THE ROLE OF PU.1 AND IRF4 INTERACTION IN THE BIOLOGY AND FUNCTION OF T HELPER 2 CELLS

Ayélé-Nati Ahyi

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Mark H. Kaplan, Ph.D. - Chair

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Janice S. Blum, Ph.D.

Doctoral Committee

_____

Michael J. Klemsz, Ph.D.

December 5th,2008

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Mervin C. Yoder, M.D.

DEDICATION

To my husband, my daughter and my family

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ABSTRACT

Ayélé-Nati Ahyi

THE ROLE OF PU.1 AND IRF4 INTERACTION IN THE BIOLOGY AND FUNCTION OF T-HELPER 2 CELLS

Adaptive and innate immune responses play a critical role in the protection against extracellular or intracellular pathogens. The function of these two types of immune responses is coordinated by CD4+ T-helper (Th) cells. Depending on the cytokine environment, Th progenitor (Thp) cells differentiate into three functionally different effector subsets. T-helper-1 (Th1) cells which mediate cell-mediated immunity, T-helper-2 (Th2) which orchestrates humoral immunity and T-helper-17 (Th17) cells key players in autoimmunity response. Cytokine induced transcription factors that are differentially expressed in Th cells are required for the development and commitment to a specific Th lineage. The population of Th2 cells can be subdivided in subpopulations depending on the level of a cytokine and the subsets of cytokines they produce. Very limited information is available about the regulation of cytokine production in this array of Th2 cells. We have recently identified the ETS family transcription factor PU.1 as regulating heterogeneity in Th2 populations. To define additional factors that might contribute to Th2 heterogeneity, we examined the PU.1 interacting protein IFN-regulatory factor (IRF)-4, a transcription factor expressed in lymphocytes and

macrophages. When Th2 cells are separated based on levels of IL-10 secretion, IRF4 expression segregates into the subset of Th2 cells expressing high levels of IL-10. To investigate the role of IRF4 in cytokine heterogeneity, Th2 cells were infected with retrovirus expressing IRF4. The cells overexpressing IRF4 secreted significantly higher levels of IL-10 and IL-4 compared to cells infected with a control vector at the same time the level of IL-9 decreases. To understand the mechanism by which IRF4 regulates IL-10 expression in various Th2 cell subpopulations we used co-immunoprecipitation assays to determine transcription factors that interact with IRF4. Our data shows that PU.1, IRF4 and NFATc2 form a complex in Th2 nuclear extract. We also demonstrated by ChIP assay that IRF4 directly binds the *Il10* and *Il4* loci in a time dependent manner. The role of these protein-protein and protein-DNA complexes and their contribution towards Th2 heterogeneity will be further defined. Understanding the regulation of the anti-inflammatory cytokine IL-10 in Th2 cells may give us a tool to control inflammation.

Mark H. Kaplan, Ph.D. - Chair

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LIST OF ABBREVIATIONS

AcH3	acetylated histone H3
Ab	antibody
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CNS	conserved noncoding sequence
DAPA	DNA affinity precipitation assay
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FACS	Fluorescence Activated Cell Sorting
FBS	fetal bovine serum
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
hCD4	human CD4
hIL-2	human interleukin-2
IFNγ	interferon gamma
IL	interleukin
IgG	immunoglobulin G

IRES	internal ribosome entry site
IRF-4	IFN regulatory factor-4
JAK	Janus Kinase
kb	kilo base pairs
kDa	kilo Daltons
LPS	lipopolysaccharide
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
STAT	signal transducer and activator of transcription
VISTA	VISualization Tools for Alignments
WT	wild-type

FOOTNOTES

1. A gene-deficient genotype is shown as “-/-”.

2. A gene symbol consists of italic letters with initial letter capitized.

3. The position of the transcriptional start site (TSS) is designated as +1. A nucleotide position upstream to the TSS is designated as a number with a prefix “-”. A nucleotide position downstream to the TSS is designated as a number with a prefix “+”.

4. A sequence starts with “5’-” and ends with “-3’ ”.

5. A plasmid name starts with a prefix “p”.

INTRODUCTION

Innate and adaptive immune response

Innate and adaptive immune responses are the two components of immunity against pathogens. Innate immunity includes epithelial barriers in the skin (1), gut (2) and lungs (3) that prevent infection while innate immune responses are carried out by granulocytes, phagocytes and natural killer (NK) cell. NK cell function in the organism is to identify and destroy infected or transformed cells that lose MHC I expression. Upon stimulation by pathogens, mast cells and eosinophils release anti-microbial mediators and cytokines (4). Phagocytes engulf microorganisms to eliminate them from the body (5). The other cell types responsible for phagocyting microbes are macrophages and dendritic cells which recognize specific microbial motifs called pathogen associated molecular patterns (PAMPS) through Toll-like and other innate immune receptors (6).

To complement the role of innate immune response, the adaptive immune response is triggered and provides an antigen-specific reaction to the pathogen that is challenging the organism. Macrophages and dendritic cells have the ability to present unprocessed pathogens or engulfed pathogens processed into particles called antigens to T-cells. Activated T-cells, especially Th2 cells, in turn trigger the proliferation and class switching of B cells. Both T-cells and B cells mediate adaptive response and a subset of these cells maintain long-term memory that provides a rapid response when the body re-encounters the same pathogen (7, 8).

Antigen presenting cells (APC) (macrophages, dendritic cells and B cells) do not have the ability to distinguish between foreign organisms, commensal organisms or cells coming from the body (self). As a result they generate both foreign and self-antigens in complex with MHC while T cells will only be activated by foreign antigen. The way the organism prevents an immune response against itself is by deleting self-activated lymphocytes in the bone marrow and the thymus while repressing them in the periphery (9). Any defect in eliminating or regulating lymphocytes that recognize self-antigens results in auto-immune diseases.

The functional interaction between B cells, T cells and APC relies on a series of co-stimulatory or inhibitory signals and the production of immunomodulatory factors named cytokines which affect the function of other immune cells in the microenvironment. To prevent an inapropriate response to any stimulus upon interaction with an APC, especially when a self-antigen is presented, lymphocytes require a second “wave” of signals coming from co-stimulatory receptors (10). In the absence of these signals, the lymphocyte response is turned off and these cells become anergic or tolerant to self. However, a tolerant response could correspond to an immunosuppressive state of the organism which favors infections and cancer (11). Immunoregulation can also be mediated by a subset of T cells called T regulatory cells (Tregs) which secrete anti-inflammatory cytokines including interleukin-10 (IL-10) (12), IL-9 (13) and transforming growth factor-beta (TGFβ) .

Depending on the site of infection of the pathogen, adaptive immune responses promote specific subpopulations of T-cells depending on the array of cytokines they secrete. Cytokines are intercellular mediators that promote precise responses from target cells upon binding to their specific receptors. Cytokine-receptor interactions induce the expression of genes involved in inflammatory or anti-inflammatory responses (6). Uncontrolled expression of pro-inflammatory cytokines can promote chronic or inflammatory diseases in the organism. Three main subpopulations of effector T-cells have been identified: Th1, Th2 and Th17 cells (14).

T helper cell subsets

T cells are comprised of three different subsets: the $\alpha\beta$ T cells which include CD4+ T cells and CD8+ T cells, the $\gamma\delta$ T cells involved in cutaneous and mucosal immunity (15) and the NKT cells which recognize lipid antigens in the context of CD1d an MHC class I like molecule (16). CD4+ T helper (Th) cells play a critical role in modulating both innate and adaptive immune responses. Cytokines direct the differentiation of precursor CD4+ T-helper cells (Thp) into one of the defined committed Th phenotypes, T-helper-1 (Th1) cells, T-helper-2 (Th2) cells and T-helper-17 (Th17) cells which are defined by their function and the array of secreted cytokines (Figure 1). The transcriptional regulation that directs the differentiation of Thp cells is based on the expression and activation of the members of the Signal Transducers and Activators of Transcription (STAT) family.

The presence of IL-12 secreted by macrophages and dendritic cells in the microenvironment induces the skewing of Thp to Th1 cells. IL-12 signals to the nucleus of T cells through STAT4 which induces the development of Th1 cells (17) and the production of Th1-type cytokines including IFN-γ, IL-2 and lymphotoxin-α. IFNγ triggers the expression of T-bet a transcription factor controlling the differentiation of Th1 cells (18-20). T-bet also inhibits the expression of Th2 specific gene thus maintaining the stability of Th1 cells. Th1 cells promote cell-mediated immunity against intracellular infections, inflammation disease such as atherosclerosis (21) and tumor development (22).

IL-4 induces Th2 development and Th2-type cytokine production through STAT6. However the first source of IL-4 that induces Th2 differentiation is still not clear. Naïve CD4+ T cells have be reported to provide this initial source of IL-4 (23) and Th2 are able to effect their differentiation in an autocrine manner. Wang *et al*. reported that basophils and mast cells are early sources of IL-4 in allergic response (24). Th2-type cytokines include IL-4, IL-5, IL-9, IL-10 and IL-13 (25). The stimulation of Thp by IL-4 triggers the expression of GATA-3 a transcription factor that epigenetically modifies Th2 cytokine genes for expression (26) and inhibits Th1 differentiation (27, 28). Th2 cells mediate humoral immune response by protecting against extracellular pathogens. Th2 cells have been linked extensively to allergy but also modulate the function of Th1 cells (29).

More recently, Th17, an IL-17 secreting subset has been described and found to play a critical role in tissue inflammation. IL-6 and TGFβ as well as IL-23 signal to CD4+ T cells to induce the production of IL-17 through the transcription factor RORγt. Th17 cells secrete IL-17F, IL-17A (30), IL-21 (31, 32) and IL-22 a member of the IL-20 family of cytokines (33). IL-17A and IL-17F induce the production of pro-inflammatory cytokines, chemokines and metalloproteinases. The skewing of CD4+ T cells to Th17 involves TGFβ, and IL-6, IL-1β to induce proliferation while IL-23 to maintain the phenotype. This differentiation is mediated through STAT3 (31, 34). It is not clear which class of pathogens stimulate Th17 response since some Gram-positive and Gram-negative bacteria or fungi can induce this response (35). Th17 cells have been mainly reported to be involved in autoimmune reaction along with Th1 cells. Experimental autoimmune encephalomyelitis (EAE) a mouse model for multiple sclerosis has been reported to be mediated by Th17 cells.

Two additional subsets of T-cells have been recently described. The first one is a subset of T cells secretor of IL-9 (Th9) described as an unstable cell type derived from Th2 cells in the presence of TGFβ and IL-4 expressed by Th2 cells (36). This finding suggests that Th cells have plasticity *in vivo* which might allow an adaptation of the immune response to the challenges of the organism. The second subset termed T follicular helper cells (Tfh) are non-Th1, non-Th2 effector cell which express CXCR5 allowing them to home to B cells follicles. These cells are primed by IL-21, require STATs and express several B cells

associated molecules like BCL6, CXCR5, IL-6 receptor and CD84 (37-39). The study by Chtanova *et al.* showed that BCL6 is preferentially expressed by Tfh cells compared to other subsets of T effector cells, suggesting that BCL6 might be a major regulator of Tfh cells maturation (37).

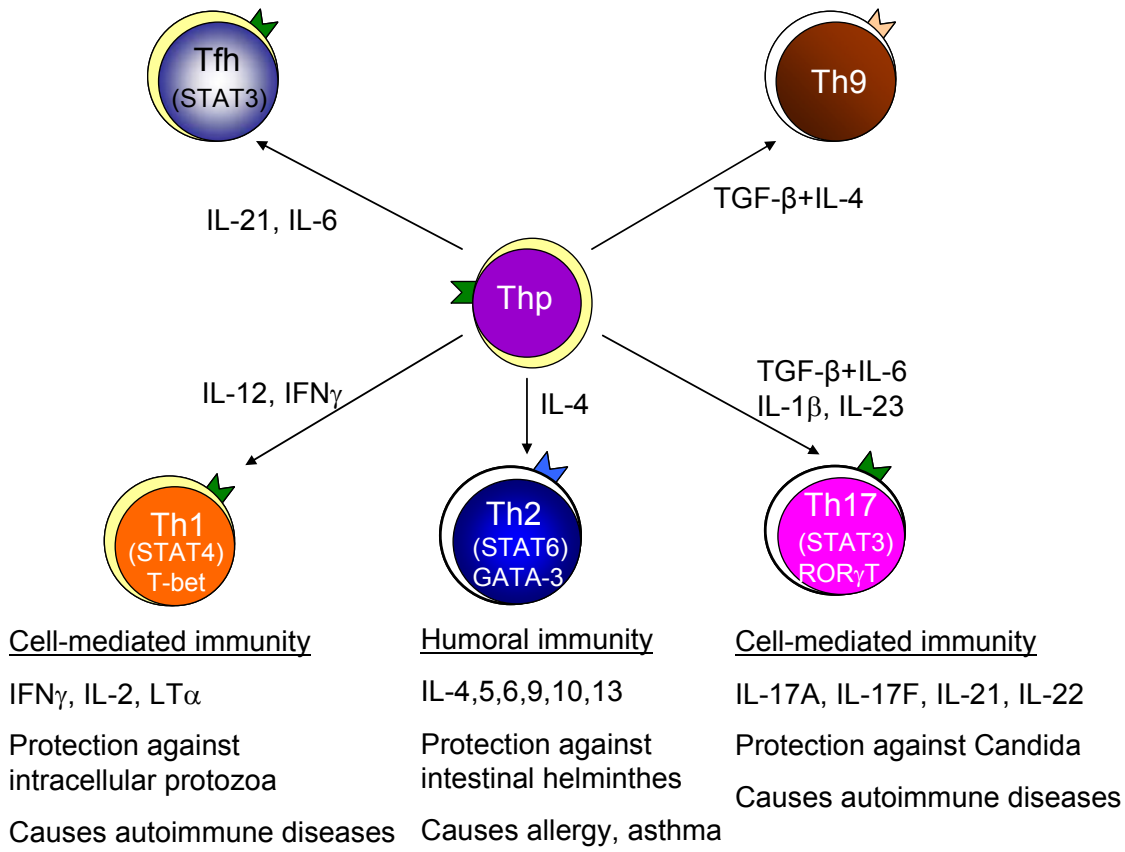


Figure 1: T helper cell development
Naïve CD4+ T cells differentiate into Five subsets of Th cells as a result of the cytokines present in the microenvironment. IL-12 promotes Th1 development; IL-4 triggers Th2 development, whereas TGFβ and IL-6 drives Th17 development. IL-21/IL-6 and TGFβ/IL-4 respectively promote Tfh and Th9 differentiation. Each of these subpopulations is characterized by the array of cytokines they secrete and their biologic functions.

IL-4 promotes Th2 development

IL-4 plays a critical role in the differentiation of Th2 cells. IL-4 is an autoregulatory cytokine secreted by T cells, mast cells and basophils. It acts on different cell types including T and B cells by regulating their proliferation and differentiation. IL-4 triggers biological effects by binding the IL-4 heterodimer receptor (IL-4R) which consists of an IL-4 receptor (IL-4R) α chain and the γ common (γc) chain subunit. The two subunits of the IL-4R do not have intrinsic tyrosine kinase activity. Two cytoplasmic protein tyrosine kinases, JAK1 and JAK3, members of the Janus kinase family, are associated respectively with the IL-4Rα and γc chains. The Janus kinase proteins bind to the intracellular portion of the receptor where they are activated and phosphorylate IL-4R to favor the anchoring, through the Src homology domain 2 (SH2), of STAT6 (Figure 2). STAT6 molecules are phosphorylated on Y-641 and form a homodimer which translocates to the nucleus and transactivates the expression of target genes. STAT6 triggers the expression of Th2-restricted transcription factors, including GATA-3. The requirement for STAT6 in IL-4 induced functions has been demonstrated using STAT6 deficient mice (40, 41). In the absence of STAT6, IL-4 is unable to promote the stable development of Th2 cells.

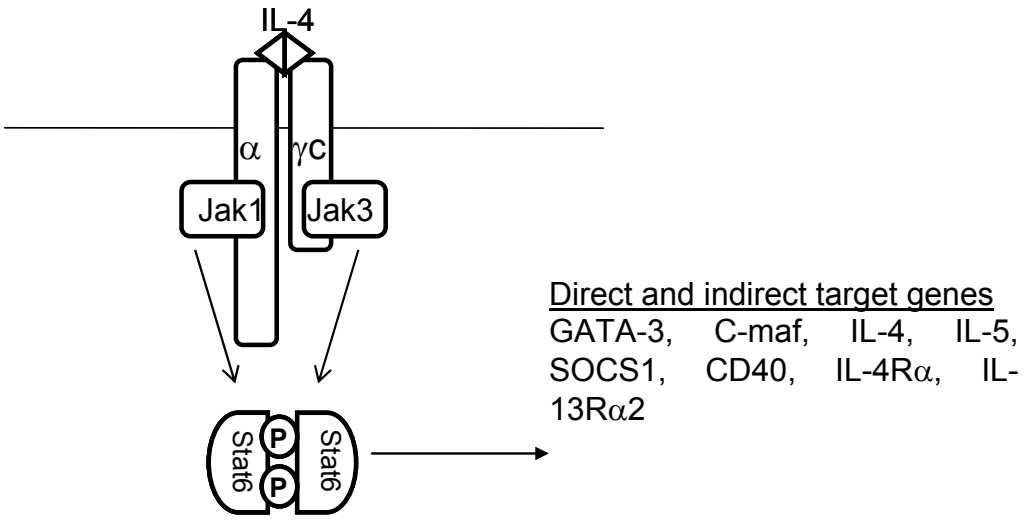


Figure 2: IL-4/STAT6 signaling pathway
The binding of IL-4 to its receptor triggers the autophosphorylation of the α chain, the subsequent binding of STAT6 and STAT6 phosphorylation. STAT6 homodimers are formed and translocate to the nucleus were target genes are transactivated.

Transcription factors involved in Th2 differentiation

Immunity against microorganisms is orchestrated by specialized CD4+ T helper cells which are subdivided in populations of Th1, Th2 and Th17 cells (42, 43). Th2 cells are involved both in immunity against helminth worms and the generation of allergic responses via secretion of IL-4, IL-5 and IL-13 (44). *In vivo* Notch plays a central role as a receptor for parasitic antigens. After binding its ligand Notch intracellular domain is cleaved and translocated to the nucleus where it binds RBP-J already at the target DNA site (45-47). One of the target genes transactivated by Notch signaling in Th2 cells is the Gata-3 gene (48) while IL-10 is transactivated in Th1 cells (49).

Upon activation of Th2 cells one of the early transcription factor induced is the special AT-rich sequence binding protein 1 (SATB1), a factor required for the formation of a compact transcriptionally active chromatin structure at the Th2 cytokine locus (50). This structure is important for STAT6 and GATA-3 function (50). GATA-3 is a pivotal transcription factor in the differentiation and the maintenance of Th2 phenotype; it also prevents the differentiation of CD4+ T cells into Th1 cells as well as IFNγ production by these cells (28, 51, 52). Both Notch (48) and IL-4/STAT6 pathways induce GATA-3 expression in Th2 cells. Like GATA-3, C-maf is a transcription factor induced by STAT6 (53) which specifically regulates the expression of IL-4 in CD4+ T cells and NK T cells (54, 55). IL-4 produced by the differentiating Th2 cells regulates in an autocrine manner production of other Th2 cytokines (55). This positive feedback loop

triggers upregulation of *Il4* gene by extracellular IL-4 and correlates with ectopic GATA-3 ability to induce the expression of endogenous GATA-3 gene. JunB complex, nuclear factor of activated T cells (NFAT) c1 and c2 also regulate the expression of Th2 cytokine genes (56, 57). Contrary to JunB, NFAT proteins are directly controlled by the calcium- and calmodulin-dependent phosphatase calcineurin (58). NFATc1 and NFATc2 (59) cooperate with IRF4 in the induction of Th2 cytokines (59, 60).

Concept of Th2 heterogeneity

In 1989, when Fiorentino *et al.* were working on long-term mouse Th cell clones, the notion of heterogeneity in the T cell population was limited to the 2 types of Th cell clones they characterized based on the profile of cytokines secreted: Th1 and Th2 (61). At the present time we define heterogeneity in every subset of Th cells based on the amount of signature cytokines secreted by each cell. The concept evolved from Hu-Li *et al*. using mice heterozygous at the *Il4* locus (*Il4*/GFP mice) (62). While assessing the allelic regulation of IL-4 in Th2 cells, they demonstrated that each cell had the same probability to express IL-4. On the contrary Calado *et al*. reported that IL-10 expression in T cells arises from a stochastic regulation mechanism dependent on TCR signaling strength (63).

The differentiation of Th2 cells *in vivo* upon infection or *in vitro* generates a heterogenous population of Th2 cells that express and secrete various subsets of Th2-type cytokines at different levels (64). The proportion of Th2 cells that

secrete a single cytokine is significantly higher than the fraction of double-positive cytokine secretors (65). Even at the level of a subpopulation of Th2 cells that secrete the same cytokine, not all cells secrete similar levels of cytokines (66). Th2 specific transcription factors have been shown to regulate the expression level of specific Th2 cytokines and by consequence define the phenotype of the individual Th2 cells. Some of these regulators are C-maf for IL-4-single positive cells, NFATc2 for IL-10-single positive cells (55, 59) and Pias1 which increases IL-13 production without affecting IL-4 or IL-5 expression (67). It has been previously reported that the pattern of high and low secretion of IL-4 is a heritable and stable event and recently Chang *et al.* demonstrated the role of the PU.1 transcription factor in establishing these phenotypes (66). BOB.1/OBF.1 a regulator of PU.1 expression in Th2 cells also affects the potential for Th2 cytokine production (68).

Biology of PU.1

PU.1 also called *Sfpi1* (spleen focus forming -1) was discovered due to its activation and overexpression in the erythroid leukemia induced by spleen focus forming virus upon insertion of this virus upstream of the *Sfpi1* gene and by cloning a lineage specific transcription factor (69, 70). PU.1 is an oncogenic transcription factor member of the *ets* gene family which shares a conserved ETS DNA binding domain. This domain recognizes a purine-rich core containing the sequence 5'-C/AGGAA/T-3'. PU.1 was the first member of the ETS protein to be crystallized (71). It was originally reported to be specifically expressed in B

cells, macrophages, mast cells and neutrophils (69), but recently our group found that it was also expressed in Th2 cells secreting low levels of IL-4, IL-5 and IL-13.

PU.1 is required for lymphoid and myeloid cell development. It consists of three domains: the transactivation domain (TAD), the Proline-Serine-Threonine (PEST) rich domain and the DNA binding (ETS) domain (71). Klemsz *et al*. demonstrated that the TAD domain located at the amino-terminal consists of an acidic subdomain and glutamine rich subdomain required for the PU.1 maximal transactivation function (Figure 3) (72). PU.1 regulates the expression of the following genes in B cells: kappa, lambda, J chain light-chain and heavy-chain. Macrophage colony-stimulating factor receptor, scavenger receptor, interleukin 1β, FcγRIIIA and FcγR1β are among the genes targeted by PU.1 in macrophages (73-76). Studies using trichostatin A suggested that PU.1 expression was blocked by histone acetylation in the promoter region (77).

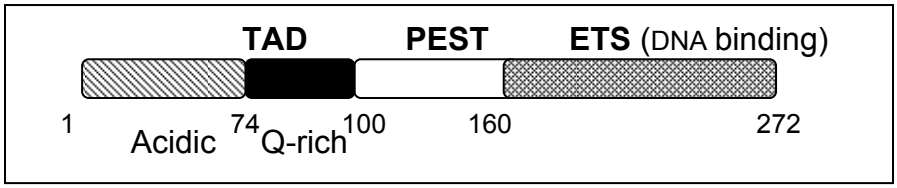


Figure 3: PU.1 protein representation

The regulation of PU.1 expression is critical for the normal development of hematopoietic cells and their function during immune response. Two *Sfpi1*-/- mouse models were developed to assess the importance of this transcription factor. The first, a null model was embryonic lethal by day 18 with an impaired development of B cells, granulocytes and monocytes in the embryos. The second PU.1 knockout model allowed the development of embryos, however, mice died soon after birth unless they are maintained on antibiotics in which case they survived for 14 days. In the second model mice showed a total loss of macrophage and B cells (78, 79). Even though neutrophil morphology and marker expression including Gr-1 and chloroacetate esterase were normal, PU.1 null neutrophils fail to terminally differentiate, to respond to chemokines, to generate superoxide ions, to phagocytose and kill bacteria. PU.1 deficient neutrophils are unable to express gp91 subunit of nicotinamide adenine dinucleotide phosphate oxidase which could explain some functional impairment (80). PU.1 is also required for the normal development of T cells as T cells development was delayed until birth in the second model with the number of these cells reaching only a fraction of their size in the wild-type mice thymus (78). The study of PU.1 significance in adult hematopoietic cells was made possible with the PU.1 conditional knockout mice developed by the Tenen group (81) and Stephen Nutt's group (82, 83). The Nutt group reported that PU.1 ablation in adult hematopoietic cells significantly enhanced granulopoiesis with increased expansion of granulocytic progenitors and the absence of macrophage-CSF, granulocytes/macrophages-CSF and IL-6 responsiveness due to the reduced

expression of M-CSFR, GM-CSFRα and IL-7Rα. This PU.1 conditional knockout showed a deficiency in lymphoid and myeloid progenitors (84). Common lymphoid progenitors, common myeloid progenitor, granulocyte-macrophage progenitors as well as megakaryocyte-erythrocyte progenitor lineages were not detected in the absence of PU.1. The study demonstrated that PU.1 regulates B and T cells lymphopoeisis by controlling multipotent progenitor commitment.

The absence of PU.1 disrupted the balance regulating the maturation of multipotent myeloid progenitors, as a result the majority of the PU.1-deficient adult mice developed myeloid leukemia. These leukemia cells were transplantable and acquired the autocrine capability to produce the growth factor IL-3 (85).

PU.1 regulates early T cell development

Early T cell development requires the extremely careful regulation of multiple transcription factors both temporally and quantitatively. T cells go through different stages of development from double-negative 1 to pre-T cells (Figure 4). As a result lineage-specific genes are activated while lineage-inappropriate genes are inhibited all in an asynchronous manner. The PU.1 and GATA-3 interaction in a dose-dependent manner is essential for early T cell development. PU.1 expression is highest in the DN1 stage. It decreases by 7 fold in DN2 and reaches background level at DN3, while GATA-3 level increases by 2 fold after β-selection during DN4 (86). Studies assessing the effect of GATA-3 and PU.1 overexpression in fetal liver precursors or fetal thymocytes revealed the

developmental stage controlled by each of them (86). The overexpression of PU.1 prevents the progression of lymphocytes precursors through β-selection but favor their differentiation into myeloid cells (87). In addition C-myb, HES-1 and GATA-3 expression is blocked. Cells transduced with low level of PU.1 are able to become DN2 or DN3. GATA-3 overexpression severely blocked early stage T cell development with PU.1 and IL-7Rα expression inhibition and thymocytes are unable to mature into into DN2 or DN3 (Figure 4) (88, 89). Ikaros and PU.1 are very important in thymocytes before birth while C-*myb* and GATA-3 are central for the emergence of thymic T-lineage precursors. Once the early T cells go through β-selection the transcription factors required change. GATA-3, a key transcription factor in the differentiation of Th2 cells, has previously been shown to block the expression of PU.1 and its target genes (86). PU.1 is expressed in T cell progenitors but expression is rapidly extinguished during the double-negative (CD4-CD8-) stage as GATA-3 expression is increasing. Both the decrease of PU.1 and the increase of GATA-3 are required for the normal development of T cells in the thymus (27, 28, 51, 86, 88-93). In differentiated Th2 cells, PU.1 can interfere with GATA-3 function (66).

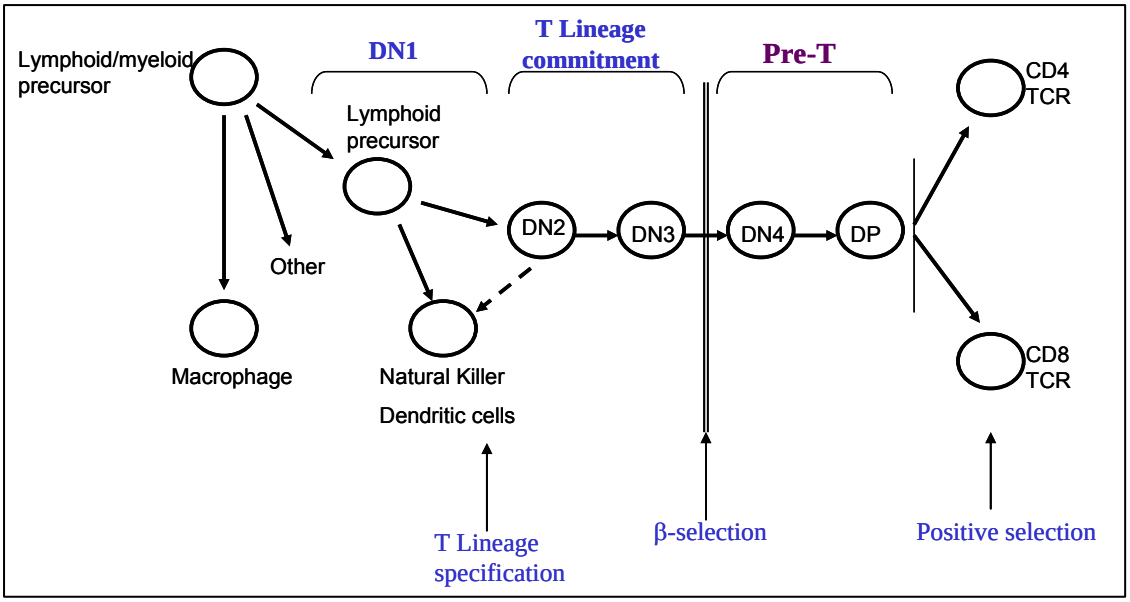


Figure 4: Lymphoid development pathway

PU.1 regulates Th2 heterogeneity

As mature T cells differentiate into Th2 cells, GATA-3 expression is further induced and is critical in the expression of Th2 cytokines (51). However, while GATA-3 is expressed in all Th2 cells, not all Th2 cells express equal amounts of cytokine. This heterogeneity could be explained by the presence of factors that modulate GATA-3 activity. We demonstrated that PU.1 expression in CD4+ T cells is restricted to Th2 cells especially to IL-4 non secreting cells (66). Evidence supports a role for PU.1 as a modulator of GATA-3 activity by preventing GATA-3 binding to the *Il4* locus in IL-4 low and IL-5 low cells, defining the phenotypes present in the heterogeneous Th2 population (66). Whether GATA-3 is the only factor targeted by PU.1 to regulate this phenotype is still

unclear. However, our understanding of the mechanism by which PU.1 regulates Th2 heterogeneity is incomplete since our data using mutant PU.1 suggests that in addition to its regulatory effect on GATA-3, PU.1 also targets other transcription factors in Th2 cells (66).

Development of IL-10-secreting cells

Different subsets of Th cells have been reported to secrete IL-10. Both Th1 and Th2 cells express IL-10, however, Th2 cells express more IL-10 than Th1 cells. After repeated restimulation in the presence of IL-4, extensive histone acetylation of the *Il10* gene was detected and determined in IL-10 memory Th2 cells (94). This mark of gene activation which correlates with the development of IL-10 memory was not detectable in Th1 cells (94). Recently IL-27, a member of the IL-12 family has been reported to increase IL-10 production in Th1 and Th2 cells via STAT3 (95, 96).

Another subset of T cells, T regulatory type 1 cells (Tr1) is also known for IL-10 secretion through which they exert a suppressive effect on T helper cell proliferation. Tr1 differentiation and IL-10 expression is triggered by the transforming growth factor-β and IL-27 produced by dendritic cells after their interaction with TGF-β T regulatory cells (97). Tr1 are different from Foxp3-expressing T regs which can also secrete IL-10 (98). This subset of Tregs is generated *in vitro* in the presence of dexamethasone and vitamin D3.

Biology of interleukin-10 and its signaling

IL-10 is a regulatory cytokine produced by a number of cells including macrophages, B cells, dendritic cells, mast cells and T cells (99). In T cells IL-10 expression is generally monoallelic with a transcriptional independence between both alleles (63). IL-10 plays a critical role in controlling inflammation *in vivo* by selectively suppressing the expression of pro-inflammatory cytokines including IL-8, IL-12, IL-6 and TNF-α (100).

IL-10 regulation

Type I interferon, IFNα, was reported to induce the binding of transcription factors including IRF1 and STAT3 to the promoter region of the human IL-10 gene locus and regulate IL-10 expression (101). In Th1 and Th2 cells, IL-10 expression is also regulated by Smad-4 (102) and Jun (103), while Sp1 and Sp3 regulates the mRNA level of IL-10 (104). The transcription factor GATA-3 plays a critical role in the regulation of IL-10 expression by remodeling of IL-10 locus in Th2 cells (26). The transduction of non-polarized Th cells and Th1 cells with ectopic GATA-3 significantly increased the number of cells secreting IL-10 demonstrating the instructive role of this factor. Analysis of Th2 cells by ChIP assays revealed GATA-3 binding to its consensus sequence in the 5' region and intron 4 of IL-10 locus. Ectopic GATA-3 also increased the chromatin accessibility as well as histones H3 and H4 acetylation across the IL-10 locus which correlates with increased transcription (26) while sustained IL-4 stimulation

is required for extensive histone acetylation of the *Il10* gene in IL-10 memory Th2 cells (94).

IL-10 signaling

IL-10 receptor consists of two chains, IL-10R1 and IL-10R2 which bind Jak1 and Tyk2 upon activation by IL-10 allowing the recruitment of STAT3, STAT5 and STAT1. As a result, STAT3 and STAT1 are phosphorylated, however, STAT3 is necessary and sufficient to mediate IL-10 anti-inflammatory effect (105, 106). Indeed IL-10 inhibits the transcription of inflammatory cytokines including IL-6 and TNFα as well as CD40 expression via the suppressor of cytokine signaling 3 (SOCS3) (107, 108). In addition, IL-10 exposed macrophages inhibit IFNγ production by Th1 cells (109) while *in vivo* CD4+ T cells produced IL-10 prevents Th1 from secreting IFNγ in response to helminthic infection (110). The function of IL-10 induced STAT1 phosphorylation is still unclear.

IL-10 has been associated with a positive clinical outcome in cardiac surgery as well as mouse carotid injury through a sustained inhibition of NFκB in addition to a decrease in the expression of chemokines and growth factors involved in pro-inflammatory response *in vivo* (111). In the context of airway hyperreactivity and asthma, IL-10 producing dendritic cells and T regulatory cells have been associated with antigen-specific CD4+ T cell unresponsiveness which protects against the development of the disease (12).

Biology of interleukin-9

Interleukin-9 (IL-9) is a pleiotropic cytokine produced specifically by Th2 cells that was discovered because of its proliferative activity on murine T helper cell clones (112), murine mast cells (113, 114) and human megakaryoblastic leukemia line (115). Murine fetal thymocytes (116), murine erythroid progenitors (117), human T cell lines and human myeloid and erythroid precursors were later found to be IL-9 biological targets (118, 119). The human and mouse IL-9 genes share a similar structure with 5 exons that span -4 kb and that share 56% to 74% homology. While IL-9 does not have any effect on freshly isolated CD4+ T cells even in the presence of anti-CD3 stimulation, mouse IL-9 is preferentially expressed by activated T cells and Th2 clones *in vitro*.

IL-9 signaling

IL-9 induces recruitment of eosinophils and lymphocytes to the lung, mucus hypersecretion, mast cells hyperplasia. It is important to note that IL-9 cannot induce these asthma pathologic and physiologic changes in the absence of IL-4, IL-5 and IL-13 (120). IL-9 plays a central role in the regulation of airway hyperreactivity and asthma. Studies performed in IL-9 transgenic mice (Tg5) demonstrated that IL-9 promotes mucus glycoprotein (Muc2 and Muc5a) expression by airway epithelial cells. The treatment of human primary lung cultures and human muccoepidermoid NCI-H292 cell line with recombinant IL-9 also induced the upregulation of the same mucus glycoproteins (121). This effect was similar to the one induced by IL-13 (121). Like the IL-4 receptor, the

IL-9 receptor is a member of the hematopoietin receptor superfamily identified by cDNA expression cloning (122). IL-9 effects were shown to be mediated by the JAK-STAT pathway. JAK1 and JAK3 tyrosine kinases are pre-associated with IL-9Rα and the IL-2 receptor γ-chain respectively (123). JAK-1 and JAK-3 kinases phosphorylates the IL-9Rα on tyrosine 367 allowing the recruitment, phosphorylation and activation of STAT1, STAT3 and STAT5 (124). STAT1 and STAT3 play a specific role in the induction of differentiation genes including granzyme A, L-selectin and Ly-6A/E, while STAT5 or both STAT1 and STAT3 mediate IL-9 anti-apoptotic effect (124).

Biology of Interferon Regulatory Factor 4

The first protein shown to interact with PU.1 was IRF4. IRF4 is also referred to in the literature as lymphoid specific interferon regulatory factor (LSIRF) (125), PU.1 interacting protein (Pip) (47), DNA binding motif EM5 binding nuclear factor (NF-EM5) (126) or interferon (IFN) consensus sequence-binding protein in adult T-cell leukemia cell lines or activated T cells (ICSAT) (127). IRF4 protein structure consists of 2 major domains: a DNA binding domain at the amino terminal and a PU.1 interacting domain at the carboxyl terminal. The C-terminal and N-terminal regions contain auto-inhibitory domains that prevent IRF4 from binding to DNA (Figure 5). The binding of IRF4 to the immunoglobulin kappa3' enhancer was dependent upon its protein-protein interaction with PU.1 as well as protein-DNA interactions (126). Perkel *et al.* demonstrated that PU.1 recruitment of IRF4 to the DNA is a two step mechanism which first involves the interaction of IRF4 with

PU.1 ETS domain in solution and PU.1 PEST phosphorylation on serine 148 triggering a covalent structural change (128). This PU.1 structural change causes IRF4 conformational modification that allows its recruitment to the DNA (128). Escalante and coworkers crystallized the PU.1/IRF4/DNA ternary complex using the DNA binding domains of theses transcription factors and a 21-mer DNA site from λB element containing PU.1 binding site (AGGAA) and IRF4 binding site (GAAA) (129, 130). This study also revealed that PU.1 and IRF4 interaction releases the autoinhibitory effect of the N-terminal and C-terminal region of IRF4 allowing its recruitment to the lambdaB element of immunoglobulin light chain lambda enhancer and transactivation of the gene in a cooperative manner (126, 129-132). Casein kinase II phosphorylates PU.1 on serine 132 and serine 148 in the PEST domain (132, 133), as a result phosphorylated PU.1 increases the stability of a ternary complex composed of PU.1 and IRF4 bound to DNA. Using quantitative hydroxyl radical footprinting, Gross *et al.* determined that the DNA binding domains of both PU.1 and IRF4 interact with the DNA (λB element) in the major groove while protein-protein interactions happen near the intervening minor groove (132, 134).

Upon binding to DNA PU.1 and IRF4 bend DNA to an S-shape which bring the DNA binding domains closer together, enhancing the interaction between the proteins by 20 to 40 fold across the minor groove (130). The mutation of R328 to glutamic acid in IRF4 abrogates the interaction with PU.1 and recruitment of IRF4 to the DNA. The deletion of residues 410 to 450 in IRF4 prevented the binding of

IRF4 to NFATc2 and decreased the IRF4-dependent synergy of NFATc2-dependent IL-4 transactivation. The central region of IRF4 is a proline-threonine rich region susceptible to FK506 Binding Protein (FKBP52) isomerization through its peptidyl-propyl isomerase (PPIase) activity. While IRF4 interaction with the PU.1 PEST domain or NFATc2 blocked the function of the auto-inhibitory domains in IRF4 DNA binding (59, 130), IRF4 and PU.1 interaction was abrogated by the PPIase activity of FKBP52 (135).

IRF4 is an important transcription factor in the differentiation of Th2, Th17 and some functions of Treg cells (136-138). While IRF4 induces cytokine production in memory T cell populations, it triggers the opposite effects in naïve T cell populations (139). In Th2 cells IRF4 binds to a site adjacent to a NFAT binding element and cooperates with NFATc1 or NFATc2 in the transcription of IL-4 (59, 60). These interactions are important as the activation of T cells triggers a rapid induction of IRF4 which enhances NFATc2 and c-maf-dependent IL-4 expression or NFATc1-dependent IL-2 expression (125, 127). IRF4-deficient T cells secrete decreased levels of Th2 cytokines including IL-4, IL-5 and IL-13 (136). The analysis of Th2 specific transcription factor GATA-3 in the *Irf4*-/- Th2 cells showed a decrease in the level of GATA-3 which in part explains the absence of Th2 cytokines expression (136).

Even though PU.1/IRF4 interaction and function have been extensively examined in B cells, their importance has never been assessed in the differentiated T cells.

IRF4 overexpression has been shown to increase the level of IL-10 secreted by Jurkat cells and IRF4 knockout cells have an impaired Th2 cytokine expression including an impaired IL-10 expression. The mechanism by which IRF4 controls the expression of IL-10 *in vivo* or *in vitro* is still unknown.

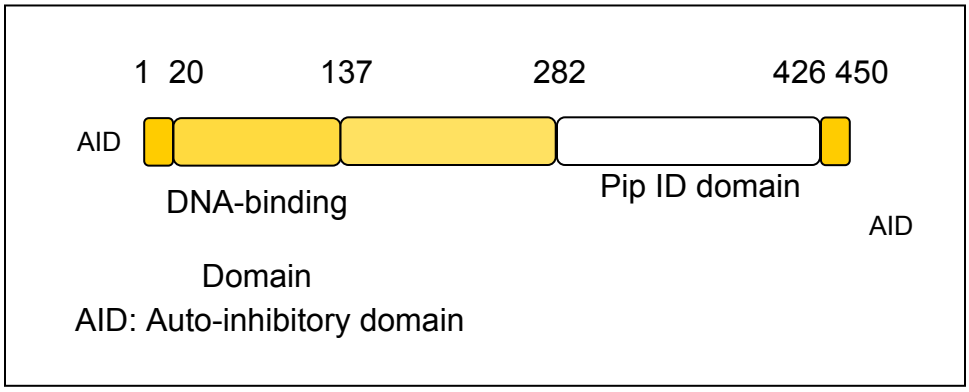


Figure 5: IRF4 Protein structure

The goal of this research

Previous work has demonstrated that IRF4 was required for the development of T cells and dendritic cells, since *Irf4*-/- mice have shown a defect in the development of these cells (136). These phenotypes may explain the impaired potential of the *Irf4*-/- T cells to express the expected hallmark cytokines after skewing under Th1 or Th2 conditions. In the case of Th2 cells the decrease in GATA-3 expression brings the question of whether IRF4 is affecting Th2 cytokine genes directly or indirectly. Our goal for this study was to decipher the role of IRF4 during Th2 differentiation versus its role during development. To achieve this aim only mice expressing wild-type IRF4 were used allowing us to assess the indirect effect of IRF4 on GATA-3 expression versus IRF4 direct effect on an array of Th2 related genes.

Th2 cells promote allergic inflammation and modulate inflammatory disease caused by Th1 and Th17 cells. The airway infiltration of Th2 lymphocytes has been associated with human asthma and the production of IL-4, IL-5 and IL-13 correlates with asthma-like symptoms in mouse model of asthma (140). It was demonstrated by Finotto and her group that T-bet deficiency induced airway remodeling as well as increased amount of IL-4 and IL-13 in the lung both characteristic of asthma (141, 142) and a study by Eisner *et al*. reported that asthma was associated with a modest increase in the risk of heart disease in women (143). Since IL-10 production has been associated with protection against the development of the heart disease, understanding the mechanisms

that regulate the heterogeneity of IL-10 producing Th2 cells would be an important tool in the development of therapies for suppressing allergic disease and increasing the modulation of Th1 mediated inflammation involved in atherosclerosis, vascular injury and heart disease. Our approach to achieve these goals was to decipher the mechanisms controlling the function and balance of Th2 cytokines that promote allergic inflammation and cytokines that are anti-inflammatory. We have previously demonstrated that PU.1 acts as a modulator of Th2 cell subset development by interfering with GATA-3 function. However, these data also suggested that additional targets regulating PU.1 activity exist. The goal of this study was to define the importance of IRF4 in the phenotype of subpopulations of IL-10 producing Th2 cells. Manipulating the level of this transcription factors offers a potential tool for switching the balance between immunity and allergy (or pathology).

MATERIALS AND METHODS

Mice

Wild-type C57BL/6 and Balb/c mice (Harlan Bioscience, Indianapolis, IN) were used for Th1 and Th2 differentiation. Conditional mutant *Sfpi1* mice on the C57BL/6 background (84) and were mated to lck-Cre expressing mice (noted as *Sfpi1*$^{lck-/-}$). Mice were maintained in pathogen-free conditions and all studies were approved by the Indiana University School of Medicine Animal Care and Use Committee.

CD4 Cell Isolation

Spleen and lymph nodes were collected from mice and homogenized in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, 1mM glutamine, 100 Units/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes buffer, 0.5 x nonessential amino acids, 1mM sodium pyruvate, and 50 μM β-mercaptoethanol. The extract suspension was sieved in a conical tube through a strainer (BD Biosciences, San Jose, CA) to remove debris. The cell suspension was centrifuged at 1500 rpm for 5 minutes at 4ºC and the supernatant was discarded. The cells were resuspended in MACS buffer [phosphate buffered saline (PBS) supplemented with 2mM EDTA and 0.5% bovine serum albumin (BSA)] (3 ml). Anti-mouse CD4 magnetic beads from Miltenyi Biotec (75 μl per 12-14x10^7 cells) were added to the cell suspension followed by 15 minutes incubation at 4ºC. Cold MACS buffer (12 ml) was added to the cell suspension followed by centrifugation at 1,500 rpm. The strong magnetic field surrounding the column

retained the CD4 positive cells while other cells were washed three times with MACS buffer (3ml). The column was then removed from the magnetic field and placed in a 15-ml tube. Six milliliters of MACS buffer were added to the column and flushed out with a plunger to elute CD4 cells. The purity of the CD4 cells was determined to be more than 97% by FACS.

In vitro T helper Cell Differentiation

CD4+ T cells were purified from spleen and lymph nodes by positive selection using magnetic beads (Miltenyi Biotech, Auburn, CA) with purity greater than 97% by FACS analysis. Cells were activated in 6-well plate coated with 2 μg/ml of anti-CD3 mAb (145-2C11) in 1 ml PBS and incubated at 37ºC, 5% CO_2, 100% humidity for 2 hours with 2 μg/ml plate-bound anti-CD3. For Th2 differentiation 1 μg/ml anti-CD28, 10 ng/ml IL-4 and 10 μg/ml anti-IFNγ mAb (R4/6A2 or XMG) were added to the CD4+ T cells while 1 μg/ml anti-CD28, 10 ng/ml IL-12 and 10 μg/ml anti-IL-4 mAb (11B11) were added for Th1 differentiation. The cells were plated at a density of $1x10^6$ cells/ml. After 3 days of incubation, cells were expanded for a total of 5 to 10 days. Differentiated cells were restimulated with 2 μg/ml anti-CD3 at a concentration of 10^6 cells/ml for real-time PCR and ELISA as previously described (34, 66). Statistics were performed using a t-test with SPSS software.

FACS Analysis of Th2 Cytokine Expression

Intracellular cytokine staining was performed using fluorochrome conjugated antibodies to stain cells that had been restimulated either with 50 ng/ml phorbol myristate acetate (PMA; Sigma) and 750 ng/ml ionomycin (P+I) or 4 μg/mL α-CD3 for 3 hours prior to treatment with 3 μM monensin. Restimulation was continued for 2h when P+I were used and 3h for α-CD3. Cells ($1x10^6$) in RPMI 1640 were centrifugated at 1,500 rpm for 5 minutes in a 12x75 mm tube. The supernatant was poured out and the cells were washed once with 2 ml FACS buffer (1x PBS, 0.1% sodium azide, 0.1% BSA). The cells were incubated in a fixative buffer (FACS buffer 48ml containing 4% paraformaldehyde) (1ml) for 10 min at room temperature (RT). The fixation buffer is washed with FACS buffer (1ml) followed by a second wash with a permeabilization buffer (0.1% saponin, FACS buffer) (1ml). The fluorochrome conjugated antibodies for IL-4, IL-5, IL-10, IL-13, IL-10Rα, IL-4Rα (BD Biosciences, San Jose, CA) and the fluorochrome conjugated anti-human CD4 antibody for transduced cells were added to the tubes followed by 30 minutes incubation at 4ºC. After this incubation time cells were washed once with a permeabilization buffer then resuspended in FACS buffer. In each sample 10,000 events were collected using LSRII machine and data was analyzed using Cellquest software.

Intracellular Staining for Transcription Factors

Differentiated cells ($1x10^6$) were restimulated, fixed and permeabilized as previously described. Cells were treated with anti-IRF4 or normal IgG (Santa

Cruz Biotechnology) as control for 30 minutes at 4ºC then washed with FACS/ELISA buffer. The secondary donkey anti-goat antibody (Jackson Immunoresearch) conjugated with Cy5 (Cyanine 5) (0.125 μL) was added to the cells for 15 minutes then washed with 1 mL permeabilization buffer.

The IRF4 stained cells were incubated with anti-GATA-3-FITC (Fluorescein isothiocyanate), anti-IL-10 PE (Phycoerythrin) and anti-IL-4 PE-Cy7 for 30 minutes, washed with 1mL permeabilization buffer then resuspended in FACS/ELISA buffer. LSRII machine was used to collect 50-100 $x10^3$ events after gating on live cells.

Generating Total/Nuclear Cell Lysates and Measuring Protein Concentration

Total cell extracts were prepared by lysing Th2 cells or Phoenix cells with lysis buffer (10% glycerol, 1% Igepal, 50 mM tris-pH 7.4, 150 mM NaCl, 1 mM EDTA-pH8) for 15min on ice before centrifugation at 14,000 rpm for 15 min at 4ºC. Nuclear and cytoplasmic proteins were prepared from differentiated Th2 cells using Nuclear and Cytoplasmic Extraction Reagents from Pierce Biotechnology. Differentiated cells ($20x10^6$) were harvested and washed with PBS. The supernatant was carefully removed and the cell pellet was resuspended in 200 μL of ice-cold cytoplasmic extraction reagent I (CER I) by vortexing for 15 seconds. The cell suspension was incubated on ice for 10 minutes. Ice-cold CER II (11 μL) was added to the cells, vortexed for 5 seconds and incubated on ice for 1 minute. The lysate was centrifugated at 14.000 rpm for 15 minutes at

4ºC and the supernatant was collected as the cytoplasmic extract. The nuclear fraction was pelleted down and resuspended in 100 μL of ice-cold nuclear extraction reagent (NER) and incubated for 40 minutes with 15 seconds vortexing every 15 minutes. The tube was centrifuged for 10 minutes at 14,000 rpm and the supernatant (nuclear extract) was transferred to a clean pre-chilled tube. For long-term storage samples were kept at -80ºC.

The protein assay dye reagent (Bio-Rad) was diluted 1:4 in ddH_2O. The standard protein BCG and the samples were serially diluted in 100 μl H_20 per well of a flat-bottom 96-well plate and 100 μL of diluted protein dye was added to each well. The color change was measured at 595 nm either with a microplate reader model 550 (Bio-Rad) or microplate reader model 680.

SDS-PAGE and Western blot

Cell lysates containing 25 to100 μg of protein were added to SDS-PAGE loading buffer (200mM Tris HCl pH 6.8, 40% glycerol, 8% SDS, 4% β-mercaptoethanol, 0.04% bromophenol blue) and boiled for 5 minutes to denature the proteins. NUPAGE 4-12% gradient Bis-Tris 12-well pre-cast polyacrylamide gels (Invitrogen) were used to separate proteins in both samples and Precision Plus marker (BioRad) by electrophoresis. Power pack from BioRad was used to run the gel at 150V for 90 minutes. The proteins were transferred overnight to a nitrocellulose membrane (Schleicher & Schuell) at 30V, 4ºC in a 2X NUPAGE transfer buffer containing 20% methanol. The membrane was blocked in 5%

nonfat powdered milk/1x TBST (Tris-Base, NaCl, Tween-20) on a shaker for 1 hour. The Primary antibodies to detect GATA-3 (R& D systems, Minneapolis, MN), IRF4, PU.1and NFATc2 (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (Calbiochem, La Jolla, CA) were used to immunoblot the membrane for 2 hours at RT on a shaker. The membranes were washed 3 times for 15 minutes with 1x TBST before incubation with the secondary antibody horse-radish peroxidase (HRP, BioRad). Three TBST-washes at RT were performed to remove the excess secondary antidody. To detect the protein of interest western lightning chemiluminescence reagent (PerkinElmer Life Sciences, Wellesley, MA) was used as a substract for HRP and exposed to CL-Xposure film (Pierce, Rockford, IL). The membranes were stripped with stripping buffer (44.65 mL 1xTBST, 5 mL 20% SDS and 350 μL β-mercaptoethanol) for 30 minutes, covered with saran wrap in the 56ºC water bath and washed with 1x TBST for 10 minutes. Membranes were blocked in 5% milk and immunoblotted for another protein.

Chromatin Immunoprecipitation Assay (ChIP)

CD4 T cells cultured under Th2 conditions for five days were restimulated with 2 μg/ml anti-CD3 for 2 to 4h before cross-linking the protein-DNA complexes by adding formaldehyde to the cell cultures. Unstimulated Th2 cells were used as control and 5 x 10^6 cells were used per ChIP reaction. Cells were resuspended in nuclear lysis buffer (50nM Tris pH 8.0, 10mM EDTA pH 8.0, 1% SDS, and protease inhibitor) at 4ºC for 15 minutes and the DNA was sheared by ultrasonication. The precleared cell lysates were incubated with anti-PU.1 or

anti-IRF4 overnight and the next day DNA was immunoprecipitated. For this experiment anti-histone H3 antibody was used as positive control and IgG antibody was used as negative control for the ChIP assay. The DNA bound beads were washed with a low salt buffer, a high salt buffer, a LiCl buffer (0.25 M LiCl, 1% Igepal, 1% sodium DeoxyCholate,1mM EDTA pH 8.0 and 10 mM Tris pH 8.0), and two TE buffer washes (1mM EDTA and 10 mM Tris pH 8.0). Real-time PCR was done with 2 μl (1.7 X 10^5 cells) of immunoprecipitated DNA for 30 cycles. DNA was then analyzed using PCR or qPCR. To calculate percent input, ChIP results from the control IgG were subtracted from ChIP results for the specific antibody and divided by input values as determined by an input standard curve.

Semiquantitative Real-Time RT-PCR

Total RNA was isolated from total Th2 cells, flow cytometry sorted IL-10-high and IL-10-low cells, retrovirally transduced or siRNA transduced Th2 cells. RNA was reverse transcribed with Super Script First-strand kit (Applied Biosystems, Foster City CA). PCR was performed on an Applied Biosystems ABI PRISM 7500 Realtime PCR system using Taqman Universal PCR Master Mix with FAM-labeled primers from the same company. Relative quantitation was performed using CT (threshold cycle) method. Assays were performed in duplicate and the target gene ΔCT values were derived by subtraction of the CT value from β-2-microglobulin. $\Delta\Delta$CT values were calculated relative to the chosen calibrator sample and relative gene expression levels were determined from the equation

2-ΔΔCT. Error bars represent the range in relative gene expression level based on the ΔΔCT standard deviation.

Detection of Cytokines using ELISA

The level of cytokines secreted by restimulated cells was measured from cell free supernatants using ELISA (Enzyme-Linked Immunosorbent Assay). To perform this experiment 96 well ELISA plate were coated with 2μg/mL of anti-IL-4, anti-IL-5 and anti-IL-13 capture antibodies (BD Biosciences) diluted in 0.1M NaHCO3 (pH 9). The plates were incubated at 4ºC overnight and blocked the next day in 250 μl FACS/ELISA buffer (2% BSA and 0.01% NaN_3 in PBS, pH 7.4) for at least 2 hours at room temperature. The blocking buffer was washed away with ELISA wash buffer (0.1% Tween-20 in PBS). The supernatants diluted 50 fold as well as the standards (R& D systems, Minneapolis, MN) were added to the plate and incubated overnight at 4ºC. After washing the samples off the plate three times 1 μg/mL of biotinylated detection antibodies in FACS/ELISA buffer was added to the plates for a minimum of 2 hours. The plates were washed three times with ELISA wash buffer and incubated with streptavidin alkaline phosphatase (Sigma-Aldrich, St Louis MO) in FACS/ELISA buffer for one hour in minimum.

IL-9 detection using ELISA (BD protocol)

Briefly, the plates were coated with 2 μg/mL of anti-IL-9 capture antibody (R& D systems, Minneapolis, MN) in 0.1M $NaHPO_4$ at 4ºC overnight and blocked the

next day in 200 μl of BD blocking buffer (10% fetal bovine serum in PBS) for 2 hours at RT. The blocking buffer was washed away with ELISA wash buffer. The supernatants were diluted 4 fold and added to the plates as well as the standards (R& D systems, Minneapolis, MN) for a an overnight incubation. After washing off the samples three times, 0.5 μg/mL of biotinylates detection antibodies in 0.05% Tween-20 of BD blocking buffer was added to the plates for a minimum of 1 hour. The plates were washed three times with ELISA wash buffer and incubated with streptavidin alkaline phosphatase for another hour (Sigma-Aldrich, St Louis MO). BIO-RAD microplate reader model 550 or 680 were used to measure the color change following the addition of sigma 104 phosphatase substrate (5 mg/mL) diluted in ELISA substrate buffer (10% diethanolamine,0.05 mM $MgCl_2$, 0.02% NaN_3, pH9.8).

Cytokine Selection of IL-10 -High and -Low Populations

IL-10 secreting cells were generated by culturing mouse CD4+ cells in Th2 skewing conditions. CD4+ T cells were differentiated into Th2 cells for 7 to 10 days of culture Th2 cells were harvested and rested in fresh medium for 6 hours to allow the cells to be more responsive to subsequent stimulation. After this resting period the cells were restimulated for 6 h with 10 ng/ml PMA and 1 μg/ml ionomycin at 10^7 cells/ml in RPMI-1640. Cells were harvested and washed with MACS buffer. The Th2 cells were labeled using the mouse IL-10 Secretion Assay (Miltenyi Biotech, Auburn, CA). The restimulated Th2 cells were labeled on their surface with IL-10-specific high affinity matrix bispecific, i.e., Ab-Ab

conjugates of anti-CD45 mAb (30-F11) with an anti-IL-10 mAb (JES5-2A5) from Miltenyi Biotec. The labeled cells were incubated at 37ºC to allow the secretion of the expressed cytokines which were captured by the conjugated antibodies bound to the surface of the secreting cells (Figure 6). During this step 10^7 Th2 cells were incubated in 100 ml of warm complete RPMI. The dilution of the secreting cells prevented false-positives that can arise from IL-10 high secretors cytokines binding to low secretors surface bound Ab. After 45 min to 1h, these cells were treated with IL-10 detection Ab conjugated with PE which bind the IL-10 cytokine immobilized on the cell surface by the bispecific Ab. IL-10 low secretors and high secretors were sorted in function of the fluorescence using FACSVantage SE or BD FACSAria from Becton Dickinson. The cells were rested for 1 or 2 days before restimulation, analysis or transduction.

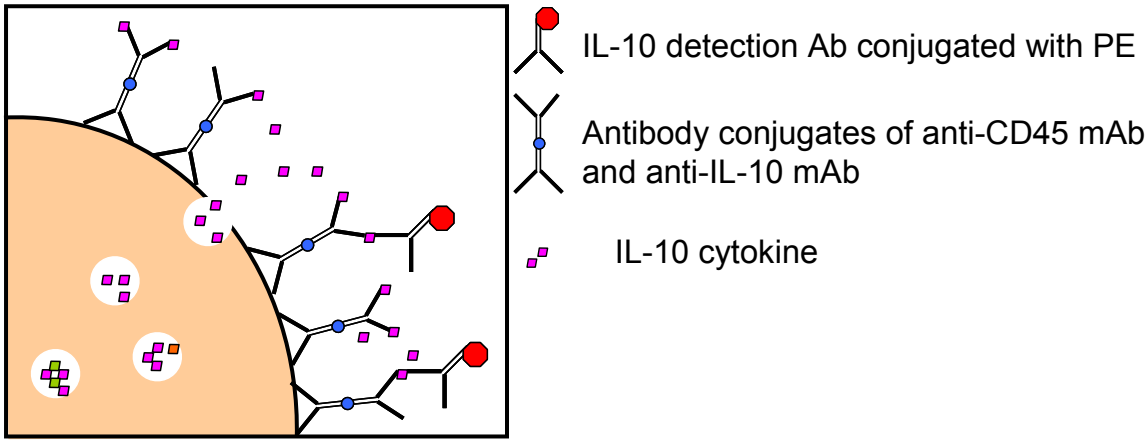


Figure 6: Schematic of IL-10 secretion assay

Co-immunoprecipitation of IRF4, PU.1 and GATA-3

For immunoprecipitation (IP) nuclear cell lysates (1 mg Th2 extract) were incubated with control antibody (normal mouse IgG), anti-IRF4, anti-PU.1 or anti-GATA-3 conjugated with protein G beads (Santa Cruz Biotechnology, Santa Cruz CA) overnight at 4ºC. The next day the immunocomplex was precipitated with protein G beads (except for anti-GATA-3), and released from the beads by boiling in non-reducing loading dye. The beads were centrifuged for 1min at 14,000rpm and washed three times with IP wash buffer containing 0.1% tween-20 and protease inhibitors. Proteins were separated on NUPAGE 4-12% Bis-Tris SDS-PAGE gel from invitrogen, and transferred onto Optitran cellulose nitrate membrane (Whatman, Dassel, Germany). The blots were blocked for 1 h in 5% dry nonfat milk in TBST at RT then probed with anti-IRF4, anti-PU.1 or anti-GATA-3 and appropriate secondary antibody (Biorad, Hercule, CA or Santa Cruz Biotechnology, Santa Cruz CA) for 1 h at RT. The signal was developed with Western blot Lightning Chemiluminescence Reagent Plus (Perkin-Wellesley, MA). The blots were stripped and reprobed with anti-actin (Calbiochem, La Jolla, CA) and Immunoblots were re-probed with the precipitating antibodies

DNA Affinity Precipitation Assay (DAPA)

CD4+ T cells were isolated and cultured under Th2 skewing condition for 5 days. Four mgs of Th2 cell extract were incubated overnight at 4ºC with 10 μl biotin-conjugated PU.1 consensus oligonucleotide, 10 μl of protease inhibitors and completed to 1 ml with oligo DNA pull down buffer-low salt (25 mM HEPES , 10%

glycerol, 15 mM NaCl, 0.5 mM DTT, 0.5% Igepal, 0.1 mM EDTA-pH 7.5). The oligonucleotides (PU-forbiotin, tgaattaaggaagtaagaag and PU-rev, cttcttacttccttaattca (5'-Biotin)) were annealed by incubating 45 μl (100 pmole/μl) of each oligo with 10 μl Roche buffer M for 5 min at 100ºC and letting the mixture cool down slowly to RT. Th2 nuclear lysate (250 μg) was incubated with double stranded biotinylated IL-10 promoter oligonucleotide (tgaggtctgaagaaaatcagccct ctcggg (5'-Biotin) and the reverse complement). For the competition assay the competitor oligonucleotide was incubated with the nuclear protein for 15min at RT before the addition of the biotinylated IL-10 promoter oligonucleotide. The IRF4 mutant competitor had a deletion of the IRF4 consensus binding site and the sequence was tgaggtctatcagccctctcggg and the reverse complement. The sequence for the Igλ (lambda B) IRF4 competitor and GATA-3 oligonucleotide were previously described (66, 130). The next day the protein-DNA complex was incubated for 6 h with streptavidin conjugated beads (1.2 mg/ml streptavidin conjugated to sepharose 4B, Millipore-Upstate). The DNA-bound protein was released by boiling in non-reducing loading dye before loading on SDS gel. Anti-PU.1, anti-IRF4 or anti-NFATc2 antibodies were used to detect the proteins pulled down with the oligonucleotide.

Retroviral Vectors and Transduction

The retroviral vector MIEG-hCD4 was made as previously described (66). Briefly, the EGFP gene contained in the MIEG-EGFP vector was replaced with hCD4 cDNA amplified from a cloned cDNA (provided by G. Alkhatib, Indiana

University). The coding region for human IRF4 cDNA was amplified by PCR and cloned into MIEG-hCD4. The Phoenix-Eco packaging cells line was transiently transfected with 15 μg of purified plasmid by calcium phosphate precipitation. The next day DMEM medium was replaced with RPMI-1640 complete medium. The supernatant containing retrovirus was collected after 1 and 2 days of incubation, filtered through 0.45 μm filter and stored at -80ºC. Th2 cells differentiated for 2 days were transduced by centrifugation at 1,800 rpm, 20ºC for 2 h with 1.5 ml of retroviral supernatant containing 8 μg/ml of polybrene, 100 U/ml of human IL-2 and cytokines and antibodies for Th2 differentiation. Two hours after centrifugation, the cells were supplemented with complete RPMI-1640 medium. The next day cells were expanded with RPMI-1640 containing IL-4, human IL-2 and anti-IFNγ. Transduced Th2 were stained with human CD4-PE from BD and purified by sorting before restimulation for real-time PCR or ELISA.

IRF4 siRNA and shRNA assays

IRF4 siRNA

Balb/c CD4+ T cells were skewed under Th2 condition for 5 days as described above and transfected by Amaxa nucleofection with srambled siRNA (cugagaguauuucgagacgaaaa) or IRF4 specific siRNA, a pool of two different siRNA constructs (siRNA2 (gaggaagaacauugagaagtugc) and siRNA3 (cuccgucauucuuccauccaaga)) described by Brüstle et *al*.(137). The treated cells were rested for 4h at 37ºC and restimulated with 5 μg/ml, anti-CD3, 1 μg/ml anti-

CD28, 10 ng/ml of IL-4 and 10 μg/ml XMG for 40h. Cells were then harvested and either analyzed by RT-PCR or restimulated with 2 μg/ml anti-CD3 for ELISA.

IRF4 shRNA

The sequences for IRF4 siRNA1 (uugggaauuguuuaaaggcaagu) and siRNA2 described by Brüstle et *al* were converted to DNA oligonucleotides and annealed following Clontech protocol before ligation into an RNAi-Ready pSIREN vector (Clonetech Laboratories, Inc. Mountain View, CA). The negative control shRNA annealed oligonucleotide provided with the kit was used to generate the negative shRNA. These vectors (2 μl) were used to transform 50 μl of DH5α and LB-ampicillin selective medium was used to grow the cells. The plasmids purified were used to transfect Phoenix GP cells and obtain viral supernants. CD4+ T cells cultured under Th2 conditions were transduced with IRF4 shRNA or negative shRNA and harvested two days later for FACS analysis.

Reporter Assay

EL4 cells were split on day 0, and on day 3 10^6 cells were transfected by electroporation with 0.9 μg of plasmid, 0.9 μg of reporter and 0.2 μg of β-galactosidase in a total volume of 100 μl of DMEM. Cells were immediately transfered to 6 well plates. After 24h the cells were harvested, washed with PBS and restimulated with 0.2 μg/ml of Ionomycin and 20 ng/ml of PMA for 24h. Harvested cells were lyzed with 100 μl of reporter lysis buffer (Promega) and the

luciferase activity was measured for each sample and divided by the protein concentration and the β-galactosidase activity of the sample.

RESULTS

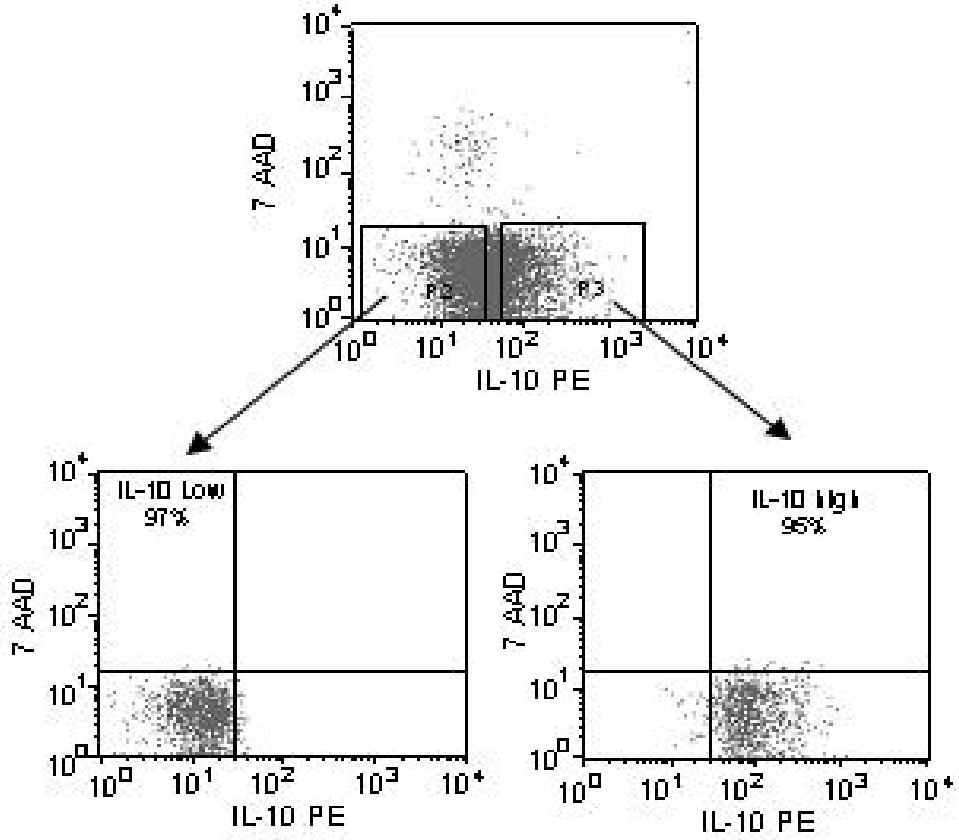
IRF4 expression defines IL-10 low and IL-10 high phenotype

Our first goal was to assess Th2 cytokine heterogeneity in IL-10 low and IL-10 high cells after separation of Th2 cells based on IL-10 secretion. Th2 cells were harvested after 10 days of differentiation and restimulated before labeling for IL-10 secretion and cell death with 7-Amino-Actinomycin D. The gates for IL-10 non-secreting cells and IL-10 secreting cells were defined respectively by the negative control (unstained cells) and the positive control (mouse CD4 PE stained cells). The purity of these populations was higher than 90% (Figure 7A). *Il10* RNA level was assessed after 4h of restimulation. IL-10 high cells expressed greater levels of *Il10* RNA than IL-10 low cells, however, IL-10 enrichment was insufficient to see a great segregation of *Il4* expression (Figure 7B). IL-10 high cells secreted more IL-10 than IL-10 low cells and this phenotype was maintained at least a week after sorting suggested that the cytokine profile of the IL-10 secreting or IL-10 non-secreting cells was not transient phenotype but stable phenotype (Figure 7C).

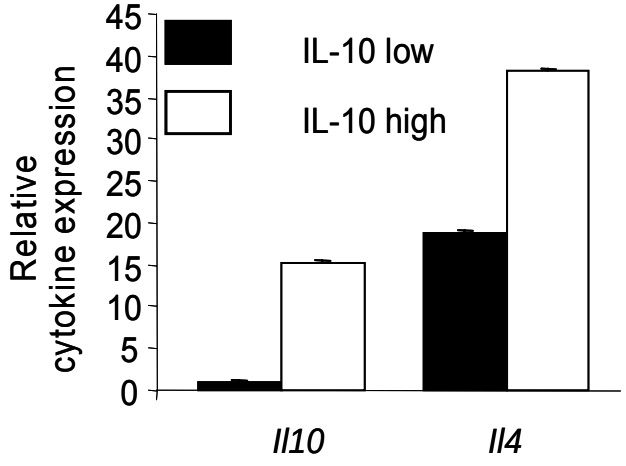
The levels of other Th2 cytokines including IL-4, IL-5 and IL-9 were analyzed in these subsets of Th2 cells. As shown in Figure 7D the production of IL-4 and IL-5 were respectively 4 and 6 fold higher in IL-10 high than in IL-10 low cells and similar patterns was observed at the RNA level. In contrast to the enrichment for IL-4 and IL-10 levels, the level of IL-9 is 9-fold higher in IL-10 low compared to IL-

10 high cells (Figure 7D). This data suggests that Th2 cytokines gene expression in IL-10 high secretor cells is controlled and not ubiquitously induced.

A


7 AAD
IL-10 PE
R2
R3
IL-10 Low
97%
IL-10 High
96%

B


Relative cytokine expression
IL-10 low
IL-10 high
Il10
Il4

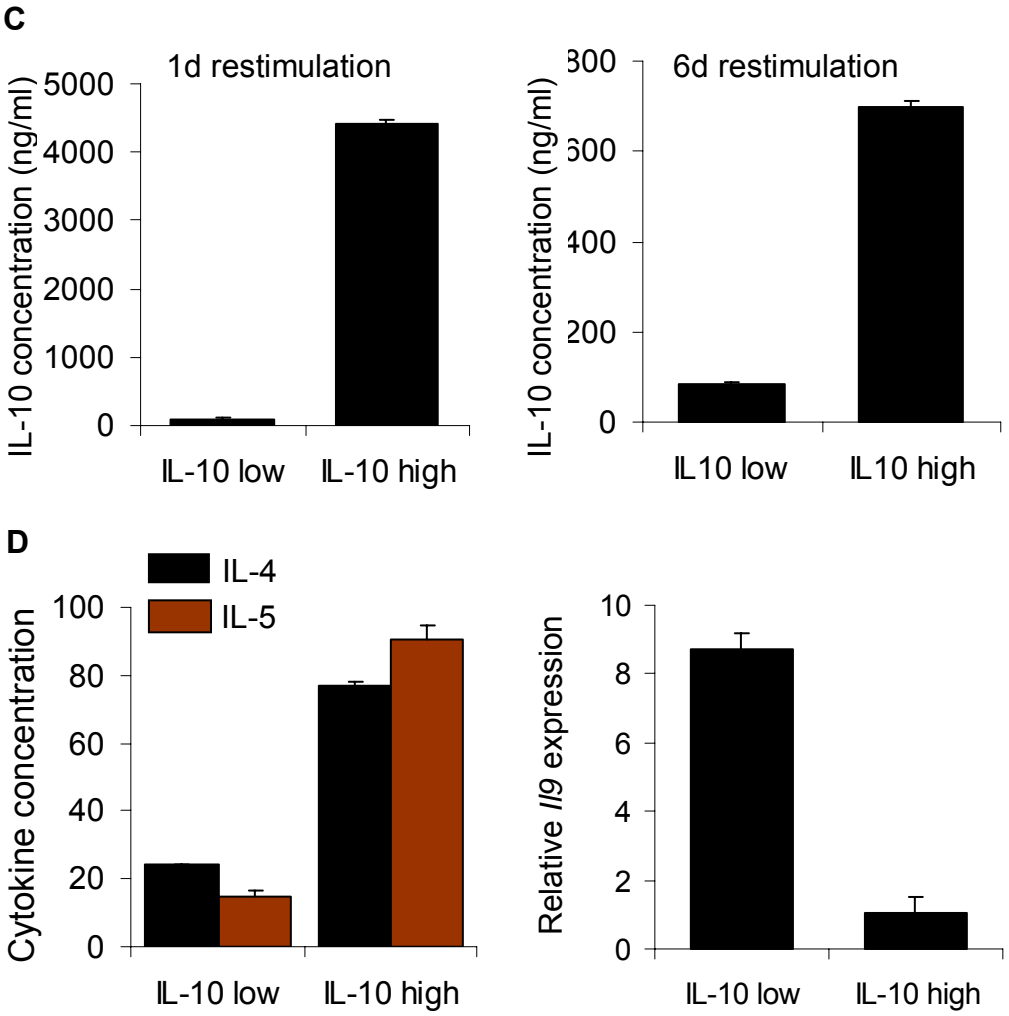
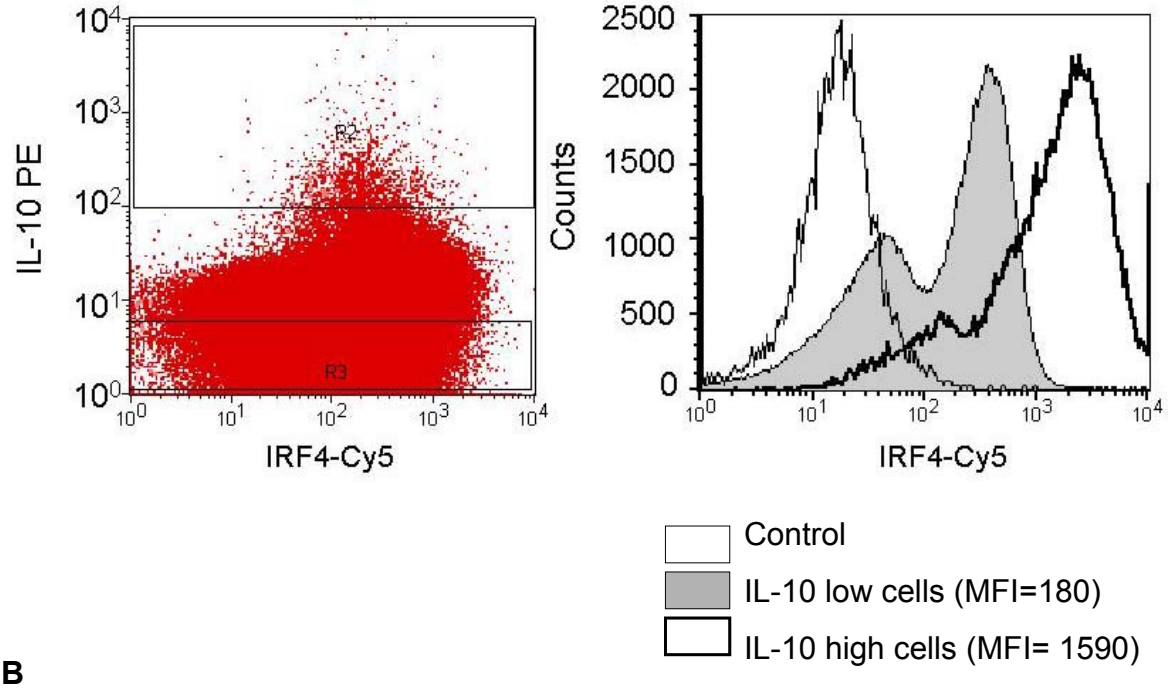


Figure 7: IL-10 high and low Th2 cells phenotype
CD4+ T cells were cultured under Th2 conditions for 7-10 days and separated into IL-10 high and IL-10 low populations using cytokine capture and sorting by flow cytometry. Based on the negative and positive control the gates were set respectively for IL-10 low and IL-10 high cells (A). IL-10 high and low cells, after sorting, were stimulated with anti-CD3 for 4 h and RNA was isolated for qPCR analysis of *Il4* and *Il10* (B). IL-10 high and low cells supernatants were tested for IL-10 concentration 1d or 6d after sorting and 1d restimulation using ELISA (C). Cells cultured and sorted as in A were stimulated with anti-CD3 for 1d for IL-4, IL-5 detection by ELISA or 4 h before RNA isolation for qPCR analysis of *Il9* (D). Data in all panels are representative of at least four experiments.

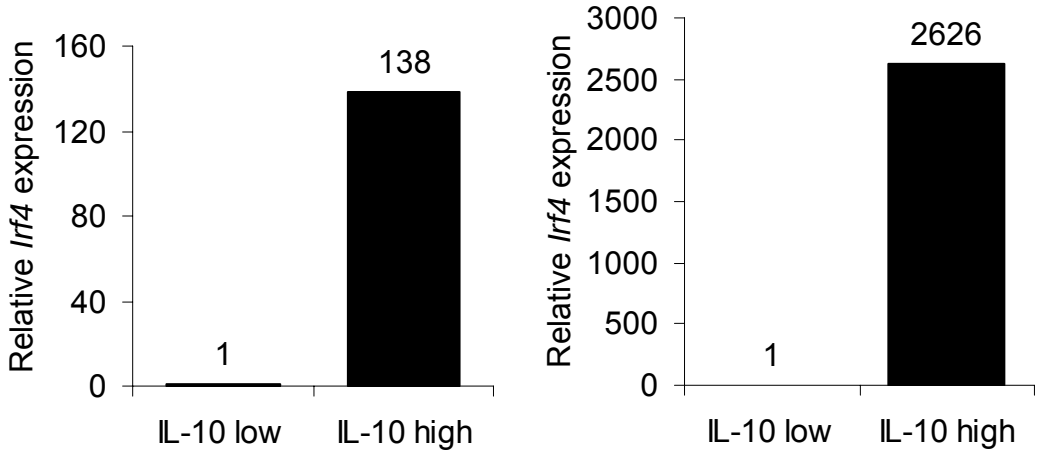
An array of Th2-associated transcription factors including IRF4, GATA-3 and PU.1 were tested after segregation in IL-10 high and IL-10 low cells. GATA-3 was reported to remodel the *Il10* promoter region (26) while *Irf4*-/- Th2 cells secreted significantly lower level of IL-10 (60). To verify IRF4 protein segregation in IL-10 high and low cells we performed intracellular staining in CD4+ T cells skewed toward Th2 for 5 days using anti-IL-10PE antibody and anti-IRF4 antibody (Santa Cruz Biotechnology). We gated on both the cells that secreted the lowest and the highest level of IL-10 so that the buffer region between these two gates is as big as possible. There was an 8-fold difference in IRF4 expression in cells gated for IL-10 high or IL-10 low expression (Figure 8A). *Irf4* expression was greater in unstimulated IL-10 secreting than IL-10 non-secreting cells (Figure 8B). Upon restimulation the fold difference in *Irf4* RNA level increased from 138 to 2626 (Figure 8B). In general IRF4 expression is induced by α-CD3 stimulation in both subsets of cells (data not shown). Interestingly, cells that express high levels of GATA-3 and IRF4 secrete 10-fold more IL-10 than GATA-3/IRF4 low expressing cells (Figure 8C).

In our 2005 paper, we reported that PU.1 is expressed in IL-4 low cells where it sequesters GATA-3 from binding the *Il4* and *Il5* loci and induces the expression of these genes. The absence of PU.1 in IL-4 high cells allows them to secrete IL-4 at a higher level than IL-4 low cells (66). By contrast, we see 5 times more *Sfpi1* RNA in 4h restimulated IL-10 high than in IL-10 low cells (Figure 8D).

A


IL-10 PE
IRF4-Cy5
R2
R3
Counts
IRF4-Cy5
Control
IL-10 low cells (MFI=180)
IL-10 high cells (MFI= 1590)

B


Relative Irf4 expression
160
120
80
40
0
138
1
IL-10 low
IL-10 high
Relative Irf4 expression
3000
2500
2000
1500
1000
500
0
2626
1
IL-10 low
IL-10 high

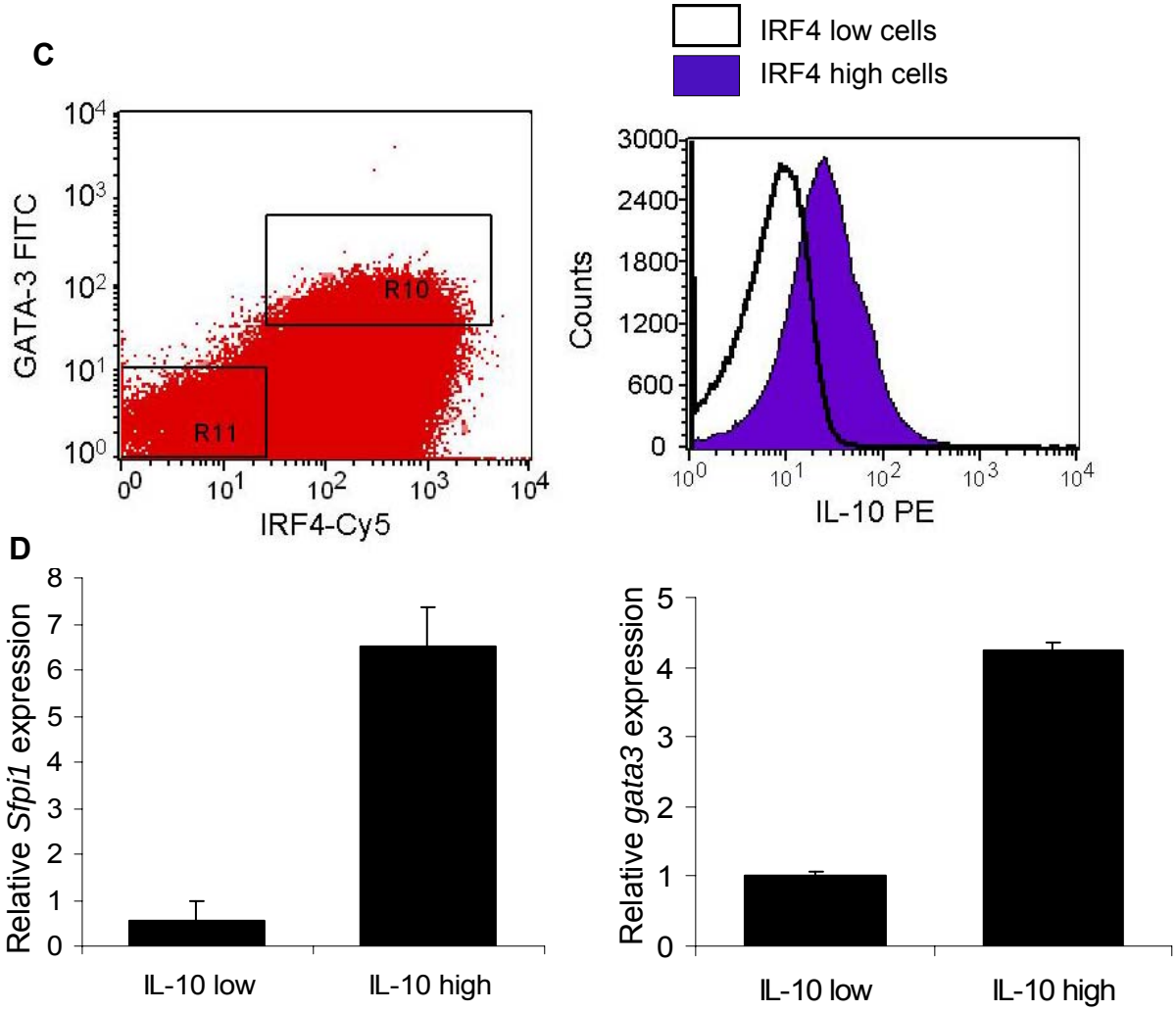


Figure 8: IRF4 expression segregates between IL-10 high and −low Th2 cells

CD4+ T cells were cultured under Th2 conditions for 7-10 days and separated into IL-10 high and IL-10 low populations (see Figure 7). Th2 cells were treated with monensin for the last three hours of a six hour stimulation with anti-CD3 before intracellular staining for IL-10, IRF4 and GATA-3 or IgG control. The fluorescence intensity histogram (FI) of IRF4 was plotted for the negative control which are Th2 cells treated with normal IgG and Cy5 conjugated donkey anti goat antibody (dashed line), IL-10 secreting (bold line) and IL-10 non secreting cells (gray area) (A). IL-10 high and low cells, after sorting, were left unstimulated or stimulated with anti-CD3 for 4 h and RNA was isolated for qPCR analysis of *Irf4* (B), *Sfpi1* and *gata3* (D). Th2 cells treated with monensin and stained as in (A) were analyzed for IRF4 and GATA-3 expression and backgated for IL-10 expression. GATA-3 and IRF4 double negative cells (bold line) and the double positive cells (purple area) FI was plotted (C). Data in all panels are representative of at least three experiments.

IRF4 expression segregates with IL-10 secreting Th1 cells

Th1 cells also secrete IL-10, though in the absence of other stimulatory cytokines, at levels much lower than those produced by Th2 cells (Figure 9A) (94). To determine whether IRF4 expression correlates with the production of IL-10 by Th1 cells, *Irf4* RNA level and IRF4 protein level were determined in Balb/C Th1 versus Th2 cells. As shown in Figure 9B, Th1 and Th2 cells expressed similar nuclear levels of IRF4, even though, only 3% of Th1 cells express IL-10 versus 30% in the Th2 subset cells (Figure 9B versus Figure 8A). To determine whether Th1 cultures also display IL-10 heterogeneity and if IRF4 expression segregated with IL-10 high Th1 cells, CD4+ T cells were cultured under Th1 skewing conditions (IL-12+α-IL-4) and were used for intracellular staining for IL-10 and IRF4. Th1 cell analysis by flow cytometry confirmed that the IRF4 expression pattern was similar to the pattern in Th2 cells where higher IRF4 expression was observed in IL-10 high cells (Figure 9C). This data suggested that in the Th1 population, IRF4 may contribute to IL-10 production but is not a determining factor for expression.

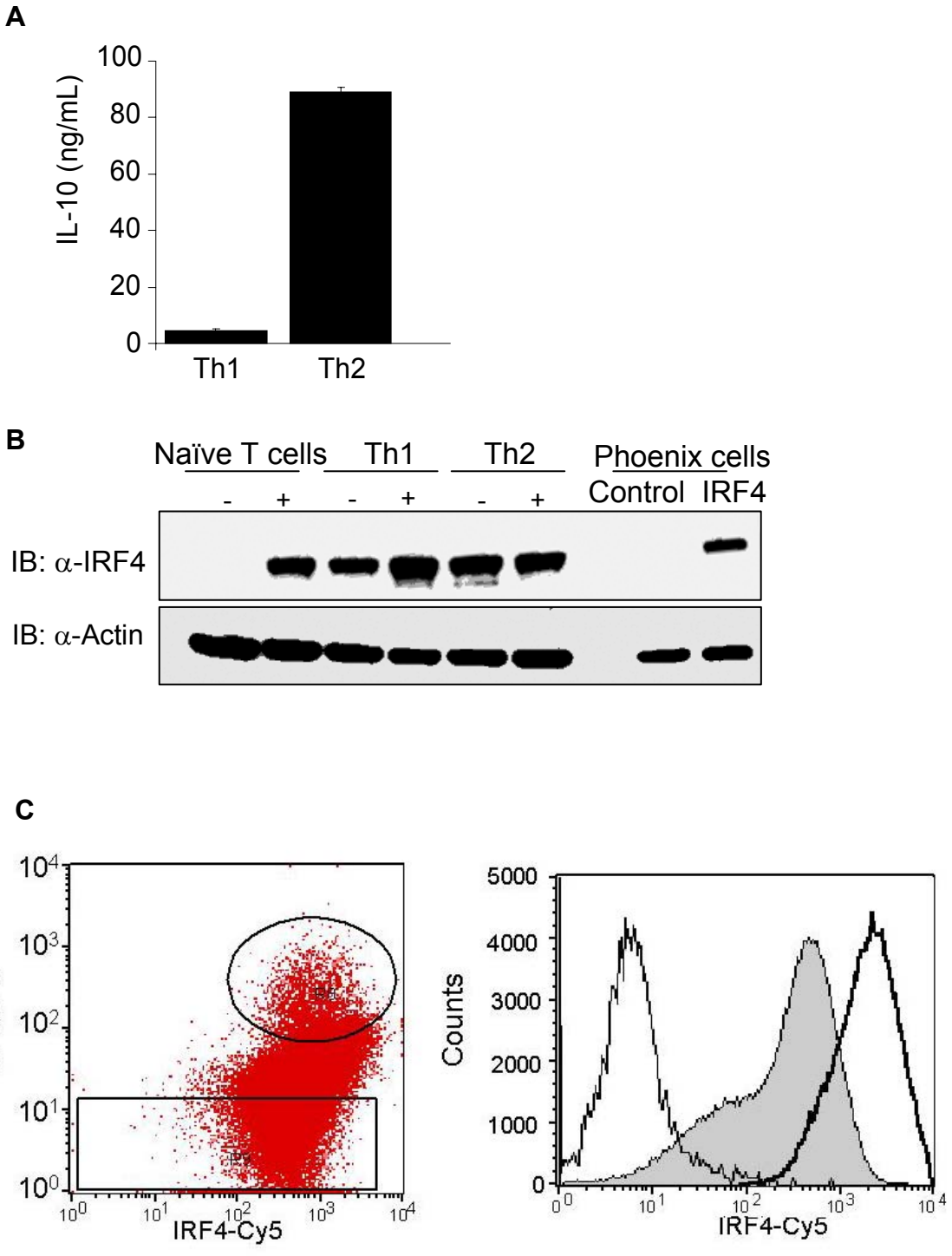

A
IL-10 (ng/mL)
100
80
60
40
20
0
Th1
Th2
B
Naïve T cells
Th1
Th2
Phoenix cells
- + - + - +
Control IRF4
IB: α-IRF4
IB: α-Actin
C
IL-10PE
IRF4-Cy5
Counts
5000
4000
3000
2000
1000
0

Figure 9: IRF4 expression segregated with IL-10 secreting Th1 cells
Balb/c CD4+ T cells were cultured for 5 days under Th1 or Th2 skewing conditions and restimulated with α-CD3 for 1 day. The concentration of IL-10 in the supernatant was assessed by ELISA (A) and the level of IRF4 protein in unstimulated or 6h stimulated naïve T, Th1 and Th2 cells as well as control or IRF4 transfected phoenix cells was assessed by western blot (B). Th1 cells were also restimulated for intracellular staining as described in Figure 8. The cells stained with α-IL-10PE and α-IRF4/DαG-Cy5 were analyzed by flow cytometry (C). The control cells were treated with normal IgG/DαG-Cy5. The histogram plot represents the mean fluorescence intensity of Cy5. The dark line represents the IgG control, the gray area represents the IL-10 low gated cells (R9) and the bold line represents the IL-10 high gated cells (R8). This data is representative of three independent experiments.

IL-10 induction in T helper cells other than Th2 is not mediated by IRF4

Various types of T helper cells secrete IL-10 including Th1 cells, T regulatory 1 and Tregs (95, 96). In addition to the known IL-10 inducing cytokines including IL-4, IL-12 and IL-2, IL-27 has been recently added to the list (95, 96). In order to determine whether IRF4 expression correlates with IL-10 level in T helper cell types other than Th2 cells and assess the involvement of IRF4 in IL-27 pathway we cultured CD4+ T cells under non polarizing (no cytokine), Th1 (IL-12+ α-IL4), Th2 (IL-4+α-IL12), Th17 (TGF-β+α-IL-4+α-IL-12+ IL-6+IL-1β) and Treg (TGFβ+α-IL-4+α-IL-12) skewing conditions. These cells were cultured in the presence or absence of 50 ng/ml recombinant IL-27 (rIL-27). After 3 days of culture the cells were expanded, supplied with half the concentration of skewing cytokines and 25 ng/ml of rIL-27. On day 5 the cells were harvested for assessment of *Il10* and *Irf4* RNA level by real-time PCR after 2h of α-CD3 restimulation. As shown in Figure 10, most of the T helper cell types express similar levels of *Irf4* RNA in the presence or absence of rIL-27 except Th2 cells which IRF4 expression decreased upon treatment with rIL-27. The effect of rIL-27 on *Il10* RNA levels was different in various T helper cell types. Th2 cells expressed the highest level of *Il10* RNA followed by Tregs and Th17 cells. The addition of rIL-27 to T helper cells during differentiation induced a dramatic increase in *Il10* expression in Th1, a significant increase in Th17 cells and modest increases in unskewed, Th2 and Treg (Figure 10). The treatment with rIL-27 increased IL-10 secretion in Th17 cells and Treg while it decreased IL-10 production in unskewed and Th2 cells. These data demonstrated that IRF4 level

did not correlate with IL-10 expression in T helper cell types other than Th2 cells and suggested that additional factors contribute to IL-10 production in other Th cells. The fact that IL-10 level increased in Th1, Th17 and Treg cells upon rIL-27 treatment with no change in *Irf4* level demonstrated that IRF4 is not mediating the IL-27 effect (Figure 10). In unskewed and Th2 cells rIL-27 has the opposite effect by inducing the decrease of *Irf4* level and the reduction of total IL-10 secreted after 24h of restimulation. The presence of rIL-27 may be inducing this decrease in Th2 IL-10 production by triggering the expression of an IRF4 inhibitor, by preventing the release of the expressed IL-10 into the medium since *Irf4* RNA level was stable or by decreasing the half-life of the IRF4 protein. The treatment of T helper cells with rIL-27 demonstrated that IRF4 does not mediate IL-10 production in response to this cytokine with the exception of Th2 cells where any change in IL-10 expression correlates with IRF4 level. In Th1, Th17 and Treg cells other transcription factors may regulate IL-10 expression.

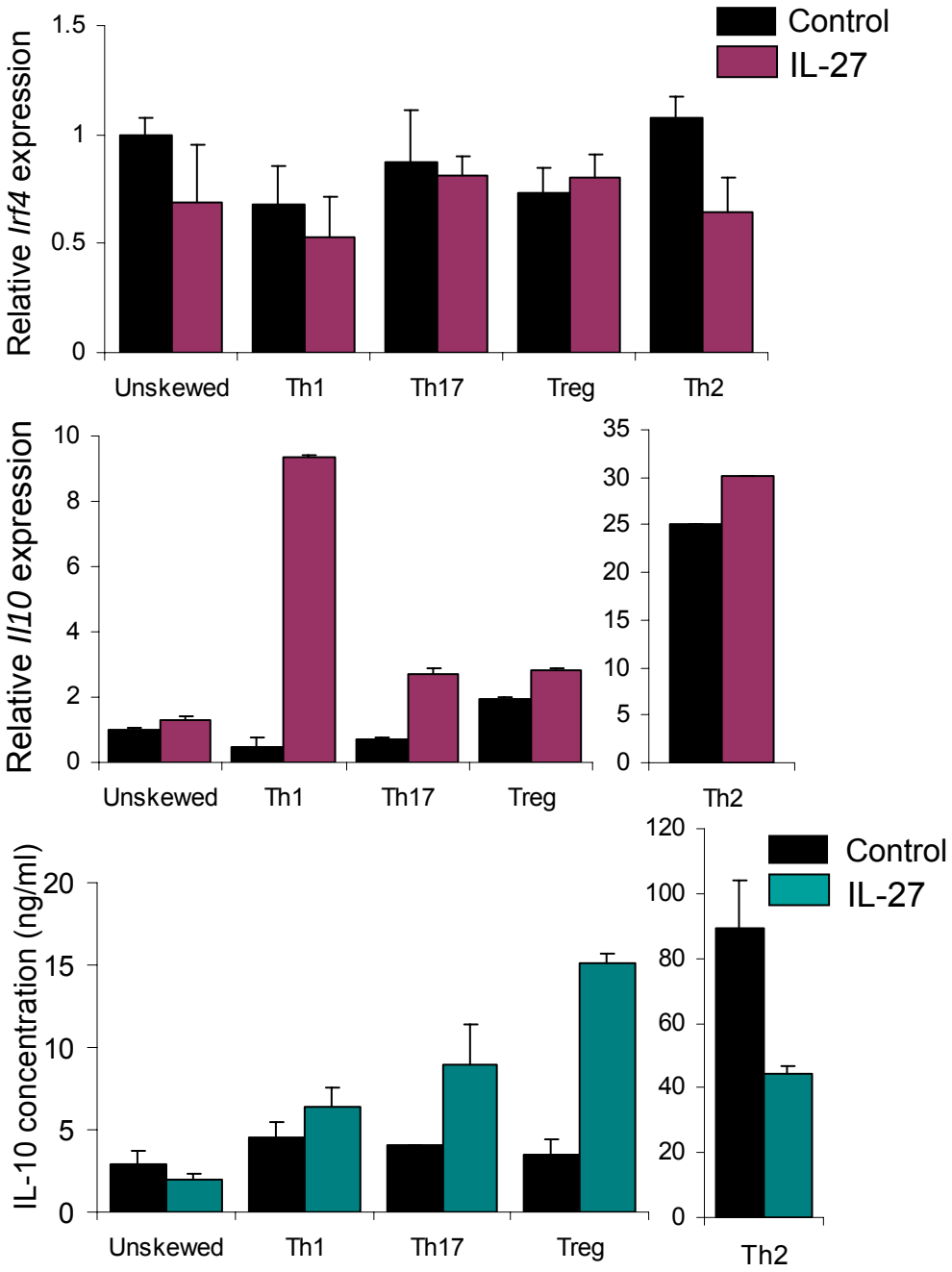


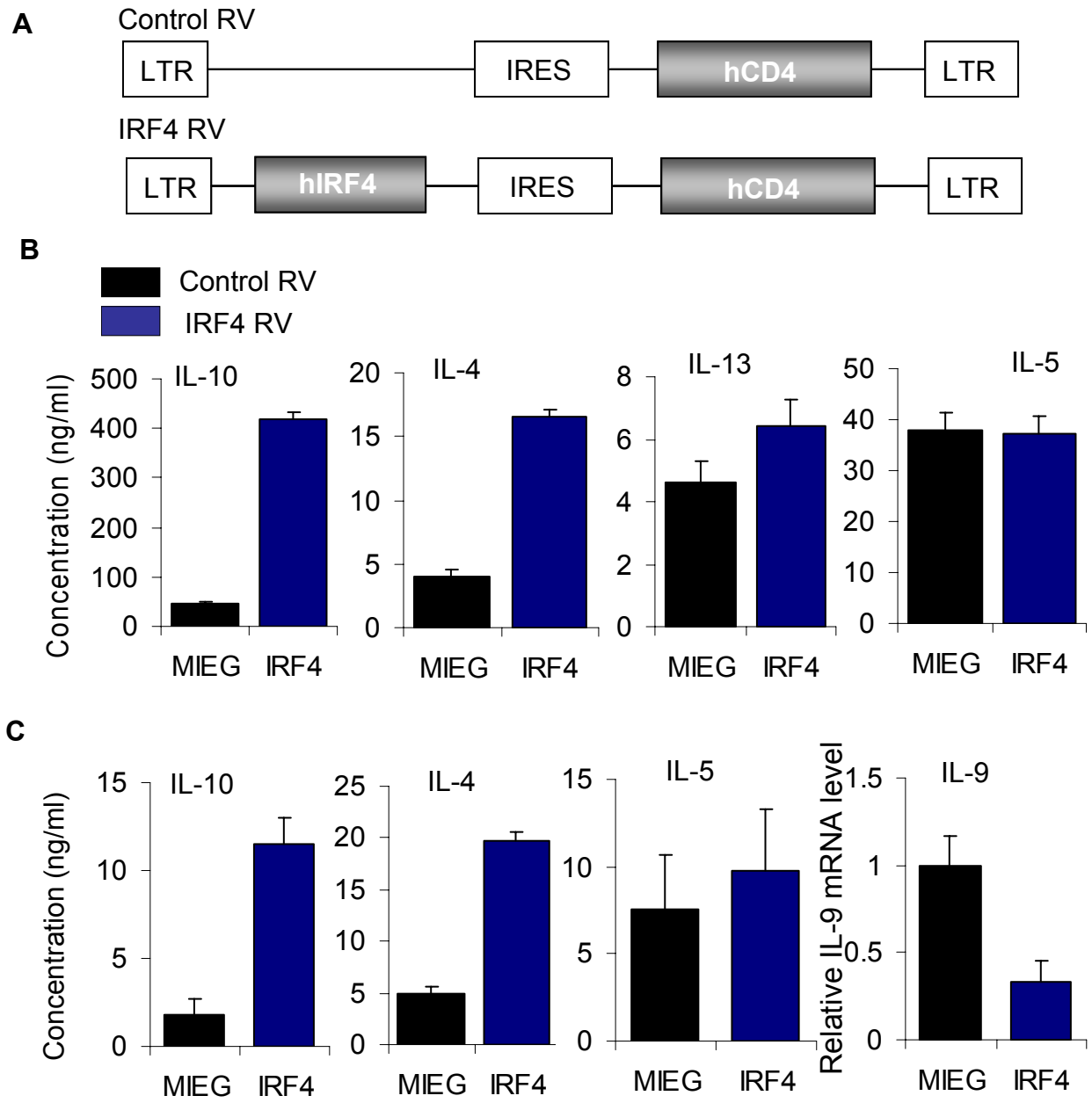
Figure 10: IL-27 induction of IL-10 in T helper cells is not IRF4 dependent
Balb/c CD4+ T cells were skewed under unskewed (no cytokine), Th1 (IL-12+ α-IL-4), Th2 (IL-4+α-IL12), Th17 (TGF-β+α-IL-4+α-IL-12+ IL-6+IL-1β) and Treg (TGFβ+α-IL-4+α-IL-12) conditions for 5 days, in the presence or absence of rIL-27. Differentiated cells were restimulated for 1 day with α-CD3 for 2h before assessing *Irf4* and *Il10* RNA levels by real-time PCR. The data is the representation of 2 experiments.

IRF4 function in Th2 cells

IRF4 was reported to be important for Th2 and Th17 development since IRF4-deficient T cells secrete decreased levels of Th2 and Th17 cytokines while IFNγ levels increase in Th2 cells (136, 137). However the role of IRF4 during Th2 differentiation in mature CD4+ T cells has not been clearly defined. The defect in Th2 cytokine expression suggested that ectopic expression of IRF4 in Th2 cells during differentiation would increase the level of secreted Th2 cytokines. To test this, we generated an IRF4-expressing bicistronic retroviral vector (Figure 11A) and transduced differentiating Th2 cells on the second day of a five-day culture period. Cells sorted for hCD4 expression were restimulated with α-CD3 and evaluated for cytokine production using ELISA. Our data demonstrated that ectopic expression of IRF4 in Th2 cells increased production of IL-10 and IL-4 by 8-fold and 4-fold, respectively, with no significant effect on IL-5 and IL-13 (Figure 11B).

Since transduction of IRF4 increases specific cytokines in differentiating Th2 cells, we next tested whether it would alter the phenotype of IL-10 low cells isolated from differentiated Th2 populations. To assess the change in IL-10 low phenotype, we sorted IL-10 low cells from cells cultured under Th2 conditions for 10 days as described in Methods. IL-10 low cells were transduced with IRF4 RV, cultured for two days before hCD4+ cells sorting and restimulation with anti-CD3 to assess the level of cytokine production. Transduction of IRF4 in IL-10 low cells enhanced the production of IL-10 and IL-4 by 6-fold and 4-fold, respectively,

with no significant effect on IL-5, but a decrease in *Il9* mRNA (Figure 11C). These results demonstrate that IRF4 specifically increases IL-4 and IL-10 production from Th2 cells but does not induce other Th2 cytokines. The infection of IL-10 low cells also demonstrated that the introduction of exogenous IRF4 increased their IL-10 production to the level of IL-10 high cells (Figure 11D), even though the level of IL-10 in MIEG infected IL-10 high cells is lower than IL-10 level in untreated IL-10 high cells (Figure 7B).


A
Control RV
LTR
IRES
hCD4
LTR
IRF4 RV
LTR
hIRF4
IRES
hCD4
LTR
B
Control RV
IRF4 RV
Concentration (ng/ml)
IL-10
IL-4
IL-13
IL-5
MIEG
IRF4
C
Concentration (ng/ml)
IL-10
IL-4
IL-5
Relative IL-9 mRNA level
IL-9
MIEG
IRF4

D

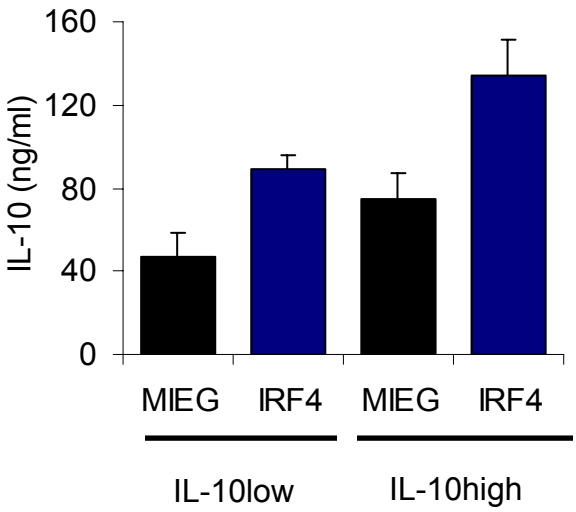


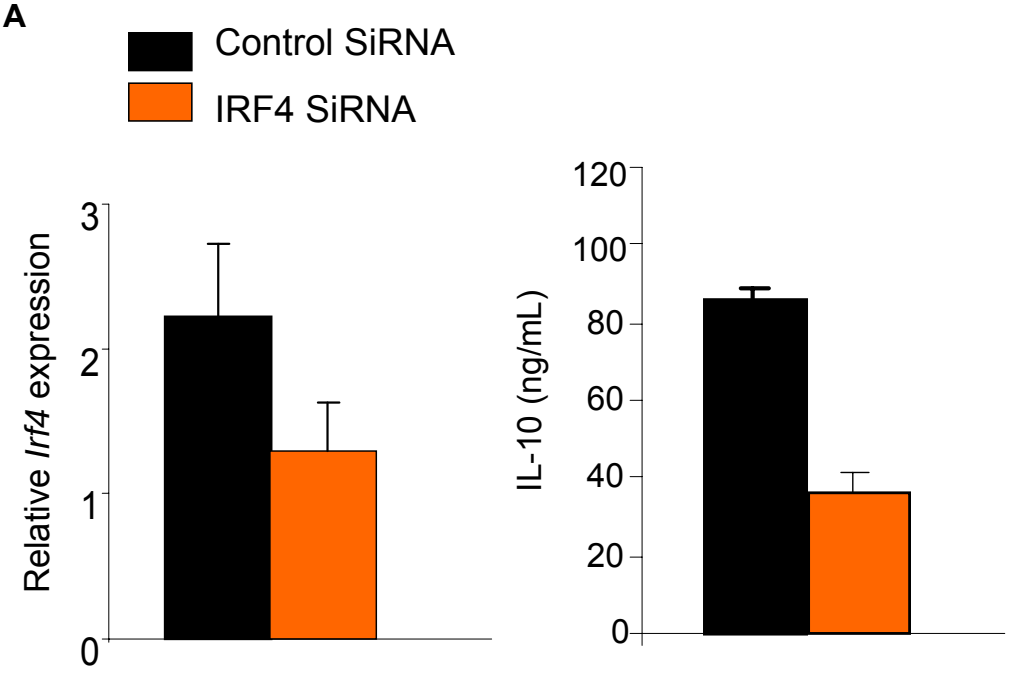
Figure 11: Effect of ectopic IRF4 expression in total Th2 cells and subsets of Th2 cells

IRF4 retroviral vector was generated as described in the material and methods section using an MIEG vector containing hCD4 gene as a marker (A). Total Th2 cells were transduced with viral supernatant generated by transfecting packaging Phoenix-GP cells cultured in DMEM medium. Th2 cells differentiated for 2d were transduced and cultured for 3 additional days. Cells were sorted for hCD4 expression and restimulated for 1 day before ELISA for IL-4, IL-5, IL-10 and IL-13 (B). Th2 cells differentiated for 7d were restimulated with PMA and ionomycin for 6h before capturing IL-10 on the cell surface and detecting the IL-10-anti-IL-10 complex with an anti-IL-10 antibody conjugated with PE. The IL-10 low secretors and IL-10 high secretors were rested for 24h before transduction with the retroviral supernatants generated with the control RV or the IRF4 RV(C and D). The level of Il9 RNA was determined by real-time PCR. These experiments were representative of 2-5 experiments.

IRF4 down-regulation decreases IL-10 and IL-4 expression

CD4+ T cells were skewed under Th2 condition for 5 days and transducted with control siRNA or IRF4 specific siRNA using Amaxa. After 40h cells were either analyzed by RT-PCR or restimulated with 2 μg/ml anti-CD3 for ELISA. *Irf4* mRNA level decreased by 2 fold while IL-10 production decreased by 60% (Figure 12A). The siRNA oligonucleotide was cloned into a Ready green fluorescent vector to generate IRF4 shRNA. Th2 cells cultured for 5 days were rested and transduced with control and IRF4 shRNA. After 2 days cells were restimulated for intracellular staining. The mean fluorescent intensity shows that IRF4 level decrease by 32%, IL-10 by 24% and IL-4 by 75% (Figure 12B). These data suggest that decrease in IRF4 level was not enough to induce a drastic IL-10 decrease but was sufficient for IL-4 decrease.

A



B

	IRF4 MFI	IL-10 MFI	IL-4 MFI
No target	2541	30.29	267
IRF4 ShRNA	1739	23	42

Figure 12: IRF4 down-regulation in Th2 cells
Balb/c CD4+ T cells were cultured for 5 days under Th2 skewing conditions and tranduced with control siRNA or IRF4 siRNA. After 40h the Cells were harvested and either analyzed by RT-PCR or restimulated with 2 μg/ml anti-CD3 for ELISA. (A). Th2 cells cultured for 5 days were rested and transduced with control and IRF4 shRNA. After 2 days cells were restimulated for intracellular staining. The mean fluorescence intensity (MFI) of IRF4, IL-10 and IL-4 was assessed by flow cytometry.

IRF4 regulates IL-4 and IL-10 expression by binding to the *Il4* and *Il10* loci

To assess whether IRF4 regulates IL-4 and IL-10 expression by directly binding to regulatory regions in *Il4* and *Il10* gene loci, we tested the direct binding of IRF4 to the *Il4* and *Il10* loci (Figure 13) using DNA affinity precipitation assay and chromatin immunoprecipitation. We first used a biotinylated oligonucleotide corresponding to an IRF4 consensus binding site in the Il10 promoter. Strepavidin-agarose was able to precipitate IRF4 in the presence but not the absence of oligonucleotide using a DAPA protocol (Figure 13B). To demonstrate specificity for this interaction IRF4 was competed with an oligonucletide from the Igλ enhancer that contains an IRF-4 binding site, but not with the *Il10* promoter oligonucleotide with the IRF-4 binding site deleted or with a GATA-3 consensus oligonucleotide. Chromatin immunoprecipitation experiments in CD4+ T cells cultured for 5 days under Th2 differentiating conditions and restimulated with α-CD3 for different amount of time. The amount of DNA immunoprecipitated was quantified as the percent input. ChIP results for the specific antibody were determined using a standard curve of input DNA from the same cells. Both the *Il10* and *Il4* promoters were enriched in IRF4 immunoprecipitates compared to control antibody precipitates. Similarly regulatory regions including *Il4V*$_A$ and *Il10* conserved non-coding sequence (CNS3) previously shown to regulate IL-10 (103) were enriched in IRF4 immunoprecipitates compared to the IgG control (Figure 13C). Interestingly IRF4 binding to these regions decreased as a function of restimulation time in *Il10* locus while IRF4 binding did not significantly change for *Il4Va*.

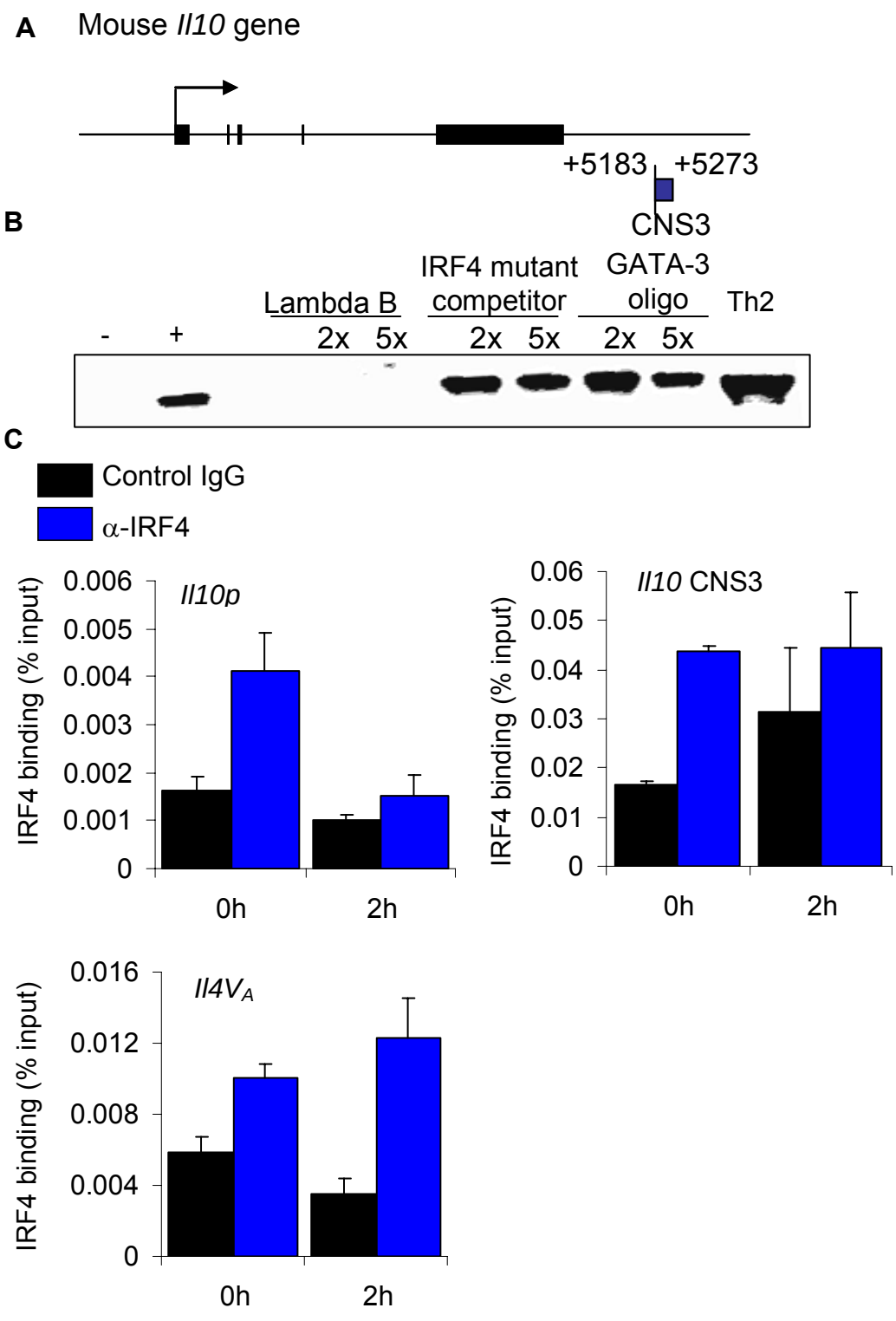

A
Mouse Il10 gene
+5183
+5273
CNS3
B
IRF4 mutant competitor
GATA-3 oligo
Lambda B
Th2
-
+
2x
5x
2x
5x
2x
5x
C
Control IgG
α-IRF4
Il10p
IRF4 binding (% input)
0.006
0.005
0.004
0.003
0.002
0.001
0
0h
2h
Il10 CNS3
0.06
0.05
0.04
0.03
0.02
0.01
Il4V_A
0.016
0.012
0.008
0.004

Figure 13: Chromatin immunoprecipitation demonstrate IRF4 binding to *Il10p*, *Il10*CNS3 and *Il4*V_A

Mouse IL-10 gene is represented with the arrow indicating the translational start and black boxes representing exons. The regions used for promoter and CNS3 ChIP assays are indicated with nucleotide positions corresponding to the transcriptional start site (A). Th2 nuclear extract was incubated with (+) or without (-) a biotinylated oligonucleotide corresponding to an IRF4 consensus binding site in the Il10 promoter and used for DAPA and IRF4 immunoblot. The following competitors were used at 2 and 5 fold exces to demonstrate specificity of the oligonucleotide-IRF4 interaction: oligonucleotides containing an IRF4 binding site from the Igλ gene, the *Il10* promoter oligonucleotide containing a deletion of IRF4 binding site, and an oligonucleotide containing a GATA-3 consensus site. Th2 extract immunoblotted without DAPA is indicated as Th2 (B). Balb/c CD4+ T cells were differentiated intoTh2 cells for 5 days. These cells were either restimulated with anti-CD3 for 2h or unstimulated before cross-linking the protein-chromatin complexes by adding formaldehyde to the cell cultures. For one ChIP reaction $20x10^6$ cells were used. The nuclei were resuspended in nuclear lysis buffer and the genomic DNA is sheared by ultrasonication. The precleared cell lysates is incubated with anti-IRF4 or IgG antibody as negative control for the ChIP assay and 0.3% of the total amount of DNA was used in each immunoprecipitation was determined in the input. ChIP assay of IRF4 binding to *Il10* promoter (*Il10*p), *Il10* CNS3 or *Il4* enhancer (*Il4* V_A) regions. Quantification of control IgG and IRF4 binding was performed using qPCR and the amount of DNA immunoprecipitated was quantified as the percent input (C). The data is representative of 3 experiments.

IRF4 transactivates both the *Il10* promoter and *Il10* CNS3 regions

IRF4 was previously shown to transactivate a luciferase reporter containing the *Il4* promoter in M12 B lymphoma cell and to synergize with NFATc2 and C-maf in the induction of this promoter activity (59). To determine if IRF4 could transactivate gene expression from the *Il10* regulatory elements we used a luciferase reporter containing either the *Il10* promoter region (*Il10p*) or the *Il10* CNS3 region (Figure 14A) (103). Upon co-transfection of EL4 T cells with either of the *Il10* reporters and IRF4-expressing or control pCEP4 vectors, the transactivation was measured by assessing luciferase activity. Our data showed that co-transfection of IRF4 with *Il10p* or *Il10* CNS3 significantly increased luciferase activity compared to the pCEP4 control (Figure 14B). Stimulation of the transfected cells with PMA + ionomycin increased basal reporter activity and IRF4 co-transfection was able to further increase reporter activity (Figure 14B). Thus, IRF4 binds and directly transactivates *Il10* regulatory elements.

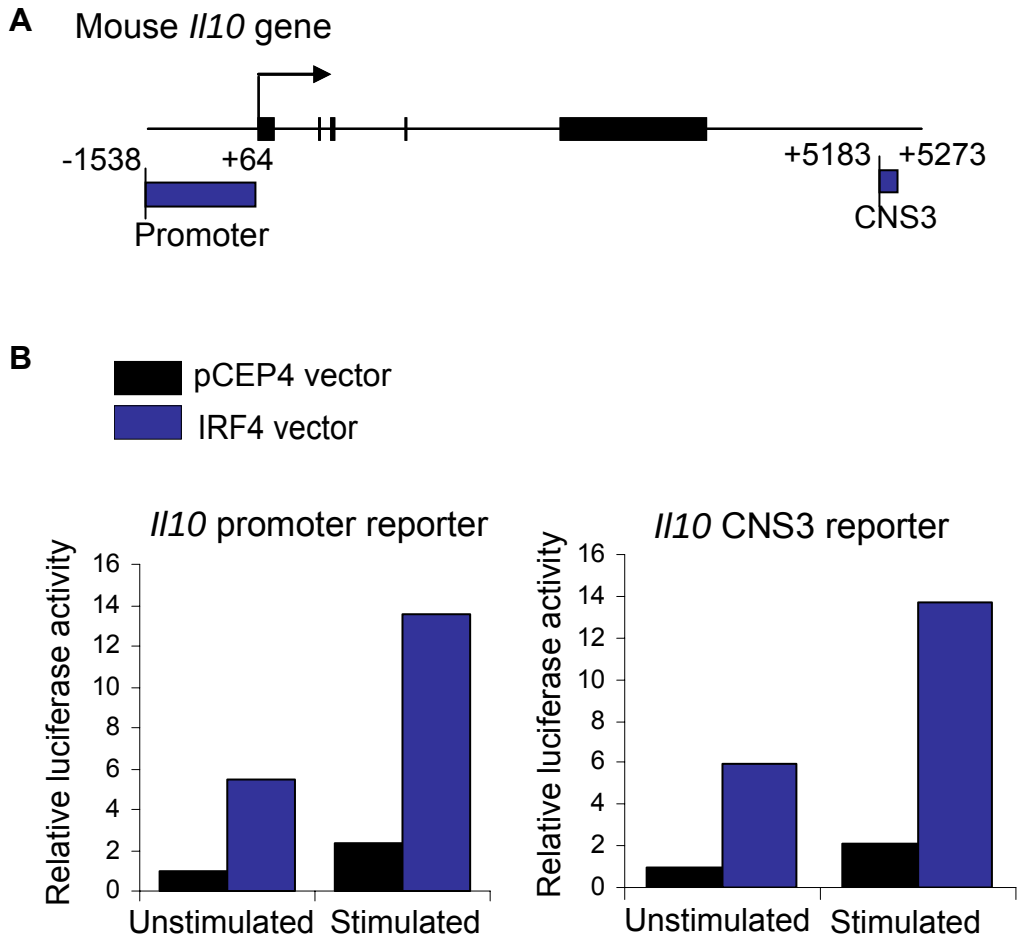


Figure 14: IRF4 transactivates *Il10* driven reporters
Representation of the mouse IL-10 gene with the arrow indicating the translational start and black boxes representing exons. The regions used for promoter and CNS3 reporters and ChIP are indicated with nucleotide positions corresponding to the transcriptional start site (A). EL4 cells were transfected by electroporation with pCEP4 or IRF4 vectors, Il10p or Il10 CNS3 reporter and β-galactosidase gene containing vector (internal control) in a total volume of 100 μl of DMEM. Cells were immediately transfered to 6 well plates containing DMEM medium at 37 ºC. After 24h the cells were harvested, washed with PBS and restimulated with 0.2 μg/ml of Ionomycin and 20 ng/ml of PMA for 24h. The plots are the representation of the luciferase activity and divided by the protein concentration and the β-galactosidase activity of each sample. The data is representative of 3 experiments.

Th2 cytokine temporal expression pattern following anti-CD3 restimulation

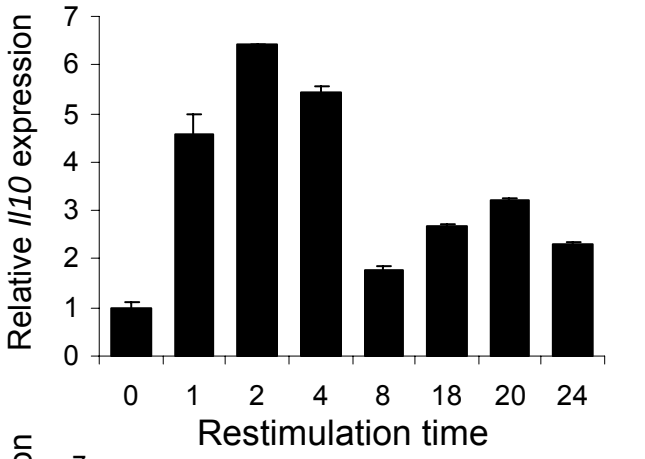
The defense mechanism against microbial invasion mimicked *in vitro* by LPS treatment, involves the activation and function of macrophages which rapidly secrets proinflammatory cytokines. The presence of these cytokines in the microenvironment dictates the differentiation pathway for lymphoid cells. To prevent damage of the host from excess secretion of pro-inflammatory cytokines, the anti-inflammatory cytokine IL-10 is almost simultaneously induced in macrophage cells by type I interferons (144). The regulation and kinetic of IL-10 expression therefore determines the outcome of the local immune response. The analysis of human and murine Kupffer's cells upon endotoxin challenge revealed a significant increase in *Il10* RNA after 2h. To assess the importance of these process in Th2 cells we investigate time course and autoregulation of IL-10 (145).

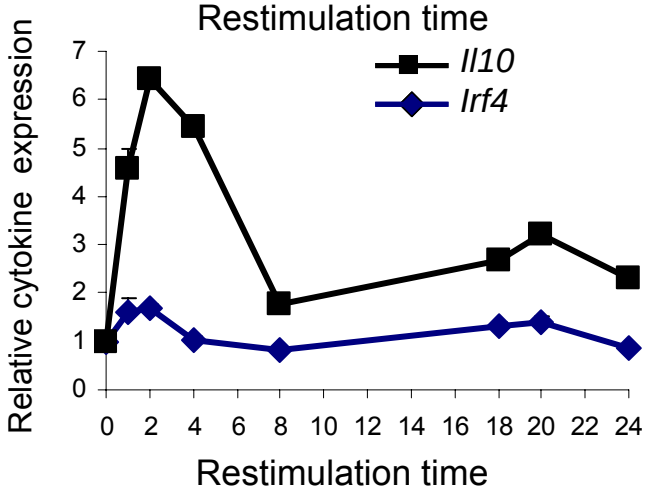
To determine the pattern of expression of hallmark Th2 cytokines and IRF4, CD4+ T cells were cultured for 5-days under Th2 skewing condition, and then washed before restimulation with anti-CD3 for increasing periods of time. We examined the expression of Th2 cytokines including *Il4*, *Il5*, *Il9*, *Il10*, *Il13* and the transcription factor *Irf4* by real-time PCR. Our data indicates that both *Il10* and *Irf4* expression reach peak expression after 2h and 20h of restimulation (Figure 15A). Interestingly this biphasic *Il10* expression was also reported in natural killer (NK) cells treated with IL-2 and IL-12 with similar kinetic for the IL-10 expression spikes (146). In these NK cells IL-10 expression was regulated by

STAT4 binding to the CNS region in the 4th intron of the *Il10* gene (146). The similar pattern of expression for Irf4 supports that this transcription factor is the key regulator of IL-10 expression in Th2 cells.

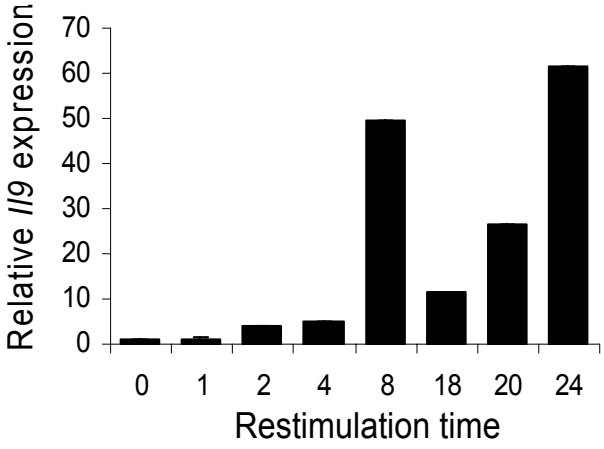
In contrast, *Il9* expression was relatively low until 8h and 24h of restimulation when it drastically increased (Figure 15B). These time points correspond to a low *Irf4* and *Il10* expression levels, suggesting a regulatory function of IRF4 and IL-10 on the expression of IL-9. *Il4* and *Il5* reached their peak expression at 4h and 24h respectively 2h and 4h after *Irf4* and *Il10* peaks (Figure 15C). Surprisingly, these data suggest that *Il10* induction precedes *Il4* expression and not the opposite, whether IL-10 is regulating IL-4 expression in differentiated Th2 cells was not addressed in this study. Figure 15C also shows a sustained *Il13* expression with a constant increase from 1h to 24h restimulation with a small plateau between 6h and 8h. The *Il13* expression profile supports other data that this cytokine is not controlled by IRF4.

A


Relative *Il10* expression
0
1
2
3
4
5
6
7
0
1
2
4
8
18
20
24
Restimulation time


Relative cytokine expression
Il10
Irf4
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16
18
20
22
24
Restimulation time

B


Relative *Il9* expression
0
10
20
30
40
50
60
70
0
1
2
4
8
18
20
24
Restimulation time

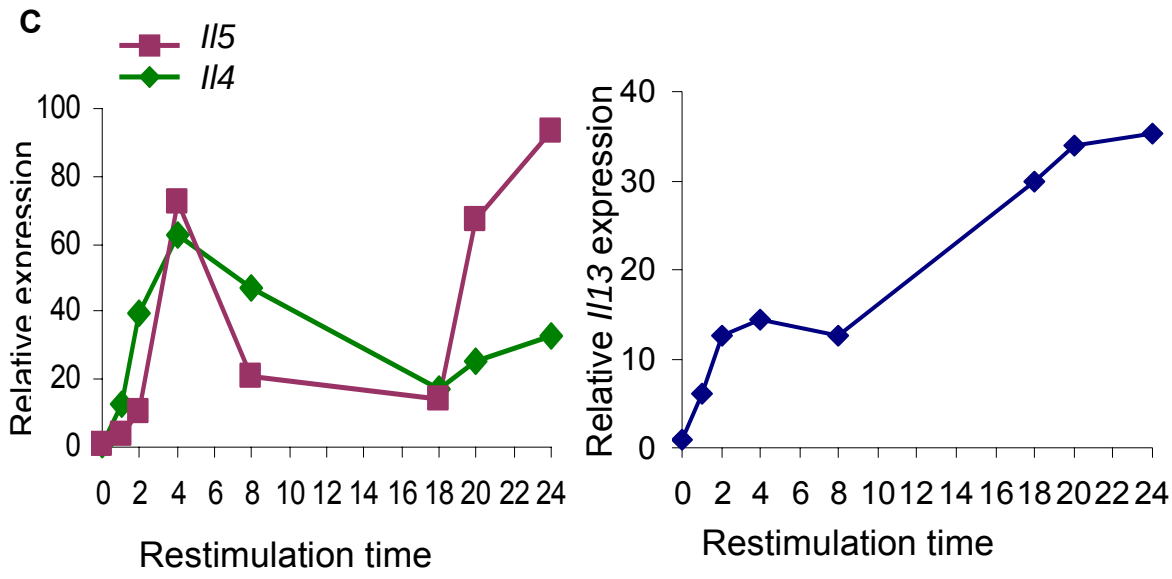


Figure 15: *Irf4*, *Il10* and *Il9* expression pattern as function of restimulation time

Balb/c CD4+ T cells were cultured for 5 days under Th2 skewing conditions and restimulated or not with α-CD3 for different amount of time. The total RNA level for *Irf4*, *Il4*, *Il5*, *Il13*, *Il10* and *Il9* were determined by real-time PCR. The data is representative of 2 experiments

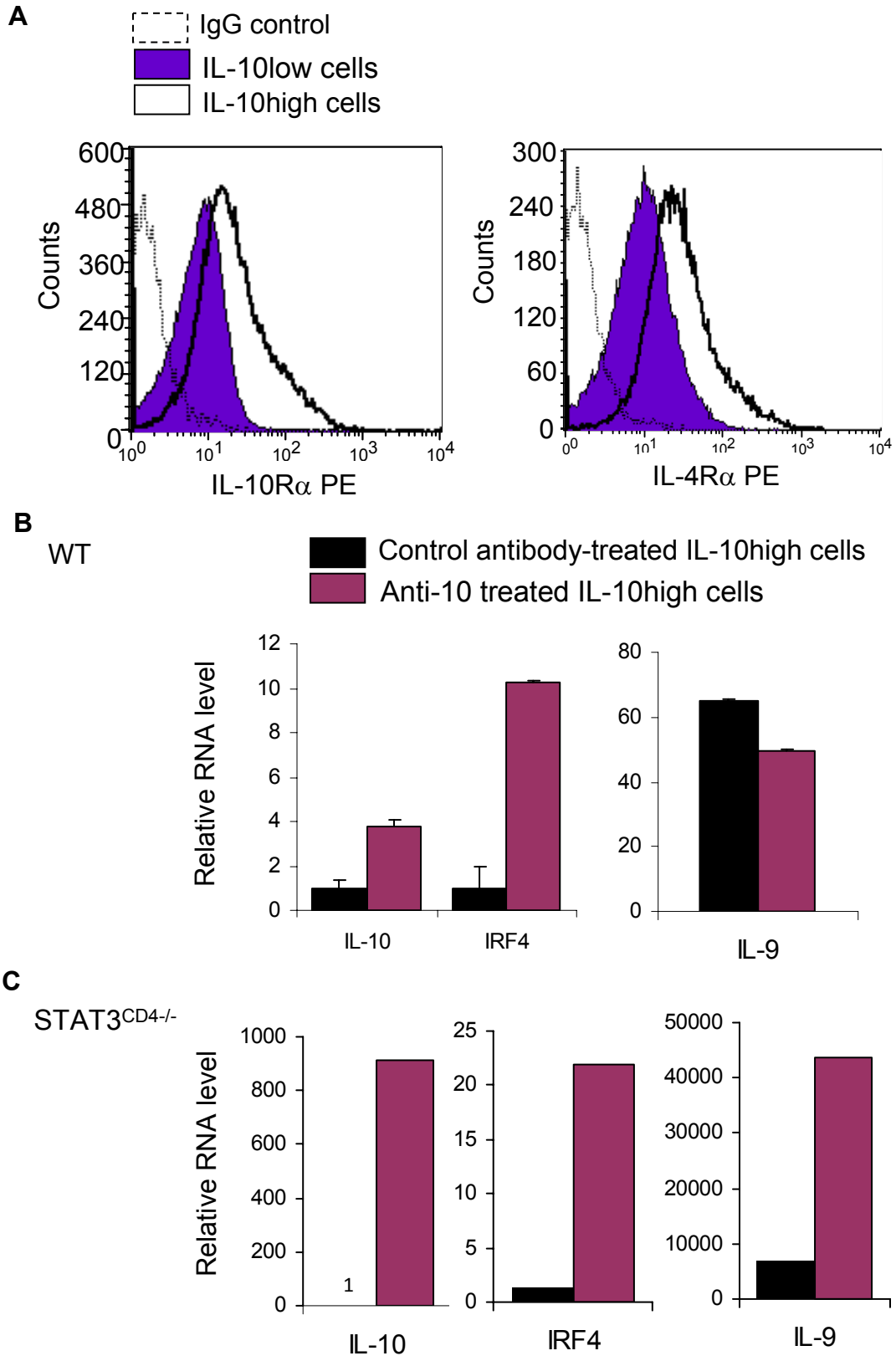
IL-10 regulates its expression in an autoregulatory feedback loop

An IL-10 autoregulatory feedback loop was reported in myeloid cell types including Kuffer cells (145) and primary macrophages (108) through a downregulation by exogenous or endogenous IL-10 protein. The observation of a biphasic IL-10 expression with a smaller peak at 20h compared to the peak at 2h may also be explained by this autoregulatory loop. Our approach to assess this pathway in Th2 cells was to change IL-10 concentration in the culture medium and assess the effect of the modification on the expression level of *Il10* and *Irf4*. We cultured CD4+ T cells for 9 days under Th2 skewing conditions and sorted IL-10 non-secreting and IL-10 secreting cells. The IL-10 low cells were then treated for 3 days with PBS or recombinant IL-10 (rIL-10), while IL-10 high cells were treated with control IgG1 or neutralizing α-IL-10 antibody. IL-10R and IL-4Rα levels were assessed on IL-10 low and IL-10 high cells after 5 days of Th2 differentiation (Figure 16A). IL-10 low cells expressed 3-fold less cytokine receptor compared to IL-10 high cells suggesting that IL-10 low cells are less susceptible to IL-10 or IL-4 in the microenvironment. The addition of rIL-10 to IL-10 low cells decreased the level of endogenous *Il10* and *Irf4* which were already low in this subset of cells. On the contrary, *Il9* levels increased in the rIL-10 treated cells compared to the control (Figure 16D). In IL-10 high cells treated with α-IL-10, *Il10* and *Irf4* levels increased compared to the control cells treated with IgG1. The level of *Il9* on the other hand slightly decreased in the α-IL-10 treated cells (Figure 16B).

To determine whether STAT3 is mediating IL-10 autoregulatory feedback loop, we performed the same experiments using $STAT3^{CD4-/-}$ Th2 cells. As shown in Figure 16C and Figure 16E, the deficiency in STAT3 did not prevent IL-10 from regulating its expression. Overall Figure 16 demonstrates that the IRF4 level correlates with IL-10 expression suggesting that IL-10 is negatively regulating IRF4 expression which in turn affects IL-10 level in the cells. Whether other transcription factors are involved in this process is unknown.

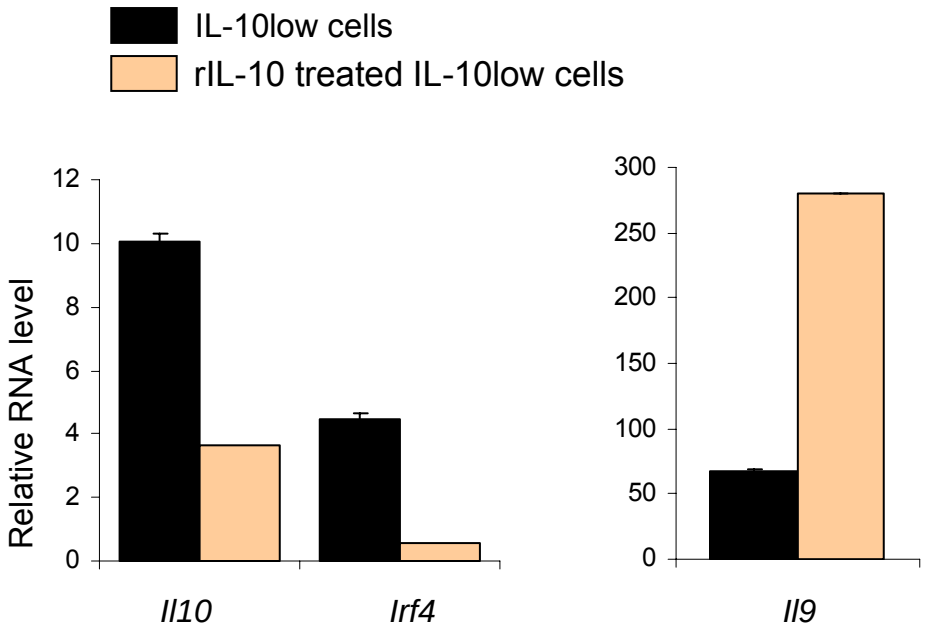
IL-9 is a pleiotropic cytokine known to induce the recruitment of eosinophils and mucus hypersecretion in asthma. Dexamethazone a strong inducer of IL-10 expression was reported to inhibit IL-9 expression (147). Our results in Figure 15A, B and Figure 11C suggest that IRF4 negatively regulates IL-9 expression. Figure 16 further supports a role for IRF4 in IL-9 regulation since rIL-10 decreased *Irf4* level while increasing *Il9* expression (Figure 16D) and the neutralization of IL-10 triggered *Irf4* expression in a STAT3-independent manner while decreasing Il9 expression (Figure 16B).

The early expression of IL-10 in Th2 cells in response to α-CD3 stimulation plays a critical role *in vivo* in the downregulation of inflammatory response upon infection to prevent organ damage. On the other hand, negative autoregulation of IL-10 expression may be the mechanism by which the immune system recovers sensitivity to new proinflammatory stimuli from the microenvironment.


A
IgG control
IL-10low cells
IL-10high cells
Counts
IL-10Rα PE
IL-4Rα PE
B
WT
Control antibody-treated IL-10high cells
Anti-10 treated IL-10high cells
Relative RNA level
IL-10
IRF4
IL-9
C
STAT3CD4-/-
Relative RNA level
IL-10
IRF4
IL-9

D

WT



E

STAT3$^{CD4-/-}$

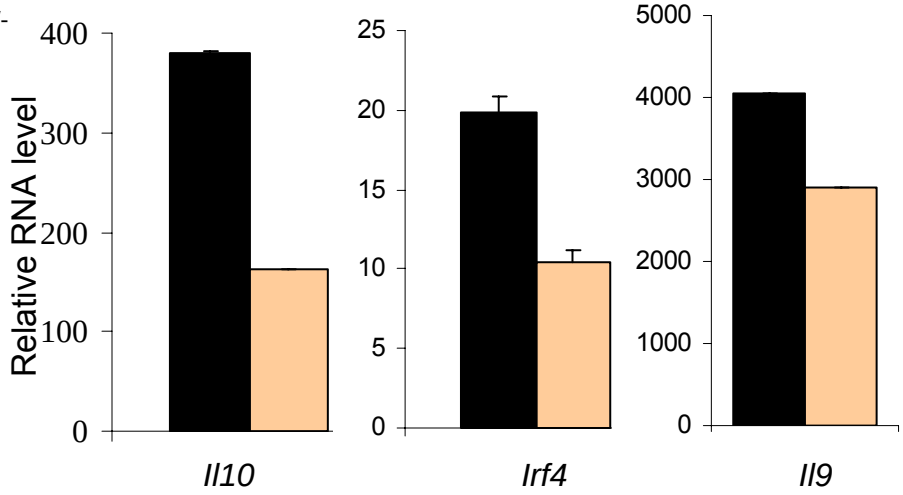


Figure 16: Effect of IL-10 neutralizing antibody on IL-10 high cells and recombinant IL-10 on IL-10 low cells

Balb/c CD4+ T cells were skewed under Th2 condition for 5 days and treated with monensin for the last three hours of a six-hour stimulation with anti-CD3 before surface staining for IL-4R or IL-10R in addition to IL-10 and IL-4 intracellular staining. The histograms in (A) are respectively the overlays of IL-10R MFI and IL-4R MFI in IL-10 low and IL-10 high cells (A). The Balb/c CD4+ T cells were cultured for 9 days under Th2 skewing conditions before resting and restimulation for IL-10 low and IL-10 high sorting. IL-10 high ($1x10^5$) cells were plated in 200 μl per well of 48 well plate and either treated with 10 μg/ml IgG1 or anti-IL-10. The next day 2 μg/ml of the antibodies were added to the cells to neutralize the IL-10 secreted by these cells. On day 3 of culture cells were harvested and restimulated for 3h and 8h with 2 μg/ml of α-CD3 (B). The same experiment was performed in $STAT3^{CD4-/-}$ IL-10 high cells (C). IL-10 low ($2x10^5$) cells were plated per well of 48 well plate and either left untreated or treated with 10 ng/ml of rIL-10. After 3 days of culture cells were harvested and restimulated for 8h with 2 μg/ml of α-CD3. The RNA level of *Irf4*, *Il10* and *Il9* were measured by real-time PCR (D). The same experience was performed in $STAT3^{CD4-/-}$ IL-10 low cells (E). The data is representative of 2 experiments.

PU.1 and IRF4: part of the same protein-protein or protein-DNA complexes

PU.1 was demonstrated to interact with GATA-3 using recombinant protein binding assays (148) and in Th2 cell co-immunoprecipitates (66). In B cells PU.1 is also known to interact with IRF4 and recruit it to the DNA to form a ternary complex (132, 134, 149, 150). To confirm the interaction of PU.1 and IRF4 in a different cell system, we transfected Phoenix cells either with an empty vector, a PU.1 vector, an IRF4 vector or both PU.1 and IRF4 vector (Figure 17A). Anti-PU.1 or anti-IRF4 was used to immunoprecipitate complexes from total Phoenix cell lysate. A small amount of PU.1 was immunoprecipated with IRF4 when either of the antibodies were used (Figure 17B). The western blot of the immunoprecipitates also showed that anti-PU.1 or anti-IRF4 were respectively able to pull down PU.1 and IRF4 when these transcription factors are expressed by themselves (Figure 17B).

To investigate the interaction of PU.1 and IRF4 in Th2 cells, we used anti-PU.1, anti-IRF4 or anti-GATA-3 to immunoprecipitate complexes from Th2 nuclear lysate. PU.1, IRF4, and a small amount of GATA-3 were immunoprecipitated from Th2 cells with anti-IRF4 and anti-PU.1 (Figure 17C). Immunoprecipitation with anti-GATA-3 confirmed the interaction between PU.1 and GATA-3, though little IRF4 was precipitated with this complex (Figure 17C). We also assessed IRF4 interaction with NFATc2 using anti-NFATc2 and found that NFATc2 binds to IRF4, GATA-3 and PU.1 while NFATc1 did not interact with PU. 1 (Figure 17D). Thus, although PU.1 interacts with IRF4 and GATA-3, these data suggest

that PU.1-IRF4 and PU.1-GATA-3 are largely separate complexes, as little IRF4 was associated with GATA-3.

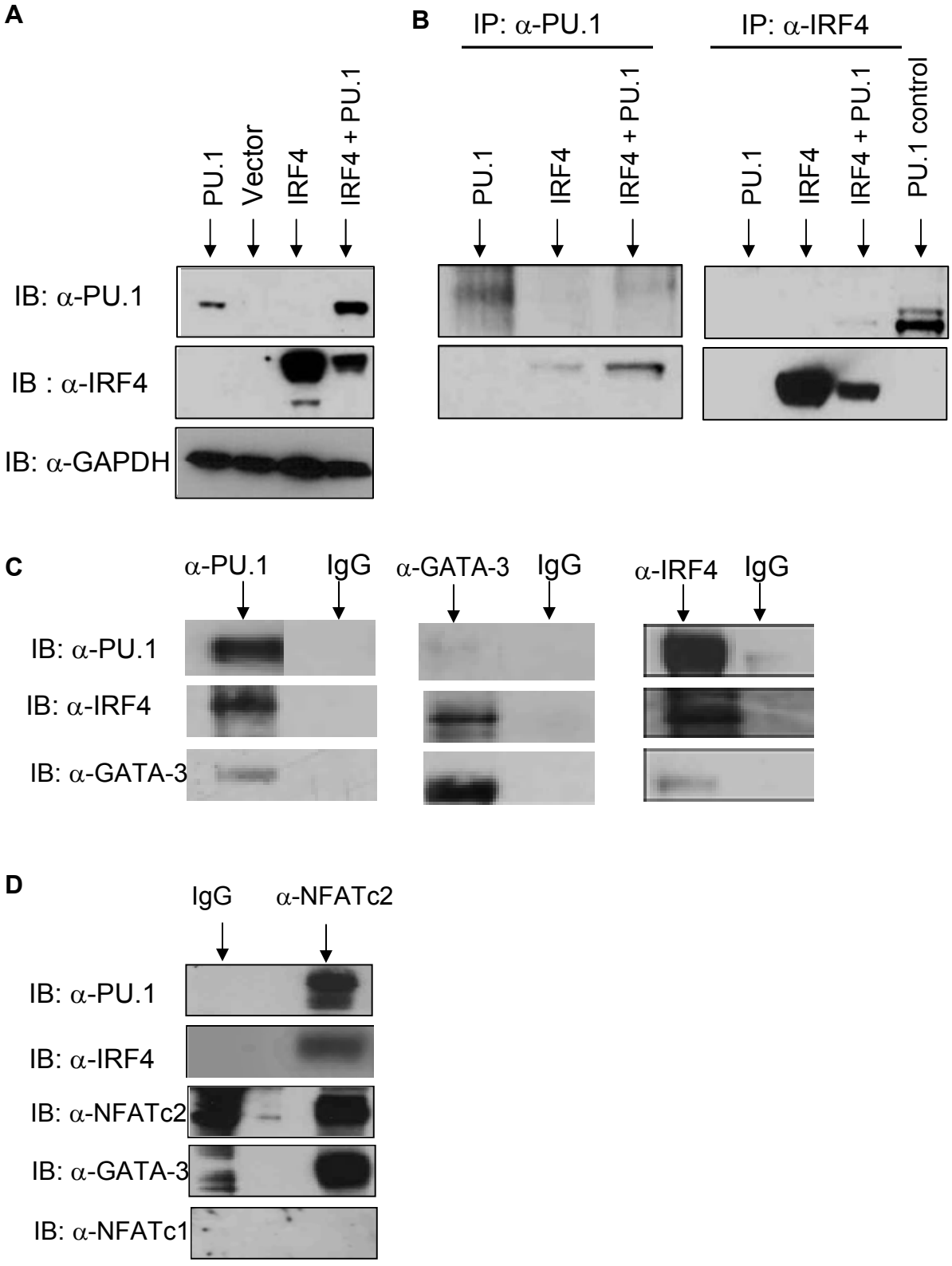

A
PU.1
Vector
IRF4
IRF4 + PU.1
IB: α-PU.1
IB : α-IRF4
IB: α-GAPDH
B
IP: α-PU.1
IP: α-IRF4
PU.1
IRF4
IRF4 + PU.1
PU.1
IRF4
IRF4 + PU.1
PU.1 control
C
α-PU.1
IgG
α-GATA-3
IgG
α-IRF4
IgG
IB: α-PU.1
IB: α-IRF4
IB: α-GATA-3
D
IgG
α-NFATc2
IB: α-PU.1
IB: α-IRF4
IB: α-NFATc2
IB: α-GATA-3
IB: α-NFATc1

Figure 17: PU.1 and IRF4 protein-protein interactions
Phoenix cells were transfected with empty, PU.1 and/or IRF4 expressing vectors. After 2d of incubation at 37ºC, PU.1, IRF4 and GAPDH expression was assessed in one hundred μgs of cell lysate by western blot (A). CD4+ T cells were cultured for 5 days under Th2 skewing conditions. Four mgs of total Phoenix cell lysate (B) or Th2 cell nuclear protein (C) were incubated overnight at 4°C with either anti-IRF4, anti-PU.1 or anti-GATA-3 conjugated (for Th2 cells) (C). Th2 nuclear cell lysates were incubated with control antibody IgG (Santa Cruz, CA) overnight at 4ºC. The immunocomplex was precipitated with protein G beads and released from the beads by boiling in non-reducing loading dye before loading on SDS gel. Immunoblots were re-probed with the precipitating antibodies (C). The data is representative of at least 4 experiments.

To determine whether PU.1 and IRF4 can form a ternary complex with an oligonucleotide containing PU.1 binding consensus, we performed a DNA affinity precipitation assay using total cell lysate from PU.1 and IRF4 expressing Phoenix cells. When PU.1 was expressed alone or co-expressed with IRF4, it formed a protein-DNA complex with the PU.1 oligonucleotide in the Phoenix cell line (Figure 18A). One limitation for this experiment was that IRF4 alone was immunoprecipitated by PU.1 oligonucleotide suggesting that IRF4 binding is not specific. The same experiment was performed in Th2 cells differentiated with 0.5 μg/ml α–CD3 (Th2 low), 2 μg/ml α–CD3 (Th2), IL-4 non secreting cells (IL-4 low) or EL4 cells transfected with PU.1. Only a small amount of PU.1 was immunoprecipitated with the biotin-labelled oligonucleotide and IRF4 when different Th2 cell subpopulations or EL4 cells expressing exogenous PU.1 were used (Figure 18B). This can be explained by the fact that PU.1 is only detectable in the nucleus of Th2 cells (66). Compared to PU.1 transfected Phoenix cells, PU.1 concentration in Th2 total cell lysate was probably too low for a strong detection by western blot after DNA affinity precipitation assay. To determine if IRF4 is directly regulating *Il10* and *Il4*, we tested the direct binding of IRF4 to the *Il10* locus (Fig. 4A) using chromatin immunoprecipitation.

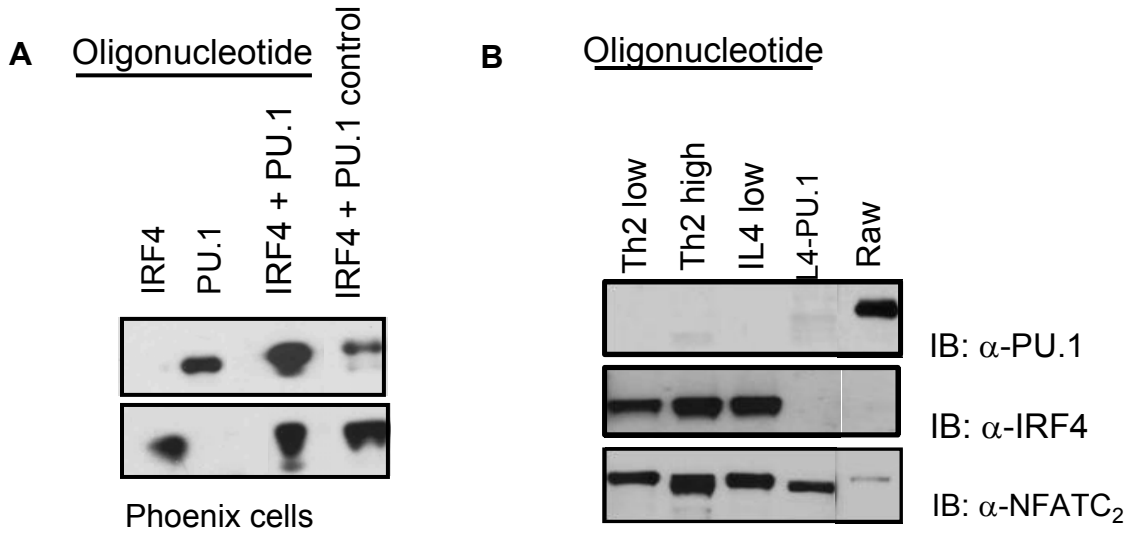


Figure 18: PU.1 and IRF4 protein-DNA interactions
Biotin-conjugated PU.1 consensus oligonucleotide was incubated overnight with Phoenix cell or Th2 cell lysates. The next day the protein-DNA complex was incubated for 2h with streptavidin conjugated beads. SDS-PAGE lysate loading buffer was added to the DNA-protein complex before loading on SDS gel (A). The same experiment was performed in different subsets of Th2 cells or EL-4 cells (B). The data is representative of 3 experiments.

Functional interactions of PU.1 and IRF4

To determine if the association of IRF4 and PU.1 had functional consequences, we used mice carrying a conditional allele of the *Sfpi1* gene, encoding PU.1, crossed to lck-Cre transgenic mice (denoted as *Sfpi1*$^{lck-/-}$). We then compared the function of IRF4 in wild-type and *Sfpi1*$^{lck-/-}$ Th2 cells. Chromatin immunoprecipitation demonstrated that IRF4 binding to the *Il10* promoter is greater in *Sfpi1*$^{lck-/-}$ Th2 cells than in C57BL/6 Th2 cells (Figure 19A). A similar trend of IRF4 binding was observed for the *Il4* promoter. The level of *Il4* DNase hypersensitivity site V_A (151, 152) in the IRF4 precipitates was also greater in *Sfpi1*$^{lck-/-}$ Th2 cells than in WT Th2 cells though binding to *Il10* CNS3 was only modestly affected by PU.1-deficiency. Concomitant with increased IRF4 binding to the *Il10* locus, transduced IRF4 induced more IL-10 in *Sfpi1*$^{lck-/-}$ Th2 cells than in WT Th2 cells. We did observe that IRF4 transduction had a less robust increase in IL-10 production in C57BL/6 background cells, compared to Balb/c Th2 cells (Figure 11B vs. Figure19B). Overall, these results suggest that PU.1 interactions limit the ability of IRF4 to transactivate Th2 cytokines. The deficiency in PU.1 expression in 5 days differentiated Th2 cells allowed these cells to secrete more IL-10 than WT. This phenotype was more pronounced in the cells cultured with 0.125 μg/ml α-CD3 compared to the cells differentiated with 2 μg/ml α-CD3. The mechanism linking PU.1 function and the strength of T cells stimulation is still under investigation in the lab.

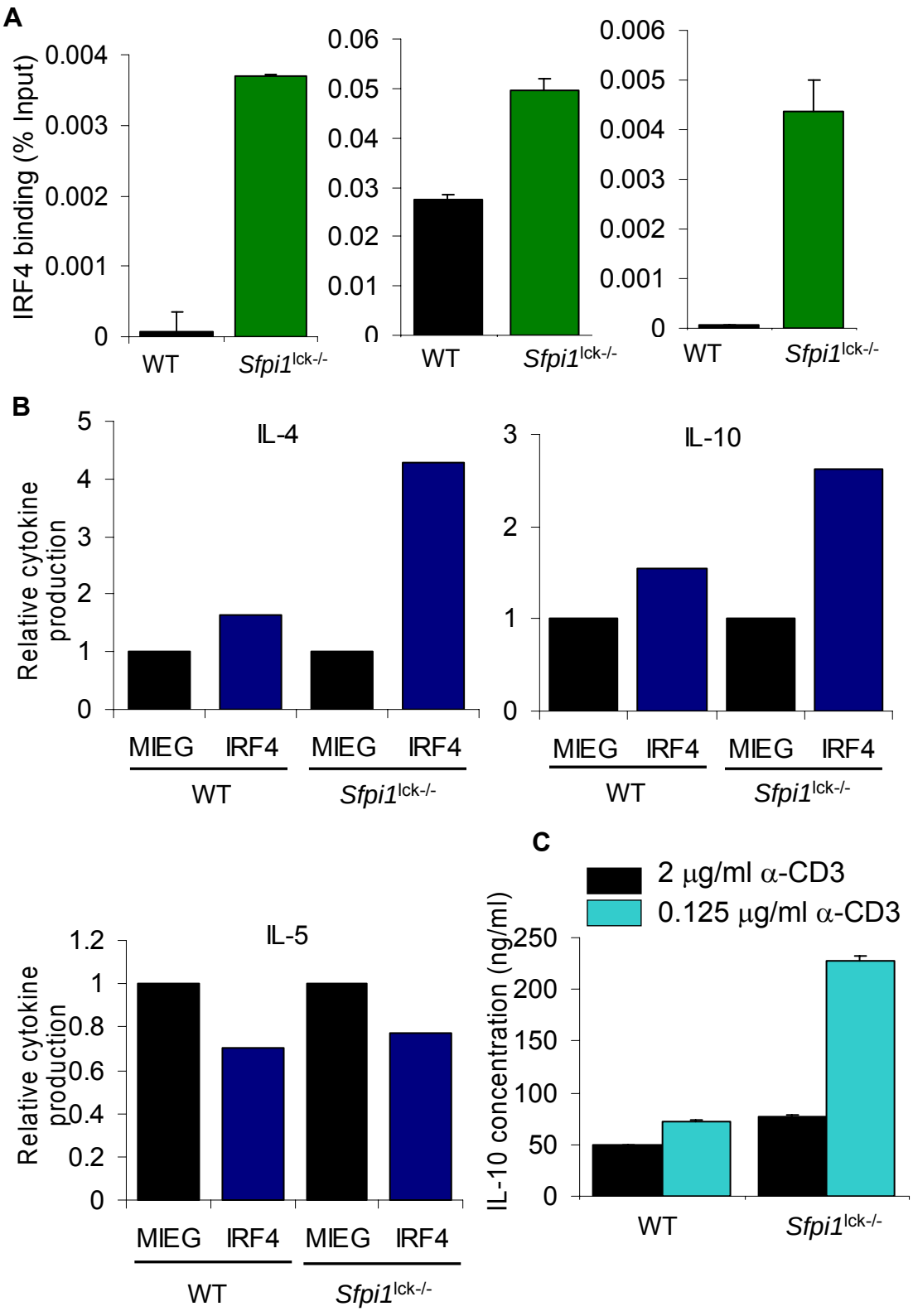

A
IRF4 binding (% Input)
0.004
0.003
0.002
0.001
0
WT
Sfpi1lck-/-
0.06
0.05
0.04
0.03
0.02
0.01
0
WT
Sfpi1lck-/-
0.006
0.005
0.004
0.003
0.002
0.001
0
WT
Sfpi1lck-/-
B
IL-4
Relative cytokine production
5
4
3
2
1
0
MIEG
IRF4
MIEG
IRF4
WT
Sfpi1lck-/-
IL-10
3
2
1
0
MIEG
IRF4
MIEG
IRF4
WT
Sfpi1lck-/-
IL-5
Relative cytokine production
1.2
1
0.8
0.6
0.4
0.2
0
MIEG
IRF4
MIEG
IRF4
WT
Sfpi1lck-/-
C
2 μg/ml α-CD3
0.125 μg/ml α-CD3
IL-10 concentration (ng/ml)
250
200
150
100
50
0
WT
Sfpi1lck-/-

Figure 19: PU.1 interacts with and limits the activity of IRF4
ChIP assay for IRF4 binding to *Il10* promoter and CNS3 regions, and *Il4* V_A regions was performed as described in Figure 13 with chromatin from WT and *Sfpi1*$^{lck-/-}$ CD4+ T cells cultured under Th2 conditions (A). WT and *Sfpi1*$^{lck-/-}$ CD4+ T cells were cultured under Th2 conditions and transduced with control or IRF4-expressing retroviruses as described in Figure 11B. IL-10 levels were determined by ELISA and normalized to control transduced cells. Results are representative of three experiments. WT and *Sfpi1*$^{lck-/-}$ CD4+T cells were differentiated under Th2 condition for 5 days in the presence of 2 μg/ml or 0.125 μg/ml α-CD3 and restimulated for 1 day with 2 μg/ml α-CD3 for ELISA (C).

PU.1 function depends on its cooperative state with other proteins.

Our previous work demonstrated that PU.1 was a regulator of Ccl22 expression in Th2 cells (66). Ccl22 is a Th2 chemokine expressed in Th2 related pathologies. It is highly expressed in IL-4 low cells compared to IL-4 high cells and the transduction of PU.1 in Th2 cells dramatically increase the expression of this chemokine (66). To determine whether PU.1 is directly regulating the expression of *Ccl22* gene by binding to the DNA we performed a ChIP assay in Balb/c Th2 cells before and after restimulation with 2 μg/ml anti-CD3 for (Figure 20A and B). Our data demonstrated that PU.1 binds to multiple regions of the *Ccl22* gene. Data in Figure 20C demonstrated that PU.1 binding to the *Ccl22* promoter region is higher after 2h restimulation compared to unstimulated cells. This result suggests that PU.1 promotes the expression of certain Th2 specific genes despite negatively regulating the binding of IRF4 or GATA-3 to target genes.

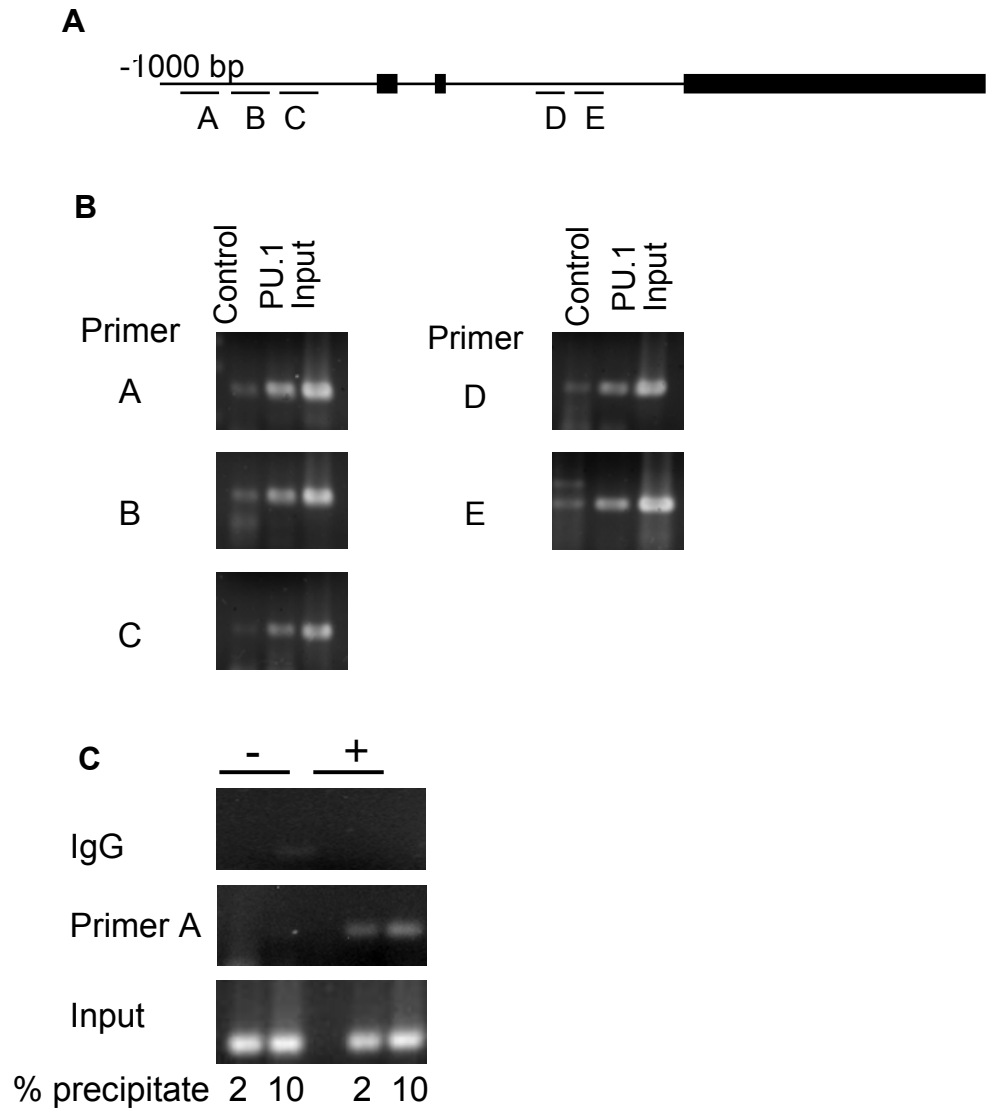


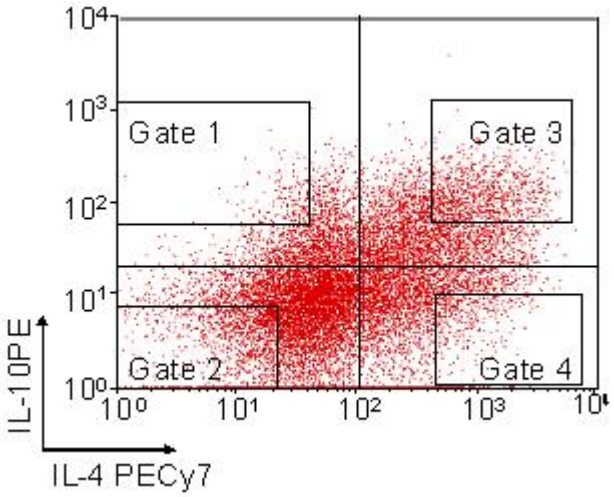
Figure 20: PU.1 directly binds to *Ccl22* promoter internal regions
Representation of the mouse *Ccl22* gene with black boxes representing exons (A). ChIP assay for PU.1 binding to *Ccl22* promoter and internal regions was performed as described in Figure 13 with chromatin from Balb/c CD4+ T cells cultured under Th2 conditions and restimulated for 2h (B). The binding of PU.1 was assessed before and after restimulation. The precleared cell lysate from unstimulated (-) or 2h stimulated (+) Th2 cells is incubated with anti-IRF4 or IgG antibody as negative control for the ChIP assay and the amount DNA bound to PU.1 was amplified by PCR in 10% or 2% of the total amount of DNA (C).

IRF4 expression profile in subpopulations of Th2 cells

Our study demonstrated that IRF4 is a key regulator of IL-4 and IL-10 expression with GATA-3. To directly visualize the level of this transcription factors in different subsets of Th2 cells we analyzed these cells based on two-state separation of cells into IL-10 high and low cells. However, IL-10 high and low cells can be further divided based on co-expression of other cytokines. Since IRF4 induced production of IL-4 and IL-10, we examined the expression of IRF4 in populations of IL-4- and IL-10-positive cells by intracellular staining. IRF4 expression was highest in Th2 cells that were double-positive for IL-4 and IL-10, and lowest in cells that did not secrete either cytokine (Figure 21A). Interestingly, expression was intermediate but similar in IL-4- and IL-10-single positive cells. Thus while IRF4 promotes IL-4 and IL-10 production, other factors also contribute to the decision of a cell to make one or both cytokines.

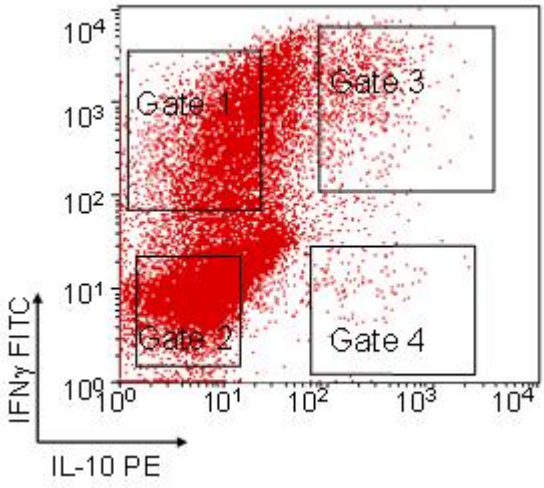
The analysis of IL-10 low and IL-10 high Th1 cells showed that most of the cells that secrete IL-10 also secrete IFNγ and express 3-fold more IRF4 than the other subsets of Th1 cells. Contrary to Th2 cells IFNγ/IL-10 non secretors or single secretors express similar levels of IRF4 (Figure 21B).

A



	IRF4 MFI	GATA-3 MFI
Gate 1	1538	7.4
Gate 2	658	4
Gate 3	3768	27.4
Gate 4	1987	8

B



	IRF4 MFI
Gate 1	1184
Gate 2	963
Gate 3	3374
Gate 4	973

Figure 21: IRF4 heterogeneity in Th2 cells
CD4+ T cells were cultured under Th2 or Th1 conditions for 5d. IRF4, GATA-3 and PU.1 protein levels were analyzed in Th2 cells treated with monensin for the last three hours of a six-hour stimulation with anti-CD3 before intracellular staining for IL-4, IL-10 and the proteins (A). The mean fluorescence intensity (MFI) of IRF4 staining is shown in the table for each of the gated populations. Th1 cells were stained with IRF4 and IFNγ and the MFI of IRF4 staining is shown in the adjacent table (B).

DISCUSSION

Role of IRF4 role in T-helper cell development

IRF4 plays an important role in the development of Th2 and Th17 cells (136, 137). In *Irf4-/-* mice, development of Th2 cells is decreased, suggesting that it plays a role in the differentiation process (60). A recent study reported that contrary to its role in effector/memory cells, IRF4 is a negative regulator of Th2 cytokine expression in naïve T cells (139). The deficiency of IRF4 in T helper cells also prevented the differentiation of Th17 cells both *in vitro* and *in vivo* by decreasing of the IL-21-induced IL-23 receptor expression, as well as the expression the lineage-specific orphan nuclear receptors RORgammat and RORalpha (153). As a result *Irf4*-/- mice are protected from experimental autoimmune encephalomyelitis (EAE) (137). However, a role in differentiation does not preclude involvement in the regulation of specific cytokines in differentiated Th2 cells. In this study we demonstrate that IRF4 contributes to the heterogeneity of Th2 populations by increasing expression of IL-4 and IL-10 and decreasing expression of *Il9*, while having no effects on IL-5 or IL-13. IRF4 expression segregates in Th2 cells between IL-10 high and IL-10 low cells. IRF4 triggered cytokine expression by directly binding to and transactivating the *Il4* gene (59) as well as the *Il10* gene, and ectopic expression of IRF4 can increase IL-10 production from IL-10 low cells. Thus, IRF4 is an instructive factor in establishing Th2 heterogeneity.

T helper cell heterogeneity

Increasing evidence suggests that the establishment of Th2 heterogeneity is not stochastic, but rather instructive, based on the expression of specific factors. As such, a growing list of transcription factors has specific effects on Th2 cytokines. IRF4 is induced following T cell activation, and expression is further increased following Th2 differentiation. Importantly, the level of IRF4 in IL-10-low cells is increased compared to that in recently activated T cells. As we have shown, IRF4 activates IL-10 and IL-4 production, while decreasing *Il9* and having little effect on IL-5 or IL-13. C-maf regulates IL-4 production but is not required for production of other Th2 cytokines (55). Similarly, Pias1 increases IL-13 production without affecting IL-4 or IL-5 expression (67). We have shown that PU.1 decreases expression of many Th2 cytokines, but increases expression of Ccl22, a chemokine associated with Th2 inflammation (66). BOB.1/OBF.1 regulates PU.1 expression in Th2 cells and also affects the potential for Th2 cytokine production (68). Moreover, the expression levels of each of these factors, and other factors that contribute to Th2 cytokine production exist in gradients that correlate with cytokine producing phenotypes (Figure 21). The similar level of expression of IRF4 in both IL-4- and IL-10-single positive cells supports the idea that other positive- or negative-acting factors overlay on the IRF4 gradient to generate the specific patterns of cytokine secretion. Some of these regulators might be C-maf for IL-4-single positive cells or NFATc2 for IL-10-single positive cells (55, 59). The mosaic of transcription factor gradients ultimately results in the heterogeneity observed in cytokine production from

individual cells. The regulation of the epigenetic modification, the transcription and the translation of the cytokine genes are all involved in defining the phenotype of the Th2 cells.

Th2 Transcription factors involved in the regulation of IL-10 expression

In Th2 cells, GATA-3 induces the expression of the cytokines IL-10, IL-4, IL-5 and IL-13 by modifying epigenetically the *Il10* locus (26), transactivating the *Il4*, *Il5*, and *Il13* loci (51, 154, 155). Contrary to IL-4 gene, memory Th2 cells need repeated restimulation in the presence of IL-4 before detection of extensive histone acetylation of the *Il10* gene. This epigenetic imprinting which correlates with the development of IL-10 memory is not detectable in Th1 cells (94).

IL-10 is a regulatory cytokine produced by a number of cells including Th2 cells and it's regulation in each cell type may be distinct. IL-10 plays a critical role in controlling inflammation *in vivo* by selectively suppressing the expression of pro-inflammatory cytokines. In various cell types, the molecular mechanism regulating the expression of IL-10 involves binding of IRF1 and STAT3 to the promoter region of the *Il10* gene locus (26, 94), and the regulation of the level of IL-10 mRNA by Sp1 and Sp3 (156). In Th2 cells, GATA-3 remodels the *Il10* locus (26), and Jun family proteins bind the CNS3 region to induce *Il10* transcription (103). Moreover, IL-10 production requires repeated stimulation to be completely imprinted within the Th2 population (157). We show that IRF4 contributes to *Il10* expression in Th2 and Th1 cells. The IL-12-dependent

production of IL-10 in Th1 cells has been documented in human and mouse cells (94, 158, 159). Clearly, additional factors contribute to the difference in IL-10 production by Th1 and Th2 cells, including that c-jun and JunB bind to the *Il10* CNS3 in Th2 but not Th1 cells (103), and that Ets-1 is a negative regulator of IL-10 production in Th1 cells (160). We demonstrate that in Th2 cells IRF4 binds directly to the *Il10* locus and is able to transactivate *Il10* regulatory element reporter plasmids. Our results parallel the recent description of a role for IRF4 in Treg cells where *Il10* was one of the prominently regulated genes (138). The fact that IRF4 binding sites exist in the *Il10* promoter and CNS3 regions, and that it binds to the same regulatory element as Jun containing complexes suggests that these factors may cooperate.

Our data demonstrated that Th1 cells express a similar level of IRF4 compared to Th2 cells, this data suggests that in addition to regulating IL-10 expression, IRF4 might be regulating the expression of additional genes in Th1 cells. The overexpression of IRF4 in Th2 cells in addition to enhancing the expression of Th2 cytokines also induced an increase in IFNγ production (136). It suggested that IRF4 can promote modifications in IFNγ gene even in Th2 cells with the presence of GATA-3 and inducing the transactivation of the gene. In Th1 cells IRF4 might contribute to IFNγ expression.

IRF4 role in naïve versus differentiating or differentiated Th2 cells

A recent study by Homna *et al.* reported a differential role of IRF4 in the regulation of Th2 cytokines in naïve CD4+ T cells versus effector/memory CD4+ T cells (139). Knocking down IRF4 promoted IL-4, IL-5 and IL-10 production in naïve cells while inhibiting it in effector/memory CD4+ T cells. This work did not address the molecular aspect of this opposite regulatory function but the authors suggested that different sets of transcription factors specific to these cells might be dictating the role of IRF4 (139). It is also possible that Th2 cytokine DNA loci in effector/memory cells were already epigenetically modified by transcription factors like GATA-3 which allows enhanceosomes containing IRF4 to bind to positive regulatory regions (26, 51). However, in naïve cells the inaccessibility of these areas of the DNA directs IRF4 repressor complexes to bind inhibitory regions.

The role of IRF4 in differentiating versus differentiated Th2 cells is very similar since it has a positive regulatory function in Th2 cytokines expression and production. CD4+ T cells skewed under Th2 condition in the absence of IRF4 demonstrated an impaired differentiation with “Th2” cells that had the cytokine profile of Th1 cells (136). Even though IL-4 signaling was not affected, GATA-3 was not upregulated (136). When we knocked down IRF4 in differentiated Th2 cells, the expression of Th2 cytokines like IL-10 and IL-4 decreased (Figure 22) correlating with the results reported in effector/memory CD4+ T cells (139). We also observed an upregulation of Th2 cytokines when Th2 cells are transduced

with exogenous IRF4 with no significant effect on GATA-3 expression suggesting that IRF4 regulates GATA-3 expression only during the priming process of Th2 cells.

Role of IRF4 and GATA-3 role in regulating IL-10

IRF4.

Our data demonstrated that IRF4 is directly regulating *Il10* gene expression by binding to the promoter and the CNS3 region of the gene (Figures 12 and 19). In the absence of PU.1 in *Sfpi1-/-* Th2 cells, IRF4 binding to it target genes increased dramatically (Figure 19A) and the expression of exogenous IRF4 in *Sfpi1* -/- Th2 cells enhanced IL-10 and IL-4 production to a greater extent than in WT Th2 cells (Figure 19B). The interaction of IRF4 and PU.1 at the protein level suggests that PU.1 sequesters IRF4 from binding to its target genes, *Il10* and *Il4*. A similar mechanism was reported for PU.1 and GATA-3 by our lab (66). The segregated expression of PU.1 in IL-4 low cells prevented GATA-3 from binding to the *Il4* gene locus while GATA-3 was bound to this locus in IL-4 high cells (66). Contrary to IL-4 low cells, IL-10 low cells expressed lower levels of PU.1 than IL-10 high cells. We propose that in IL-10 high cells, the high expression of IRF4 and GATA-3 overcome the elevated level of PU.1.

Interestingly, knocking down PU.1 increased the number of IL-10, IL-4 and IL-5 secreting cells, while exogenous wild-type PU.1 strongly decreased Th2 cytokine expression (66). In this report a PU.1 DNA binding mutant was less efficient in

decreasing Th2 cytokine production compared to the wild-type PU.1, suggesting that PU.1 regulatory effect may be mediated through other transcription factors such as IRF4, which are known to be recruited to the DNA by PU.1 in B cells (74, 126, 161) or T cells (Figure 18B). However, in B cells PU.1/IRF4 enhanced the transcriptional activity of *Il1β* (74), class II transactivator promoter III (CIITA-PIII) (161) and kappa 3' enhancer (126) while in T cells PU.1/IRF4 might have an inhibitory effect on the transcription of the target genes including *Il10* and *Il4*. In the case of *Il1β*, PU.1 forms an enhanceosome with IRF4, IRF1 and IRF2 (74). The presence of cell specific transcription factors in the protein complex may dictate an enhancing or inhibitory regulatory function of PU.1/IRF4 in the cells. Whether PU.1 binds IRF4 on the *Il10* and *Il4* loci in Th cells and prevents the transactivation of these genes still needs to be investigated.

GATA-3.

GATA-3 is a key transcription factor in the differentiation and development of Th2 cells. Farrar *et al.* using a retroviral-based tagging technique to monitor the fate of individual T cell progenitors demonstrated that IL-4 was able to shift the phenotype of already committed non-IL-4-producing via GATA-3-induced epigenetic modification *in vitro* (28, 92) and *in vivo* (27). This data confirmed that Th cell skewing is through an instructive transcriptional programming driven by the cytokines present in the cell microenvironment (92).

The genetic programming of each cell may predispose it to a differential expression of the instructive factors. For example Figure 16A shows that IL-10 high cells express 3 times more IL-4Rα and IL-10Rα than IL-10 low cells. This data suggests that IL-10 high cells may be more responsive to IL-4 stimulation which in turn induces a high level of GATA-3 and IRF4 expression in these cells. We propose that even though IL-10 high cells express a significant level of PU.1 (Figure 8D) the elevated level of GATA-3 and IRF4 overcome the presence of PU.1 in the IL-10 secreting cells so that only a fraction of these factors is sequestered from their target genes. PU.1 might be preventing Th2 cells from excessive production of Th2 cytokines upon stimulation, as a result Th2 cells can quickly turn off cytokine production in a feed back loop. In this report, we have identified IRF4 as a regulator of Th2 heterogeneity by enhancing or decreasing the production of specific cytokines. IRF4 function, like GATA-3 as described in our previous report (66), is limited by the expression of PU.1 in Th2 cells, which binds IRF4 and decreases binding to target genes including *Il10*. Future work will examine how these factors interact to generate the population phenotype and what signals determine the expression of each factor within individual cells.

We did not observe strong interactions of IRF4 with GATA-3. In contrast, we did observe interactions with PU.1, which we previously demonstrated decreased IL-10 production (66). In the absence of PU.1, IRF4 had greater potential to bind *Il10* and increase IL-10 production, suggesting that at least some of the ability of PU.1 to modulate *Il10* is through the ability to interfere with IRF4 activity. Further

analysis of how these factors interact at the functional level will discern more precise roles.

In addition to its indirect regulatory role on Th2 cytokines expression, PU.1 directly binds to *Ccl22* gene upon stimulation (Figure 20) and regulates its expression since PU.1 overexpression increases Ccl22 production in Th2 cells (66). It is possible that PU.1 is recruiting other factors to the promoter region of *Ccl22* or that PU.1 is recruited to the promoter by another factor since its binding was detected after 2h of restimulation. This aspect of PU.1 function was not assessed in this study. The model in Figure 22 depicts the different pathways for PU.1 regulatory activity.

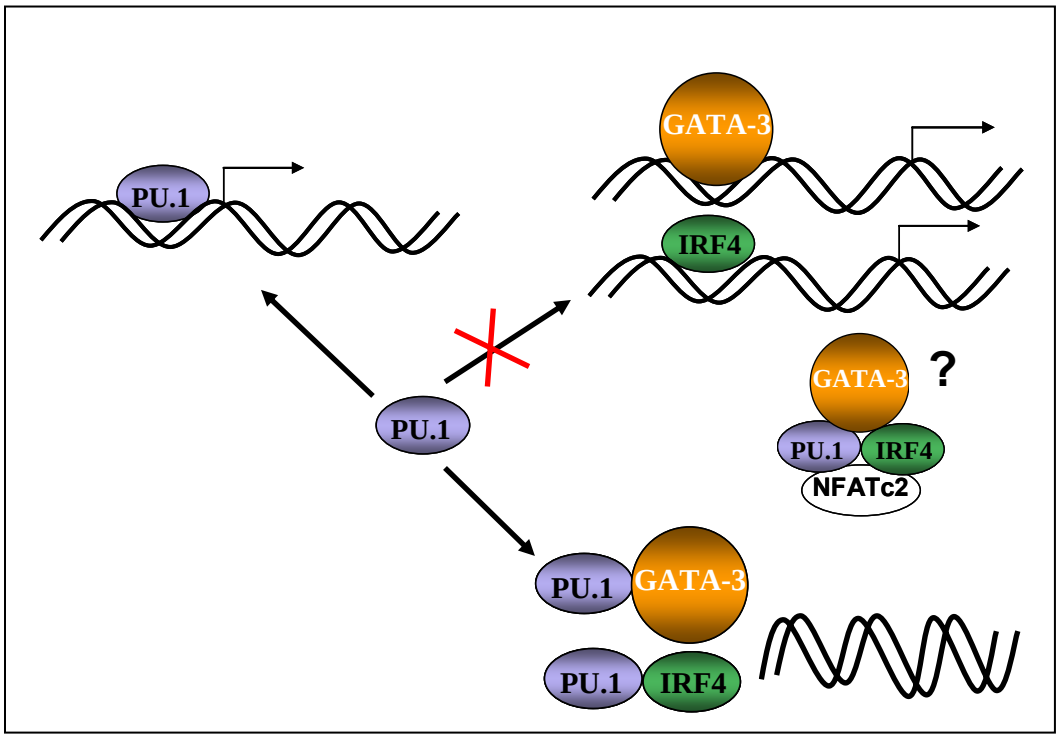


Figure 22: PU.1 regulatory function in Th2 cells
IRF4 and GATA-3 transcriptional activities are regulated by the presence of PU.1 in Th2 cells. When IRF4 and GATA-3 are expressed in excess they bind their target DNA whether the Th2 subsets express PU.1 or not but the absence of PU.1 favor the transcriptional activity of GATA-3 and IRF4. PU.1 is also an inducer of Th2 genes including *Ccl22* by directly binding to its promoter region.

Reciprocal regulation between, IL-10, IRF4 and IL-9

The reciprocal regulation of *Il10* and *Il9* is striking and distinct from the IL-9 and IL-10-producing cells present in cultures primed with TGFβ and IL-4 (36). While transduction of IRF4 in Th2 cells decreases *Il9* expression, we did not observe IRF4 binding to the *Il9* promoter in a ChIP assay (data not shown). This suggested that the effects of IRF4 could be indirect, through the induction of IL-10. Indeed, neutralizing IL-10 in IL-10 high cells, that express low levels of *Il9*, modestly decreased *Il9* mRNA (Figure 16B) while addition of exogenous IL-10 to IL-10 low cells increase *Il9* expression. However, it is not clear which IL-10 activated pathways might be responsible for this regulation (model summarized in Figure 24). It is also not clear why a cell would be specialized to express only one of these cytokines. IL-9 is a pleiotropic cytokine involved in the pathologic and physiologic evolution of asthma by recruiting eosinophils and lymphocytes to the lung, inducing mucus hypersecretion, mast cells hyperplasia in concert with IL-4, IL-5 and IL-13 (120), while IL-10 is a suppressive cytokine that may modulate many of these processes. It is possible that secretion of IL-9 by Th2 cells would only be effective if target cells did not receive a conflicting signal generated by IL-10. In this manner, Th2 heterogeneity may reflect functional specialization of cell types within the inflammatory microenvironment.

The role of IL-10 in inducing late expression of IL-9 through this regulatory loop may have different roles depending on the disease. Contrary to its proinflammatory role, IL-9 has a immunosuppressive functions which allow

allograft tolerance (13). Indeed mast-cell-deficient mice do not display immune tolerance (13). Studies reported that CD4+CD25+Foxp3+ regulatory T cells secrete IL-9 which recruits and activates mast cells to mediate regional immune suppression (13). The IL-9 secreted by Th2 cells could also complement IL-10 immunosuppressive function by recruiting mast cells to area of the organism where tolerance is established.

In addition to the effect on IL-9 expression there is evidence that IL-10 expression is controlled by a regulatory feedback loop which allows the lymphocytes and the microenvironment to respond to subsequent infection through the production of inflammatory cytokines (summarized in Figure 23). The effect of this feedback loop can be observed in Th2 cells restimulated for increasing times. Figure 15 A shows that IL-10 expression peaks at 2h, followed by a smaller peak at 20h restimulation and suggests that the IL-10 negative regulatory feedback loop prevents Th2 cells from a second strong “wave” of anti-inflammatory response. It is interesting to note that IL-10 high cells express higher level of IL-10R which might make these cells more prone to rapidly turning off IL-10 production in response to IL-10 stimulatory feedback loop. Apparently STAT3 is not mediating the IRF4-dependent feedback regulation of IL-10; however, Irf4 gene expression is a target for this pathway.

Our data suggests that IL-10 is negatively regulating *Il9* expression in wild-type Th2 cells via IRF4, however, in the absence of STAT3 IL-10 induces *Il9*

expression even though *Irf4* mRNA level increases in response to the feedback loop. We propose two mechanisms for *Il9* regulation by IL-10. In the first mechanism IRF4 may be forming an *Il9* repressor complex with STAT3, as a result, other transcription factors can induce *Il9* expression in STAT3$^{CD4-/-}$ Th2 cells. In the second mechanism we propose the IL-10/STAT3 pathway to trigger the expression of an inhibitory factor which is not expressed in STAT3$^{CD4-/-}$ Th2 cells upon IL-10 stimulation.

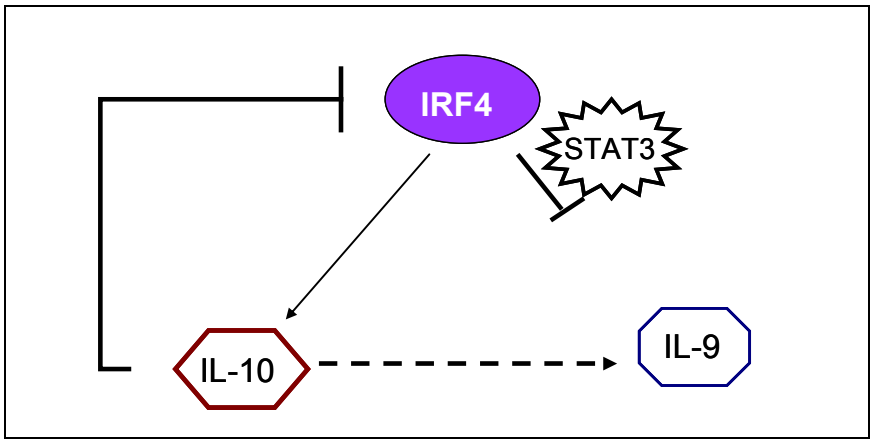


Figure 23: Model of the IL-10 autoregulation feedback loop and the indirect regulation of IL-9 expression

OVERALL CONCLUSION

Cytokine induced transcription factors that are differentially expressed in Th cells are required for the development and commitment to a specific Th lineage. Our study confirmed that heterogeneity in Th2, as well as in Th1, populations results from the instructive function of transcription factors in the regulation of cytokine expression at a single-cell level. Indeed, we have recently identified the ETS family transcription factor PU.1 as regulating heterogeneity in Th2 populations and we examined the PU.1 interacting protein IFN-regulatory factor (IRF)-4, a transcription factor expressed in lymphocytes and macrophages. We were able to understand the role of IRF4 in this process and its relation with other instructive transcription factors including GATA-3 and PU.1. When Th2 cells are separated based on levels of IL-10 secretion, IRF4 expression segregates into the subset of Th2 cells expressing high levels of IL-10. To investigate the role of IRF4 in cytokine heterogeneity, Th2 cells were infected with retrovirus expressing IRF4. The cells overexpressing IRF4 secreted significantly higher levels of IL-10 and IL-4 compared to cells infected with a control vector at the same time the level of IL-9 decreases. Down regulation of IRF4 by shRNA or siRNA decreased IL-10 and IL-4 expression. We used co-immunoprecipitation assays to determine transcription factors that interact with IRF4. Our data shows that PU.1, IRF4 and GATA-3 form a complex in Th2 nuclear extract while NFATc2 forms a complex with each of these factors; whether all 4 factors are part of the same complex was not clear. We also demonstrated by ChIP assay that IRF4 directly binds the *Il10* and *Il4* loci in a time dependent manner and transactivates *Il10* regulatory

regions. The binding of IRF4 to these loci is regulated by PU.1 which is a sequestering factor for both GATA-3 (66) and IRF4. The IRF4 dependent regulation of IL-10 was specific to Th2 cells and may be partial in Th1 cells. Understanding the molecular mechanism dictating the cytokine profile of Th2 cells is an important tool in modulating the function of these cells in a disease model by transiently affecting their ratio of anti-inflammatory cytokines to inflammatory cytokines.

FUTURE DIRECTIONS

Stability and function of IL-10 –low and –high cells *in vivo*

We demonstrated that different subpopulations of Th2 cells (IL-10 secreting and IL-10 non-secreting cells) have a stable phenotype *in vitro* where they are not challenged by a fluctuating cytokine microenvironment. Determining the stability of IL-10 –low and –high cell phenotype *in vivo* will confirm its heritability as well as the epigenetic modifications defining each subset of cell. The second aspect of this question will be to assess the effects of these cells on the progression of an allergic disease like asthma. To address these questions Th2 cells will be generated from I-Ad-binding ovalbumin (OVA) 323-339-specific T-cell receptor-transgenic (TCR-Tg) mouse spleen cells by culturing with OVA323-339 peptide, APC, IL-4 and α-IL-12 antibody. IL-10 secreting and non secreting cells will be sorted from the OVA co-culture and injected by tail vein injection in sensitized Balb/c mice. Two different groups of mice will be analyzed in this study, the non-sensitized mice and the mice sensitized with ovalbumin for 14 days which favor recruitment of lymphoid cells to the lungs. These mice will be challenged with aerosolized OVA or bovine serum albumin (BSA) and analyzed for changes in lung resistance, airway responsiveness to inhaled methacholine. The analysis of donor CD4+T cells in bronchoalveolar lavage by flow cytometry after IL-10 intracellular staining will assess the stability of IL-10-low and IL-10 -high cells phenotype as well as their ability to mediate allergic inflammation.

Effect of PU.1 and IRF4 direct DNA binding on IRF4 transcription activity

We demonstrated that IRF4, like Jun proteins, transactivates the IL-10 promoter and the IL-10 CNS3 driven luciferase reporters. We also showed that IRF4 directly binds to *Il10* gene regulatory regions. Interestingly PU.1 also binds to these regions with a different kinetic of binding (data not shown). One mechanism by which PU.1 can also inhibit IRF4 transcription activity could be through the formation of PU.1/IRF4/DNA ternary complex. This complex has been shown to enhance λB transactivation after substantial conformational changes of DNA shape in B cells, but it could play the opposite role in T cells due to the presence of other transcription factors specific to Th2 cells. Our approach to address this question is to transfect EL4 cells with both PU.1 and IRF4 vectors along with *Il10*p or *Il10* CNS3 driven luciferase reporters and assess luciferase activity. The Controls for this experiment will consist of cells transfected with empty vector, PU.1, IRF4 or JunB vectors alone the earlier been the negative controls and the later been the positive controls. The function of the different domains of PU.1 in the transactivation of IL-10 will be assessed by using PU.1 mutants in the reporter assays.

IRF4 effect on IL-10 transcription might be mediated through its DNA binding domain and/or its PU.1 interacting domain. To determine the role of the different IRF4 domains on the induction of IL-4 and IL-10, we propose to engineer IRF4 mutant/hCD4 bicistronic retroviral vectors which will be used to transduce both WT and *Sfpi1-/-* Th2 cells as described in Figures 11 and 19. The binding of full-

length IRF4 in the presence of mutant PU.1 or IRF4 mutants to IL-10 promoter or IL-10 CNS3 oligonucleotides will be assessed by DNA affinity precipitation assay using Th2 cell nuclear extract.

REFERENCES

1. Braff, M. H., A. Bardan, V. Nizet, and R. L. Gallo. 2005. Cutaneous Defense Mechanisms by Antimicrobial Peptides. *J Investig Dermatol* 125:9-13.
2. Kraehenbuhl, J.-P., and M. Corbett. 2004. Immunology: Keeping the Gut Microflora at Bay. *Science* 303:1624-1625.
3. Martin, T. R., and C. W. Frevert. 2005. Innate Immunity in the Lungs. *Proc Am Thorac Soc* 2:403-411.
4. Lazarus, S. C. 1998. Inflammation, inflammatory mediators, and mediator antagonists in asthma. *J Clin Pharmacol* 38:577-582.
5. Marshall, J. S. 2004. Mast-cell responses to pathogens. *Nat Rev Immunol* 4:787-799.
6. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 5:987-995.
7. Stockinger, B., C. Bourgeois, and G. Kassiotis. 2006. CD4+ memory T cells: functional differentiation and homeostasis. *Immunol Rev* 211:39-48.
8. Kalia V., S. Sarkar , T. Gourley, B. Rouse , and R. Ahmed. 2006. Differentiation of memory B and T cells *Curr Opin Immunol* 18: 255-264.
9. Medzhitov, R., and C. A. Janeway Jr. 1998. Innate immune recognition and control of adaptive immune responses. *Semin Immunol* 10:351-353.
10. Nurieva R., S. Thomas, T. Nguyen, N. Martin-Orozco , Y. Wang , M.K. Kaja, X.Z. Yu, and C. Dong. 2006. T-cell tolerance or function is determined by combinatorial costimulatory signals *EMBO J* 25:2623-2633.
11. Song J., F.T. Lei , X. Xiong, and R. Haque. 2008 Intracellular signals of T cell costimulation. *Cell Mol Immunol* 5:239-247.
12. Urry, Z., E. Xystrakis, and C. Hawrylowicz. 2006. Interleukin-10-Secreting regulatory T cells in allergy and asthma. *Curr Allergy Asthma Rep* 6:363-371.

13. Lu, L.-F., E. F. Lind, D. C. Gondek, K. A. Bennett, M. W. Gleeson, K. Pino-Lagos, Z. A. Scott, A. J. Coyle, J. L. Reed, J. Van Snick, T. B. Strom, X. X. Zheng, and R. J. Noelle. 2006. Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* 442:997-1002.
14. Zhu, J., and W. E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112:1557-1569.
15. Holtmeier W. 2003. Compartmentalization gamma/delta T cells and their putative role in mucosal immunity. *Crit Rev Immunol.* 23:473-488.
16. Van Kaer, L. 2007. NKT cells: T lymphocytes with innate effector functions. *Curr Opin Immunol* 19:354-364.
17. Kaplan, M. H., Y.-L. Sun, T. Hoey, and M. J. Grusby. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature* 382:174-177.
18. Yang, Y., J. C. Ochando, J. S. Bromberg, and Y. Ding. 2007. Identification of a distant T-bet enhancer responsive to IL-12/Stat4 and IFN{gamma}/Stat1 signals. *Blood* 110:2494-2500.
19. Afkarian, M., J. R. Sedy, J. Yang, N. G. Jacobson, N. Cereb, S. Y. Yang, T. L. Murphy, and K. M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naïve CD4+ T cells. *Nat Immunol* 3:549 - 557.
20. Lighvani, A. A., D. M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B. D. Hissong, B. V. Nguyen, M. Gadina, A. Sher, W. E. Paul, and J. J. O'Shea. 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci* 98:15137-15142.
21. Ayada, K., K. Yokota, K. Kobayashi, Y. Shoenfeld, E. Matsuura, and K. Oguma. 2008 Chronic Infections and Atherosclerosis. *Ann N Y Acad Sci* 1108:594-602.
22. Murphy, K. M., W. Ouyang, J. D. Farrar, J. Yang, S. Ranganath, H. Asnagli, M. Afkarian, and T. L. Murphy. 2000. Signaling and Transcription in T Helper Development. *Annu Rev Immunol* 18:451-494.

23. Noben-Trauth, N., J. Hu-Li, and W. E. Paul. 2000. Conventional, Naive CD4+ T Cells Provide an Initial Source of IL-4 During Th2 Differentiation. *J Immunol* 165:3620-3625.
24. Wang, M., A. Saxon, and D. Diaz-Sanchez. 1999. Early IL-4 Production Driving Th2 Differentiation in a Human in Vivo Allergic Model Is Mast Cell Derived. *Clin Immunol* 90:47-54.
25. Renauld, J. C. 2001. New insights into the role of cytokines in asthma. *J Clin Pathol* 54:577-589.
26. Shoemaker, J., M. Saraiva, and A. O'Garra. 2006. GATA-3 Directly Remodels the IL-10 Locus Independently of IL-4 in CD4+ T Cells. *J Immunol* 176:3470-3479.
27. Nawijn, M. C., G. M. Dingjan, R. Ferreira, B. N. Lambrecht, A. Karis, F. Grosveld, H. Savelkoul, and R. W. Hendriks. 2001. Enforced Expression of GATA-3 in Transgenic Mice Inhibits Th1 Differentiation and Induces the Formation of a T1/ST2-Expressing Th2-Committed T Cell Compartment In Vivo. *J Immunol* 167:724-732.
28. Ouyang, W., S.H. Ranganath, K. Weindel, D. Bhattacharya, T.L. Murphy, W.C. Sha, and K.M. Murphy. 1998. Inhibition of Th1 Development Mediated by GATA-3 through an IL-4-Independent Mechanism. *Immunity* 9:745-755.
29. Hansen, G., G. Berry, R. H. DeKruyff, and D. T. Umetsu. 1999. Allergen-specific Th1 cells fail to counterbalance Th2 cell–induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest* 103:175-183.
30. Liang, S. C., A. J. Long, F. Bennett, M. J. Whitters, R. Karim, M. Collins, S. J. Goldman, K. Dunussi-Joannopoulos, C. M. M. Williams, J. F. Wright, and L. A. Fouser. 2007. An IL-17F/A Heterodimer Protein Is Produced by Mouse Th17 Cells and Induces Airway Neutrophil Recruitment. *J Immunol* 179:7791-7799.

31. Wei, L., A. Laurence, K. M. Elias, and J. J. O'Shea. 2007. IL-21 Is Produced by Th17 Cells and Drives IL-17 Production in a STAT3-dependent Manner. *J Biol Chem* 282:34605-34610.
32. Korn, T. 2007. IL-21 initiates an alternative pathway to induce proinflammatory TH17 cells. *Nature* 448:484-488.
33. Liang S. C., X-Y Tan , D.P. Luxenberg, R. Karim, K. Dunussi-Joannopoulos, M. Collins, and L.A. Fouser. 2006. Interleukin (IL)-22 and IL-17 are coexpressed by TH17 cells and cooperatively enhance expression of antimicrobial peptides 1. *J Exp Med* 203:2271-2279.
34. Mathur, A. N., H.-C. Chang, D. G. Zisoulis, G. L. Stritesky, Q. Yu, J. T. O'Malley, R. Kapur, D. E. Levy, G. S. Kansas, and M. H. Kaplan. 2007. Stat3 and Stat4 Direct Development of IL-17-Secreting Th Cells. *J Immunol* 178:4901-4907.
35. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-[beta] induces development of the TH17 lineage. *Nature* 441:231-234.
36. Veldhoen, M., C. Uyttenhove, J. van Snick, H. Helmby, A. Westendorf, J. Buer, B. Martin, C. Wilhelm, and B. Stockinger. 2008. Transforming growth factor-[beta] 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9:1341 - 1346
37. Chtanova, T., S. G. Tangye, R. Newton, N. Frank, M. R. Hodge, M. S. Rolph, and C. R. Mackay. 2004. T Follicular Helper Cells Express a Distinctive Transcriptional Profile, Reflecting Their Role as Non-Th1/Th2 Effector Cells That Provide Help for B Cells. *J Immunol* 173:68-78.
38. Nurieva RI, Y. Chung, D. Hwang, X.O. Yang, H.S. Kang, L. Ma, Y.H. Wang, S.S. Watowich, A.M. Jetten, Q.Tian, and C. Dong. 2008. Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages. *Immunity* 29:138-149.

39. Vogelzang A., H.M. McGuire, D. Yu, J. Sprent, C.R. Mackay, and C. King. 2008. A fundamental role for interleukin-21 in the generation of T follicular helper cells. *Immunity* 29:127-137.

40. Kaplan M.H., U. Schindler, S.T. Smiley, and M.J. Grusby. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 4:313-319.

41. Shimoda, K., J. van Deursent, M. Y. Sangster, S. R. Sarawar, R. T. Carson, R. A. Tripp, C. Chu, F. W. Quelle, T. Nosaka, D. A. A. Vignali, P. C. Doherty, G. Grosveld, W. E. Paul, and J. N. Ihle. 1996. Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted State6 gene. *Nature* 380:630-633.

42. Reiner S.L. 2007. Development in motion: helper T cells at work. *Cell* 129:33-36.

43. Mosmann, T. R., and R. L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145-173.

44. Abbas, A. K., K.M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787-793.

45. Tu, L., T. C. Fang, D. Artis, O. Shestova, S. E. Pross, I. Maillard, and W. S. Pear. 2005. Notch signaling is an important regulator of type 2 immunity. *J. Exp. Med.* 202:1037-1042.

46. Maillard, I., T. Fang, and W. S. Pear. 2005. Regulation of lymphoid development, differentiation and function by the notch pathway. *Annu Rev Immunol* 23:945-974.

47. Fang, T. C., Y. Yashiro-Ohtani, C. Del Bianco, D. M. Knoblock, S. C. Blacklow, and W. S. Pear. 2007. Notch Directly Regulates Gata3 Expression during T Helper 2 Cell Differentiation. *Immunity* 27:100-110.

48. Amsen, D., A. Antov, D. Jankovic, A. Sher, F. Radtke, A. Souabni, M. Busslinger, B. McCright, T. Gridley, and R. A. Flavell. 2007. Direct Regulation of Gata3 Expression Determines the T Helper Differentiation Potential of Notch. *Immunity* 27:89-99.
49. Rutz, S., M. Janke, N. Kassner, T. Hohnstein, M. Krueger, and A. Scheffold. 2008. Notch regulates IL-10 production by T helper 1 cells. *Proc Natl Acad Sci* 105:3497-3502.
50. Cai, S., C. C. Lee, and T. Kohwi-Shigematsu. 2006. SATB1 packages densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes. *Nat Genet* 38:1278-1288.
51. Zheng W., and R.A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89:587-596.
52. Ferber, I. A., H.-J. Lee, F. Zonin, V. Heath, A. Mui, N. Arai, and A. O'Garra. 1999. GATA-3 Significantly Downregulates IFN-[gamma] Production from Developing Th1 Cells in Addition to Inducing IL-4 and IL-5 Levels. *Clin Immunol* 91:134-144.
53. Kurata H., H.J. Lee, A. O'Garra, and N. Arai. 1999. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity* 11:677-688.
54. Ho, I. C., D. Lo, and L. H. Glimcher. 1998. c-maf Promotes T Helper Cell Type 2 (Th2) and Attenuates Th1 Differentiation by Both Interleukin 4-dependent and -independent Mechanisms. *J. Exp. Med.* 188:1859-1866.
55. Kim, J. I., I. C. Ho, M. J. Grusby, and L. H. Glimcher. 1999. The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Immunity* 10:745-751.
56. So T., J. Song, K. Sugie, A. Altman, and M. Croft. 2006. Signals from OX40 regulate nuclear factor of activated T cells c1 and T cell helper 2 lineage commitment. *Proc Natl Acad Sci* 103:3740-3745.

57. Diehl, S., C.-W. Chow, L. Weiss, A. Palmetshofer, T. Twardzik, L. Rounds, E. Serfling, R. J. Davis, J. Anguita, and M. Rincon. 2002. Induction of NFATc2 Expression by Interleukin 6 Promotes T Helper Type 2 Differentiation. *J. Exp. Med.* 196:39-49.

58. Garcia-Cozar, F. J., H. Okamura, J. F. Aramburu, K. T. Shaw, L. Pelletier, R. Showalter, E. Villafranca, and A. Rao. 1998. Two-site Interaction of Nuclear Factor of Activated T Cells with Activated Calcineurin. *J Biol Chem* 273:23877-23883.

59. Rengarajan, J., K. A. Mowen, K. D. McBride, E. D. Smith, H. Singh, and L. H. Glimcher. 2002. Interferon Regulatory Factor 4 (IRF4) Interacts with NFATc2 to Modulate Interleukin 4 Gene Expression. *J Exp Med* 195:1003-1012.

60. Hu, C.-M., S. Y. Jang, J. C. Fanzo, and A. B. Pernis. 2002. Modulation of T cell cytokine production by interferon regulatory factor-4 (IRF-4). *J Biol Chem* 277:49238-49246.

61. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 170:2081-2095.

62. Hu-Li, J., C. Pannetier, L. Guo, M. Lohning, H. Gu, C. Watson, M. Assenmacher, A. Radbruch, and W. E. Paul. 2001. Regulation of Expression of IL-4 Alleles: Analysis Using a Chimeric GFP/IL-4 Gene. *Immunity* 14:1-11.

63. Calado, D. P., T. Paixao, D. Holmberg, and M. Haury. 2006. Stochastic Monoallelic Expression of IL-10 in T Cells. *J Immunol* 177:5358-5364.

64. Guo, L., J. Hu-Li, J. Zhu, C. J. Watson, M. J. Difilippantonio, C. Pannetier, and W. E. Paul. 2002. In TH2 cells the Il4 gene has a series of accessibility states associated with distinctive probabilities of IL-4 production. *Proc Natl Acad Sci* 99:10623-10628.

65. Karulin, A. Y., M. D. Hesse, M. Tary-Lehmann, and P. V. Lehmann. 2000. Single-Cytokine-Producing CD4 Memory Cells Predominate in Type 1 and Type 2 Immunity. *J Immunol* 164:1862-1872.

66. Chang, H.-C., S. Zhang, V. T. Thieu, R. B. Slee, H. A. Bruns, R. N. Laribee, M. J. Klemsz, and M. H. Kaplan. 2005. PU.1 Expression Delineates Heterogeneity in Primary Th2 Cells. *Immunity* 22:693-703.

67. Zhao, X., B. Zheng, Y. Huang, D. Yang, S. Katzman, C. Chang, D. Fowell, and W. P. Zeng. 2007. Interaction between GATA-3 and the Transcriptional Coregulator Pias1 Is Important for the Regulation of Th2 Immune Responses. *J Immunol* 179:8297-8304.

68. Brunner C., A. Sindrilaru, I. Girkontaite, K-D. Fischer, C. Sunderkötter, and T. Wirth. 2007. BOB.1/OBF.1 controls the balance of Th1 and Th2 immune responses. *EMBO J* 26:3191-3202.

69. Klemsz, M. J., S.R. McKercher, A. Celada, C. Van Beveren, and R. A. Maki. 1990. The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell* 61:113-124.

70. Moreau-Gachelin F., D. Ray , M.G. Mattei, P. Tambourin, and A. Tavitian. 1989. The putative oncogene Spi-1: murine chromosomal localization and transcriptional activation in murine acute erythroleukemias. *Oncogene* 4:1449-1456.

71. Pio, F., C. Z. Ni, R. S. Mitchell, J. Knight, S. McKercher, M. Klemsz, A. Lombardo, R. A. Maki, and K. R. Ely. 1995. Co-crystallization of an ETS Domain (PU.1) in Complex with DNA. *J. Biol. Chem.* 270:24258-24263.

72. Klemsz M.J., and R. A. Maki. 1996. Activation of transcription by PU.1 requires both acidic and glutamine domains. *Mol Cell Biol.* 16:390-397.

73. Nishiyama, C., M. Hasegawa, M. Nishiyama, K. Takahashi, Y. Akizawa, T. Yokota, K. Okumura, H. Ogawa, and C. Ra. 2002. Regulation of Human Fc{epsilon}RI {alpha}-Chain Gene Expression by Multiple Transcription Factors. *J Immunol* 168:4546-4552.

74. Marecki, S., C. J. Riendeau, M. D. Liang, and M. J. Fenton. 2001. PU.1 and Multiple IFN Regulatory Factor Proteins Synergize to Mediate Transcriptional Activation of the Human IL-1{{beta}} Gene. *J Immunol* 166:6829-6838.

75. Moulton, K. S., K. Semple, H. Wu, and C. K. Glass. 1994. Cell-specific expression of the macrophage scavenger receptor gene is dependent on PU.1 and a composite AP-1/ets motif. *Mol Cell Biol* 14:4408-4418.

76. Henkel, G. W., S. R. McKercher, and R. A. Maki. 2002. Identification of three genes up-regulated in PU.1 rescued monocytic precursor cells. *Int Immunol* 14:723-732.

77. Li, Y., Y. Okuno, P. Zhang, H. S. Radomska, H.-m. Chen, H. Iwasaki, K. Akashi, M. J. Klemsz, S. R. McKercher, R. A. Maki, and D. G. Tenen. 2001. Regulation of the PU.1 gene by distal elements. *Blood* 98:2958-2965.

78. McKercher S.R., B.E. Torbett, K.L. Anderson, G.W. Henkel, D.J. Vestal, H. Baribault, M. Klemsz, A.J. Feeney, G.E. Wu, C.J. Paige, and R.A. Maki. 1996 Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J* 15: 5647–5658.

79. Scott E.W., M.C. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265:1573-1577.

80. Anderson, K. L., K. A. Smith, F. Pio, B. E. Torbett, and R. A. Maki. 1998. Neutrophils Deficient in PU.1 Do Not Terminally Differentiate or Become Functionally Competent. *Blood* 92:1576-1585.

81. Iwasaki, H., C. Somoza, H. Shigematsu, E. A. Duprez, J. Iwasaki-Arai, S. I. Mizuno, Y. Arinobu, K. Geary, P. Zhang, T. Dayaram, M. L. Fenyus, S. Elf, S. Chan, P. Kastner, C. S. Huettner, R. Murray, D. G. Tenen, and K. Akashi. 2005. Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* 106:1590-1600.

82. Polli, M., A. Dakic, A. Light, L. Wu, D. M. Tarlinton, and S. L. Nutt. 2005. The development of functional B lymphocytes in conditional PU.1 knock-out mice. *Blood* 106:2083-2090.

83. Cook, W. D., B. J. McCaw, C. Herring, D. L. John, S. J. Foote, S. L. Nutt, and J. M. Adams. 2004. PU.1 is a suppressor of myeloid leukemia, inactivated in mice by gene deletion and mutation of its DNA binding domain. *Blood* 104:3437-3444.

84. Dakic, A., D. Metcalf, L. Di Rago, S. Mifsud, L. Wu, and S. L. Nutt. 2005. PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis. *J Exp Med* 201:1487-1502.

85. Metcalf D., A. Dakic, S. Mifsud, L. Di Rago, L. Wu, and S. Nutt. 2006 Inactivation of PU.1 in adult mice leads to the development of myeloid leukemia. *Proc Nat Acad Sci* 103: 1486-1491.

86. Anderson, M. K., G. Hernandez-Hoyos, C. J. Dionne, A. M. Arias, D. Chen, and E. V. Rothenberg. 2002. Definition of Regulatory Network Elements for T Cell Development by Perturbation Analysis with PU.1 and GATA-3. *Dev Biol* 246:103-121.

87. Anderson, M. K., A. H. Weiss, G. Hernandez-Hoyos, C. J. Dionne, and E. V. Rothenberg. 2002. Constitutive Expression of PU.1 in Fetal Hematopoietic Progenitors Blocks T Cell Development at the Pro-T Cell Stage. *Immunity* 16:285-296.

88. Rothenberg, E. V., and M. K. Anderson. 2002. Elements of Transcription Factor Network Design for T-Lineage Specification. *Dev Biol* 246:29-44.

89. Rothenberg, E. V. 2002. T-lineage specification and commitment: a gene regulation perspective. *Semin Immunol* 14:431-440.

90. Ting, C. N., M. C. Olson, K. P. Barton, and J. M. Leiden. 1996. Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature* 384:474-478.

91. Hattori, N., H. Kawamoto, S. Fujimoto, K. Kuno, and Y. Katsura. 1996. Involvement of transcription factors TCF-1 and GATA-3 in the initiation of the earliest step of T cell development in the thymus. *J Exp Med* 184:1137-1147.

92. Farrar, J. D., W. Ouyang, M. Lohning, M. Assenmacher, A. Radbruch, O. Kanagawa, and K. M. Murphy. 2001. An Instructive Component in T Helper Cell Type 2 (Th2) Development Mediated by GATA-3. *J Exp Med* 193:643-650.

93. Hendriks R.W., M.C. Nawijn , J.D. Engel, H. van Doorninck, F. Grosveld, and A. Karis. 1999. Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. *Eur J Immunol* 29:1912-1918.

94. Chang, H.-D., C. Helbig, L. Tykocinski, S. Kreher, J. Koeck, U. Niesner, and A. Radbruch. 2007. Expression of IL-10 in Th memory lymphocytes is conditional on IL-12 or IL-4, unless the IL-10 gene is imprinted by GATA-3 *Eur J Immunol.* 37:807-817.

95. Fitzgerald, D. C., G.-X. Zhang, M. El-Behi, Z. Fonseca-Kelly, H. Li, S. Yu, C. J. Saris, B. Gran, B. Ciric, and A. Rostami. 2007. Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. *Nat Immunol* 8:1372-1379.

96. Stumhofer, J. S., J. S. Silver, A. Laurence, P. M. Porrett, T. H. Harris, L. A. Turka, M. Ernst, C. J. M. Saris, J. J. O'Shea, and C. A. Hunter. 2007. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat Immunol* 8:1363-1371.

97. Awasthi, A., Y. Carrier, J. P. S. Peron, E. Bettelli, M. Kamanaka, R. A. Flavell, V. K. Kuchroo, M. Oukka, and H. L. Weiner. 2007. A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells. *Nat Immunol* 8:1380-1389.

98. Maynard, C. L., L. E. Harrington, K. M. Janowski, J. R. Oliver, C. L. Zindl, A. Y. Rudensky, and C. T. Weaver. 2007. Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10. *Nat Immunol* 8:931-941.

99. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.

100. D'Andrea A, Aste-Amezaga M, M. X. Valiante NM, Kubin M, and T. G. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* 178:1041-1048.

101. Ziegler-Heitbrock, L., M. Lotzerich, A. Schaefer, T. Werner, M. Frankenberger, and E. Benkhart. 2003. IFN-{alpha} Induces the Human IL-10 Gene by Recruiting Both IFN Regulatory Factor 1 and Stat3. *J Immunol* 171:285-290.

102. Kitani A., I. Fuss, K. Nakamura, F. Kumaki, T. Usui, and W. Strober. 2003. Transforming Growth Factor (TGF)-β1-producing Regulatory T Cells Induce Smad-mediated Interleukin 10 Secretion That Facilitates Coordinated Immunoregulatory Activity and Amelioration of TGF-β1-mediated Fibrosis. *J Exp Med* 198:1179-1188.

103. Wang, Z.-Y., H. Sato, S. Kusam, S. Sehra, L. M. Toney, and A. L. Dent. 2005. Regulation of IL-10 Gene Expression in Th2 Cells by Jun Proteins. *J Immunol* 174:2098-2105.

104. Tone, M., M. J. Powell, Y. Tone, S. A. J. Thompson, and H. Waldmann. 2000. IL-10 Gene Expression Is Controlled by the Transcription Factors Sp1 and Sp3. *J Immunol* 165:286-291.

105. Finbloom, D. S., and K. D. Winestock. 1995. IL-10 induces the tyrosine phosphorylation of tyk2 and Jak1 and the differential assembly of STAT1 alpha and STAT3 complexes in human T cells and monocytes. *J Immunol* 155:1079-1090.

106. Ding, Y., L. Qin, D. Zamarin, S. V. Kotenko, S. Pestka, K. W. Moore, and J. S. Bromberg. 2001. Differential IL-10R1 Expression Plays a Critical Role in IL-10-Mediated Immune Regulation. *J Immunol* 167:6884-6892.

107. Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 147:3815-3822.

108. Qin, H., C. Wilson, K. Roberts, B. Baker, X. Zhao, and E. N. Benveniste. 2006. IL-10 Inhibits Lipopolysaccharide-Induced CD40 Gene Expression through Induction of Suppressor of Cytokine Signaling-3. *J Immunol* 177:7761-7771.

109. Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 146:3444-3451.

110. Sher, A., D. Fiorentino, P. Caspar, E. Pearce, and T. Mosmann. 1991. Production of IL-10 by CD4+ T lymphocytes correlates with down-regulation of Th1 cytokine synthesis in helminth infection. *J Immunol* 147:2713-2716.

111. Zimmerman, M. A., L. L. Reznikov, C. D. Raeburn, and C. H. Selzman. 2004. Interleukin-10 attenuates the response to vascular injury. *J Surg Res* 121:206-213.

112. Uyttenhove, C., R. J. Simpson, and J. V. Snick. 1988. Functional and Structural Characterization of P40, a Mouse Glycoprotein with T-Cell Growth Factor Activity. *Proc Natl Acad Sci* 85:6934-6938.

113. Moeller, J., L. Hultner, E. Schmitt, M. Breuer, and P. Dormer. 1990. Purification of MEA, a mast cell growth-enhancing activity, to apparent homogeneity and its partial amino acid sequencing. *J Immunol* 144:4231-4234.

114. Hültner L., C. Druez, J. Moeller, C. Uyttenhove, E. Schmitt, E. Rüde, P. Dörmer, and J. Van Snick. 1990. Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). *Eur J Immunol* 20:1413-1416.

115. Yang, Y. C., S. Ricciardi, A. Ciarletta, J. Calvetti, K. Kelleher, and S. C. Clark. 1989. Expression cloning of cDNA encoding a novel human hematopoietic growth factor: human homologue of murine T-cell growth factor P40. *Blood* 74:1880-1884.

116. Suda, T., R. Murray, M. Fischer, T. Yokota, and A. Zlotnik. 1990. Tumor necrosis factor-alpha and P40 induce day 15 murine fetal thymocyte proliferation in combination with IL-2. *J Immunol* 144:1783-1787.

117. Williams, D. E., P. J. Morrissey, D. Y. Mochizuki, P. de Vries, D. Anderson, D. Cosman, H. S. Boswell, S. Cooper, K. H. Grabstein, and H. E. Broxmeyer. 1990. T-cell growth factor P40 promotes the proliferation of myeloid cell lines and enhances erythroid burst formation by normal murine bone marrow cells in vitro. *Blood* 76:906-911.

118. Donahue, R. E., Y. C. Yang, and S. C. Clark. 1990. Human P40 T-cell growth factor (interleukin-9) supports erythroid colony formation. *Blood* 75:2271-2275.

119. Renauld, J. C., A. Goethals, F. Houssiau, E. Van Roost, and J. V. Snick. 1990. Cloning and expression of a cDNA for the human homolog of mouse T cell and mast cell growth factor P40 *Cytokine* 2:9-12.

120. Temann, U.-A., P. Ray, and R. A. Flavell. 2002. Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. *J Clin Invest* 109: 29-39.

121. Louahed, J., M. Toda, J. Jen, Q. Hamid, J.-C. Renauld, R. C. Levitt, and N. C. Nicolaides. 2000. Interleukin-9 Upregulates Mucus Expression in the Airways. *Am. J. Respir. Cell Mol. Biol.* 22:649-656.

122. Renauld J.C., C. Druez, A. Kermouni, F. Houssiau, C. Uyttenhove, E. Van Roost, and J. V. Snick. 1992 Expression cloning of the murine and human interleukin 9 receptor cDNAs. *Proc Natl Acad Sci* 89:5690-5694.

123. Demoulin, J. B., C. Uyttenhove, E. Van Roost, B. DeLestre, D. Donckers, J. Van Snick, and J. C. Renauld. 1996. A single tyrosine of the interleukin-9 (IL-9) receptor is required for STAT activation, antiapoptotic activity, and growth regulation by IL-9. *Mol. Cell. Biol.* 16:4710-4716.

124. Demoulin, J.-B., E. Van Roost, M. Stevens, B. Groner, and J.-C. Renauld. 1999. Distinct Roles for STAT1, STAT3, and STAT5 in Differentiation Gene Induction and Apoptosis Inhibition by Interleukin-9. *J. Biol. Chem.* 274:25855-25861.

125. Matsuyama, T., A. Grossman, H. W. Mittrucker, D. P. Siderovski, F. Kiefer, T. Kawakami, C. D. Richardson, T. Taniguchi, S. K. Yoshinaga, and T. W. Mak. 1995. Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). *Nucleic Acid Res* 23:2127-2136.

126. Pongubala J.M., Nagulapalli S., Klemsz M.J., McKercher S.R., Maki R.A, and Atchison M.L. 1992. PU.1 recruits a second nuclear factor to a site important for immunoglobulin kappa 3' enhancer activity. *Mol cell Biol* 12:368-378.

127. Yamagata, T., J. Nishida, S. Tanaka, R. Sakai, K. Mitani, M. Yoshida, T. Taniguchi, Y. Yazaki, and H. Hirai. 1996. A novel interferon regulatory factor family transcription factor, ICSAT/Pip/LSIRF, that negatively regulates the activity of interferon- regulated genes. *Mol Cell Biol* 16:1283-1294.

128. Perkel, J. M., and M. L. Atchison. 1998. A Two-Step Mechanism for Recruitment of Pip by PU.1. *J Immunol* 160:241-252.

129. Escalante, C. R., L. Shen, M. C. Escalante, A. L. Brass, T. A. Edwards, H. Singh, and A. K. Aggarwal. 2002. Crystallization and characterization of PU.1/IRF-4/DNA ternary complex. *J Struct Biol* 139:55-59.

130. Escalante, C. R., A. L. Brass, J.M. Pongubala, E. Shatova, L. Shen, H. Singh, and A.K. Aggarwal. 2002. Crystal Structure of PU.1/IRF-4/DNA Ternary Complex. *Mol Cell* 10:1097-1105.

131. Eisenbeis, C. F., H. Singh, and U. Storb. 1995. Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator. *Genes Dev* 9:1377-1387.

132. Yee, A. A., P. Yin, D. P. Siderovski, T. W. Mak, D. W. Litchfield, and C. H. Arrowsmith. 1998. Cooperative interaction between the DNA-binding domains of PU.1 and IRF4. *J Mol Biol* 279:1075.

133. Pongubala J.M., C. Van Beveren, S. Nagulapalli, M.J. Klemsz, S.R. McKercher, R.A. Maki, and M.L. Atchison. 1993. Effect of PU.1 phosphorylation on interaction with NF-EM5 and transcriptional activation. *Science* 259:1622-1625.

134. Gross, P., A. A. Yee, C. H. Arrowsmith, and R. B. Macgregor. 1998. Quantitative Hydroxyl Radical Footprinting Reveals Cooperative Interactions between DNA-Binding Subdomains of PU.1 and IRF4. *Biochem* 37:9802-9811.

135. Mamane, Y., S. Sharma, L. Petropoulos, R. Lin, and J. Hiscott. 2000. Posttranslational regulation of IRF-4 activity by the immunophilin FKBP52. *Immunity* 12:129-140.

136. Lohoff, M., H.-W. Mittrücker, S. Prechtl, S. Bischof, F. Sommer, S. Kock, D. A. Ferrick, G. S. Duncan, A. Gessner, and T. W. Mak. 2002. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc Natl Acad Sci* 99:11808-11812.

137. Brustle, A., S. Heink, M. Huber, C. Rosenplanter, C. Stadelmann, P. Yu, E. Arpaia, T. W. Mak, T. Kamradt, and M. Lohoff. 2007. The development of inflammatory TH-17 cells requires interferon-regulatory factor 4. *Nat Immunol* 8:958-966.

138. Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J. M. Kim, T.-T. Chu, L. Corcoran, P. Treuting, U. Klein, and A. Y. Rudensky. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control TH2 responses. *Nature* Advance publication.

139. Honma, K., D. Kimura, N. Tominaga, M. Miyakoda, T. Matsuyama, and K. Yui. 2008. Interferon regulatory factor 4 differentially regulates the production of Th2 cytokines in naive vs. effector/memory CD4+ T cells. *Proc Natl Acad Sci* 105:15890-15895.

140. Robinson, S. D. 2000. Th-2 cytokines in allergic disease. *Br Med Bull* 56:956-968.

141. Finotto, S., M. F. Neurath, J. N. Glickman, S. Qin, H. A. Lehr, F. H. Y. Green, K. Ackerman, K. Haley, P. R. Galle, S. J. Szabo, J. M. Drazen, G. T. De Sanctis, and L. H. Glimcher. 2002. Development of Spontaneous Airway Changes Consistent with Human Asthma in Mice Lacking T-bet. *Science* 295:336-338.

142. Finotto, S., M. Hausding, A. Doganci, J. H. Maxeiner, H. A. Lehr, C. Luft, P. R. Galle, and L. H. Glimcher. 2005. Asthmatic changes in mice lacking T-bet are mediated by IL-13. *Int Immunol* 17:993-1007.

143. Iribarren, C., I. V. Tolstykh, and M. D. Eisner. 2004. Are patients with asthma at increased risk of coronary heart disease? *Int J Epidemiol* 33:743-748.

144. Chang, E. Y., B. Guo, S. E. Doyle, and G. Cheng. 2007. Cutting Edge: Involvement of the Type I IFN Production and Signaling Pathway in Lipopolysaccharide-Induced IL-10 Production. *J Immunol* 178:6705-6709.

145. Knolle PA., A. Uhrig, U. Protzer, M. Trippler, R. Duchmann, K-H. Meyer zum Büschenfelde, and G. Guido. 1998. Interleukin-10 expression is autoregulated at the transcriptional level in human and murine kupffer cells. *Hepatology* 27:93-99.

146. Grant, L. R., Z. J. Yao, C. M. Hedrich, F. Wang, A. Moorthy, K. Wilson, D. Ranatunga, and J. H. Bream. 2008. Stat4-dependent, T-bet-independent regulation of IL-10 in NK cells. *Genes Immun* 9:316-327.

147. Holz, L., K. Jakobsen, J. Van Snick, F. Cormont, and W. Sewell. 2005. Dexamethasone inhibits IL-9 production by human T cells. *J Inflamm (Lond)* 2:3.

148. Rekhtman, N., F. Radparvar, T. Evans, and A. I. Skoultchi. 1999. Direct interaction of hematopoietic transcription factors PU.1 and GATA-1: functional antagonism in erythroid cells. *Genes Dev* 13:1398-1411.

149. McKercher, S. R., C. R. Lombardo, A. Bobkov, X. Jia, and N. Assa-Munt. 2003. Identification of a PU.1-IRF4 protein interaction surface predicted by chemical exchange line broadening. *Proc Natl Acad Sci* 100:511-516.

150. Escalante C.R., Brass A. L., Pongubala J.M. R., Shatova E., Shen L., Singh H., and A. A. K. 2002. Crystal Structure of PU.1/IRF-4/DNA Ternary Complex. *Molecular cell* 10:1097-1105.

151. Baguet, A., and M. Bix. 2004. Chromatin landscape dynamics of the Il4-Il13 locus during T helper 1 and 2 development. *Proc Natl Acad Sci* 101:11410-11415.

152. Avni, O., D. Lee, F. Macian, S. J. Szabo, L. H. Glimcher, and A. Rao. 2002. Th cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nat Immunol* 3:643-651.

153. Huber, M., A. Brustle, K. Reinhard, A. Guralnik, G. Walter, A. Mahiny, E. von Low, and M. Lohoff. 2008. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc Natl Acad Sci* 105:20846-20851.

154. Ranganath, S., W. Ouyang, D. Bhattarcharya, W. C. Sha, A. Grupe, G. Peltz, and K. M. Murphy. 1998. Cutting Edge: GATA-3-Dependent Enhancer Activity in IL-4 Gene Regulation. *J Immunol* 161:3822-3826.

155. Kishikawa, H., J. Sun, A. Choi, S.-C. Miaw, and I. C. Ho. 2001. The Cell Type-Specific Expression of the Murine IL-13 Gene Is Regulated by GATA-3. *J Immunol* 167:4414-4420.

156. Brightbill, H. D., S. E. Plevy, R. L. Modlin, and S. T. Smale. 2000. A Prominent Role for Sp1 During Lipopolysaccharide- Mediated Induction of the IL-10 Promoter in Macrophages. *J Immunol* 164:1940-1951.

157. Lohning, M., A. Richter, T. Stamm, J. Hu-Li, M. Assenmacher, W. E. Paul, and A. Radbruch. 2003. Establishment of memory for IL-10 expression in developing T helper 2 cells requires repetitive IL-4 costimulation and does not impair proliferation. *Proc Natl Acad Sci* 100:12307-12312.

158. Gerosa, F., C. Paganin, D. Peritt, F. Paiola, M. T. Scupoli, M. Aste-Amezaga, I. Frank, and G. Trinchieri. 1996. Interleukin-12 primes human CD4 and CD8 T cell clones for high production of both interferon-gamma and interleukin-10. *J Exp Med* 183:2559-2569.

159. Mo, C., W. Chearwae, J. T. O'Malley, S. M. Adams, S. Kanakasabai, C. C. Walline, G. L. Stritesky, S. R. Good, N. B. Perumal, M. H. Kaplan, and J. J. Bright. 2008. Stat4 Isoforms Differentially Regulate Inflammation and Demyelination in Experimental Allergic Encephalomyelitis. *J Immunol* 181:5681-5690.

160. Grenningloh, R., B. Y. Kang, and I. C. Ho. 2005. Ets-1, a functional cofactor of T-bet, is essential for Th1 inflammatory responses. *J. Exp. Med.* 201:615-626.

161. van der Stoep, N., E. Quinten, M. M. Rezende, and P. J. van den Elsen. 2004. E47, IRF-4, and PU.1 synergize to induce B-cell-specific activation of the class II transactivator promoter III (CIITA-PIII). *Blood* 104:2849-2857.

CURRICULUM VITAE

Ayélé-Nati Ahyi

Education

2009	**Ph.D.** Department of Microbiology and Immunology, Indiana University, Indianapolis, IN
2004	**M.S.** Molecular Pharmacology and Medicinal Chemistry, Purdue University, Lafayette, IN
1998	**M.S.** (non-thesis) Life Sciences: Université de Lomé, Togo
1997	**B.S.** Biology: Université de Lomé, Togo

Honors, Awards and Fellowships

2006 IUPUI travel fellowship award

Abstracts Presented and Conferences Attended

2008 American Association of Immunologists (AAI) Annual Meeting. San Diego, CA. Ahyi AN, Chang H-C and Kaplan MH “IRF4 regulates the expression of specific Th2 cytokines”.

2007 Midwest Autumn Immunology Conference. Chicago, IL. Ahyi AN, Chang H-C and Kaplan MH “IRF4 regulates the expression of specific Th2 cytokines”.

2007 American Association of Immunologists (AAI) Annual Meeting. Miami, FL. AN Ahyi, HC Chang and MH Kaplan. IRF4 Expression Defines Th2 Populations Secreting High Levels of IL-10.

2006 Midwest Autumn Immunology Conference. Chicago, Ahyi AN, Chang H-C and Kaplan MH “IRF4 regulates the expression of specific Th2 cytokines” IL.

Publications

1. Interferon Regulatory Factor (IRF)-4 regulates the expression of specific Th2 cytokines. **Ayele-Nati N. Ahyi**, Hua-Chen Chang, Stephen L. Nutt and Mark H. Kaplan. (resubmitted).

2. Transcription factor-dependent chromatin remodeling of Il18r1 during Th1 and Th2 differentiation. Qing Yu, Hua-Chen Chang, Ayele-Nati N. Ahyi and Mark H. Kaplan, ***The Journal of Immunology,*** **181(5):3346-3352.**

3. IL-4 Is a Critical Determinant in the Generation of Allergic Inflammation Initiated by a Constitutively Active Stat6. Sarita Sehra, Heather A. Bruns, Ayele-Nati N. Ahyi, Evelyn T. Nguyen, Nathan W. Schmidt, E. Grace Michels, Götz-Ulrich von Bülow, and Mark H. Kaplan, ***The Journal of Immunology,*** **2008, 180(5):3551–3559.**

4. Bruton's Tyrosine Kinase Is Required for TLR-Induced IL-10 Production. Nathan W. Schmidt, Vivian T. Thieu, Brandon A. Mann, Ayele-Nati N. Ahyi, and Mark H. Kaplan, ***The Journal of Immunology,*** **2006, 177:7203–7210.**