5**(8): p. 617.
334. Gunn, M.D. *Chemokine mediated control of dendritic cell migration and function*. in *Seminars in immunology*. 2003. Elsevier.
335. Charo, I.F. and R.M. Ransohoff, *The many roles of chemokines and chemokine receptors in inflammation.* New England Journal of Medicine, 2006. **354**(6): p. 610-621.
336. Ley, K., et al., *Getting to the site of inflammation: the leukocyte adhesion cascade updated.* Nature Reviews Immunology, 2007. **7**(9): p. 678.
337. McEver, R.P. and R.D. Cummings, *Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment.* The Journal of clinical investigation, 1997. **100**(3): p. 485-491.
338. DeGrendele, H.C., P. Estess, and M.H. Siegelman, *Requirement for CD44 in activated T cell extravasation into an inflammatory site.* Science, 1997. **278**(5338): p. 672-675.
339. Goodison, S., V. Urquidi, and D. Tarin, *CD44 cell adhesion molecules.* Molecular Pathology, 1999. **52**(4): p. 189.
340. Aruffo, A., et al., *CD44 is the principal cell surface receptor for hyaluronate.* Cell, 1990. **61**(7): p. 1303-1313.
341. Huet, S., et al., *CD44 contributes to T cell activation.* The Journal of Immunology, 1989. **143**(3): p. 798-801.
342. Haynes, B.F., et al., *CD44—a molecule involved in leukocyte adherence and T-cell activation.* Immunology today, 1989. **10**(12): p. 423-428.
343. Ponta, H., L. Sherman, and P.A. Herrlich, *CD44: from adhesion molecules to signalling regulators.* Nature reviews Molecular cell biology, 2003. **4**(1): p. 33.
344. Pepper, M. and M.K. Jenkins, *Origins of CD4+ effector and central memory T cells.* Nature Immunology, 2011. **12**(6): p. 467-471.
345. Sallusto, F. and A. Lanzavecchia, *Exploring pathways for memory T cell generation.* Journal of Clinical Investigation, 2001. **108**(6): p. 805-806.
346. Unsoeld, H. and H. Pircher, *Complex memory T-cell phenotypes revealed by coexpression of CD62L and CCR7.* J Virol, 2005. **79**(7): p. 4510-3.
347. Berard, M. and D.F. Tough, *Qualitative differences between naive and memory T cells.* Immunology, 2002. **106**(2): p. 127-138.
348. Fearon, D.T., P. Manders, and S.D. Wagner, *Arrested differentiation, the self-renewing memory lymphocyte, and vaccination.* Science, 2001. **293**(5528): p. 248-50.
349. Butcher, E.C. and L.J. Picker, *Lymphocyte homing and homeostasis.* Science, 1996. **272**(5258): p. 60-67.
350. Mackay, C.R., W.L. Marston, and L. Dudler, *Naive and memory T cells show distinct pathways of lymphocyte recirculation.* Journal of Experimental Medicine, 1990. **171**(3): p. 801-817.

351. Hogan, R.J., et al., *Protection from respiratory virus infections can be mediated by antigen-specific CD4+ T cells that persist in the lungs.* Journal of Experimental Medicine, 2001. **193**(8): p. 981-986.
352. Gebhardt, T., et al., *Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus.* Nature immunology, 2009. **10**(5): p. 524.
353. Klicznik, M.M., et al., *Human CD4+ CD103+ cutaneous resident memory T cells are found in the circulation of healthy individuals.* Science immunology, 2019. **4**(37): p. eaav8995.
354. Wilk, M.M. and K.H. Mills, *CD4 TRM cells following infection and immunization: implications for more effective vaccine design.* Frontiers in immunology, 2018. **9**.
355. Sathaliyawala, T., et al., *Distribution and compartmentalization of human circulating and tissue-resident memory T cell subsets.* Immunity, 2013. **38**(1): p. 187-197.
356. Bankovich, A.J., L.R. Shiow, and J.G. Cyster, *CD69 suppresses sphingosine 1-phosophate receptor-1 (S1P1) function through interaction with membrane helix 4.* Journal of Biological Chemistry, 2010. **285**(29): p. 22328-22337.
357. Spiegel, S. and S. Milstien, *The outs and the ins of sphingosine-1-phosphate in immunity.* Nature Reviews Immunology, 2011. **11**(6): p. 403.
358. Gebhardt, T., et al., *Different patterns of peripheral migration by memory CD4+ and CD8+ T cells.* Nature, 2011. **477**(7363): p. 216.
359. Gattinoni, L., et al., *A human memory T cell subset with stem cell–like properties.* Nature medicine, 2011. **17**(10): p. 1290.
360. Ahmed, R., et al., *Human stem cell-like memory T cells are maintained in a state of dynamic flux.* Cell reports, 2016. **17**(11): p. 2811-2818.
361. Mpande, C.A., et al., *Functional, antigen-specific stem cell memory (TSCM) CD4+ T cells are induced by human Mycobacterium tuberculosis infection.* Frontiers in immunology, 2018. **9**: p. 324.
362. Cone, R.E., J. Sprent, and J.J. Marchalonis, *Antigen-binding specificity of isolated cell-surface immunoglobulin from thymus cells activated to histocompatibility antigens.* Proceedings of the National Academy of Sciences, 1972. **69**(9): p. 2556-2560.
363. Benoist, C. and D. Mathis, *Regulation of major histocompatibility complex class-II genes: X, Y and other letters of the alphabet.* Annual review of immunology, 1990. **8**(1): p. 681-715.
364. Bona, C., et al., *Transfer of antigen from macrophages to lymphocytes: II. Immunological significance of the transfer of lipopolysaccharide.* Immunology, 1973. **24**(5): p. 831.
365. Hudson, L. and J. Sprent, *Specific adsorption of IgM antibody onto H-2-activated mouse T lymphocytes.* Journal of Experimental Medicine, 1976. **143**(2): p. 444-449.
366. Sharrow, S., B. Mathieson, and A. Singer, *Cell surface appearance of unexpected host MHC determinants on thymocytes from radiation bone marrow chimeras.* The Journal of Immunology, 1981. **126**(4): p. 1327-1335.
367. Nepom, J., B. Benacerraf, and R. Germain, *Acquisition of syngeneic IA determinants by T cells proliferating in response to poly (Glu60Ala30Tyr10).* The Journal of Immunology, 1981. **127**(3): p. 888-892.
368. Baba, E., et al., *Functional CD4 T cells after intercellular molecular transfer of 0X40 ligand.* J Immunol, 2001. **167**(2): p. 875-83.
369. Shi, M., et al., *CD4(+) T cells stimulate memory CD8(+) T cell expansion via acquired pMHC I complexes and costimulatory molecules, and IL-2 secretion.* J Leukoc Biol, 2006. **80**(6): p. 1354-63.

370. Hudrisier, D., et al., *Capture of target cell membrane components via trogocytosis is triggered by a selected set of surface molecules on T or B cells.* J Immunol, 2007. **178**(6): p. 3637-47.
371. Adamopoulou, E., et al., *Human CD4+ T cells displaying viral epitopes elicit a functional virus-specific memory CD8+ T cell response.* J Immunol, 2007. **178**(9): p. 5465-72.
372. Umeshappa, C.S., et al., *CD4+ Th-APC with Acquired Peptide/MHC Class I and II Complexes Stimulate Type 1 Helper CD4+ and Central Memory CD8+ T Cell Responses.* The Journal of Immunology, 2008. **182**(1): p. 193-206.
373. Hudrisier, D., et al., *CTLs rapidly capture membrane fragments from target cells in a TCR signaling-dependent manner.* J Immunol, 2001. **166**(6): p. 3645-9.
374. Riond, J., et al., *Capture of membrane components via trogocytosis occurs in vivo during both dendritic cells and target cells encounter by CD8(+) T cells.* Scand J Immunol, 2007. **66**(4): p. 441-50.
375. Gary, R., et al., *Antigen-specific transfer of functional programmed death ligand 1 from human APCs onto CD8+ T cells via trogocytosis.* J Immunol, 2012. **188**(2): p. 744-52.
376. Uzana, R., et al., *Trogocytosis Is a Gateway to Characterize Functional Diversity in Melanoma-Specific CD8+ T Cell Clones.* J Immunol, 2012. **188**(2): p. 632-40.
377. Espinosa, E., et al., *Synaptic transfer by human gamma delta T cells stimulated with soluble or cellular antigens.* J Immunol, 2002. **168**(12): p. 6336-43.
378. Aucher, A., et al., *Capture of plasma membrane fragments from target cells by trogocytosis requires signaling in T cells but not in B cells.* Blood, 2008. **111**(12): p. 5621-8.
379. Gardell, J.L. and D.C. Parker, *CD40L is transferred to antigen-presenting B cells during delivery of T-cell help.* Eur J Immunol, 2017. **47**(1): p. 41-50.
380. Poupot, M., J.J. Fournie, and R. Poupot, *Trogocytosis and killing of IL-4-polarized monocytes by autologous NK cells.* J Leukoc Biol, 2008. **84**(5): p. 1298-305.
381. Nakayama, M., et al., *Natural killer (NK)-dendritic cell interactions generate MHC class II-dressed NK cells that regulate CD4+ T cells.* Proc Natl Acad Sci U S A, 2011. **108**(45): p. 18360-5.
382. Miner, C.A., et al., *Acquisition of activation receptor ligand by trogocytosis renders NK cells hyporesponsive.* J Immunol, 2015. **194**(4): p. 1945-53.
383. Daubeuf, S., et al., *The direction of plasma membrane exchange between lymphocytes and accessory cells by trogocytosis is influenced by the nature of the accessory cell.* J Immunol, 2010. **184**(4): p. 1897-908.
384. Sarvari, A.K., et al., *Interaction of differentiated human adipocytes with macrophages leads to trogocytosis and selective IL-6 secretion.* Cell Death Dis, 2015. **6**: p. e1613.
385. Li, K.J., et al., *Membrane Transfer from Mononuclear Cells to Polymorphonuclear Neutrophils Transduces Cell Survival and Activation Signals in the Recipient Cells via Anti-Extrinsic Apoptotic and MAP Kinase Signaling Pathways.* PLoS One, 2016. **11**(6): p. e0156262.
386. Valgardsdottir, R., et al., *Human neutrophils mediate trogocytosis rather than phagocytosis of CLL B cells opsonized with anti-CD20 antibodies.* Blood, 2017. **129**(19): p. 2636-2644.
387. Mercer, F., et al., *Neutrophils kill the parasite Trichomonas vaginalis using trogocytosis.* PLoS Biol, 2018. **16**(2): p. e2003885.
388. Zhang, Q.J., et al., *Trogocytosis of MHC-I/peptide complexes derived from tumors and infected cells enhances dendritic cell cross-priming and promotes adaptive T cell responses.* PLoS One, 2008. **3**(8): p. e3097.

389. Bonaccorsi, I., et al., *Membrane transfer from tumor cells overcomes deficient phagocytic ability of plasmacytoid dendritic cells for the acquisition and presentation of tumor antigens.* J Immunol, 2014. **192**(2): p. 824-32.
390. Ambudkar, S.V., et al., *A novel way to spread drug resistance in tumor cells: functional intercellular transfer of P-glycoprotein (ABCB1).* Trends Pharmacol Sci, 2005. **26**(8): p. 385-7.
391. Haastert, B., et al., *T cells at the site of autoimmune inflammation show increased potential for trogocytosis.* PLoS One, 2013. **8**(12): p. e81404.
392. Hudrisier, D., et al., *T cell activation correlates with an increased proportion of antigen among the materials acquired from target cells.* Eur J Immunol, 2005. **35**(8): p. 2284-94.
393. Patel, D.M., R.W. Dudek, and M.D. Mannie, *Intercellular exchange of class II MHC complexes: ultrastructural localization and functional presentation of adsorbed I-A/peptide complexes.* Cell Immunol, 2001. **214**(1): p. 21-34.
394. Hwang, I., et al., *T cells can use either T cell receptor or CD28 receptors to absorb and internalize cell surface molecules derived from antigen-presenting cells.* J Exp Med, 2000. **191**(7): p. 1137-48.
395. Hudrisier, D. and P. Bongrand, *Intercellular transfer of antigen-presenting cell determinants onto T cells: molecular mechanisms and biological significance.* Faseb J, 2002. **16**(6): p. 477-86.
396. Sabzevari, H., et al., *Acquisition of CD80 (B7-1) by T cells.* J Immunol, 2001. **166**(4): p. 2505-13.
397. Huang, J.F., et al., *TCR-Mediated internalization of peptide-MHC complexes acquired by T cells.* Science, 1999. **286**(5441): p. 952-4.
398. Wetzel, S., et al., *Trogocytosis efficiency is dependent upon TCR affinity for MHC: Peptide ligands (APP4P. 115)*. 2014, Am Assoc Immnol.
399. Li, G., et al., *T cell antigen discovery via trogocytosis.* Nat Methods, 2019. **16**(2): p. 183-190.
400. Daubeuf, S., et al., *Preferential transfer of certain plasma membrane proteins onto T and B cells by trogocytosis.* PLoS One, 2010. **5**(1): p. e8716.
401. Zimmer, J., V. Ioannidis, and W. Held, *H-2D ligand expression by Ly49A+ natural killer (NK) cells precludes ligand uptake from environmental cells: implications for NK cell function.* Journal of Experimental Medicine, 2001. **194**(10): p. 1531-1539.
402. Rechavi, O., et al., *Intercellular transfer of oncogenic H-Ras at the immunological synapse.* PLoS One, 2007. **2**(11): p. e1204.
403. Eisenberg, G., et al., *Imprinting of lymphocytes with melanoma antigens acquired by trogocytosis facilitates identification of tumor-reactive T cells.* J Immunol, 2013. **190**(11): p. 5856-65.
404. Beadling, C. and M.K. Slifka, *Quantifying viable virus-specific T cells without a priori knowledge of fine epitope specificity.* Nature medicine, 2006. **12**(10): p. 1208.
405. Hwang, I. and J. Sprent, *Role of the actin cytoskeleton in T cell absorption and internalization of ligands from APC.* The Journal of Immunology, 2001. **166**(8): p. 5099-5107.
406. Stinchcombe, J.C., et al., *The immunological synapse of CTL contains a secretory domain and membrane bridges.* Immunity, 2001. **15**(5): p. 751-761.
407. Sowinski, S., et al., *Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission.* Nature cell biology, 2008. **10**(2): p. 211.

408. Patel, D.M., R.W. Dudek, and M.D. Mannie, *Intercellular exchange of class II MHC complexes: ultrastructural localization and functional presentation of adsorbed IA/peptide complexes.* Cellular immunology, 2001. **214**(1): p. 21-34.
409. Boes, M., et al., *T-cell engagement of dendritic cells rapidly rearranges MHC class II transport.* Nature, 2002. **418**(6901): p. 983.
410. Game, D.S., N.J. Rogers, and R.I. Lechler, *Acquisition of HLA-DR and costimulatory molecules by T cells from allogeneic antigen presenting cells.* Am J Transplant, 2005. **5**(7): p. 1614-25.
411. Martinez-Martin, N., et al., *T cell receptor internalization from the immunological synapse is mediated by TC21 and RhoG GTPase-dependent phagocytosis.* Immunity, 2011. **35**(2): p. 208-22.
412. Reed, J. and S.A. Wetzel, *Trogocytosis-Mediated Intracellular Signaling in CD4(+) T Cells Drives TH2-Associated Effector Cytokine Production and Differentiation.* J Immunol, 2019. **202**(10): p. 2873-2887.
413. Tomaru, U., et al., *Detection of virus-specific T cells and CD8+ T-cell epitopes by acquisition of peptide-HLA-GFP complexes: analysis of T-cell phenotype and function in chronic viral infections.* Nat Med, 2003. **9**(4): p. 469-76.
414. Mukherjee, S., et al., *Trypanosoma cruzi invasion is associated with trogocytosis.* Microbes Infect, 2015. **17**(1): p. 62-70.
415. LeMaoult, J., et al., *Immune regulation by pretenders: cell-to-cell transfers of HLA-G make effector T cells act as regulatory cells.* Blood, 2007. **109**(5): p. 2040-8.
416. Ahmed, K.A. and J. Xiang, *Mechanisms of cellular communication through intercellular protein transfer.* J Cell Mol Med, 2011. **15**(7): p. 1458-73.
417. Brown, R., et al., *Trogocytosis generates acquired regulatory T cells adding further complexity to the dysfunctional immune response in multiple myeloma.* Oncoimmunology, 2012. **1**(9): p. 1658-1660.
418. Shao, Z., et al., *Trogocytic CD137 transfer causes an internalization of CD137 ligand on murine APCs leading to reduced T cell costimulation.* J Leukoc Biol, 2015. **97**(5): p. 909-919.
419. Barinov, A., et al., *CD4/CD8/Dendritic cell complexes in the spleen: CD8+ T cells can directly bind CD4+ T cells and modulate their response.* PLoS One, 2017. **12**(7): p. e0180644.
420. Zhou, G., et al., *Presentation of acquired peptide-MHC class II ligands by CD4+ regulatory T cells or helper cells differentially regulates antigen-specific CD4+ T cell response.* J Immunol, 2011. **186**(4): p. 2148-55.
421. Xiang, J., H. Huang, and Y. Liu, *A new dynamic model of CD8+ T effector cell responses via CD4+ T helper-antigen-presenting cells.* J Immunol, 2005. **174**(12): p. 7497-505.
422. Amiot, L., N. Vu, and M. Samson, *Biology of the immunomodulatory molecule HLA-G in human liver diseases.* J Hepatol, 2015. **62**(6): p. 1430-7.
423. Nolte-'t Hoen, E.N., et al., *Uptake of membrane molecules from T cells endows antigen-presenting cells with novel functional properties.* Eur J Immunol, 2004. **34**(11): p. 3115-25.
424. Nakayama, M., *Antigen Presentation by MHC-Dressed Cells.* Front Immunol, 2014. **5**: p. 672.
425. Romagnoli, P.A., et al., *CD8 T cell memory recall is enhanced by novel direct interactions with CD4 T cells enabled by MHC class II transferred from APCs.* PLoS One, 2013. **8**(2): p. e56999.

426. Romagnoli, P., D. Hudrisier, and J.P. van Meerwijk, *Molecular Signature of Recent Thymic Selection Events on Effector and Regulatory CD4+ T Lymphocytes.* J Immunol, 2005. **175**(9): p. 5751-8.
427. Tsang, J.Y., J.G. Chai, and R. Lechler, *Antigen presentation by mouse CD4+ T cells involving acquired MHC class II:peptide complexes: another mechanism to limit clonal expansion?* Blood, 2003. **101**(7): p. 2704-10.
428. Ferlazzo, G., et al., *T lymphocytes express B7 family molecules following interaction with dendritic cells and acquire bystander costimulatory properties.* European journal of immunology, 2002. **32**(11): p. 3092-3101.
429. Helft, J., et al., *Antigen-specific T-T interactions regulate CD4 T-cell expansion.* Blood, 2008. **112**(4): p. 1249-58.
430. Ford McIntyre, M.S., et al., *Cutting edge: in vivo trogocytosis as a mechanism of double negative regulatory T cell-mediated antigen-specific suppression.* J Immunol, 2008. **181**(4): p. 2271-5.
431. Hao, S., et al., *Antigen specificity acquisition of adoptive CD4+ regulatory T cells via acquired peptide-MHC class I complexes.* J Immunol, 2008. **181**(4): p. 2428-37.
432. Bahcheli, D., et al., *Transfer of cell membrane components via trogocytosis occurs in CD4+ Foxp3+ CD25+ regulatory T-cell contact-dependent suppression.* Autoimmunity, 2011. **44**(8): p. 607-15.
433. Kranc, K.R., et al., *Control of autoimmunity by "epitope theft".* Trends Mol Med, 2005. **11**(1): p. 1-4.
434. Dhainaut, M. and M. Moser, *Regulation of immune reactivity by intercellular transfer.* Front Immunol, 2014. **5**: p. 112.
435. Umeshappa, C.S. and J. Xiang, *Tumor-derived HLA-G1 acquisition by monocytes through trogocytosis: possible functional consequences.* Cell Mol Life Sci, 2010. **67**(23): p. 4107-8.
436. Chung, B., et al., *Antigen-specific inhibition of high-avidity T cell target lysis by low-avidity T cells via trogocytosis.* Cell reports, 2014. **8**(3): p. 871-882.
437. Hsu, P., et al., *Expansion of CD4(+) HLA-G(+) T Cell in human pregnancy is impaired in pre-eclampsia.* Am J Reprod Immunol, 2014. **71**(3): p. 217-28.
438. Gonzalez, A., et al., *The immunosuppressive molecule HLA-G and its clinical implications.* Crit Rev Clin Lab Sci, 2012. **49**(3): p. 63-84.
439. Zhou, J., et al., *Physiological relevance of antigen presentasome (APS), an acquired MHC/costimulatory complex, in the sustained activation of CD4+ T cells in the absence of APCs.* Blood, 2005. **105**(8): p. 3238-46.
440. Yuan, Y., Y. Yang, and X. Huang, *IL-21 is required for CD4 memory formation in response to viral infection.* JCI Insight, 2017. **2**(7): p. e90652.
441. Seder, R.A., et al., *The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice.* J Exp Med, 1992. **176**(4): p. 1091-8.
442. Faroudi, M., et al., *Cutting edge: T lymphocyte activation by repeated immunological synapse formation and intermittent signaling.* J Immunol, 2003. **171**(3): p. 1128-32.
443. Lee, J.M., C.M. Kay, and T.H. Watts, *Conformational changes in mouse MHC class II proteins at acidic pH.* Int Immunol, 1992. **4**(8): p. 889-97.
444. Simms, P.E. and T.M. Ellis, *Utility of flow cytometric detection of CD69 expression as a rapid method for determining poly- and oligoclonal lymphocyte activation.* Clin Diagn Lab Immunol, 1996. **3**(3): p. 301-4.

445. Chan, A.C., et al., *The zeta chain is associated with a tyrosine kinase and upon T-cell antigen receptor stimulation associates with ZAP-70, a 70-kDa tyrosine phosphoprotein.* Proc Natl Acad Sci U S A, 1991. **88**(20): p. 9166-70.
446. Straus, D.B. and A. Weiss, *The CD3 chains of the T cell antigen receptor associate with the ZAP-70 tyrosine kinase and are tyrosine phosphorylated after receptor stimulation.* J Exp Med, 1993. **178**(5): p. 1523-30.
447. Kupfer, A., T.R. Mosmann, and H. Kupfer, *Polarized expression of cytokines in cell conjugates of helper T cells and splenic B cells.* Proc Natl Acad Sci U S A, 1991. **88**(3): p. 775-9.
448. Fairfax, K.C., et al., *IL-4-secreting secondary T follicular helper (Tfh) cells arise from memory T cells, not persisting Tfh cells, through a B cell-dependent mechanism.* J Immunol, 2015. **194**(7): p. 2999-3010.
449. Schmidt-Weber, C.B., A. Rao, and A.H. Lichtman, *Integration of TCR and IL-4 signals through STAT6 and the regulation of IL-4 gene expression.* Mol Immunol, 2000. **37**(12-13): p. 767-74.
450. Cook, K.D. and J. Miller, *TCR-dependent translational control of GATA-3 enhances Th2 differentiation.* J Immunol, 2010. **185**(6): p. 3209-16.
451. Krawczyk, C.M., H. Shen, and E.J. Pearce, *Functional plasticity in memory T helper cell responses.* J Immunol, 2007. **178**(7): p. 4080-8.
452. Magombedze, G., et al., *Cellular and population plasticity of helper CD4(+) T cell responses.* Front Physiol, 2013. **4**: p. 206.
453. Howard, J.G., C. Hale, and W.L. Chan-Liew, *Immunological regulation of experimental cutaneous leishmaniasis. 1. Immunogenetic aspects of susceptibility to Leishmania tropica in mice.* Parasite Immunol, 1980. **2**(4): p. 303-14.
454. Conboy, I.M., et al., *Novel genetic regulation of T helper 1 (Th1)/Th2 cytokine production and encephalitogenicity in inbred mouse strains.* J Exp Med, 1997. **185**(3): p. 439-51.
455. Tubo, N.J. and M.K. Jenkins, *TCR signal quantity and quality in CD4(+) T cell differentiation.* Trends Immunol, 2014. **35**(12): p. 591-596.
456. Keck, S., et al., *Antigen affinity and antigen dose exert distinct influences on CD4 T-cell differentiation.* Proc Natl Acad Sci U S A, 2014. **111**(41): p. 14852-7.
457. Brogdon, J.L., D. Leitenberg, and K. Bottomly, *The potency of TCR signaling differentially regulates NFATc/p activity and early IL-4 transcription in naive CD4+ T cells.* J Immunol, 2002. **168**(8): p. 3825-32.
458. Vella, A.T., et al., *Cytokine-induced survival of activated T cells in vitro and in vivo.* Proc Natl Acad Sci U S A, 1998. **95**(7): p. 3810-5.
459. Ebihara, M., M. Hattori, and T. Yoshida, *Distinctly different sensitivity in the induction and reversal of anergy of Th1 and Th2 cells.* Biosci Biotechnol Biochem, 2007. **71**(1): p. 130-7.
460. Morris, S.C., W.C. Gause, and F.D. Finkelman, *IL-4 suppression of in vivo T cell activation and antibody production.* J Immunol, 2000. **164**(4): p. 1734-40.
461. Pfeiffer, C., et al., *Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo.* Journal of Experimental Medicine, 1995. **181**(4): p. 1569-1574.
462. Allison, K.A., et al., *Affinity and dose of TCR engagement yield proportional enhancer and gene activity in CD4+ T cells.* Elife, 2016. **5**.
463. Gottschalk, R.A., E. Corse, and J.P. Allison, *TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo.* J Exp Med, 2010. **207**(8): p. 1701-11.
464. King, C.G., et al., *T cell affinity regulates asymmetric division, effector cell differentiation, and tissue pathology.* Immunity, 2012. **37**(4): p. 709-20.

465. Corse, E., R.A. Gottschalk, and J.P. Allison, *Strength of TCR-peptide/MHC interactions and in vivo T cell responses.* J Immunol, 2011. **186**(9): p. 5039-45.
466. Winstead, C.J. and C.T. Weaver, *Dwelling on T cell fate decisions.* Cell, 2013. **153**(4): p. 739-41.
467. Lane, P., *Role of OX40 signals in coordinating CD4 T cell selection, migration, and cytokine differentiation in T helper (Th) 1 and Th2 cells.* Journal of Experimental Medicine, 2000. **191**(2): p. 201-206.
468. McAdam, A.J., et al., *Mouse inducible costimulatory molecule (ICOS) expression is enhanced by CD28 costimulation and regulates differentiation of CD4+ T cells.* The Journal of Immunology, 2000. **165**(9): p. 5035-5040.
469. Simpson, T.R., S.A. Quezada, and J.P. Allison, *Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS).* Current opinion in immunology, 2010. **22**(3): p. 326-332.
470. Tai, X., et al., *CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2.* Nature immunology, 2005. **6**(2): p. 152.
471. Guo, F., et al., *CD28 controls differentiation of regulatory T cells from naive CD4 T cells.* The Journal of Immunology, 2008. **181**(4): p. 2285-2291.
472. Bouguermouh, S., et al., *CD28 co-stimulation down regulates Th17 development.* PloS one, 2009. **4**(3): p. e5087.
473. Watanabe, M., et al., *ICOS-mediated costimulation on Th2 differentiation is achieved by the enhancement of IL-4 receptor-mediated signaling.* The Journal of Immunology, 2005. **174**(4): p. 1989-1996.
474. Rulifson, I.C., et al., *CD28 costimulation promotes the production of Th2 cytokines.* The Journal of Immunology, 1997. **158**(2): p. 658-665.
475. Suh, W.K., *Life of T follicular helper cells.* Mol Cells, 2015. **38**(3): p. 195-201.
476. Fahey, L.M., et al., *Viral persistence redirects CD4 T cell differentiation toward T follicular helper cells.* J Exp Med, 2011. **208**(5): p. 987-99.
477. Nakayama, T. and M. Yamashita, *The TCR-mediated signaling pathways that control the direction of helper T cell differentiation.* Semin Immunol, 2010. **22**(5): p. 303-9.
478. Aasheim, H.C., J. Delabie, and E.F. Finne, *Ephrin-A1 binding to CD4+ T lymphocytes stimulates migration and induces tyrosine phosphorylation of PYK2.* Blood, 2005. **105**(7): p. 2869-76.
479. Weinstein, J.S., et al., *TFH cells progressively differentiate to regulate the germinal center response.* Nat Immunol, 2016. **17**(10): p. 1197-1205.
480. Coffman, R.L., et al., *The role of helper T cell products in mouse B cell differentiation and isotype regulation.* Immunological reviews, 1988. **102**(1): p. 5-28.
481. Sahoo, A., S. Wali, and R. Nurieva, *T helper 2 and T follicular helper cells: Regulation and function of interleukin-4.* Cytokine Growth Factor Rev, 2016. **30**: p. 29-37.
482. Baumjohann, D. and K.M. Ansel, *Tracking early T follicular helper cell differentiation in vivo.* Methods Mol Biol, 2015. **1291**: p. 27-38.
483. Chevalier, N., et al., *CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses.* J Immunol, 2011. **186**(10): p. 5556-68.
484. Pepper, M., et al., *Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells.* Immunity, 2011. **35**(4): p. 583-95.
485. !!! INVALID CITATION !!! {Choi, 2013 #371;MacLeod, 2011 #234}.

486. Kaka, A.S., et al., *Genetic modification of T cells with IL-21 enhances antigen presentation and generation of central memory tumor-specific cytotoxic T-lymphocytes.* J Immunother, 2009. **32**(7): p. 726-36.
487. Cui, W., et al., *An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells.* Immunity, 2011. **35**(5): p. 792-805.
488. Tangye, S.G., *Advances in IL-21 biology - enhancing our understanding of human disease.* Curr Opin Immunol, 2015. **34**: p. 107-15.
489. Novy, P., et al., *Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection.* The Journal of Immunology, 2011. **186**(5): p. 2729-2738.
490. Harrington, L.E., et al., *Memory CD4 T cells emerge from effector T-cell progenitors.* Nature, 2008. **452**(7185): p. 356-60.
491. Nish, S.A., et al., *CD4+ T cell effector commitment coupled to self-renewal by asymmetric cell divisions.* J Exp Med, 2017. **214**(1): p. 39-47.
492. Akiba, H., et al., *The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo.* The Journal of Immunology, 2005. **175**(4): p. 2340-2348.
493. Weinstein, J.S., et al., *B cells in T follicular helper cell development and function: separable roles in delivery of ICOS ligand and antigen.* J Immunol, 2014. **192**(7): p. 3166-79.
494. Moser, B., *CXCR5, the Defining Marker for Follicular B Helper T (TFH) Cells.* Front Immunol, 2015. **6**: p. 296.
495. Schaerli, P., et al., *CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function.* J Exp Med, 2000. **192**(11): p. 1553-62.
496. Haynes, N.M., et al., *Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation.* The Journal of Immunology, 2007. **179**(8): p. 5099-5108.
497. Ise, W., et al., *T follicular helper cell-germinal center B cell interaction strength regulates entry into plasma cell or recycling germinal center cell fate.* Immunity, 2018. **48**(4): p. 702-715. e4.
498. Choi, Y.S., et al., *ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6.* Immunity, 2011. **34**(6): p. 932-946.
499. Kaji, T., et al., *CD4 memory T cells develop and acquire functional competence by sequential cognate interactions and stepwise gene regulation.* Int Immunol, 2016. **28**(6): p. 267-82.
500. Choi, Y.S., et al., *Bcl6 expressing follicular helper CD4 T cells are fate committed early and have the capacity to form memory.* J Immunol, 2013. **190**(8): p. 4014-26.
501. Hale, J.S. and R. Ahmed, *Memory T follicular helper CD4 T cells.* Front Immunol, 2015. **6**: p. 16.
502. MacLeod, M.K., et al., *CD4 memory T cells: what are they and what can they do?* Semin Immunol, 2009. **21**(2): p. 53-61.
503. Tanel, A., et al., *Cellular and molecular mechanisms of memory T-cell survival.* Expert Rev Vaccines, 2009. **8**(3): p. 299-312.
504. Dalai, S.K., et al., *Resolution of infection promotes a state of dormancy and long survival of CD4 memory T cells.* Immunol Cell Biol, 2011. **89**(8): p. 870-81.
505. Haynes, L., et al., *Interleukin 2, but not other common γ chain–binding cytokines, can reverse the defect in generation of CD4 effector T cells from naive T cells of aged mice.* Journal of Experimental Medicine, 1999. **190**(7): p. 1013-1024.

506. Marrack, P., J. Kappler, and T. Mitchell, *Type I interferons keep activated T cells alive.* Journal of Experimental Medicine, 1999. **189**(3): p. 521-530.
507. Vella, A.T., et al., *Cytokine-induced survival of activated T cells in vitro and in vivo.* Proceedings of the National Academy of Sciences, 1998. **95**(7): p. 3810-3815.
508. Gossel, G., et al., *Memory CD4 T cell subsets are kinetically heterogeneous and replenished from naive T cells at high levels.* Elife, 2017. **6**.
509. Lo, Y.-C., M.A. Edidin, and J.D. Powell, *Selective activation of antigen-experienced T cells by anti-CD3 constrained on nanoparticles.* The Journal of Immunology, 2013. **191**(10): p. 5107-5114.
510. Mueller, D.L., et al., *Differential regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 receptor in cloned CD4+ helper T cells. A model for the long-term survival of memory cells.* The Journal of Immunology, 1996. **156**(5): p. 1764-1771.
511. Wojciechowski, S., et al., *Bim/Bcl-2 balance is critical for maintaining naive and memory T cell homeostasis.* Journal of Experimental Medicine, 2007. **204**(7): p. 1665-1675.
512. Oliaro, J., et al., *Asymmetric cell division of T cells upon antigen presentation uses multiple conserved mechanisms.* J Immunol, 2010. **185**(1): p. 367-75.
513. Chen, Y.H., et al., *Asymmetric PI3K Activity in Lymphocytes Organized by a PI3K-Mediated Polarity Pathway.* Cell Rep, 2018. **22**(4): p. 860-868.
514. Mosenden, R., et al., *Effects of type I protein kinase A modulation on the T cell distal pole complex.* Scand J Immunol, 2011. **74**(6): p. 568-73.
515. Chang, J.T., et al., *Asymmetric proteasome segregation as a mechanism for unequal partitioning of the transcription factor T-bet during T lymphocyte division.* Immunity, 2011. **34**(4): p. 492-504.
516. Lin, W.H., et al., *Asymmetric PI3K Signaling Driving Developmental and Regenerative Cell Fate Bifurcation.* Cell Rep, 2015. **13**(10): p. 2203-18.
517. Brander, C., et al., *Peptide immunization in humans: a combined CD8+/CD4+ T cell-targeted vaccine restimulates the memory CD4 T cell response but fails to induce cytotoxic T lymphocytes (CTL).* Clinical & Experimental Immunology, 1996. **105**(1): p. 18-25.
518. Van, V.Q., et al., *CD47(high) expression on CD4 effectors identifies functional long-lived memory T cell progenitors.* J Immunol, 2012. **188**(9): p. 4249-55.
519. Asrir, A., et al., *Interconnected subsets of memory follicular helper T cells have different effector functions.* Nat Commun, 2017. **8**(1): p. 847.
520. Prendergast, C.T., et al., *CD4+ T cell hyporesponsiveness after repeated exposure to Schistosoma mansoni larvae is dependent upon interleukin-10.* Infection and immunity, 2015. **83**(4): p. 1418-1430.
521. Fazilleau, N., et al., *The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding.* Nat Immunol, 2009. **10**(4): p. 375-84.
522. Metz, P.J., et al., *Regulation of Asymmetric Division by Atypical Protein Kinase C Influences Early Specification of CD8(+) T Lymphocyte Fates.* Sci Rep, 2016. **6**: p. 19182.
523. Xiang, J., H. Huang, and Y. Liu, *A new dynamic model of CD8+ T effector cell responses via CD4+ T helper-antigen-presenting cells.* The Journal of Immunology, 2005. **174**(12): p. 7497-7505.
524. Riteau, B., *Exosomes bearing hla-g are released by melanoma cells.* Human Immunology, 2003.
525. Zitvogel, L., et al., *Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes.* Nature medicine, 1998. **4**(5): p. 594.

526. Raposo, G., et al., *B lymphocytes secrete antigen-presenting vesicles.* Journal of Experimental Medicine, 1996. **183**(3): p. 1161-1172.
527. Geuze, H.J., *The role of endosomes and lysosomes in MHC class II functioning.* Immunology today, 1998. **19**(6): p. 282-287.
528. Satta, N., et al., *Monocyte vesiculation is a possible mechanism for dissemination of membrane-associated procoagulant activities and adhesion molecules after stimulation by lipopolysaccharide.* The Journal of Immunology, 1994. **153**(7): p. 3245-3255.
529. Admyre, C., et al., *Direct exosome stimulation of peripheral humanT cells detected by ELISPOT.* European journal of immunology, 2006. **36**(7): p. 1772-1781.
530. Thery, C., et al., *Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes.* Nat Immunol, 2002. **3**(12): p. 1156-62.
531. Kovacs, B., et al., *Ligation of CD28 by its natural ligand CD86 in the absence of TCR stimulation induces lipid raft polarization in human CD4 T cells.* The Journal of Immunology, 2005. **175**(12): p. 7848-7854.
532. Pandiyan, P., et al., *CD152 (CTLA-4) determines the unequal resistance of Th1 and Th2 cells against activation-induced cell death by a mechanism requiring PI3 kinase function.* Journal of Experimental Medicine, 2004. **199**(6): p. 831-842.
533. Akkoc, T., et al., *Increased activation-induced cell death of high IFN-γ–producing TH1 cells as a mechanism of TH2 predominance in atopic diseases.* Journal of Allergy and Clinical Immunology, 2008. **121**(3): p. 652-658. e1.
534. Gertner, J., et al., *Human gammadelta T lymphocytes strip and kill tumor cells simultaneously.* Immunol Lett, 2007. **110**(1): p. 42-53.
535. Machlenkin, A., et al., *Capture of tumor cell membranes by trogocytosis facilitates detection and isolation of tumor-specific functional CTLs.* Cancer Res, 2008. **68**(6): p. 2006-13.
536. Meli, A.P., et al., *T Follicular Helper Cell-Derived IL-4 Is Required for IgE Production during Intestinal Helminth Infection.* J Immunol, 2017. **199**(1): p. 244-252.
537. Corsiero, E., et al., *Ectopic lymphoid structures: powerhouse of autoimmunity.* Frontiers in immunology, 2016. **7**: p. 430.
538. Hutloff, A., *T follicular helper-like cells in inflamed non-lymphoid tissues.* Frontiers in immunology, 2018. **9**.
539. MacLennan, I.C., et al., *Extrafollicular antibody responses.* Immunological reviews, 2003. **194**(1): p. 8-18.
540. Khattar, M., et al., *Interleukin-21 is a critical regulator of CD4 and CD8 T cell survival during priming under Interleukin-2 deprivation conditions.* PLoS One, 2014. **9**(1): p. e85882.
541. Peluso, I., et al., *IL-21 counteracts the regulatory T cell-mediated suppression of human CD4+ T lymphocytes.* J Immunol, 2007. **178**(2): p. 732-9.
542. Gullicksrud, J.A., et al., *Differential Requirements for Tcf1 Long Isoforms in CD8(+) and CD4(+) T Cell Responses to Acute Viral Infection.* J Immunol, 2017. **199**(3): p. 911-919.
543. Miron, M., et al., *Human Lymph Nodes Maintain TCF-1(hi) Memory T Cells with High Functional Potential and Clonal Diversity throughout Life.* J Immunol, 2018. **201**(7): p. 2132-2140.
544. Danilo, M., et al., *Suppression of Tcf1 by inflammatory cytokines facilitates effector CD8 T cell differentiation.* Cell reports, 2018. **22**(8): p. 2107-2117.
545. Fernandez-Cabezudo, M.J., et al., *Evidence for a dual pathway of activation in CD43-stimulated Th2 cells: differential requirement for the Lck tyrosine kinase.* Int Immunol, 2004. **16**(8)

The end