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Memory T cells and the endothelium in allograft rejection

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Memory T Cells and the Endothelium in Allograft Rejection

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Abstract

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Organ transplantation has become increasingly important as a treatment for many human diseases. Despite the dramatic improvements in immunosuppression in recent years, acute and chronic rejection remain significant problems. It has become increasingly evident that the presence of T cell memory correlates with both acute and chronic rejection episodes. Endothelial cells (EC) have been shown to preferentially activate memory T cells and, as the lining of every transplanted organ, they are in a unique position to provide signals to alloreactive memory T cells. EC activation of memory T cells depends in part on the costimulatory molecule LFA-3 in addition to other signals. The nature of these other signals is not well understood. In this dissertation I further explore the mechanisms by which EC activate memory T cells and investigate the role that these mechanisms play in a model of memory T cell mediated allograft rejection.

Several newly described costimulatory pathways, ICOS-ICOSL, 4-1BB-41BBL, and OX40-OX40L, have recently been recognized as important players in the generation and function of memory T cells. EC can express all three of the ligands from these pathways and also increase expression in response to the cytokine tumor necrosis factor (TNF) and in co-cultures with T cells. Furthermore, blockade of these pathways using

monoclonal antibodies in co-cultures causes reductions in T cell production of IFN-γ and IL-2 as well as decreased T cell proliferation.

To examine the contribution of memory T cells to allograft rejection *in vivo*, I describe a chimeric model of human skin grafted onto SCID mice that are adoptively transferred with various subsets of human T cells. In this model, rejection of the human skin by the transferred T cells is mediated by memory T cells alone. Using this model, the effect of different costimulatory pathways on allograft rejection is tested. Interestingly, blockade of any of the three costimulatory pathways can diminish the T cell response to the allograft. The role of the B7-CD28 pathway is also examined and found to partially contribute to the memory T cell response.

Finally, using observations that memory T cells can be further divided into two functionally distinct subsets known as central and effector memory T cells, the response of these two subsets to EC is explored. Central memory T cells respond to EC by producing IL-2 while effector memory T cells generate IFN-γ. The basis for this difference may be due to the differential expression and sensitivity of the two subsets to costimulation by CD27 and CD28. Further, in the chimeric human-SCID model effector memory T cells alone can mediate allograft rejection while central memory T cells cannot.

Our results strongly support the idea that the EC capacity to activate memory T cells and its subsets depends in part on the CD28-B7 pathway and memory T cell specific costimulatory molecules. Furthermore, targeting CD28 or these molecules *in vivo* can attenuate allograft rejection mediated by memory T cells.

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List of Abbreviations

Amo	Adherent Monocyte
APC	Antigen Presenting Cell
BLC	B Lymphoblastoid Cell
BSA	Bovine Serum Albumin
CMV	Cytomegalovirus
EBV	Epstein Barr Virus
EC	Endothelial Cell
ECGS	Endothelial Cell Growth Supplement
FBS	Fetal Bovine Serum
HDMEC	Human Dermal Microvascular EC
HLA	Human Leukocyte Antigen
HUVEC	Human Umbilical Vein EC
i.p.	intraperitoneal
ICAM	Intercellular Adhesion Molecule
IDO	Indolemine-2,3-Dioxygenase
IFN	Interferon
IL	Interleukin
LCMV	Lymphocytic Choriomeningitis Virus
LFA-3	Lymphocyte Function-Associated Antigen 3
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
Mo	Monocyte
NK	Natural Killer
PBMC	Peripheral Blood Mononucler Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase-PCR
s.c.	Subcutaneous
SCID	Severe Combined Immunodeficiency
Tcm	Central Memory T Cells
Tem	Effector Memory T Cells
TNF	Tumor Necrosis Factor

VCAM	Vascular Cell Adhesion Molecule
VSMC	Vascular Smooth Muscle Cell

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CHAPTER I - INTRODUCTION

More than 25,000 organ transplants are performed annually with over 93,000 patients waiting to receive a transplant in the United States alone (1) and what was once an experimental exercise has now become the treatment of choice for many diseases. The technical aspects of organ transplantation were resolved by 1964 when Alexis Carrel won the Nobel prize in 1964 for the description of anastomotic technique and experimental organ transplantation (2). However, the understanding of the biological barriers to transplantation lagged far behind the surgical technique.

Peter Medawar was the first to propose the idea that the immune system could reject a transplanted organ (3, 4). The basis for this idea came from Medawar's work using skin grafts on burns. He discovered that skin grafted from a given donor lasted about 10 days, but a second graft was rejected immediately. Medawar suggested that the initial and accelerated rejection were an immunological process whereby the immune system "remembered" what the graft looked like and promptly rejected it. Medawar's experiments coincided with Frances Macfarlane Burnet's work on a second critical observation. Burnet found that chick embryos and fetal animals did not produce antibodies and that immunocompetence developed slowly over time (5). This finding led to the concept that during embryonic development there is a period of "self-recognition" where tolerance to a given antigen is developed by fetal exposure to "non-self antigens." In other words, the immune system had to be clonally selected and taught about self and non-self (2). Combining Burnet's observation about self versus non-self and his own work with skin grafts, Medawar with his postdoctoral fellows Rupert Billingham and Leslie Brent demonstrated that neonatally acquired transplant tolerance could be achieved

in mice by inoculating embryos or newborn mice with allogeneic cells (6). These critical experiments led to the initial understanding that the immune system was responsible for rejection due to its ability to identify which cells were self and which were not. The molecular basis of self recognition came only a few years later with the description of the first leukocyte antigen called MAC in 1958 by Jean Dausset, which became known as the major histocompatability complex (MHC) (7). These critical experiments formed the foundation for the biological understanding of transplant rejection, however, it would take several decades before the discovery of a fungal metabolite known as cyclosporin made transplantation a clinical reality (8, 9).

Despite the introduction of new protocols and immunosuppressive reagents over the past twenty years, incidences of rejection at 1 year remain at 20% for kidneys, 30% for livers and nearly 40% for hearts (10). Furthermore, current immunosuppressants also fail to control long-term immune responses to the organ which can lead to more chronic forms of rejection such as coronary allograft vasculopathy in heart transplants and chronic allograft nephropathy in renal transplants. The current drugs also have toxic side effects that can damage the transplanted organ (such calcineurin inhibitory mediated nephrotoxicity) as well as contribute to the incidence of malignancy, infection, diabetes, hypercholesterolemia and cardiovascular disease (11). Therefore, finding better and more specific means of immunosuppression continues to be a major goal of transplantation.

In the search for better immunosuppression, it is important to understand why current immunosuppressive regimens fail. One hypothesis is that there are portions of the immune system that are resistant to immunosuppression and there is increasing

recognition that one reason for the failure of immunosuppression in both the acute and chronic settings may be the presence of immunological memory. In the setting of transplantation it has long been recognized that the presence of preformed alloreactive antibodies may result in hyperacute allograft rejection (12). Experimental models as early as the 1970s also recognized the potential for alloreactive memory T cells to elicit allograft rejection (13, 14). Since then, the role of T cell immunologic memory was largely forgotten because of the initial successes with immunosuppression. However, with advances in our understanding of the immune system it has now become evident that memory T cells play a critical role in the immune response to allograft and understanding their biology has become a major focus of transplantation research.

1.1 T CELL MEMORY

One of the defining features of the immune system is the concept of immunologic memory. Immunological memory can be defined as the faster and stronger response of the immune system in a secondary response to an Ag compared to a primary response to the same Ag (15, 16). Immunologic memory is also thought to last for the lifetime of an organism. The faster and stronger secondary response of the immune system results from quantitative as well as qualitative changes in the cell populations that respond to a given Ag. Among these changes are increases in the frequency of responding cells, changes in the activation requirements and alterations of their migratory patterns. For example, in humoral responses, there is a greater frequency of antigen reactive cells specific for an antigen, as well as antibody products that are clearly different from the initial response (17). For T cells, there is a similar increase in frequency of antigen reactive cells and a

more rapid acquisition of effector function in a recall response. In order to more closely examine the properties of memory T cells it is important to understand the primary response as a comparison.

The primary response

Naïve T cells may be defined as T cells that have not encountered antigen and the primary response describes their response to the first exposure. Before contacting antigen, naïve T cells circulate between secondary lymphoid organs such as the spleen and lymph nodes by traveling in the blood or lymph (18, 19). The continuous migration of naïve T cells through the secondary lymphoid tissues is important for T cells to efficiently encounter antigens. To encounter antigens, naïve T cells must make contact with specialized antigen presenting cells (APCs) such as dendritic cells (DC) that present antigens in the form of peptide fragments bound to major histocompatibility complex (MHC) molecules (20). In animals that have not encountered antigen, the MHC complex is bound to self peptides which are largely ignored by the naïve T cells. Naïve T cells live for extended periods of time in a metabolically quiescent state, and experiments in mice have demonstrated that their survival in this state depends on continually receiving low-levels signals from self-peptide/MHC (21-24) and IL-7 (25, 26).

Without the presence of foreign antigen, naïve T cells circulate through the secondary lymphoid organs contacting self-peptide/MHC indefinitely. However, once naïve T cells recognize an APC bearing a foreign peptide/MHC complex, the naïve T cells undergo a series of changes in migration and function in response to the foreign antigen. T cells recognize antigens through highly-specific interactions between their T

cell receptor and peptide/MHC complexes. Upon recognition of a foreign peptide, naïve T cells stop circulating and increase contact between the T cell and the APC in part through the formation of structures known as synapses (27-30).

A synapse refers to the grouping of molecules formed at the contact site between the T cell and the APC and reflects the organization of the signaling molecules used in T cell activation (31, 32). The synapse is composed of a cluster of TCR molecules and accumulation of signaling molecules such as LCK, LAT, and PKCθ and costimulatory/adhesion molecules including CD28, CD2 and LFA-1. The costimulatory and adhesion molecules are thought to provide key second signals as well as enhance TCR triggering by stabilizing the synapse or recruiting other intracellular signaling molecules (33). Once the T cell has been triggered, the T cells undergo substantial proliferation and differentiation into effector cells that generally lead to the elimination of the source of the foreign antigen. Once the source of the antigen is eliminated, the effector cells are rapidly eliminated and memory T cells are formed (34).

When the organism re-encounters an antigen, the response of memory T cells differs from that of naïve T cells in several significant ways. The first major difference is in the size and speed of the response. The secondary response is generally both larger and more rapid than the primary response. The second major difference is the conversion to effector cells. Naïve T cells when they are activated produce IL-2 initially and require several rounds of proliferation before they start to express effector molecules. In response to antigen, memory T cells become effector T cells much more quickly. Identifying the mechanisms that are responsible for these differences in the secondary response is the basis for studies of T cell memory.

Surface Markers of Naïve, Effector and Memory T cells

Many of the differences in activation potential and homing between memory and naïve T cells are reflected in the surface molecules used to distinguish them. These molecules include those involved in trafficking and adhesion (CD44, CD11a, CD62L); TCR signaling (CD45) and cytokine and chemokine responses (CD25 (IL-2Rα), CD122 (IL-2/15Rβ), CCR7) (see Table I-1).

In humans, naïve T cells are CD45RA positive and CD45RO negative whereas memory T cells are the converse, CD45RO positive and CD45RA negative (35, 36). The antibody used to identify CD45RO (UCHL1) identifies an epitope that is present in the absence of all the other CD45R isoforms (A, B, and C). In the mouse, there is no equivalent of CD45RO that has been studied and so some investigators use CD45RB, however CD45RB has been shown to change with activation or cytokine treatment (37, 38). Therefore, in the mouse, unlike in humans, memory T cells are most commonly defined using the adhesion molecule CD44 with naïve T cells being CD44 low and memory T cells being CD44 high. The only concern is that this pattern is only true for the C57BL/6 strain and is less clear for other strains (39). The rat does not upregulate CD44 on its memory T cells and therefore loss of CD45RC (OX-22) has been used (37). In other large mammals such as sheep, adhesion molecules such as CD62L and CD11a have also been used to identify memory T cells (40).

			Memory	
	Naïve	Effector	Effector	Central
CD11a	Lo	Hi	Hi	Hi
CD25	Lo	Hi	Lo	Lo
CD44	Lo	Hi	Hi	Hi
CD62L	Hi	Lo	Lo	Hi
CD69	Lo	Hi	Lo	Lo
IL-2/15R	Lo	Hi	Hi	Hi
CCR7	Hi	Hi	Lo	Lo
CD45RA	Hi	Lo	Lo	Lo
CD45RO	Lo	Hi	Hi	Hi
CD45RB	Hi	Lo	Lo	Lo

Table I-1 *Surface markers of human naïve, effector and memory T cells.*

Relative expression of several commonly used markers for distinguishing naïve from effector and both central and effector subsets of memory T cells.

As evidenced by the diversity of different markers used to identify memory cells, there are a number of complications to isolating a true memory T cell population. One problem with identifying memory T cell populations is that effector cells often share some of the same surface molecules as memory T cells most particularly elevated CD44. However, it is possible to use other markers of activation such as CD25 and CD69 to distinguish effector cells from bona fide memory T cells (41, 42). Another problem is that the memory phenotype can be recapitulated in certain situations without having seen foreign antigen including lymphopenia-induced proliferation and extrathymic development. In both of these situations, naïve T cells acquire the phenotype of memory T cells and in some cases even memory T cell functions like rapid IFN-γ production (43-45). However, all these studies have been carried out in mice and it is unclear what role these two factors play in determining the memory T cell pool in humans.

In addition to distinguishing between a naïve and memory T cell pool, it has become increasingly clear that memory T cells are heterogeneous and can be further divided into different subsets based on the expression of the adhesion molecule CD62L (L-selectin) and the chemokine receptor CCR7 (46). These two markers define two subsets that are distinct in their homing potential and functional capacity. Central memory T cells (T_{CM}), CD62L+CCR7+, are thought to circulate through the secondary lymphoid organs like naïve T cells and have higher proliferative potential but lower immediate effector function. In contrast, effector memory T cells (T_{EM}), CD62L-CCR7-, circulate to the periphery and can rapidly secrete cytokines and other effector molecules.

Thus, despite some confusion about which markers to employ in distinguishing the different subsets especially between species, it is possible to differentiate between

naïve, effector and memory T cells as well as different subsets within memory T cells using surface markers. Studies carried out on these distinct populations have indicated that as suggested by their different surface phenotypes, that the three groups possess significantly different functional capacities.

Functions of Naïve vs. Memory T cells

Following clearance of a given antigen in a primary response, there is a massive culling of effector cells. Despite this massive extinction of responding T cells, the precursor frequency of Ag-specific memory T cells is significantly greater compared to naïve T cells (47, 48). This increased frequency in part explains the increased speed and size of secondary responses. This can be seen in studies done in mice, where treatments that decrease the numbers of Ag specific memory CD8+ T cells showed diminished secondary responses to those Ag (49, 50).

In addition to greater numbers, memory T cells also possess different homing potentials. As discussed above, memory T cells can be subdivided into two separate subsets based on expression of CD62L and CCR7 (46). T_{CM} have a circulation pattern that closely resembles naïve T cells in terms of their distribution in the lymphoid tissues. Expression of CD62L and CCR7 allows both T_{CM} and naïve T cells to enter lymph nodes via high endothelial venules (HEV). Like naïve T cells, T_{CM} appear to require stimulation in secondary lymphoid organs in order to acquire effector function (46, 51, 52). By contrast, T_{EM}, like effector cells, are CD62L and CCR7 low and consequently migrate through non-lymphoid tissues. They also display constitutive CTL function and cytokine synthesis *in vivo* (34, 51, 53, 54). The positioning of T_{EM} allows them to

provide immediate immunity in a secondary response. This homing potential may in part account for the faster secondary responses compared to naïve T cells.

In addition to quantitative differences in precursor frequency and homing, memory T cells are also qualitatively different from naïve T cells. In general, memory T cells have a lower activation threshold than naïve T cells. They possess the ability to respond to low affinity/avidity interactions thus allowing them to respond to a much lower amount of antigen compared to naïve T cells (23, 55-57). Part of this lower threshold is related to the fact that memory T cells also have a reduced reliance on APC provided costimulation (58, 59). In fact, memory T cells have been shown to be less dependent on the B7/CD28 pathway and independent of the CD40/CD154 pathway (60). Memory T cells can also become activated in the absence of secondary lymphoid organs (61). Furthermore, T_{CM}, despite their similar migratory potential, are clearly less quiescent than their naïve counterparts. For instance, CD8+ T_{CM} express high levels of mRNA suggesting that they are in the G1 phase of cell cycle (62) and show a much shorter lag time for entering the cell cycle (56, 63-65). Additionally, both T_{CM} and T_{EM} also have much faster kinetics for synthesizing cytokines, differentiating into CTL and migrating to non-lymphoid tissues (56, 62, 63, 65-68).

In sum, memory T cells can detect lower concentrations of their cognate antigen, expand far more rapidly, produce a greater amount and broader array of cytokine on a per-cell basis, and survive longer than either naïve or effector cells. It is these unique properties of memory T cells that not only serve as the basis for both the beneficial effects as in the case of vaccines but also the harmful effects as in autoimmunity and transplant rejection.

1.2 T CELL COSTIMULATION

In order for T cells to see antigen, there is significant evidence supporting the concept of the two-signal hypothesis of T cells activation (69, 70). The two-signal hypothesis results from observations that peptide/MHC complexes alone are in general insufficient to activate T cells (71). Therefore, in order for T cells to be activated, another signal is required to amplify the weak signal coming from the T cell receptor (TCR). This second signal, known as the co-stimulatory signal, is a critical feature of competent APCs that provide this signal in addition to the antigen specific signal (signal one) to initiate an immune response. Many stromal and epithelial cells lack the expression of these co-stimulatory molecules and thus even in the situation where they express functional peptide/MHC complexes they are unable to activate T cells. Costimulatory molecules can be divided along structural lines into two families, the Ig superfamily and the tumor necrosis factor (TNF) family. Included in the Ig superfamily are the well-known CD28/CTLA-4/B7 family and CD2/LFA-3. The TNF family includes CD40/CD40L (CD154), CD27/CD70, CD134 (OX40)/OX40L, and CD137 (4-1BB)/4-1BBL (See Table II-1).

The Ig superfamily of costimulatory molecules

One of the first costimulatory pathways to be identified in mice and thus perhaps the best characterized is the CD28/B7 family (72-77). The B7-1/B7-2:CD28/CTLA-4 pathway consists of two B7 family members B7-1 (CD80) and B7-2 (CD86) that both bind the same two receptors, CD28 and CTLA-4 (78). Both receptors have distinct kinetics and binding affinities for B7-1 and B7-2 (79). CD28 is constitutively expressed

on the surface of T cells while CTLA-4 is upregulated with activation. CTLA-4 also has a much higher affinity for both B7-1 and B7-2. This difference in kinetics and affinity is also reflected in the function of these two receptors in T cell activation. Engagement of CD28 in conjunction with TCR engagement allows T cells to make IL-2 and survive. The higher affinity CTLA-4 gets expressed later and both competes with CD28 and delivers a negative signal to down regulate the T cell response. Professional APCs like dendritic cells and B cells express low levels of B7-2 constitutively, but rapidly upregulate both B7-2 and B7-1 on their surface when they become activated (i.e. through TLR signals or cytokines). The interaction between B7s and CD28 are critical for the activation of naïve T cells (80).

There have also been several recently described new members of the B7 family including ICOS-ICOSL and PD-1/PD-L1/2 (81). ICOS is upregulated on both CD4+ and CD8+ T cells following activation and is present on both effector and memory T cells (82-84). ICOSL is expressed on the surface of B cells, macrophages, dendritic cells, and endothelial cells (85, 86). ICOS-ICOSL has been shown to play an important role in T cell activation, differentiation, and effector responses. The key differences between the ICOS-ICOSL pathway and the CD28-B7 pathway are that ICOS signaling can only be delivered after T cells have been activated and that signaling does not upregulate IL-2 production (87). Therefore, it seems that ICOS plays a role in propagating effector function, but does not have a role in the primary response.

PD-1 is induced on peripheral CD4+ and CD8+ T cells, B cells and monocytes upon activation (88). The ligands for PD-1, PD-L1 and PD-L2, are widely expressed in both lymphoid and non-lymphoid tissues including B, T, myeloid and DC, but also EC

and other epithelial cells (89, 90). The PD-1/PD-L1/PD-L2 pathway has a critical role in regulating T cell activation and tolerance. PD-1 appears to regulate inflammatory responses in peripheral tissues and help maintain tolerance particularly in CD8+ T cell responses (91, 92). In transplantation, PD-1 stimulation by PD-L1Ig in combination with other blockade significantly inhibited naïve T cell responses in cardiac and islet allografts (93, 94).

The CD2/LFA-3 (CD58) interaction was the first costimulatory pathway to be identified in T cell activation and remains an important costimulatory pathway (95-97). Originally known as LFA-2, CD2 is expressed on the majority of T cells while LFA-3 can be found on dendritic cells, macrophages, B cells, fibroblasts and ECs (98-101). The CD2-LFA-3 interaction has been shown to be an important pathway independent of CD28 to activate T cells and part of its costimulatory function may be mediated by its adhesive properties (102, 103). No rodent homologue of LFA-3 has been identified, however the related molecule CD48 (blast-1, BCM1, OX45) has been characterized as a CD2 ligand in both mice (104) and rats (105). Humans also express CD48, but it has such a low affinity binding to CD2 that it is not clear that the CD2-CD48 interaction is physiologically relevant in humans (106). CD2-CD48 interactions in mice, like their human counterpart, appear to be important in T cell activation as demonstrated in a mouse allograft model (107, 108), but as the ligand is not equivalent it is not clear that murine results can be accurately compared to humans. However, studies have been done using a chimeric human SCID mouse model and these demonstrated that inhibition of the CD2/LFA-3 pathway with blocking antibodies against LFA-3 or an LFA-3Ig fusion protein can significantly diminish allograft rejection (109).

The TNFR family of costimulatory molecules

The TNFR family members can be divided into three groups, death domain (DD)-containing receptors, decoy receptors and TNF receptor associated factor (TRAF) binding receptors (110, 111). TNFR1 and Fas are examples of DD-containing receptors. They activate caspase cascades leading to apoptosis. TRAF binding receptors do not contain DDs, but possess motifs of 4-6 amino acids that recruit TRAF proteins (112). All costimulatory members of the TNFR family are TRAF binding receptors and in particular they all bind TRAF2 in conjunction with various other TRAF proteins (112) leading to NF-κB (113-115) translocation downstream of all the receptors. CD27 and 4-1BB have also been shown to lead to p38 MAPK (116) and JNK activation (117, 118). The result of this signaling is to enhance survival signaling, cytokine production and cellular proliferation of T cells.

The most well-known TNFR receptor pair in T cell responses is CD40/CD40L (CD154). CD40 is expressed on B cells (119), DC, monocytes, macrophages, EC (120) and VSMC (121) and is inducible by cytokines such as TNF and interferons (122-124). Its ligand CD154 is expressed on activated CD4+ and CD8+ T cells, NK cells, monocytes, B cells, activated DC, EC and VSMC (119, 121, 124). CD40-CD154 interactions play a critical role in humoral immunity, activation of APC costimulatory function as well as activation of macrophages and the endothelium (124, 125). The broad reciprocal expression of the receptor and ligand pairs suggests that this pair may be used for bi-directional signaling, for example APC activate T cells through CD40L (126) while the T cells simultaneously modify the activity of the APC through CD40 (127).

Thus, the CD40-CD40L pathway may play a more complex role in the immune response beyond its pure costimulatory function.

CD27 is expressed on NK cells, B cells, naïve and T_{CM} CD4+ and CD8+ T cells. CD27 expression increases transiently with activation and gradually decreases with successive rounds of division (128, 129). Its loss is thought to correlate with acquisition of effector function (130, 131). In addition to correlating with effector function, engagement of CD27/CD70 costimulatory pathway has been shown to enhance $CD8^+$ effector/memory T cell functions (132, 133). Its ligand, CD70, is found on activated T cells, B cells and DC, but not resting EC (134, 135). In mice, CD70 can be induced on B cells and DC by anti-CD40 or LPS and on DC with GM-CSF (136) and it is unknown whether it is similarly inducible in humans.

OX40 (CD134), originally identified in the rat (137), has its expression restricted to activated T cells in both mice and humans (138). In a number of conditions OX40 is preferentially expressed on activated CD4+ T cells rather than CD8+ T cells suggesting that there may be a larger role for OX40 in CD4 mediated processes (139). Engagement of CD28 enhances the kinetics of OX40 expression and expression of OX40 can be prolonged in a number of inflammatory conditions (140-143). OX40 does not have the ability to replace CD28 or affect the initial rate of cell division in naïve T cells, but does allow for recovery of greater numbers of T cells later in the response likely due to effects of OX40 signaling on induction of key survival molecules like Bcl-2 and Bcl-X_L (140, 144, 145). In part through the induction of these survival molecules OX40 signaling plays a critical role in the generation and function of memory T cells, particularly CD4+ memory T cells (146, 147). OX40L is expressed on activated B cells (148) and DC (149,

150), plasmacytoid DC (151), and EC (152, 153). It can be induced by CD40L-activation on DC (154).

4-1BB was originally characterized as a molecule expressed on activated T cells (155), though it has since been found on a number of other cell types including DC (156), Mo (157), follicular DC (158), and NK cells (159). Current evidence indicates that both CD4+ and CD8+ T cells can express 4-1BB, but in several circumstances CD8+ T cells can upregulate 4-1BB more rapidly and to higher levels than CD4+ T cells (139, 160). 4-1BB provides signals in a CD28 independent manner that lead to enhanced cell division and improved survival (161, 162). Studies in mice and humans have shown that 4-1BB engagement prevents activation-induced cell death in T cells and agonistic antibodies to 4-1BB both *in vitro* and *in vivo* show prolonged T cell survival post-activation with greater effects on CD8+ T cells (163-167). Signaling through the 4-1BB pathway is also critical for the survival and function of memory CD8+ T cells (147). The ligand for 4-1BB, 4-1BBL, is expressed on activated macrophages, DC and B cells (168, 169). 4-1BBL expression can be upregulated on both B cells and DC via CD40 stimulation (170, 171). There are also reports that 4-1BBL engagement on human monocytes with 4-1BB fusion proteins induces reverse signaling implying that like CD40L there may be multiple bi-directional effects of receptor-ligand engagement (172, 173).

Structural Family	Costimlatory Molecule	Expression of Costimulatory Molecule	Ligand	Ligand Expression
Ig Superfamily	CD28	$T_{4, 8, N, E, M}$	B7-1 (CD80), B7-2 (CD86)	B, Mo, DC, T_E
	CTLA-4	$T_{4, 8, E, M}$	B7-1 (CD80), B7-2 (CD86)	B, Mo, DC, T_E
	ICOS	$T_{4, 8, E, M}$, NK	ICOSL	B, Mo, DC, T, EC
	PD-1	$T_{4, 8}$, B, Mo	PD-L1, PD-L2	B, Mo, DC, T, EC
	CD2	T	LFA-3 (CD58)	B, Mo, DC, T, EC
TNF Superfamily	CD154 (CD40L)	$T_{4, 8}$, NK, Mo, B, Eo, EC, V, DC	CD40	B, DC, Mo, EC, V
	OX40 (CD134)	$T_{4, 8, E, M}$	OX40L	B, DC, T, EC
	4-1BB (CD137)	$T_{4, 8, E, M}$, B, Mo, NK, DC, Eo	4-1BBL	B, Mo, DC
	CD27	$T_{4, 8, N, CM}$, NK, B	CD70	B, DC, T_E
	GITR	T, Treg	GITRL	B, Mo, DC, EC

Table II-1. *Costimulatory molecules and their expression.*

The costimulatory molecules discussed in the introduction are listed here along with their known expression patterns. T = T cell, 4 = CD4+ T cells, 8 = CD8+ T cells, N = Naïve T cells, E = Effector T cells, M = Memory T cells, CM = Central Memory T cells, Treg = Regulatory T cell, NK = Natural killer cell, B = B cell, Mo = monocyte/macrophage, Eo = Eosinophil, DC = Dendritic cell, V = Vascular Smooth Muscle Cell

1.3 ENDOTHELIAL CELLS (EC) AS ANTIGEN PRESENTING CELLS

Endothelial cells (EC), the cells that line the entire vascular tree, are specialized cells that have an important role in regulating the communication of oxygen, nutrients, different proteins and cells between the different organs of the body. With regards to the immune system, they play a pivotal role in the recruitment of immune cells to sites of inflammation. As such, they are uniquely positioned to serve as sentinels of the peripheral tissue conveying to immune cells the status of the organs beyond them. It is this proximity to immune cells such as T cells and the need to for tissues to communicate to T cells within blood vessels that led to the hypothesis that EC serve as intermediaries in this process and that an important part of this role is the ability of EC to activate T cells.

EC can express MHC molecules in vivo and in vitro

Antigen presenting cells (APC) are specialized cells that express surface molecules that synergize with specific antigens and present them to T cells. Among these specialized molecules are the major histocompatability complex (MHC) molecules whose only known function is to present peptides to T cells. Professional antigen presenting cells include dendritic cells, macrophages and B cells and have the capacity to activate naïve T cells.

In humans and other large mammals (not including mice or other rodents), all endothelial cells strongly express MHC class I. MHC Class II molecules are also constitutively expressed, but only on some ECs including postcapillary venules, veins, some arteries and most of the microvasculature (174-177). Expression of the MHC molecules *in vivo* may depend on basal levels of circulating IFN-γ. This idea is

supported by the fact that artery segments or skin transplanted into immunodeficient mice show decreased MHC class I and loss of expression of MHC class II both of which can be restored by the addition of exogenous IFN-γ (178, 179). *In vitro*, a similar phenomenon is observed, whereby under standard culture conditions, human EC express MHC class I, but not MHC class II. MHC class I can be augmented by tumor necrosis factor (TNF) and Type I interferons, while IFN-γ can induce class II (180-184). Consistent with the expression of MHC, EC can also express key proteins involved in the assembly of the MHC and peptide processing such as invariant chain and TAP1/2 (181, 185). Experiments using cultured human EC to activate peptide-specific T cell clones further demonstrates that EC can process and present functional peptide-MHC complexes (186), though recent data has suggested that EC may present a distinct, less immunogenic repertoire in their peptide-MHC complexes compared with other types of APCs or epithelial cells (187).

EC express costimulatory molecules

EC can also provide costimulation via cell-surface signaling molecules, adhesion molecule and cytokines in addition to the TCR signal they provide via peptide-MHC complexes. EC have been shown to express several members of both the Ig superfamily and TNFR family on their cell surface.

In general, human EC do not express B7 molecules (188, 189) which in part explains their inability to activate naïve T cells. However, there is evidence that EC may induce B7 expression on T cells and provide B7 signaling via a *trans* costimulatory mechanism (190). There is also some suggestion that EC from different locations such as

cardiac microvascular EC and brain EC may be capable of expressing B7 molecules (191, 192). ICOSL is constitutively expressed on the surface of HUVEC and has been shown to be induced by TNF and IL-1 on HUVEC in an NF-κB dependent manner (86, 193). HUVEC expressing ICOSL *in vitro* can activate memory CD4+ and CD8+ T cells (86, 193). In addition to the costimulatory molecules B7 and ICOSL, EC have also been demonstrated to express the inhibitory B7 molecule PD-L1 (89, 194) and to upregulate expression of PD-L1 with treatment from any of the interferons (89, 195). EC expression of PD-L1 has a role in inhibiting CD4+ T cell cytokine synthesis and CD8+ T cell cytolytic function (89, 90, 194). EC also constitutively express LFA-3 (100). There is much evidence that CD2-LFA-3 interactions between EC and T cells is a critical component of the costimulatory signals delivered by EC to T cells (188, 196, 197).

EC also have been described to express two of the TNFR family members including CD40-CD154 (124) and OX40L (152). EC have been shown to express CD40 constitutively both *in vitro* and *in vivo* and upregulate its expression when treated with TNF, IL-1, IFN-γ and IFN-β (120, 122). There have also been reports of EC expressing CD154 which is upregulated with IL-1, TNF and IFN-γ (121). CD70 is not expressed on resting endothelium and it is not known if it is inducible on EC (135). EC also express OX40L constitutively, but there is not much known about its regulation (152). Expression of 4-1BBL on EC has not been reported, though 4-1BBL expression has been reported on cardiac myocytes and aortic tissue (198, 199).

In sum, many EC have been previously reported to express the known costimulatory molecules ICOSL, PD-L1, CD40/CD154 and OX40L. Therefore, while most EC do not express the B7 molecules and thus cannot fully activate naïve T cells,

they possess sufficient alternative costimulatory molecules which may play a large role in the activation of memory T cells. The CD2/LFA-3 and ICOS/ICOSL interactions in particular have been demonstrated to be important in EC capacity to activate memory T cells and will be discussed later. EC also express adhesion molecules such as ICAM-1, ICAM-2 and hyaluronan (200) and cytokines such as IL-1α (201), IL-15 (202), IL-6 (203), IL-11 (204) and IL-18 (205) that may provide additional costimulation to T cells.

EC can costimulate T cells in vitro

Compared to other stromal or epithelial cell types, cultured human EC have a unique capacity in that they can activate proliferation of allogeneic T cells freshly isolated from peripheral blood (206, 207). The kinetics of this activation is comparable to that observed with professional APC such as allogeneic blood mononuclear cells, but the magnitude of the response in co-cultures between allogeneic T cells and endothelial cells is generally smaller (180). EC have been shown to activate both CD4+ and CD8+ T cells *in vitro* (196, 208, 209). Both CD8+ and CD4+ T cells secrete IFN-γ in response to co-culture with EC. Isolated CD8+ T cells co-cultured with allogeneic EC make some IL-2 and proliferate, but purified CD4+ T cells make much more IL-2 and proliferate to a much greater extent (209, 210). However, the CD4+ response depends upon pretreatment of the ECs with IFN-γ (or preincubation with natural killer or CD8+ T cells) to cause class II MHC molecule expression, whereas constitutive levels of MHC class I expression are sufficient to activate CD8+ T cells (209).

Studies using limiting dilution assays to quantify the number of T cells that respond to allogeneic EC have indicated that the frequency of responding T cells is

significantly lower than the frequency of T cells responding to professional APC like adherent monocytes or B cells (211, 212). Part of this difference in activation capacity may be attributed to the fact that for both CD4+ and CD8+ T cells EC preferentially activate memory T cells whereas professional APC can activate both naïve and memory T cells (213-215). *In vitro* experiments suggest that naïve T cells ignore and can even become anergized in response to antigens presented by endothelial cells (216-218). In contrast, co-cultures of memory T cells with allogeneic ECs show T cell cytokine production and proliferation, though even among memory CD4+ or CD8+ T cells the EC response is less compared to adherent monocytes (213). One reason for this difference may be that EC present a different, less immunogenic repertoire than professional APC (187). Another part of the difference between EC and APC may lie in the distinct costimulatory signals provided by EC as compared to professional APCs.

T cell proliferation in response to EC is also dependent on costimulatory interactions between the T cell and the EC. However, unlike professional APC, most human EC do not express B7 molecules and consequently cannot activate naïve T cells to proliferate in allogeneic reactions. Mouse EC which do express B7.2 can activate CD8+, but not CD4+, naïve T cells *in vitro* and *in vivo* (216, 219). As discussed previously, the costimulatory requirements of T cells change as T cells mature and differentiate. In particular, memory T cells require less costimulation than naïve T cells (58, 59). Naïve T cells need the assistance of professional APC, like dendritic cells, to be activated, while memory T cells can be activated by less specialized (or "semi-professional") APC such as EC. Early experiments suggested that the costimulatory capacity of EC appears to be contact dependent as paraformaldehyde-fixed cells were demonstrated to costimulate T

cells (98, 197). Interestingly, EC also seems to be capable of providing costimulation in trans to cells such as fibroblasts that ordinarily cannot activate T cells (220, 221).

The costimulatory functions of EC were initially studied using polyclonal activators such as phytohemagglutinin (PHA) or anti-CD3 antibodies (98, 196, 222, 223). These studies demonstrated that EC like B lymphoblastoid cells could stimulate the transcription and secretion of both IL-2 and IFN-γ from T cells whereas fibroblasts or smooth muscle cells could not. In examining the alloresponse, blocking interactions between CD2/LFA-3 partly inhibited proliferation whereas antibodies to ICAM-1/2, VCAM-1 or B7.1/2 had little effect on EC induced T cell proliferation despite having significant effects on allogeneic blood adherent cell induced proliferation (209, 213, 224, 225). This suggests a strong role for the CD2/LFA-3 pathway in EC-mediated T cell activation. However, since blockade of the CD2/LFA-3 pathway did not completely inhibit cytokine production or proliferation (98, 197, 209) this suggests that other costimulators expressed by EC may play a role in mediating T cell activation.

The consequence of EC interactions with T cells is to generate specific changes in T cell signaling. ECs can trigger activity from the transcription factors AP-1, NFAT and NF-κB (226) as well increased the levels of AP-1 (227) in part due to LFA-3 dependent aggregation of lipid rafts (226). In addition to increasing mRNA of IL-2 and IFN-γ in T cells, ECs can increase production of IL-4 (223) and CD154 (228, 229) on the surface of T cells via LFA-3 dependent mRNA stabilization. CD4+ T cell adhesion, cytokine production in response to polyclonal activators and IL-2 mRNA stabilization depends upon signaling via OX40-OX40L (152, 153, 230, 231). The change in signaling leading to IL-2 synthesis mediated by EC appears to be cyclosporine resistant. This effect is EC-

specific and cannot be replicated by B lymphoblastoid cells or blood adherent cells suggesting that EC provide a unique constellation of costimulation (188, 197).

One caveat to studies done on costimulatory molecules are that T cell clones have even less costimulatory requirements than resting memory T cells and can often be stimulated by cells bearing peptide/MHC complexes without the assistance of costimulatory molecules. Many of the studies done initially using EC as APCs utilized T cell clones (186, 232-234) and thus results interpreted from earlier studies may not truly assess costimulatory function of EC. Another important caveat is that there are significant species differences in the distribution of costimulatory molecules. For example, mice do not possess the gene for LFA-3 (CD58) and in fact do not display a known CD2 ligand on the surface of their EC (104, 235, 236). In addition, murine and porcine EC express B7.2. In humans, some early reports suggested that HUVEC and some types of microvascular EC express B7.2 (191, 237), however, since then several groups have shown by multiple methods that purified human EC populations largely do not express B7 molecules (219, 238, 239).

Unique features of antigen presentation by EC

EC-mediated T cell activation appears to provide signals that are distinct from other APCs. In comparison to B lymphoblastoid cells from the same donor, EC activate a smaller number of both CD4+ and CD8+ T cells in an MLR or with superantigen (212, 240). As discussed previously, EC also selectively activate memory but not naïve T lymphocytes (214, 223, 240), There are several possibilities as to why EC are less

efficient activators of T cells and cannot activate naïve T cells in comparison to conventional APC.

One possibility is that EC generate a different contact site compared to professional APC. T cells are known to form aggregates of TCR, adhesion and costimulatory molecules at the contact site with professional APC known as the immunological synapse (33). Studies of T cell-EC contact regions suggest that adhesion molecules at the T cell-EC synapse may be different from the one T cells form with professional APC (241, 242). Further evidence to support this comes from studies done using PHA in which EC can activate naïve T cells (223). PHA is known to provide adhesion molecule-independent bridging between the T cell and the APC and thus with PHA EC can activate naïve T cells whereas in the alloresponse or using other polyclonal activators like superantigen EC are limited to activating memory T cells (240). As mentioned previously, memory T cells also have elevated levels of β1 and β2 integrins as well as specialized chemokines receptors that may contribute to their capacity to be activated by EC (46, 243).

A second possibility is that EC may express molecules that actively inhibit T cell activation. Recent work has demonstrated that EC express a large amount of the inhibitory molecule PD-L1 and that this molecule is increased by IFN-γ and TNF (194). Blockade of this interaction can increase T cell cytokine synthesis and proliferation *in vitro* (89, 194). EC have also been shown to express an activated form of TGF-β (244, 245) which is known to have significant inhibitory effects on T cells (246). In addition to direct inhibitory effects on T cells, EC may also modulate regulatory T cell activity

through the expression of GITR-L (247) and IL-6 (203) both of which have been demonstrated to decrease the suppressor function of regulatory T cells (248-251).

The final possibility is that EC do not express the appropriate costimulatory molecules and therefore provide less effective activation which would enable only a fraction of memory T cells and no naïve T cells to reach the activation threshold. This is consistent with the idea that naïve T cells have higher costimulatory requirements than memory T cells (58). As described above, EC do not express B7 molecules which are important costimulatory molecules in activating naïve T cell (188, 189). However, EC do express other costimulatory molecules, notably LFA-3 as well as CD40, OX40L and ICOSL which are costimulators known to be important in T cell activation (124, 140, 145, 252, 253).

In addition to activating fewer T cells compared to professional APCs, EC-activated T cells also possess a distinct phenotype from those activated by conventional APCs. For instance, CD8+ T cells activated by EC retain expression of CD69, CD25 and CD62L at 7 days, but have a lower level of perforin compared to CD8+ T cells activated by B lymphoblastoid cells (BLC) which upregulate then downregulate all three of these markers and express high levels of perforin (214). Additionally, allogeneic EC-activated CTL can kill both EC and BLC targets from the same donor, whereas BLC-activated CTL can only kill BLC, but not EC (215, 254). CD4+ T cells that have been activated by EC demonstrate a more rapid increase in CD154 upon activation (228) and cyclosporine resistance (188).

In vivo evidence for EC antigen presentation

EC occupy a unique niche in that they are in continual contact with T cells in the periphery. As such, EC have a particular opportunity to influence the T cell response. T cells may encounter antigen presented on EC and recruit antigen-specific memory T cells to sites of infection. This is supported by the observation that intradermal injection of PPD antigen can elicit a response within hours in sensitized, but not naïve test subjects (255). The rapid kinetics of this response suggests that EC may be presenting antigen to PPD-specific T cells and activating them *in situ*.

Further evidence for EC as APC *in vivo* comes from transplantation models. CTL specific for EC can be found in rejecting kidneys and heart transplants (256-258). Furthermore, depletion of passenger leukocytes using anti-CD45 monoclonal antibodies from human kidneys, which have MHC class II expressing EC, has no effect on allograft rejection (259), whereas in rats, whose EC do not express MHC class II, depletion of passenger leukocytes can significantly delay or prevent rejection (260, 261). Experiments done in mice also suggest the importance of EC antigen presentation in mediating transplant rejection. Kreisel et al showed in a model of cardiac allograft rejection that non-hematopoetic cells such as EC can mediate rejection by direct recognition of allogeneic MHC class I by naïve CD8+ T cells. In this model, Kreisel et al used a bone marrow chimera where the donor bone marrow was syngeneic to the recipient and in this situation the cardiac allograft was still rejected indicating that rejection depended on MHC expressed by nonhematopoetic cells (262). In a model of skin rejection, CD8+ T cells could recognize the graft only when EC in the graft had been

replaced by recipient EC demonstrating that EC can cross-present antigen and that EC alone can sufficiently activate T cells to mediate rejection (263). Another mouse model utilizing cardiac allografts showed CD4+ T cell allorecognition could also be mediated by EC. In this model, a TCR transgenic mouse specific for HY antigen presented on MHC class II molecules was transplanted with an HY positive heart bearing another MHC class II molecule and rejection only occurred when the donor heart was replaced by recipient EC (264). This suggests that like the case for CD8+ T cells, EC are also capable of presenting antigen to CD4+ T cells as well though there is conflicting evidence. Unlike the previous experiment, experiments using bone marrow chimeras where the EC do or do not express MHC class II in a cardiac allograft show that MHC class II expression on EC, and therefore CD4+ T cell recognition of EC, does not influence allograft rejection (216).

The final piece of evidence for EC presentation *in vivo* comes from another mouse model of diabetes. In this model, it was observed CD8+ T cells specific for insulin could cause diabetes in mice strains that had no preexisting inflammation. It was found that these T cells could recognize pancreatic endothelium in *in vitro* islet cultures. The recognition triggered T cell integrins and EC expression of chemokines, especially CCL21 or SLC, which mediated firm adhesion of the specific T cells to the endothelium (265). This suggests that antigen presentation by EC in addition to chemokine signaling may be important to in mediating appropriate T cell homing.

Thus, EC express molecules critical for antigen presentation including MHC molecules and costimulatory molecules. EC have also been demonstrated to activate both memory CD4+ and CD8+ T cells *in vitro* and *in vivo*, though this activation appears

to be qualitatively and quantitatively distinct from activation mediated by other APCs such as BLC and monocytes. EC antigen presentation may have an important role in the homing to sites of inflammation and regulation of the local immune response.

1.4 MEMORY T CELLS IN TRANSPLANTATION

Identification of alloreactive memory T cells

Alloreactive memory T cells have been demonstrated to play a role in the rejection of heart, kidney and liver transplants. This has been demonstrated by correlating the presence of pre-transplant memory T cells with increased incidence and severity of rejection (266-269). Furthermore, two groups have reported a correlation between pre-transplant frequency of alloreactive memory T cells as measured by *in vitro* IFN-γ production to post-transplant risk of rejection (270, 271). Reduced numbers of pre-transplant memory T cells also correlated with improved function of kidney allografts 12 months following transplantation (270, 272). Late episodes of rejection in patients 2 to 18 years after transplantation were also associated with an increase in cells with a memory phenotype (CD45RO+) in the circulation (273). In fact, several studies have shown that donor-reactive T cells with a memory phenotype can be generated in the presence of immunosuppression (271, 274). All these studies suggests that memory T cells may contribute to both initial and late acute rejection episodes. They also suggest that despite immunosuppression, existing memory T cells and those generated during early rejection episodes may contribute to late or chronic graft loss.

Generation of alloreactive memory T cells

Memory T cells, by definition, are T cells that have previously encountered antigen. In the case of allografts, the major source of antigen exposure would be via pregnancy, blood transfusion or prior organ transplant. However, these situations do not apply to many patients which raises the question of how memory T cells arise in non-sensitized patients. Two hypotheses for the generation of this memory population are heterologous immunity and homeostatic proliferation.

Heterologous immunity refers to the concept that the T cells (or B cells) responding to one antigen may be cross-reactive with other related or unrelated antigens. This is applicable to transplantation in that T cells generated from infectious agents may be capable of responding to alloantigen. This has been supported by work done in both mice and humans. Adams et al provided strong evidence that multiple viral infections were capable of generating significant numbers of alloreactive memory T cells that prevented the induction of tolerance (275). Memory CD8+ T cells that can react with alloantigen have also been found in mice infected with lymphocytic choriomeningitis virus (LCMV) (275). Memory CD4+ T cell cross reactivity with alloantigen was observed in mice infected by *Leishmania major* (276). Studies in humans have also correlated the presence of alloreactive cytotoxic T cells with seropositivity for Epstein Barr virus (EBV) and cytomegalovirus (CMV) (277, 278). Specifically, HLA-B8 restricted CD8+ T cells specific for an EBV epitope showed cross reactivity with alloantigens, in particular HLA-B44 (277). Interestingly, prior to this study B8 and B44 were identified as an immunological pairing that predicted worse prognosis for allograft survival compared with pairings between other MHC haplotypes (279). Taken together,

these studies provide significant evidence in humans to suggest that memory T cells generated by infection can respond to alloantigen and may contribute to allograft rejection.

Memory T cells may also be generated by the process known as homeostatic proliferation. Homeostatic proliferation refers to the observation that donor T cells undergo proliferation when transferred into lymphopenic recipients. This phenomenon has been well-documented in mice (280) and, as mentioned above, this lymphopenia-induced proliferation can generate T cells with a memory phenotype from naïve T cells (44, 281, 282). Memory T cells generated in this fashion possess properties similar to antigen-experienced memory T cells; for instance, they can mediate rejection without secondary lymphoid organs (61). Homeostatic proliferation occurs naturally in neonates during the first wave of naïve T cell release from the thymus into the empty lymphoid compartment (283). More relevant for transplantation, homeostatic proliferation can occur during infection (284), and following chemotherapy in older patients where the thymus has involuted (285). Another situation of lymphopenia relevant for transplantation is the use of T-cell depletion strategies such as rabbit anti-human thymocyte globulin or anti-CD52 (286). Homeostatic proliferation following any of these situations to replenish the T cell pool may generate memory T cells that contribute to allograft rejection.

While alloreactive memory T cells can be generated from heterologous immunity and homeostatic proliferation, it is not clear that these mechanisms generate memory T cells that function like true antigen-specific memory T cells. More specifically, it is not known if the affinity and frequency of memory T cells generated by both these

mechanisms reflect properties of a true memory T cell response. Some studies suggest that homeostatically generated T cells are not as efficient at rejecting grafts compared with alloantigen experienced memory T cells (61). Studies of heterologous immunity have also suggested that the frequency of alloreactive memory T cells generated by infection is lower than that seen with exposure to alloantigen (275, 276). However, the same studies also demonstrated that while the frequency of alloreactive memory T cells may be lower, rejection of a skin graft by cross-reactive memory T cells generated from infection had the same kinetics as second set rejection in mice that had previously been primed with donor-type alloantigens (276).

Memory T cells and inhibition of tolerance

In addition to contributing to increased episodes of allograft rejection and poorer long-term graft survival, memory T cells may also stymie the development of transplant tolerance. The quest to induce tolerance to foreign organ transplants has been the holy grail of the transplant field. A number of successful protocols have recently emerged in rodent models where recipients will tolerate donor organs indefinitely using combinations of costimulatory blockade (287-289). However, the same protocols in large animal and non-human primate models have been largely unsuccessful (290). One of the key differences between rodents and humans/large animals is the significant population of memory T cells in the circulation of the latter.

This idea of memory T cells being a barrier to tolerance has been tested in several rodent models. For instance, mice that have been sensitized to a donor antigen using a donor-type skin graft could reject a cardiac allograft in the presence of the anti-CD154

which induces tolerance in unsensitized mice (291-293). Memory T cells generated from infectious sources have also been shown to cause rejection even with a known tolerance inducing protocol (275). In those experiments, Adams et al showed that co-infection with LCMV, vaccinia virus, and vesicular stomatitis virus resulted in the generation of alloreactive memory T cells that prevented tolerance with a protocol involving treatment with CTLA-4Ig, anti-CD154 mAb, busulfan and donor bone marrow infusion. That protocol has been demonstrated by several groups to induced donor-specific tolerance in naïve animals and Adams et al shows that the presence of virally-specific CD8+ memory T cells prevented the induction of tolerance in the majority of mice that had received the tolerance protocol.

Wu et al have also shown that memory T cells generated from homeostatic proliferation were similarly resistant to tolerance inducing protocols (281). Blockade of either CD28/B7, CD40/CD154 interactions or a combination of both agents which has been shown to induce tolerance in naïve animals was ineffective at preventing cardiac allograft rejection when homeostatically generated memory T cells were present. They further show that the mechanism for this graft rejection relied in part on a reduced necessity of memory T cells for CD28 costimulation.

The evidence discussed above highlights the importance of memory T cells in mediating allograft rejection and in particular how the unique properties of memory T cells can not only contribute to enhanced rejection responses, but also to prevention of tolerance. Transplant recipients more than likely have had numerous infections, particularly from EBV and CMV, and thus it is likely that any attempt to manipulate the

immune response that focuses on T cell activation will need to account for memory T cells.

Targeting memory T cells

Current clinical therapies for organ transplants that generate global immunosuppression have revolutionized the success of organ transplantation. However, it is not clear what effects global immunosuppression has on T cell memory. Studies correlated pretransplant alloreactive memory T cells with acute rejection episodes that occurred despite immunosuppression using tacrolimus or sirolimus (270, 271). Memory T cells may also be resistant to lymphocyte depletion strategies using rabbit anti-thymocyte globulin or anti-human CD52 mAb. A recent study from Pearl et al showed that memory CD4+ T cells are the dominant remaining subtype following depletion (286). The responses of these memory CD4+ T cells were resistant to steroids, deoxyspergualin, and sirolimus, but not tacrolimus or cyclosporin A. Other reports have indicated that memory T cells may be tolerized using a combination of deoxyspergualin and costimulation blockade (275) and that cyclosporine instead of inhibiting memory T cells actually promotes memory T cell formation and may result in an enhanced recall response (294, 295).

In addition to global immunosuppression, another strategy to target memory T cells is the induction of donor-specific tolerance. One study in mice showed that memory CD8+ T cells specific for hemagglutinin A (HA) were tolerized by administration of soluble HA peptide or via cross-presentation of antigen from pancreatic islets engineered to produce HA. Both methods seemed to produce tolerance via induction of peripheral

apoptosis (296). Memory CD4+ T cells can also be anergized using by low avidity TCR stimulation (297). Memory T cells may also be subject to regulation from regulatory T cells. CD4+CD25+ T cells can suppress CD8+ memory T cells responses to *Listeria monocytogenes* and, when adoptively transferred, Tregs can also delay allograft rejection in a model of CD8+ T cell memory (298, 299).

Another strategy, and perhaps the one with the most promise, is to target memory T cell activation. Targeting the CD28/B7 and CD40/CD154 pathways has been demonstrated in numerous rodent models to successfully induce tolerance (288, 289, 300, 301). However, memory T cells have been shown to be less reliant on APC mediated costimulation (58) and in particular they have a reduced requirement for CD28/B7 and no requirement for CD40/CD154 signaling (60). Despite being less reliant on CD28/B7 and CD40/CD154 singaling, it has become increasingly clear that memory T cells may depend on other costimulatory pathways to become fully activated. Some newly described pathways that have been implicated in memory T cell function include ICOS/ICOSL, 4-1BB/4-1BBL, OX40/OX40L, CD30/CD30L and CD27/CD70. ICOS/ICOSL blockade has been shown to prolong allograft survival in rodents, particularly if administration of the blocking antibody is delayed suggesting that it interferes with effects that occur after T cells have been primed (302). Blocking ICOS-ICOSL interaction has also been shown to prevent chronic rejection and potentiate tolerance when combined with other immunosuppressive treatments (303). 4-1BB/4-1BBL has been demonstrated in murine models to be important in CD8+ T cells mediated rejection of allogeneic intestine (304), cardiac (305), and skin (305) allografts. Blockade of 4-1BB/4-1BBL pathway prolonged graft survival for all organs by

decreasing the number of alloreactive CTL, however blockade did not induced tolerance to grafts in any of these models. Despite success in inhibiting naïve T cells responses, inhibition of 4-1BB did not prolong graft survival in a murine model of memory T cell mediated skin allograft rejection (306). OX40-OX40L interactions have been shown to be important in CD4+ memory T cell responses to allografts (307). In this rat model of transplant rejection, blockade of OX40-OX40L in combination with B7 blockade prolonged cardiac allograft survival in recipients receiving primed T cells (307). Vu et al also showed that OX40-OX40L blockade in conjunction with B7 and CD40/CD154 blockade could induce long term survival of skin grafts in mice possessing memory T cells that were generated by either donor-specific priming or homeostatic proliferation (306). Finally, targeting CD27-CD70 interactions could lead to indefinite allograft survival in the presence of memory CD8+ T cells (308). All the above evidence suggests that memory T cells do require costimulation distinct from naïve T cells to achieve full activation and that targeting these costimulatory pathways can affect the response of memory T cells to allografts though the specific mechanisms by which they work remain unclear.

Memory models and the SCID/beige mouse-human skin chimeric transplant model

Murine models of T cell memory have contributed significantly to our understanding of memory and allograft rejection. The existence of allospecific memory T cells *in vivo* was initially suggested using a murine minor Ag (HY) model confirmed that allosensitized memory T cells mediate an accelerated form of rejection known as second set rejection (309). Since that time, murine models have shown that allospecific

memory T cells can be generated by heterologous immunity or homeostatic proliferation can mediate transplant rejection (275, 281). These murine models using presensitized mice or mice with homeostatically generated memory T cells demonstrated that memory T cell mediated rejection is independent of secondary lymphoid organs (61) and resistant to CD28 and CD154 blockade (281, 291, 292, 306). However, studying memory in transplantation remains limited by fact that mice used in these models are either too young or too antigen inexperienced to have significant populations of memory T cells. Almost all of the current mouse models used to study memory must generate memory T cells through specific infection or donor-specific priming which may not accurately recapitulate the numbers and diversity found in large animals and humans. As mentioned above, this in part may explain why protocols developed in mice for tolerance have not been successfully translated into large animals or humans. Therefore, one strategy to improve our understanding of memory may be to study the human immune response and one method to do so *in vivo* is the human mouse chimera model.

The ability to study a functional human immune system in an *in vivo* model was made possible by the development of immunodeficient mouse hosts. Mice bearing the severe combined immunodeficiency (SCID) mutation or lacking recombinase activating gene (RAG) have been successfully used as hosts for human cells. In the absence of mouse T and B cells, stable long-lived reconstitution of human T cells can be achieved with interperitoneal injection of human peripheral blood leukocytes in the SCID mouse (310). These mice have been used to study the human immune system in particularly in HIV and transplantation. Early studies demonstrated that skin could be engrafted onto SCID mice and that if these mice were reconstituted with human T cells that the

recruitment of these T cells are limited to human tissue and that this recruitment depends on specific cytokines (179, 311). In addition to mediating allograft rejection, studies using tetanus toxin demonstrated that human T cells derived from the mouse spleen could be activated *in vitro* which indicated that the human T cells retained function in their murine hosts (312). Furthermore, T cells from human donors immunized with tetanus toxin, but not naïve donors, homed specifically to the human skin graft when tetanus toxin was injected into the skin graft, but not when it was injected into the adjacent mouse skin suggesting that recruitment depended on a human microenvironment.

The model used in our laboratory employs SCID/beige mice which have an additional mutation in a lysosomal transporter that is homologous to the Chediak-Higashi gene found in humans (313). The effect of this gene is to effectively eliminate NK cell function in the mice, which is to ensure human graft acceptance by preventing xenograft recognition by murine NK cells. The protocol developed by Murray et al involves grafting SCID/beige mice with human skin allowing the skin to heal in for several weeks and then adoptively transferring PBMC allogeneic to the skin into the peritoneal cavity of the mice (179). CD3+ T cells can be detected in the circulation within one week and no significant levels of CD19+ B cells, CD14+ macrophages, or CD16+ NK cells can be detected in the circulation. Human B and T cells can be found in the lymph nodes in low numbers and while the mice do produce circulating human immunoglobulin there is no evidence of complement deposition or neutrophil invasion (314). Depletion of B cells also has no effect on the infiltration or microvascular destruction observed in the transplanted human skin grafts (315). Graft versus host disease is also negligible in this model because the human antibodies produced do not appear to be specific for murine

antigens and the MHC restriction between mice and humans is sufficiently different to prevent direct recognition. There is also no evidence of human macrophage or dendritic cell engraftment (314). Thus, this model describes a largely T cell mediated allograft response.

The initial studies of this model characterizing the response demonstrated that this model recapitulates first set skin rejection observed in humans (179). Human skin grafts on mice reconstituted with allogeneic PBMC demonstrate progressive leukocyte infiltration and destruction of the microvasculature that has equivalent kinetics and histologic appearance to human allogeneic skin rejection. Experiments showing significant graft protection using mouse anti-LFA-3 mAb or human LFA-3-IgG1 fusion protein have demonstrated the utility of using this model to study human allograft rejection *in vivo* (109). Expansion of this model to human arteries has also demonstrated successful engraftment and further supports the use of this model to study human alloresponses (316, 317).

1.5 THESIS OBJECTIVES

The basis for the studies in this thesis derives from three observations. First, memory T cells are a significant hindrance to allograft rejection and long-term allograft survival (318). Second, EC antigen presentation has been shown to play an important role in transplant rejection (197, 263, 264). Third, EC have been shown to selectively activate memory T cells (213, 214) through LFA-3 and other as yet unknown costimulatory molecules expressed on EC (197). The questions of how EC activate

memory T cells and what role this activation may play in transplant rejection have not been explored.

Thus, I hypothesize that EC express molecules that uniquely enable them to activate memory T cells and that presentation of antigen by EC using these costimulatory molecules contributes to memory T cell mediated allograft rejection. In this thesis, I address this question with the following four aims. First, I will detail the human memory T cell response to human EC *in vitro* and describe an *in vivo* model to study human memory T cell mediated allograft rejection (Chapter 3). Second, using this model, I will investigate the role of B7 costimulatory molecules on EC-mediated T cell activation *in vitro* and *in vivo* using a novel CD28 blocking reagent (Chapter 4). Third, I will examine the role of memory T cell specific costimulators on EC-mediated T cell activation *in vitro* and *in vivo* (Chapter 5). Finally, I will study the response of different memory T cell subsets to EC (Chapter 6). Completion of these aims will enhance the understanding of how EC activate memory T cells and elucidate the role that interactions between EC and memory T cells play in allograft rejection.

CHAPTER II – MATERIALS AND METHODS

2.1 MATERIALS

Cytokines

Recombinant human TNF (TNFα) was purchased from R&D Systems (Minneapolis, MN) and recombinant human IFN-γ from Biosource International (Camarillo, CA).

Antibodies

Mouse anti-human CD4, CD8, CD80, and CD86 conjugated to phycoerythrin were obtained from BD Biosciences (San Jose, CA). Azide-free IgG1 was obtained from eBioscience (San Diego, CA) and mouse anti-human CD58 (LFA-3) (clone TS2.9) was purchased from Pierce Biotechnology (Rockford, IL). Mouse anti-CD28 (clone 28.2) and CD27 (clone M-T271) were purchased from BD Bioscience (San Jose, CA). Mouse anti-human 4-1BB and mouse anti-human OX-40 mAbs were purchased from Ancell (Bayport, MN). Mouse anti-human ICOS mAb was purchased from eBioscience (San Diego, CA). Mouse anti-human 4-1BBL mAb was a gift from X.G. Zhang (Suzhou, China), mouse anti-human ICOSL mAb was a gift from H.W. Mages (Berlin, Germany). Mouse anti-human OX40L mAb and recombinant human 4-1BBFc were purchased from R&D Systems (Minneapolis, MN). Biotin-conjugated goat and donkey anti-mouse antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

FK734 was produced by Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharma Inc.). FK734 is a humanized IgG2κ anti-human CD28 antibody that has the complementary determining regions (CDRs) of the mouse anti-human monoclonal antibody TN228 (319) and the Fc domain of human IgG2M3, in which 2 amino acid

mutations (V234A, G237A) have been introduced into the human γ2 chain to eliminate binding of the antibody to FcγR. The original TN228 cell line was generated by immunizing BALB/c mice with human CD28-transfected mouse fibroblast L cells and fusing immune splenocytes with P3 U1 myeloma cells. The purified molecule consists of two heavy chains and two light chains, which are 447 amino acid residues ($C_{2177}H_{3358}N_{575}O_{669}S_{19}$; *M*W 48898.64) and 218 amino acid residues ($C_{1043}H_{1628}N_{279}O_{342}S_7$; *M*W 23772.21) in length, respectively. FK734 binds to a human CD28-mouse IgG Fc fusion protein ($Kd=3.72\times10^8$) and inhibits proliferation of human T cells stimulated with anti-CD3 and P815/human CD80+ cells in a concentration dependent manner with the half inhibitory concentration at 74.6 ng/mL (unpublished data, K.K.). FK734 does not cross-react with mouse CD28.

2.2 *IN VITRO* METHODS

Isolation and culture of human cells

All human cells and tissues were obtained under protocols approved by the Yale Human Investigations Committee. PBMCs were isolated from by density gradient centrifugation of leukapharesis products from healthy adult volunteers by using Lymphocyte Separation Medium (Gibco BRL, Grand Island, N.Y.). Isolated cells were stored in 10% DMSO/90% FBS in liquid N_2 (-196°C) and were thawed and washed before use (320). CD4+ and CD8+ T cells were isolated from PBMCs by positive selection by using Dynabeads (Dynal (now Invitrogen), Lake Success, N.Y.) as described previously (189). The selected populations obtained by this procedure were routinely >97% CD4+ or CD8+ by FACS analysis (data not shown). Activated T cells were

removed by negative selection with an anti-HLA-DR antibody at a concentration of 5 μg/ml (LB3.1; gift of J. Strominger, Harvard University, Cambridge, Mass.) for 20 min, washing twice, and depleting by using magnetic beads conjugated to goat anti-mouse antibody (Dynal). Naïve and memory subsets of T cells were isolated from the total T cell population by further negative selection by using anti-CD45RA or anti-CD45RO antibodies at a concentration of 2 μg/ml (Biosource, Camarillo, Calif.). To obtain memory T cell subsets, CD4+ T cells were isolated from PBMCs by positive selection by using BD IMag CD4+ magnetic particles (BD Biosciences, San Jose, CA). The selected population obtained by this procedure was routinely >95% CD4+ by flow cytometry (data not shown). Cells were then stained with mouse antibodies to mouse anti-human CD62L (BD Biosciences San Jose, CA), mouse anti-human CD45RO (BD Biosciences, San Jose, CA) and mouse anti-human CCR7 (R&D Systems, Minneapolis, MN) and subjected to FACS sorting on a FACSAria (BD Biosciences, San Jose, CA) to isolate T_{CM} (CD45RO+, CCR7+ and CD62L+) from T_{EM} (CD45RO+, CCR7-, CD62L-).

Monocytes (Mo) were obtained by incubating PBMCs on gelatin-coated tissue culture plates in RPMI 1640 medium containing 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/liter L-glutamine (RPMI/FBS) for 1 hour then washing away the non-adherent cells. Mo were then harvested by washing the plates with PBS and incubating the flasks with ice-cold 10 mM EDTA in RPMI 1640 for approximately 5-10 minutes. Detached cells were then collected, centrifuged at 1200 rpm and plated onto 24-well gelatin-coated plates in RPMI/FBS. The selected population obtained by this procedure was routinely >80% CD14+ by FACS analysis and <10% CD3+ (data not shown). Alternatively Mo were negatively selected using the Monocyte Negative

Isolation kit from Dynal (Lake Success, NY). Whole PBMC were incubated with the blocking and antibody reagents for 15 minutes at 4°C and then washed with PBS/0.5% BSA/2 mM EDTA. The cells were then incubated with the supplied beads at the ratio recommended by the manufacturer. The bead/cell mixture was then incubated at 4°C for 15 minutes and then placed on the magnet. The cells left in the supernatant were then harvested for use. The population obtained by this procedure was routinely >90% CD14+ by FACS analysis and <5% CD3+ (data not shown).

HUVECs, which are uniformly positive for CD31 and negative for CD45 by FACS analysis, were isolated and cultured as described previously (321). CD86-transduced HUVEC were generated using a retroviral vector containing the full-length mRNA of human CD86 using methods described previously (322). Transduced EC, control EC (EC transduced with retroviral vector containing LacZ or EGFP), and Mo expression of CD80 and CD86 were compared by staining cells with mouse anti-human CD80 and CD86 and subjected to FACS analysis (Figure 4.3s). Human dermal microvascular endothelial cells (HDMEC) were isolated from discarded human skin, purified using anti-CD31 mini MACS beads (Miltenyi Biotec, Auburn, CA) and cultured in EGM2-MV growth medium (Clonetics, San Diego, CA) as previously described (323). When indicated, the cells were treated with 50 ng of IFN-γ per ml for 3 days prior to cocultivation. After pretreatment with IFN-γ, the ECs were uniformly HLA-DR positive (data not shown).

Monocyte (Mo) and HUVEC co-cultures with allogeneic T cells

For the co-cultures, 1.5×10^5 HUVEC or HDMEC at subculture level 1 or 2 were plated into gelatin-coated wells of a 24-well culture plates (Falcon, Becton Dickinson, Bedford, MA) and grown to visual confluence. EC cultures were treated with 50 ng/mL of IFN-γ (Biosource, Camarillo, CA) per ml for 3 days prior to the addition of T cells. After pretreatment with IFN-γ, the ECs were uniformly HLA-DR positive by FACS analysis (data not shown). 1×10^5 Mo were collected and plated into gelatin-coated wells of a 24-well culture plates and cultured for three days in RPMI/FBS. Purified T cell pupulations were then added to each well (~1×10^6 per well) (See Figure 2.1). All cultures were maintained in 5% CO_2 at 37°C. The medium for co-culture consisted of RPMI 1640 supplemented with 10% FBS serum (Gibco BRL, Grand Island, N.Y.), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

For cell surface immunostaining, T cells from co-cultures were collected by washing wells twice with Hanks-buffered saline solution (HBSS) and centrifugation at 1000 x *g* for 5 min, washed twice with ice-cold PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBS/BSA). T cells were then incubated with the indicated antibodies or isotype controls at 2 ug/ml in PBS/BSA for 2 h at 4°C and then analyzed using a FACSort and Cellquest software (BD Biosciences). To measure proliferation by carboxy-fluorescein diacetate succinimidyl ester (CFSE) dilution, the T cells were stained with 250 nM CFSE (Molecular Probes, Eugene, OR) for 15 min prior to co-culture with ECs. Cells were collected after the indicated times and subjected to FACS analysis.

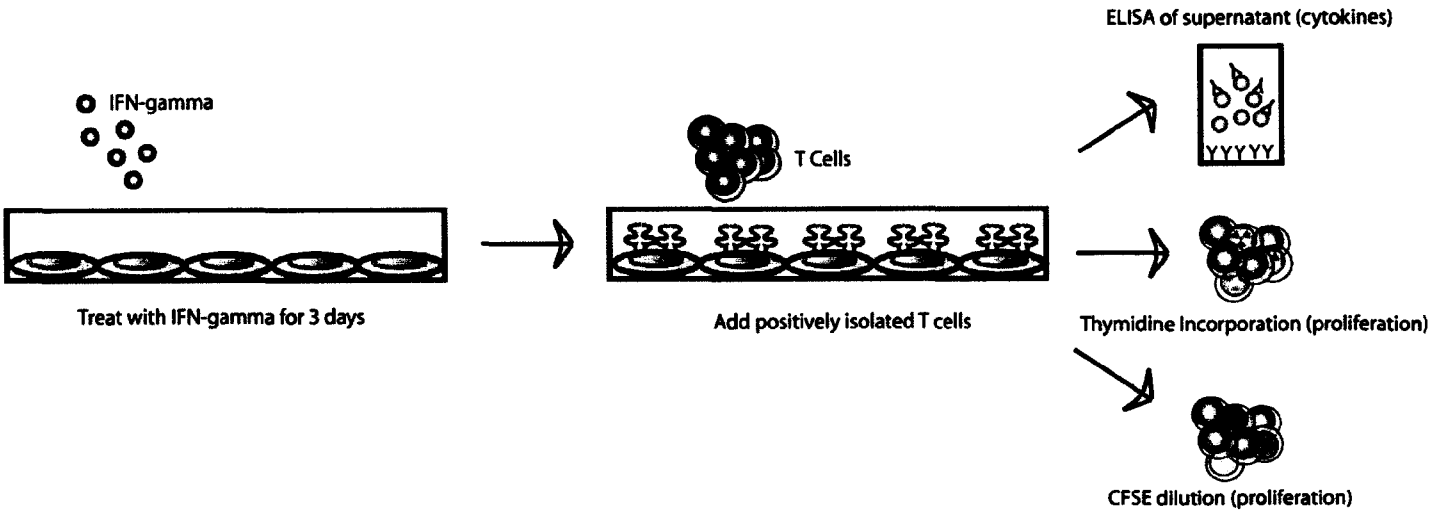

Figure 2.1. ***EC-T cell co-cultures.*** For co-cultures with CD4+ T cells, EC are treated with IFN-γ for three days to upregulate MHC class II. T cells are then added to the EC. After 24h supernatants are collected for analysis of cytokine production by ELISA. T cells are then either subject to FACS or radiolabeled thymidine to analyze their proliferation.

Preparation of RNA, cDNA, and Procedure for Quantitative PCR

RNA was isolated from T cells and EC using RNeasy mini kits (Qiagen, Valencia, CA) with on-column DNase treatment following the manufacturer's supplied instructions. RNA was isolated from skin grafts using the RNeasy fibrous tissue mini kit (Qiagen) again with on-column DNase treatment and following the manufacturer's supplied instructions. cDNA was synthesized using Taqman RT reagents (Applied Biosystems), following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) for 4-1BBL, ICOSL, OX40L, FasL, Granzyme B and Perforin and CD3ε was performed as using primers shown in Table I-2. The PCR reaction mixture (final volume 25 μl) contained 5 μl of cDNA, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 2.5 μl of PCR 10x SYBR Green PCR buffer, 3 μl of 25mM $MgCl_2$, 2 μl of dNTP mix (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, and 5 mM dUTP), 0.125 μl of AmpliTag Gold DNA polymerase (5 units/μl AmpliTag Gold DNA polymerase), and 10.375 μl of H_2O. The PCR reaction was performed in triplicate (3 wells of C96 well plate). The reaction was amplified with iCycler iQ Multicolor Real Time PCR Detector (Bio-Rad) for 37 cycles with melting at 94 °C for 30 s, an annealing at 58 °C for 30 s, and extension at 72 °C for 1 min in iCycler iQ PCR 96-well plates (Bio-Rad). Samples were analyzed by generating a standard curve from plasmids containing the PCR fragment and expressed as copy number where indicated or by calculating a fold induction. The relative quantification values for the gene expression was calculated from the accurate C_T, which is the PCR cycle at which an increase in reporter fluorescence from SYBR Green dye can be first detected obtained above a baseline signal. C_T values for GAPDH (or CD3ε) cDNA were subtracted from C_T values for the cDNA of the gene of interest for each well to calculate

Δ-C_T. The triplicate Δ-C_T values for each sample were averaged. To calculate the fold induction of mRNA in experimental cells compared with control cells, the averaged Δ-C_T values calculated for control cells was subtracted from Δ-C_T values calculated for experimental cells to calculate $\Delta\Delta$-C_T. Then, the fold induction for each well was calculated by using $2^{-(\Delta\Delta\text{-CT})}$ formula. The fold induction value for triplicate wells was averaged, and data are presented as the mean ± S.E. of triplicate wells.

Flow cytometric analysis

For cell surface immunostaining, HDMEC/HUVEC were washed twice with Hanks-buffered saline solution (HBSS) and incubated with trypsin/EDTA for 1 min. Detached cells were collected by centrifugation at 1000 x *g* for 5 min, washed twice with ice-cold PBS containing 1% bovine serum albumin and 0.1% sodium azide (PBS/BSA). Either detached cells, monocytes or T cells were then incubated with the antibodies or isotype controls at 2 μg/ml in PBS/BSA for 2 h at 4°C. After two further washes cells were incubated with 2 μg/ml biotin-conjugated secondary antibodies in PBS/BSA for 1 h at 4 °C. Following another two washes, cells were incubated with 1 μg/mL strepavidin-R phycoerythrin (Molecular Probes, Eugene, OR) in PBS/BSA for 30 minutes at 4°C, washed a further two times and analyzed on a FACSort using Cellquest software (BD Biosciences).

ELISA

Supernatants were collected from co-cultures of T cells with HUVEC or with Mo after 24h or 48h of co-culture. The samples were then assessed using an ELISA kit for

IFN-γ (Biosource, Camarillo, CA) or IL-2 (Ebioscience, San Diego, CA). Both ELISAs were performed as described by the respective manufacturers.

Proliferation Assays

T cell proliferation was measured by [^{3}H]-thymidine incorporation. In brief, HDMEC or HUVEC were cultured in 96-well U bottom plates (Falcon) as described previously (323) and confluent monolayers were then pretreated with 50 ng IFN-γ (Biosource, Camarillo, CA) prior to co-culture with CD4+ T cells. All HUVEC were then treated with mitomycin C (50 μg/ml in PBS, 30 min; Sigma-Aldrich, St. Louis, MO) prior to co-culture to prevent their proliferation as described previously (209). 24h prior to each indicated time point, 1 μCi [^{3}H]-thymidine (Amersham, Arlington Heights, IN) was added to each well. Plates were frozen, thawed and then harvested on a 96-well harvester (Tomtec, Hamden, CT) and counted on a Microbeta scintillation counter (Wallac, Gaithersburg, MD). The mean of the replicates (n=16) was calculated, and the mean [^{3}H]-thymidine incorporation into wells containing only EC was subtracted.

To measure proliferation by carboxy-fluorescein diacetate succinimidyl ester (CFSE) dilution, the cells were stained with 250 nM CFSE (Molecular Probes, Eugene, Oreg.) for 15 min prior to coculture with ECs. Cells were then collected after co-culture and subjected to FACS analysis.

2.3 *IN VIVO* METHODS

Animals

C.B-17 SCID/beige female mice (Taconic Farms, Germantown, NY) were used at 5–8 wk of age. All protocols involving animals were approved by the Yale Animal Care and Use Committee. The animals were housed individually in microisolator cages and were fed autoclaved food and water. Prior to each experiment, serum mouse IgG levels were determined by sandwich ELISA using reagents from Cappel (Durham, NC) as previously described (179). SCID/beige animals were considered "leaky" at IgG levels >1 μg/ml and excluded from experimental use.

Skin Grafting

Human skin was obtained from cadaveric donors through the Yale University Skin Bank under a protocol approved by the Yale Human Investigations Committee. Human skin was orthotopically transplanted to SCID/beige mice as previously described (179). In brief, 0.5-mm-thick sheets were divided into 1-cm^2 pieces, kept at 4°C in RPMI 1640 medium (GIBCO/BRL) and fixed onto similar sized defects on the dorsum of C.B-17 SCID/beige recipients using staples (3M, St. Paul, MN). The resultant surface area of healed grafts was kept constant between animals when possible. The skin reproducibly grafted with a >95% success rate and was allowed to heal for 4 wks before manipulating the graft. Rare animals that did not successfully engraft were excluded from the experimental groups before treatments.

PBMC and T cell subset adoptive transfer

For adoptive transfer of PBMC, SCID/beige mice were reconstituted with 3×10^8 human PBMC by i.p. inoculation 4-5 weeks after skin engraftment. Animals demonstrated no signs of graft-vs.-host disease. Rare animals that failed to reconstitute with human T cells were, by prior design, excluded from analysis.

For memory or naïve T cell subsets, CD45RA+ or CD45RO+ T cells were isolated as described above and cells were >95% pure populations and both populations contained approximately a 2:3 ratio of CD8+ to CD4+ cells. SCID/beige mice were reconstituted with 3×10^8 human PBMC by i.p. inoculation 4 wk after skin engraftment. Animals demonstrated no signs of graft-vs.-host disease. Rare animals that failed to reconstitute with human T cells were, by prior design, excluded from analysis. Alternatively, separated naïve or memory T cells were then counted using a Coulter particle counter (Beckman Coulter, Fullerton, CA) and 1.5×10^8 cells were i.p. inoculated into SCID/beige mice 4 weeks after skin engraftment.

For memory T cell subsets, CD62L-CCR7- (T_{EM}) or CD62L+CCR7+ (T_{CM}) T cells were isolated as described above and the resulting cells were >95% pure upon reanalysis. SCID/beige mice bearing human skin from a donor allograft to the PBMC were adoptively transferred with 1×10^6 cells from either subset by tail vein injection 4 wk after skin engraftment. None of the animals demonstrated signs of graft-vs.-host disease.

Circulating human T cells were evaluated by flow cytometry as previously described (320). In brief, heparinized retro-orbital venous samples were obtained 14 days after reconstitution, and the erythrocytes were lysed. The leukocytes were incubated with

FITC-conjugated mouse anti-human CD3 (Immunotech, Westbrook, ME) and PE-conjugated rat anti-mouse CD45 (Sigma-Aldrich, St. Louis, MO) mAbs. Samples were then analyzed using a FACSort (BD Biosciences, San Jose, CA). (See Figure 2.2)

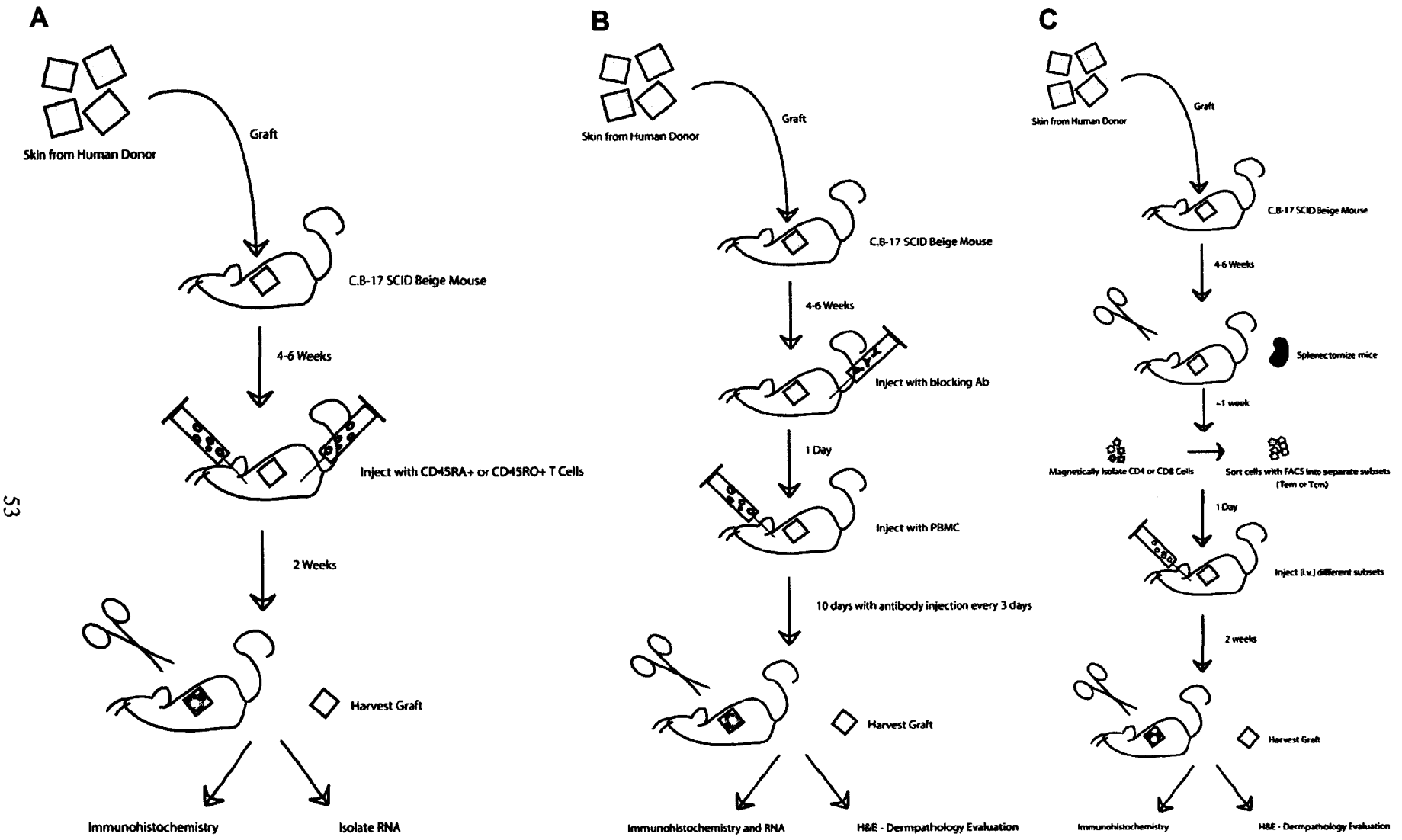

Figure 2.2 ***The human-SCID mouse chimera.*** SCID/beige mice are grafted with split thickness human skin. The grafts are allowed to heal for 4-6 weeks. The mice are then injected with either CD45RA+ or CD45RO+ T cells (A), blocking antibodies and PBMC (B), or purified central or effector memory T cells (C). The grafts are then harvested and taken for analysis.

Histology and Immunohistochemistry

Human skin grafts, harvested at indicated times, were processed for paraffin-embedded or frozen sections as previously described (320). The degree of graft microvascular damage was evaluated from H&E-stained sections by a dermatopathologist (J.M.M.) blinded to treatment protocols as previously described (109). In brief, the percentage of dermal vessels showing injury, defined as EC loss or sloughing, and thrombosis were assessed from an average of three high-power (x200) fields using the following semiquantitative grading scale: grade 0, all vessels patent and uninvolved; grade 1, <25% of vessels show injury; grade 2, ~50% of vessels show injury; and grade 3, >75% of vessels show injury.

Immunostaining was performed using isotype-matched, nonbinding control Abs or the following Abs: mouse anti-human CD3 (PS1, IgG1) and mouse anti-human CD31 (HC1/6, IgG1) (Novocastra Ltd., Newcastle Upon Tyne, UK). The number of human $CD3^+$ T cell infiltrates and $CD31^+$ vessels were assessed by counting and averaging the number of T cells/complete lumens in 10 random, 40x high-power fields (HPF) using the ImageJ program (NIH).

Preparation of RNA, cDNA, and Procedure for Quantitative PCR

RNA was isolated from skin grafts using the RNeasy fibrous tissue mini kit (Qiagen, Valencia, CA) again with on-column DNase treatment and following the manufacturer's supplied instructions. Quantitative real-time PCR (qRT-PCR) was performed exactly as described above, using primers shown in Table I-3. Samples were

either analyzed by examining threshold cycles and comparing threshold cycles between the two groups using a one-tailed t-test with a Bonferroni correction or by calculating the amount of mRNA from a standard curve and expressing it as ± S.E.

Statistics

Statistical differences between groups with respect to T cell infiltrates were evaluated using a two-tailed t-test. Statistical difference between the pathology scores were evaluated using a nonparametric analysis Mann-Whitney *U* test. [^{3}H]-thymidine analysis was done with a two-tailed t-test.

Target	Primers (5'→3')
4-1BBL	ACTGCCCAGCTGGTACATTC
	CACAGGTCCTTTGTCCACCT
ICOSL	AAACCTTGCAGGCAACAATC
	CACTTGGCTTGGATCAGTCA
OX40L	GTGAATGGCGGAGAACTGAT
	ATGCTGGTGCCTGGTTTTAG
IL-2	AACTCACCAGGCTGCTCACA
	GCACTTCCTCCAGAGGTTTG
IL-4	AGCTGATCCGATTCCTGAAAC
	ACTCTGGTTGGCTTCCTTCA
IFN-γ	GTCCAACGCAAAGCAATACA
	TGCTCTTCGACCTTGAAACA
IL-10	TGAGAACCAAGACCCAGACA
	CAGGGAAGAAATCGATGACA
CD28	CTGCTCTTGGCTCTCAACTT
	TTGTCGTACGCTACAAGCAT
CD80	CCTGGCTGAAGTGACGTTAT
	TCCAGAGGTTGAGCAAATTA
CD86	TCCACCAGATGAATTCTGAAC
	CCGTGTATAGATGAGCAGGTC
CD152	ATGTACCCACCGCCATACTA
	TCCAGAGGAGGAAGTCAGAA

Table I-2. *PCR primers used for studies.*

Each primer is listed 5' →3'. The forward primer is above and the reverse primer below. In each case, the identity of the amplified fragment was confirmed by sequencing.

CHAPTER III – IN VITRO AND IN VIVO STUDY OF MEMORY AND NAÏVE T CELL RESPONSES TO THE ENDOTHELIUM

3.1 INTRODUCTION

Long-lived memory T cells may play an important role in early events following transplantation. Alloreactive memory T cell populations are not typically present in the young rodents commonly used to study transplantation. However, in adult humans, about one half of the T cells capable of responding to allogeneic cells in limiting dilution assays express CD45RO, a marker of memory cells (209, 324). However, there is no direct evidence that human memory T cells, like their murine counterparts, can mediate rejection.

Memory T cells also have a special relationship to vascular endothelial cells (EC). Central memory T cells express chemokine receptors (e.g. CCR7) and adhesion molecules (e.g. CD62L) that preferentially interact with high endothelial venules (46). Effector memory cells express chemokine receptors like CXCR3 that respond to inflammatory chemokines and express high levels of adhesion molecules (e.g. LFA-1 or VLA-4) (325) whose ligands are preferentially expressed by cytokine-activated peripheral vascular EC (200). This phenotype allows effector memory cells to be directly recruited to sites of peripheral inflammatory reactions such as allograft rejection. Furthermore, resting memory T cells can be activated by alloantigens presented by EC but not other stromal or parenchymal cells *in vitro* (206, 213).

It has become increasingly evident that T cell memory may pose a significant challenge to transplantation. Memory T cells specific for and expanded by microbial Ag, like naïve T cells, may cross-react with allogeneic MHC-peptide complexes with very high frequency (275). Recent evidence has suggested that the number of cross-reactive

anti-donor memory T cells correlates with increasing rejection rates (271) and may prevent the ability to achieve tolerance (291). This phenomenon has largely been overlooked in the past because alloreactive memory T cell populations are not typically present in the young rodents commonly used to study transplantation. However, adoptive transfer of memory T cells in rodents can rapidly reject a cardiac graft in the absence of secondary lymphoid organs whereas transfer of naïve T cells cannot (61). I have developed a model in which I can test the capacity of human memory T cells to mediate allograft rejection (179, 314). Specifically, I engraft C.B-17 SCID/beige mice with split thickness human skin, containing the superficial vascular plexus of the papillary dermis. The healed graft is perfused through retained human EC-lined microvessels. At this point, usually 3-4 weeks post-skin transplantation, I adoptively transfer human allogeneic PBMC or naïve or memory T cell subpopulations by i.p. inoculation. Within seven days, human T cells are present in the mouse circulation and by 12-14 days infiltrate the human graft. Between 14-21 days, human graft EC are progressively destroyed and the graft frequently ulcerates, indicative of rejection. Previous studies have shown that this response depends upon LFA-3 (109).

The data in this chapter demonstrate memory, but not naive T cells can proliferate in response to microvascular EC derived from human skin. I also use the chimeric human-SCID mouse model described above to show that the graft EC injury is mediated by human memory but not naïve T cells.

3.2 RESULTS

HDMEC activate memory but not naïve T cells

Cultured HUVEC have been shown to preferentially activate allogeneic memory T cells compared to naïve T cells and they do so in an MHC and costimulation dependent manner (209, 223). Cultured HUVEC are the most widely used model of human EC, but the behaviors of microvascular EC, the targets of graft injury, are not always reflected accurately by this cell type. I therefore began my study by examining the capacity of EC isolated from human skin microvessels (i.e. HDMEC) to activate memory and naïve T cells in co-culture. Cultured HDMEC, like HUVEC, express HLA-A/B but not HLA-DR and increase expression of both type of molecules in response to IFNγ (326). In my experiments, HDMEC were treated with IFN-γ for 3 days (to induce HLA-DR) for optimal CD4+ T cell stimulation and then co-cultured with allogeneic, CFSE-labeled T cell subsets. Media were collected at 24h for measurement of cytokines and T cells were collected after 7 days for analysis of proliferation. These time points were judged optimal in pilot experiments. Significant production of both IFN-γ and IL-2 was observed in co-cultures containing memory but not naïve T cells (Figure 3.1A). By FACS analysis purified memory (CD45RO+) T cells, but not naïve (CD45RO-) T cells, proliferated in co-culture with HDMEC (Figure 3.1B). When further separated into CD4+ and CD8+ memory and naïve cells, again memory cells, but not naïve cells proliferated in response to HDMEC (Figure 3.1C). In control experiments CD4+ T cells did not respond to HDMEC that were not pretreated with IFN-γ (data not shown). Thus, HDMEC, like HUVEC, selectively activate allogeneic memory T cells and require pre-treatment with IFNγ to stimulate CD4+ T cells.

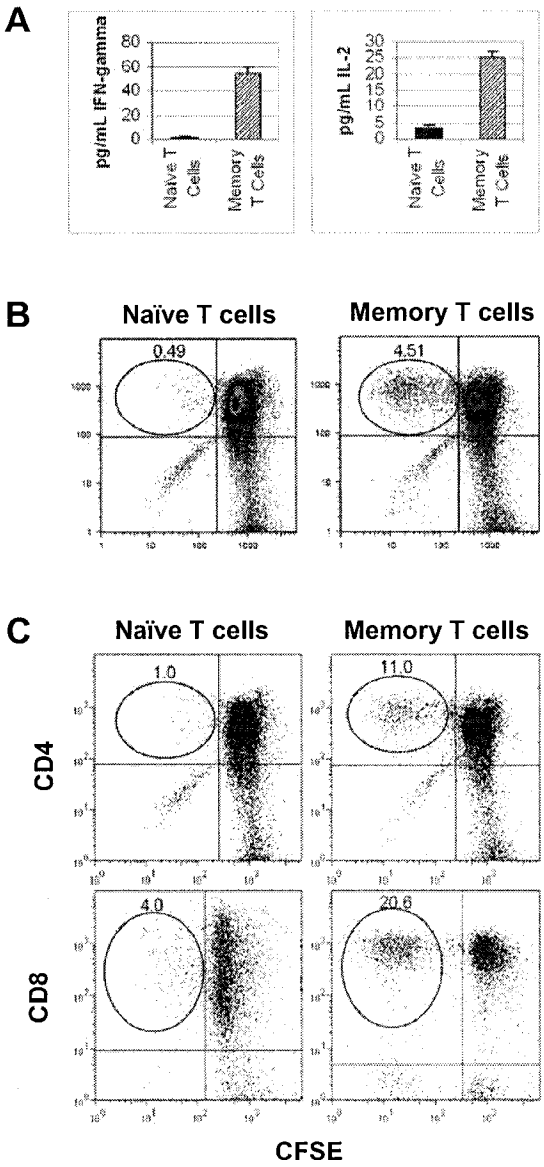

Figure 3.1. ***HDMEC activate memory T cells.*** HDMEC were treated with IFN-g to upregulate MHC class I/II and subsequently co-cultured with either naïve (CD45RA+) or memory (CD45RO+) T cells. (a) Media was taken for ELISA from HDMEC cultured with either naïve or memory T cells for 24 hours. Memory T cells cultured with HDMEC showed significant production of both IFN-g and IL-2 (*hatched bars*) when compared to naïve T cells (*black bars*). (b) After one week of co-culture, CFSE-labeled T cells were stained with anti-CD3 and subjected to FACS analysis. Memory T cells demonstrated significant proliferation (4.51%, *right panel, upper left quadrant, circled population*), whereas naïve T cells showed very little proliferation (0.49%, *left panel, upper left quadrant, circled population*) as judged by CFSE dilution. (c) Following one week of co-culture, purified and CFSE-labeled CD4+ or CD8+ naïve and memory T cells were stained with CD4 or CD8, respectively, and analyzed using FACS. Again, purified memory T cells (*left panels, upper left quadrants, circled population*)) or either subset showed significantly greater proliferation than their purified naïve counterparts (*right panels, upper left quadrants, circled population*). The data shown represent one of four experiments with similar results.

Memory T cells cause human skin allograft rejection in human PBL-SCID

I have previously described a model of human T cell responses to allogeneic skin microvascular EC *in vivo* involving human skin grafts and adoptive transfer of human PBMC in immunodeficient mice (179). To determine which T cell subsets contribute to graft EC injury, I adoptively transferred purified T cell subpopulations and compared the results to those observed following transfer of PBMC. CD4+ plus CD8+ T cells were isolated from whole PBMC and then further fractionated into memory and naïve subsets based on negative selection of CD45 isoform expression (Figure 3.2A). Memory (CD45RO+) or naïve (CD45RA+) T cells were then injected i.p. into SCID/beige mice engrafted with human skin. One week later circulating CD3+ T cells were present in similar numbers following adoptive transfer of either CD45RO+ or CD45RA+ T cell subsets as shown by FACS analysis of mouse blood (Figure 3.2B). Interestingly, mice reconstituted with CD45RA+ T cells developed some circulating CD45RO+ T cells (Figure 3.2B) in addition to circulating CD45RA+ T cells, consistent with observations that naïve T cells acquire some memory markers during homeostatic proliferation (44). In contrast, mice receiving purified CD45RO+ cells displayed exclusively CD45RO+ cells in their circulation. Similar to effects following adoptive transfer of whole PBMC, neither human T cell subset appeared to infiltrate or injure any mouse tissue (data not shown).

Human skin grafts were harvested from the mice at 15 days after adoptive transfer of T cells and analyzed using histology, immunohistochemistry and quantitative real-time PCR (qRT-PCR). Hematoxylin and eosin (H&E) (Figure 3.3A) and anti-CD3 (Figure 3.3B) staining showed a significantly different pattern of infiltration and tissue

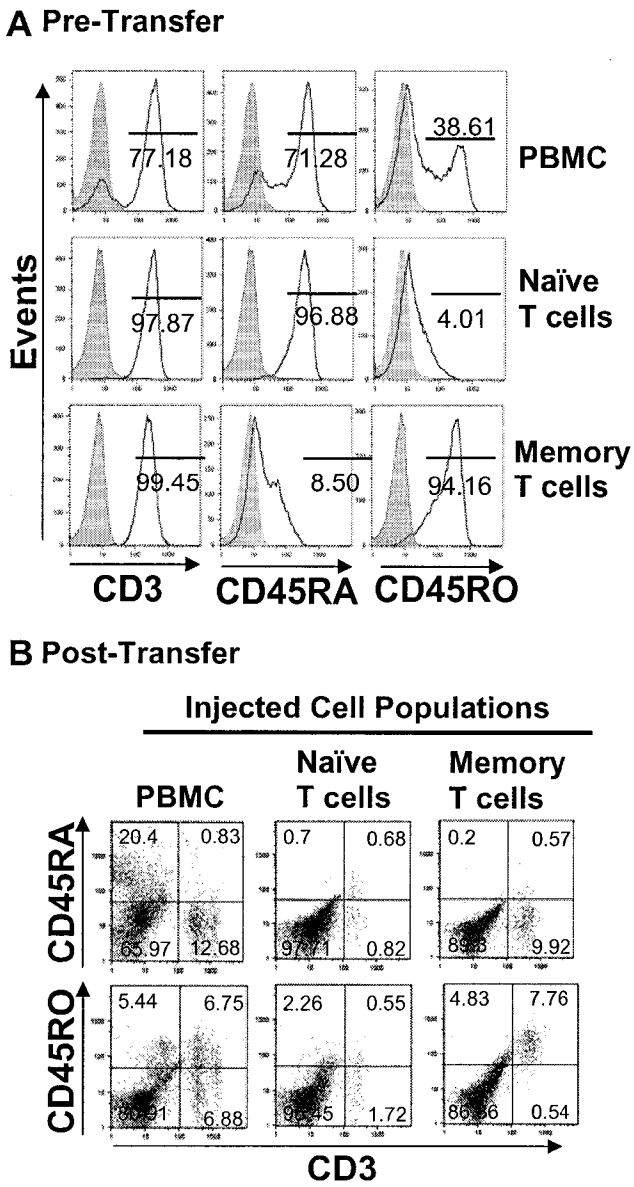

Figure 3.2. ***Injection of CD45RO+ (memory) T cells or CD45RA+ (naïve) T cells results in circulating human T cells in SCID-beige mice.*** CD45RA+ T cells (naive), CD45RO+ T cells (memory) (1.5×10^8 cells) or whole PBMC (3×10^8) were injected i.p. into SCID mice that had been grafted with skin allogeneic to the mononuclear cells. After 7 days, peripheral blood lymphocytes where then stained with anti-human CD3-FITC Ab and either anti-human CD45RA-PE (top panel) or anti-human CD45RO-PE and analyzed by flow cytometry. As seen in the top panel, only mice injected with naïve T cells or PBMC have circulating naive cells, whereas mice receiving only memory cells do not. The bottom panel shows that mice receiving naïve T cells, memory T cells or whole PBMC all have circulating memory T cells. The data shown represent one of five experiments with similar results (n=15 per group).

destruction following transfer of naïve T cells compared to memory T cells. Transferred CD45RA+ T cells produced only limited perivascular infiltrates whereas CD45RO+ T cells, like whole PBMC, infiltrated the dermis in a more diffuse pattern. Quantitatively, naïve T cells showed less total infiltration of CD3+ T cells compared to memory T cells (Figure 3.3C). Naïve T cells did not produce histological evidence of EC injury, whereas memory T cells, like PBMC, did do so (Figure 3.3C), even when mice given naïve cells were followed out to five weeks (data not shown). RNA transcripts encoding effector molecules (FasL, granzyme B, and perforin) were minimally expressed in the CD45RA+ inoculated mice compared to expression following either CD45RO+ T cells or unfractionated PBMC inoculation (Figure 3.3D). These data suggest that memory T cells largely account for the rejection response observed with whole PBMC since naïve T cells, though they can enter the circulation and focally infiltrate human skin grafts, do not express effector molecules or cause graft injury. Thus, my model provides a means to study human memory T cell alloresponses *in vivo*.

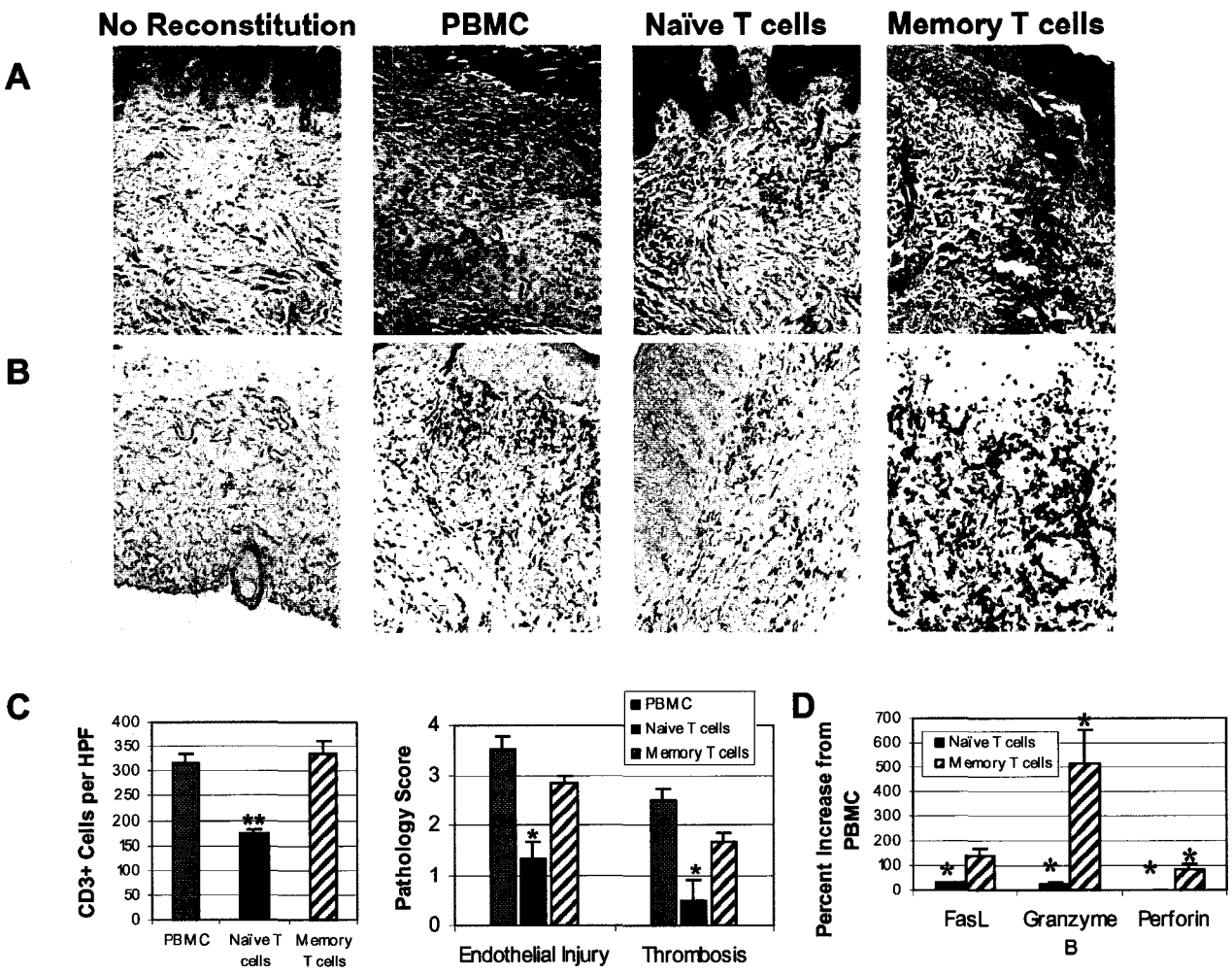

Figure 3.3. ***Reconstitution of mice with memory T cells, but not naïve T cells, exhibits a rejection response comparable to whole PBMC.*** Mice were reconstituted with CD45RA+ (naïve) T cells, CD45RO+ (memory) T cells, or PBMC and then grafts were then harvested at 21 days for analysis. (a) H&E staining demonstrates that mice reconstituted with memory cells exhibit an infiltration pattern (full dermis, ablation of the dermal papillae architecture) similar to whole PBMC, while naïve cells exhibit a much more focal, perivascular infiltrate that does not damage the dermal architecture (n=18) (b) CD3 staining of the infiltrated dermis confirms that the infiltrate observed in the H&E is human T cells. (c) CD3+ T cell counts showed a decreased number of naïve T cells infiltrating the skin graft compared to memory T cells and only the mice reconstituted with memory T cells or whole PBMC show significant endothelial damage and vascular thrombosis. (d) qRT-PCR of RNA isolated from grafts at Day 10 indicate that memory cells express much higher levels of FasL, granzyme B and perforin than naïve cells, though less than whole PBMC (n=9). Data are analyzed using standard curves generated from plasmids and normalized to CD3ε transcripts.

3.3 DISCUSSION

First, I show that human microvascular EC, in this instance HDMEC, preferentially activate allogeneic CD4+ and CD8+ T memory T cells, defined by expression of CD45RO, but not naïve T cells. Second, I demonstrate that following adoptive transfer in immunodeficient mice, memory but not naïve T cells can attack human microvessels in skin grafts allogeneic to the T cells.

Many previous studies examining the role of EC in T cell activation have focused on EC derived from large vessels (namely HUVEC) (208, 209, 327). However, in allograft rejection and many other inflammatory situations, it is not the macrovasculature, but rather the microvasculature, that may be responsible for the recruitment of inflammatory cells, initiation of the immune response and eventually the target of the alloresponse. Microvascular EC in rejecting grafts express adhesion molecules important for T cell recruitment, namely ICAM-1, VCAM-1 and E-selectin (191, 328) and may have prolonged expression compared to HUVEC (323). Furthermore, MHC class II was shown to be expressed basally on microvasculature in unstimulated, untransplanted hearts and was up-regulated following rejection episodes (329). The microvasculature has also been shown to activate CD4+ and CD8+ T cells, however they appear to possess different requirements for activating T cells compared to macrovascular EC (208, 215, 330). This difference may be due to a difference in costimulator expression. For example, HUVEC express the costimulatory molecules LFA-3 and CD40, but lack both B7 molecules, CD80 and CD86 (122); however, unlike HUVEC, microvascular EC from cardiac tissue express CD80 after CD40 ligation (191). I have not detected CD80 or CD86 on the HDMEC used in my experiments. Finally, microvascular EC are the primary target of

rejection in vascularized organ transplants (179). Due to the important role of the microvasculature in the alloimmune response outline above I focused my study on their behavior. I show in my study that HDMEC, like HUVEC, have the capacity to activate memory (CD45RO+) T cells, but not naïve (CD45RO-) to proliferate and produce cytokines.

Memory T cells are thought to play an important role in allograft rejection. In particular, memory T cells may have important roles in rejection resistant to immunosuppressants because the activation requirements of memory T cells appear to be less stringent that those of naïve T cells (318), and may be more difficult to inhibit and to tolerize (275, 293). Several previous studies have reported the presence of memory T cells (CD45RO+) in heart and kidney allograft biopsies and in the peripheral circulation correlated with the incidence and intensity of rejection (267, 331). Heeger et al. also showed that pretransplant frequency of donor-specific memory T cells correlates with risk of rejection post-transplantation despite high levels of immunosuppression (271). All these studies suggest that memory cells could play a large role in determining the survival of human allografts; however direct experimental evaluation in humans systems has not been possible. I establish here using purified human T cell subsets that the human PBL-SCID mouse model of skin allograft injury is an example of memory T cell-dependent allograft rejection. At three weeks following adoptive transfer of memory T cells, the grafts had intense mononuclear infiltrates and significant injury to the microvasculature, however naïve cells transferred to the same mice demonstrated no evidence of injury even when followed out to five weeks after the adoptive transfer.

In sum, I show that *in vitro* that HDMEC activate resting memory T cells and demonstrate that memory T cells are the major effector population of human allograft rejection following adoptive transfer into immunodeficient mice bearing human skin grafts. This chapter presents data introducing a small animal model to study human memory T cell reactions to allografts.

CHAPTER IV – TARGETING CD28 IN EC-MEDIATED T CELL ACTIVATION

4.1 INTRODUCTION

Optimal T cell activation requires the engagement of two signals, the T cell receptor (TCR) and a costimulatory molecule. The CD28/CTLA-4/B7 is perhaps the best-characterized costimulatory pathway. CD28 is a receptor expressed on resting CD4+ T cells, some CD8+ T cells, NK cells, neutrophils and eosinophils and binds to either B7.1 (CD80) or B7.2 (CD86), ligands which are expressed on professional antigen presenting cells (APC) (80). Binding of either of these ligands to CD28 increases IL-2 production, provides a survival signal for T cells, and regulates entry of T cells into the cell cycle (332, 333). Both B7.1 and B7.2 also bind to CTLA-4, an alternate receptor, with higher affinity than they do to CD28. CTLA-4 is primarily expressed by activated CD4+ and CD8+ T cells and, in contrast to CD28, CTLA-4 is thought to provide negative costimulation (80, 334), functioning to shut off T cell responses and perhaps contributing to the induction of tolerance. Soluble CTLA-4Ig can effectively inhibit T cell activation by competing with CD28 for B7.1 and B7.2 molecules (335). However, since CTLA-4Ig also prevents signaling via CTLA-4, it may prevent the acquisition of tolerance (336). Therefore, a reagent that selectively targets CD28 could be a more desirable immunotherapeutic than CTLA-4Ig because it would allow inhibitory CTLA-4 signals to be received in the absence of positive CD28 signals.

Human EC are not known to express B7 molecules and several studies demonstrated that their capacity to activate CD4+ and CD8+ T cells depends on the CD2-LFA-3 pathway (98, 209, 239). However, B7 molecule expression can be triggered on CD4+ T cells co-cultured with EC and these molecules may provide a costimulatory

signals to other T cells in trans (190). It is not known what role if any these trans B7 signals play *in vivo* nor for CD8+ T cells. In mice, EC express CD80, but not CD86 which is critical for the murine EC capacity to activate CD8+ T cells, but insufficient for murine EC to activate CD4+ T cells (216, 219).

One approach to selectively target CD28 is to use an antibody to this receptor. Using monovalent recombinant antibodies, Vanhove et al. showed that selectively targeting CD28 could in fact block a human MLR reaction (337). A rat model of transplantation using an antibody that induces CD28 internalization demonstrated that functional blockade of the CD28 pathway could induce tolerance (338). While effective *in vitro*, monovalent antibodies are unlikely to display sufficient avidity or half-life to be useful *in vivo*. Most intact antibodies to human CD28 have been agonistic, replacing and often exceeding rather than inhibiting CD80 and CD86 signals. Moreover, the properties of anti-CD28 mAb have been unpredictable, showing different effects *in vitro* than *in vivo*. In this chapter, I describe the properties of a humanized IgG2 antibody, designated FK734 (Astellas Pharma Inc.), that incorporates the variable regions derived from mouse antibody against human CD28 (clone TN228) and the constant regions from human IgG2 antibody with mutations in the Fc portion of the heavy chain of human IgG2 to prevent binding of the antibody to FcγR. These modifications are designed to make FK734 a candidate for use as an immunosuppressant in humans. I find that FK734 is a partial agonist *in vitro*. More importantly, this reagent reduces the extent of T cell-mediated damage to a skin graft in a chimeric human-mouse model of human allograft rejection *in vivo*.

4.2 RESULTS

FK734 stimulates T cell cytokine production and proliferation in co-culture with allogeneic EC or Mo.

I began my analysis of FK734 (anti-CD28 antibody) by examining its effect on CD4+ and CD8+ T cells separately co-cultured with allogeneic EC (HUVEC), APCs that do not express B7 and FcRγ molecules. HUVEC were treated with IFN-γ for 3 days to induce HLA-DR expression prior to co-culture with allogeneic, CFSE-labeled CD4+ T cells. HUVEC for co-culture with CD8+ T cells were untreated. Media was collected at 24 hours for measurement of cytokines and T cells were collected after 7 days for analysis of proliferation. Supernatants collected at 24 hours demonstrated that addition of FK734 to the T-EC co-culture increased production of both IFN-γ and IL-2 in comparison to a control IgG1 for both CD4+ (Figure 4.1A) and CD8+ (Figure 4.1C) T cells. In contrast, T cells co-cultured with EC and an anti-LFA-3 mAb (TS2.9), a reagent that blocks costimulation of human T cells via CD2, showed significantly less IFN-γ and IL-2 production (Figure 4.1A and C). I additionally examined the effects of FK734 in CD4+ and CD8+ T cell co-cultures with allogeneic Mo, a cell type that basically expresses low levels of HLA-DR, CD86 and FcRγ (339, 340) . T cells co-cultured with Mo show the same pattern of upregulation of IFN-γ and IL-2 secretion by FK734 and inhibition by TS2.9 compared to IgG1 (Figure 4.1B and D). Similar results were obtained at 48 hours for both CD4+ and CD8+ T cells (data not shown).

Proliferation of the T cells in T-EC or T-Mo co-cultures as assessed by CFSE dilution mirrored 24-hour cytokine production. Positively isolated CD4+ and CD8+ T cells were collected from co-cultures at day 7 and stained with anti-CD4 or anti-CD8

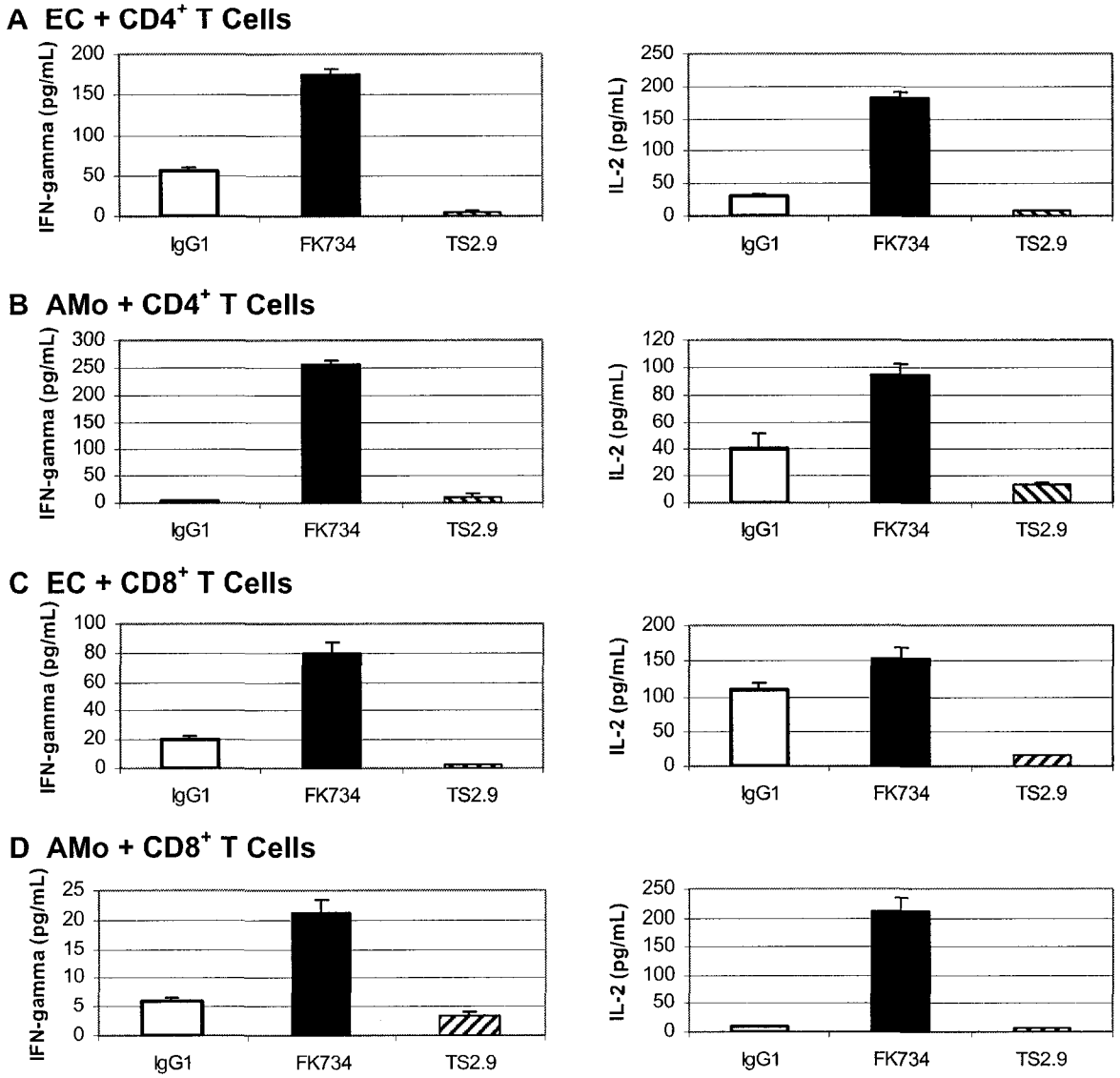

Figure 4.1. ***T-EC and T-Mo co-culture shows increased IFN-γ and IL-2 production in the presence of FK734.*** Positively isolated CD4+ T cells (A, B) or CD8+ T cells (C, D) were cultured in the presence of endothelial cells that were pre-treated with IFN-γ (A, C) or adherent Mo (B, D) and incubated with either IgG1 (2 ug/mL), anti-CD28 (FK734) (2 ug/mL), or anti-LFA-3 (TS2.9) (2 ug/mL). Supernatants were collected at 24 hours and assessed for IFN-γ and IL-2 by ELISA. T cells, EC and Mo alone all produced less than 5 pg/mL of IFN-γ and IL-2. Data represents one of five similar experiments.

antibodies and analyzed by FACS. Addition of FK734 significantly increased the number of proliferated cells in both the T-EC and T-Mo co-cultures compared to an IgG control (Figure 4.2A and B). TS2.9 was mildly inhibitory in the same assays. Thus, in allogeneic co-cultures, FK734 has agonist activity.

T cells co-cultured in the presence of B7.2-transduced endothelial cells show inhibition of IL-2 but not IFN-γ production and reduced proliferation with FK734.

Since addition of FK734 to standard T-EC or T-Mo co-cultures demonstrated agonistic effects, I sought to understand if the antibody could be inhibitory in a condition where strong signals via CD28 would be present at the time of initial activation. As EC do not express B7-molecules I transduced endothelial cells with retrovirus encoding B7.2 (CD86) and then examined the effects of adding FK734. B7.2 transduced EC plus IgG1 or FK734 were either pre-treated with IFN-γ for three days then co-cultured with positively isolated CD4+ T cells or directly co-cultured with positively isolated CD8+ T cells. When compared to EGFP-transduced EC, both CD4+ and CD8+ T cells co-cultured with B7.2-transduced EC produced greater amounts of IFN-γ (Figure 4.3A) and IL-2 (Figure 4.3B). Addition of FK734 further increased the level of IFN-γ production (Figure 4.3A) for CD4+ T cells, but inhibited IFN-γ secretion by CD8+ T cells (Figure 4.3D). Both CD4+ and CD8+ T cells showed a marked decrease in IL-2 production (Figure 4.3B and E). Consistent with the decrease in IL-2 there was also a significant decrease in proliferation of CD4+ and, to a lesser extent, CD8+ T cells co-cultured with B7.2 transduced EC and FK734 as assessed by CFSE dilution (Figure 4.3C and F). Therefore, it appears that in the absence of B7 signals (as on control EC) or in

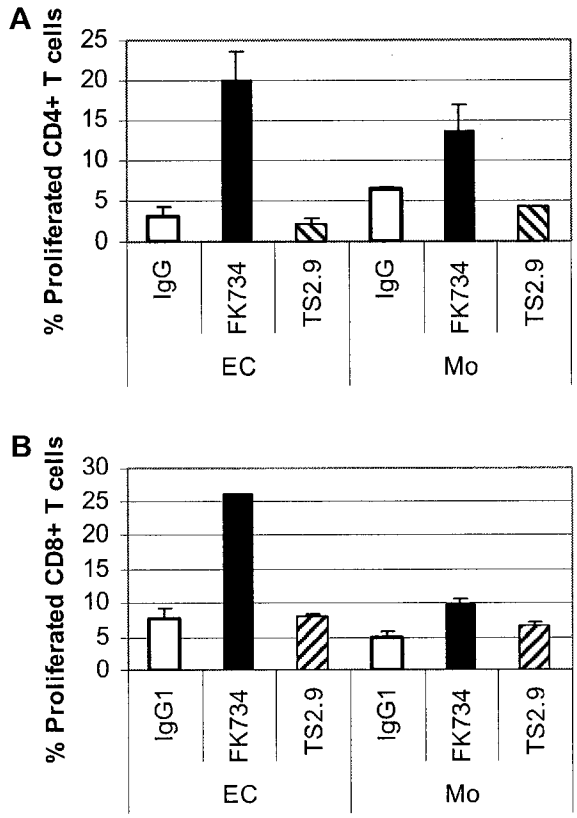

Figure 4.2. ***T-EC and T-Mo co-cultures show increased proliferation in the presence of FK734.*** Positively isolated CD4+ T cells (A) or CD8+ T cells (B) stained with CFSE were cultured in the presence of endothelial cells that were pre-treated with IFN-γ or adherent Mo and incubated with the indicated antibody. Co-cultures were harvested at Day 7, stained with anti-CD4 and subjected to FACS analysis. Data from five experiments were pooled and plotted.

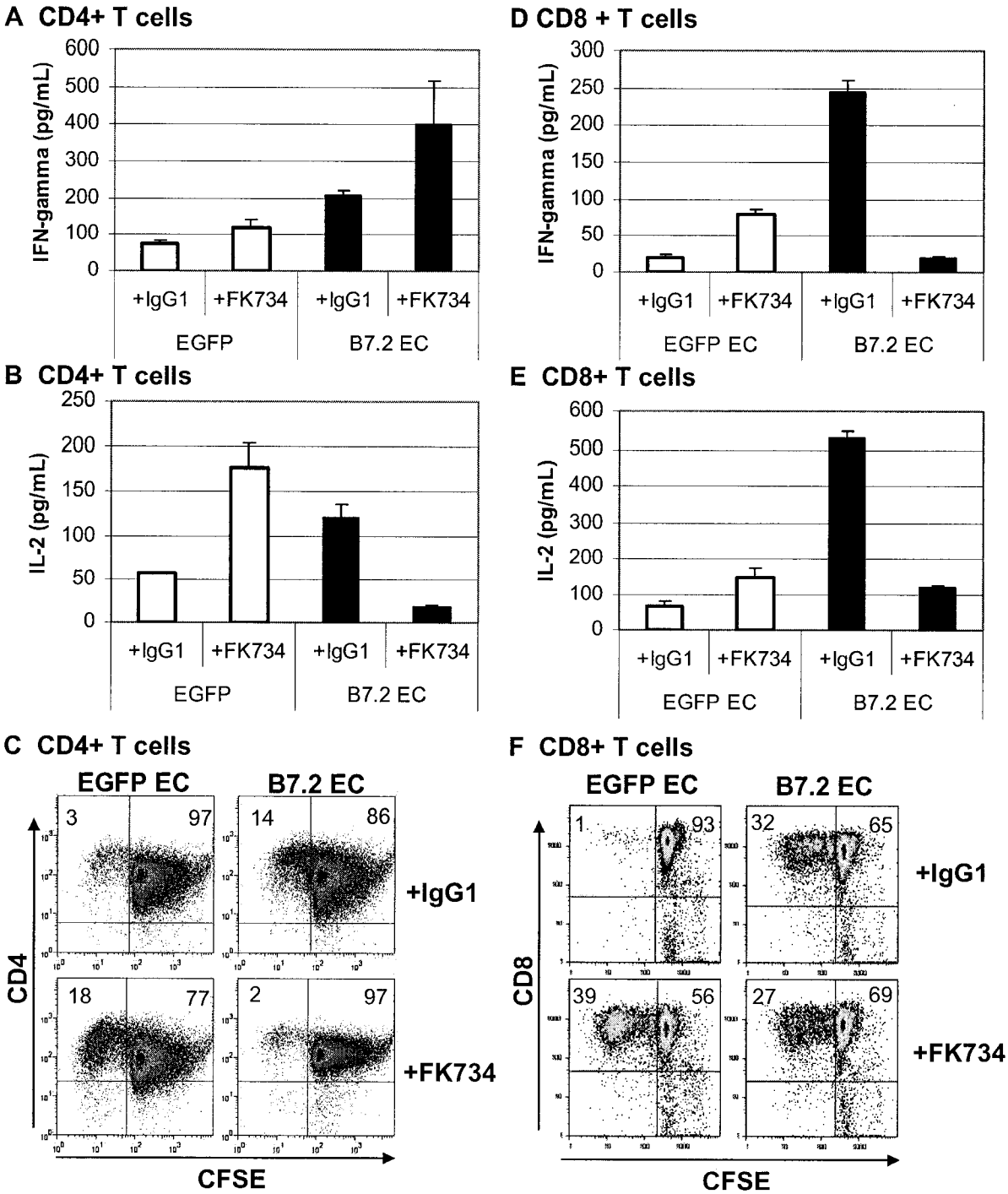

Figure 4.3. ***T cells co-cultured with B7.2 transduced EC in the presence of FK734 demonstrate decreased IL-2 production and proliferation.*** Positively isolated CD4+ (A, B, and C) or CD8+ (D, E, and F) T cells were co-cultured in the presence of EC transduced with retrovirus encoding human B7.2 (CD86) and pre-treated with IFN-γ and either with or without FK734 (2 ug/mL). Supernatants were obtained at 24 hours and assessed for IFN-γ (A, D) and IL-2 (B, E). Co-cultures were then harvested at Day 7, stained with anti-CD4 and subjected to FACS analysis (C, F). Data represents one of four similar experiments.

the presence of modest B7 signals (as on Mo, Figure 4.3S) FK734 has agonistic effects, however in the presence of a strong B7 signal (as on transduced EC), the same reagent has some antagonistic actions on CD8+ T cell IFN-γ production and both CD4+ and CD8+ T cell IL-2 production as well as proliferation.

FK734 stimulates less cytokine production and proliferation in comparison to another costimulatory anti-CD28 antibody (28.2).

I next compared FK734 to another anti-CD28 antibody with known costimulatory activity (clone 28.2). When either CD4+ or CD8+ T cells were co-cultured with allogeneic HUVEC, FK734 stimulated IFN-γ production at low doses, but did not stimulate further IFN-γ production with increasing concentrations. In contrast, 28.2 induced more IFN-γ production at similar concentration and this effect increased with higher levels of mAb (Figure 4.4A and D). FK734 stimulated significantly less IL-2 at all concentrations tested compared to 28.2 (Figure 4.4B and E). Consistent with the stimulation of less IL-2 production, FK734 also stimulated less proliferation at all concentrations when compared to 28.2 (Figure 4.4C and F).

FK734 inhibits the rejection response in the hu-SCID chimera.

Finally, I examined the effect of FK734 on an allogeneic response *in vivo*. I have previously described a model of human T cell responses to allogeneic skin microvascular EC *in vivo* involving human skin grafts and adoptive transfer of human PBMC, allogeneic to the skin donor in immunodeficient mice (179). I used qRT-PCR to compare the levels of expression of various components of the CD28 system in these

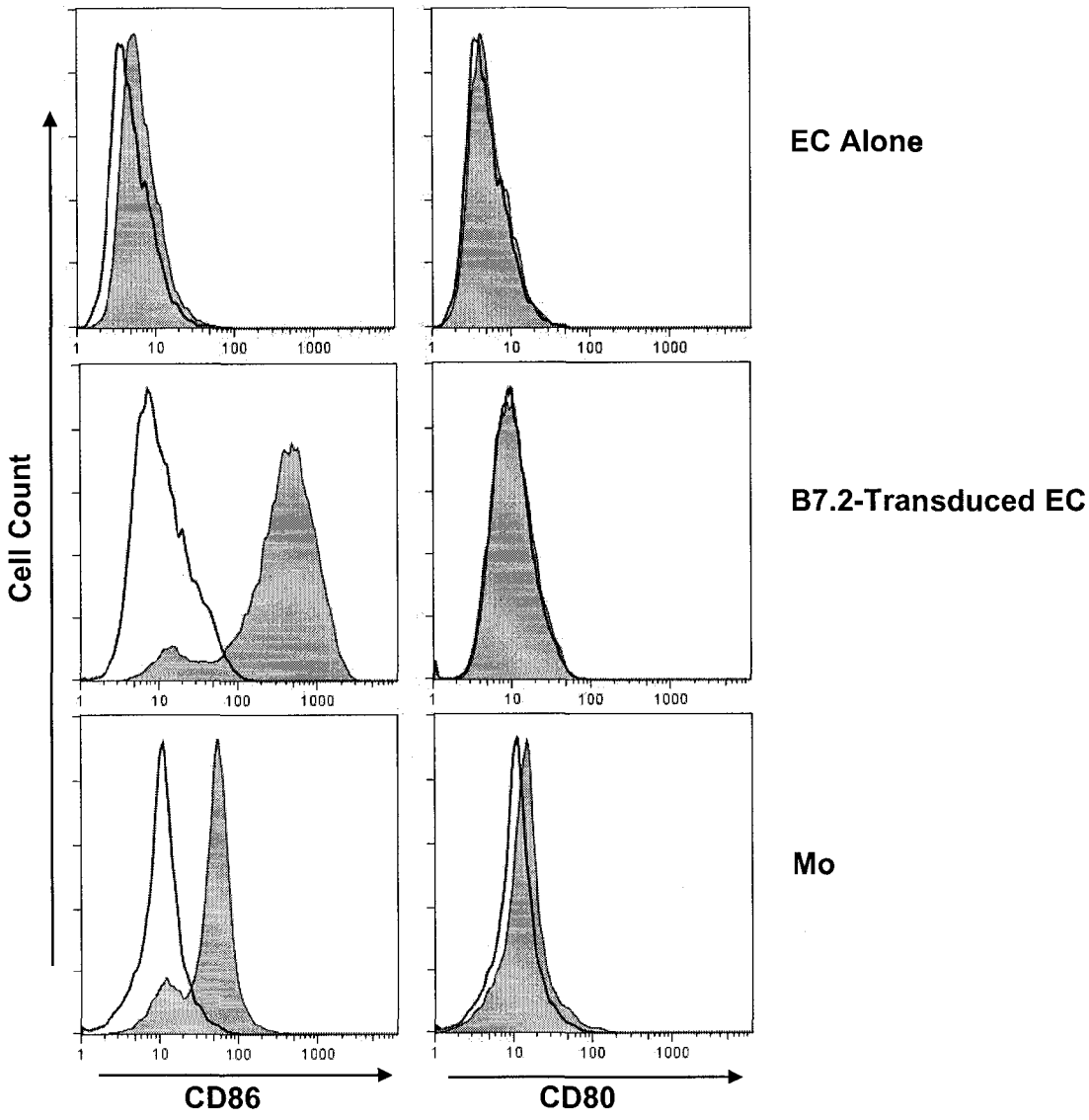

Figure 4.4. ***CD80 and CD86 expression on EC, transduced EC, and Mo.*** EC (top), EC-transduced with a retrovirus encoding B7.2 (middle) and Mo (bottom) were stained with anti-CD86 (filled histograms, left side of panels) and anti-CD80 (filled histograms, right side of panels) and subjected to FACS analysis. The open histograms are IgG1 for the indicated cell types. Data shown is one of three similar experiments.

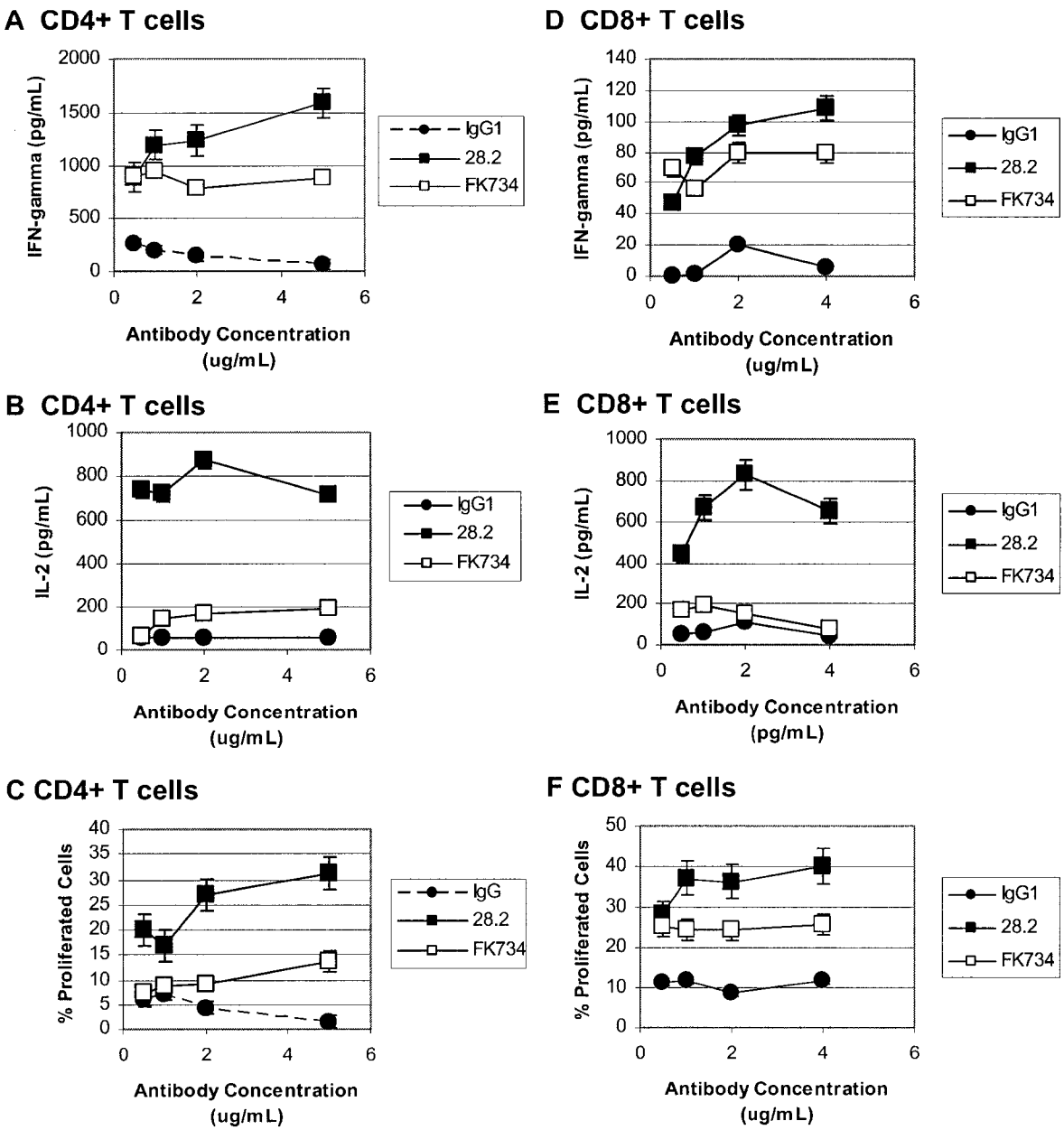

Figure 4.5. ***FK734 exhibits less costimulatory activity than another anti-CD28 antibody at similar concentrations.*** Either FK734 or 28.2 were added to co-cultures of CD4+ (A, B nd C) or CD8+ (D, E, and F) T cells and IFN-g pre-treated EC in the concentrations indicated. IFN-g (A, D) and IL-2 (B, E) production were assessed by ELISA after twenty-four hours of co-culture. Proliferation was assessed by CFSE dilution at 5 days using FACS analysis. The percentage of $CFSE^{lo}CD4+$ cells were plotted for each antibody concentration (C, F). Data represents one of three similar experiments.

human skin grafts (Table I-4) in the absence of PBMC inoculation. Levels of CD3ε, CD28, CTLA-4 (CD152) and B7.1 (CD80) were all very low, requiring approximately 30 cycles of amplification to reach the threshold of detection. B7.2 (CD86) was somewhat higher. The addition of PBMC led to a significant increase in all of these molecules except B7.2. These data suggest that CD28 and its principal ligands are present within rejecting skin grafts.

Previous experiments using blocking antibodies in this model demonstrated that some inhibitory antibodies could be given prior to adoptive transfer without effecting circulating T cell levels. However, initial pilot experiments with FK734 showed that administration of this antibody either immediately before or shortly after adoptive transfer (within 3 days) inhibited the successful transfer of human PBMC (Figure 4.5A). However, if I injected 3×10^8 human PBMC i.p. into mice and began administering 100 ug (2.5 mg/kg) of FK734, anti-LFA-3 (TS2.9) or IgG1 s.c. once every three days starting one week after adoptive transfer, then the inhibitory effect on adoptive transfer was minimized (Figure 4.5B).

After determining that delayed administration of FK734 does not significantly affect adoptive transfer of human PBMC, I examined the effect of the antibody on graft rejection mediated by the transferred PBMC. Split thickness human skin was grafted onto SCID-beige mice and allowed to heal for 4-6 weeks. Human PBMC were then transferred into mice at day 0, one week later on day 7 100 ug of IgG1, FK734 or TS2.9 were given to the mice. Another dose was given at day 10 and the grafts were harvested at day 14. Grafts were collected for RNA isolation or fixed in formalin, sectioned and taken for H&E staining. The resulting slides were scored blindly by a dermatopathlogist

	-PBMC	+PBMC	Fold Induction	p value
GAPDH	19.5±0.4	18.6±0.5	1.9	0.032
CD3e	31.4±1.0	28.4±0.4	8.1	0.002
CD28	29.5±0.7	27.6±1.3	3.8	0.45
CD80	31.3±1.2	28.8±0.7	5.4	0.013
CD86	27.2±1.1	27.6±0.7	0.7	0.58
CD152	31.1±1.0	28.8±1.1	4.9	0.013

Table I-4. *Expression of the CD28 family of molecules in skin grafts.*

RNA was isolated from skin grafts on mice that had either not received any PBMC (-PBMC) or received 3×10^8 PBMC (+PBMC) and were assessed for expression of the indicated mRNA by real time qRT-PCR as described in the methods. Values listed are threshold cycles and are the average from twelve mice (n=12).

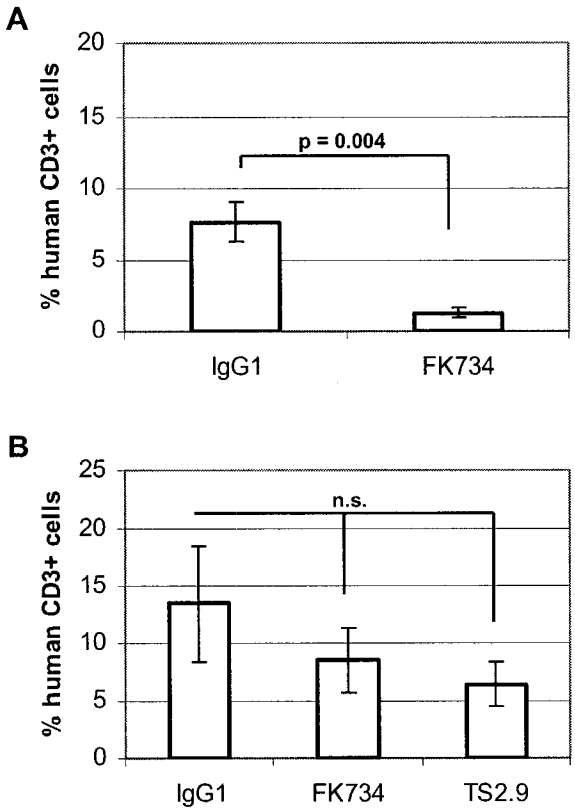

Figure 4.6. ***Delayed administration of FK734 in mice does not affect adoptive transfer of human T cells.*** Mice were given 250 ug of either IgG1, FK734 or TS2.9 at either the same time as the adoptive transfer (A) or one week following adoptive transfer (B). On Day 14, adoptive transfer was assessed by staining blood obtained via retroorbital bleed for human CD3 and mouse CD45. The percentage of CD3+ and mCD45- cells was assessed by FACS and plotted. Data represents twelve mice (n=12) per group.

(JMM). Administration of FK734 reduced endothelial injury and thrombosis when compared to the IgG control at one week (Figure 4.6A) and only endothelial injury at two weeks (Figure 4.6B). The reduction in injury was comparable to that observed with TS2.9, whose protective effects have been described previously (109).

Further analysis of the grafts using immunohistochemistry to quantitate the number of CD3+ cells and CD31+ vessels showed an increased presence of CD31+ vessels at one week ($p<10^{-5}$, Figure 4.6D) and two weeks ($p<0.002$, Figure 4.6D) compared to IgG1 and decreased infiltration of CD3+ cells at one week ($p<10^{-4}$, Figure 4.6C) and two weeks ($p<0.008$, Figure 4.6C) compared IgG1. I have previously shown that rejection in this model is largely dependent on memory T cells in that when PMBC are fractionated into memory (CD45RO+) and naive (CD45RA+) subsets that only memory T cells can mediate graft rejection (see chapter III). Thus, the data presented here suggests that CD28 may play a role in this largely memory T cell-dependent response.

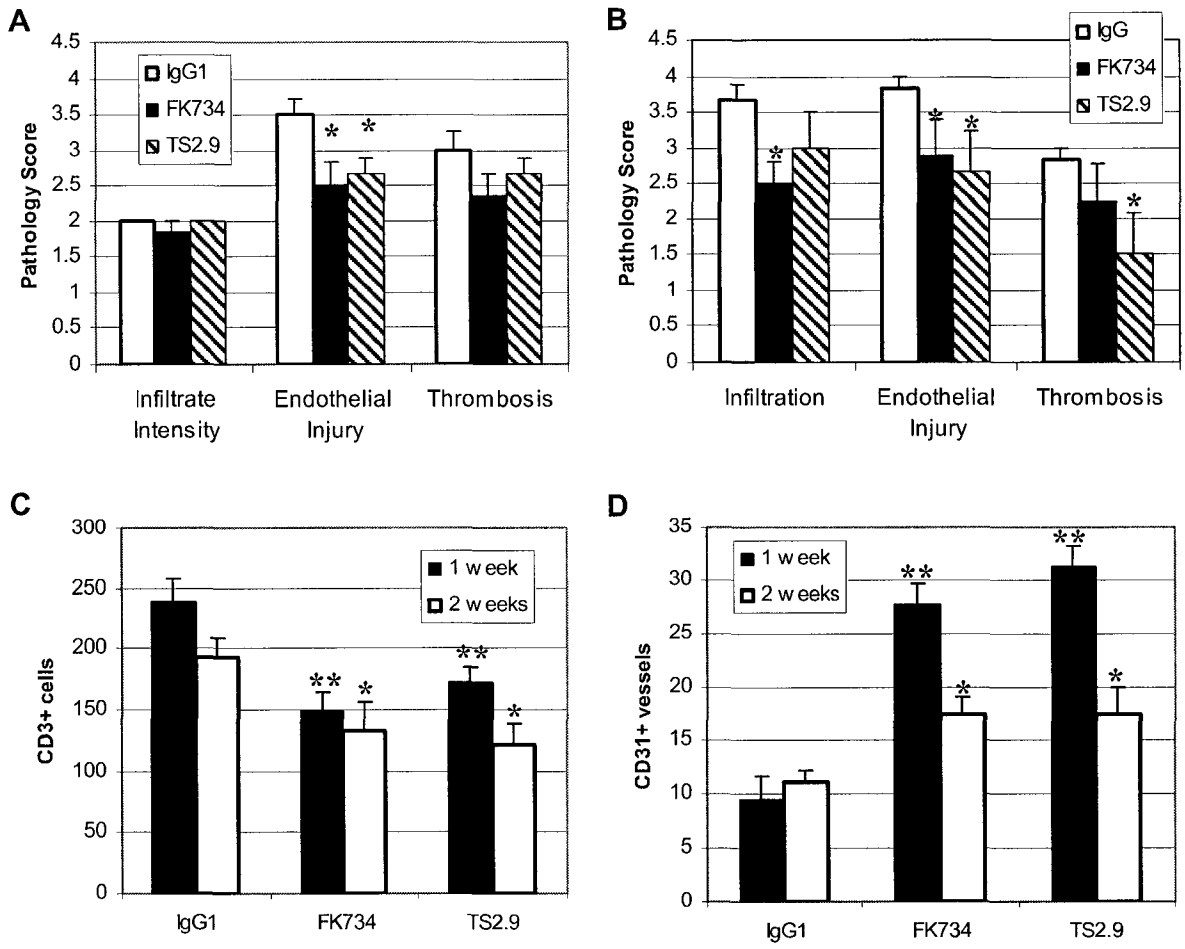

Figure 4.7. ***Pathology scoring, vessel counts and cell counts of day 7 and 14 grafts demonstrates decreased endothelial injury and significant reduction in infiltrating T cells with administration of FK734.*** Grafts from either day 7 (A) or day 14 (B) were scored blindly by a dermatopathologist on three criteria, infiltration, endothelial injury, and thrombosis using a semiquantitative scoring scale with 4 indicating 100% graft involvement and 0 indicating 0% of graft involvement. Data shown is pooled data from four separate experiments. Grafts harvested at either day 7 or day 14 were sectioned and stained for CD3 (C) and CD31 (D). Human CD31 positive vessels and human CD3 positive T cells were scored by counting 10 high powered fields per graft.

4.3 DISCUSSION

The primary goal of the studies in this chapter was to characterize a humanized antibody to human CD28 that may have utility as an immunosuppressive in a model of memory T cell mediated allograft rejection. This reagent, designated FK734, has both agonistic and antagonistic effects *in vitro*, depending on the strength of the CD80/CD86 signals provided by the APCs. Interestingly, the antagonistic effects of FK734 are most evident on production of IL-2 and proliferation in CD4+ T cells whereas the antibody seems agonistic for IFN-γ production even in the presence of strong B7 signals. However, under the same conditions, FK734 antagonizes both IFN-γ and IL-2 production in CD8+ T cells. The basis of these differences is unknown. Since human CD8+ T cells in peripheral blood are heterogeneous for expression of CD28, my data suggest that the CD28 expressing subpopulation of CD8+ T cells contribute significantly to cytokine production in allogeneic responses of this subset. Most importantly, I demonstrate that in an *in vivo* model of human T cell-mediated allograft rejection this antibody can reduce infiltration and endothelial damage, hallmarks of graft rejection.

Understanding the role of CD28/B7 interactions *in vivo* initially came from studies performed in the transplant setting. CTLA-4Ig was one of the first reagents identified that could selectively target the B7 pathway. Part of the mechanism of this reagent depended on the ability of CTLA-4 to bind with much higher affinity to B7 molecules, effectively blocking CD28 interactions (335). In mice, CTLA-4Ig treatment was described to prolong graft survival and in some cases could even induce donor specific tolerance (341). However, CTLA-4Ig and other reagents designed to block the interactions of B7 with CD28 may simultaneously prevent negative or regulatory signals

from being transmitted via CTLA-4. Signaling through CTLA-4 independent of its competition with CD28 has been demonstrated to decrease signaling from the CD3 complex in a transgenic rodent model (342). Also, it has been shown that CTLA-4 has a role in the development of regulatory T cells in the transplant setting (343). Thus, considering the importance of the signals from CTLA-4, targeting CD28 alone may prove more effective. While there is extensive murine and non-human primate data studying the effects of reagents targeting CD28, human data has been limited to mostly *in vitro* studies (336, 337). My chimeric mouse model provides *in vivo* data with human T cells using a unique human specific reagent. I find that despite retaining some agonist activity, a CD28-selective antibody can reduce T cell infiltration and endothelial damage in an acutely rejecting organ. My *in vitro* data suggest this may be due to decreased T cell proliferation secondary to the effects of FK734 on IL-2 production in CD4+ and CD8+ T cells receiving a strong B7 signal.

One concern is that the partial agonist activity may boost levels of IFN-γ present in animals treated with FK734. Previous data from my lab has shown that microvascular endothelial cells in human skin grafts retain both MHC class I and II expression in situ in the absence of exogenously added PBMC (179), perhaps due to low levels of IFN-γ produced by the small number of T cells resident in skin (see Table I-4 for basal CD3ε). Increased IFN-γ production provided by PBMC could further enhance expression on endothelial cells and induce de novo expression on other cell types. Such an effect could theoretically amplify the allogeneic T cell response to the skin graft. Despite this concern, the data show that the overall effect of FK734 *in vivo* is inhibitory and

equivalent to that of the anti-LFA-3 antibody, TS2.9, which inhibits IFN-γ production *in vitro*.

An anti-CD28 mAb may function through several different mechanisms. First, the antibody might block interaction between CD28 and its natural ligands, B7.1 and 2, either by steric hindrance or by down modulating CD28 expression. Second, the antibody could act as an agonist and stimulate CD28. Third, especially *in vivo*, the antibody may deplete CD28+ cells. The vast majority of intact antibodies reactive with human CD28 described to date have been agonistic with the extent of signaling downstream of CD28 related to the degree of crosslinking of the receptor (337). Therefore, it is not surprising that a modified monovalent antibody that selectively targets human CD28 and does not crosslink was shown to inhibit T cell activation in a mixed lymphocyte reaction (337). Further, murine models have shown that CD28 Fab fragments, which inhibit CD28 signals, ameliorate EAE and uvoretinitis (344, 345). In fact, selective blockade of CD28 in the rat was shown to have greater immunosuppressive effects than CTLA-4Ig in part through the generation of non-T regulatory cells and the induction of indolemine-2,3-dioxygenase (IDO) and iNOS (338). In addition to blockade of CD28, there have been reports that suggest the agonistic effects of a CD28 antibody may also be paradoxically immunosuppressive through depletion of activated T cells (334) or, in the case of superagonists, selective activation of regulatory T cells (346). My mouse model cannot support T cell depletion via ADCC, presumably because of defects in NK cell-mediated killing due to the beige mutation. FK734 also contains structural modifications to prevent FcR binding. I cannot rule out inhibition of tissue injury by induction of regulatory T cells and my data indicates the presence of mRNA for CTLA-4

in rejecting grafts. This possibility warrants further investigation although I do not yet know if regulatory T cells are generated or function in my model.

In chapter III, I show that the mouse model of skin graft rejection I utilized in this study is mediated principally by memory T cells. Memory T cells have special properties compared to naïve T cells including lower activation threshold, faster acquisition of effector function, enhanced proliferation potential and increased survival (16). Many of the properties of memory cells reflect changes in the TCR mediated activation of memory T cells part of which includes changes in the costimulation requirements (318). The CD28/B7 pathway has been studied in the generation and function of memory T cells and mouse models have indicated that CD28 signals are not required to mount a CD8+ T cell response, but may be important in the CD4+ T cell response (347). My data suggest that CD28 signals do play a role in the ability of human memory T cells to mount an immune response in a skin graft rejection model. This finding is interesting in that microvascular human EC, the primary target of this alloresponse, do not express detectable levels of B7.1 or B7.2. Thus the cellular source of the CD28 signals in this model is unknown, though my data shows the presence of B7.2 molecules in the graft at baseline and induction of B7.1 with the introduction of PBMC. It is possible that T cells themselves provide B7 signals to other T cells (190). Attempts to confirm a role for CD28 by using CTLA-4Ig in the same model were confounded by an unexpected enhancement of T cell engraftment and skin graft infiltration and injury (SS and JSP, data not shown). This effect is under investigation.

FK734 has been engineered to be a candidate for use in humans as an immunosuppressant. My *in vivo* experiments provide evidence that this antibody may

function as an immunosuppressant despite its partial agonist actions *in vitro*. Preclinical studies in a humanized mouse model may be particularly important given recent clinical trials that demonstrated severe, unanticipated toxicity for another CD28 antibody, deemed a "superagonist" that was recently tested in a phase I trial in the UK (348). In that trial, all six patients given an experimental anti-CD28 antibody TGN1412 became extremely ill even though the antibody had been tested and deemed safe in non-human primates (349). It would be interesting to examine if TGN1412 induces cytokine release or exacerbates tissue injury in my model of human allograft rejection.

In summary, I describe a humanized antibody that targets CD28 and has both agonist and blocking capability. Furthermore, I demonstrate that this reagent has the ability to reduce endothelial cell injury in a model of human memory T cell mediated graft rejection. This both suggests the potential of such a blocking reagent in human disease and demonstrates a role for CD28 in human memory T cell responses.

CHAPTER V – MEMORY T CELL SPECIFIC COSTIMULATORS IN EC-MEDIATED T CELL ACTIVATION

5.1 INTRODUCTION

I have shown in the previous chapter that human dermal EC can preferentially activate memory T cells and that memory T cells, but not naïve can mediate allograft rejection in a chimeric human-SCID mouse model of allograft rejection. The capacity for EC to activate memory, but not naïve T cells, may arise from differences in the co-stimulatory signals provided by EC to T cells. This possibility is explored further in this chapter.

The T cell response to antigen depends both on TCR signals (provided by peptide-MHC molecule complexes) and antigen-independent costimulatory molecules. The best described T cell receptors for costimulation are CD28 (which binds B7-1 and -2, also known as CD80 and CD86, respectively and is discussed in the previous chapter) and CD2. CD2 binds CD48 in rodents, but is preferentially activated in humans by LFA-3 (CD58), a molecule missing from the rodent genome (350). Human EC express LFA-3 (but generally not B7-1 or -2) and the LFA-3-CD2 pathway seems to be particularly important in human allogeneic responses to this cell type. However, antibody blocking experiments have suggested that LFA-3 does not account for all of the costimulation that human EC provide to memory T cells (208, 209). Several newly discovered receptors for costimulators in both the B7 family (such as ICOS) and the TNF family (such as 4-1BB and OX40) are important for the generation of effector/memory T cells (140, 160, 351, 352) and ligands for these molecules can be expressed at high levels on cytokine-activated human umbilical vein EC (HUVEC) (86, 152). It is not know if

any of these molecules also contribute to the reactivation of resting memory T cells by human microvascular EC.

The above observations suggest that circulating human alloreactive memory T cells may effectively recognize graft EC *in vivo* and may do so in a manner dependent upon costimulators other than LFA-3, CD80 or CD86. I demonstrate here that ICOS ligand (ICOSL), OX40 ligand (OX40L), and 4-1BB ligand (4-1BBL) are inducible on cultured human dermal microvascular EC (HDMEC) and that all three molecules contribute to EC-dependent memory T cell activation *in vitro* and graft rejection *in vivo*.

5.2 **RESULTS**

HDMEC inducibly express ICOSL, 4-1BBL, and OX40L

LFA-3 is an important costimulator expressed by HUVEC, but blocking the LFA-3 interaction with its ligand, CD2, does not fully inhibit proliferation or cytokine production by allogeneic T cells (98). To characterize additional molecules involved in costimulation, I initially examined HDMEC with or without treatment with pro-inflammatory cytokines (at doses and times shown to be optimal for other HDMEC responses), for the expression of three molecules proposed to act preferentially on activated T cells, namely 4-1BBL, ICOSL, and OX40L (CD134L). Specifically HDMEC were mock- treated or treated with either TNF (10 ng/mL) or IFN-γ (50 ng/mL) for 24, 48, or 72 hours and collected for quantitative real-time (qRT)-PCR and FACS analysis. Mock-treated HDMEC expressed minimal transcript levels and surface expression for all three of these memory-selective costimulators. Transcript levels of all three molecules were increased many-fold at 24 hours and remain elevated for 4-1BBL and OX40L, but not ICOSL, over 72 hours (Figure 5.1A). There were some differences between surface expression and mRNA expression. 4-1BBL surface expression only increased modestly, while ICOSL surface expression increased substantially with TNF treatment (Figure 5.2B). TNF treatment shifted OX40L, which has high basal expression, from a heterogeneous population of low and high expressors to a mostly high expressing population leading to an overall increase in OX40L expression assessed as corrected mean fluorescence intensity (Figure 5.2B). IFN-γ did not affect costimulator mRNA or protein for any of the molecules (data not shown).

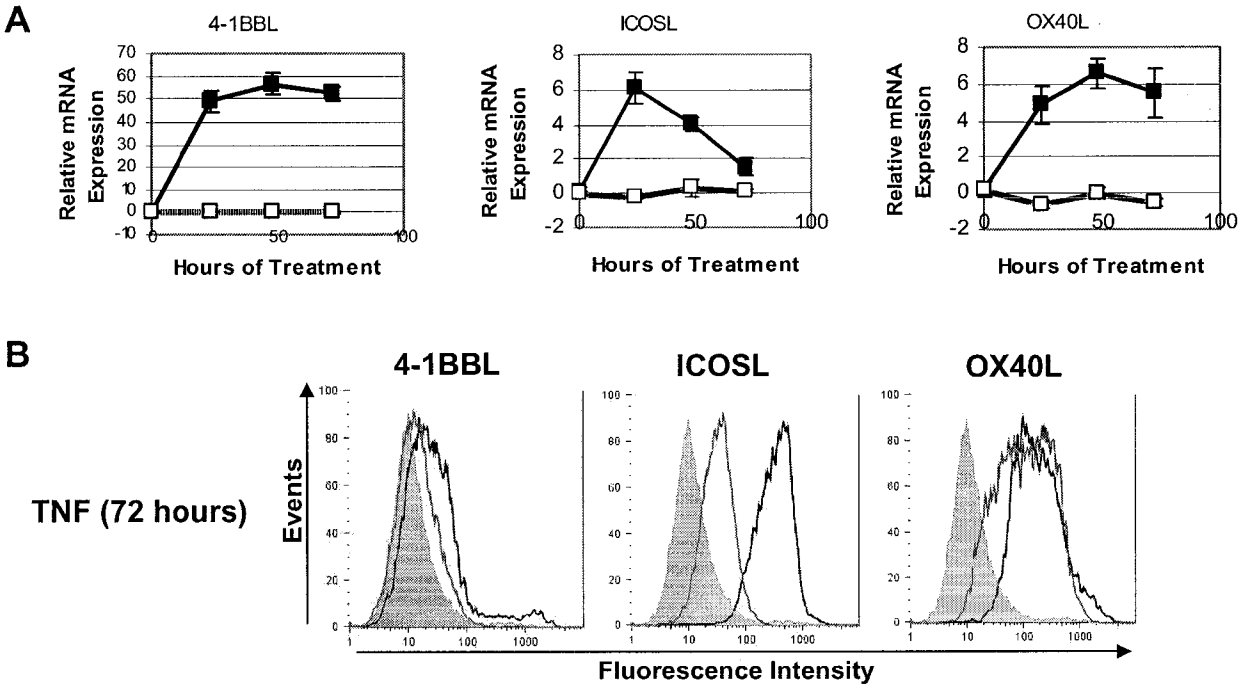

Figure 5.1. ***HDMEC express inducible memory T cell-selective costimulatory molecules.*** HDMEC were cultured with cytokines for the indicated times and mRNA levels were calculated as a percent change from untreated cells. (a) HDMEC cultured with TNF (10 ng/mL) showed a 50-fold increase in mRNA for 4-1BBL at 24 hours that remained elevated for 72 hours. 6-fold and 8-fold increases in mRNA were observed for ICOSL and OX40L respectively at 24 h that remained elevated for 72h for OX40L, but slowly declined for ICOSL over the same time period (*filled squares*). HDMEC cultured with IFN-g (50 ng/mL) showed no increase in mRNA for either 4-1BBL, ICOSL or OX40L over 72 hours (*empty squares).* The data shown are pooled from four separate experiments. (b) By FACS analysis, 4-1BBL, ICOSL, and OX40L all show increased surface expression (*black line*) with 72 hours of TNF treatment compared to control, untreated EC (*dark grey line*). IgG controls which do not change with cytokine treatment are shown as filled histograms. The data shown represent one of four experiments with similar results.

I next evaluated whether these three costimulators molecules are induced when CD4+ T cells are co-cultured with IFNγ-pretreated allogeneic HDMEC. Using qRT-PCR analysis, I found that mRNA for 4-1BBL, ICOSL and OX40L all increase in HDMEC after co-culture with allogeneic T cells. The induction of 4-1BBL and OX40L was most pronounced at 48h with almost a hundred-fold induction, while ICOSL showed a more modest 20-fold induction (Figure 5.2A). Surface expression was then assessed by FACS. 4-1BBL increased modestly at day 1 and steadily increased through day 7. ICOSL showed immediate increase by day 1 and also continued to increase throughout the co-culture. OX40L, in contrast, increased at day 1, but then returned to baseline by day 3 (Figure 5.2B). T cells recovered from the same co-cultures at day 1, 3, 5 and 7 express increasing levels of the corresponding receptors (4-1BB, ICOS, and OX-40) to these EC ligands on cells that had proliferated, although OX40 expression actually declined by day 7. Surprisingly, these molecules were also increased in expression on some T cells that had not proliferated, as assessed by dilution of CFSE fluorescence (Figure 5.2C). I do not know if this is a bystander response to cytokines or a response to suboptimal TCR stimulation.

Blocking 4-1BBL, ICOSL or OX40L in T cell-EC co-cultures decreases T cell activation

The parallel induction of the T cell receptors and their EC costimulators suggest that these interactions could contribute to memory T cell activation by allogeneic HDMEC. I therefore examined the effect of blocking these pathways using antibodies and/or fusion proteins in T cell-EC co-cultures. In my experiments, HDMEC were treated with IFN-γ for 3 days (to induce HLA-DR) for CD4+ T cell stimulation or mock

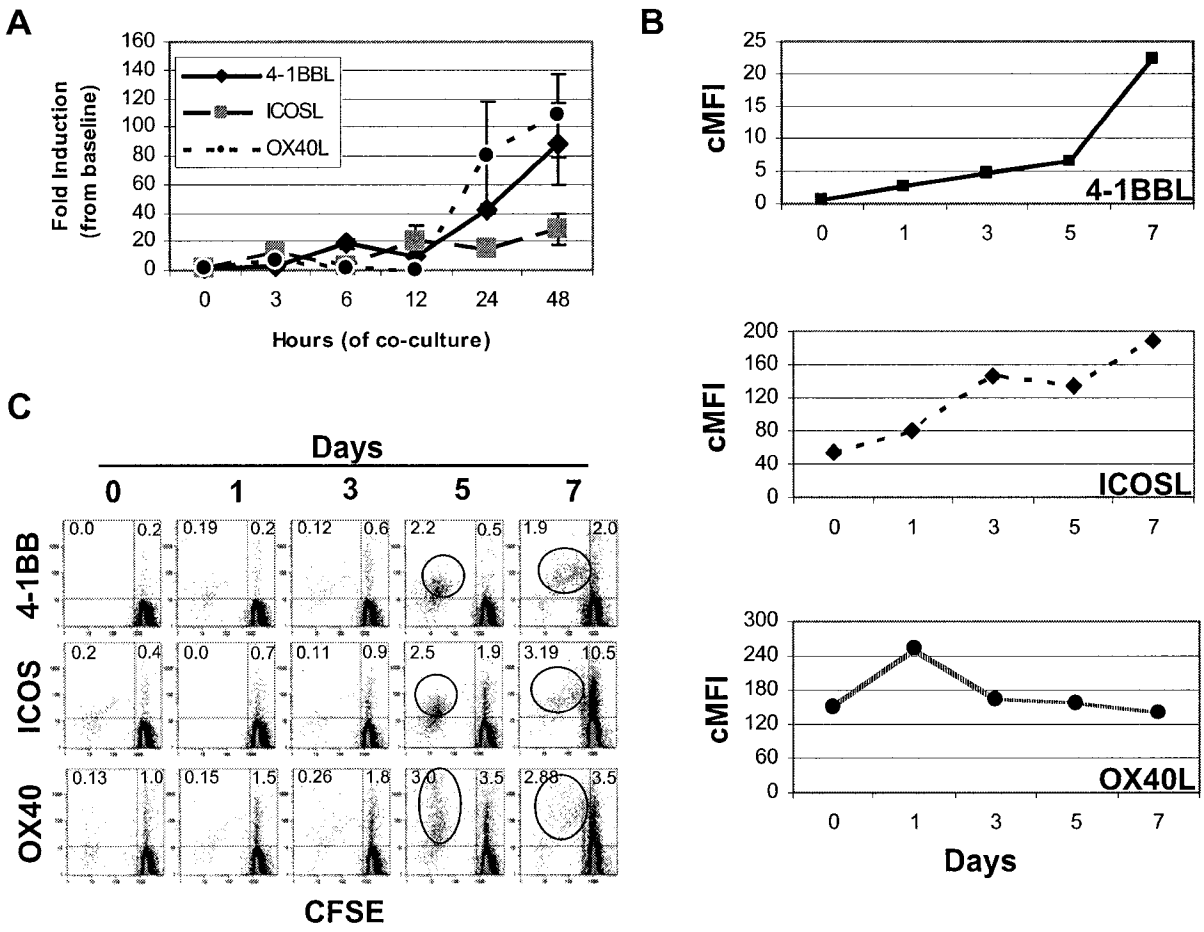

Figure 5.2. ***HDMEC upregulate memory costimulatory molecules in co-culture with T cells and stimulate T cells to express the corresponding ligands.*** HDMEC were co-cultured with allogeneic T cells and cells were harvested at 3, 6, 12, 24, and 48 hours for RNA and at Day 7 for FACS analysis. (a) HDMEC upregulate for mRNA 4-1BBL (*filled circle*), ICOSL (*grey square*), and OX40L (*filled diamond*) over the course of 48 hours. (b) 4-1BBL, ICOSL and OX40L protein expression on HDMEC after 1, 3, 5, and 7 days of co-culture with allogeneic T cells (cMFI = corrected mean fluorescent intensities, i.e. cMFI = Ab MFI - IgG control). (c) Both proliferating T cells (*circled population)* and non-proliferating T cells identified by CFSE dye dilution, show increasing expression of 4-1BB, ICOS, and OX-40 at day 1, 3, 5, and 7 of co-culture (numbers represent percentage of total T cell population). The data shown represent one experiment of four with similar results.

treated for CD8+ T cell stimulation and then co-cultured with allogeneic T cell subsets. Pilot titrations were done in T cell-EC co-cultures to determine maximally inhibiting doses of each antibody and fusion protein typically between 4-10 μg/mL (data not shown). Cytokines were assessed by ELISA in the supernatants collected from the co-cultures at 24 hours. By ELISA, I found that blocking either ICOSL or OX40L with specific antibodies led to a substantial decrease in IFN-γ production by CD4+ T cells, while blockade of 4-1BBL with a fusion protein had little effect (Figure 5.3A). IL-2 production by CD4+ T cells showed similar decreases (Figure 5.3A). In contrast, blockade of any of the three costimulators in CD8+ T cell-EC co-cultures led to at least a 70% decrease in IFN-γ production (Figure 5.3A) when compared to control antibody. Overall, CD8+ T cells produce substantially less IL-2 and exhibited a lesser decrease in IL-2 production than CD4+ T cells with blockade of any of the three costimulators (Figure 5.3A). T cell proliferation was also reduced by blocking each of these costimulators. For CD4+ T cells, blocking 4-1BBL or OX40L decreased proliferation by almost 35% and blocking ICOSL led to a decrease of almost 45% at day 5 (Figure 5.4A). CD8+ T cells also demonstrated decreased proliferation, with 4-1BBL blockade reducing proliferation by 40% and ICOSL or OX40L blockade causing lesser, but significant reductions of 26% and 20% (Figure 5.4B). My results suggest that these costimulators can contribute to resting memory T cell activation by allogeneic HDMEC. In several experiments, combined blockade did not appear more effective than blockade of individual costimulators (data not shown). The effects of blocking these pathways were comparable in magnitude to those attained with blockade of LFA-3 (Figure 5.3 and data not shown). Overall, my *in vitro* experiments demonstrate that allogeneic HDMEC, like

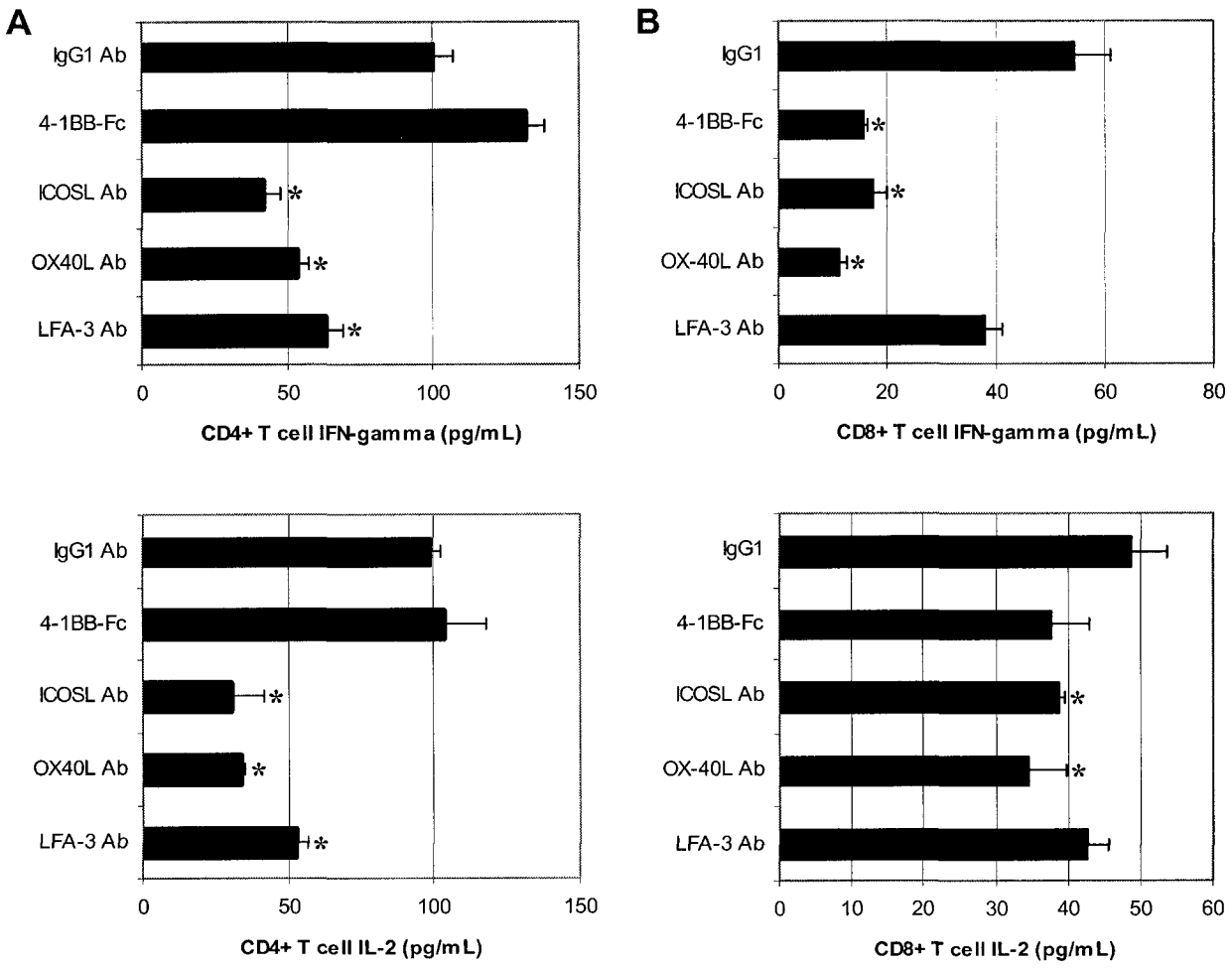

Figure 5.3. ***CD4+ and CD8+ T cell production of IFN-g and IL-2 in allogeneic T-EC co-cultures is decreased when memory costimulator molecules are blocked.*** CD4+ (a) or CD8+ (b) T cells were co-cultured with allogeneic HDMEC pre-treated with IFN-g for 3 days (to upregulate MHC Class II). Cytokine production was then assessed by ELISA on supernatant collected at 24 hours. (a) CD4+ T cells exhibit >50% reduction of both IFN-g and IL-2 production in the presence of blocking abs to ICOSL or OX40L, but not in the presence of the blocking fusion protein 4-1BB-Fc. Combinations of antibodies do not show additive blocking effects. (b) CD8+ T cells demonstrate a large decrease in IFN-g production in the presence of all three blocking reagents, but only modest a decrease in IL-2 production. The data shown represents one of four experiments with similar results.

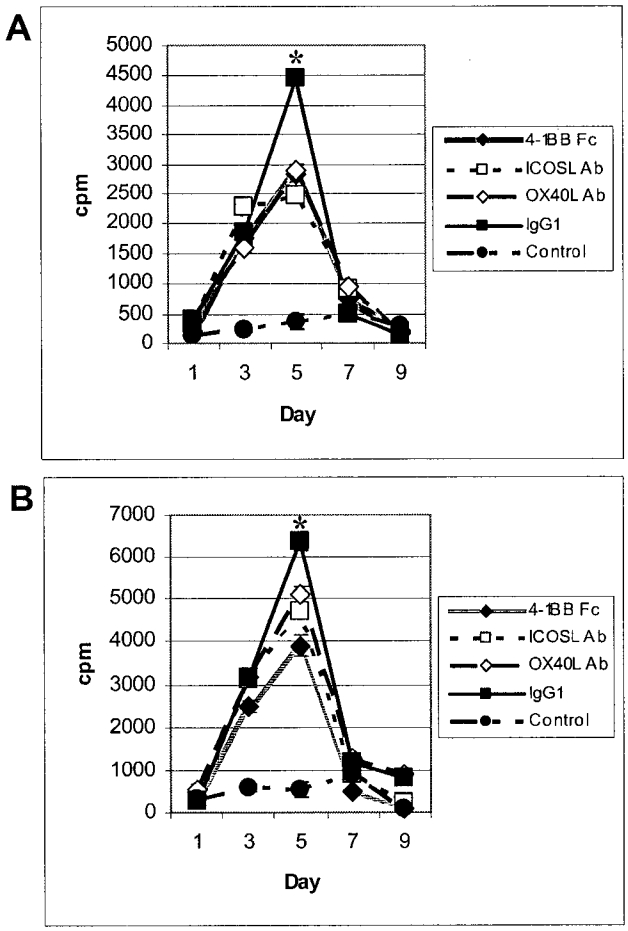

Figure 5.4. ***CD4+ and CD8+ T cells show decreased proliferation in the presence of blocking reagents to the memory costimulatory molecules.*** Thymidine was added to the co-cultures 24 hours prior to the time points indicated and the control is T cells alone. (a) CD4+ T cells were co-cultured with allogeneic HDMEC that had been pre-treated with IFN-g for 3 days ($p<0.02$ for all points at Day 5). (b) CD8+ T cells were co-cultured with allogeneic HDMEC ($p<0.02$ for all points at Day 5). The data shown represent one of five experiments with similar results.

HUVEC, selectively activate memory T cells and that several newly described costimulators, which are inducible on HDMEC, contribute to the alloresponse.

Memory T cell-selective costimulators contribute to human allograft injury in vivo

To evaluate the function of the memory T cell-EC costimulatory interaction *in vivo*, I again used my model of human skin allograft rejection in immunodeficient mice. I first investigated whether skin grafts in my alloimmune response express these ligands. Although basal expression was low, I found a large increase in the mRNA expression of 4-1BBL and ICOSL following PBMC transfer; OX40L showed a more modest increase (Figure 5.5A). These increases may be explained by the presence of TNF in the circulation following adoptive transfer of human PBMC (353). Unfortunately, the reagents available for FACS analysis of cultured cells are unable to detect these molecules in tissues so I could not directly assess protein expression in the skin grafts.

To investigate the role of memory T cell-selective costimulators in the progression of immune-mediated damage in human skin grafts, i.p. injections of blocking antibodies or IgG control were administered three times a week to groups of animals with healed human skin grafts starting on the day before i.p. inoculation with PBMC. The dosing schedule used was one that gives circulating trough levels of Ig sufficient to match *in vitro* levels. Within each experiment, all skin grafts were obtained from a single donor and all PBMC came from a second donor allogeneic to the skin graft donor. Data from five independent experiments were pooled for analysis; in each experiment, additional control animals bearing skin grafts were injected with blocking antibodies or IgG control and were not inoculated with PBMC to be certain that injury was not caused by the

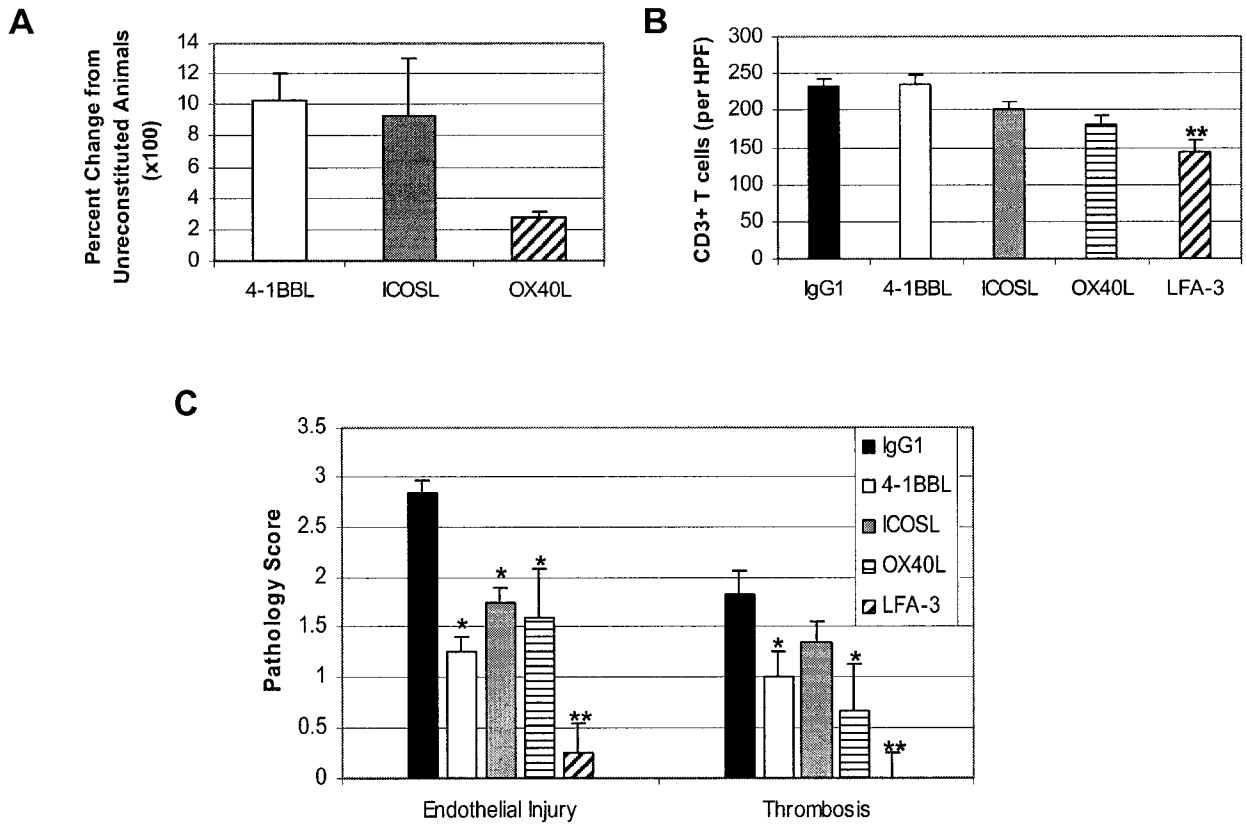

Figure 5.5. ***Rejecting skin grafts in the human-SCID chimera upregulate mRNA for the memory costimulatory ligands and blocking these pathways decreases rejection pathology.*** mRNA was harvested from skin grafts that had been on mice reconstituted for 10 days with PBMC. Quantitative PCR on the mRNA harvested from these grafts showed an induction of mRNA for 4-1BBL, ICOSL and OX40L (n=12) (a). Similar grafts were then harvested from mice that had been reconstituted for 10 days with PBMC that had also been receiving i.p. injections of blocking ab (100 ug) every three days. (b) 10 random high-powered fields (HPF) were taken of slides cut from the grafts and stained for human CD3e for assessment. Counts of the CD3e+ T cells showed similar levels of T cell infiltration except for LFA-3, which showed a significantly decreased number of infiltrating T cells ($p<0.001$). H&E slides were generated from these grafts and subsequently evaluated by a dermatopathologist blinded to the treatment. Blocking 4-1BBL, ICOSL or OX40L reduced endothelial injury ($p<0.004$, 0.05, 0.0003, respectively) and thrombosis ($p<0.01$, 0.1, 0.002), despite having similar levels of infiltration (c). LFA-3, as described previously, showed decreases in both endothelial injury ($p<10^{-6}$) and thrombosis ($p<0.0004$) corresponding with its lower level of infiltration (n=9 per treatment group).

antibodies. Injury to graft EC was evaluated as microvascular lumenal occlusion or shedding of EC, initial steps in human vessel loss (179). Blockade of ICOSL, 4-1BBL or OX40L all failed to reduce T cell infiltration while blockade of LFA-3 did do so (Figure 5.5B). ICOSL blockade decreased endothelial injury and thrombosis, but the difference in thrombosis did not achieve statistical significance. Blockade of the 4-1BBL or OX40L pathway reduced both endothelial injury and thrombosis to a significant degree (Figure 5.5C). However, the decrease in endothelial damage was not as effective as that observed with blockade of LFA-3.

In a final series of experiments, I used qRT-PCR to examine RNA obtained from grafts on animals treated with blocking antibodies. Blockade of 4-1BBL, ICOSL or OX40L all significantly decreased FasL and perforin mRNA production in T cells, though blockade had little effect on granzyme B expression (Table I-5). Analysis of cytokine mRNA expression within skin grafts was less clear cut. Blockade of 4-1BBL caused a decrease in IFN-γ, IL-2 and IL-10 mRNA expression compared to control grafts, but no change in IL-4 mRNA expression (Table I-5). In contrast, ICOSL blockade showed an increase in IFN-γ, a decrease in IL-4 mRNA and a modest decrease in IL-2 and IL-10 mRNA levels. OX40L blockade, when compared to IgG control, showed dramatic reductions in IFN-γ, IL-2 and IL-4 expression, but a comparable level of IL-10 mRNA. Overall there is a better correlation of reduction in cytotoxic effector molecule than on cytokine expression with protection of grafts from injury, consistent with the correlation of effector molecule transcripts with rejection of human allografts (354, 355).

	Percent inhibition of expression						
Treatment	**FasL**	**Granzyme B**	**Perforin**	**IFN-γ**	**IL-2**	**IL-4**	**IL-10**
4-1BBL	85.6±9.8	8.8±8.0	32.3±21.9	42.1±1.7	59.6±8.6	(-4.0±5.1)	47.4±2.4
ICOS-L	98.3±0.7	(-28.3±14.9)	80.5±4.9	(-51.8±10.0)	30.4±15.0	63.1±7.7	59.7±11.9
OX-40L	81.8±14.5	0.0±8.2	70.8±10.3	59.3±5.0	92.8±3.5	95.8±4.2	6.3±14.3
LFA-3	98.7±0.01	82.3±5.9	74.8±8.9				

Table I-5. *Effect of antibody blockade on expression of effector molecules and cytokines.*

mRNA from human skin grafts harvested from mice that had been reconstituted for 10 days with PBMC and receiving i.p. injections of blocking mAbs (100 ug) every three days was obtained for qRT-PCR. Data was analyzed by comparing threshold cycles to a standard curve generated using plasmid DNA and then expressed as a percentage of IgG control.

5.3 DISCUSSION

In this chapter I present two new observations relevant for human transplantation. First, I show that three recently described costimulatory molecules, namely ICOSL, OX40L and 4-1BBL, are inducible on HDMEC by TNF or by culture with allogeneic T cells, and that these ligands contribute to activation of resting memory T cells by allogeneic HDMEC. Second, I show that the three costimulators I have determined as inducible on HDMEC in culture are also inducible in human skin grafts and contribute to human memory T cell-mediated injury during an alloreaction. Protection correlates with reduction of effector molecule transcripts within the infiltrated graft.

I began by looking at the expression of several newly described molecules 4-1BBL, ICOSL (also known as B7Rp1, B7h, GL-50 or LICOS) and OX40L (gp34) in response to cytokines and T cell co-culture. Previous studies have demonstrated that activation of T cells by EC involves the interaction of LFA-3, ICOSL and OX40L (86, 98, 153), however no data exists regarding 4-1BBL on endothelial cells and only ICOSL has been studied on microvascular EC (86). In addition to EC, 4-1BBL, OX40L and ICOSL are expressed on most classical antigen presenting cells (APCs) including dendritic cells, B cells, activated monocytes (356-358). ICOS is induced following initial activation of T cells and ligation of ICOS leads to augmented proliferation (85) and secretion of many effector cytokines (including IL-4, IL-10, IFNγ) from CD4+ T cells (359). Like ICOS, in humans, OX40 is expressed by T cells after ligation of the T-cell receptor and was initially identified as a marker of T cell activation (360) and appears to be important for proliferation and cytokine production by CD4+ T cells when stimulated with HUVEC (231) or allogeneic DC (153). 4-1BB, like ICOS and OX40, augments

cytokine production and proliferation, however it does so for both CD4+ and CD8+ T cells (160).

In this chapter, I demonstrate that all three of these molecules are inducible on HDMEC in response to TNF, but not IFNγ. This is consistent with a previous report of ICOSL on HDMEC (86); the expression of OX40L and 4-1BBL has not been previously reported on this cell type. Furthermore, previous studies have not examined expression in co-cultures of T cells with human endothelial cells. I show that co-culture of HDMEC and allogeneic CD4+ T cells induces expression of both the costimulator on T cells and their corresponding ligands on HDMEC. The mRNA for the ligands is induced early on EC for all three ligands, however their surface expression differs markedly with 4-1BBL peaking and persisting at Day 7, ICOSL peaking and persisting at Day 3 and OX40L peaking at Day 1 and then returning to baseline by Day 3 (Figure 5.1B). This difference in expression may explain the reduction on 24 hour cytokine production in CD4+ T cells when ICOSL and OX40L are blocked, but the lack of effect when 4-1BB is blocked. Surface expression of OX40L did not increase to the degree anticipated by the mRNA. The reason for this difference is unclear and under current investigation. Blockade of any of the three costimulator molecules seems to have moderate effects on both CD8+ T cell cytokine production and proliferation. Preliminary data regarding combinations of blockade indicate that the effects are less than additive, consistent with overlapping activity (data not shown). CD4+ T cells in co-culture with EC showed increasing expression of 4-1BB, ICOS, and OX40 in both the proliferated and unproliferated population as measured by CFSE dilution. The increased expression on the proliferating cells is consistent with earlier data that showed that these molecules are upregulated on T

cells upon activation (361, 362), however the increased expression on unproliferated cells has not been observed previously and may be a response to cytokines produced by the activated cells or may represent responses of T cells that are incompletely activated by TCR signals.

Using the human-SCID model I examined the role of 4-1BBL, ICOSL and OX40L in human allograft injury. Several recent studies using rodent transplant models have described roles for 4-1BB, OX40, and ICOS *in vivo*. Genetic ablation or antibody blockade of the 4-1BB-4-1BBL pathway prolongs graft survival by reducing T cell proliferation and cytotoxicity (305, 363, 364). ICOS deficiency or blockade have also been shown in rodent models to improve transplant survival and, in conjunction with B7 blockade, even induce tolerance (303, 365), although not in all cases (366). OX40L blockade, also leads to increased graft survival in presensitized mice in combination with B7 blockade (307, 366). When any of these pathways are blocked in my chimeric model of human memory I show significantly decreased endothelial damage, vascular destruction and effector molecule mRNA at 10 days, though none seems to be as effective as blockade of LFA-3. One reason for this is that LFA-3 may have some effects on T cell infiltration into the grafts in addition to any of its potential costimulatory effects although the extent of infiltration was reduced by less than 50%. Thus, my model is the first to demonstrate a role for 4-1BBL, ICOSL and OX40L in human allograft rejection *in vivo*. To date, previous data suggested a correlation between these costimulators and graft rejection in human transplantation (367, 368) as well as in other human diseases (362), but none have explored the effects of targeting these pathways in humans.

Though 4-1BBL, ICOSL, and OX40L appear to have similar potential to reduce the allograft rejection, the effects on cytokine transcript expression *in vivo* reveals differences among the functions of these costimulators *in vivo*. Blockade of 4-1BBL decreased mRNA production of IFN-γ, IL-2, as well as IL-10, but had no effect on IL-4. This is consistent with observations that 4-1BBL activates both CD4+ and CD8+ human T cells to produce IL-2 and IFN-γ independently of B7 interactions (160). In comparison to 4-1BBL, blockade of ICOSL increased IFN-γ and only minimally reduced IL-2 production, but showed a much larger decrease in IL-10 and IL-4 production. My *in vivo* data correlates with *in vitro* studies that have indicated a limited role for ICOS in IFN-γ regulation, while playing a large part in the regulation of IL-10 (369) and IL-4 (370, 371). OX40L blockade had no effect on the level of IL-10 mRNA, while reducing all the other cytokines, suggesting a distinct mechanism of action different from either 4-1BBL or ICOSL blockade and may be related to the role that the OX40 interaction plays in the control of regulatory T cells (T_{reg}) (372, 373)

In conclusion, I have shown that memory T cell-mediated activation by HDMEC depends in part on the costimulatory molecules 4-1BBL, ICOSL and OX40L. Further that memory T cell-selective costimulation plays a significant role in this model of memory T cell-dependent allograft rejection. My studies extend the understanding of the mechanism by which human memory T cells may be activated by the endothelium and identify targets for reducing the memory T cell participation in clinical allograft rejection.

CHAPTER VI – THE *IN VITRO* AND *IN VIVO* RESPONSE OF MEMORY T CELL SUBSETS TO THE ENDOTHELIUM

6.1 INTRODUCTION

A cardinal feature of the adaptive immune response is memory, i.e. a second immune response to a given Ag that has been encountered previously is faster and more vigorous compared to the initial (primary) immune response. In cell-mediated immune responses, memory arises from changes in the populations of T lymphocytes that respond to the specific Ag. When compared to naïve response, a memory response is characterized by a higher frequency of Ag-specific T cells and by a series of changes in the individual T cells that respond to that Ag (16). Such changes include an altered requirement for costimulators and a change in receptors involved in homing. Memory T cells also have a special relationship to vascular endothelial cells (EC) in that resting memory T cells can be activated by alloantigens presented by EC, but not by other stromal or parenchymal cells *in vitro* (206, 213).

It has recently been discovered that memory T cells exist in at least two subsets based on their homing characteristics and effector functions (46, 51, 54). Central memory T cells (T_{CM}) express chemokine receptors (e.g. CCR7) that bind lymph node-derived chemokines like SLC and adhesion molecules (e.g. CD62L) that preferentially interact with ligands expressed by high endothelial venules of lymph nodes (46). Consequently, T_{CM} migrate preferentially to the T cell areas of lymphoid organs where they conduct surveillance for their specific Ag displayed by professional APCs such as DC (374). Effector memory T cells (T_{EM}) have enhanced effector functions, such as secretion of effector cytokines like IFN-γ, and a corresponding preference for migration to inflamed peripheral tissues (54). This migration pattern is explained by observations

that T_{EM} express chemokine receptors (e.g. CXCR3) that respond to inflammatory chemokines instead of CCR7 and express high levels of adhesion molecules (e.g. LFA-1 or VLA-4) (325) whose ligands (ICAM-1 and VCAM-1, respectively) are preferentially expressed by cytokine-activated peripheral vascular EC (200). These homing properties are shared with activated effector T cells and may allow circulating effector memory cells to be directly recruited to sites of peripheral inflammatory reactions without first requiring Ag recognition and activation in secondary lymphoid organs.

As discussed previously, the T cell response to antigen depends both on TCR signals (provided by peptide-MHC molecule complexes) and antigen-independent costimulatory molecules. The majority of costimulatory molecules fall into two structural families, those that are part of the Ig superfamily and those that are part of the tumor necrosis factor (TNF) superfamily. The best described T cell receptors for costimulation are those from the Ig superfamily, particularly CD28 (which binds B7-1 and -2, also known as CD80 and CD86, respectively) and CD2 (which binds CD58 in humans and CD48 in rodents) (350). Human EC express LFA-3 (but generally not B7-1 or -2) and the LFA-3-CD2 pathway seems to be particularly important in human allogeneic responses to this cell type. However, antibody blocking experiments have suggested that LFA-3 does not account for all of the costimulation that human EC provide to memory T cells (208, 209). Several newly discovered receptors for costimulators in both the B7 family (such as ICOS) and the TNF receptor family (such as CD27, 41BB and OX40) are important for the generation of effector/memory T cells (140, 160, 352, 375). Ligands for these molecules are expressed on cytokine-activated human EC cultured from umbilical vein (86, 152) and dermal microvessels (See chapter

V). Results from Chapter V demonstrate that ICOS, 41BB and OX40 can contribute to EC mediated activation of memory T cells, but the specific costimulatory requirements of the different memory T cell subsets has not been fully explored.

In addition to addressing whether naïve or memory T cells can mediate allograft rejection, the subset of memory T cells responsible for this phenomenon is not clear. Work by Adams et al has shown that CD8+ central memory T cells are principally responsible for memory cell-mediated rejection (275) while others have described a role for CD4+ T effector cells (376). Little is known about differences between alloreactive human T_{CM} and T_{EM}.

In this chapter I demonstrate that both T_{CM} and T_{EM} subsets from human peripheral blood respond can proliferate in response to allogeneic HUVEC *in vitro*, but that these two subsets respond differently to EC vs. professional APC. Further, I also demonstrate in the human-SCID chimeric model of allograft rejection that adoptively transferred T_{EM} are capable of mediating rejection whereas human T_{CM} cannot. These observations may be important for the design of new therapies to address the problem of immunological memory in transplant rejection.

6.2 RESULTS

T_{CM} proliferate more and produce greater amounts of IL-2 in response to EC compared to T_{EM}, while T_{EM} proliferate less and produce more IFN-γ.

Memory T cells in humans have been defined by expression of CD45RO (377). These memory T cells can be further divided into separate subsets based on CD62L and CCR7 expression (46). I took positively isolated CD4+ T cells from human peripheral blood and sorted then for the CD45RO+CCR7+CD62L+ population ("central" memory T cells, T_{CM}) and the CD45RO+CCR7-CD62L- ("effector" memory T cells, T_{EM}) (Figure 6.1). T cell subsets were sorted with >95% purity. Previous work from Chapter III demonstrated that CD4+ memory T cells but not naïve T cells respond to class II MHC-expressing HDMEC *in vitro*. To assess the responses of T_{CM} vs T_{EM}, I used the FACS-sorted memory CD4+ T cells subsets described above and co-cultured them with EC pre-treated with IFN-γ for 72 h to induce class II MHC molecules, assaying the supernatants for IFN-γ and IL-2 after 24 h. I found that T_{EM} cultured with allogeneic EC produce significantly more of IFN-γ but less IL-2 than do T_{CM} (Figure 6.2A and B). The increased production of IL-2 by T_{CM} correlates with increased proliferation of T_{CM} in response to allogeneic EC compared to T_{EM} assessed by ^{3}H-thymidine incorporation at days 5, 6, and 7 (Figure 6.2C). In contrast I did not observe a difference in IFN-γ production when these same subsets are co-cultured with allogeneic Mo. However, both IL-2 production and T cell proliferation are greater for T_{CM} compared to T_{EM} in co-cultures with allogeneic Mo (Figure 6.2A, B, and D). In other words, EC appear to provide a signal that selectively augments IFN-γ production by T_{EM} or, conversely, lack a signal needed for optimal IFN-γ production by T_{CM}.

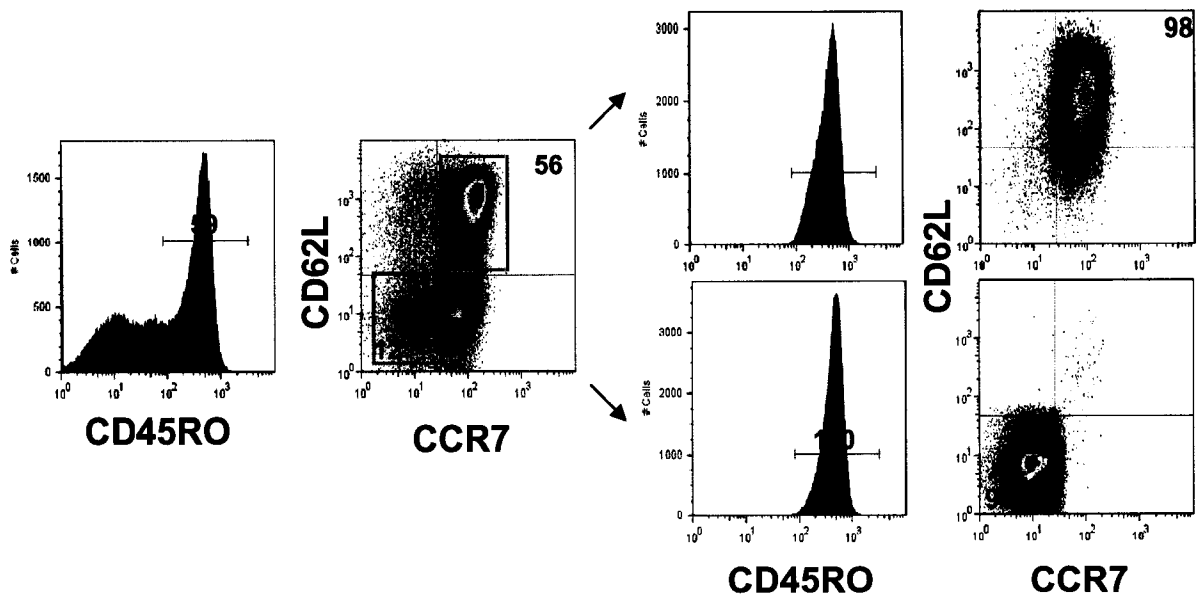

Figure 6.1. ***FACSort of human T cells demonstrates good purity of subsets.*** CD4+ T cells were isolated using magnetic beads prior to being stained with antibodies to CD45RO, CD62L and CCR7. Cells were then sorted into T_{CM} and T_{EM} by collecting only the gated regions.

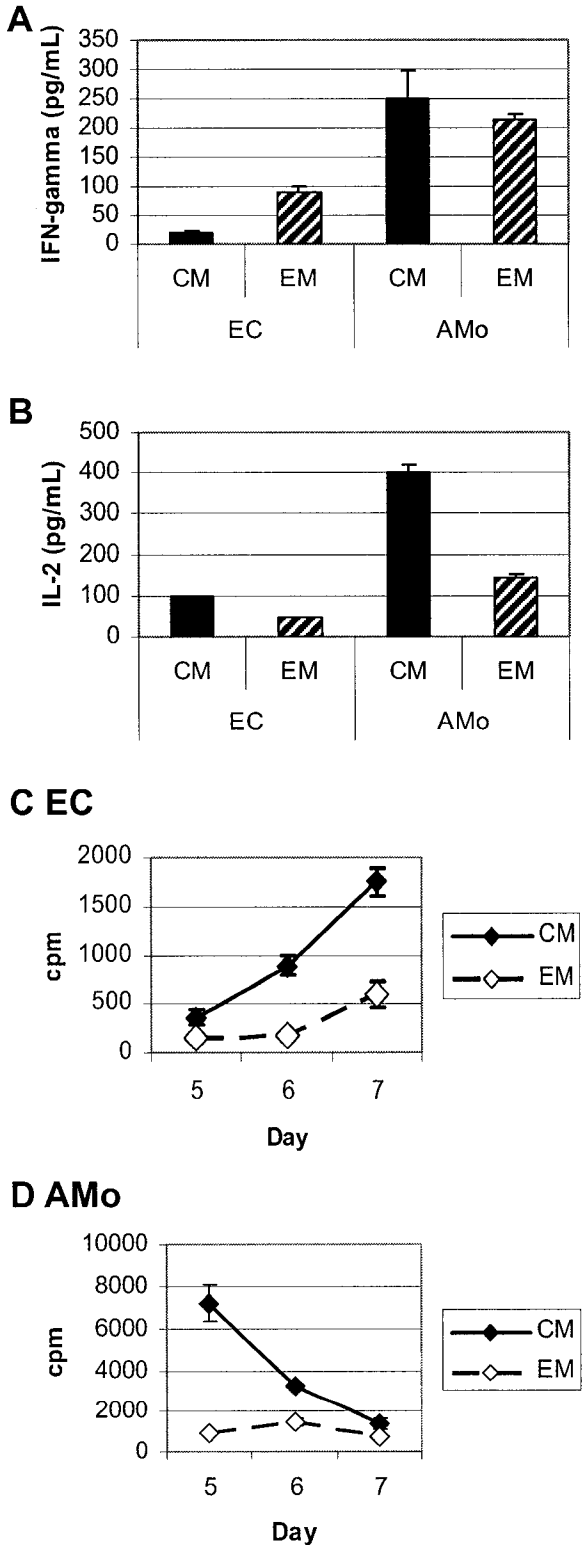

Figure 6.2. ***Effector memory T cells exhibit greater IFN-g production and less IL-2 production than central memory T cells in response to EC, but not monocytes.*** T_{CM} or T_{EM} were cultured with either EC or AMo and supernatant was collected at 24 hours. Supernatants were then assessed for IFN-g (A) or IL-2 (B) using ELISA. T cell subsets were also assessed for proliferation at day 5, 6, and 7 using ^{3}H-thymidine incorporation when cultured with EC (C) or AMo (D).

T_{CM} express different levels of costimulatory and adhesion molecules compared with T_{EM}.

In order to explore the differences that underlie the varying responses of T_{CM} and T_{EM}, I examined the expression level of several known costimulatory and adhesion molecules on the surface of the two subsets. I found that for the costimulatory molecules that T_{CM} express significantly more CD27 and slightly more CD28, CD152 (CTLA-4), ICOS and 4-1BB compared to T_{EM} (Figure 6.3A). Expression of CD27 on T_{EM} was, however, heterogeneous with two populations of cells, a small population with an intermediate level of CD27 expression and much larger population of T_{EM} expressing low levels of CD27. Unexpectedly, I found slightly higher levels of expression of LFA-1 on T_{CM} than on T_{EM} (Figure 6.3A). HUVEC lack ligands that may engage CD28, but do express both ICOSL and 4-1BBL (189). They also express ICAM-1 and 2, the ligands for LFA-1 (200). Expression of CD70, the ligand for CD27, has not been previously described. As shown in Figure 3B, EC do not express CD70 or B7 molecules at rest nor do they increase CD70 or B7 molecules with IFN-γ treatment or co-culture with T cells. Adherent Mo, however, express low levels of CD70 and B7 molecules at baseline.

Effects of costimulation blockade on T_{CM} and T_{EM} response.

Due to the differential expression of CD70 on Mo compared to EC and CD27 on T_{CM} compared to T_{EM}, I examined the effect of adding a blocking antibody to this T cell receptor in my allogeneic co-culture experiments. Blocking CD27 decreased production of IL-2 by T_{CM} but not by T_{EM} in co-cultures with EC or Mo (Figure 6.4C and D, $p < 0.05$). Surprisingly, blocking CD27 inhibited IFN-γ production in co-cultures of

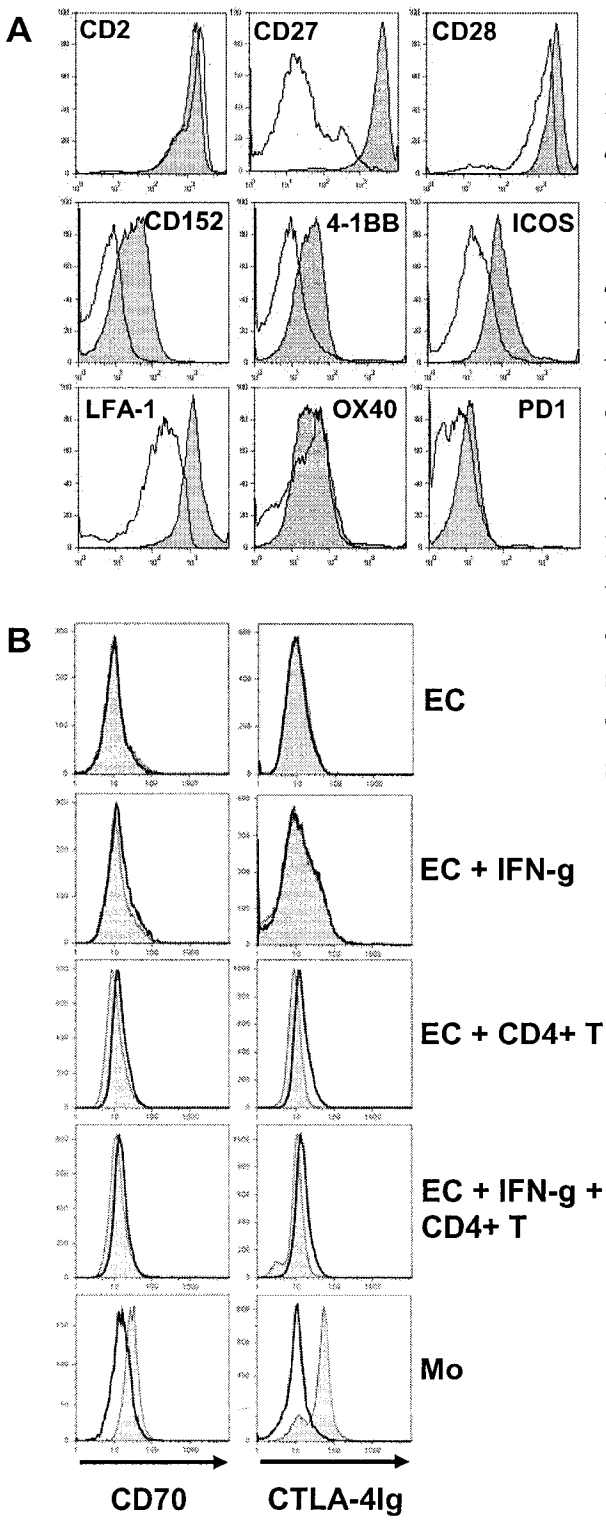

Figure 6.3. ***Effector memory T cells express lower levels of CD27, CD152 (CTLA-4), 4-1BB and ICOS compared to central memory T cells and EC do not express CD70 or B7, but Mo express both.*** CD4+CD45RO+ T cells were isolated from PBMCs using magnetic beads and subsequently stained with CCR7 and CD62L. The T_{CM} CCR7+CD62L+ (gray filled) population was gated on and compared with the T_{EM} CCR7-CD62L- (black line) population (A). HUVEC or negatively isolated Mo were either treated with IFN-g for 3 d, co-cultured with CD4+ T cells or both and assessed for CD70 expression or binding to CTLA-4Ig by FACS analysis (B). Data represents one of three experiments.

EC with T_{CM} or T_{EM}, but has no effect on co-cultures of either subset with Mo (Figure 6.4A and B).

I also examined the effects of signaling through CD28 since human Mo but not EC express B7 (189). I found that if B7 signaling is blocked using CTLA-4Ig that there is no effect on IFN-γ production in either subset with EC (Figure 6.4A), but that there is a significant reduction in both subsets when they are cultured with Mo (Figure 6.4B, $p < 0.01$). For IL-2 production I find that CTLA-4Ig inhibits T_{CM}, but not T_{EM} IL-2 production in co-cultures with EC (Figure 6.4C, $p < 0.04$), but inhibits IL-2 production from both subsets in co-cultures with Mo (Figure 6.4D, $p < 0.005$). To further test if B7-mediated costimulation accounts for the decreased IFN-γ production in T_{CM} cultured with EC I generated EC that express B7.2 using retrovirus (Figure 6.5A). When co-cultured with these EC, T_{CM} produce greater amounts of both IFN-γ ($p < 0.002$) and IL-2 ($p < 1\times10^{-4}$). Interestingly, T_{EM} do not produce any additional IFN-γ, but do produce significantly more IL-2 ($p < 0.001$), similar to the response when T_{EM} are cultured with AMo (Figure 6.5B and C). These data suggest that the absence of B7-ligands on EC may contribute to the reduced capacity of EC to activate T_{CM}. The ability of CTLA-4Ig to reduce IL-2 synthesis by T_{CM} cultured with EC is consistent with a previous study in which T cells proved to be the source of B7 ligands (190).

T_{EM} alone can mediate graft rejection in a hu-SCID model of allograft rejection.

I have recently shown that human skin injury observed in a human mouse chimera can be mediated by isolated memory T cells but not naïve T cells (189). Knowing that both central and effector memory T cells can respond to EC but that

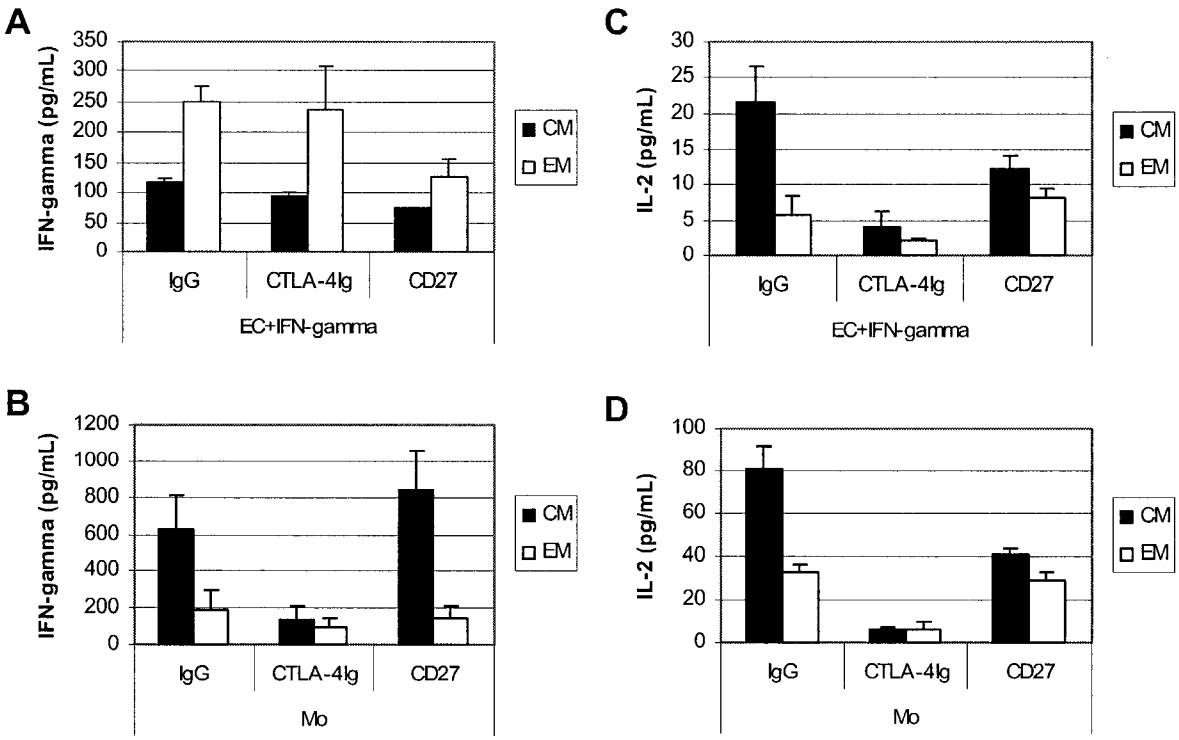

Figure 6.4. ***CD27 and CD28 have different roles for EC and Mo in stimulating memory T cell subset cytokine production.*** T_{CM} and T_{EM} were then co-cultured with EC (A, C) or Mo (B, D) and blocking antibody to CD27, CTLA-4Ig or IgG1. ELISAs were performed for IFN-gamma (A, B) or IL-2 (C, D). Data represents four experiments with similar results.

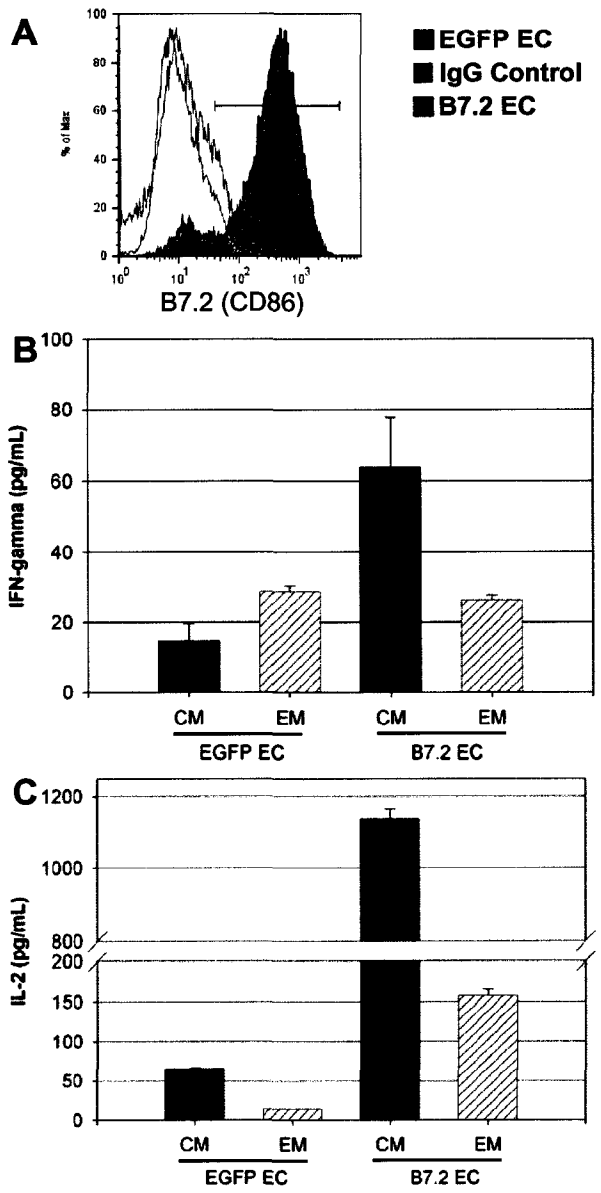

Figure 6.5. ***Differences in central and effector memory T cell IFN-g production are abolished in the presence of CD86 (B7.2).*** EC were transduced with a retrovirus containing the full-length mRNA of B7.2 and checked for expression (A). T_{CM} and T_{EM} were cultured with either B7.2 or EGFP transduced EC and supernatants were assessed for IFN-g (B) and IL-2 (C).

only effector memory T cells can produce high amounts of IFN-γ, I wanted to see which memory T cell subset was responsible for the rejection response. I adoptively transferred either central or effector memory T cells into SCID/beige mice that had been grafted with human skin. After three weeks, the grafts were harvested and analyzed. I found in three separate experiments that effector memory T cells (Figure 6.6C and D), but not central memory T cells (Figure 6.6A and B) could infiltrate and destroy graft microvessels. When scored by a blinded dermatopathologist, mice receiving adoptively transferred effector memory T cells showed significantly more infiltration and endothelial damage ($p > 0.0001$) compared to mice receiving central memory T cells (Figure 6.6E).

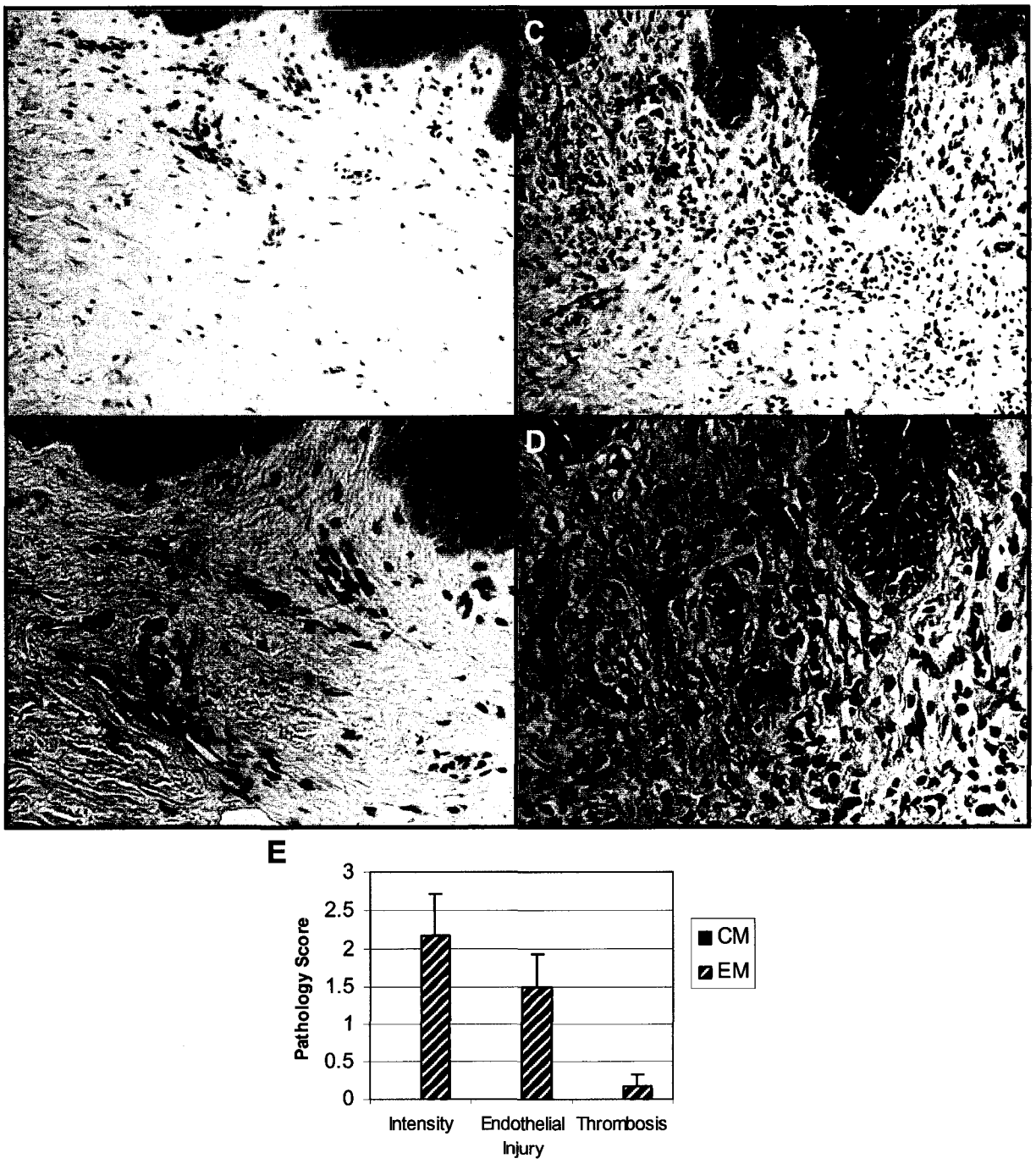

Figure 6.6. ***Effector memory T cells alone can mediate graft rejection.*** Grafts from mice that received either T_{CM} (20x-A, 40x-B) or T_{EM} (20x-C, 40x-D) for 14 days were harvested and stained with H&E. Grafts were then scored blindly by a dermatopathologist (E). The data represent n=12 mice per group.

6.3 DISCUSSION

In this chapter, I present two new observations about memory T cell subsets and their relationship to EC. First, I demonstrate that human T_{CM} and T_{EM} subsets, defined by the expression of CCR7 and CD62L, produce different levels of cytokine in response to allogeneic endothelial cells. Specifically, T_{CM} produce less IFN-γ and more IL-2 compared to T_{EM} when co-cultured with EC but not with allogeneic peripheral blood Mo. Second, I show that both T cell CD27 and CD28 may be involved in the different response of memory T cell subsets to allogeneic EC vs. Mo. Third, I demonstrate that following adoptive transfer, T_{EM} but not T_{CM} can mediate graft injury in a hu-SCID chimera model of skin allograft rejection.

The current conception of T cell memory proposes that the memory T cell pool may be divided into central memory (T_{CM}) and effector memory (T_{EM}) (46). This model proposes that T_{CM} cells which are CCR7+ preferentially migrate into secondary lymphoid organs and lack immediate effector functions such as IFN-γ production or cytolytic activity. However, T_{EM}, which lack expression of CCR7, more rapidly acquire effector function and have a migratory pattern that favors extralymphoid sites. Studies in mice have demonstrated that a large number of CD4+ memory T cells can be found in extralymphoid sites and that there is a functional difference between memory T cells residing in the lymphoid tissue compared to those of the extralymphoid tissues (51, 54). However, several human and mouse studies have not been able to confirm the relationship between immediate effector function and the potential to migrate to extralymphoid sites (as defined by the expression of CCR7). In fact, these studies suggest that the majority of cytokine producing memory T cells express CCR7 and that

immediate effector function did not correlate with CCR7 or CD62L expression (378-380). My data shows that human memory T cell subsets do show such differences but only when responding to allogeneic EC. One consideration, however, is the definition of T_{CM} and T_{EM}, some groups use CCR7 alone and others have used CCR7 in combination with CD62L. As seen in figure 6.1 there is a significant population that is CCR7- but CD62L+; the contributions of this subset are unclear.

It is now become evident that the existence of memory T cells presents a significant challenge to long-term graft survival. The presence of alloreactive CD4+ memory T cells correlates with increased episodes of acute rejection and diminished graft function (270, 271). However, the role of memory T cells in allograft rejection has not been well-studied due to the lack of appropriate *in vivo* models. Mouse models have been described that use donor-specific alloreactive memory T cells (376) or those that have been generated from viral infection (275), but these still lack the diversity of the human memory T cell pool. My study using human memory T cells fractionated into their different subsets shows a role for T_{EM} in allograft rejection, but not T_{CM}. One key difference between my human-mouse chimera and the current mouse models is that, though SCID/bg mice possess lymph nodes, human T cells cannot effectively circulate to the mouse secondary lymphoid organs thus providing an isolated model of peripheral rejection only. I did not find any human T cells in any lymph nodes examines and this lack of ability to circulate in the secondary lymphoid organs may explain why, in contrast to experiments with mice, T_{CM} in my model appear unable to mediate graft rejection. This is supported by my *in vitro* data suggesting that T_{EM} can produce more IFN-γ in response to the endothelium compared to T_{CM} which produce significantly more IL-2.

This difference *in vitro* is abolished with sufficient B7-signals (i.e. from monocytes or transduced EC) which further supports the idea that T_{CM} may need to circulate through the secondary lymphoid organs and encounter B7 bearing APC to attain full functional capacity.

Recent data have suggested that memory T cells may have different costimulatory requirements for activation when compared to their naïve counterparts. Early work in mice (60) and humans (381) demonstrated that memory T cells can be activated independently of CD28 and more recently it has been appreciated that there may be a role for memory specific costimulators in memory T cell activation (361, 362). In accordance with other studies that have shown decreased CD27 and CD28 in the effector memory T cell population (131, 382), I have also found decreases in CD27 and CD28 in the effector memory populations used in my experiments. To explore whether the differential expression of these costimulatory molecules on the two different subsets is responsible for the difference in their response to EC I used a blocking antibody to CD27 and CTLA4-Ig to block the B7-interaction. My results were surprising in that blockade of CD27 showed decreased IFN-γ production from the effector memory T cell subset as well as decreased IL-2 production from the central memory T cell subset. One possible explanation is that the majority of the IFN-γ may be produced by the sub-population in the effector memory T cells that are expressing CD27 at an intermediate level and thus blockade of CD27 decreases their IFN-γ production. The other possible explanation is that blockade of CD27 of this intermediate population may be affecting the production of IFN-γ production from the CD27- population. The other surprising result from my blocking experiments was the effect that CTLA-4Ig had on IL-2 production in the central

memory T cell population co-cultured with EC. As EC do not express B7 molecules it is unclear where the source of CD28 stimulation for the central memory T cells is originating. One source of CD80 and CD86 may be the T cells themselves. Previous work has demonstrated that interaction of CD4+ T cells with EC can induce expression of CD80 and CD86 on the T cells *in vitro* (190). The increased IL-2 production in central memory T cells may depend on this source of B7 stimulation and my experiments with B7-transduced endothelial cells supports this hypothesis in that central memory T cells seem to be more responsive to B7 signals than their effector memory counterparts.

There has also been evidence showing that the memory T cell subsets are not fixed, but rather retain some flexibility in terms of their differentiation and that differentiation into the different subsets may be dependent on the microenvironment (383, 384). Endothelial cells may play a central role in the determination of this microenvironment. My data supports this hypothesis in that interaction between the different memory T cell subsets and EC produces different responses. Further examination of memory T cell expression of costimulatory and adhesive molecules whose ligands are known to be expressed on surface of EC suggests that these molecules may play an integral role in the differential stimulation of T_{CM} and T_{EM}.

In conclusion, I demonstrate here that when the different memory T cell subsets are co-cultured with EC, T_{CM} favor IL-2 production and T_{EM} produce more IFN-γ. The difference in IFN-γ production is abolished in the presence of AMo or B7-transduced EC, but the difference in IL-2 production is retained. I also show that in a chimeric hu-SCID model of allograft rejection that T_{EM} alone can mediate rejection whereas T_{CM} cannot. This study introduces the idea that EC may activate different memory T cell subsets

differently and that this differential in activation may have a functional consequence in allograft rejection.

CHAPTER VII – CONCLUSIONS AND FUTURE DIRECTIONS

This thesis describes several new observations. First, I demonstrate that human dermal microvascular endothelial cells (HDMEC) like other human EC preferentially activate allogeneic memory T cells. Second, I also show that allograft rejection in my chimeric human skin-SCID mouse model is mediated by memory T cells. Third, I demonstrate that the CD28-B7 pathway may have a role in memory T cell mediated rejection by using a novel reagent that blocks CD28. Fourth, I show that HDMEC can express memory T cell specific costimulatory molecules 4-1BBL, OX40L and ICOSL and that these molecules have a role in human memory T cell mediated allograft rejection. Finally, I describe the different responses of central memory and effector memory T cells to the endothelium and provide evidence that T_{EM} alone can mediate allograft rejection.

T cell activation by EC

The question of whether EC can activate CD4+ and CD8+ T cells *in vitro* has been controversial. The first reason is species differences between humans and other animals such as mice. My lab has previously demonstrated that HUVEC activate resting allogeneic CD8+ T cells to proliferate, produce IL-2 and acquire cytolytic potential (209, 215). In the human system, HUVEC preferentially activate memory CD8+ T cells (214). Studies done in the mouse have yielded conflicting results. It was initially reported that murine cardiac EC are capable of directly activating naïve CD8+ T cells (385), however a subsequent study using a model of indirect presentation suggested that IFN-γ activated lung microvascular EC fail to induce proliferation or activation of naïve CD8+ T cells

(386). Further studies using murine aortic EC have shown that both resting and IFN-γ activated EC are capable of activating naïve CD8+ T cells (219). Since EC in those studies derived from different organs, the conflicting findings among the murine studies may be due to the fact that EC from different locations have been shown to display significant heterogeneity in function which may include their capacity to activate T cells (387). Differences between the human and mouse capacity to activate naïve allogeneic CD8+ T cells may be attributable to the different types of costimulatory molecules provided by mouse EC, which express B7, and human EC which do not (388). Despite these differences, both murine and human data support the idea that EC possess the capacity to directly activate allogeneic CD8+ T cells. The results from Chapter 3 agree with these findings in that HDMEC, like the large vessel-derived HUVEC, are capable of activating allogeneic memory CD8+ T cells preferentially over naïve CD8+ T cells.

This situation for CD4+ T cells is similar to that of the CD8+ T cells. My lab and others have previously demonstrated that HUVEC can activate CD4+ T cells (208, 389) and that HUVEC also preferentially activate memory CD4+ T cells (223). McDouall et al further showed that EC derived from large vessels and microvessels of the heart can activate CD4+ T cells (390). Vora et al verified the capacity of HDMEC to present specific Ag in experiments using CD4+ T cells clones (233). Other groups have questioned the ability of human EC to activate CD4+ T cells (217, 327) and one reason may be the insufficient sensitivity of their assays to detect the initial primary activation. Marelli-Berg at al demonstrate that culture of EC with CD4+ memory T cells does enable them to be subsequently activated by EC in the presence of B7 costimulation suggesting that the T cells in their assays did receive some signal from the EC (327). I confirm in

this thesis that HDMEC possess the capacity to activate CD4+ T cells and provide evidence that HDMEC specifically activate resting memory CD4+ T cells and not naïve CD4+ T cells (Chapter 3). Similar to the human data, several groups have provided convincing evidence that murine EC do not possess the capacity to activate naïve CD4+ T cells (216, 385), but may possess the capacity to activate CD4+ memory T cells (391). In sum, I show that HDMEC can activate both memory CD4+ and CD8+ T cells, but not naïve T cells of either subset. This observation is consistent with previous data derived from large-vessel human EC, but distinct from murine data in that EC from mice can activate naïve CD8+ T cells.

Memory T cell models in transplant rejection

Much of the progress in my understanding of memory has come from studies that make use of murine models. Most of the mice used in these studies do not possess significant numbers of memory T cells due in part to the relatively pathogen-free environments in which mice are housed and the young age of the mice. Thus, in using mice to study memory T cells, the two most common approaches are to generate memory T cells prior to a given study (priming) or to adoptively transfer memory T cells that have been generated in another source.

As discussed in the introduction, one of the major limitations of the murine models of memory used to study transplantation is the lack of the diversity and numbers of memory T cells found in mice compared to those in found in large animals. It is this significant population of memory T cells that is thought to underlie the failure to translate strategies to inhibit rejection from mice to other animals. In this thesis, I provide evidence that the rejection response seen in my previously described model of adoptively

transferring human PBMC in to human skin-SCID mouse chimeras actually (179) represents a model of memory T cell mediated destruction (Chapter 3). Thus, my system provides a small animal model to test strategies that target human memory T cells. This chimera provides a useful bridge between pure murine models and large animal models to test hypotheses developed in mice before embarking on the more costly large animal models as shown in Chapter 4 where I test a novel reagent against CD28.

Before discussing how the model can be used, there are several technical considerations that may be relevant to the understanding of this model. The first consideration is the repertoire of the memory T cells used in this system. Murine models of memory because of their requirement for generating memory T cells and the use of T cell receptor transgenics have very precise control of the specificity of the memory T cells generated. Thus, it is possible to study the rejection in models of memory with T cells that are limited to cross-reactivity from specific infectious agents (275) or are specific for minor antigens (392). My model has much less control of the specificity in that my HLA mismatches are random and likely involve multiple major MHC mismatches between the skin and the adoptively transferred leukocytes. The implication of this is that my model may have an alloresponse that is more difficult to inhibit because of the multiple mismatches and that this model may be more vigorous than a typical clinical transplant where the MHC alleles between donor and host, particularly in kidney and bone marrow transplants (but not liver or hearts), would be HLA matched (393). Therefore, any effects seen in my model may be diminished in comparison to a model where the specificity is more limited. This may explain why costimulatory blockade in

my system, while partially ameliorating graft rejection, does not fully recapitulate the results seen using murine models of allograft rejection (305, 306, 365).

A second consideration to note is the role that homeostatic proliferation plays in my model. Homeostatic proliferation occurs as a result of low-affinity recognition of MHC-peptide ligands and cytokine in a setting where the T cell compartment has been depleted, i.e. lymphopenia (24, 48, 280). These dividing cells appear to transiently acquire memory markers and have memory-like function during the period during which they are undergoing homeostatic proliferation (43, 44, 394, 395). In T cells undergoing homeostatic proliferation there is decreased CD62L, increased CD44 and further these cells, like bone fide memory cells, show a decreased reliance on CD28 signaling (396). The lymphocytes in my model exhibit this type of change and this is most pronounced in the CD45RA group that when adoptively transferred appears to transiently acquire CD45RO positivity (See Figure 3.2) suggesting that the T cells in my model are undergoing homeostatic proliferation in the mouse. However, in contrast to evidence that this conversion of a naïve to memory phenotype during homeostatic proliferation leads to enhanced rejection (281) it appears that in my model this is not the case. The adoptively transferred naïve T cells despite acquisition of a memory marker do not seem capable of mediating a rejection response. This may be because naïve T cells require activation in secondary lymphoid organs whereas memory T cell do not (61). In my model there are no human dendritic cells in addition to the fact that human T cells may not possess the appropriate cross-reacting chemokines and/or adhesion molecules to enter the secondary lymphoid organs of mice. Thus, while there is significant homeostatic proliferation going

on in my model, it is not clear that it contributes to the observed allograft response, which appears to depend solely on memory T cells.

My model provides the unique opportunity to study the role of human memory T cells in allograft rejection. I chose to focus on the effects of costimulation blockade, but certainly there are a number of studies that could be carried out in my model to study the regulation of memory T cells. In particular, the role of regulatory T cells or peripheral tolerance in memory T cell mediated rejection would be interesting topics to pursue in future studies.

CD28 costimulation in memory T cell mediated rejection

Costimulation of T cells through CD28 by EC has been a contentious issue largely because of differences in results between experiments with human cells compared with other animals. In humans, HUVEC initially activate T cells independently of CD28 (98, 188, 197) and in fact do not express any B7 molecules (188, 397). In contrast, mouse and porcine EC express CD80 and CD86 respectively (239, 388) and can utilize these molecules to activate naïve T cells (219). Despite these differences, it is clear that agents targeting the CD28-B7 pathway have no effect on the initial stimulation that human EC provide to T cells. However, the question of whether CD28 mediated signaling plays any role in the ensuing reaction remains unclear. Denton et al have shown that while HUVEC themselves do not express B7 molecules, HUVEC can stimulate CD4+ T cells to express CD86 and CD80 in culture (190). Thus, in Chapter 4 I used a novel reagent that blocks CD28 to try and identify the effects of this reagent on possible B7 interactions in co-cultures of T cells and EC. In characterizing this reagent, I discovered that the reagent, a monoclonal antibody, is actually partially costimulatory

except in the presence of high levels of B7 expression. However, the level of costimulation is less than the typical antibodies used for costimulation (Chapter 4). The partial costimulatory activity made it difficult to assess the effects of CD28 blockade *in vitro* other than the interesting point that in the presence of excess B7.2 that the antibody did not inhibit CD4+ T cell IFN-γ production, while it did inhibit IL-2 production. This may be due to the fact that CD4+ IFN-γ production may be much more sensitive to the level of B7 stimulation and the small amount of blockade that is not inhibited by the antibody may augment IFN-γ production.

Initial studies done in mice with CTLA-4Ig, which binds B7 molecules with high affinity and blocks their interaction with CD28, demonstrated much promise for inhibiting T cell reactions (288). In combination with anti-CD154 blockade this combination proved to be very effective in preventing allograft rejection (289). As discussed in Chapter 4, one of the problems with CTLA-4Ig is that it has become increasingly evident that signals delivered from CTLA-4 binding B7 molecules is important for the function of regulatory T cells (398). Therefore, this antibody with the ability to specifically target CD28 provides an opportunity to deliver inhibitory CTLA-4 signals while simultaneously preventing activating CD28 signals. Unfortunately, the two reagents could not be compared *in* vivo because CTLA-4Ig augmented the adoptive transfer leading to dramatic increases in the level of circulating human cells in my model (data not shown). With regards to FK734, despite the partial agonist activity *in vitro* I found that blocking CD28 *in vivo* is able to ameliorate memory T cell mediated allograft rejection. However, the effect of blockade is modest at best and this is consistent with my understanding of the resistance of memory T cells to CD28-B7 blockade (291).

There is ample evidence in mice suggesting that memory T cells are less reliant on CD28-B7 signals than naïve T cells (58, 60). While these studies suggest that other pathways may compensate for the loss of CD28-B7 costimulation making memory T cells resistant to CD28-B7 blockade, it is important to note that in the normal memory T cell response the CD28-B7 pathway retains an large role because blockade of these alternative pathways alone do not delay allograft rejection (306). My findings further support this idea and suggest a role for CD28 mediated signals in memory T cell activation.

Thus, while memory T cells may be resistant to CD28 blockade, it may be that blockade of this pathway is needed for the other pathways to become prominent. To test this idea, again, combinations of blockade would be useful to identify the precise hierarchy of signals mediating the allograft rejection seen in my model. Based on the murine data, the most useful combination should be CD28 and OX40 blockade (306), though my data suggests that blockade of all three costimulatory pathways in addition to B7 and/or LFA-3 blockade would likely have the greatest effect.

Memory specific costimulators in memory T cell- mediated rejection

In contrast to CD28, which is expressed constitutively on naïve T cells, the memory specific costimulators, ICOS, 4-1BB and OX40, are expressed only upon T cell activation. This suggests that their function is likely related to the effector/memory phase of T cell activation rather than the initial phase of T cell activation. As discussed above, EC also have a unique ability to activate memory T cells in partly LFA-3 dependent manner. In Chapter 5, I examined whether the EC capacity to activate memory T cells

depended on their expression of these memory T cell-specific costimulators and if targeting these interactions could ameliorate graft rejection.

EC have been shown to express functional ICOS-L which is important for EC reactivation of effector/memory T cells (86). In that study, Khayyamian et al examined HUVEC and demonstrated that HUVEC express ICOSL and that blockade of EC ICOSL partially inhibits the EC capacity to activate CD4+ T cells. My data with HDMEC corroborates the evidence from this study showing that HDMEC express ICOSL constitutively, upregulate it with TNF and that blocking ICOSL inhibits CD4+ and CD8+ cytokine production and proliferation (Chapter 5). The reduction in T cell cytokine production and proliferation was approximately 50% suggesting that ICOSL plays a significant, but partial role in EC mediated T cell activation.

ICOS-ICOSL interactions have been shown to play a significant role in transplant rejection. Studies in mice using an anti-ICOS mAb, an ICOS-Ig fusion protein, or transfer into an ICOS deficient recipient show prolonged survival of a mismatched cardiac allograft, though to a lesser extent than administration of CTLA-4Ig or an anti-CD40L mAb (303). Treatment with both CTLA-4Ig or anti-CD40L mAb and ICOS-Ig resulted in the long-term survival of a cardiac allograft (302, 399) suggesting there may be a synergistic effect between the ICOS-ICOSL pathway and the CD40-CD154 or CD28-B7 pathways. The timing of the administration of blockade of the ICOS-ICOSL pathway can also appears to influence transplant rejection. Harada et al demonstrated in a major mismatch cardiac allograft model that delayed ICOS blockade was more effective that early ICOS blockade in prolonging allograft survival because early blockade targeted only CD4+ T cells while delayed blockade inhibited both CD4+ and

CD8+ T ell expansion (365). All these studies focused on rejection mediated by mostly naïve T cells and recent work using memory T cells generated by homeostatically proliferated memory T cells or by donor Ag priming suprisingly showed no effect of blocking the ICOS-ICOSL pathway alone or in combination with CD28/CD154 blockade on skin allograft rejection (306). My studies using the hu-SCID chimera showed an increase in intragraft ICOSL mRNA during rejection and that blocking the ICOS-ICOSL pathway could inhibit T cell mediated endothelial injury and intragraft expression of IL-2, 4, and 10 mRNA (Chapter 5). This contradicts the mouse data with memory T cells, but is consistent with studies done in mice using naïve T cell recipients. One explanation for this difference is access of the memory T cells to the secondary lymphoid organs. While memory T cells apparently do not need secondary lymphoid organs to mediate rejection, those derived from homeostatic proliferation in the absence of the secondary lymphoid organs have been shown to be inferior to antigen experienced memory T cells (61). My model does not allow memory T cells access to functional secondary lymphoid organs and as such this may explain the greater effect of ICOS blockade in my system where T cells may rely more on ICOSL provided in the graft for activation without access to the secondary lymphoid organs.

4-1BBL expression on EC has not been described previously. I demonstrate in this thesis that 4-1BBL can be induced at a low level on the surface of HDMEC both in response to TNF and co-culture with allogeneic T cells (Chapter 5). It has been shown to be induced by CD40 and IL-1 signaling on DC, but these stimuli have not been tested in EC (169). Blockade of this pathway inhibits CD8+ T cell, but not CD4+ T cell, IFN-γ and IL-2 production (Chapter 5). However, blockade does decrease both CD4+ and

CD8+ T cell proliferation in response to EC. These data are in agreement with data showing that 4-1BB signaling affects both CD4+ and CD8+ T cells, but has a stronger effect on the activation of CD8+ T cells (160, 400). One consideration to the data regarding 4-1BB signaling is that 4-1BBL is also known to induce reverse signaling. In human monocytes, stimulating 4-1BBL enhances their activation and survival (172, 173). The effects of 4-1BBL signaling in EC have not been examined and will be important to explore further.

With regard to transplantation, using 4-1BBIg (401), blocking mAbs, or genetic ablation (305) to eliminated 4-1BB signals demonstrated prolonged graft survival in murine cardiac allograft models showing decreased T cell infiltration and vascular damage. In skin allografts, however, deletion of 4-1BBL had no effect unless it was combined with ablation of CD28 (364) or CD154 and CD45RB blockade (402). Thus, for naïve T cells targeting 4-1BB can prolong graft survival in both skin and cardiac allografts possibly affecting CD8+ T cell effector function. In one study looking at memory T cells derived from homeostatic proliferation or donor specific priming there was no effect of an anti-4-1BBL mAb (306). My data again contradicts the results in the murine model of memory and instead supports the data for naïve T cells. In Chapter 5, administration of a blocking 4-1BBL mAb ameliorated memory T cell mediated allograft rejection and showed decreased intragraft IFN-γ and IL-2 (Chapter 5). There was no change in T cell infiltration or perforin/granzyme B mRNA so it is not clear if decreased cytolytic function of CD8+ T cells is the mechanism of protection. The discrepancy between my model and the murine model may again lie in the lack of access to functional secondary lymphoid organs in my model compared to the murine model. Blockade of the

4-1BB-4-1BBL pathway in EC-T cell co-cultures showed effects on CD4+ and CD8+ T cells and without access to other signals in the secondary lymphoid organs this pathway may be more important in my model than in the murine model.

The final pathway I examined in Chapter 5 was the OX40-OX40L pathway. OX40L expression on HUVEC has been described previously and been shown to mediate both adhesion to EC as well as costimulation with a polyclonal activator (152, 153). I demonstrate in Chapter 5 that HDMEC constitutively express significant levels of OX40L and that stimulation with TNF or co-culture with T cells increased those levels slightly. Blockade of OX40L in co-cultures led to decreased IFN-γ and IL-2 production from both CD4+ and CD8+ T cells. This supports observations from other groups that have shown that in mice agonistic anti-OX40 antibodies enhance the expansion of both CD4+ and CD8+ T cells *in vivo* (403, 404).

In allograft responses, OX40 has emerged as a key player in the activation of memory T cells. Initial work in mice with OX40L showed that in alloresponses DC deficient in OX40L induced less effector cytokine production and weaker CTL generation (150, 405). Work from GVHD murine models have suggested that OX40 deficiency has a much greater impact on CD4+ T cells compared to CD8+ T cells (406, 407). The Sayegh group further demonstrated a critical role for OX40-OX40L signaling in both CD28/CD154 independent rejection and memory T cell mediated rejection in conjunction with CD28/CD154 blockade (306, 366). This data supports the observation that blockade of OX40L has significant impact on memory T cell mediated allograft rejection. OX40L blockade produced the greatest reduction in intragraft IFN-γ, IL-2, IL-4, FasL and perforin mRNA expression in comparison to the other memory costimulators

suggesting that blockade of OX40L in my model likely has strong effects on both CD4+ and CD8+ T cells.

In sum, I found that blockade of any of these three memory T cell specific costimulators can reduce allograft injury significantly, but not completely. Interestingly, all three of these costimulatory molecules can be found on the surface of HDMEC and blockade of any of them can partially inhibit EC-mediated memory T cell activation for both CD4+ and CD8+ T cells. From my studies, it is not clear if the effects that I have observed here are redundant and testing blockade in different combinations will be important to understand if they have overlapping function. It may also be useful to know if they have additive function with LFA-3, which is the original costimulatory molecule described on EC. More detailed data about the numbers and phenotype of CD4+ and CD8+ T cells infiltrating the graft might also shed some light as to whether these costimulatory molecules work by similar or distinct mechanisms.

Another interesting issue to pursue is one raised in the discussion of 4-1BBL, the idea that reverse signaling of the costimulatory molecule to their respective ligand may be occurring in EC. While it is not clear if ICOSL has signaling function, there is evidence to suggest that ligation and signaling through B7-molecules may have important regulatory function in DC (408, 409) and B cells (410). Evidence for reverse signaling in the TNFR family is much stronger with evidence supporting bi-directional signaling for CD40/CD154 (411) and 4-1BB/4-1BBL (172, 173). Recent evidence using EC have shown that OX40L ligation can induce the production CXCR5 (412) and RANTES (413) suggesting that OX40L may also have signaling capabilities. As all my antibodies involve blockade of these ligands, it is possible that the effects that I have observed may

be mediated by positive signaling in EC or other cells expressing the appropriate ligand in addition to blockade of the signal delivered to T cells. This issue requires further study to identify whether reverse signaling is occurring in EC and, if it is, what are the functional consequences of such signaling on the EC capacity to activate memory T cells.

The response of memory T cell subsets to EC and their role in allograft rejection

As described in the Introduction and Chapter 6, human and mouse memory T cell can be broadly divided into two groups based on effector function and homing capacity (46, 51, 54). T_{CM} home to lymph nodes and have limited effector function, but have a large proliferative capacity and become effector cells upon restimulation. T_{EM} on the other hand can home to peripheral tissues, rapidly produce IFN-γ upon antigenic stimulation, but have limited proliferative potential. These distinctions were first described by Sallusto et al using polyclonal activators and little is known about the response of these different subsets to the endothelium. In considering how the two subsets would respond, there are two likely possibilities to consider. The first is given that T_{EM} would typically see EC in the periphery I expected T_{EM} to respond as they would to polyclonal stimulation and since T_{CM} likely depend upon DC mediated activation in the lymph nodes that, like naïve T cells, they would not respond to allogeneic EC. The second possibility is that both subsets would respond similarly as they do to polyclonal activators with T_{EM} favoring effector cytokines and T_{CM} favoring IL-2. It is the second hypothesis that proved to be true, that in response to EC T_{EM} produced more IFN-γ and less IL-2, whereas T_{CM} produced more IL-2 and less IFN-γ. Their corresponding proliferative capacity is also consistent with the original observations that like the

response to polyclonal activators in response to EC, T_{CM} possess greater proliferative capacity than T_{EM}. Co-culture of the different subsets with Mo showed a different pattern with T_{CM} producing equivalent amounts of IFN-γ as T_{EM} though T_{EM} still produce less IL-2. One explanation for this difference may be differences in the costimulatory capacity of EC compared to Mo.

In examining the two subsets for a variety of costimulatory and adhesion molecules, I found that T_{EM} express less CD27, CD28, CD152 (CTLA-4), 4-1BB, ICOS, LFA-1 and PD-1 when compared to T_{CM}. I decided to examine the role that CD27 and CD28 played in this difference in activation because of the corresponding difference in expression of the ligand on EC versus Mo. For IFN-γ, about 50% of T_{CM} and T_{EM} production depended on signals from CD27 in co-cultures with EC, whereas co-cultures with Mo did not depend on CD27 signaling at all. For IL-2, about 50% of T_{CM} IL-2 production depended on CD27 costimulation for both EC and Mo, but T_{EM} IL-2 production seems to be independent of CD27 signals. Studies from the Lipsky group showed that CCR7-CD27+ T cells produce more IFN-γ compared to the CCR7+CD27+ or CCR7-CD27- and my T_{EM} population does have a small percentage of CD27+ cells (Figure 6.3A) and this may explain why both T_{CM} and T_{EM} IFN-γ production may depend on CD27 signaling (131). The fact that IFN-γ production was not affected in co-cultures with Mo could be because CD28 signals in Mo may mask any effect of CD27 signals on IFN-γ production. IL-2 production is consistent with observations from the Lipsky group that T_{CM}, which express CD27 highly, produce a greater amount of IL-2 than T_{EM}, and it appears that CD27 signaling may mediate part of the difference in IL-2 production between T_{CM} and T_{EM} in response to EC or Mo.

Due to the differences in B7 expression on the surface of EC compared Mo and the relative importance of B7 signaling for T cell activation, a likely explanation for the different responses of the T cell subsets may lie in the ability of the two different APC to deliver CD28 mediated signals. The slight reduction in CD28 expression on T_{EM} also suggests that T_{EM} may not be as sensitive to B7 mediated signals. The observations in Chapter 6 are consistent with work in Rhesus macaques where Pitcher et al found that T_{CM} express CD28, whereas T_{EM} do not (414) and experiments from Fontenot et al who studied beryllium specific T cells in humans and found a gradual decrease in CD28 expression and dependence when memory T cells convert from T_{CM} to T_{EM} (415). Given my observations and the data from these other groups I was surprised to find that CTLA-4Ig blockade of B7-signaling affected both T_{CM} and T_{EM} cytokine production co-cultures with Mo, however consistent with data from other groups I found that the percent reduction in T_{CM} cytokine production was several fold higher than that of T_{EM} suggesting that T_{CM} have a greater reliance on CD28 signaling. It was also interesting to find that CTLA-4Ig also had significant effects on IL-2 production from both subsets in cultures with EC though again there was a much greater effect on T_{CM} compared to T_{EM}. It is not clear where the B7 signals are coming from in co-cultures with EC and the reduction in IL-2 may be related to the trans-costimulation effect observed in co-cultures with EC from Denton et al (190). When the different subsets were co-cultured with EC expressing B7.2, the difference in IFN-γ production was abolished while the difference in IL-2 production was retained. Thus, addition of B7.2 signals on the surface of EC enhanced the T_{CM} production of IFN-γ suggesting that T_{CM} are more sensitive to B7-

signals. Therefore CD28 signaling may underlie the difference between EC and Mo activation of the T_{CM} subset.

In conclusion, I find that T_{EM} produce more IFN-γ versus T_{CM} in response to allogeneic EC but not with Mo. I believe that this difference in activation may be due to the ability of Mo to deliver B7 signals to the T_{CM} whereas EC cannot. It appears that Mo activate both subsets to produce IFN-γ via CD28 without needing CD27 signals and the increased sensitivity of T_{CM} to CD28 signaling allows them to produce equivalent or more IFN-γ compared to T_{EM} in response to Mo. Since EC do not express B7 molecules, it appears that EC rely on CD27 to enhance T_{EM} IFN-γ production in combination with some other unknown signals. It seems that CD27 works in conjunction with other signals to augment cytokine production for IL-2 as well. From my data it appears that both EC and Mo stimulate T_{CM} and T_{EM} to produce IL-2 at a certain level likely through CD28 and that additional IL-2 production by T_{CM} relies on CD27 signals.

Using my model of allograft rejection I next sought to identify which of the memory T cell subsets could mediate allograft rejection. Interestingly, only T_{EM} and not T_{CM} could mediate allograft rejection in my model. This does not agree with Adams et al who studied heterologous memory in mice and showed that CD8+ T_{CM} were significantly more effective at mediating allogeneic skin graft rejection than CD8+ T_{EM} (275). One reason for the differences in my results is again that my model lacks functional secondary lymphoid organs as the adoptively transferred human T cells do not effectively circulate through the murine lymph nodes or spleen. While it has been shown that both CD4+ and CD8+ memory T cells of both subsets can survive and function without secondary lymphoid organs (416), one hypothesis is that once T_{CM} in my model are deprived of key

signals like B7 that they would ordinarily receive in secondary lymphoid organs they cannot attain full effector function. Another related possibility is that the T_{CM} may be able to mediate rejection, but can not appropriately home to a peripheral organ without first encountering secondary lymphoid organs. Studies from my lab have shown that T_{EM} can migrate in response to inflammatory chemokines presented on EC whereas T_{CM} cannot. This may indicate that even though T_{CM} can produce IL-2 and some IFN-γ in response to EC, they might never get the chance to mediate rejection in my model because of their failure to transmigrate across the endothelium (417). Thus, in my model T_{EM}, but not T_{CM}, can mediate allograft rejection and I speculate that this may be due to deficiencies in costimulation and/or homing.

Final Conclusions

In this thesis I have provided evidence that expands my current understanding of both the mechanisms and details of EC-mediated activation of memory T cells. I find that EC derived from human skin (HDMEC) can activate memory T cells and that EC use newly described memory T cell specific costimulators to do so. I also describe a model to study memory T cell mediated allograft rejection *in vivo* using human T cells. Using this model I find that memory T cell specific allograft rejection can be mediated in part by both B7 mediated costimulation and the memory T cell specific costimulators. I also characterize the response of difference memory T cell subsets to the endothelium and find that T_{CM} respond differently than T_{EM} to EC compared to Mo. I provide evidence to suggest that this difference may be due to differences in CD28 and CD27 signaling provided by EC versus Mo. Finally I show that in my model of peripheral rejection that T_{EM} but not T_{CM} can mediate an allograft response.

Recognizing that EC mediated activation of memory T cells plays a critical role in allograft rejection, I propose that EC use the newly described memory specific costimulatory molecules, ICOSL, 4-1BBL, and OX40L to activate memory T cells. These pathways in addition to the classic B7 pathway are important in memory T cell mediated rejection. The mechanism by which the costimulatory molecules interact with each other is unclear. In particular, the effects of reverse signaling on the endothelium or the signaling pathways they stimulate in T cells and EC remain poorly understood. These are concepts that will need to be explored further.

In addition to the unique costimulatory properties of EC, I also propose that the subset of memory T cells known as effector memory T cells through their capacity to express effector molecules in response to EC can directly mediate peripheral rejection whereas central memory T cells in response to EC do not fully differentiate and therefore lack the ability to mediate peripheral rejection. It is not clear whether it is lack of costimulation, difficulties in homing or some other mechanism like differences in T cell repertoire that prevents the T_{CM} from mediating rejection *in vivo* and these will be an important questions to address in future studies.

The identification of important costimulatory pathways and the introduction of a new model of allogeneic memory T cell responses may have important implications for studying and ameliorating allograft rejection.

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