Molecular mechanisms for the regulation of the *Il-10* gene in CD4 T cells : comparison of IL-10-T_{reg} and CD25+T_{reg}.

Thesis

Molecular mechanisms for the regulation of the *Il-10* gene in CD4 T cells: comparison of IL-10-T_{reg} and CD25+T_{reg}

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Abstract

This thesis outlines and provides direct evidence for transcription factor-mediated instructive regulation of the *Il-10* gene in CD4 T cells. Firstly, I describe transcription factors that are differentially expressed in populations of IL-10-producing CD4 T cells. Genome-wide mRNA profiles (GeneChip) are used from six populations of CD4 T populations which represent both temporal and lineage-specific time-points within naïve, effector and regulatory CD4 T cell populations. Secondly, validation experiments (qPCR) verified whether transcription factors isolated from the genome-wide profiling experiments (GeneChip) were quantitatively expressed in IL-10-producing populations. Thirdly, comparative genomics was used to analyze *in silico* the *Il-10* locus for conserved putative transcription factor binding sites at potential regulatory regions. Selected transcription factors were retro-virally transduced into primary naïve CD4 T cells and analyzed for their ability to induce IL-10 expression in a variety of differentiation conditions. Finally, the role of functionally validated transcription factors inducing IL-10 production was investigated at the molecular level, via their presence at specific locations at the *Il-10* locus *in vivo* and in chromatin modifications. One example, GATA-3, shown here to be differentially expressed in IL-10-producing CD4 T cell populations, instructing modifications at the *Il-10* locus and inducing IL-10 production in CD4 T cells.

The second aim of this thesis was to investigate -- at the transcription factor-level -- the comparison of two phenotypically different CD4 regulatory T cell populations ($CD25+T_{reg}$ and $IL\text{-}10\text{-}T_{reg}$). On one hand, both CD4 T regulatory cell populations are similar in that they do not produce effector upon secondary stimuli, both are anergic, and

both suppress CD4 T cell proliferation *in vitro* and *in vivo* (as shown by their ability to abrogate multiple autoimmune and allergic disease animal models). On the other hand, CD25+T_{reg} are phenotypically described by their expression of the lineage-specific transcription factor, FoxP3, and IL-10-T_{reg} do not express FoxP3. Therefore, the molecular mechanisms as to how IL-10-T_{reg} do not express effector cytokines, are anergic and suppress CD4 T cell proliferation *in vitro* and *in vivo* without the expression of FoxP3 is investigated in this thesis. This thesis concludes by providing strong evidence that IL-10-T_{reg} down-regulate key NF-κB and AP-1 family transcripts and inhibit NFAT transcriptional activity, thereby compensating for FoxP3 expression, which is necessary in natural occurring CD25+T_{reg}.

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INTRODUCTION

1. Perspective

The host immune response is competitively selected for mechanisms to protect the host from a variety of potential invading pathogens and intrinsic cellular defects. During evolution, as multi-cellular organisms developed from land-borne to migratory species the pressure from new pathogenic microorganisms increased, and therefore the host immune system played an increasing complex role in protecting species from new infectious threats. Today, the human immune system is a highly developed, complex mix of specialized cells, which serve multi-faceted functions in multi-cellular compartments throughout the host. As a continuously adapting organ, the immune system remains a vital driver for human survival in respect to eliminating or co-existing with an array of old and new infectious pathogens from a constantly changing natural environment.

Not surprisingly, evolution likely selected individuals that not only developed vigilant immune responses which aggressively protected the host, but also individuals who mounted balanced immune responses that reduced excessive or pathogenic self-destruction. Examples of extreme cases of these situations are immunodeficiency on the one hand (e.g., susceptibility to *Mycobacterium tuberculosis* (MTb)) or autoimmune and inflammatory pathologies on the other hand (e.g., inflammatory bowel disease (IBD), allergy or atopic reactions) (1). In order for the immune response to find the appropriate balance during an immunogenic challenge a system of negative feedback mechanisms are hardwired into the host immune response that co-coordinately lower the level of pro-inflammatory forces at the peak of immune activity. One key anti-inflammatory molecule that plays a central role in balancing the immune response is the

immunosuppressive cytokine, IL-10. IL-10 acts to lower the sensitivity of the immune response directly at the interface between the innate and adaptive arms of the host immune response. The molecular mechanism in which this crucial immunomodulatory cytokine is regulated at the molecular level remains an important immunological question today and will be the focus of this thesis.

1.2 The immune response-an overview

The immune system defends the host against infection. The immune response is orchestrated by two main mechanisms: innate immunity and adaptive immunity (2). Innate immunity is the first line of defense against infectious pathogens where initiation of cellular and molecular mechanisms occurs within minutes of pathogen recognition (2). The innate response is initiated by antigen presenting cells (APCs), which distinguish infectious structures through germ-line encoded receptors, generally termed pattern recognition receptors (PRRs) (2). Upon activation, PRRs on APCs will undergo phagocytosis and/or signal via distinct intrinsic networks to trigger the production of pro- or anti-inflammatory cytokines and up-regulation of co-stimulatory molecules to activate and initiate the adaptive immune response (2). The adaptive immune response (> 96 hours after initial antigen recognition) is dependent on the innate response to present specific signals that guide the differentiation of the subsequent antigen-specific adaptive response (2). A pool of naïve T and B cells is located throughout the host, but mainly in primary and secondary lymph nodes that serve as the reservoir of cells which rapidly expand into the adaptive immune response. A CD4 T cell adaptive response can be achieved when antigen-specific naïve CD4 T cells differentiate into antigen-specific CD4

T helper (Th) cells (3), which assist or "help" other immune cells presenting similar antigen-specific motifs during the course of an immune response and secondly are able to secrete pro- or anti-inflammatory effector molecules (4). Molecules released by differentiated T cells act at local sites of secretion or systemically throughout the host to maintain the immune response (5).

1.2.1 Adaptive immune response: CD4 T cells

CD4 and CD8 T cells comprise the T cell mediated arm of the adaptive immune response. Naïve T cells circulate throughout the body and can be "primed" to clonally expand and mount an adaptive immune response via presentation of specific antigen and co-stimulatory molecules from an APC. This antigen specific "priming" occurs when naïve CD8 T cells recognize and clonally expand after APCs present specific antigen and major histocompatibility complex (MHC) class I molecules, while CD4 T cells are triggered via specific APCs (Dendritic Cell (DC)) expressing antigen and MHC class II molecules (6). Subsequent factors can further influence the expansion of recently primed naïve CD4 T cells which include the cytokine microenvironment, the avidity for the initial antigen:T cell receptor (TCR) "triggering" and the strength of co-stimulatory molecules provided by the DC (6). The rapid expansion of clonally antigen-specific T cells provides a key mechanism in the adaptive immune response.

CD4 T cells have been shown to be required for the host response to an array of pathogens. For example, an antigen-specific CD4 T cell response is necessary for the control or clearance of various bacterial, viral and intra- and extra-cellular parasitic

pathogens by the host. A CD4 T cell response is not only required for immediate cell-mediated immunity, but a small population of previously expanded CD4 T cells are long-lived in the host and necessary for immunological recall or a "memory" response witnessed during rapid re-activation of the immune response by subsequent exposure to a previous pathogen. The memory response is common to all adaptive arms of the immune response and commonly clinically targeted as a necessary feature in developing an effective therapeutic vaccine (7). In summary, the molecular mechanism in which naïve CD4 T cells clonally expand from a common naïve precursor cell to a phenotypic effector and/or memory population, distinguished by their ability to recognize specific antigens and produce large quantities of specific molecules, is essential to their dynamic ability within the immune response (8). Figure 1 illustrates a simplistic-view of an immune response initiating and receiving "help" or maintenance from a CD4 Th population.

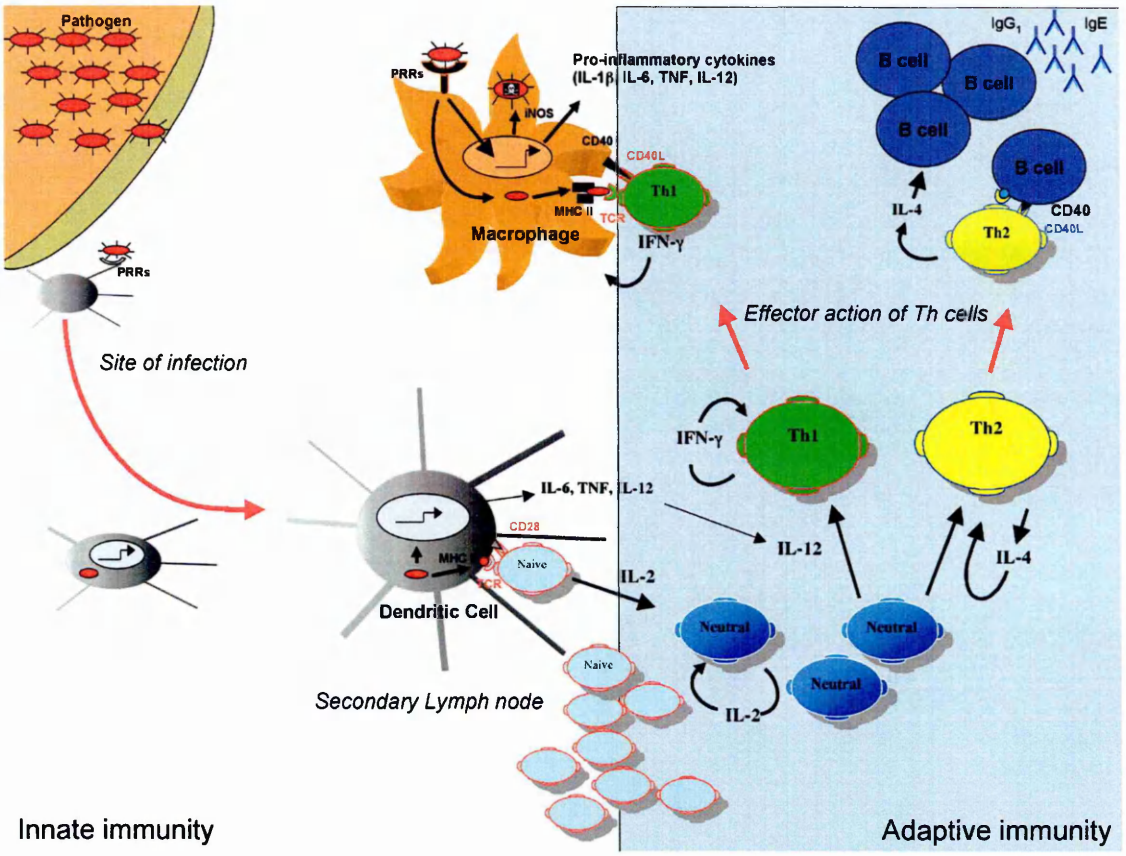


Figure 1.1. Initiation, generation and effector mechanism achieved via two separate lineage of CD4 Th cell during an immune response: Th1 and Th2.
A simplified cartoon of the initiation, generation and effector mechanism realized through a Th1 or Th2 immune response.

1.3 IL-10

An effective immune response is sometimes accompanied by host collateral damage elicited by the strong reaction to control infection. In order to minimize host damage, the activation of the anti-inflammatory networks within the course of an immune response is necessary. To accomplish this central role, the immune response must find the appropriate balance to coordinately fight infectious pathogens while preventing self-induced immune pathology. Here in this essential "niche" is where the immunosuppressive cytokine IL-10, plays a key role in modulating immune homeostasis

during an immune response, ensuring the immune response is able to eradicate pathogens while achieving minimum self-induced damage to the host.

1.3.1 Immune cells and conditions in which IL-10 is produced

To understand the role of IL-10 in the immune response, it is necessary to consider both the cell-types and biological conditions whereby IL-10 is produced. In immune cells, major producers of IL-10 are effector or regulatory CD4 T cells (discussed more later) (8, 9). However, other immune cells produce IL-10 including: Natural Killer (NK) T cells, B cells, mast cells, DCs, and macrophages (however not to the quantitative level of IL-10-producing CD4 T cells) and collectively it seems necessary that multiple cell types are able to produce IL-10 in the course of an infection (9). This further illustrates the regulation of IL-10 within various immune cell-types and temporal layers within the course of an immune response. For example, under certain conditions B cell-derived IL-10 has been shown to inhibit pathology in the Th1-driven autoimmune disease, experimental autoimmune encephalomyelitis (EAE), demonstrating the non-redundant role of B cell-derived IL-10 (10). In contrast, CD4 T cell-derived IL-10 has been well-described to selectively protect the host against uncontrolled inflammatory responses, particularly with respect to the gastrointestinal tract (9) (discussed more below). Accordingly, IL-10 is produced in different quantitative levels by a variety of immune cells depending on multiple variables including: host area of infection, level of infection, and temporal stages during the immune response.

Within CD4 T cell populations, IL-10 is produced by effector T helper and regulatory T (T_{reg}) cells (8). Within effector populations, Th2 cells produce large amounts of IL-10, whereas a Th1 cell can produce low amounts of IL-10 (9). In addition, CD4 T_{reg} can produce IL-10 (8). Two types of CD4 T_{reg} are defined to date, IL-10–producing CD4 T_{reg} (IL-10-T_{reg}) and natural occurring CD25+T_{reg}. Both function to regulate immune responses and inflammatory pathology (discussed more below) (11). Importantly, both these CD4 T_{reg} populations produce IL-10 and little to no effector cytokines, whereas the effector populations produce IL-10 in combination with other pro-inflammatory cytokines, which direct maintenance of the immune response (8). In summary, the CD4 T cells compartment is a major producer of IL-10 and will serve as the cell-type for the study of *Il-10* gene regulation in this thesis.

1.3.2 IL-10 mediated function

How does IL-10 regulate the immune response? IL-10 potently inhibits the innate immune response from activating a strong immune response via modulating the inflammatory signals produced by DCs (12), macrophages (13) and monocytes (9) (Figure 2). Specifically, IL-10 directly limits the innate immune response to promote killing upon antigen recognition, and further inhibits the adaptive immune response via down-regulation of: key co-stimulatory receptors CD80 and CD86, pro-inflammatory cytokines, MHC presentation directed by DCs, macrophages and monocytes (9). Intrinsically, IL-10 mediates signals within APCs to limit cytokine production and action (9). One mechanism is via IL-10 inhibition of the transcriptional activity of Nuclear Factor (NF)-kappaB (κB) complexes, which selectively activate target expression of key

pro-inflammatory cytokines (IL-1β, IL-6, IL-12, TNF) (14, 15). Furthermore, IL-10-mediated signaling in APCs directly effects STAT signaling, as IL-10 induces suppressor of cytokine synthesis SOCS-3, which act to attenuate pro-inflammatory cytokine signaling (16, 17). IL-10 directly down-regulates intrinsic networks within APCs and inhibits the release of inflammatory molecules with important consequences for their ability to activate and sustain the immune response.

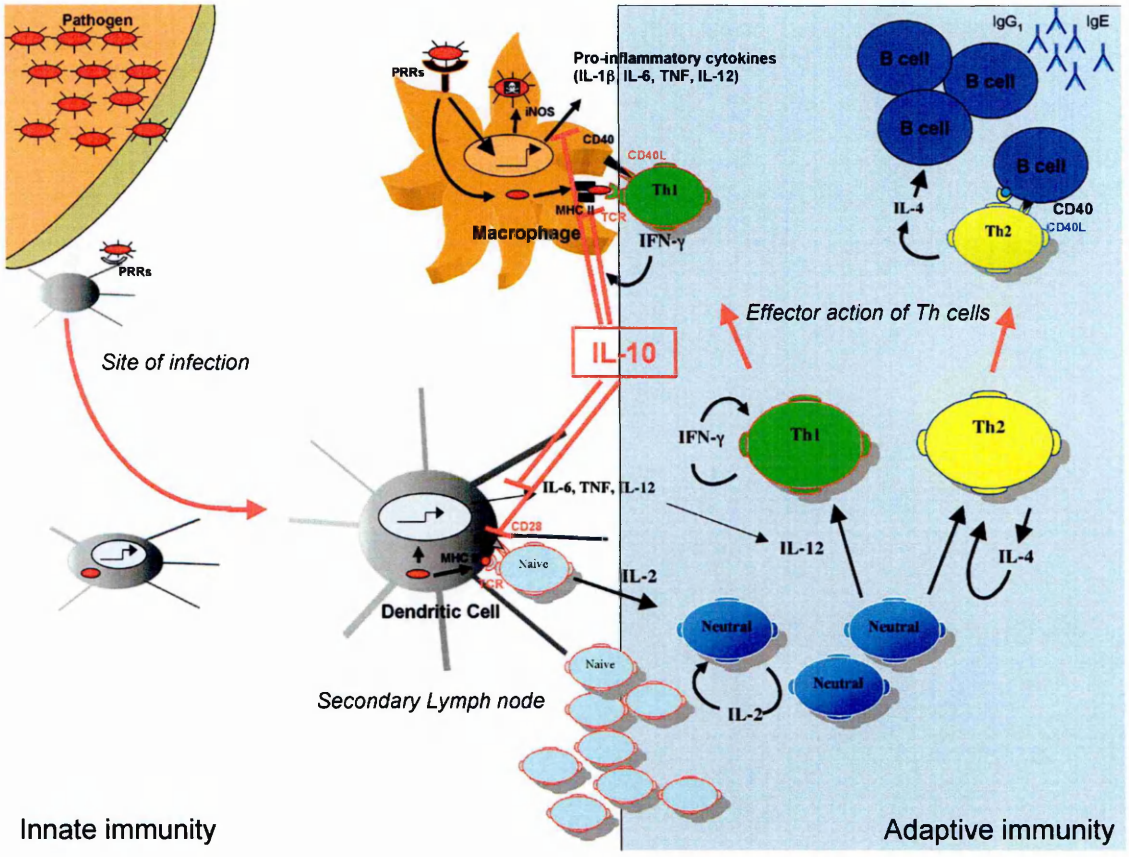


Figure 1.2. IL-10 inhibits the immune response.
This figure represents an adaptation on Figure 1.1, showing explicitly where IL-10 directly acts to inhibit the immune response.

Additional clues to the role of IL-10 in the balance of the immune response are seen with IL-10-deficient mice, IL-10(-/-), which were reported to spontaneously develop a severe Th1-driven pathology in the gut, chronic enterocolitis (IBD) (18, 19). Interestingly, the

spontaneous inflammatory bowel phenotype was attenuated when IL-10(-/-) mice were housed under germ-free conditions (20). This suggested that the IL-10(-/-) IBD phenotype was dependent on the generation of an immune response to enteric bacteria and consequently with the loss of the immunosuppressive IL-10 a subsequent Th1-mediated uncontrolled immune response is elicited. This scenario was classically shown with protection of colitis in IL-10(-/-) animal models with administration at early time-points and continued treatment of recombinant IL-10 (rIL-10); however colitis developed after treatment stopped, suggesting that IL-10 down-regulates the activation and regulation of the immune response at the innate and adaptive interface (14, 21). Another key study demonstrated that IL-10 plays a non-redundant role in CD4 T_{reg} suppressive ability to control colitis, as co-transfer of CD4 T cells and IL-10-deficient $CD45RB^{low}$, which contain $CD25+T_{reg}$ and other CD4 T_{reg} and effector cells, or treatment of monoclonal IL-10 antibodies were unable to regulate intestinal inflammation in immunodeficient hosts; however co-transfer CD4 T cells and WT $CD25+T_{reg}$ were able to regulate pathology, independently of IL-10 (22). This group further demonstrated that in the IBD model, IL-10 was essential for $CD25+T_{reg}$ mediated suppression of "antigen-experienced" (effector or memory) CD4 T cells, but not required for $CD25+T_{reg}$ suppression of naïve CD4 T cells (23). Finally, in a somewhat similar designed study, the adoptive transfer of $CD25+T_{reg}$ was shown to inhibit both the innate and T cell-mediated intestinal inflammation, and critically the innate immune mechanisms were suppressed in part due to T cell-derived IL-10 (discussed in greater detail below) (24). IL-10 has been shown to protect the host against pathology induced by strong pro-inflammatory responses with experimental group B streptococcal arthritis (25) and

preventing lethal endotoxic shock (26). The experimental group B streptococcal arthritis study clearly described that the neutralization of endogenous IL-10 by administration of αIL-10 at the time of infection resulted in mortality associated with early sustained production of pro-inflammatory cytokines: IL-6, IL-1β and TNF (25). IL-10 has also been shown to limit pro-inflammatory collateral damage in other animal models including: pancreatitis, diabetes mellitus, allergy, asthma, and experimental endotoxemia (14, 27).

1.3.3 Clinical perspective

IL-10 regulation (over and under production) with respect to clinical applications is currently of great interest (14, 27). Dysregulated expression levels of IL-10 are implicated in numerous diseases including: autoimmune disorders, AIDS progression, advanced alcoholic liver disease, Epstein-Barr virus infection, gastric cancer, cervical cancer, multiple myeloma, cutaneous malignant melanoma, and resistance to anti-viral therapy (14). Alternatively, activating the anti-inflammatory activities of IL-10 have been attempted by clinicians through administration of human rIL-10 to patients with Crohn's disease, rheumatoid arthritis, psoriasis, hepatitis C infection, and patients undergoing organ transplantation (14). IL-10 administration has not been successful for IBD and autoimmune diseases, likely due to: short half-life, delivery to the wrong place, or treatment at the wrong time. A better approach is to find ways of activating production of IL-10, particularly from antigen-specific IL-10-producing T cells. For example, immunosuppressive drugs such as the glucocorticoid receptor agonist, dexamethosone, are known to induce IL-10 production from immune cells (28-30) and

remain a widely prescribed drug for dampening pro-inflammatory immune responses. In summary, over-production of IL-10 can cause pathophysiological conditions seen primarily via a suppressed immune response and conversely a deficiency in IL-10 production can cause a hyper-immune response inducing pathology or excessive collateral damage during infection. The neutralization or induction of IL-10 provides evidence that this cytokine could serve as a promising clinical target.

IL-10 administration or neutralization with respect to tumor therapy is another area of intense study (14). The results from this area of therapy are far from straight forward. Early cancer model studies described IL-10 to be a tumor-promoting molecule. Direct effects of IL-10 enhancement of tumor survival were shown through IL-10-induced down-regulation of MHC class I presentation on tumor cells, rendering them resistant to CD8 cytotoxic T cells (CTL)-mediated lysis (31). Transgenic mice expressing IL-10 under control of the IL-2 promoter were described to be unable to limit the growth of immunogenic tumors and the administration of αIL-10 restored the ability for the immune response to control tumor growth (32). Finally, the use of αIL-10 in parallel with TCR ligation was shown to promote an anti-tumor response, probably by activation of tumor antigen-specific T cells (33). Alternatively several more recent studies have shown IL-10 to have a negative overall effect on tumor growth or survival, albeit a clear mechanism is not understood. Briefly, IL-10 tumor suppressing effect has been shown via at least three mechanisms (1) IL-10 directly inhibiting tumor-associated macrophages (TAMs) from angiogenesis via down-regulated expression of vascular endothelial growth factor (VEGF) (34, 35), (2) IL-10-mediated anti-metastatic activity via IL-10 activated

NK cells (36) and (3) more generally from IL-10 anti-inflammatory effect on the immune response, specifically via its down-regulation of NF-κB transcriptional activity in APCs, which in turn, is considered to be tumor suppressing feature (15, 37). In conclusion, depending on the experimental model and probably the underlying differences between the models that are not fully appreciated, IL-10 effect seems to inhibit survival and growth of tumors, although certain conditions exist where it is considered a tumor-promoting cytokine as a result from its pleiotropic activities.

Taken together, IL-10 is a complex cytokine predominately inducing anti-inflammatory signals at different layers throughout the immune response and this effect can be dependent on multiple factors during the immune response. The role of IL-10 illustrates the challenge faced by the host immune system to respond to infectious pathogens while minimizing host collateral damage. From the clinical perspective the relevant questions for IL-10 being: (1) can specific IL-10-producing cell-types be targeted and (2) more globally, due to the multi-faceted role of IL-10 function in respect to specific cells, is it inherently too diverse a molecule to target therapeutically?

To address these questions we will review the functional and molecular mechanisms regulating various CD4 T cell populations (major IL-10 producer) and current studies reported with respect to *Il-10* gene regulation.

1.4 Function of CD4 T helper populations

CD4 Th populations are necessary in mediating the development of the host-protective response and are divided into at least two populations termed: Th1 and Th2 (3, 5). Functionally, a Th1 cell response is essential in the host cell-mediated protection to intracellular pathogens including viruses, parasites and bacteria; whereas a Th2 cell response (classified by high IgG_1 and IgE antibody isotypes) can instruct a humoral-mediated response to eradicate nematodes, helminthes and other extra-cellular pathogens (4, 5, 38, 39). Both Th1 and Th2 responses can initiate the humoral immune response by activating naïve B cells to produce IgM antibodies (4), and Th1 cells can instruct the production of opsonizing antibodies IgG2a (4, 38). The effector action of a Th1 response is primarily achieved through the activation of infected macrophages, whereas a Th2 response is primarily responsible for activation and maintenance of mast cells, eosinophils and B cell growth factors (some of which is outlined in Figure 1) (4). The mechanisms by which Th populations achieve these describe effector actions are through the ligand/receptor interactions with APCs and short or long-range delivery of differential profiles of immunological proteins, of which, one key group is cytokines (Figure 1) (5). The molecular basis for the differentiation of a common naïve precursor cell to distinct effector CD4 T cell populations able to produce distinct signatures of cytokines (which can be measured in serological samples) is the hallmark of the CD4 T cell-mediated adaptive immune response (40).

1.4.1 Th1

Th1 cells differentiate to produce high amounts of the type II interferon, IFN-γ, upon secondary TCR stimulation (5). In the course of an infection, IFN-γ is initially produced at high levels by NK cells and as the adaptive response develops IFN-γ production also becomes a T cell effector-mediated cytokine (38). Th1 cells, as well as CTLs, maintain IFN-γ production that drive an activated immune response towards intracellular and some extra-cellular pathogens (5, 38). IFN-γ is a key cytokine playing a central role in the induction of a cell-autonomous, microbicidal state in macrophages against intracellular pathogens and more generally for APCs to process antigen and co-currently present co-stimulatory molecules (5, 41). Triggered CD4 T cells canonically consume IFN-γ, which intrinsically signal and regulate Th1 differentiation through directly up-regulating key Th1 specific transcription factors and cross-regulating Th2 associated transcription factors (42) (discussed more below). Together, IFN-γ producing Th1 CD4 T cells ensure and stabilize an efficient cell-mediated immune response (5, 41). A lack of an efficient CD4-mediated response is seen in human patients with depleted CD4 T cells (for example: AIDS) where a common cause of death is caused by the opportunistic resident-microorganism *Pneumocytis carinii* and MTb which is a major reason they succumb to death; due in part to the lack of effector CD4 response in supporting macrophage activation (43, 44). It is important to note that if uncontrolled, a Th1-mediated immune response can also induce unnecessary host pathology and initiation of certain autoimmune diseases (41). For instance, IBD stems from the establishment of an uncontrolled CD4-driven immune response attacking host-resident microbial flora interactions and causing unwanted host-pathology (41, 45). In summary, a Th1-driven

immune response is essential for the clearance or control of various intracellular and extra-cellular pathogens; however if the Th1 response is not properly regulated it can cause serious undue host pathology.

1.4.2 Th2

A Th2 response is required for control and or clearance of infection with certain extra-cellular pathogens (nematodes and helminthes parasites) not generally recognized by known innate PRRs (5, 46). Th2 cells differentiate to produce high amounts of the signature cytokine IL-4 (as well as IL-5 and IL-13) upon secondary TCR triggering (5). IL-4 is a pleiotropic immunomodulatory cytokine produced by Th2 lymphocytes, mast cells and eosinophils (38). Functionally, IL-4-producing Th2 cells act to inhibit macrophages while activating B cells and mast cell growth (38). IL-4 instructs subsets of DCs to produce IL-12 (a key Th1-driving cytokine in early naïve CD4 T cell differentiation) via IL-4-direct intrinsic ability to down-regulate IL-10 in DCs through a STAT6 mechanism (47). This may explain why paradoxically, IL-4 acts on DCs to prime CD4 T cells towards a Th1 cell lineage (47). A Th2 response is defined by high IL-4, IL-5 and IL-13 cytokine levels and is necessary for eradication of certain extra-cellular pathogens.

Th2 differentiation from a naïve CD4 T cell is dependent on early IL-2 mediated intrinsic signaling initially stabilizing the *Il-4* locus for low-level CD4 T cell IL-4 production which becomes under autocrine control via the IL-4R signaling network (48). In the initial stages of infection low-level IL-4 production by recently primed CD4 T cells and

non-T cells, including eosinophils and basophils (38) (especially seen in the absence of IFN-γ and IL-12/IL-18) can allow Th2 development. Similar to the CD4 Th1-mediated intrinsic signaling the IL-4R triggered network cross-regulates recently triggered CD4 cells to inhibit Th1-associated receptors and transcription factors (4, 5, 49). Several *in vivo* mouse models have shown the essential and non-redundant role of IL-4-producing Th2 cells in the host protection against extra-cellular intestinal parasites (specifically: nematode and helminthes) (46). The inability of the host immune system to recognize and control extra-cellular parasites (especially nematode varieties) is suggested to have led to the evolution of a necessary effector mechanism (Th2 response) to protect against most intestinal nematode parasites (46). However, uncontrolled Th2-associated cytokine production (IL-4, IL-5, IL-13) can drive allergic inflammation and atopic reactions in the host (50, 51). If uncontrolled Th2-associated cytokines cause undue pathology via mechanisms including: IL-4 activates allergen-specific Th2-lineage cell differentiation, IL-5 promotes eosinophil infiltration, and IL-13 plays a central role hyper-secretion of mucus and regulation of AHR (51). Therefore, the combination or individual dysregulation of either of these potent cytokines within CD4 T cells can trigger long-term disease (allergic rhinitis, asthma, and atopic dermatitis) or death (anaphylaxis). It is likely that the host needs a combination of a Th1 and Th2 response for a balanced immune response.

1.5 Molecular mechanisms in effector cytokine gene regulation

Cytokine gene regulation with respect to IFN-γ and IL-4 expression has been extensively studied to determine the molecular mechanism behind the ability of common naïve CD4

T cell precursors to differentially produce these key molecules in either Th1 or Th2 cells, respectively. The early moments after naïve CD4 T cell "triggering" in the periphery are critical to initiating early intrinsic Th1 versus Th2 differentiation (52). The avidity of the TCR to the antigen/MHC class II complex, the nature of co-stimulatory molecules, and the cytokine microenvironment all play a key role in subsequent differentiation of a naïve CD4 T cells into a proficient effector population (3, 5). Extrinsically, the presence of IL-12 and IL-18 on recently primed naïve CD4 T cells drives differentiation towards a Th1 population, whereas early IL-2 and subsequent IL-4 will drive Th2 differentiation (5, 41). After, the initial ubiquitous burst of IFN-γ and IL-4 expression in recently primed naïve CD4 T cells, the balance of *Ifn-γ* and *Il-4* gene regulation becomes highly cross-regulated at the level of chromatin, transcription factor milieu and cytokine receptor networks (52). This is not surprising considering their divergent effects of these powerful cytokines in the context of the host immune response.

Effector cytokine locus control can be conveniently divided into two mechanisms: epigenetic chromatin re-modeling and the up-regulation of specific transcription factors - or ubiquitous transcription factors - that bind to DNA regulatory regions of the re-modeled locus to enhance or repress transcription (53). Naïve CD4 T cells exhibit heterochromatic or condensed chromatin structure at both effector cytokine, *Ifn-γ* and *Il-4*, loci and therefore are inaccessible to nuclear factors (54) in these cells. "Triggering" naïve CD4 T cells, can induce epigenetic, chromatin re-modeling (54) and induction of cycling covalent modifications of histone tails (suggested to indicate "active" or "silenced" regulatory regions), which are best currently described through acetylation,

methylation, and phosphorlylation at the targeted locus region (55, 56). Chromatin re-modeling is directly induced by the presence of transcription factors at specific DNA-binding regions near or at long-range sites from the target gene locus (52, 54). The location of these potential regulatory regions can be observed via assays involving the study of DNase I Hypersensitive Sites (HSS) within the targeted (probed) genomic region (53). DNase I HSS studies were used to identify both *Ifn-γ* and *Il-4* gene regulatory regions for further study (53, 57, 58). Secondly, a specific group of transcription factors can home to the exposed or re-modeled locus to enhance or repress gene expression (54). These transcription factors can themselves be up-regulated through similar epigenetic differentiation through extra-cellular signals and further reinforced through autocrine or paracrine signaling (54). Key transcription factors act to further stabilize cytokine gene euchromatic architecture and consequently further exposing regulatory DNA binding regions for the rapid gene expression upon sequestration of nuclear factors after secondary TCR stimulus (54). Other key types of CD4 transcription factors are present in the cytosol and activated via TCR-mediated activation thereby translocating or shuttling to the nucleus to act at exposed or euchromatic DNA regulatory regions (59). The specific signature epigenetic transcription factors for Th populations will be discussed below, followed by a review of three key families of transcription factors that form important combinatorial complexes after TCR-mediated activation: NF-κB, NFAT (Nuclear factor of activated T cells) and AP-1 (Activator protein-1).

1.5.1 Th1

Th1 cells differentiate to produce high amounts of the signature cytokine IFN-γ upon secondary TCR stimulation (5). Priming of naïve T cells in the presence of the cytokines, IL-12 and IL-18, can drive Th1 differentiation through intrinsic CD4 T cells IL-12Rβ(1/2)/STAT4 and IL-18R/IL-1 receptor-associated kinase (IRAK)/NF-κB network mediated signaling (41, 60-62). Secondly, initial CD4 T cell production of IFN-γ canonically acts, via the IFN-γR/STAT1 signaling network, to specifically target T-bet transcription, a T-box family transcription factor (variant of the *Tbx21* gene) (63). Finally, T-bet, serves as the terminal, master regulator for the Th1 population (63).

The transcription factor, T-bet, acts via three mechanism: (1) it drives Th1 differentiation via re-modeling and *trans*-activating the *Ifn-γ* gene and inducing IL-12Rβ2 (62) expression which leads to increased Th1 driven signaling (64), (2) intrinsic cross-regulation of Th2 signaling (64, 65), and (3) repression of *Il-2* gene activation (66). The initial *Ifn-γ* locus re-modeling features induced by T-bet and subsequent *trans*-activation of the *Ifn-γ* gene is via T-bet binding to a conserved (mouse and human) putative binding site located at a distant -2.0 kbp 5' location from the IFN-γ start site (64). Further studies, demonstrated that T-bet potently *trans*-activates the IFN-γ promoter in at least one other region described as the "Brachiury site" at the proximal -66 bp position relative to the *Ifn-γ* gene start site for transcription (67). Secondly, T-bet physically interacts with GATA-3 (discussed more below) via interaction of tyrosine phosphorylated T-bet and GATA-3 (65) and thereby down-regulating Th2 signaling. Thirdly, T-bet interacts with RelA to form non-functional nuclear heterodimers and regulate the binding of RelA to

the IL-2 promoter, and hence, *trans*-activation of the *Il-2* gene (66). Finally, T-bet gene-deficient mice, exhibit impaired Th1 development, increased Th2-associated cytokine production, and up-regulated IL-2 production in CD4 and CD8 cells (64, 68). In terms of an immune response, T-bet(-/-) mice succumb to *Salmonella* infection (69) and increased host-susceptibility to infection with MTb (70).

At least two other transcription factors act to enhance IFN-γ expression after the locus is made accessible: Hlx (71) and Ets-1 (72). Ets-1, E26 transformation-specific-1, has recently been shown to be an essential co-factor in IFN-γ production via its binding to the proximal IFN-γ promoter and cooperation with T-bet at the "Brachiury site" (72). Furthermore, Ets-1 is necessary for mounting certain Th1 responses *in vivo* (72). Hlx, a homeobox 2.0 gene member, is an early transcriptional target gene of T-bet and acts synergistically with T-bet to increase IFN-γ production in Th1 cells, shown to be especially critical in the early establishment of a Th1 response (71). Together, T-bet, Ets-1, and Hlx gene expression provide a firm marker in resting Th1 cells and their subsequent ability to produce a Th1 cytokine profile upon secondary stimulation.

1.5.2 Th2

Th2 cells produce high amounts of signature cytokines IL-4, IL-5, and IL-13 upon secondary TCR triggering. Priming of naïve CD4 T cells in the presence of early IL-2/STAT5 signaling has been shown to be necessary in the early re-enforcement of IL-4 production (48). The early IL-2-mediated signaling effect has been shown through the binding of STAT5a to an intronic region of the *Il-4* locus and thereby allowing for early

re-enforced IL-4 production (48). At the molecular level, the expression of IL-4 is subsequently, strongly controlled by the transcription factor GATA-3 (73-78). GATA-3, a zinc finger transcription factor, acts indirectly to repress the Th1 cytokine, IFN-γ, via direct down-regulation of STAT4 expression (75, 77, 79). The up-regulation of basal GATA-3 expression in recently primed CD4 T cells is controlled largely by the IL-4R(a/b)/STAT6 (80) signaling network (73); however other networks as yet undefined may induce independent IL-4R-mediated GATA-3 induction (74). During Th2 differentiation, GATA-3 induces changes in the chromatin structure at the *Il-4* locus (74, 78) by binding to two sites: (1) the proximal 5' region of the *Il-4* locus and (2) the *Il-4/Il-13* intergenic region (58, 76, 81). Furthermore, GATA-3 binds and strongly *trans*-activates the IL-5 (78, 82, 83) and IL-13 promoters (84). Since GATA-3(-/-) mice are embryonic lethal other knock-down approaches to inhibit GATA-3 have demonstrated its effect of Th2-associated cytokines. Inhibition by antisense transfection (73) or conditional deletion (85, 86) of GATA-3 *in vitro* and *in vivo* have further demonstrated the instructive role of GATA-3 in initiating (naïve CD4 T cells) and maintaining (effector CD4 T cells) the expression of the cytokines: IL-4, IL-5, and IL-13.

GATA-3 weakly *trans*-activates the IL-4 promoter (73, 76, 81) and an additional transcription factor, c-Maf (basic region-leucine zipper family member) (78, 87), is required to strongly *trans*-activate the IL-4 promoter. Additionally, Growth Factor Independent (GFI)-1, a zinc finger transcription factor, is induced rapidly via the IL-4R/STAT6 signaling network (88) and promotes proliferation and co-activates with GATA-3, especially seen at early time-points to terminally differentiate CD4 T cells

towards a Th2 phenotype (86). GFI-1 transcriptional up-regulation provides an early expression marker indicating STAT6 dependent signaling after initial TCR triggering has been initiated (88). Taken together, the GATA-3/c-Maf transcription factor combination provide a firm marker for the *Il-4, Il-13, Il-5* gene regulation and GFI-1 expression provides a clear marker for IL-4 driven CD4 T cell differentiation.

Further action of TCR-activated transcription factors is necessary for optimal Th1-and Th2-associated cytokine production. These factors are not generally considered to be epigenetic, lineage specific transcription factors (in terms of differential up-regulated expression during Th cell differentiation) but instead are regulated after TCR activation via mechanisms such as protein-protein interactions (59). The dimerization of these TCR activation specific factors can regulate cytokine gene regulation (59). Of these nuclear factors, various distinct NFAT:AP-1 dimers have been shown in TCR-mediated secondary "triggering" to be necessary for optimal Th1 and Th2 cytokine production (discussed more below) (59, 89).

1.5.3 NF-κB family

Nuclear factor(NF)-kappaB(κB) transcription factors form functionally active nuclear proteins that individually or synergistically bind and activate gene transcription necessary for activation, proliferation, and apoptosis in CD4 T cells. The NF-κB family is comprised of NF-κB1(p105), NF-κB2(p100), c-Rel, RelA(p65), and RelB (90). Together, the IKK-β (inhibitor of NF-κB (IκB) kinase-β) - dependent NF-κB activation pathway - is closely associated with targeting pro-inflammatory immune response genes

after receiving external stimuli (90). There are at least two distinct NF-κB activation pathways: the classical and alternative pathway (90). We will focus and outline the classical pathway below. Briefly, the IKK complex is composed of two catalytic components IκBα and IκBβ and a regulatory subunit, IκBγ (NEMO) (90). Classically, the IKK complex phosphorylates NF-κB-bound IκBs, where IκBs are channeled for proteasomal degradation thereby activating NF-κB dimers (p65/RelA, c-Rel, p50) to target gene transcription (90) (Fig. 1.3).

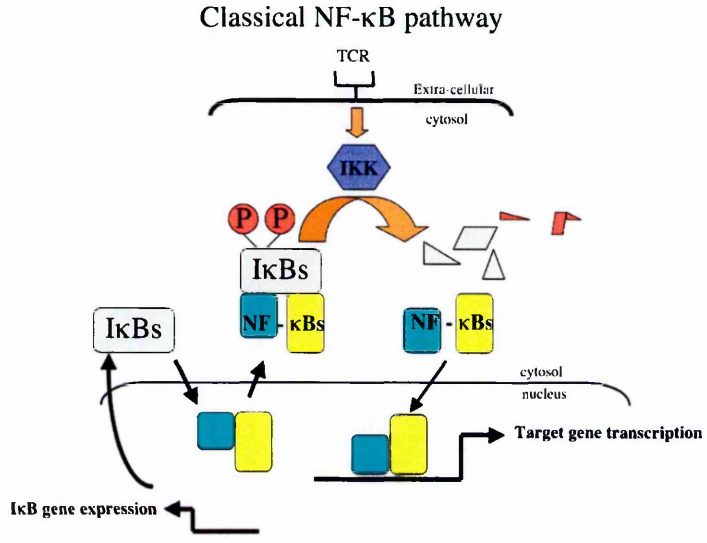


Figure 1.3. NF-κB activation in CD4 T cells.
Represents a simplified cartoon of the classical NF-κB pathway in CD4 T cells. A TCR stimulus activates the classical IKK pathway in CD4 T cells and induces the phosphorylation (P) (and subsequent polyubiquitination (not shown)) of IκBs, which are then targeted for degradation by the proteasome. NF-κB dimers are released and translocate to the nucleus, binding to DNA regulatory regions and initiating target gene expression. New gene transcription is initiated and translation of new IκBs shuttling nuclear NF-κB dimers back to the cytosol for further induction.

In T cells, IκBα degrading signals are rapidly initiated via the triggering of the TCR to activate p65/RelA activation and rapid target gene activation (91, 92). IκBβ degradation is initiated only after TCR-mediated gene expression and protein translation by a pathway that can be suppressed by calcineurin inhibiting drugs (cyclosporin A) which, in turn

leads to the release of bound IκBβ complexes for activation and target gene expression (93). Consequently, IκBβ-dependent gene expression is slower than IκBα-dependent new gene transcription (91, 92). In unstimulated naïve CD4 T cells, c-Rel binds primarily to IκBβ (91, 92); however a body of literature now exists demonstrating how the innate immune system may pre-activate naïve CD4 T cells with cytokines (IL-1β and TNF) to generate rapid TCR-triggered expression of high-abundance IL-2 and low-abundance IFN-γ (94). The mechanism for this lies in pre-activated naïve CD4 T cells ability to shuttle c-Rel from cytoplasmic-bound IκBβ to nuclear IκBα that in turn can more rapidly initiate cytokine expression (IL-2 and IFN-γ) after TCR triggering during the primary immune response (94). This mechanism is not achieved in effector CD4 T cells, as these genes (IFN-γ) are controlled via c-Rel independent mechanism or not expressed to the same abundance as naïve or terminal Th populations (discussed above). c-Rel provides an essential role in re-modeling the 300-base pair region of the IL-2 promoter (95) and *trans*-activating the *Il-2* gene in activated CD4 T cells (96). c-Rel(-/-) mice versus WT mice demonstrate a 50-fold decrease in IL-2 production when T cells are stimulated via the TCR (α-CD3/CD28) (97). In view of these studies, it is not surprising that studies performed *in vivo* in CD4 T cell-specific IκBα gene-defective cells describe these mice as being unable to mount sufficient Th1 immune response due to low-abundance proliferation, Th1 differentiation, and low concentration of IFN-γ production (98). IκBα gene-defective cells were also shown to be intrinsically unable to proliferate and produce IFN-γ in IL-12-supplemented CD4 T cell cultures *in vitro* (98). Lastly, NF-κB1 (p50) expression is important in CD4 T cells as illustrated by NF-κB1(-/-) being susceptible to Th1-driven infectious pathogens such as the intracellular parasite

Leishmania major (99). Their susceptibility was due to the intrinsic inability to proliferate directly due to reduced levels of IL-2R expression on the T cell surface after activation and indirectly reduced IFN-γ production (99). Importantly, these genetic studies illustrate the role for individual NF-κB genes and proteins and collectively the activation of NF-κB complexes plays an essential, non-redundant role in CD4 T cell function.

1.5.4 NFAT family

The NFAT family plays an important role in the regulation of CD4 T cell cytokine production (59). The functional enhancement (or repression) of a variety of effector cytokine genes is mediated by NFAT transcriptional activity (100). NFAT proteins function via rapid calcineurin-dependent translocation to the nucleus to induce regulation of multiple cytokine genes upon T cell activation (101) (Fig. 1.4). The NFAT family is comprised of four key calcineurin-dependent members, termed NFATc1-4 (102). NFATc1 and NFATc2 both play an active role in the regulation of various Th1 and Th2 cytokine genes: including TNF, IFN-γ, IL-4, IL-5 and IL-13 (59). NFAT can act synergistically with AP-1 (Jun:Fos) proteins on composite DNA binding elements to form stable ternary complexes to regulate multiple inducible genes (100). Additionally, GATA proteins have also been hypothesized in CD4 T cells to interact with NFAT (59), following known examples of interactions of NFATc4:GATA-4 (103) and NFATc1:GATA-2 (104) in cardiomyocytes and skeletal muscle tissue, respectively. In summary, NFAT proteins are activated via calcium/calcineurin-dependent signaling

which promotes NFAT dephosphorylation, nuclear translocation, and gene activation required for multiple productive immune responses in CD4 T cells.

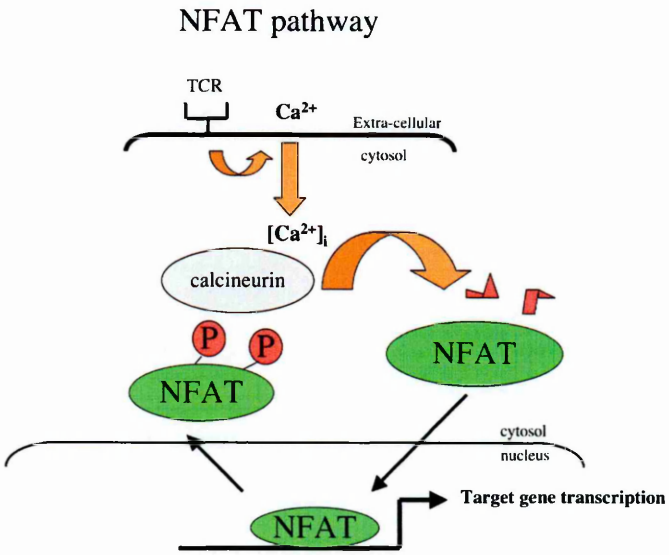


Figure 1.4. NFAT activation in CD4 T cells.
Represents a simplified cartoon of the activation of the NFAT pathway in CD4 T cells. NFAT proteins are phosphorylated **(P)** and reside in the cytosol of resting CD4 T cells. Upon stimulus NFAT proteins are dephosphorylated by calcineurin, translocate to the nucleus and become transcriptionally active DNA binding proteins. This pathway provides a direct link between Ca^{2+} flux and gene expression.

Considerable information is known about individual NFAT members involved in CD4 T cells cytokine regulation through studies involving genetic knock-outs and constitutively active NFAT mutants. IL-4 production in NFATc1(-/-) (105) and NFATc2(-/-) (106) mice is both enhanced and repressed in CD4 cells *in vivo* and *in vitro*, depending on the temporal stage at which the T cells were studied. Overall, data from NFATc1(-/-) mice show a significant decrease in a CD4 T cell IL-4–driven immune response (105). Conversely, ectopic expression of a constitutively active mutant version of NFATc1 into naïve CD4 T cells skew T cell differentiation towards a Th1 phenotype, by inhibiting IL-4 production and preferentially enhancing IFN-γ producing in neutral, Th1, and Th2 polarizing conditions (107). A direct mechanism for the constitutively active NFATc1 to

skew a naïve CD4 T cell differentiation towards a Th1 cell-fate is unclear. NFATc2(-/-) CD4 T cells are described to have early defects in their ability to produce IL-4; however under Th2 culture conditions, IL-4 production is higher then corresponding driven WT cells after day 7; whereas production of IL-2 and IFN-γ were similar to wild-type conditions throughout all time-points (106). Importantly, this differential effect on early and late levels of IL-4 production in NFATc2(-/-) CD4 T cells was shown both *in vitro* and in the context of an Th2-driven immune response *in vivo* (106). NFATc2 expression is directly targeted by IL-6 in CD4 T cells, especially by IL-6-producing Th2 cells (108). The direct effect of IL-6 on NFATc2 expression in differentiating CD4 T cells was shown to directly enhance IL-4 expression (108). Collectively, the NFATc1 or NFATc2 transcriptional activity within CD4 T cells is clearly temporal and dependent on multiple factors (for example with the accessibility of a target regulatory region).

1.5.5 AP-1 family

Although the AP-1 family members were first discovered over twenty years ago the biological basis and physiological functions of this highly interactive family is still not clear, especially in terms of individual complexes regulating CD4 T cells (109). Whereas, NFAT are activated by calcineurin, AP-1 members are activated by protein kinase C (PKC)/Ras signaling which promote the synthesis, phosphorylation and activation of AP-1 members (109). After activation, protein-protein interactions play an essential role in downstream gene expression targeting mediated by AP-1 family members (109). AP-1 complexes are basic region-leucine zipper (bZIP) elements, comprising of Jun (c-Jun, JunB, JunD, JDP1, JDP2), Fos (c-Fos, FosB, Fra-1, Fra-2) and

closely related activating transcription factors (ATF2, ATF3, and B-ATF) subfamilies (109). In CD4 T cells, differential AP-1 complexes form transcriptional enhancesomes with non-related proximal transcription factors to regulate various cytokine gene expression (59). Some key cytokine-mediated transcription factors known to dimerize with AP-1 members include Maf (c-Maf and v-Maf) and NFAT (NFATc1 and NFATc2) (59). Therefore, it is not surprising that several studies have described the recruitment of AP-1 complexes to enhance cytokine gene expression for IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-10, and IL-13 genes (59). In summary, specific target genes for different AP-1 complexes and the mechanisms that govern the subsequent co-activation of AP-1 complexes at the DNA binding-level demonstrate that AP-1 members play an important role as CD4 T cell transcriptional regulators.

For this thesis the focus will be on the AP-1-associated Jun subfamily, which has recently been shown to regulate IL-10 expression in CD4 T cells (110). c-Jun is a well-described component of the AP-1 family, in that c-Jun can mediate different-types of downstream gene expression profiles based upon its dimerization upon TCR triggering (100, 109). c-Jun forms the strongest heterodimers with c-Fos (Jun:Fos) to transcriptionally activate target tumor suppressor genes in various cell-types (100). In CD4 T cells, Jun:Fos dimers are known to enhance various pro-inflammatory/effector cytokines: IFN-γ, IL-2, IL-3, IL-4, IL-5 and IL-13 (100, 111). Conversely, c-Jun can heterodimerize with JunB (Jun:Jun), which attenuates Jun:Fos transcriptional capacity and activates a separate array of target genes (111, 112). Additionally, JunB:c-Maf can interact and at the *Il-4* locus there co-

operation was shown to *trans*-activate the IL-4 promoter (113). In conclusion, different AP-1 complexes can mediate activation of different gene expression profiles.

The ability of AP-1 complex (Jun:Fos) to bind other "third-party" complexes is also critical for functional downstream gene expression activation (109). Various DNA regulatory regions within cytokine genes are occupied by AP-1 and composite NFAT putative binding sites. Furthermore, the Jun:Fos:NFAT ternary complex was elegantly described to be necessary to enhance IFN-γ, IL-2, IL-3, IL-4, IL-5 and IL-13 gene expression in activated CD4 T cells (100). Conversely, TNF and IL-6 cytokine expression is exclusively dependent on NFAT-mediated signaling, without AP-1:NFAT interactions (100). These studies used various mutated NFAT proteins - that upon activation were unable to bind AP-1 complexes - in order to investigate whether the ternary AP-1:NFAT complex was required to enhance cytokine gene transcription (100). Importantly, in this study (100), the differentiation of CD4 T cell populations was established (i.e. the re-modeling of regulatory regions of effector cytokine loci was established) before TCR-mediated AP-1:NFAT interactions were analyzed (Jurkat cell-lines). This method was in contrast to gene-deficient models (AP-1 and NFAT) where differentiation from a primary naïve CD4 T cell precursor cell is likely disrupted at early-time-points (due to deficiency in AP-1 or NFAT proteins) and further specific-effector cytokine gene regulation becomes disrupted. In conclusion, it is speculated that specific inhibitory interactions via Jun:Fos heterodimers and also AP-1:NFAT ternary interactions could lead to a degree of differential cytokine expression in the primary differentiation stage as well as in the secondary stimulus.

1.6 Molecular mechanisms in *Il-10* gene regulation

Despite the central role of IL-10 in the immune response not a great deal is known in terms of the molecular mechanisms regulating *Il-10* gene expression in CD4 T cells or other IL-10-producing immune cells. When I started my thesis in August 2003, no reports on the molecular mechanism of *Il-10* gene regulation within primary CD4 T cells were published. However, as I have began to finish my thesis some initial reports of *Il-10* gene regulation have been published that describe specific chromatin re-modeling regions (DNase I HSS) and potential regulatory regions for control of *Il-10* gene transcription (110, 114, 115). I will outline the findings of these first papers detailing *Il-10* gene regulation at the chromatin-level and secondly transcription factors that have been associated with *Il-10* gene regulation.

1.6.1 Regulation of the *Il-10* gene at the chromatin-level

The first report on chromatin-level regulation of the *Il-10* gene (performed in D5 (Th1) and D10 (Th2) cell-lines and primary Th1/Th2 cells) clarified several regions of DNase I HSSs (114). The Im *et al.* group showed that the *Il-10* locus is not re-modeled in naïve CD4 T cells and a differential degree of re-modeling is established between Th1 and Th2 cells at the chromatin-level (cell-lines and primary cells). Importantly, this group used probes spanning either IL-10 exon 3 or exon 5 to show HSS in 16-kb BamHI fragments containing regions weighted primarily towards the 3' end of the murine *Il-10* locus (114). This group suggests the proximal 5' region (promoter) of the *Il-10* gene is both exclusively (1) re-modeled and (2) transcriptionally "active" in Th2 cells as opposed to

Th1 cells. This was determined by analyzing the promoter region in both Th1 and Th2 cells for DNase I HSS patterns (cell-lines) and status of acetylation of histone H4 using Chromatin immunoprecipitation (ChIP) with primers spanning the promoter region (cell-lines) (114). Together, the group identified 6 potential HSSs located throughout the *Il-10* locus (summarized in Figure 1.5). This was the first chromatin-level study to be performed in murine CD4 T cells and provided the first evidence of differential chromatin-level regulation for *Il-10* gene regulation.

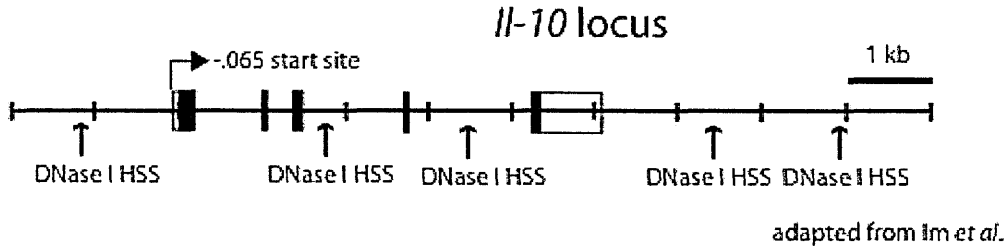


Figure 1.5. Initial *Il-10* locus chromatin-level results performed in CD4 T cells.
The *Il-10* locus at the chromatin-level in CD4 T cells as first described by Im *et al.* DNase I HSS were described in either Th1 or Th2 cells. Black boxes represent exons (5 total) and white boxes represent the untranslated regions (UTRs) of the *Il-10* gene.

The second DNase I HSS study was performed in similar cell-lines from CD4 Th cells and further added to the previous study by defining a regulatory region within the *Il-10* locus and putative transcription factors that enhanced IL-10 production at this site (110). Wang *et al.*, defined a conserved noncoding sequence (CNS) region expressed in both Th2 (D10 cell-line) cells and Th1 (AE7 cell-line) cells located in the 3' region of the *Il-10* gene, termed CNS-3. This region of the *Il-10* locus contained a conserved (human and mouse) putative AP-1 binding site. They further showed that AP-1 family members, c-Jun and JunB, were recruited *in vivo* to this site in stimulated primary Th2 cells and not in stimulated primary Th1 cells (ChIP). Furthermore, retro-viral transduction of c-Jun or

JunB into naïve CD4 T cells was shown to enhance IL-10 production in primary cells in the absence of other effector cytokines 3 days post-transduction (ELISA). Finally, they show the CNS-3 sequence can enhance *Il-10* expression in reporter assays when cloned together with the full-length minimal IL-10 promoter (1.5 kb), transfected into D10 cells and triggered with PMA/Ionomycin. This is in sharp contrast to parallel experiments where the full-length minimal promoter by itself was unable to enhance *Il-10* transcription after PMA/Ionomycin triggering in D10 cells. Wang *et al.*, were the first group to collectively show a molecular mechanism for *Il-10* gene regulation by DNase I HSS, *in vivo* recruitment of putative transcription factors to the corresponding HSS and enhancement of IL-10 production in primary CD4 cells with RV-mediated transduction of the putative transcription factors (c-Jun and JunB).

Up-to-date the most complete study on chromatin-level *Il-10* gene regulation was performed by Saraiva *et al.* within primary naïve CD4 T cells, effector CD4 T cells, IL-10-T_{reg} CD4 T cells and bone marrow (BM)-macrophages (115). In this study, "common" HSS within the *Il-10* gene are described at the 5' proximal region (promoter), intron 3 and intron 4 within both primary IL-10-producing CD4 T cells: Th2 and IL-10-T_{reg} cells (the HSS described by Wang *et al.* were not analyzed in this study, as Saraiva *et al.* used a probe located 7 kb upstream of the *Il-10* start site of transcription). This study was the first to describe chromatin-level regulation of the *Il-10* gene in IL-10-T_{reg}. The authors go further and describe shared HSS in IL-10-producing (stimulated with LPS (lipopolysaccharide), or other stimuli) BM-macrophages (5' proximal region, intron 3, and intron 4) and a novel BM-macrophages specific HSS (-4.5 kb upstream of the IL-10

start site, HSS-4.5) not shared with IL-10-producing CD4 T cell populations. Within the novel BM-macrophage HSS a conserved (human and mouse) putative NF-κB binding site was shown to recruit the NF-κB p65/RelA subunit in stimulated BM-macrophages (115). HSS-4.5 was further shown to have increased acetylated histone H3 (ChIP) in either resting or activated BM-macrophages and enhanced *Il-10* gene expression in the *trans*-activation studies when HSS-4.5 was cloned up- or down-stream of the full-length minimal IL-10 promoter (115). Finally, primary BM-macrophages where shown to exhibit a dose-dependent decrease in IL-10 mRNA and protein secretion when treated with an IKK inhibitor, indicating NF-κB activation is necessary for optimal IL-10 expression in BM-macrophages (115). Together, this study demonstrates that different IL-10-producing immune cells can have different potential regulatory regions that influence *Il-10* gene regulation in a cell-type specific manner. Conversely, they also show that "core" HSSs (5' proximal region (IL-10 promoter), intron 3 and intron 4) are conserved across IL-10-producing cells: BM-macrophages, Th2 cells, and IL-10-T_{reg} cells. The summary of all three chromatin-level studies across the *Il-10* locus is shown in Figure 1.6.

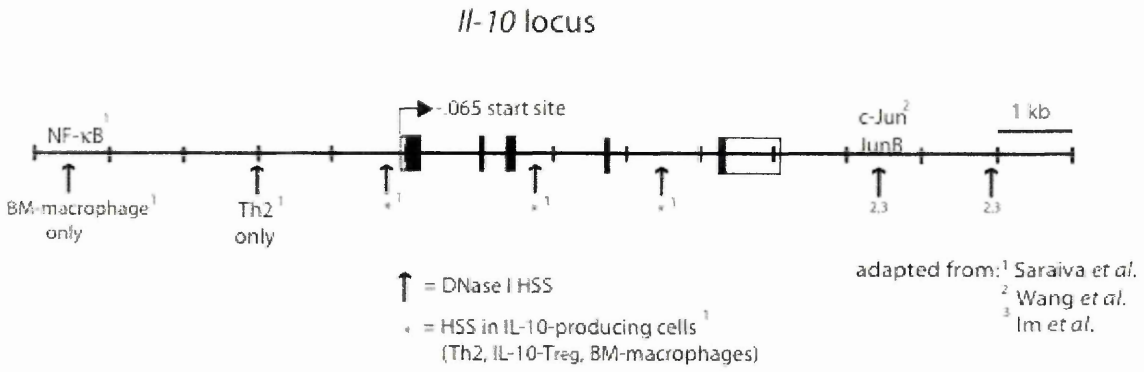


Figure 1.6. Chromatin-level analysis of the *Il-10* locus.
The *Il-10* locus at the chromatin-level in CD4 T cells as first described by Im *et al.*, Wang *et al.* and Saraiva *et al.* Transcription factors binding and regulating gene transcription are shown with the corresponding investigative authors. "Core" HSSs for high-IL-10-producing cells are labeled (*) at the 5' proximal region, intron 3, and intron 4.

1.6.2 IL-10-associated transcription factors

A comprehensive picture of all the necessary transcription factors to evoke instructive and essential enhancing or repressive regulation of the *Il-10* gene is not clear. Recent chromatin-level studies have determined as discussed in detail earlier transcription factors (c-Jun and JunB in CD4 T cells (112) and NF-κB in BM-macrophages (115)) acting directly at exposed regulatory regions within the *Il-10* gene to enhance IL-10 production. In macrophages, the proto-oncogene c-Maf is reported to act to regulate the *Il-10* gene based on the following observations: (1) c-Maf *trans*-activates the "minimal" IL-10 promoter (-.190 kb from *Il-10* gene start codon) in RAW264.7 cells and (2) c-Maf was shown by ChIP to be associated with the IL-10 promoter in IL-10-producing human monocytes (-.190 kb from *Il-10* gene start codon) and (3) transfection of c-Maf into human monocytes up-regulates IL-10 transcription stimulated by LPS (116). SMAD-4 is functionally shown to enhance CD4 T cell IL-10 production in Th1-driven conditions (117). Transduction of SMAD-4 into naïve primary CD4 T cells differentiated under Th1

conditions induces differential gene expression of IL-10; however a clear direct molecular mechanism for this effect is not understood (117). Several IL-10-enhancing transcription factors have been shown - only in cell-lines – to *trans*-activate the minimal IL-10 promoter. For instance, non-cell-type specific transcription factors Sp1/Sp3 (118) and c/EBP-β (119) have been shown to *trans*-activate the minimal IL-10 promoter in reporter assays performed in various non-immune derived cell-lines. In conclusion, whether these molecules are involved in enhancing *Il-10* gene expression via instructive events leading to IL-10 modifications, at the level of the chromatin, or at site-specific regions accessible in differentiated IL-10 producers remain unclear.

Two recent transcription factors have been implicated in CD4 T cells as possible direct repressors of *Il-10* gene regulation (although to our knowledge no clear direct molecular mechanism is yet understood). Transcription factor-mediated repression of the *Il-10* gene is intriguing because it may help explain why some populations of CD4 T cells with similar HSS differentially bind enhancing transcription factors (ex: AP-1 family members in the 3' region) and produce IL-10 whereas other do not. The initial chromatin report by Im *et al.* suggested that both intron 3 and intron 4 was re-modeled but "silenced" in Th1 cells (D5 cells) as opposed to being acetylated at histone H3 and H4 ("active") in Th2 cells (D10 cells). This was shown primarily via increased histone deacetylase HDAC1 in Th1 cells at positions within intron 3 and intron 4 (ChIP). They further show that NFATc2 binds at a conserved (human and mouse) putative binding site within intron 4, exclusively in Th1 cells (D5 cells) and not in Th2 cells (D10 cells) (ChIP). They speculate that functionally, NFATc2 may act as a key repressor-like molecule for IL-10

expression by binding within intron 4, "silencing" IL-10 expression as shown by increased histone deacetylase HDAC1 at this position. Another transcription factor functionally described to repress IL-10 expression is the proto-onco transcription factor, Ets-1 (72). Knock-out Ets-1(-/-) CD4 T cells produce significantly higher levels of IL-10 in both effector populations, although especially seen in Th1 cells (1% (WT) to 57% (Ets-1 (-/-)) by ICS FACS) (72). Although the exact molecular mechanism by which Ets-1 may repress IL-10 production is not known, it may act directly on a transcriptionally active region of the *Il-10* locus or conversely re-model a region of the *Il-10* locus allowing for repressing factors to bind. Together, the likely interaction of several transcription factors in "silencing" potential regulatory region remains an intriguing possibility but a clear mechanism is still not understood.

A collective cartoon of *Il-10* gene regulation (chromatin-level and transcription factors-mediated) is shown here in Figure 1.7.

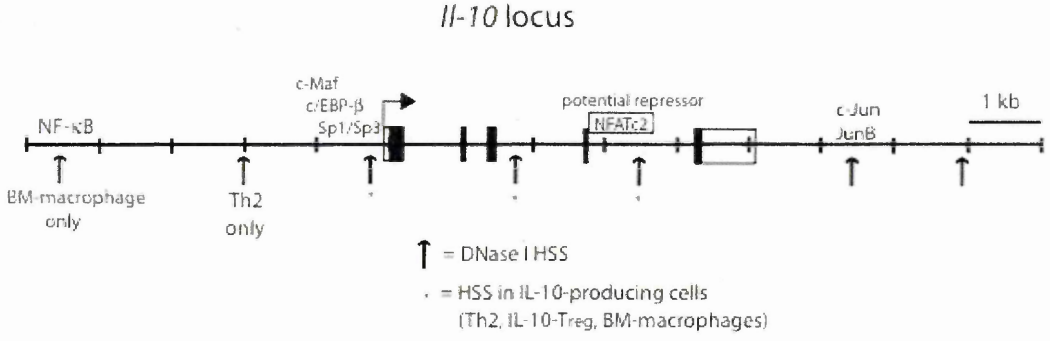


Figure 1.7. Gene regulation of the *Il-10* locus in various IL-10-producing immune cells.
Represents a collective cartoon of the regulatory mechanism understood to regulate the *Il-10* gene outlined throughout this chapter.

1.7 CD4 T_{reg}: cellular and molecular properties

1.7.1 CD25+T_{reg}

Natural occurring CD25+(IL-2Rα) T_{reg} represent a CD4 T cell population that have shown the capacity to regulate immune response to auto- and alloantigens, autoimmune diseases, allergy and infections (27, 120-125). CD25+T_{reg} were first discovered in mice (120, 121), and later an identical phenotypic and functional population of regulatory T cells has been defined in humans (126). In both mice and humans the CD4+CD25+ population comprises approximately ~10% of the peripheral CD4 T cell compartment (125). The naturally occurring immunosuppressive population of CD4 T cells is not only involved in the regulation of various autoimmune diseases, allergy, or atopy but can also provide effective anti-tumor immunity and tolerance to transplantation (27, 127). Below, I will introduce the lineage specific transcription factor for these natural occurring CD4 T_{reg}, FoxP3, and possible cellular mechanisms for CD25+T_{reg} suppression.

1.7.1.1 FoxP3

FoxP3, a member of the forkhead-winged-helix family, has been classified as the signature lineage specific transcription factor in natural occurring T_{reg} (122, 124, 128, 129). Functional studies involving FoxP3 over-expression initially showed that FoxP3 induced suppressive properties in naïve CD4 T cells to inhibit naïve T cell proliferation *in vitro* (122). Further studies confirmed *in vivo* that FoxP3 acts as the regulatory T cell lineage specification factor, irrespective of CD25+ expression (129). The induction of FoxP3 expression is speculated to at least partly occur within the thymus (130) and

further FoxP3 induction in peripheral naïve CD4+CD25- T cells, is speculated but not yet clearly understood (127, 131). The overall maintenance or homeostasis of CD25+T_{reg} in the periphery is supported or "fuelled" by critical IL-2 uptake (132). Another clear pathway maintaining FoxP3 expression in peripheral CD25+ T_{reg} is via the presence of transforming growth factor (TGF)-β (133). Specifically; TGF-β1 has been shown to support the expression of FoxP3 within peripheral CD25+T_{reg} and therefore, the homeostasis of peripheral CD25+T_{reg} population (133, 134).

The role of FoxP3 in the regulation of the immune response is shown clearly by genetic diseases in mice and humans that are tracked to defects in the *foxp3* locus (135). Mice deficient for the DNA-binding motif for *foxp3*, Scurfy (*sf*) mice, exhibit an activated T cell phenotype, produce excessive amounts of a number of effector cytokines upon activation, lack differentiated T regulatory cells and die by week three of age (128, 136, 137). This further supports the crucial role of FoxP3 in the regulation of peripheral CD4 T cells. Humans suffer from a similar disorder clinically termed immunodysregulation, polyendocrinopathy and enteropathy, X-linked syndrome (IPEX) brought about by various point mutations within the *foxp3* gene (135, 138). Interestingly, the severity of the phenotype between humans differs based on the location of the point mutations within the *foxp3* gene (139). Mutations in both the DNA-binding region (FKH) and to a milder degree the protein-protein interaction domain of the *foxp3* gene predispose humans with symptoms clinically associated with IPEX (135, 139). These IPEX-mediated point mutations within the *foxp3* gene clearly illustrate that FoxP3 regulates the expression of target genes via DNA-binding regions and also to some degree through

protein-protein interactions (135, 139).

The exact role of FoxP3 in CD25+T_{reg} is currently being defined by several groups. It is clear that ectopic expression of FoxP3 in naïve CD4 T cells is sufficient to confer regulatory function (122, 128, 129). Another clue to the direct mechanism of FoxP3 function in CD25+T_{reg} is the apparent direct repression of pro-inflammatory (IL-2) (137) and effector (IFN-γ, IL-4) cytokine expression and production via inhibition of NF-κB and NFAT proteins to bind target DNA regions (137, 140). More investigation into other transcription factors either interacting with FoxP3 or independently adding to CD25+T_{reg} ability to survive without the ability to produce proliferative cytokines upon TCR triggering will be discussed later.

1.7.1.2 Cellular mechanisms for *in vitro* or *in vivo* suppression by CD25+T_{reg}

Multiple mechanisms of *in vitro* or *in vivo* suppression by CD25+T_{reg} have been addressed; although a universal mode of immunosuppression by CD25+T_{reg} is still not established (127). Some postulated mechanisms for suppression by CD4 T_{reg} include: the local *in vivo* secretion of cytokines such as TGF-β or IL-10, direct cell-cell interactions, and/or transcriptional control of the *Il-2* gene in CD4+CD25- T cells (127). Well-described assays and disease models for systemically understanding the multiple levels of suppression by CD25+T_{reg} *in vitro* and *in vivo* have been essential to investigating their possible suppressive properties.

Throughout the numerous studies on the mechanism for CD25+T_{reg} suppression, three clear themes with regards to potential suppressive mechanisms are universally recognized (127). First, *in vitro* studies using defined systems where cells are separated by permeable membranes have illustrated that *in vitro* T_{reg} suppression of naïve or effector T cells is mediated via a cell-contact-dependent mechanism (141). Secondly, the presence of APCs is not required as *in vitro* APC-free mixed cultures of CD4 T_{reg} and naïve or effector cells are suppressed to levels equivalent to APC-driven -naïve and CD4 T_{reg} culture systems - although these studies are not widespread and do not rule out an effect of CD25+T_{reg} on APC (127). Lastly, *in vitro* studies have clearly shown that activation of CD4 T_{reg} via TCR ligands or antibody to CD3 is essential for CD4 T_{reg} ability to suppress naïve or effector T cell proliferation (11).

1.7.1.1.1. Cell-cell contact

The CD28 receptor family members, cytotoxic T lymphocyte-associated antigen (CTLA)-4, inducible T-cell co-stimulator precursor (ICOS) and programmed cell death (Pcd)-1 (also known as programmed death (PD)-1), have all been postulated to exhibit proximal suppressive properties in direct cell-cell interactions via CD25+T_{reg} and naïve or effector T cells (142). A sufficient amount of data describing CD4 T_{reg} immunosuppressive properties *in vitro* centers on the expression of CTLA-4. CTLA-4 is actively ligated by two ligands termed: CD80 (B7-1) and/or CD86 (B7-2), and these ligands are recently shown to be expressed on effector CD4 T cells as well as multiple APCs (143) (discussed more below). The systematic deletion of these B7 ligands, CD80 and/or CD86, within *in vitro* systems has conclusively shown that cell-cell contact

mediated suppression by CD25+T_{reg} of CD4 effector cells is significantly reduced as compared to wild-type CD4 effector T cells in APC-free CD4 T_{reg} suppression assays (127). These studies imply that cell-cell immunosuppression *in vitro,* is dependent on activated CD25+T_{reg} expressing CTLA-4 that can interact directly with the ligand CD80 and/or CD86 expressed on effector CD4 T cells. Additionally, CTLA-4 expression on CD4 T_{reg} has been shown to transfer suppressive function, for the activation of naïve and effector CD4 T cells, to CD80 and/or CD86 expressing APCs (142) (discussed more below).

CTLA-4 can exert inhibitory effects directly to professional APCs or via direct cell-cell contact. Interestingly, a ligand independent form of CTLA-4 (liCTLA-4) has been recently shown to exist predominately in CD4 memory/T_{reg} cells. Furthermore, the liCTLA-4 form is shown to be differentially expressed in resting T cells as opposed to the full-length version of CTLA-4 being up-regulated on activation (144). *In vivo* studies showed that the liCTLA-4 form was responsible for susceptibility to type 1-diabetes in a mouse model (145). This dichotomy of resting versus activation expression profiles leads to the postulate that liCTLA-4 inhibits initial low-threshold T cell activation; whereas the full length CTLA-4 is regulated by the strength of the TCR stimulus and thus serves to dampen high-affinity T cells (146).

CTLA-4 is found on a variety of T cells including CD4 T_{reg}, which have been shown to exert suppressive function by interacting with dendritic cell derived CD80 and/or CD86 and thereby inducing indoleamine 2,3-dioxygenase (IDO) activation (125). IDO further

metabolizes tryptophan and lowers the amounts of free tryptophan within DCs, which leads to the lowered capacity of DCs to activate CD4 T cells (142). However, this pathway has been shown to be not essential in CD4 T_{reg} suppression assays in an *in vitro* setting, as APC-free T_{reg} cultures also exert suppressive properties (127, 143). Cell-cell ligand/receptor binding of CTLA-4 and CD80 or CD86 has been reported in two separate environments: CD4 T_{reg}-effector cells and CD4 effector-effector cells (127, 143). Importantly, CD4 T cells up-regulate CD80 and CD86 ligands upon activation, and these ligands have been shown to be required for *in vitro* CD25+T_{reg} suppression. CD4 T_{reg}-effector cell interaction has been postulated to occur on recent TCR triggering allowing CTLA-4 to engage the effector cell CD80 and/or CD86 ligand (127). This interaction triggers an 'outside in' suppression of the target effector cell (146). Other reports show that certain effector cells express CTLA-4 (142). Therefore, CD4 effector-effector cell interactions are suggested to regulate each other via cell autonomous CTLA-4 signaling.

The mechanism by which CTLA-4 exhibits suppression is somewhat unclear. One role that was been postulated is CTLA-4 antagonizing effects on the necessary secondary T cell receptor, CD28 (142). Full length CTLA-4 may competitively remove CD80 and/or CD86 available for activating necessary proliferative secondary signals through CD28 (144). Secondly, ligation of CTLA-4 may directly inhibit the TCR pathway (142). One clear mechanistic role of CTLA-4 is within DCs where activation of CTLA-4 by CD80 and/or CD86 induces IDO signaling within DCs, which indirectly reduces the ability for a DC to activate subsequent naïve T cells (125). Taken together, the multiple forms of CTLA-4 and cell-type interaction dynamics that govern CTLA-4 various pathway-

specific functions elude to its robust ability to suppress proliferation within the T cell compartment.

Another CD28 homolog shown to exhibit tolerance on CD4 T cells is ICOS. ICOS has one natural B7 family ligand, ICOSL, which is expressed in T cells, B cells, DCs, macrophages, and other non-lymphoid tissues (142). ICOS is up-regulated on both CD8 and CD4 T cells upon activation and is present in all types of effector and memory cells (142). Both Th1 and Th2 cells express ICOS; however ICOS is present in higher levels on Th2 versus Th1 cells (147). ICOS is similar to CD28 - but not dependent on CD28 - in that it can augment cytokine expression and drive differentiation in both Th1 and Th2 effector cells as shown with various *in vivo* disease models performed in CD28 gene-deficient mice (148, 149). One clear difference between CD28 and ICOS lies in the fact that ICOS ligation is not able to drive early production of IL-2, while CD28 is required for maximum naïve T cell activation and further downstream IL-2 production (150). Another clear role for ICOS lies in its ability to drive B cell differentiation, immunoglobulin class switching, germinal center formation, and memory B cell development (147, 149). Definitive studies have illustrated that ICOS(-/-) mice are severely affected in their ability to mount sufficient T cell-dependent B cell responses (151-153).

Several studies have clearly shown the effect of ICOS during the course of an effector Th2 response. Firstly, Coyle *et al.* showed *in vitro* that blocking ICOS, with ICOS-Ig, in recently activated primary Th2 cells reduced cytokine production of IL-4 and IL-5, by

over 50%, independently of CTLA-4 or CD28 activation (147). This group went on further to test blockade of ICOS in an *in vivo* hyper-active Th2-associated disease model, by administrating blocking ICOS-Ig to mice before transferring highly polarized Th2 cells (147). ICOS-Ig recipient mice exhibited a significant reduction in clinical signs of pathology and this was associated to production with IL-5 being reduced by over 80% versus control mice (147). In a separate study, researchers treated mice with antagonists or blocking antibodies for ICOS (ICOS-Ig and/or α–ICOS) at day 0, 8, or 21 days after airway priming in a similar Th2-driven disease model (154). Blocking antibodies for ICOS had no effect on cytokine production at day 0 or 8, versus control. However, at day 21, mice receiving ICOS-Ig or α–ICOS had significant reduction in IL-10, IL-5, and IL-4. Furthermore, up-regulation of Th2-associated chemokine receptors CCR3, CCR4, and CCR5 was shown to be significantly reduced at day 21, from cells pooled from the draining lymph nodes in mice receiving α-ICOS. These preliminary studies suggest, blocking ICOS can regulate an established Th2 driven immune response via reduced cytokine production and cell-surface homing markers (chemokine receptors); however early differentiation of Th2 cells is likely to be less dependent on ICOS.

ICOS expression has also been implicated in CD4 T_{reg}. Firstly, ICOS expressing CD4 T cells were isolated from secondary lymph nodes in unmanipulated mice and categorized as $ICOS^{high}$, $ICOS^{medium}$, and $ICOS^{low}$ expressing cells after re-stimulation for their cytokine profiles (155). $ICOS^{high}$ cells were linked to IL-10 production, $ICOS^{medium}$ cells to IL-4, IL-5, and IL-13, and $ICOS^{low}$ cells were loosely-linked to production of IL-2 and IFN-γ (155). One report generated CD4 T_{reg} with *ex vivo* mature pulmonary DC that

were isolated from mice primed with respiratory allergen and showed that the subsequent generation of IL-10-Treg was possible from these DCs but dependent on the ICOS:ICOSL interactions (156). This study implied that the combinations of the ICOS:ICOSL pathway and local presence of IL-10 was critically important for mature DC to drive CD4 T_{reg} in the lung and thereby prevent development of airway hyper reactivity (AHR). However, other models where mice were challenged with pathogens and CD4 T cells were collected and re-stimulated show different cytokine profiles relating to ICOS expression levels (157).

Taken together, ICOS is important in the effector phase of T cell differentiation and especially key to Th2 effector function. Furthermore, ICOS ligation on CD4 T cells does not up-regulate IL-2 production, an especially crucial feature of early differentiating CD4 T cells. Finally, high-density expression of ICOS has been implicated in stimulating IL-10 production (156), which may provide a mechanism for CD4 T_{reg} suppression.

Programmed cell death (Pcd)-1 constitutes an important pathway regulating T cell activation and tolerance (158). Pcd-1 - a CD28 family member - is related closely to CD28 and CTLA-4 (158). Pcd-1 is expressed on thymic double negative cells (158), peripheral CD4 and CD8 cells (159, 160), B cells, and monocytes upon activation (160). Unlike CD28 and CTLA-4, Pcd-1 is expressed on a broad range of lymphocyte-derived cell-types. There are two natural ligands for Pcd-1 termed: PD-L1 and PD-L2, which are expressed on B, myeloid, and DC upon activation (161). Interestingly, PD-L1 is also

expressed on naturally occurring CD25+T_{reg}, although its function within this population has not been systemically addressed (161).

Within CD4 T cells, Pcd-1 acts similarly to CTLA-4, in that, low amounts of Pcd-1 are able to potently inhibit T cell activation at an early developmental state (162). Co-localization at the cell surface level of Pcd-1 to TCR/CD28 receptor complexes is essential for Pcd-1 inhibitory function (162, 163). Although unlike CTLA-4, Pcd-1 has a well-established functional target in that it inhibits expression of the cell survival gene bcl-x_L, thereby inhibiting T cell activation and proliferation (163). T (CD4 or CD8) cells stimulated via the TCR/CD28 and PD-L1:Pcd-1 displayed dramatically decreased proliferation and the intrinsic inability to produce significant levels of IL-2 (163).

Recently, a group has isolated CD4 T_{reg} that express Pcd-1, irrespective of CD25+ (164). These cells either produce IL-10 and IL-4 upon re-stimulation, but little to no IL-2 and IFN-γ. The mechanism by which these CD4 T_{reg} inhibit naïve CD4 T cell proliferation *in vitro* was determined to be IL-10 and IL-4 independent, but partially CTLA-4 dependent.

Some members of the tumor necrosis factor (TNF) receptor family have been implicated in CD4 T_{reg} cell-mediated suppression by CD4 T_{reg} (165). The most studied member of this family is the glucocorticoid-induced tumor necrosis factor receptor (GITR). Initially, GITR was thought to be a CD25+T_{reg} specific marker, and its activation on CD4 T_{reg} was demonstrated to repress CD4 T_{reg}-mediated suppression (166), while a simultaneous independent study required activation of CD25+T_{reg} along with IL-2, in order to abrogate

GITR-mediated suppression (167). It is now understood that GITR expression is not restricted to the CD25+T_{reg} population and activated effector CD4 T cells can also express GITR to signal either TCR-driven activation or cell death (168). Triggering of another TNF receptor family member, OX40 (CD134), has also been shown to abrogate CD25+T_{reg} mediated-suppression and restore effector T cell proliferation, IL-2 transcription and effector CD4 T cell cytokine production (169, 170). However, similar to GITR, OX40 is expressed on activated effector CD4 T cells and thus is not specific for CD25+T_{reg} (169). A combinatorial *in vitro* study of both GITR and OX40 expression specific for CD25+T_{reg} revealed that α-GITR treatment abrogated CD25+T_{reg} suppression of effector CD4 T cells before and after TCR activation, while similar α-OX40 treatment of CD25+T_{reg} only abrogated suppression before CD25+T_{reg} activation (170). Together, both GITR and OX40 activation on CD25+T_{reg} can inhibit suppression under certain conditions, however since GITR and OX40 show high abundance expression in activated effector CD4 T cells this limits the ability to isolate direct GITR and/or OX40 CD25+T_{reg}-mediated effects *in vivo*.

1.7.1.1.2 Cytokines

Independent studies have suggested that the primary mechanism for *in vitro* suppression by CD4 T_{reg} is the consumption of exogenous IL-2 (141, 171, 172). While it is clear that IL-2 is essential to initiate proliferation of suppressive CD4 T_{reg} (172, 173), it is not clear whether the consumption of IL-2 by CD4 T_{reg} is the primary means to which CD4 T_{reg} translate their suppressive properties for naïve and/or effector T cells (127). Furthermore, *in vivo* studies involving gene-deficient (IL-2R(-/-)) mice have shown that competitive

CD4 T_{reg} consumption of IL-2 is not sufficient to suppress various autoimmune responses (173). Recent definitive studies have demonstrated that both CD25(-/-) and IL-2(-/-) FoxP3-expressing cells were able to fully suppress proliferation of CD4 T cells *in vitro* (132). Thus, IL-2 consumption by CD4 T_{reg} in the periphery likely plays a role in CD4 T_{reg} maintenance and proliferation; however, it cannot be considered the essential mechanism for *in vitro* or *in vivo* naïve and effector T cell suppression.

Although CD25+T_{reg} do not have the ability to produce IL-10 directly upon stimulation after *ex vivo* isolation from unmanipulated hosts, IL-10 has been considered an essential molecule for CD4 T_{reg}-mediated *in vivo* immunosuppression within certain immune responses (11). It was initially shown that generation of CD4 T_{reg} via repeated *in vivo* antigenic stimulation by specific peptide, resulted in anergic CD4 T cells that secrete large amounts of IL-10 (174-177). One area of intensive study in CD4 T_{reg} function is observations from the development of mucosal inflammation to intestinal antigens (21). Intestinal inflammation occurs spontaneously after adoptive transfer of naïve CD4 T cells from normal mice into T and B cell immunodeficient recipients (21). The resultant pathology (colitis) is due to an excessive Th1-driven immune response (21). The co-transfer of CD25+T_{reg} into these immunodeficient hosts inhibits colitis and this suppression is dependent on the production of IL-10, as $CD45RB^{low}$ cells (which contain effector cells, CD25+T_{reg} and other CD4 T_{reg}) treated to mice with IL-10R antibody or IL-10(-/-) $CD45RB^{low}$ cells were unable to control intestinal inflammation (22). These findings provided clear evidence that IL-10 plays a non-redundant role in the functioning of CD4 T_{reg} that control inflammatory responses towards enteric bacteria (22).

Interestingly, the investigators determined that the effect of IL-10 was specific for "antigen-specific" effector CD4 T cell populations, and that additional regulation of the naive CD4 T cell pool was likely via a cell-cell mechanism, and therefore IL-10 independent, since CD25+T_{reg} from IL-10(-/-) could regulate pathology induced by naïve T cells (23). An additional report with regards to adoptive transfer and intestinal inflammation in immunodeficient hosts, demonstrates that CD25+T_{reg}-derived IL-10 can inhibit pathology (colitis) in an innate immune response (24). In these studies colitis was triggered with the bacterium *Helibacter hepaticus* (not via transferred naïve CD4 T cells responding to an enteric bacteria antigen) in immunodeficient hosts, thereby driving T cell-independent intestinal inflammation via the constitutive innate immune response. *Helibacter hepaticus*-infected immunodeficient hosts receiving adoptively transferred CD25+T_{reg} were protected from development of colitis (induced in a T cell-independent fashion). This response was cytokine dependent as CD25+T_{reg} transferred into mice treated with IL-10R antibody or IL-10(-/-) CD25+T_{reg} cells completely ablated the ability of CD25+T_{reg} to inhibit intestinal inflammation via the innate immune response. In summary, IL-10 plays an essential role in some CD4 T_{reg} suppressive-mediated microenvironments; however the IL-10 effect is complex in these contexts and *in vivo* CD25+T_{reg} may use various mechanisms for suppression depending on the disease model and the level of inflammation.

Another important cytokine in the functional regulation and homeostasis of CD25+T_{reg}, is the pluripotential cytokine TGF-β (127, 134). Upon activation, CD25+T_{reg} have been shown to express high levels of membrane-bound TGF-β1 (178). Initially, the

production of TGF-β1 was thought to directly regulate CD4+CD25-T cells (178); however more recent studies have concluded that CD25+T_{reg} expression of TGF-β1 is likely to specifically suppress antigen-specific CTLs but not effector CD4 cells (179, 180). Therefore, direct suppression by CD25+T_{reg} via TGF-β1 production is likely restricted to the CD8 T cell population while microenvironments that include TGF-β1 favor generation and/or proliferation of CD25+T_{reg} (as described above) but not direct suppression of effector CD4 T cells (127).

1.7.2 Trafficking of CD25+T_{reg}

The homing of CD25+T_{reg} to sites of activated effector T cells within the host has been suggested to play a critical role in CD25+T_{reg} suppressive function (125). Chemokines or chemoattractants regulate leukocyte migration and likely play a key role during inflammation in the local recruitment of both effector T cells and CD4 T_{reg} (181, 182). Early clues to trafficking of CD25+T_{reg} have been addressed by studies showing the up-regulated expression of chemokine receptors, CCR4 and CCR8, upon TCR activation (183). These chemokine receptors are postulated to dictate regional trafficking of CD25+T_{reg} to areas where mature DCs secrete their target ligands: CCL17 and CCL22 (183). Analysis of chemokine receptor expression from antigen challenged CD25+T_{reg} populations indicated that CD4 T_{reg} selectively expressing CCR8 produced IL-10 upon *ex vivo* re-stimulation (184). Both CCR4 and CCR8 expression have also been shown to be up-regulated with TCR and CD28 stimulation in associated Th2 cells (185, 186). CCR6 is another candidate for CD4 T_{reg} specific homing in select immune responses. Mice lacking the chemokine receptor CCR6, were shown to have a 2 to 15-fold increase in T

cell populations in response to select antigens targeting the intestinal mucosal tissue; whereas other systemic immune responses to subcutaneous antigens were unimpaired (187). The same group also postulated that CCR6 function was critical for AHR, as eosinophil accumulation in the lung and cytokine production by allergen-specific Th2 cells were reduced in CCR6(-/-) mice in response to a model for allergic pulmonary inflammation (188). More recently, CCR6 was shown to be expressed on a distinct subset of CD25+T_{reg} that are shown to exhibit markers of recent activation and expansion that are indicative for what the authors refer to as regulatory effector-memory T cells or T_{rem} (189). T_{rem} were also shown to express high levels of IL-10 after re-stimulation *ex vivo* and be enriched within the central nervous system after induction of EAE suggesting homing into potentially destructive immune responses directly in inflamed tissues (189). Finally, CXCR5 expression is characterized with an anergic cytokine profile in activated CD4 T cells and up-regulated expression of CD28 family member, ICOS (190-192). CXCR5+ T cells are found localized in T-B cell follicles where induction of the chemokine target ligand, BCL (BCA-1 in humans) (181). Given their ability to provide efficient help to antibody production and localization within follicles, CXCR5 expressing T cells have been referred to as follicular B helper T cells (T_{fh}) (181). The fact that CXCR5 expression is reported to be absent on naïve CD4 T cells and CXCR5 expression leads to poor cytokine producing CD4 T cells upon activation suggests that CXCR5 could play a role in immune surveillance. Taken together, chemokine receptors - CCR4, CCR6, CCR8, and CXCR5 - have been implicated to play a homing role in populations of cells exhibiting suppressive properties demonstrated by various gene-deficient(-/-) and functional animal models.

Lastly, the adhesion molecule CD103, specifically classified as a α_E integrin, is expressed on recently activated peripheral CD4 T_{reg} (193). CD103+CD25+T_{reg} were initially shown to control IBD and peripheral T cell homeostasis (193). CD103+CD25+T_{reg} were highly enriched in gut-associated lymphoid tissue as compared to CD103-CD25+T_{reg} (193). One implication that has been put forward is CD103 expression marks recently activated CD4 T_{reg} in the periphery (194) and these CD103+CD25+T_{reg} may be homing to sites of inflammation (195, 196). Therefore, similar to chemokine expression, CD103 may act as marker of a recently homing CD4 T_{reg} and not lineage or antigen-specific CD4 T_{reg} as was first described.

1.7.3 IL-10-T_{reg}

The *in vitro* IL-10-T_{reg} population – unlike the in vivo equivalents – can be generated in large numbers for addressing molecular mechanisms for *Il-10* gene regulation and their anergic and suppressor function (197). Our lab has previously generated a second regulatory CD4 population termed: IL-10-T_{reg} (197). This homogeneous population is generated from naïve CD4 T cells differentiated with the combination of two immunosuppressive drugs, 1α,25-Dihydroxyvitamin D_3, the active form of Vitamin D_3 (vitD_3) and a synthetic glucocorticoid receptor (GR) agonist, dexamethasone (DEX) (197). Similar to the natural occurring CD25+T_{reg}, the homogenous population of IL-10-T_{reg} produce little to no proliferative (IL-2) or effector cytokines (IFN-γ and IL-4) upon secondary TCR triggering (197). Functionally, IL-10-T_{reg} have been shown to regulate naïve CD4 T cells *in vivo* and *in vitro*, in similar systems to the CD25+T_{reg} suppression,

and regulate autoimmune disease *in vivo* (174, 197, 198) although these IL-10-T_{reg} do not express the natural occurring T_{reg} linage specific transcription factor, FoxP3 (174). We believe that these cells are the equivalent of antigen-specific IL-10-producing T_{reg} that are induced by repeated challenge of mice with soluble antigen (175, 177) in that like the $vitD_3$/DEX generated IL-10-producing cells they do not express FoxP3 or IL-2 (174).

1.7.3.1 Background: DEX and $vitD_3$

The initial use of immunosuppressive drugs $vitD_3$ and/or DEX to generate populations of anergic CD4 T cells has a relatively long history in terms of modern immunology (199, 200). Today, DEX and $vitD_3$ both separately and together have been shown to induce regulatory cells that inhibit naïve T cell proliferation in mouse (174) and human (201) systems and inhibit cytokine production by human Th2 cells (201). The presence or treatment of $vitD_3$ on CD4 T cells was initially speculated (1984) as a potential model system for understanding the molecular mechanisms by which IL-2 production could be inhibited (199). Similar to initial studies on $vitD_3$, DEX was described (1992) to "interfere" with TCR-mediated activation signals and directly inhibit IL-2 transcription (200). In fact, these authors were more direct than the initial $vitD_3$ studies and described the lack of AP-1 and NFAT co-operation at the IL-2 promoter, which in turn allowed DEX to inhibit IL-2 gene transcription. As I write this thesis it is nearly 22 years since $vitD_3$ and 14 years since DEX were described in some detail in CD4 T cell biology and there remains to be defined a clear definition for their individual or combinatorial mechanisms at the molecular level in CD4 T cells.

Molecular mechanisms in which DEX suppresses CD4 T cells was initially described as direct inhibition of the NFAT:AP-1 complex to bind effectively to the known DNA binding targets (200). Additionally, the NF-κB family was shown to be targeted by DEX, as described by the inhibition of NF-κB transcriptional activity (202). Other DEX targets include the expression of receptor activator of nuclear factor-κB ligand (RANKL) (203) and interference of NF-κB subunits to bind known target genes, including areas for optimal IFN-γ transcription (204, 205)). Work by Riccardi *et al.*, identified a DEX-induced transcription factor in CD4 T cells (206), glucocorticoid-induced leucine zipper (GILZ), which was further shown to directly inhibit the expression of TCR-activated genes: Fas Ligand, IL-2, and IL-2R. The suppression of these transcripts was facilitated via GILZ interaction with specific NF-κB proteins (207). Another group has demonstrated that GILZ acts to bind and inhibit specific AP-1 family members, Jun:Fos dimers, limiting the activity of the AP-1 complex ability to bind target DNA (Jun:Fos) (208). Recently generated CD4-specific trans-genetic (TG) GILZ over-expression mice up-regulate IL-4, IL-5, IL-13 and IL-10 production on *ex vivo* stimulus as opposed to *ex vivo* production from CD4 WT cells (209). CD4 T cells from TG mice also down-regulate IFN-γ when compared to WT cells; however no significant difference in IL-2 production was witnessed in naïve CD4 T cells from either *ex vivo* stimulated TG or WT mice (209). Taken together, the inhibitory effect of DEX (or DEX induced transcription factors) on T cell activation and survival appears to be achieved through the antagonizing role of three key TCR activation pathways: AP-1, NF-κB and/or NFAT activity.

The immunosuppressive drug, vitD$_3$, has been shown to modulate T cell activation (210, 211) and inhibit secretion of cytokines, IL-2 (212) and IFN-γ (213), via vitD$_3$ direct interaction with either NFATp:AP-1 complexes (214) or NF-κB proteins (215). In terms of cytokine genes, vitD$_3$ down-regulates IFN-γ mRNA via inhibition of transcription via the minimal promoter region of the *Ifn-γ* gene {Cippitelli, 1998 #249}. VitD$_3$ has two direct mechanisms in inhibiting IL-2 mRNA transcription: (1) inhibition of the NFATc2:AP-1 complex to bind to the *Il-2* gene (214) and (2) vitD$_3$ ability to reduce levels of NF-κB proteins in treated CD4 T cells which indirectly lowers IL-2 expression (215). VitD$_3$ has also been described to enhance the ability of CD4 naïve T cells to differentiate into a Th2 population in an *in vitro* APC-free culture system (216). VitD$_3$ treated CD4 T cells were shown to express Th2-associated transcription factors, GATA-3 and c-Maf, as opposed to neutral (no polarizing cytokines) driven CD4 T cells (216). However, the vitD$_3$-induced effects of differentiating naïve CD4 T cells were further shown to be mediated through IL-4, extrinsically (216). Together, these results suggest that the skewing of differentiating naïve CD4 T cells towards an effector Th2 population in the presence of vitD$_3$ may be a direct result of early augmentation of initial TCR-triggered differentiation away from Th1 conditions (with inhibition of IFN-γ production) and minimal IL-4 production which leads to subsequent IL-4R/STAT6 autocrine transcriptional control and Th2 development.

Taken together, that both DEX and vitD$_3$ share the ability to suppress the transcriptional activity (NF-κB, NFAT) and protein-protein interactions (AP-1:NFAT) of several transcription factors provides a direct mechanism for effects on subsequent naïve CD4 T

cell differentiation. It is also clear that naïve CD4 T cell development under the individual presence of vitD$_3$ (216) or DEX-induced GILZ (209) seems to influence a Th2 differentiation patterning. The molecular mechanism for the generation of IL-10-T$_{reg}$ which exclusively express IL-10 and little to no IL-2, IL-4 and IFN-γ and their subsequent expression (in particular, transcription factor) profiles will serve as a major determinant in the results section of this thesis. This system provides a key mechanism to dissect the regulation of *Il-10* gene and the subsequent effects on function of these cells.

1.7.3.2 Cellular mechanisms for suppression by IL-10-T$_{reg}$

Similar to CD25+T$_{reg}$ the range of suppressive features mediated by IL-10-T$_{reg}$ under different physiological conditions is unclear. *In vitro* suppression assays have shown IL-10-T$_{reg}$ are equal to CD25+T$_{reg}$ in inhibiting CD4+CD25- T cell proliferation *in vitro* (174). IL-10-T$_{reg}$ suppressive ability to regulate CD4+CD25- T cell populations *in vitro*, is IL-10-independent, TGF-β-independent and CTLA-4-independent, mimicking CD25+T$_{reg}$ (11, 174). Furthermore, exogenous IL-2 abrogated IL-10-T$_{reg}$ suppressive ability (174) *in vitro* in a manner similar to abolished *in vitro* CD25+T$_{reg}$ suppression (217). Clear evidence demonstrates that IL-10-T$_{reg}$ regulate certain *in vivo* autoimmune pathologies directly through production of IL-10 (197), as α-IL-10R was shown to abrogate the ability of IL-10-T$_{reg}$ to regulate EAE (197). The role of IL-10 produced by CD4 T$_{reg}$ has been discussed in the previous CD25+T$_{reg}$ section for regulation of intestinal mucosal inflammation and studies involving regulation of allergic disease is another area of potential implications for IL-10-T$_{reg}$. Nonspecific therapies such as glucocorticoids are a common agent for allergic or asthmatic conditions in humans which

in turn is known to induce IL-10 T cell populations (30). Furthermore, allergen immunotherapy under the appropriate conditions is reported to induce protective allergen-induced IL-10-secreting T cells (218-220). The *in vivo* generation of IL-10-T_{reg} in the case of allergen-specific immunotherapy is of current interest for this novel method to provide long-lived immunological recall to allergen-specific stimuli to dampen a potentially dangerous acute pro-inflammatory immune response (27). Taken together, IL-10-T_{reg} are dependent on IL-10 production for control of certain *in vivo* autoimmune or allergic pathologies; however *in vitro* systems demonstrate that the complete mechanism for IL-10-T_{reg} suppression of CD4+CD25- T cells is not entirely IL-10 dependent and remains unclear, but it is also accompanied by lack of IL-2 production and anergy in both types of CD4 T_{reg} (CD25+T_{reg} and IL-10-T_{reg}).

Materials and Methods

2.1 Mice

Female BALB/c mice WT and BALB/c IL-4(-/-) mice, housed in specific pathogen-free animal facilities at the National Institute for Medical Research (NIMR) were used as a source for CD4 T cells. All mice were between ages 10-14 weeks of age.

2.2 Media and Reagents

Tissue culture medium used was RPMI 1640 supplemented with 10 mM HEPES buffer, 100 U/ml penicillin and 100 U/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate (all from BioWhittaker, Walkersville, MD), 0.05 mM 2-ME (Sigma-Aldrich), and 10% FCS (Labtech International). Monoclonal Antibodies (mAbs) used in cultures were anti-IFN-γ (XMG1.1) and anti-IL-4 (clone 11B11). Anti-mouse CD3 (clone 2C11) and CD28 (clone 37.51) mAbs used for T cell stimulation were purchased from BD PharMingen. mAbs used for T cell enrichment were anti-B220 (clone RA3-6A2), anti-CD8 (clone C291.2.43), anti-ClassI-A^d/I-E^d (clone 2G9), and for T cell isolation were anti-CD4-FITC, -PE or -CyChrome® (clone RM4-5), anti-CD62L-PE (clone Mel-14), anti-CD45RB-FITC or -PE (clone C363.16A) and biotinylated anti-CD25 (clone 7D4) was followed by streptavidin (SA)-CyChrome® or SA-APC, and isotype controls (all BD PharMingen). mAbs used for intracellular staining were anti-IL-2-FITC, -PE or APC (clone JES6-5H4) , anti-IL-4, PE (clone 11B1), anti-IL-5, PE (clone TRFK5), IFN-γ, FITC (clone XMG1.1) and anti-IL-10-PE or –APC (clone JES5-16E3), anti-TNF-α-PE or APC (clone MP6-XT22) and isotype controls (all BD PharMingen). Luminex conjugated beads for ELISA was purchased through UpState Signaling (Mouse Beadlyte Cytokine 9-Plex Kit).

2.3 FACS cell preparation

Female BALB/c mice were culled using schedule 1 kill guidelines and whole spleens were immediately harvested using sterile technique and placed in cRPMI (this work is done in the pathogen-free animal facilities at the National Institute for Medical Research (NIMR)). Spleens were pooled and transported to a sterile-hood environment for further processing within the laboratory. Spleens were mashed (with the sterile back-end of a 2-ml syringe) and washed through a 70-micron filter in the presence of cRPMI. Homogenized or "mashed" cells were collected and pooled into a sterile 50-ml falcon tube and spun-down (1,300 rpm for 3 minutes) in V_T= 40 ml (cRPMI). Supernatant was discarded and cells were re-suspended in 40-ml of cRPMI and spun down again. Supernatant was discarded and cells were re-suspended in Ammonium Chloride, NH_4Cl (0.83%) (V_T= 2 ml per spleen). After 3 minutes cells were spun-down and supernatant was discarded (containing the lysed re-blood cells). Cells were re-suspended in 40-ml FACS sort buffer (SB) (5% FCS, 100 U/ml penicillin and 100 U/ml streptomycin, and sterile PBS). Cells were spun-down and supernatant is discarded. Cells were re-suspended in 40-ml FACS SB and spun-down a second-time. Finally, cells were re-suspended in FACS SB at V_T= 0.5 ml per spleen.

Depleting antibodies were prepared in FACS SB for depletion of APCs and CD8 T cells (anti-B220, anti-CD8, anti-Mac-1) and combined in V_T= 0.5-ml per spleen. Each antibody was filtered though a .2-micron filter unit. Cells and depleting antibody cocktail were combined (V_T= 1-ml per spleen) and thoroughly mixed and placed on ice for 20 minutes. Magnetic beads (V_T= 2-ml per spleen) were washed with FACS SB (3x) within

a sterile 50-ml falcon tube placed on the provided magnetic rack. After the final wash the magnetic beads, are re-suspended in FACS SB, V_T = 0.5-ml per spleen. After a 20-minute staining period, cells were washed twice in 40-ml ice-cold FACS SB, and re-suspended in FACS SB, V_T= 0.5-ml per spleen. Cells and magnetic beads were combined and mixed thoroughly, V_T= 1-ml per spleen, and placed on a slow-rotating rotor for 30 minutes in 4°C temperature conditions.

Cells/magnetic beads were placed in a sterile 50-ml falcon tube in the magnetic rack provided by Miltenyi magnetic beads were allowed to collect on the magnetic strip adjacent to the falcon tube (3 minutes). Media was aspirated and placed in the second falcon tube for further collection of magnetic beads and 15-ml of FACS SB is re-placed in the first falcon tube for second enhancement of left-over non-magnetic bead-bound cells. This process is repeated 3x and a diagram is shown in Figure 2.1.

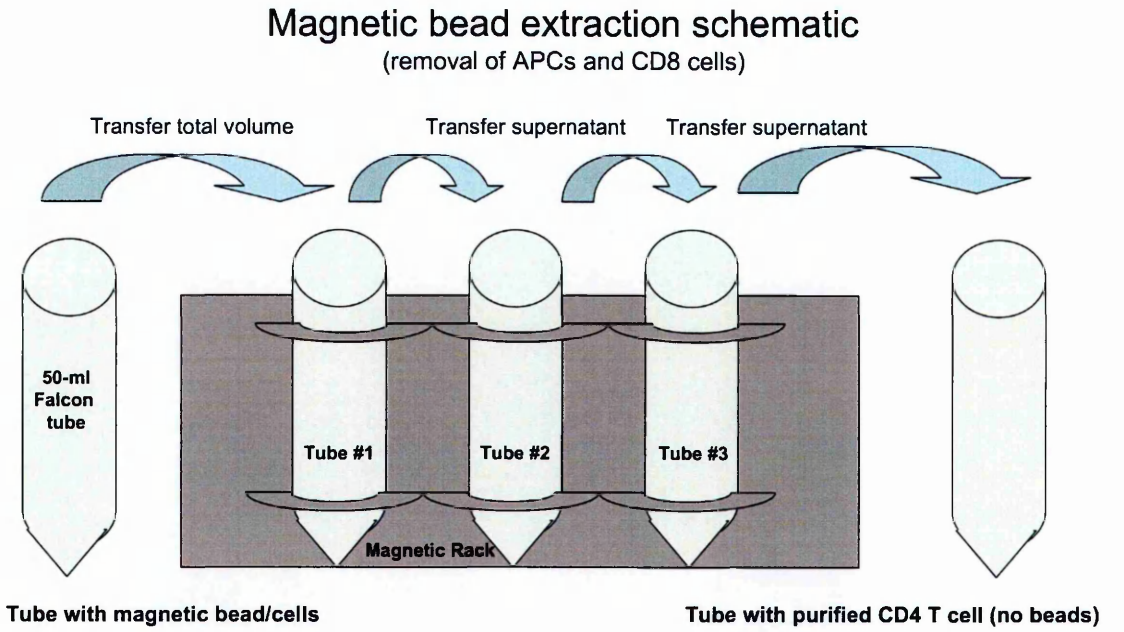


Figure 2.1. Magnetic bead depletion for CD4 T cell purification
This schematic represents the process of steps required to remove magnetic bead/cell complexes in order to purify CD4 T cells for FACS.

Following magnetic bead depletion, purified CD4 T cells were spun-down. Supernatant was withdrawn and cells were re-suspended in 40-ml of FACS SB and spun-down. Supernatant was withdrawn and cells were re-suspended in V_T= 0.5-ml per spleen in FACS SB. Sort cocktail of the necessary antibodies in FACS SB were prepared at the appropriate concentrations for mix with cells to a V_T= 1-ml per spleen. Wait 20 minutes for cells to be stained with appropriate antibodies (ice-cold conditions). Cells/antibodies were spun-down and supernatant was removed, cells were re-suspended in 40-ml of FACS SB, and re-spun-down. Supernatant was removed and cells are re-suspended in V_T= 0.5 ml per spleen, filtered through a 40-micron filter and placed in 5-ml falcon tubes for MoFlo isolation via fluorescent activated cell sorting. A typical recovery of naïve CD4 T cells is shown below as a FACS plot.

2.4 *In vitro* tissue culture conditions

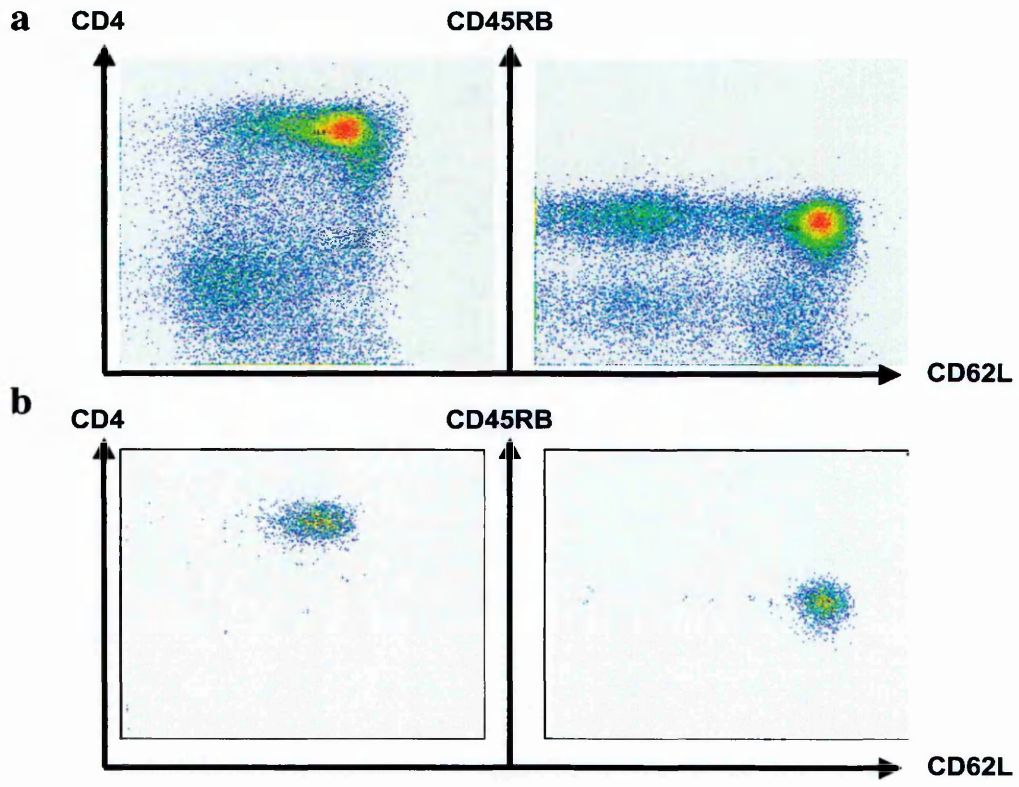


Figure 2.2. Naïve FACS isolation and re-analysis
(a) An example of a naïve T cell (CD4+CD62L+CD45RB+) FACS sort. **(b)** A typical post-sort re-analysis of the isolated CD4+CD62L+CD45RB+ population, always quality-controlled to be greater than 98% pure.

After naïve CD4 T cells were isolated MoFlo (CD4+CD62L+CD45RB+) (Figure 1), cells ($1x10^6$ cells/ml in cRPMI) are immediately plated in 24-well plates ($1x10^6$ cells per well) and stimulated in a sterile APC-free environment with plate-bound antibodies for CD3 (α-CD3) (10 μg/ml) and soluble α-CD28 (2 μg/ml) and their corresponding cytokine cocktail in cRPMI.

Table 2.1. Cytokine and Antibody Cocktails for Cultured CD4 T Cell Population

T cell population	Cytokine Cocktail
neutral	α-IL-4 (20 μg/ml), α-IFN-γ (5 μg/ml)
Th1	IL-12 (5 ng/ml), α-IL-4 (20 μg/ml)
Th2	IL-4 (10 ng/ml)
IL-10-T_{reg}	vitamin D_3 ($4x10^{-4}$ M), Dexamethosone ($4x10^{-6}$ M)

Cells are grown in a sterile incubator (37C, 5% CO_2). Each cell population was grown as described in Figure 2.3, with the key highlights being summarized below. On day 3, cells were split in a 1:3 dilution with their corresponding cytokine cocktail into new wells (24-well plate) in the absence of α-CD3 and α-CD28. On day 7, neutral and Th1 cells are collected and a portion was stimulated via the TCR pathway for intracellular cytokine production, protein production and mRNA. Th2 and IL-10-T_{reg} are re-stimulated overnight in their corresponding cytokine cocktail ($1.5x10^6$ cell/well) with plate-bound α-CD3 (1 μg/ml) and soluble α-CD28 (2 μg/ml) in the presence of IL-2 (5 ng/ml). On day 8, cells are removed from the α-CD3 and α-CD28 stimulus and moved to a new 24-well plate. On day 10, a 1:3 split is performed with their corresponding cytokine cocktail, including IL-2. On day 14 cells are re-stimulated overnight in their corresponding cytokine cocktail ($1.5x10^6$ cell/well) with plate-bound α-CD3 (1 μg/ml), soluble α-CD28 (2 μg/ml) and IL-2, similar to day 7. On day 15, cells are moved to new 24-well plate. On day 18, a 1:3 split is performed with their corresponding cytokine cocktail, including IL-2. On day 21, Th2 and IL-10-T_{reg} are collected and a portion was stimulated via the TCR pathway for intracellular cytokine production, protein production and mRNA.

	Week 1			Week 2			Week 3			
	Day 0	Day 3	Day 5	Day 7	Day 8	Day 10	Day 14	Day 15	Day 18	Day 21
Neutral	Cells: 1.0 x 10^6 Stimulus: αCD3(10 μg/ml) αCD28 (2 μg/ml)	Move cells and split 1:3	Split 1:3	Harvest cells	-	-	-	-	-	-
Th1	Cells: 1.0 x 10^6 Stimulus: αCD3(10 μg/ml) αCD28 (2 μg/ml)	Move cells and split 1:6	Split 1:3	Harvest cells	-	-	-	-	-	-
Th2	Cells: 1.0 x 10^6 Stimulus: αCD3(10 μg/ml) αCD28 (2 μg/ml)	Move cells and split 1:3	Split 1:3	Cells: 1.5 x 10^6 Re-stimulus: αCD3(1 μg/ml) αCD28 (2 μg/ml)	Move cells	Split 1:3	Cells: 1.5 x 10^6 Re-stimulus: αCD3(1 μg/ml) αCD28 (2 μg/ml)	Move cells	Split 1:3	Harvest cells
IL-10-T_{reg}	Cells: 1.0 x 10^6 Stimulus: αCD3(10 μg/ml) αCD28 (2 μg/ml)	Move cells and split 1:3	Split 1:3	Cells: 1.5 x 10^6 Re-stimulus: αCD3(1 μg/ml) αCD28 (2 μg/ml)	Move cells	Split 1:3	Cells: 1.5 x 10^6 Re-stimulus: αCD3(1 μg/ml) αCD28 (2 μg/ml)	Move cells	Split 1:3	Harvest cells

Figure 2.3. Schematic of key points throughout *in vitro* differentiation process for CD4 T cells.
This schematic highlights the cell-confluence in the 24-well format and concentration of stimulus at each time-point (day 0, day 7, day, 14) during the *in vitro* differentiation of neutral, Th1, Th2, and IL-10-T_{reg}.

2.5 Quality controls for homogeneous primary CD4 T cell populations

The isolation and/or generation of homogeneous populations of primary T cells were critical for analyzing their respective transcriptional profiles. Cytokine production at the respective time of collection is checked at the mRNA level (qPCR), and protein level: both at the single cell level (ICS FACS) and the quantitative level (ELISA). Two other important populations are collected *ex vivo*: naïve T cells and CD25+T_{reg} (only with post-sort analysis >98% purity).

2.6 ICS FACS

Intracellular staining (ICS) FACS staining was an integral part of this thesis. Each week T cell cultures are quality-controlled largely based on their ICS FACS profiles and further the RV system used the ICS to determine whether differential cytokine profiles were induced under the gene-of-interest (GFP+) RV system (discussed more below). ICS FACS was done consistently with two types of CD4 TCR pathway activation, soluble

PMA (50 ng/ml)/Ionomycin (500 mg/ml) and plate-bound αCD3 (2 μg/ml)/soluble αCD28 (2 μg/ml).

Firstly, *ex vivo* or cultured CD4 T cells are washed completely of any residual antibodies/cytokines/immunosuppressive drugs and re-suspended at a concentration of 1.5 x 10^6 cells per ml in cRPMI. 24-well plates were prepared with 3x concentrated, V_T= 340 μl, of either TCR pathway activation stimuli (α-CD3/CD28 or PMA/Ionomycin) in cRPMI. 660 μl of CD4 T ells were added to TCR stimuli prepared 24-well plate and the plate was placed in the incubator (37°C, 5% CO_2) for two hours. After two hours 10 μl of Brefeldin A (stock @ 1 mg/ml) is added to each well. After addition of Brefeldin A (which will encapsulate the cells), cells were replaced in the incubator for an additional two hours. After two hours (four hours total), cells are harvested and spun-down in ice-cold PBS. Ice-cold PBS wash is repeated. Cells were re-suspended in 0.5-ml of ice-cold PBS and then 0.5-ml 4% formaldehyde was added to fix the cells (mix vigorously). After 20 minutes, ice-cold PBS was added and cells were spun-down. Repeat the wash with ice-cold PBS, a second-time. PBS was discarded and re-suspended cells were replaced with 1-ml of PBS/BSA/Azide buffer (500 ml PBS/0.5 ml of 1.0 M Azide/2.5 grams of dissolved BSA). Store cells at 4°C or proceed immediately to ICS.

"Plate-out" approximately 2 x10^5 of cells per well in 96-well U-bottom plates. Cells were "plated-out" or spun-down by centrifuge at room temperature at 1,600 rpm for <10 sec with the 96-well U-bottom plates. After spin-down supernatant was "flicked-out" in the sink. Immediately add 150 ml of permeabilization buffer (PBS/BSA/Azide/0.5%

Saponin) per well and mix gently with pipette. Wait 10 minutes at room temperature. Cells were spun-down and supernatant has flicked-off. Add appropriate concentration of cocktail of various fluorochromes directly conjugated to cytokine antibodies, V_T= 35-ml per 96-well. Incubate for 30 minutes, RT. Add 100 ul of permeabilization buffer to each well, and spun-down. Supernatant was flicked-off, and cells were re-suspended in 200 µl of permeabilization buffer and spun-down. . Supernatant was flicked-off, and cells were re-suspended in 150 µl of PBS/BSA/Azide buffer, spin-down. . Supernatant was flicked-off, and cells were re-suspended in 150 µl of PBS/BSA/Azide buffer and transferred to ICS FACS analysis tubes. ICS FACS was performed on Beckton Dickinson FACS Caliber with CellQuest software under manufactures instructions.

2.7 Real-time quantitative RT-PCR (qPCR)

RNA from the different T cell subsets was extracted using RNeasy Mini-Kit (Qiagen), DNase treated (Roche), and reverse transcribed with oligo pdT12-18 (GE Bioscience), random hexamer primers (Promega) and Superscript II RNaseH$^-$ reverse transcriptase (Invitrogen) according to the manufacturer's instructions. cDNA was analyzed for the expression of cytokine genes with primers designed to span intronic regions. qPCR was performed in 96-well plates (Applied Biosystems) containing: cDNA, primers, and SYBR Green (ABgene) reagents and was analyzed with either the ABI Prism 7000 or 7900 Sequence Detection System (Applied Biosystems). Quantification of target gene cDNA expression (Ct value) was normalized to the ubiquitin mRNA levels (Ct value) as measured from the same corresponding biological sample (cDNA) on the same plate. cDNA was analyzed for the expression of all other genes with primer/probes

(TAM/FAMRA reporter) purchased through the inventoried stock from Applied Biosystems (with the expectation of c-Maf, for which a primer/probe was designed with the help of designmyprobe.com). Primer/probes were used with Mastermix (ABgene) and analyzed identically to SYBR Green generated Ct values for cytokine genes with either the ABI Prism 7000 or 7900 Sequence Detection System (Applied Biosystems). Quantification of target gene cDNA expression for primer/probe pairs was normalized to the corresponding cDNAs HPRT1 (Applied Biosytems) Ct value, similar to normalized cytokine gene expression as described with the control or housekeeping gene being ubiquitin Ct values. Relative expression for all genes was calculated by the following empirical equation in Microsoft Excel:

Relative expression= POWER(1.8,((Ct, housekeeping gene)–(Ct, gene of interest))) * 10

2.8 ELISA

ELISA results are shown here with the use of the Luminex xMAP 100 Instrument and Beadlyte Mouse Multi-Cytokine reagents. ELISA reactions were performed on Millipore Multiscreen 96-well plates, with UpState Beadlyte Mouse Multi-Cytokine Standard sets (Standard 2 and 4) containing 5 ng/ml for each cytokine or CD4 T cell supernatant, and Beadlyte Anti-Mouse Cytokine Bead Sets for IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and TNF-α.

Firstly, cytokine standards were re-suspended and placed in a 1:3 dilution series for a total of 7 dilution steps from the neat concentration, 5 ng/ml (5 ng/ml – 6.9 pg/ml). Duplicates of 50 µl from each dilution set were pipetted into labeled wells on the

Millipore Multiscreen plates. "Blank" wells were included (50 µl of cRPMI) as the final (base-line) data point on the standard curve. Sample supernatants were immediately pipetted into each well in single-cates and diluted from a "neat" concentration, 1:9 dilution and 1:27 dilution (3 wells for samples: neat or 1:1, 1:9, 1:27). Beadlyte Cytokine Anti-Mouse Beads (9 total) are immediately mixed together with the appropriate amount of Cytokine Assay Buffer (UpState Signaling). 25 µl was added of pooled Cytokine Anti-Mouse Beads to each well with either the standard mix or sample supernatant, vortexed briefly, and allowed to incubate on a plate-shaker at room temperature for two hours (V_T= 75 ul per well). While plate is incubating, the secondary Beadlyte Anti-Mouse Cytokine-Biotin were combined for all measured cytokines and mix thoroughly and placed on ice. After the two-hour incubation the vacuum manifold was applied to the Millipore Multiscreen plate to remove the liquid and each well was re-suspended in 50 µl of Cytokine Assay Buffer, vacuumed again, and each well was re-suspended in 75 µl of Cytokine Assay Buffer. Plate was vortexed and 25 µl of secondary Beadlyte Anti-Mouse Cytokine-Biotin was added to each well (V_T= 100 ul per well). Plate was vortexed and incubated for 1.5 hours on the plate-shaker in room temperature conditions. After 1.5 hours, 25 µl of diluted Beadlyte Strepavidin-Phycoerythrin was added to each well, mixed and incubated on the plate-shaker at room temperature for 0.5 hours (V_T= 125 ul per well). After 0.5 hours, 25 µl of Beadlyte Stop solution was added to each well, mixed and incubated on the plate-shaker at room temperature for 5 minutes (V_T= 150 ul per well). After, 5 minutes the vacuum manifold was applied to the Millipore Multiscreen plate to remove the liquid and re-suspended in 125 µl of PBS, vortexed and placed at on a plate-shaker for 1 minute. Plate was then processed on the Luminex 100 Instrument with

Multi-Plexv4.0 (Bio-Rad) software to quantify concentration amounts first generated from the known standard curves and these set amounts are then related to samples from unknown concentrations from sample supernatants for the 9 corresponding cytokines (IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ and TNF-α).

2.9 GeneChip: RNA preparation to GeneSpring v7.0 presentation

Cells were FICOLL-separated before total RNA was prepared with the RNeasy Mini Columns (Qiagen) according to the manufacturer's instructions. Cell numbers were approximately $5x10^6$ in order to generate at least 3μg of total RNA. For *ex vivo*-generated populations (naïve and CD25+T_{reg}), 20 mice were culled per condition to generate 3μg of total RNA post-sort. Total RNA is then quality controlled for integrity (quantitative and qualitative) on a Nano LabChip (Agilent) with the Agilent Bioanlyzer 2100. Upon passing the quantitative (> 3 μg of total RNA) and qualitative (>1.9 28s/18s ratio) tests between 3-5 total micrograms of total RNA was further processed with one-cycle Affymetrix-recommended cRNA protocols (Roche Applied Science). Briefly, according to the manufacturer's instructions mRNA is transcribed to cDNA, cDNA is transcribed to cRNA, and 15 micrograms of cRNA target was fragmented and hybridized onto an Affymetrix M430A or M430v2.0 GeneChip, stained with phycoerythrin (PE), washed, and scanned on a con-focal based laser scanner.

Two computational algorithms were compared when analyzing the GeneChip image generated by the con-focal laser scanner: MAS5.0 (Affymetrix) and GC-RMA (GC-

Robust Multiarray Analysis, www.bioconductor.org). Both, MAS5.0 or GC-RMA were used to generate the independent biological experiments and the replicates were pooled per T cell population and condition (12 total: 6 populations, 2 conditions). Each algorithm used the image file (.cel file) and the absolute values were processed with the support of GeneSpringv7.0 software (Silicon Genetics) for further normalizations. The MAS5.0 algorithm is run from GCOSv2.0 application (Affymetrix) and the GC-RMA algorithm was computed from the GeneSpringv7.0 (Silicon Genetics) application. Irrespective of the algorithm used to generate absolute values for each transcript, individual GeneChip generated datasets were normalized to the 50% median ($\log_2 = 1$) from each GeneChip and the for each gene. Each chip was further quality-controlled after normalizations, to check for relative average expression values in respect to other global expression values on other chips. For data presentation, normalized expression was transformed to $\log_2$ scale ($\log_2 = 1.0$, being the median) and genes are shown as reported in comparison to other normalized conditions as a differential log-based value.

2.10 Algorithm for GeneChip analysis

I evaluated various computational algorithms (MAS5.0, GC-RMA, dChip, and RMA) in order to obtain the greatest signal-to-noise ratio for the quantitative signal reported without compromising signal specificity. I determined that the algorithm that is chosen to compress the gene expression (raw probe set signal, .cel file) is critical for increasing sensitivity across the GeneChip. The Affymetrix GeneChip is designed to have 11 specific 'perfect-match' (PM) probes, each 25-basepairs long, that span the full length transcript for each gene measured, weighted towards the 3' end of the transcript

measured. Each transcript also has 11 'mis-match' (MM) probes, which are identical to each complementary PM probe expect for the 13th base pair is reversed. In the commonly used MAS5.0 algorithm (221), provided by Affymetrix, the raw probe set signal between the PM and MM probes is internally controlled for background noise and cross-hybridization. The raw probe-set signal is calculated (average difference value) between all 11 pairs and summed for the entire transcripts total probe signal using a common Tukey-weight equation. Importantly, the MAS5.0 algorithm flags and discards average value differences if less than 20% (PM – MM), or automatically if the MM signal value is higher than the PM value. This feature becomes critical when evaluating low expressing transcripts. A more recent algorithm for GeneChip processing is the GC-Robust Multiarray Analysis (GC-RMA) algorithm (222, 223). The cornerstone of the GC-RMA approach lies in its removal of the PM/MM average difference value calculation and instead GC-RMA sums only the PM probe signals to determine the raw probe set signal. To account for background noise and cross-hybridization errors, GC-RMA specifies global signal differences at the specific PM probe with two variables; the quantitative level of GC bounds in each PM probe (theoretical sensitivity), and the signal at the specific PM probe across all GeneChips assayed (experimental dynamic range). When more than 10 arrays are compared the GC-RMA algorithm reports significantly higher intensity for all relative expression transcripts, especially those genes in the lower expression spectrum (222). We also found that the GC-RMA algorithm gave higher raw probe set signal (across all levels of expression) and achieves a more accurate coverage of lower expressing genes than the MAS5.0 algorithm, when compared to quantitative qPCR results.

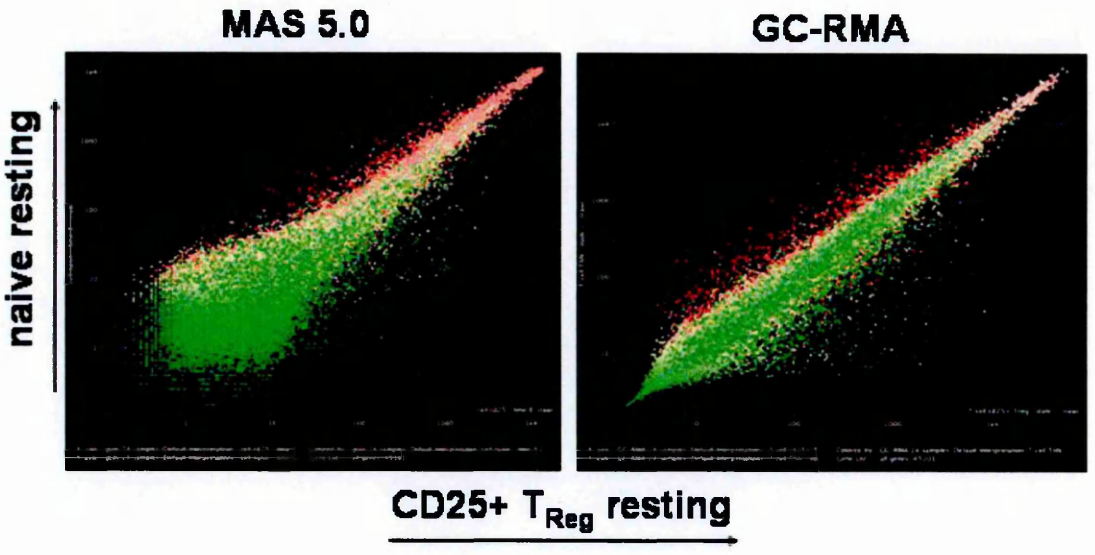


Figure 2.4. The GC-RMA algorithm reduces noise at low-expressing transcripts.
This figure represents a scatter plot illustrating the differences between normalization algorithms MAS 5.0 and GC-RMA. Both plots (MAS 5.0 and GC-RMA) are generated from the same GeneChip raw images, and raw signal for each population is displayed for all transcripts (>45,000) (x-axis = CD25+T_{reg} unstimulated, y-axis = naïve unstimulated).

2.11 *In silico Il-10* gene analysis

Comparative genomic alignments and predicted transcription factor binding sites are all performed from the website: http://dcode.org (provided freely by the Lawrence Livermore National Laboratory). The *Il-10* gene from the murine sequence (version: mm4) was used as the base sequence and aligned in http://ecrbrowser.dcode.org to other species available, using several vertebrate genomes available from the ECR browser and GALA database. The total sequence analyzed, and shown on all figures, is "-3x" zoomed out from the 5.12 kb coding region of the *Il-10* locus (RefSeq annotation), which can be formatted easily in the bottom window of the ECR browser. The total sequence analyzed is ~15.3kb, with approximately 10kb non-coding regions shown (3' and 5' non-coding regions). The human genome alignment (blastx) is shown in the results section but other

species (rat and dog) have similar homology to the murine *Il-10* locus. Evolutionary conserved regions (ECRs) between murine and human sequences are shown with default criteria settings: 70% homology over at >100 bp and images are exported via the http://zpicture.dcode.org image tool. The genome alignment portal of the ECR browser also permits fast automated transcription factor prediction alignments with generic transcription factor binding site position weight matrix's (PWMs) (>85% confidence shown) as determined by the computational algorithms from http://rvista.dcode.org which is closely interconnected with the TRANSFEC database. The ECRs and predicted transcription factor binding sites in the murine *Il-10* sequence are shown in the results section. Taken further, the individual murine *Il-10* sequence and human genomic alignments can be further annotated and distilled by selecting the link within the ECR Browser labeled: "Synteny/Alignments". Within this window you can further select individual sequences from other species that are shown to align with the base sequence (murine *Il-10* locus), and directly investigate either similarity through phylogenetic shadowing analysis (http://mulan.dcode.org) or transcription factor binding sites (http://rvista.dcode.org). In this thesis, I have chosen the two sections from the human genome that align with the murine *Il-10* locus and further investigated the conserved regions of both these sequences in http://rvista.dcode.org. Within the rvista 2.0 website you can select specific transcription factors to scan your conserved regions of multiple species sequence alignments. In this thesis, I show conserved sequence alignments between both human sequences that correspond to known and novel IL-10-associated transcription factors described from the results section. This data analysis was formatted on the http://rvista.dcode.org website and results were exported to zPicture website for

presentation purposes. In conclusion, the entire *in silico* analysis was performed within the suite of applications available on the http://dcode.org website.

2.12 Retroviral (RV) transduction of CD4 T cells

RV-mediated transduction of investigated transcription factors into primary naïve CD4 T cells cultured in various cell-type specific media is described here. Firstly, the cloned gene (cDNA) of interest is inserted into a SV40-promoter driven expressing vector that conveniently, contains a bicistronic IRES inserted between the gene of interest and the eGFP-reporter tag. On day 0, the packaging cell-line PLAT-Es were plated in 6-well plates in DMEM media (w/ 10% FCS) for a cell-confluence of around 75%. On day 1, FuGene (lipofectamine-based transfection reagent) (Invitrogen) (96µl) and the specific SV40-driven expression vector with your gene of interest (4µl from 1 mg/ml concentrated stock) are mixed in DMEM (FCS-free) according to manufacturers requirements. Then from the stock solution, 100 µl of this solution is added drop-wise to each well of PLAT-E packaging cells and gently mixed by hand for five minutes. Day 2, media is gently aspirated from the PLAT-Es and replaced with 1 ml of DMEM (w/ 10% FCS) and the packaging cells are returned to the incubator. Further, on Day 2 naïve CD4 T cells are MoFlo isolated and stimulated in normal conditions (neutral, Th1, Th2, etc.) described above. Day 3, PLAT-E media containing the RV is gently collected and pooled. PLAT-E media is replaced with 1 ml of DMEM (w/ 10% FCS) and returned to the incubator. Aspirated, pooled RV media is spun at 13,000 rpm for 45 minutes at 4°C. Concentrated RV is collected, (bottom 1/3 of volume from spin) and added drop-wise to CD4 T cells. Additionally, IL-2 is added to CD4 T cells at a corresponding concentrated

amount to achieve normal 5 ng/ml concentration for total volume. Day 4, PLAT-E media containing the RV is gently collected and pooled (same procedure as Day 3). Aspirated, pooled RV media is spun at 13,000 rpm's for 45 minutes at 4°C. Concentrated RV is collected, (bottom 1/3 volume from spin) and added drop-wise to CD4 T cells. No additional IL-2 is required on Day 4. Day 5, cells should be split 1:2 and removed from α-CD3/CD28 conditions (a portion of the cells can be checked here via FACS for transduction efficiency, GFP+). Media conditions are according to respective population conditions (Table 1) except IL-2 is supplemented (5 ng/ml). CD4 T cells are differentiated for 7 days in the respective population conditions and cytokine production is measured by ICS upon re-stimulation. RV-mediated transduction efficiency varies from experiment to experiment but usually 10-30% transduction was achieved with higher percentages in both Th1 and Th2 driven conditions (probably due to higher cell-cycle rates). For RNA preparation and ELISA, transduced cells are further purified by MoFlo isolation based on bicistronic eGFP expression.

	PLAT-E cells	CD4 T cells
Day 0	-Plate PLAT-E in 6 well plates (2×10^5 per well) -Use 2 ml of media per well (media = 10% FCS in DMEM w/ no AB)	
Day 1	-Add 100 ml total of mix into each well (add dropwise): (2ml) vector w/ GFP and gene of interest (6 ml) FuGene (92 ml) DMEM (no FCS, no AB) *Use manufactures recommended steps for mixing	
Day 2	Wash media from well and add 1 ml of fresh DMEM w/ no AB media.	-FACS Sort CD4 T cells and plate at 1×10^6 cells per well in 24-well plates. -Use 1 ml V_T in each well under normal day 0 plate-bound αCD3 (10 μg/ml) soluble αCD28 (2 μg/ml) conditions -Use regular media for T cells (cRPMI)
Day 3	Collect media from PLAT-Es and spin at 13,000 rpm for 45 minutes. Remove top 800 ml and collect the bottom 200 for transfer to T cells. Add 1 ml of fresh DMEM w/ no AB into each PLAT-E well for 2nd round transfection.	-Add 200 ml of concentrated PLAT-E supernatant per well and IL-2 (5 ng/ml)
Day 4	Collect media from PLAT-Es and spin at 13,000 rpm for 45 minutes. Remove top 800 ml and collect the bottom 200 for transfer to T cells. Discard PLAT-E cells	-Add additional 200 ml of concentrated PLAT-E supernatant per well and IL-2 (5 ng/ml)
Day 5		Move T cells and media. Split accordingly (usually I:2). Measure transduction via FACS (GFP+).

Figure 2.5. Schematic of the key points during the RV process.
This schematic highlights the parallel cell culture system key points during the first six-days. PLAT-E cells represent the packaging cell-line used to generate the high-titer viral supernatant and CD4 T cells are representative of the transduced population of cells.

2.13 Chromatin accessibility by real-time (ChART)-PCR assay

IL-4(-/-) or WT naive CD4 T cells were transduced with a "transcription factor of interest"-RV and a Mock retrovirus or cultured under normal Th condition and chromatin were isolated from unstimulated cells and non-digested or digested with 1.0 or 2.5 μg/ml of DNase I (Sigma) (224). For the digestion process cells are pelleted and washed with ice-cold PBS. Cells are re-suspended in 5-ml of ice-cold reticulocyte standard buffer (RSB), avoiding clumps. Lysis buffer (0.5% NP-40 in RSB), 3-ml, is added, mixed and cells are placed on ice for 5 minutes. Ice-cold RBS is then added to V_T= 50-ml, and spun-down at 1,100 rpm for seven minutes at 4°C. Supernatant was discarded and pellet

is re-suspended in 1-ml ice-cold RBS. Three tubes for either un-treated, 1.0- or 2.5-μg/ml of DNase I is prepared here, each tube with 1 μl of $CaCl_2$ and 100 μl of ice-cold cells in RBS. At 37°C the incubation last four minutes for untreated, 1.0-, or 2.5-μg/ml DNase I digestion and an example is illustrated in Table 2 (mixing vial contains DNase I at 0.5 μg/ml).

Table 2.2 DNase I digestion timetables, with the appropriate concentrations of DNase I. Time of addition correlates to the time-point when DNase I (0.5 μg/ml) is added and time of stopping correlates to addition of stop solution (labeled below).

[DNase I] μg/ml	Mixing Vial μl	Time of addition	Time of stopping
0	0	0	4 minute
1.0	0.2	1 minute	5 minutes
2.5	0.5	2 min. 30 sec.	6 min. 30 sec.

The 100-ml stop solution contains: 12 ml of 5 M NaCl, 2 ml of 1 M Tris pH 8.0, 2 ml of 0.5 M EDTA, 10 ml of 10% SDS, and 74 ml of H_2O. After stopping the reaction PK (70 μg) is added per tube and the tubes were then incubated at 56°C overnight. The following morning the DNA is extracted with a normal phenol extraction with EtOH and re-suspended in the appropriate volume (1 μg/ml) of TE.

The digested DNA was purified and 10 ng used as template for real-time PCR amplification with specific oligonucleotides (primers) and SYBR green as described in qPCR studies. Primers were designed throughout the *Il-10* locus under criteria discussed in the results (and according to PrimerExpressv2.1 annealing guidelines). Each set of primers was designed to have an optimal amplicon of 100 bp. Each primer set had to be further quality controlled upon arrival and further normalized by generating a standard curve (Ct values) via amplifying known amounts of template genomic DNA

(1,2,3,4,5,6,7,8,9,10 nanograms of DNA) with the corresponding set of primers. These standard curves were used to convert experimental Ct values for untreated and DNase I-treated biological samples to absolute amount of PCR product obtained in each condition. In accordance with previous reports (225) the absolute amounts of PCR products were used to calculate the percentage of chromatin accessibility. The reported % Chromatin Accessibility was calculated for each primer pair with the following equation: [(No DNase I $_{(gDNA)}$-DNase I $_{(gDNA)}$)/No DNase I $_{(gDNA)}$]*100%. Following this calculation, when a particular site is exposed to DNase I digestion, less absolute amount of PCR product is obtained with primers that span that site, and this will be reflected in a higher percentage of chromatin accessibility.

2.14 Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed with unstimulated or stimulated primary CD4 T cells (1h in the presence of PMA/Ionomycin) (226) (115). Cells were fixed by adding 1/10 volume of 10% formaldehyde in the fix solution (0.1 M NaCl, 1 mM EDTA, 0.5 M mM EGTA, 50 mM Hepes, pH 8.0). Cells and fix solution were mixed well and left for 10 minutes. Following fixation, a stock solution of glycine was used to adjust the final concentration in the cell sample to 125 nM glycine. Wait 5 minutes. Cells were harvested and washed with ice-cold PBS and re-suspended in 10-ml of lysis buffer (0.25% Triton-X, 0.5% NP-40, 10 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl pH 8.0, and 1 tablet of protease inhibitor cocktail, PIC). Cells and lysis buffer were placed on ice and swirled briefly for 10 minutes. After 10 minutes nuclei was pelleted (1,500 rpm for 5 min,) and re-suspended in post-lysis buffer (0.2 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-

HCl pH 8.0, and 1 tablet of protease inhibitor cocktail, PIC). Nuclei was pelleted (1,500 rpm for 5 min,) and re-suspended on ice in sonic buffer (1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl pH 8.0, and 1 tablet of protease inhibitor cocktail, PIC). Samples were sonicated up to 10x with a 20-second constant burst. Between each burst the suspension was cooled on ice for 1 minute. The average fragment size was achieved to be between 0.3-1 kb. Debris was removed after sonication steps by spinning in a clinical centrifuge (speed 4) for 10 minutes. Supernatant was transfer to another 50-ml falcon tube and pellet was discarded.

Chromatin extracts were precleared for 2h at 4°C with salmon sperm-blocked protein A beads (Amersham) overnight. Immunoprecipitation (IP) was performed by diluting the chromatin sample in RIPA buffer (1% Triton-X, 0.1% Na deoxycholate, 0.1% SDS, 140 mM NaCl, PIC) and adding 5 micrograms of the appropriate antibody (GATA-3, acetylated histone H3, etc.) in 0.5-ml, V_T. Complexes were incubated overnight at 4°C. Meanwhile, Protein A Sepharose beads were washed twice in RIPA buffer and incubated with sonicated salmon sperm DNA (0.1 mg/ml, final concentration), overnight at 4°C. The following morning, DNA-protein complexes were recovered by adding 20 µl of the pre-cleared Protein A Sepharose beads for three hours (4°C). After the incubation, immunoprecipitates are wash thoroughly (7x) in 1-ml of RIPA wash buffer (1% Triton-X, 0.1% Na deoxycholate, 0.1% SDS, 500 mM NaCl). Each wash was done at 8,000 rpm for 1 minute and supernatant was aspirated carefully leaving beads intact. After the final wash, the beads were re-suspended in 150 µl of H_2O add adjusted to 0.5% SDS (add 5 µl of 10% SDS). Proteinase K was added to a 0.2 mg/ml final concentration and incubated

for three hours at 55°C. Then incubate overnight at 66°C, in order to reverse the formaldehyde cross-linking. The following morning, the samples were extracted with phenol/chloroform, and then chloroform. Finally, ethanol precipitates the samples with NaOAc and 20 µg glycogen as the carrier. The samples were washed twice with ethanol, and resuspended in 50 µl of H2O. Immunoprecipitated DNA was used as a template for real-time PCR with specific oligonucleotides for the *Il-10* locus and the IL-5 promoter. Ct values for GATA-3 (or antibody of interest) and control antibody (anti-rabbit IgG) were normalized to the input Ct (with same primer pair) from the same sample preparation, and the GATA-3 versus control ratio was reported using the same formula as relative gene expression results (qPCR).

RESULTS

3. Profiling CD4 T cell populations

Six primary derived CD4 T cell populations (naïve, neutral, Th1, Th2, IL-10-T_{reg}, CD25+T_{reg}) were generated (*ex vivo* or *in vitro*) for analysis of both *Il-10* gene regulation and CD4 T_{reg}-mediated functional properties. The six populations of CD4 T cells chosen for this study represent a homogeneous repertoire of populations that exist in the peripheral T cell compartment of a host: naïve T cells, effector Th cells, and T_{reg}. In this Chapter, I have used the Affymetrix Mouse 430v2.0 GeneChip to profile their transcript-ome. Affymetrix GeneChips were chosen for two general reasons: (1) extensive coverage of the murine transcript-ome (>45,000 transcripts) and (2) confidence in reproducibility achieved through high manufacturing quality-control features and multiple probes (11) for each transcript (for more information refer to the Materials and Methods). One limitation in GeneChip experiments is the investment in both money and time and therefore one time-point was chosen for the stimulated condition. The kinetics (1 hr – 24 hr) of both cytokine and transcription factor expression was initially evaluated via qPCR and the 6 hour time-point was chosen as the profiled "stimulated" time-point. In conclusion, the GeneChip profile for each population was resting state and activated state (6 hours after stimulation via the TCR pathway).

CD4 T cells are also profiled here for expression of transcription factors outlined in regulation of effector and regulatory populations and their products and function (Introduction). The transcription factor expression profiles are described by their population-specific mRNA up-regulation, but also strongly chosen as a focus by the absence or low-abundance expression in other CD4 T cell populations. Also of note in

this chapter, I describe side-by-side activation of two different well-described CD4 T cell TCR-pathway stimuli, PMA/ionomycin and plate-bound α-CD3/soluble α-CD28, in terms of their subsequent cytokine profiles (mRNA and protein). Importantly, each population was stimulated in parallel with either PMA/ionomycin or α-CD3/CD28 to eliminate non-stimuli specific variables. PMA/ionomycin stimulation is widely used as a method to stimulate CD4 T cells; however this stimulus bypasses TCR and co-stimulation regulated signaling. Therefore, validation (qPCR) was performed via an α-CD3/CD28 stimulus, mediated via the extra-cellular receptor (more representative of APC:TCR interactions), which consequently induces TCR-pathway activation in the context of co-stimulators. Finally, this chapter validates the homogeneous nature of each CD4 T cell population through their unique cytokine and transcription factor profiles as well as setting the stage for their use in subsequent result chapters. The classic cytokine and hallmark gene expression markers from each individual population are profiled here.

3.1 CD4 T cell populations have unique cytokine profiles

3.1.1 Protein

CD4 T cell populations were stimulated for 48 hours in cytokine/antibody-free media with either soluble PMA/ionomycin or plate-bound α-CD3 and soluble α-CD28 (Fig. 3.1). Each population was stimulated in the absence of cytokines, APCs, and at a universal concentration/confluence. The protein concentration was detected using the Luminex multi-plex (xMap) system and shown here in Figure 3.1. The cytokines are clustered within four subsets based on their expression profiles across the CD4 T cell populations.

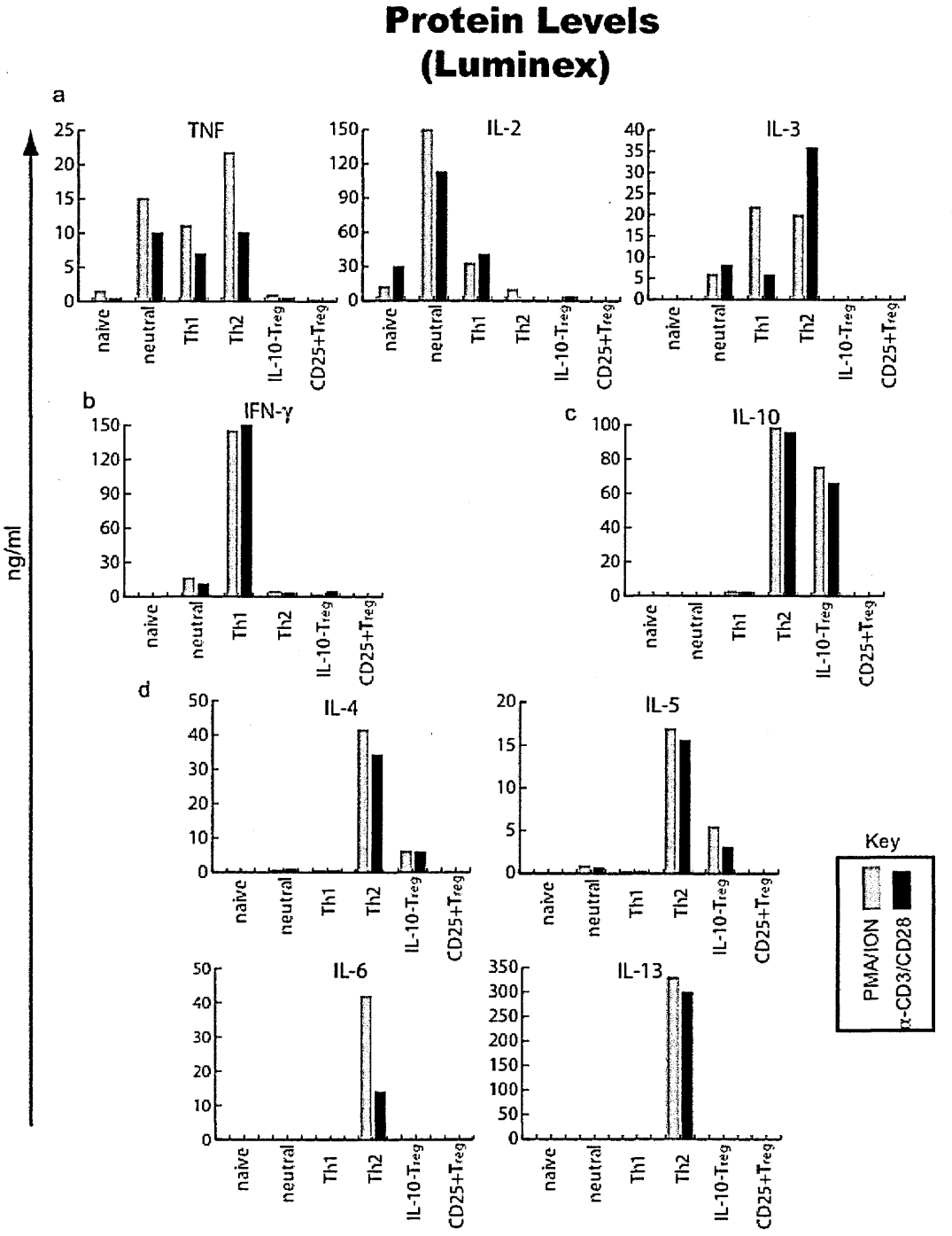


Figure 3.1. Cytokine production in CD4 T cell populations.
Cytokine protein concentration levels were measured with the Luminex xMap bead assay. Antibody-coated beads were acquired from UpState signaling. Results are a representation from one well (96-well plate) for each stimulus (α-CD3/CD28 or PMA/Ionomycin) measuring 9 cytokines simultaneously for each CD4 T cell population. Standard curves for each cytokine were generated from standards supplied by UpState Signaling. This figure is representative of at least 3 biological replicates.

IL-2, IL-3, and TNF are labeled in Figure 3.1a to signify their presence in effector and naïve populations. IL-2 production was more prominent in the naïve and neutral populations, IL-3 production more specific to effector populations, and TNF production shared to some degree between naïve and effector populations. As predicted, IFN-γ is exclusively produced by Th1 cells and represents a unique profile as opposed to the other analyzed cytokines here (Fig. 3.1b). IL-10 production is produced at high concentrations in both the Th2 and IL-10-T_{reg} populations, and relatively low concentration production in Th1 cells (Fig. 3.1c). IL-4, IL-5, IL-6, and IL-13 are produced predominately by the Th2 cells and low concentration amounts of IL-4 and IL-5 in the IL-10-T_{reg} (Fig. 3.1d). Both stimuli (PMA/ionomycin versus α-CD3/CD28) induce similar cytokine profiles; however some cytokines (TNF, IL-6 and IL-13) were detected with slightly higher-concentration amounts with PMA/ionomycin versus α-CD3/CD28 TCR triggering. In summary, protein profiles of proliferative, effector and regulatory cytokines confirm homogenous populations and both TCR-activation induce similar protein profiles within each CD4 T cell population.

3.1.1 Affymetrix GeneChip Profiles

Each CD4 T cell population was profiled for their representative gene expression profiles. Total RNA was isolated at the corresponding time-points and mRNA was further processed to a representative target cRNA for hybridization on the oligo-based GeneChip. The GeneChip data was processed with the GC-RMA algorithm to determine the quantitative abundance of each transcript (>45,000). Each individual GeneChip is further normalized with respect to the collective GeneChips quantitative signal and the

final individual transcript-abundance is profiled with the Genespringv7.0 application. I have generated various differential expression profiles and I will begin with a parallel expression profile Figure 3.2 corresponding to the protein profiles in Figure 3.1 in stimulated conditions.

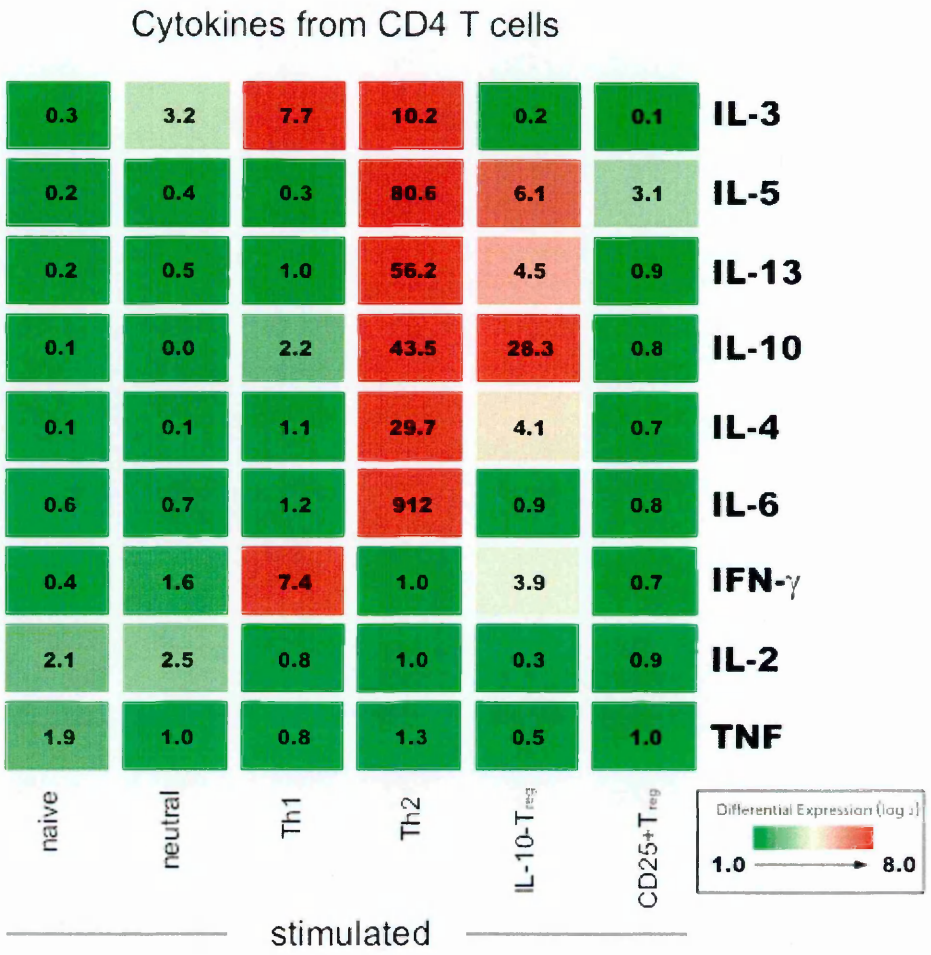


Figure 3.2. Profiling key cytokines in stimulated CD4 T cells.
GeneChip profiles for signature CD4 T cell cytokines (stimulated condition, 6 hr. post-TCR pathway activation). Cytokines are displayed in an unsupervised hierarchical cluster format. The GeneChip image was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. More information is available in Materials and Methods for the computational details.

GeneChip profiles in Figure 3.1 parallel the protein results shown in Figure 3.2. Differential expression values for individual transcripts are normalized to a medium logarithmic value, 1.0. The vertical progression of each transcript is the result of an

unsupervised hierarchical clustering technique (as described in the Materials and Methods). IL-2 and TNF are up-regulated in naïve and effector populations, whereas, IL-3 is up-regulated in effector populations (Fig. 3.2). IFN-γ forms a unique cluster with up-regulation on activation in the Th1 population and slight up-regulation in IL-10-T_{reg} (Fig. 3.2). Th2-associated cytokines, IL-4, IL-5, IL-13, cluster together with high differential up-regulation in Th2 cells and slight up-regulation in IL-10-T_{reg} (Fig. 3.2). Finally, significant and differential up-regulation of IL-10 in both Th2 and IL-10-T_{reg} populations (Fig. 3.2) correspond with the IL-10 protein results in Figure 3.1. Confirming protein results, CD25+T_{reg} do not differentially up-regulate cytokine transcripts (Fig. 3.2). GeneChip data illustrates the individual low-abundance of cytokine transcripts in naïve, neutral, and CD25+T_{reg} as compared to Th1 and Th2 high-abundance levels (Fig. 3.2). In a similar fashion, Th1 and Th2, clearly cross-regulate the cytokine expression of their corresponding hallmark cytokines, IFN-γ and IL-4, respectively, in keeping with previous reports (Fig. 3.2) (4, 5).

3.1.1 qPCR profiles

Figure 3.3, shows key cytokines validated from the GeneChip profiles with real-time quantitative PCR (qPCR) at the corresponding time-point, 6 hours after stimulus. Both pathway of stimulation are included in the qPCR data (Fig. 3.3a and 3.3b). The relative amount of expression between populations for individual cytokines shown here does not vary with stimulus. The qPCR cytokine results are in agreement with the cytokine protein results, in that either stimuli will induce a similar quantitative cytokine profile (mRNA and protein). Together, the qPCR results verify the differential mRNA results

acquired via the GeneChip (Fig. 3.3) and provide a quantitative relative expression value to compare across the CD4 T cell populations.

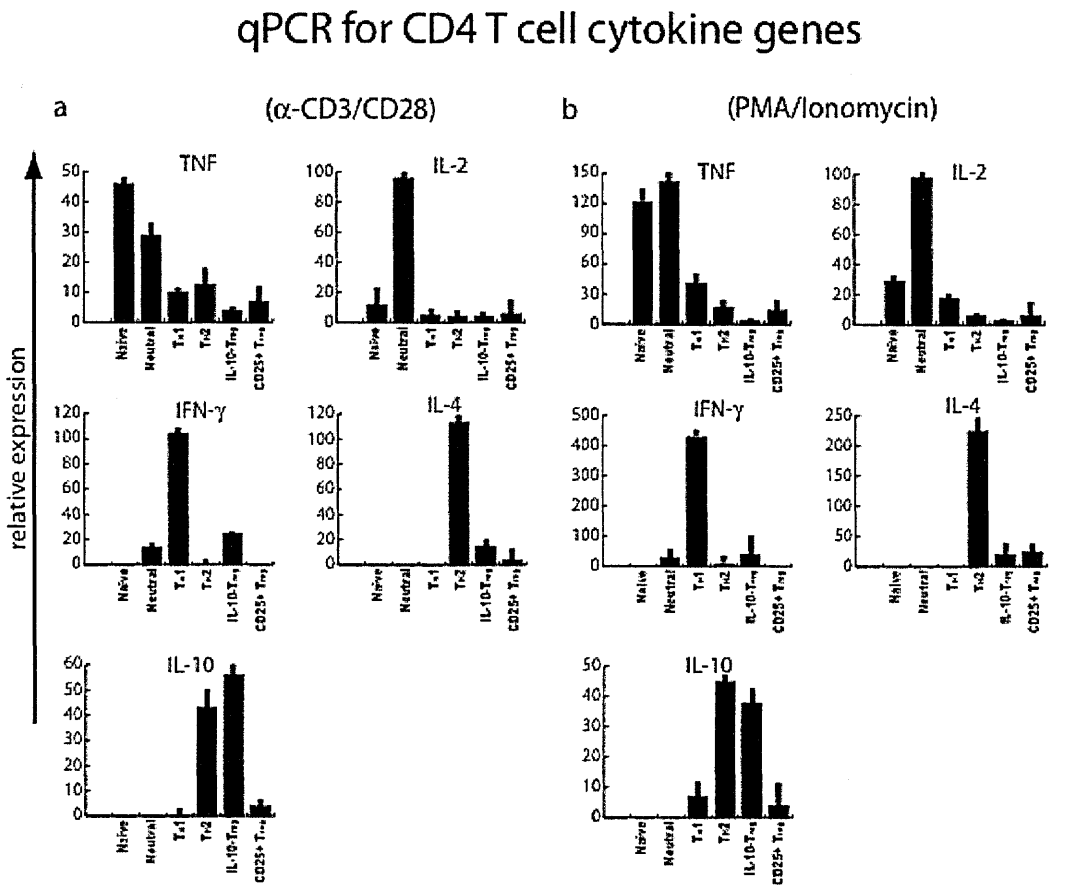


Figure 3.3. qPCR validation of key cytokine in CD4 T cell populations.
Real-time reverse transcriptase PCR (qPCR) for selected cytokine genes. (a) CD4 T cells were stimulated with α-CD3/CD28, mRNA collected at 6 hr. post-TCR activation, converted to cDNA and qPCR was performed with primers and SYBR Green mix to measure relative expression of labeled cytokine transcripts on the ABI Prism 7000. (b) CD4 T cells were stimulated with PMA/Ion, mRNA collected at 6 hr. post-TCR activation, converted to cDNA and qPCR was performed with primers and SYBR Green mix to measure relative expression of labeled cytokine transcripts on the ABI Prism 7000. Three biological samples were used to generate the displayed relative expression value for each transcript, and standard deviation (+/-) was calculated as shown.

3.2 Signature Transcription Factors in Effector Th and CD25+T_{reg}

Similar to the previous GeneChip cytokine transcript profiles (Fig. 3.2), key transcription factors are profiled here. Transcription factors were chosen here as outlined within the "Introduction" section as being essential in differentiating effector and CD4 T_{reg} populations. Specifically, T-bet, Ets-1, Hlx are profiled here for their regulation of the *Ifn-γ* gene, GATA-3, c-Maf, GFI-1, for regulation of Th2-associated genes, and the lineage-specific transcription factor for CD25+T_{reg}, FoxP3. Transcription factors profiled below are only shown at the unstimulated time-point. This was done for three reasons. Firstly, each transcription factor is regulated at its own locus via different mechanisms after TCR-mediated stimulus. Secondly, the range of kinetics for optimal transcription after stimulus is variable between transcription factor genes (whereas, the cytokine genes were similar at the 6 hr. time-point) and therefore the stimulated GeneChip data may not demonstrate the ideal time-point for up-regulation for each transcription factor. Thirdly, the achieved function of each of these profiled transcription factor genes occurs before secondary TCR triggering or very shortly after TCR-pathway stimulus and is a hallmark of each differentiated CD4 T cell population. Thus, their differential expression profile will be profiled at the unstimulated time-point.

3.2.1 Affymetrix GeneChip profiles

Key CD4 T cell transcription factors are profiled here (Fig. 3.4).

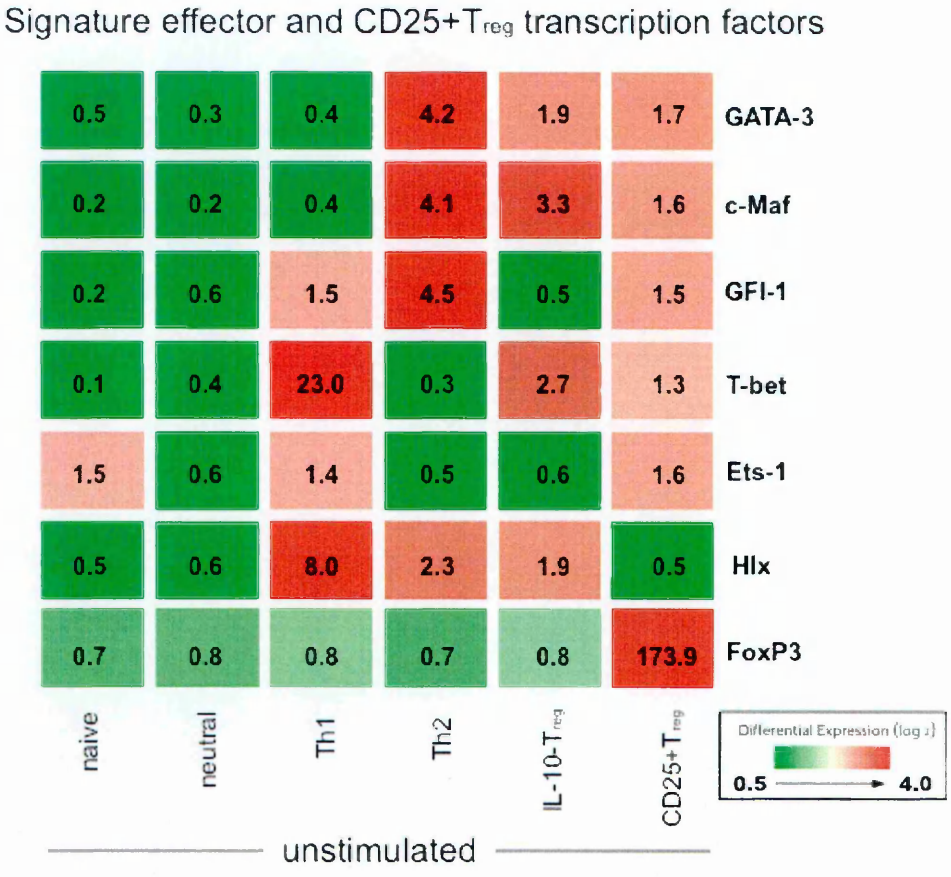


Figure 3.4. Profiling key transcription factors in CD4 T cell populations.
GeneChip profiles for signature CD4 T cell transcription factors in CD4 T cells. The GeneChip image was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. More information is available in Materials and Methods for the computational details.

Figure 3.4 demonstrates Th1, Th2 and CD25+T_{reg} express the signature transcription factors T-Bet/Ets-1/Hlx (Th1); GATA-3/c-Maf/GFI-1 (Th2); FoxP3 (CD25+T_{reg}), respectively. IL-10-T_{reg} population express low levels of both Th1- and Th2- associated transcription factors: T-Bet, Hlx, GATA-3, and c-Maf; however GFI-1 and not surprisingly FoxP3 (174) are not detected in IL-10-T_{reg} by GeneChip (Fig. 3.4). Furthermore, the CD25+T_{reg} express low amounts of T-Bet, GATA-3, c-Maf and GFI-1 (Fig. 3.4). Lastly, the transcription factors T-bet and GATA-3/c-Maf are highly cross-regulated in both Th populations, which mirrors the high cross-regulation of their target cytokine gene networks in Figure 3.4.

3.2.2 qPCR

Transcription factors shown in Figure 3.4 were validated by qPCR to show quantitative levels of expression was similar to the differential levels of expression reported via the GeneChip. Together, the qPCR results (Fig. 3.5) shown below verify the differential mRNA results acquired via the GeneChip (Fig. 3.4) and provide a quantitative relative expression value to compare across the CD4 T cell populations.

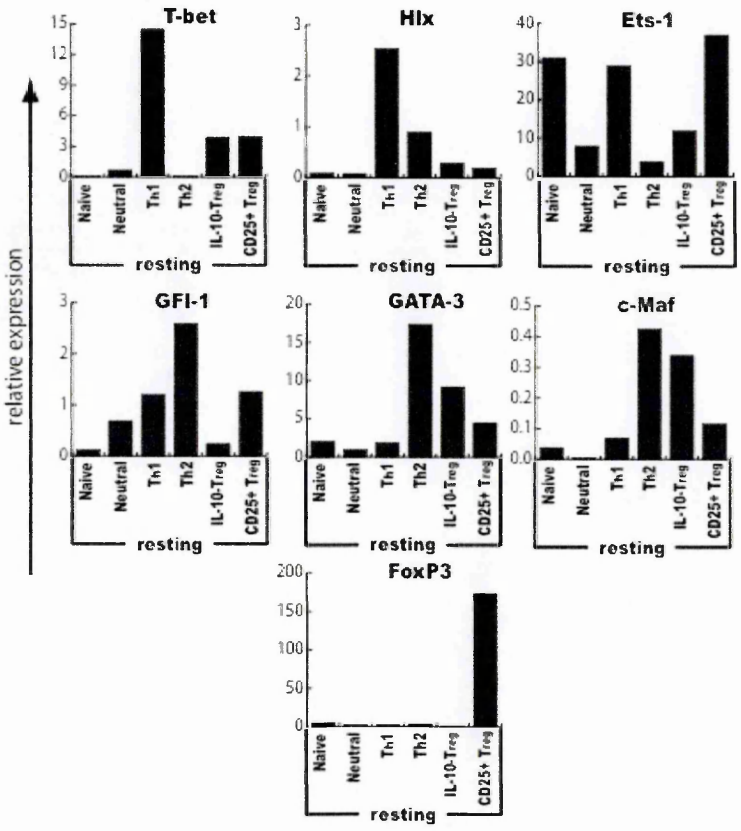


Figure 3.5. qPCR validation of key transcription factors in CD4 T cell populations.
Real-time reverse transcriptase PCR (qPCR) for signature CD4 T cell transcription factors in CD4 T cells (same as Figure 3.4). mRNA was isolated from unstimulated CD4 T cells, converted to cDNA and qPCR was performed with primer/probes acquired from ABI (inventoried: assay-on-demand) and mastermix (ABGene) was used to measure relative expression of labeled transcription factor transcripts on the ABI Prism 7900. This figure is representative of at least 3 biological replicates.

3.3 Discussion

Six populations of *ex vivo* and *in vitro* generated CD4 T cell populations were profiled to represent phenotypic CD4 populations that could exist in the peripheral-host environment after immune challenge with various pathogens, allergens or other antigens (5). The cytokine and transcription factor profiles shown here demonstrate that each CD4 T cell population is phenotypically different as described by their cytokine profile (mRNA and protein) after TCR-pathway stimuli and transcription factor profile (mRNA) at the unstimulated time-point. The differentiation process of naïve CD4 T cells towards T helper cell populations is necessary for expression and production of key effector-molecules: cytokines. This process is mediated by highly-regulated intrinsic signaling which up-regulates expression of necessary transcription factors essential in cytokine gene regulation. These key transcription factors were profiled here at the unstimulated time-point and for cytokines after stimuli. As expected the CD4 T regulatory populations express or produce little to no effector-mediated cytokines, although as expected IL-10-T_{reg} produced IL-10. However, within the CD4 T_{reg}, IL-10-T_{reg} and CD25+T_{reg} differ in their respective amounts of FoxP3 expression, which has been shown to induce strong regulatory properties as demonstrated by its transduction into naïve CD4 T cells (122, 124). This suggests that IL-10-T_{reg} acquire their regulatory phenotype without the expression of the lineage-specific "natural-occurring" CD4 T_{reg} marker FoxP3 as described (discussed later) (174). This section also validates the level of confidence to which we can compare the GeneChip generated mRNA profiles to quantitative qPCR generated mRNA profiles, for both the high-expression transcripts (cytokines) and low-expression transcripts (transcription factors). In summary, this chapter validates the

homogeneous, phenotypic profile of each CD4 T cell population while confirming the ability to compare cytokine mRNA profiles (GeneChip or qPCR) to protein profiles (Luminex) for either PMA/ionomycin or α-CD3/CD28 TCR-pathway stimuli as the basis of the future studies in this thesis.

4. IL-10-associated transcription factors in CD4 T cells

The *Il-10* gene can be regulated by several transcription factors as demonstrated by studies performed in various cell-types (mostly cell-lines); however a universal understanding of *Il-10* gene regulation is not yet clear, especially in terms of IL-10-producing CD4 T cells. A section of literature has shown enhancement of *Il-10* gene transcription using dual-transfection of (1) the minimal *Il-10* promoter region (proximal 5' region, ~ 1.5 kbp) and (2) specific transcription factors (Sp1 (118, 227), Sp3 (227), c-Maf (116) and c/EBP-β {Liu, 2003 #137}) which induce increased IL-10 expression. These studies further showed each transcription factor bound putative sites within the proximal IL-10 promoter as demonstrated when these sites were mutated the corresponding IL-10-enhancing transcription were reduced in the dual-transfection studies (performed in cell-lines). Additional *Il-10* gene regulation studies use transfection or transduction of transcription factors (c-Maf (116), SMAD-4(117), c-Jun (112) and JunB (112)) into primary immune cells to induce IL-10 expression upon stimulus. Conversely, two transcription factors (Ets-1 (72) and NFATc2 (114)) have been implicated in repression of IL-10 expression. Many questions remain in terms of *Il-10* gene regulation especially at the molecular level.

Therefore, I will start the analysis of *Il-10* gene regulation by comparing global expression profiles of two phenotypically different CD4 IL-10-producing populations (Th2 and IL-10-T_{reg}) versus four non-IL-10-producing CD4 T cell populations (naïve, neutral, Th1, CD25+T_{reg}). Th1 cells transcribe and produce small amounts of IL-10 upon stimulus; however for our purposes here I will initially consider Th1 cells to be a "non"-

IL-10-producing population in the global expression-profiling studies. To increase the computational sensitivity and specificity from the GeneChip data, I have included populations of non-IL-10-producing CD4 T cells at different levels of CD4 differentiation and secondly utilized computational techniques (GC-RMA algorithm) to amplify signal-to-noise levels of low-expressing transcripts from the GeneChip (transcription factors inherently express low quantitative levels of transcripts in comparisons to other gene families). I will establish criteria to filter all possible transcription factors (transcriptions factors are defined from gene ontology (GO) classification as "DNA-binding genes"). Any of these may be involved in *Il-10* gene regulation in CD4 T cells based upon their mRNA profile. The general assumption being, I will be able to identify novel and major instructive transcription factors involved in *Il-10* gene regulation because such transcription factors will be differentially expressed in IL-10-producing CD4 T cell populations versus a robust panel of non-IL-10-producing CD4 T cell populations.

4.1 Known IL-10 associated transcription factors

Multiple transcription factors have been reported to regulate *Il-10* gene expression as described through their corresponding studies above. Since the mechanism for some of these transcription factors, in terms of *Il-10* gene regulation, is not yet clear I will profile both unstimulated and stimulated time-points. "Known" transcription factors that may enhance or repress IL-10 production are profiled here by their mRNA expression in both non-IL-10-producing and IL-10-producing CD4 T cells (Fig. 4.1).

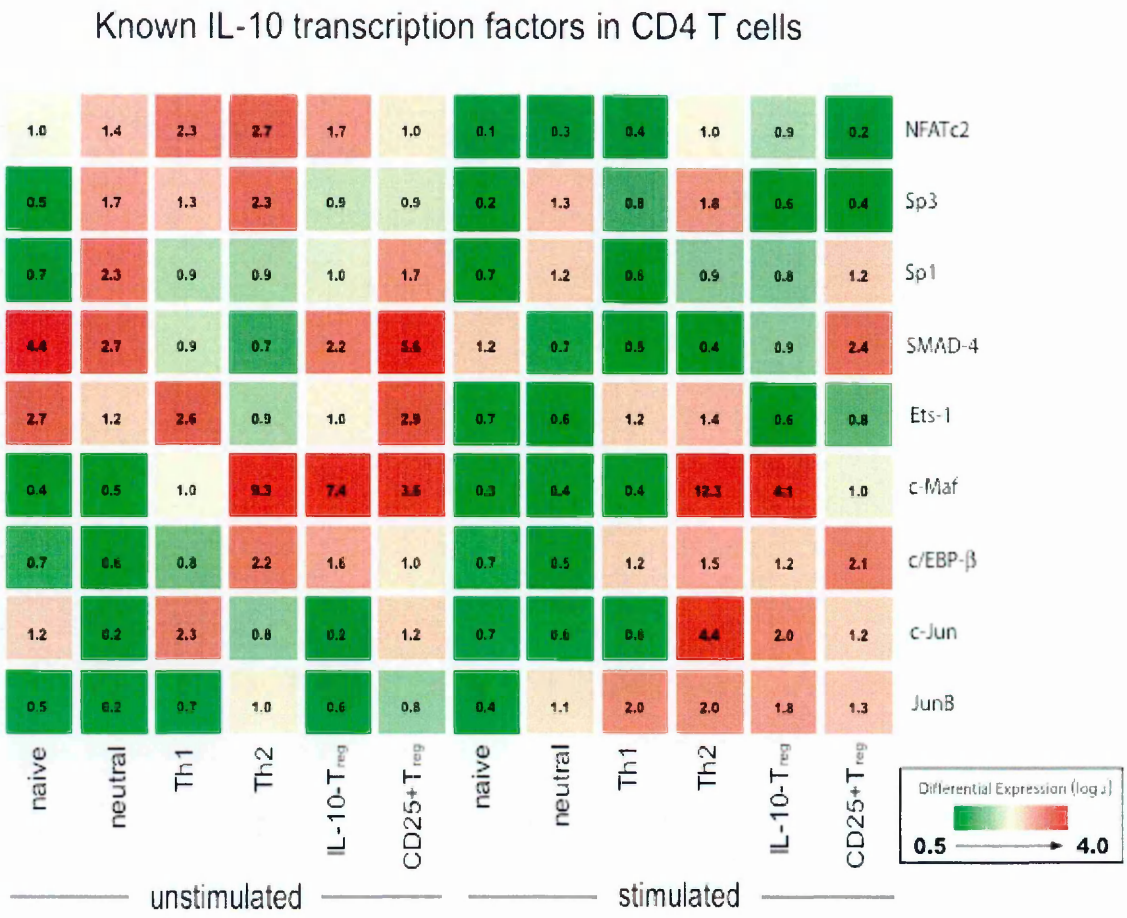


Figure 4.1. Profiling known IL-10-associated transcription factors in CD4 T cell populations. GeneChip profiles for known IL-10-associated transcription factors in CD4 T cells. The GeneChip image was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. More information is available in Materials and Methods for the computational details.

Transcription factors Sp1 and Sp3 are expressed in all CD4 T cell populations (data not shown, qPCR) and not exclusively expressed in IL-10-populations (Fig. 4.1). Interestingly, c/EBP-β and c-Maf are differentially up-regulated in unstimulated IL-10-producing populations, and c-Maf is differentially up-regulated exclusively in IL-10-producing populations (IL-10-T_{reg} and Th2) (Fig. 4.1). Jun family members, c-Jun and JunB, are shown here to be expressed in many CD4 T cell populations; however both c-Jun and JunB become up-regulated in IL-10-producing populations in stimulated conditions (Fig. 4.1). SMAD-4 is not differentially expressed in IL-10 producing

populations, but is up-regulated to a small extent in CD25+T_{reg} (Fig. 4.1). Potential IL-10 repressors: NFATc2 (114) is up-regulated in both IL-10-producers (Th2 and IL-10-T_{reg}) as well as Th1 cells at the unstimulated time-point (Fig. 4.1).

4.2 Novel IL-10 associated transcription factors

The expression of transcription factors exclusively expressed in IL-10-producing cells from the Affymetrix data is reported here. The criteria for stratifying theses "IL-10-associated transcription factors" are listed below.

(1) IL-10-associated transcription factors must have a minimum level of raw signal (>200 with GC-RMA) within the IL-10-producing populations.

(2) IL-10-associated transcription factors must be exclusively expressed above a minimum fold differential (>1.8 fold) in both IL-10-producing populations.

(3) The initial list of candidate IL-10-associated transcription factors includes genes with a known DNA-binding motif in their structure (4,000 transcripts).

I will describe three lists of IL-10-associated transcription factors, which were independently generated using the criteria: (1) all time-points, (2) unstimulated time-points and (3) stimulated time-points. It should be noted that transcription factors associated with IL-10-producing cells could be involved in directly down-regulating other cytokines absent in IL-10-producing cells; however this type of transcription factor can only be defined with subsequent functional studies. Figure 4.2 represents the IL-10-associated transcription factors isolated using the criteria above including both unstimulated and stimulated time-points.

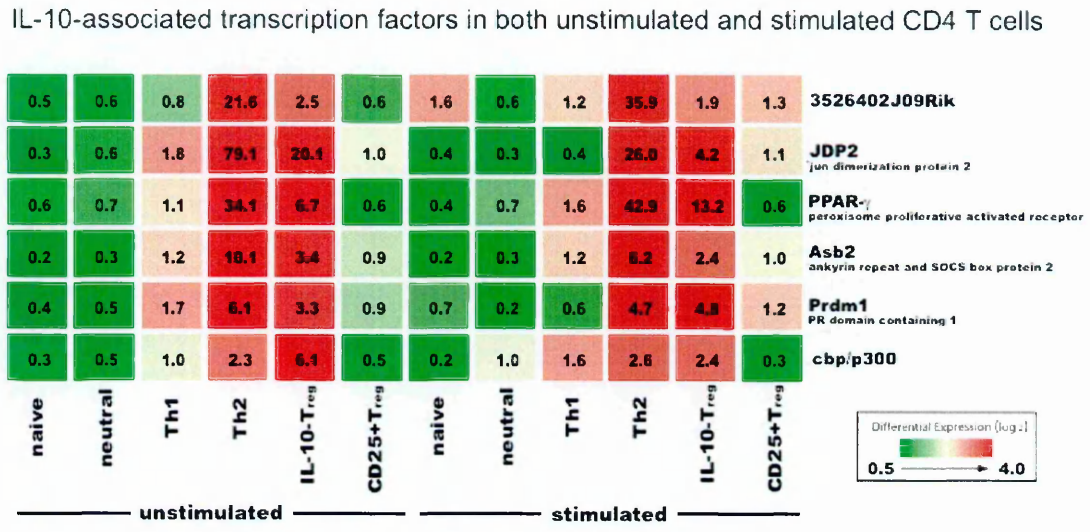


Figure 4.2. Profiling IL-10-associated transcription factors in CD4 T cell populations.
GeneChip profiles for IL-10-associated transcription factors in CD4 T cells. The GeneChip image was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. More information is available in Materials and Methods for the computational details.

These six transcription factors are highly expressed in IL-10-T_{reg} and Th2 cells before and after stimulus but some are also expressed at a low-level in Th1 cells (which produce low amounts of IL-10): JDP2, PPAR-γ, Asb2, and Prdm1 (Fig. 4.2). Transcription factors in Figure 4.2 are down-regulated in unstimulated naïve and neutral CD4 T cells. Three of these six transcription factors, JDP2 (jun dimerization protein 2), PPAR (peroxisome proliferative activated receptor)-γ and cbp/p300 (Fig. 4.2), have been described to some respect in their ability to mediate transcriptional activity. JDP2 was first described to bind to c-Jun subunits and thereby suppress c-Jun ability to form active Jun:Fos dimers (228). The functional inhibition of Jun:Fos dimers is intriguing due to the well-described role of AP-1 (Jun:Fos) dimers in regulating cytokine genes upon TCR activation (59). However, this is speculative because little is known about the function of JDP2 in CD4 T cells. PPAR-γ is a nuclear receptor that is activated through nuclear ligand/receptor interactions which induces active protein-protein interactions and DNA

binding. There is a large body of literature suggesting that activation (ligand-mediated) of endogenous PPAR-γ in precursor mesenchymal cells activates differential gene expression targets and influences subsequent differentiation (229). The mechanism by which active PPAR-γ, influences this decision-point in the precursor mesenchymal differentiation process is not clear (i.e.: via repression or enhancement of gene transcription). In CD4 T cells, two known ligands for PPAR-γ, 5-deoxy-Delta 12,14-PGJ2 (15d-PGJ2) and ciglitazone, are described to mediate inhibition of IL-4 (230) and IFN-γ (231) upon TCR activation. However, the mechanism for these results was not clear, due to mixed cultures (APCs and CD4 T cells) and the cell-specific potential effects of the potent ligands, 15d-PGJ2 and ciglitazone (230, 231). cbp/p300 is functionally classified as an important partner in the RNase II polymerase transcriptional machinery, but little is known about its differential expression being involved in lineage-dependent or target gene regulation (232). Very little is known about the general function or role of Asb1, Prdm1, and 3526402J09Rik genes to bind putative DNA motifs or functionally regulate gene transcription.

In order to expand the list of possible candidate transcription factors that would correlate with expression of IL-10, I have generated lists of hypothetical IL-10-associated transcription factors by applying the same criteria in Figure 4.2 but normalizing GeneChip profiles at unstimulated (Fig. 4.3) or stimulated (Fig. 4.4) time-points independently of each other.

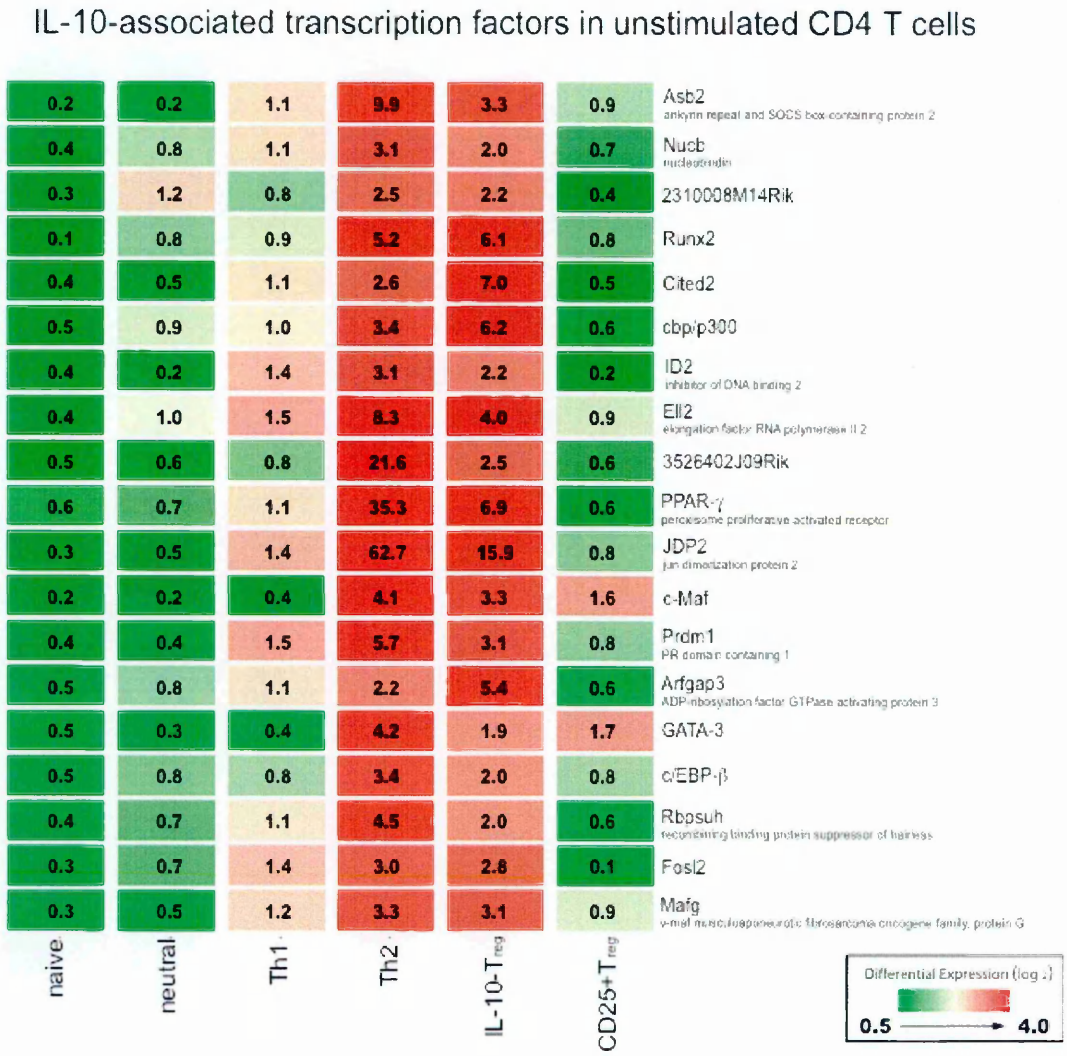


Figure 4.3. Profiling IL-10-associated transcription factors in unstimulated CD4 T cell populations. GeneChip profiles for IL-10-associated transcription factors in unstimulated CD4 T cells. The GeneChip image was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. More information is available in Materials and Methods for the computational details.

In Figure 4.3, I have isolated nineteen transcription factors by only evaluating the unstimulated time points from the GeneChip data. From this list I have validated (qPCR) six genes (four are shown in subsequent Chapters). Two genes, GATA-3 and c-Maf, already have been described to influence the chromatin structure and *trans*-activation of specific Th2-associated cytokine loci (40). GATA-3 and c-Maf are shown here in be up-

regulated the Th2 and IL-10-T_{reg} (which do not express Th2-associated cytokine genes or at least at a very low amount: IL-4, IL-5, IL-13) populations. We show here that c/EBP-β, which has been described to *trans*-activate the IL-10 promoter in minimal promoter enhancement studies (233), to be up-regulated in resting IL-10-producing CD4 T cells. The unstimulated GeneChip data will serve as important means for justifying IL-10-producing transcription factors in the upcoming Chapters.

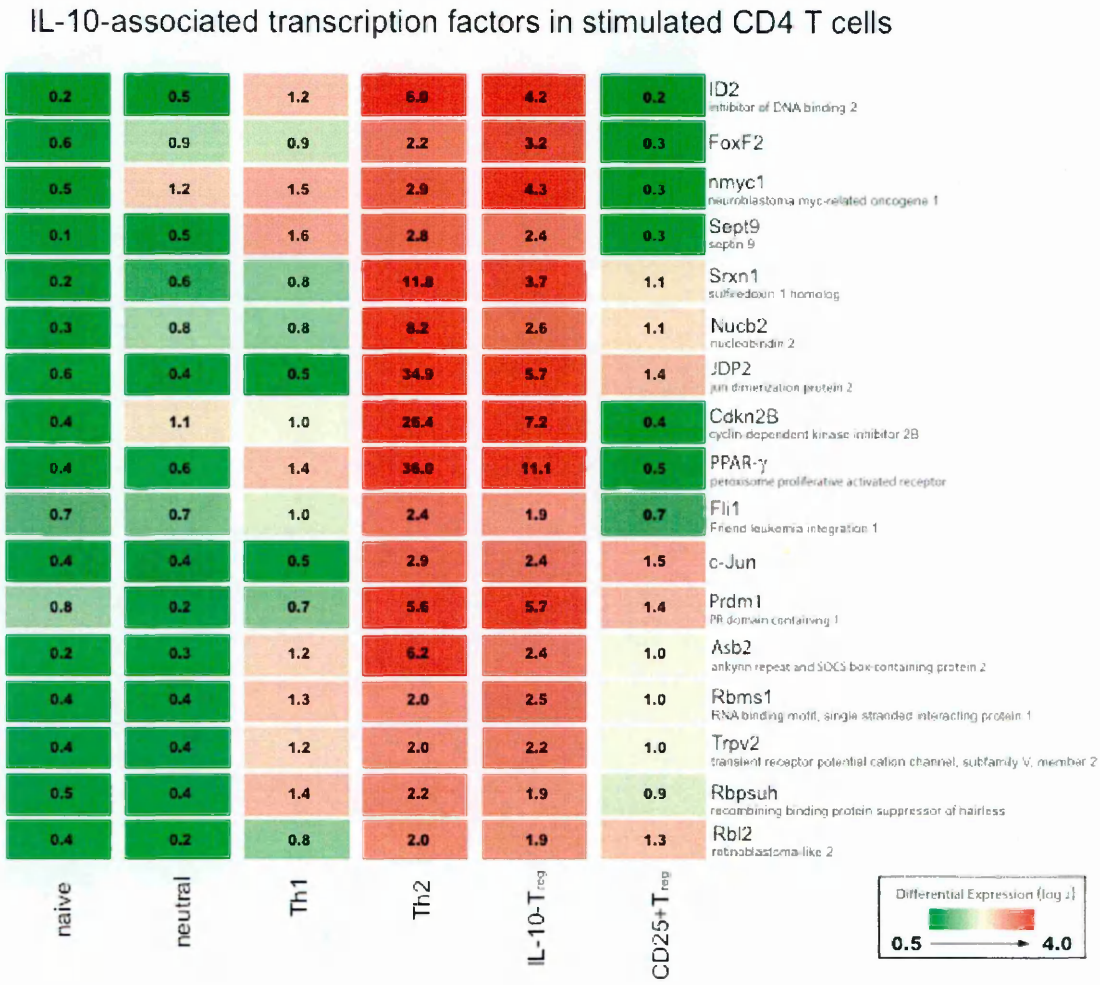


Figure 4.4. Profiling IL-10-associated transcription factors in stimulated CD4 T cell populations. GeneChip profiles for IL-10-associated transcription factors in stimulated CD4 T cells. The GeneChip image was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. More information is available in Materials and Methods for the computational details.

Finally, Figure 4.4 reveals seventeen IL-10-associated transcription factors isolated by evaluating the stimulated GeneChip data independent of the unstimulated condition.

4.3 Discussion

Figure 4.4 concludes the novel IL-10-associated transcription factor search derived the GeneChip profiles. This Chapter represents an essential part of the search for *Il-10* gene transcription factor regulators in CD4 T cells. Firstly, I have taken an "unsupervised" approach in collecting over 4,000 possible transcript profiles (based on DNA-binding motifs) and mined their expression profiles with a broad criteria developed to isolate correlating IL-10-associated expression profiles. This represents a strong hypothesis-driven approach to discovering novel IL-10-associated transcription factors. In general, the IL-10-associated transcription factors were detected in IL-10-T_{reg} and Th2 cells both of which produce high amounts of IL-10 (Fig. 3.1). This process was possible because of the format in which GeneChip (or other mRNA arrays) generate global expression profiles. However, this ambitious, global, unsupervised approach has its drawbacks in that it can increase the probability of collecting false-positives (as well as missing false-negatives that do not follow IL-10-associated expression profiles). Therefore, the lists of IL-10-associated transcription factors will be compared to (1) literature-based information available on potential function (2) comparative studies involving the *in silico* evaluation of the *Il-10* locus for potential putative regulatory binding sites (next Chapter) before they are further analyzed for functional regulation of the *Il-10* gene.

5. *In silico* and ChART-PCR of the *Il-10* gene

The combination of publicly available bioinformatic information and increasingly robust computational algorithms has allowed researchers to complement hypothesis-driven lab research performed at the bench with an equal amount of *in silico* data (234). For this thesis I have shown select transcription factors, which are differentially up-regulated in IL-10-producing CD4 T cells. In order to complement this data, an independent *in silico* analysis of the *Il-10* gene using computational algorithms (dcode.org) was made to predict putative transcription factor binding sites (motifs) throughout the *Il-10* locus. Furthermore, by highlighting genomic sequence-specific DNA orthologs from cross-species comparisons we can generate evolutionary conserved regions (ECRs) (235) which reveal conserved non-coding sequences throughout the *Il-10* locus region. ECRs from noncoding regions of mammalian genomes are generally assumed to hold several keys for sophisticated gene regulation signals that determine when, where and to what extent lineage dependent decisions are made at the transcriptional level (235). Thus, using ECRs and putative binding sites *in silico* data will enrich the search for IL-10-associated transcription factors.

ECRs have also proven to add-value to labor-intensive DNase I HSS studies (234), which have been described as the "gold-standard" in forecasting regions of gene regulation (234). Promoters, enhancers, suppressors, inducible gene regulatory regions and generalized locus control regions (LCR) have all been associated with HSS (234). DNase I HSS-generated data is advantageous for our purposes because we can use different CD4 T cell populations and assess the nucleosome-free regions of the *Il-10* locus, allowing for

identification of differentially exposed ubiquitous or lineage specific regulatory elements. In this study I used a highly sensitive DNase I digestion technique, chromatin accessibility by real-time (ChART)-PCR, to compare low-abundance IL-10-producing Th1 chromatin and high-abundance IL-10-producing Th2 chromatin for differential areas of re-modeling within the *Il-10* locus in unstimulated conditions. This technique (described in detail within the Materials and Methods Chapter) requires that the regulatory elements be first previously defined since PCR primers are then designed flanking the regulatory elements. In summary, freely available comparative genomic with predictive transcription factor binding sites programs will help profile the evolutionary content and lab research performed at the bench by the DNase I digestion ChART-PCR technique will define the architecture of the *Il-10* locus in differential IL-10-producing populations. Together, these studies help select transcription factors to pursue in functional studies.

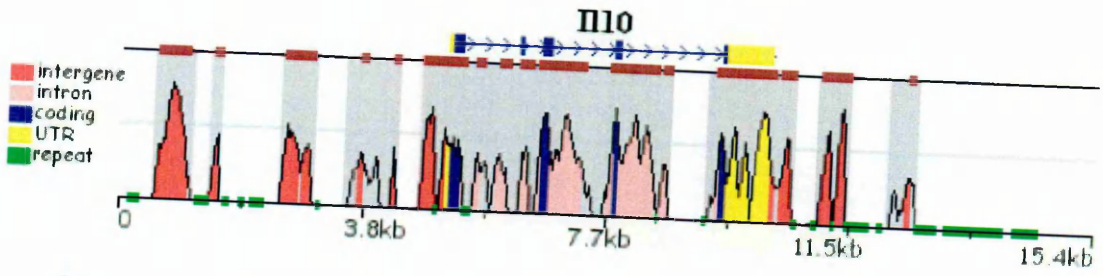
5.1 Predicted transcription factor binding sites

To analyze the *Il-10* locus *in silico* I have selected ~15.4 kbp of the IL-10 murine sequence (chr1:130884907-130900284) which includes approximately 5 kbp upstream and downstream of the *Il-10* coding region (5.12 kbp). Initially, the analysis of this sequence for putative transcription factor binding sites (with default settings) using a complete list of predicted transcription factor matrixes over 8,200 transcription factor binding sites are predicted (data not shown, rvista.dcode.org). A more supervised approach is performed by selecting regions of potential regulatory regions through analyzing ECRs (234). ECRs are identified by blastx alignment of (1) the *Il-10* gene

from a base sequence and (2) whole genomes from multiple other species. ECRs in this thesis are identified by having at least 70% identity over a span of at least 100 base-pairs. For the purpose of this study I will show the alignment of the mouse (base sequence) to human genome. This result is shown in Figure 5.1a. Figure 5.1b further predicts transcription factor binding sites (corresponding to the murine genomic sequence) in the identified ECRs.

a

ECRs within the *Il-10* locus (mouse versus human)



b

Predicted transcription factors binding in ECRs within the *Il-10* locus

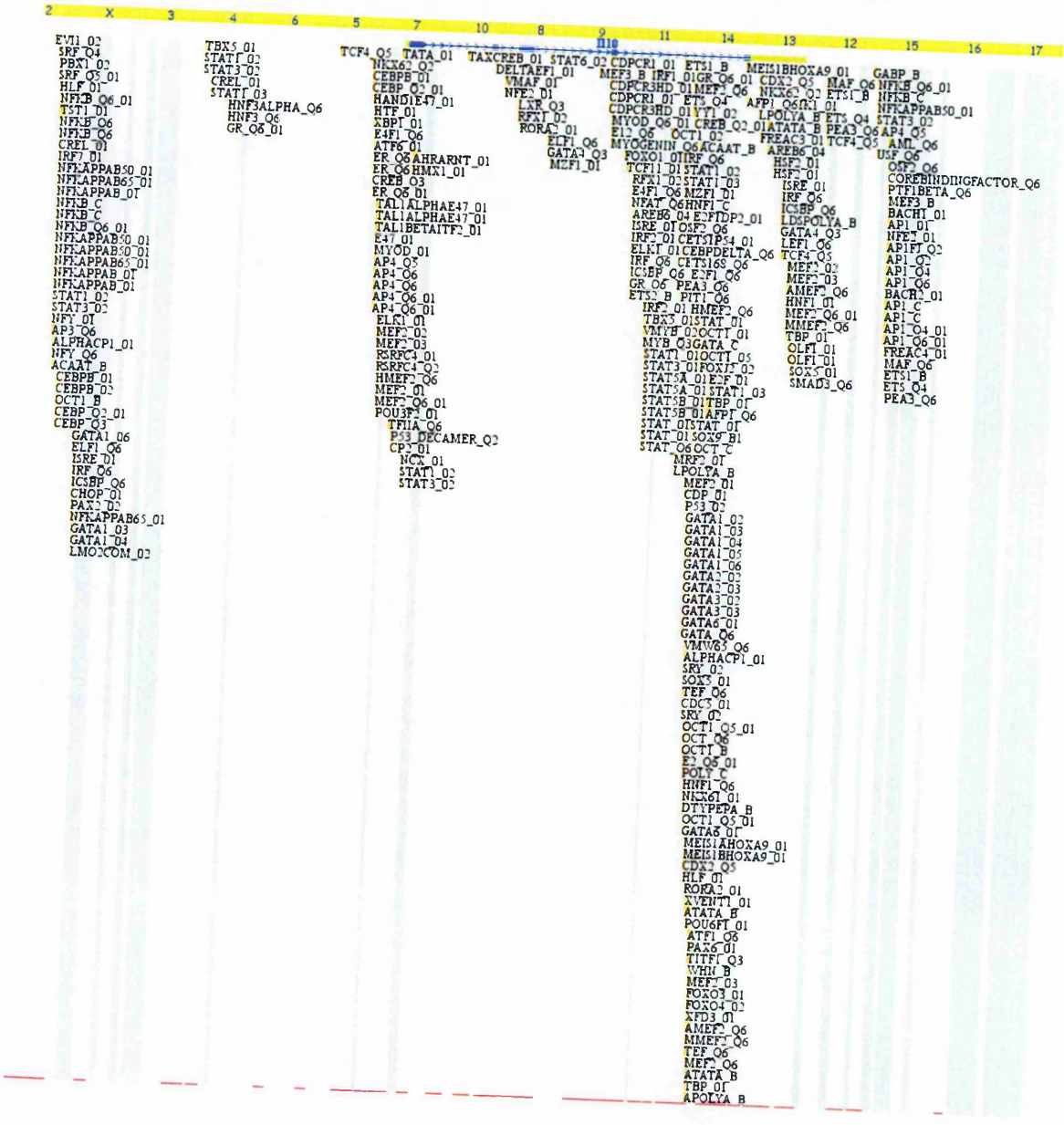


Figure 5.1. ECRs within the mouse and human *Il-10* locus and ECR-associated predicted transcription factor binding sites.
(a) *Il-10* gene alignment between the murine (base sequence) and human sequence. Homology is shown as a smooth dot plot with the range from 50% to 100% sequence-specific homology over a sliding 100 bp window in corresponding locus regions (color-coded, legend appears on the left-side of the Figure). (b) Predicted (rvista.dcode.org) transcription factor binding sites are shown (>85% confidence limit) from ECRs generated from a blastx alignment of the *Il-10* gene (mouse) and the complete human genome.

One striking feature of Figure 5.1b lies within intron 4, where a high degree of conserved transcription factor putative motifs and two conserved TATA boxes proximal to intron 5 are predicted. Both these observations suggest that this location could provide regulatory capacity for the *Il-10* gene (discussed more later).

A higher degree of biological confidence is demonstrated in Figure 5.2, where ECRs and predicted transcription factor sites are only shown for locally highly conserved regions between species. In this prediction model the computational program (rvista.dcode.org) only reports select putative transcription factors: (1) the conserved murine and human regions must share a sliding window of least 20 bp of 80% homology and (2) the core transcription factor binding motif must be 100% conserved between species. Finally, only the "known" and "novel" IL-10-associated transcription factors (as defined from the previous chapter) are included in the hypothetical list of surveyed transcription factor binding motifs.

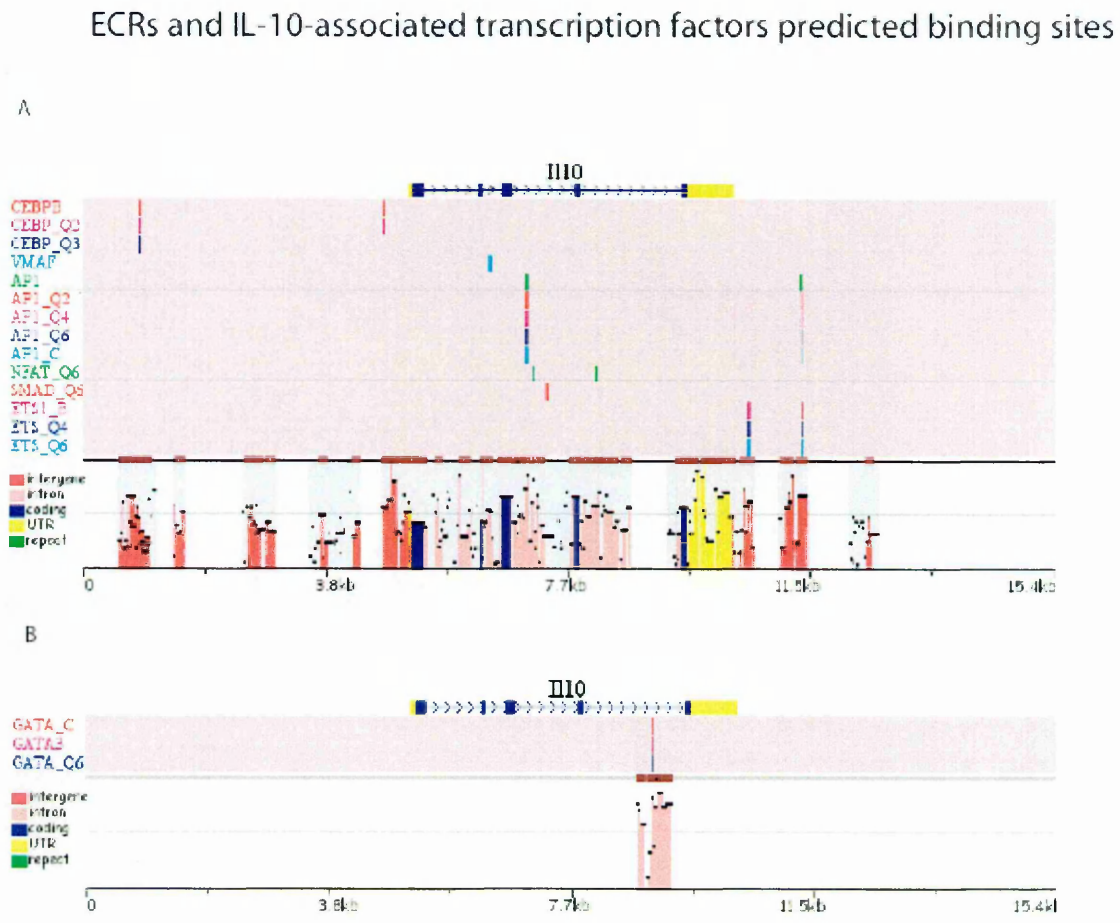


Figure 5.2. ECRs and IL-10-associated transcription factors binding sites.
(a) Murine and human *Il-10* locus alignment and the corresponding IL-10-associated transcription factors binding sites (color-coded, legend appears on the left-side of the Figure) within ECRs. (b) Murine *Il-10* locus alignment with a highly conserved non-coding sequence outside of the human *Il-10* locus and corresponding IL-10-associated transcription factors binding sites (color-coded, legend appears on the left-side of the Figure) within ECRs.

From the "known" and "novel" list of IL-10-associated transcription factors, c/EBP-β is exclusively predicted to bind in the 5' region of the conserved *Il-10* gene sequences (Figure 20a). AP-1 binding sites are conserved within intron 3 and two non-coding sequences in the 3' region. v-Maf (c-Maf homologue) and SMAD-4 are shown to putatively bind to intronic regions. A consensus NFAT binding site (114) and Ets-1 binding site (72), which have both been speculated to repress *Il-10* transcription, are shown to bind to two separate intronic and 3' regions of the *Il-10* locus, respectively.

Interestingly, one NFAT:AP-1 composite site is conserved (intron 3), one singular NFAT is conserved (intron 4), and one singular AP-1 site is conserved (3' region). GATA-3 is shown here to bind to a conserved region of DNA that is not shared within the human *Il-10* locus (Fig. 5.2b), but is shared with a consensus human genomic region 7 Mbp downstream (chr1:203324447-203339128) of the human *Il-10* gene (chr1:27322548-27323066). Interestingly, the piece of homologous human DNA, which has a strong conserved GATA-3 putative binding site and the alignment of the murine *Il-10* gene to human sequence fits "perfectly" with the non-conserved region of (intron 4) murine versus human *Il-10* loci alignment. According to this model, Sp1, Sp3, and c-Maf (within the proximal IL-10 promoter) would not be predicted to bind and enhance *Il-10* expression; however each has been demonstrated to enhance *Il-10* transcription via specific-binding sites at the murine IL-10 promoter (116, 118, 227). This is a recognized limitation of the computational conserved putative binding site model employed here. Together, the combination of the two separate human genomic sequences (chr1:203324447-203339128, chr1:27322548-27323066) in sequence alignments with the murine *Il-10* gene predicts putative binding sites for IL-10-associated transcription factors.

5.2 ChART-PCR: defining regulatory regions in the *Il-10* locus

The prediction of HSSs can be supplemented with ECR analysis and/or by using information from previous studies that reported site-specific DNase I digestion via Southern blot. In our case we have used information from a comprehensive study performed in the lab (115), and additionally by review of ECRs within the *Il-10* locus

performed *in silico*. In summary, primary Th cell populations were differentiated and ChART-PCR was performed to identify areas of chromatin re-modeling across the *Il-10* locus.

Chromatin accessibility studies are designed to access whether genomic regions of chromatin are "highly" sensitive to DNase I digestion (more so than Southern blots) and therefore suggested to be re-modeled (or nucleosome-free) and consequently exposed to/or directly by transcription factors. Southern blots are classically described as the method to describe DNase I digestion regions throughout a probed locus; however Southern blots require high-amounts of cells and lack the specificity achieved with ChART-PCR which utilizes PCR primers. ChART-PCR technique requires specific oligonucleotides to be designed across predicted regions of target chromatin that might be probed for DNase I digestion. Each primer pair (or set) (labeled on Figure 5.3 for the *Il-10* locus) was used to amplify (qPCR) 10 ng of genomic DNA from untreated or DNase I-treated chromatin samples. As an example, in Figure 5.3a I have labeled the *Il-10* locus and HSSs assayed with PCR primers and a sample graph with raw Ct curves generated from a qPCR experiment using both a DNase I-untreated sample and DNase I-treated sample from either Th1 or Th2 chromatin the primer set spanning HSS-0.860 (Fig. 5.3b). A standard curve was generated by amplifying known amounts of template genomic DNA with each set of primers and these standard curves are used to convert our Ct values for untreated and DNase I-treated samples to absolute amount of PCR product obtained in each condition (Fig. 5.3c). In accordance with previous reports (225) the absolute amounts of PCR products were used to calculate the percentage of chromatin

accessibility (Fig. 5.3d) at specific sites within the *Il-10* locus. In summary, when a particular site is exposed to DNase I digestion a higher raw Ct value is measured in the DNase I treated sample (Fig. 5.3b.), this leads to less absolute amount of PCR product being calculated when compared to the untreated sample (Fig. 5.3c.), and reflected in a higher percentage of chromatin accessibility (Fig. 5.3d.). The result for ChART-PCR with primary, unstimulated Th1 and Th2 cells is shown below.

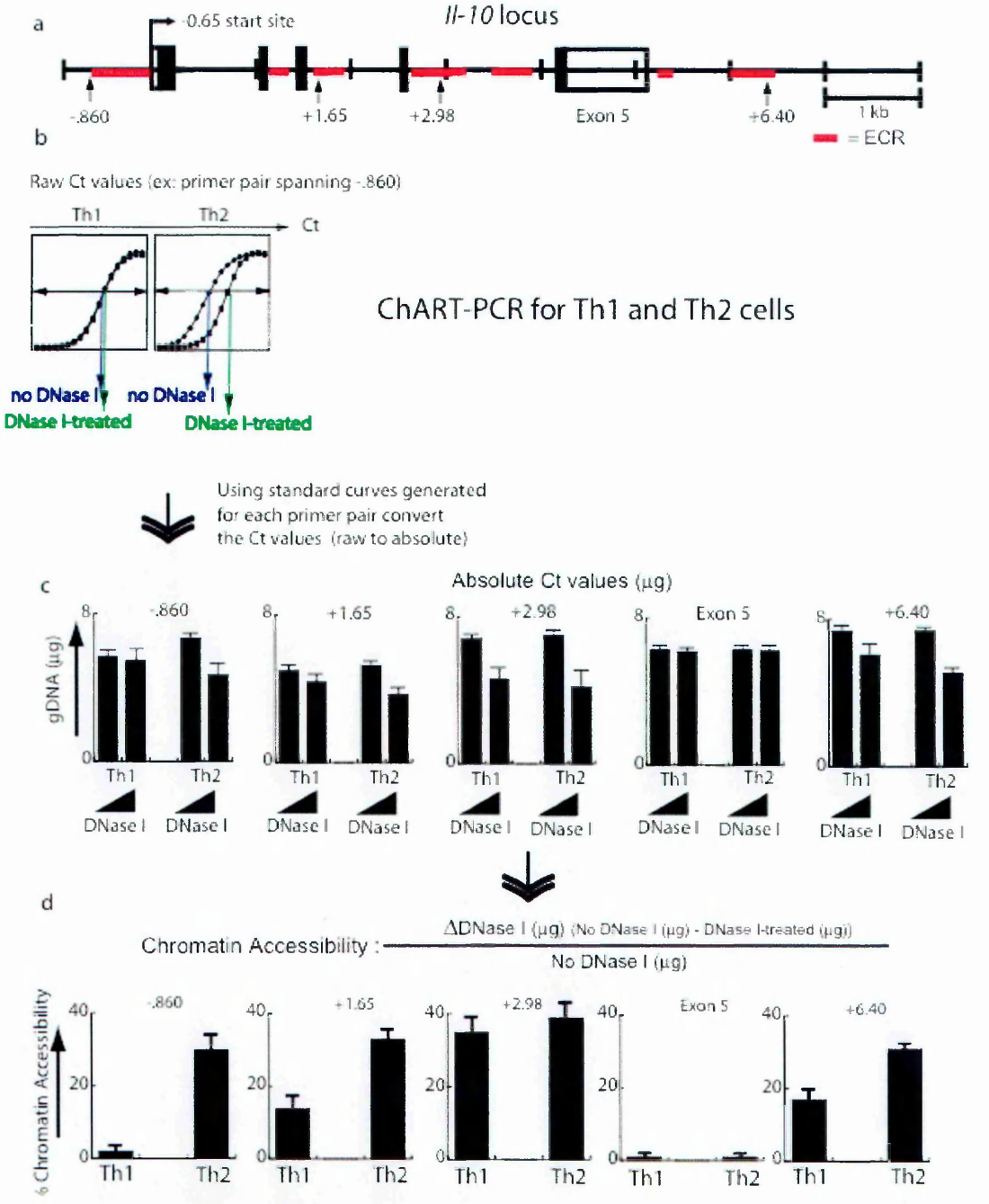


Figure 5.3. Highly sensitive DNase I digestion reveals Th2-specific HSS at the proximal 5' region in the *Il-10* locus within unstimulated Th cells

ChART-PCR was performed in unstimulated CD4 Th cells. (a) Schematic of the fragment of the *Il-10* genomic locus analysed in this study with the corresponding primer pairs used. (b) Th cells were lysed the nuclei isolated and left untreated (No DNase I) or treated with DNase I. The resulting DNA was purified and 10 ng used as template for SYBR Green real-time PCR amplification using specific oligonucleotides for the indicated sites. An example of the raw Ct curves for the primer set, .860 is shown with Th1 or Th2 chromatin (c) The Ct values obtained were converted to quantitative DNA amounts using a standard curve generated for each of the primer sets using genomic DNA as template (data not shown). (d) Chromatin accessibility was calculated and expressed as a percentage of the untreated (No DNase I) DNA.

The 5' proximal region (IL-10 promoter (primers spanning -.860 bp), for our discussion here) of the *Il-10* locus is differentially re-modeled in Th2 cells and not in Th1 cells in these highly sensitive DNase I digestion conditions. Higher concentrations of DNase I (such as the concentrations used in Southern blot studies) may induce DNase I digestion in Th1 at the IL-10 promoter (personal communication, Margarida Saraiva) but under the conditions shown here the IL-10 promoter is a Th2-specific DNase I HSS. This result (Th2-specific DNase I HSS at the IL-10 promoter) is also in agreement with Jones *et al.* that used primary Th1 and Th2 generated cells to perform Southern blots (236). In summary, it is likely that the IL-10 promoter is strongly re-modeled in Th2 cells and only weakly so in Th1 (personal communication, Margarida Saraiva), which may explain in part why these cells produce different amounts of IL-10.

One intriguing area from previous studies focused on possible differential re-modeling of intronic regions within Th population of the *Il-10* locus (114). The ChART-PCR data shows that intron 3 and 4 are not differentially re-modeled between Th populations. In fact, both Th1 and Th2 cells have DNase I digested intronic regions within the *Il-10* gene (intron 3 and intron 4), suggesting that expression of differential transcription factors enhancing or suppressing *Il-10* transcription may be differentially acting on the *Il-10* gene at these locations. The constitutive HSS in Th1 and Th2 cells is in agreement with DNase I HSS studies within the lab (personal communication, Margarida Saraiva) and with Jones *et al.* (236). However, this is in disagreement with the initial report by Im *et al.*, which described the intronic *Il-10* locus region (intron 3 and intron 4) to be a Th1-specific HSS. This difference may arise from the nature of Th1/Th2 cell used for their

study (D5 (Th1) and D10 (Th2) cell-lines), whereas we use primary Th1 and Th2 cells (114). The 3' region of the *Il-10* locus is re-modeled to a similar degree in both Th1 and Th2 cells. This is in agreement with published reports on chromatin-level regulation of the *Il-10* gene in this region (112, 114). Primers spanning exon 5 were included to control for nonspecific digestion by DNase I and show no digestion. In summary, these data shows similar degrees of DNase I digestion in both intronic regions (intron 3 and intron 4) and previously reported highly conserved (human and mouse) 3' regions of the *Il-10* locus between Th1 and Th2 cells.

5.3 Discussion

Together, identification of putative transcription factors involved in *Il-10* gene regulation can be addressed by their cell-specific expression and their potential role in function is then supported by comparative genomics and predicted transcription factor binding sites adding value to the global question of *Il-10* gene regulation investigated here. These putative factors will then be tested functionally by their ability to induce IL-10 when transduced into non-IL-10-producing populations via the RV system (next Chapter). As an additional layer, *in silico* studies clarify where potential transcription factors would theoretically bind within the *Il-10* locus and the ChART-PCR assay demonstrates areas of remodeled chromatin accessible to nuclear factors in differential IL-10-producing CD4 Th populations. The *in silico* approach independently identified three "known" sites of transcription factor binding sites: an AP-1 (JunB and c-Jun) binding site in the 3' region (112), a NFAT intron 4 binding site (114) and c/EBP-β binding site (233) in the proximal 5' region of the *Il-10* locus. Secondly, *in silico* analysis predicted a "novel" putative

transcription factor binding sites for IL-10-associated transcription factors: GATA-3 and Ets-1. The GATA-3 conserved putative binding site in intron 4 (murine), is highly related to a distant genomic sequence (~500 bp) in the human genome (7 Mb downstream from the *Il-10* locus). The murine intron 4 sequence is important in terms of potential regulatory function as previous reports describe the intron 4 region - where a consensus putative GATA-3 binding site is located - to contain a HSS. The fact that in the human genome a highly conserved sequence in comparison to the murine *Il-10* gene (which does not contain homology in the encoded human *Il-10* gene) is located 7 Mbp downstream suggests a possible evolutionary trans-location of this specific non-coding genomic sequence (~500 bp) between the mouse and human genome. Finally, the fact that there is high conservation of multiple putative transcription factors in intron 4 along with two conserved TATA boxes proximal to the exon 5 coding region suggests a possible site for an alternate promoter (discussed further in Future Perspectives) within this intronic region. Although no precedent is yet described for cytokine genes, other genes (such as the transcription factor, GATA-3) are expressed under the instruction of at least two promoters (237).

Since some transcription factors are not able to bind inaccessible or heterochromatic regions of DNA the ChART-PCR assay analyzed low- and high-level IL-10-producing Th cells to determine whether differential sites of the *Il-10* locus are accessible in these populations for these types of nuclear factors. The results in this Chapter demonstrate the 5' region of the *Il-10* locus is exclusively re-modeled in Th2 cells and not in Th1 cells. Furthermore, Saraiva *et al.* reported (Southern blot) the 5' region of the *Il-10* locus was

re-modeled in IL-10-T_{reg}. Therefore, the combination of the Southern blot data and the ChART-PCR results suggest that a transcription factor not present in Th1, but available in Th2 and IL-10-T_{reg} cells, may provide an instructive mechanism for high-level IL-10-producing populations to strongly remodel the 5' proximal region of the *Il-10* locus. The *in silico* computational model to predict conserved putative transcription factors within the 5' *Il-10* locus region only predicts c/EBP-β and no other known or novel IL-10-associated transcription factors. This suggests that the computational model may not be able to predict functional transcription factors regulating *Il-10* gene regulation at this location or the settings to predict conserved putative sites may be too strict to isolate potential regulators. On the other hand, conserved putative sites isolated from the computational model may bind transcription factors in high-IL-10-producing populations at locations outside the proximal 5' region of the *Il-10* gene and induce long-range chromatin accessibility specific to the proximal 5' region of the *Il-10* locus. In either case, it is clear that high-abundance IL-10-producing CD4 T cells differentially expose the proximal 5' region of the *Il-10* locus.

Both low-IL-10-producing (Th1) and high-IL-10-producing (Th2) Th populations exhibit similar re-modeling within intronic (intron 3 and intron 4) and 3' regions of the *Il-10* locus. This suggests Th1 and Th2 cells have a differential mechanism whereby different transcription factors may bind and act via either repressing or enhancing *Il-10* transcriptional activity in constitutively re-modeled intronic and/or 3' regions (Th1 and Th2 cells). This mechanism is attractive especially with respect to transcription factors not able to bind inaccessible or heterochromatic regions of regulatory regions. This

mechanism could involve factors that are classically shown to be inducible upon TCR activation, as there is considerable evidence that this class of transcription factors (NFAT, AP-1) preferentially bind euchromatic regulatory regions (59). The abundance or differential dimerization of these factors upon TCR activation may play a role at the exposed regions (intron 3/4 and the 3' region) of differential IL-10-producing Th populations. This type of mechanism has already been speculated by the Wang *et al.* report in which they show JunB and c-Jun can enhance IL-10 expression at their putative binding site in the 3' region (HSS+6.40). However, this mechanism did not describe why high IL-10-populations (Th2 and IL-10-T_{reg}) differentially remodel the 5' proximal region of the *Il-10* gene (which has been proposed to have enhancing regulatory elements) in comparison to low-IL-10-producing populations before TCR activation. In terms of the data presented here, it should be clear that chromatin accessibility is not a direct means to assay potential "active" or "silencing" regions of re-modeled genes and further studies assessing covalent modifications of histone tails would serve as another level of investigation into regulatory elements (within the *Il-10* gene) in these Th populations, before or after TCR activation (discussed in Future Perspective). In summary, the degree to which either of these regulatory non-coding intergenic or intronic regions within the *Il-10* locus controls *Il-10* gene expression is most likely dependent on multiple factors; however the results in this chapter in addition to the previous expression profiles provide critical insight into *Il-10* gene regulation.

In the next chapter I present evidence that the GATA-3 transcription factor plays an important role at the molecular-level in the regulation of the *Il-10* gene.

6. GATA-3 re-models the *Il-10* locus independently of IL-4

GATA-3 was selected as a potential candidate to regulate the *Il-10* gene based on a number of observations. Firstly, mRNA profiling of six distinct primary CD4 T cell populations showed differential expression of the transcription factor GATA-3 within IL-10-producing cells (Th2 and IL-10-T_{reg}) (Fig. 3.5 and 6.1a). Secondly, a conserved putative GATA-3 binding site was isolated in the *Il-10* locus as described in Chapter 5. Finally, GATA-3 has been speculated to play a role in *Il-10* gene regulation from a number of studies outlined below. Although the *Il-10* locus (chromosome 1) is at a distant location from the *Il-4/Il-5/Il-13* cluster (chromosome 11), antisense inhibition (73) or conditional deletion (85) of GATA-3 in established murine Th2 cells has been shown to reduce IL-10 production. Conversely, over-expression of GATA-3 in a transgenic murine model caused an up-regulation of IL-10 production (238). However, the direct mechanism whereby GATA-3 may regulate IL-10 expression was never established. One reason for this lack of direct evidence for GATA-3 role in *Il-10* gene regulation in these studies described above relates to the coordinate expression of IL-4, IL-5 and IL-13 in GATA-3-expressing or non-expressing CD4 T cells. Therefore, GATA-3 effect on the IL-10 production could be via indirect effects, for instance its regulation directly on IL-4 production. To begin the GATA-3 studies - in terms of IL-10 production - I will introduce the RV system and the results of differential cytokine production in GATA-3-transduced primary naïve CD4 T cells.

6.1 Ectopic expression of GATA-3 induces IL-10 production in primary CD4 T cells

The retroviral (RV) system was used to assess the functional potential of discovered transcriptional factors in terms of cytokine expression or proliferation via their transduction into naïve CD4 T cells and differentiation in an environment where the (investigated transcription factor) was not up-regulated as shown with the GeneChip and qPCR data. The RV system has advantages in that each transcription factor can be essentially "turned on" in a primary naïve CD4 T cell. Furthermore, the RV system has an internal control with parallel transduction of a Mock-RV vector alongside the transduction of a transcription factor of interest. The RV system has been shown in previous studies (77) to functionally validate and gain insight into transcription factor-mediated molecular mechanism and will be used here to functionally investigate isolated genes from the mRNA profiling and the *in silico* analysis.

To investigate the effect of GATA-3 on IL-10 production, purified naïve CD4 T cells were transduced with a recombinant retrovirus expressing GATA3-RV or with a Mock-RV and cultured under neutral or Th1 conditions. The use of a retroviral vector containing an IRES-GFP facilitated the monitoring of the expression of GATA-3 in the transduced cells at the single cell level by measuring GFP expression, which has previously been shown to reflect GATA-3 expression in transduced cells (77).

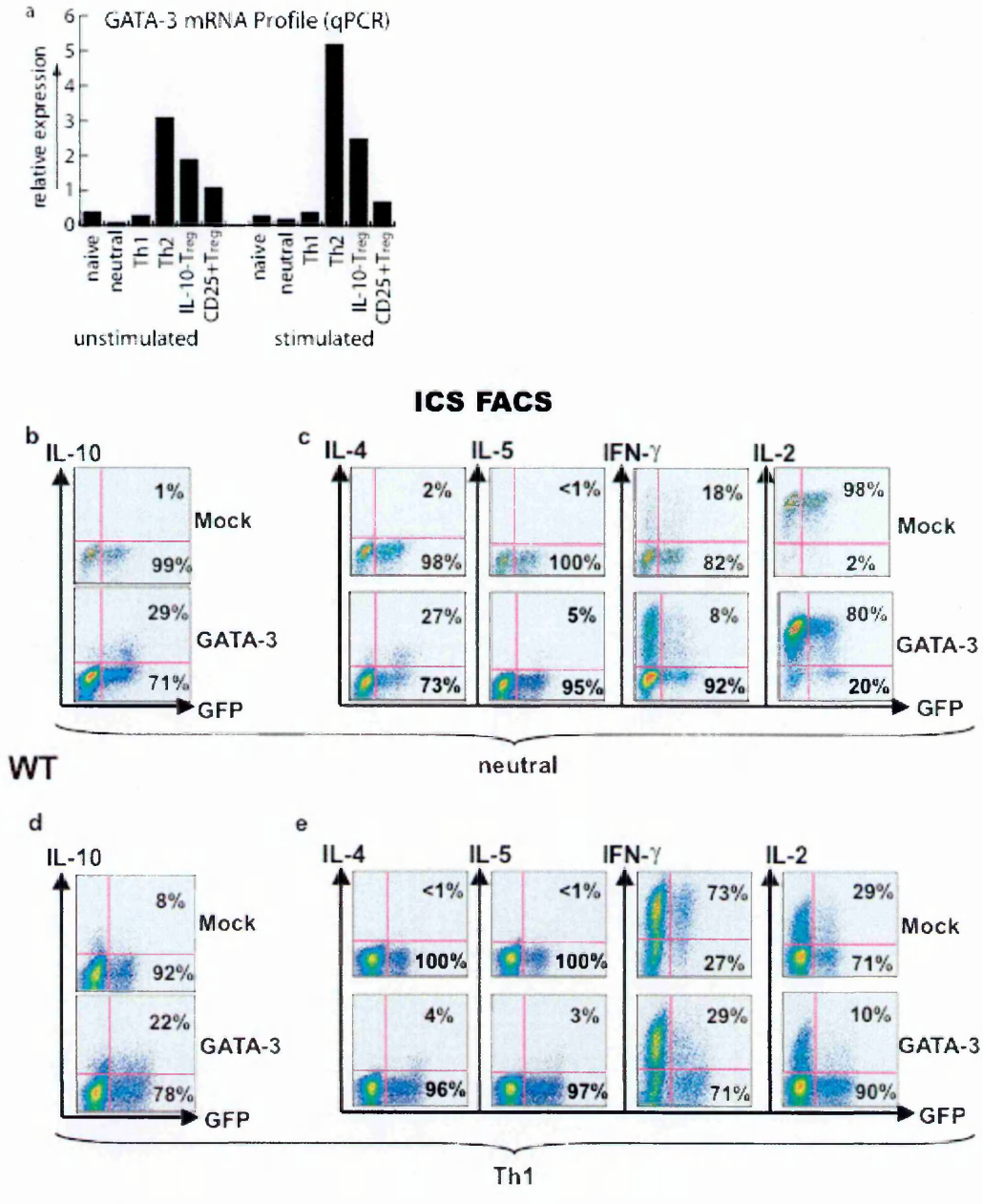


Figure 6.1. GATA-3 enhances IL-10 production in CD4 T cells.
(a) Real-time PCR was performed to validate the results from the GeneChip for IL-10 and GATA-3 expression in unstimulated or stimulated CD4 T cell populations. WT naïve (CD4+CD62L+CD45RB^{high}) T cells were infected with a Mock or a GATA-3-expressing retrovirus and differentiated under Neutral (b, c) and Th1 (d, e) conditions. Six days after infection, cells were stimulated with PMA/Ionomycin for 4 h, intracellular stained for IL-10 (b, d) or for IL-4, IL-5, IFN-γ, and IL-2 (c, e) and analyzed by FACS. Cells were gated on GFP expression (x-axis) and the percentage of GFP-positive cells expressing the respective cytokines is shown. Data shown is an accurate representation of three biological replicates.

T cells cultured under neutral conditions and transduced with the Mock retrovirus produced little to no IL-10 (Fig. 6.1b). However, ectopic expression of GATA-3 induced an increase in the number of IL-10-producing cells upon stimulation (29% versus 1% obtained with the Mock-RV) (Fig. 6.1b) and increased levels of secreted IL-10 as measured by ELISA (data not shown). In agreement with previous studies, the percentage of IL-4- and IL-5-producing cells (74, 77) was increased by the presence of GATA-3 (Fig. 6.1c). Interestingly, the percentage of IL-10-producing cells was increased to a higher degree than that of IL-4- or IL-5-producing cells, two known GATA-3 transcriptional targets. Finally, the percentage of IFN-γ– and IL-2-producers was reduced by ectopic GATA-3 expression (Fig. 6.1c). The down-regulation of IFN-γ production by GATA-3 was in keeping with previous studies (75, 77, 79).

Similarly to what was observed under neutral conditions, over-expression of GATA-3 in naïve CD4 T cells differentiated under Th1 conditions led to an increase of IL-10 production (Fig. 6.1d). Increased percentages of IL-4- and IL-5-producing cells (Fig. 6.1e) were also observed as previously reported (74, 77). Here in the Th1-driven culture conditions GATA-3 induced a much higher number of IL-10- than of IL-4- or IL-5-producing cells suggesting a potential direct effect of GATA-3 on the *Il-10* gene (Fig. 6.1d and 6.1e). The percentage of cells producing IFN-γ and IL-2 was again significantly diminished in the presence of GATA-3 as compared to controls (Fig. 6.1e).

6.2 GATA-3 induction of IL-10 production is independent of IL-4

GATA-3 has been shown to induce changes in the chromatin structure at the *Il-4/Il-5/Il-13* locus (74, 78), and to drive subsequent production of these Th2-associated cytokines (73, 74, 77). Thus, the increased production of IL-10 observed in the presence of GATA-3 could result from indirect effects of Th2-associated cytokines (specifically IL-4, since IL-5 and IL-13 do not act directly on T cells (51)). To determine whether GATA-3 enhanced *Il-10* gene expression independently of IL-4, GATA-3 was transduced into naïve CD4 T cells derived from IL-4(-/-) mice (239). The ability to use IL-4(-/-) CD4 T cells provided a key step to understanding whether or not GATA-3 was acting in a direct mechanism in regulating the *Il-10* gene.

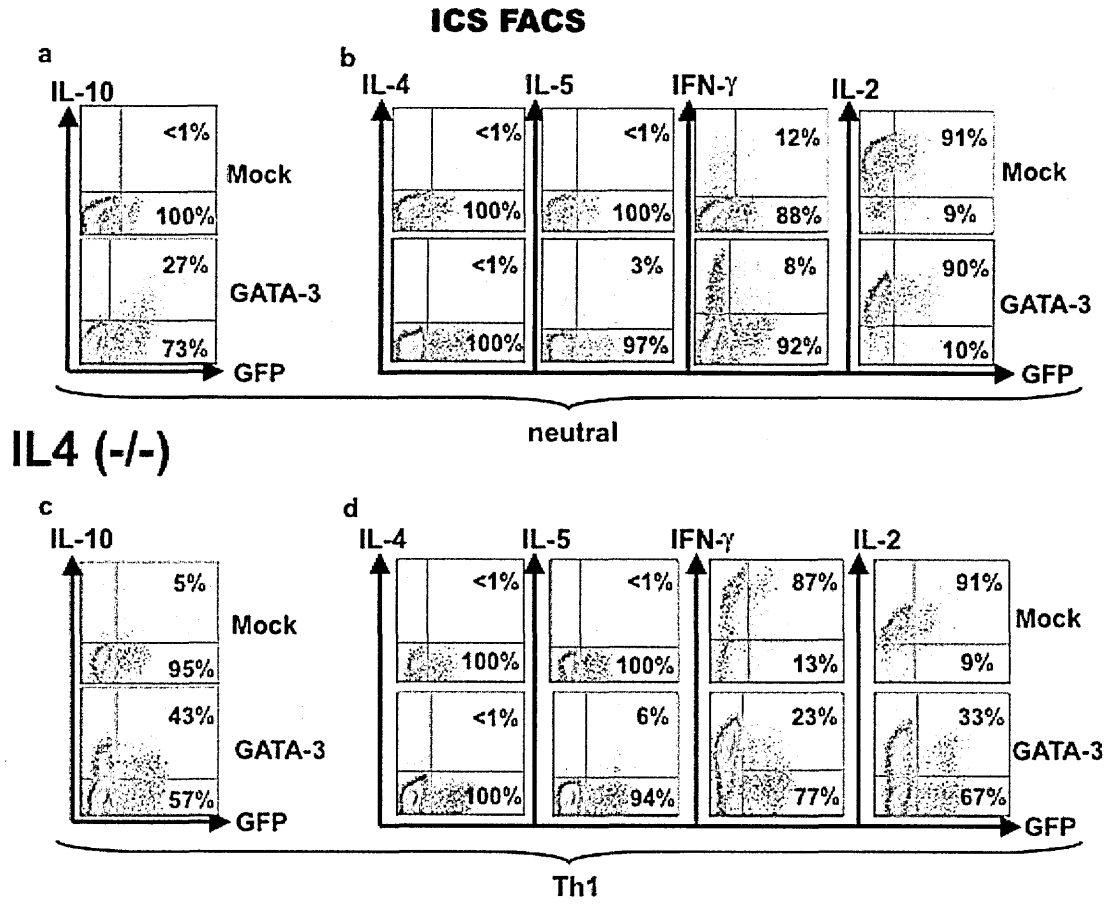


Figure 6.2. IL-10 production induced by GATA-3 is independent of IL-4.
IL-4(-/-) naïve (CD4+CD62L+CD45RB^{high}) T cells were infected with a Mock or a GATA-3-expressing retrovirus and differentiated under Neutral (a, b) and Th1 (c, d) conditions. Six days after infection, cells were stimulated with PMA/Ionomycin for 4 h, intracellular stained for IL-10 (a, c) or for IL-4, IL-5, IFN-γ, and IL-2 (b, d) and analyzed by FACS. Cells were gated on GFP expression (x-axis) and the percentage of GFP-positive cells expressing the respective cytokines is shown. Data shown is an accurate representation of three biological replicates.

Under neutral culture conditions, GATA3-RV induced a similar number of IL-10-producing cells in the presence (WT) (29%) (Fig. 6.1b) or absence of IL-4 (27%) (Fig. 6.2a), showing that IL-4 is not necessary for IL-10 induction. However, the number of IL-4- and IL-5-producing cells was much lower (Fig. 6.2b) than the WT conditions (Fig. 6.1c), with the IL-4-producers being eliminated (IL-4 (-/-) cells) and the number of IL-5-producing cells much lower in the IL-4(-/-) cells (Fig 6.2b) as compared to WT cells (Fig

6.1c). Over-expression of GATA-3 induced similar changes in the percentage of IFN-γ-producing cells (Fig. 6.2b.) as compared to WT cells (Fig. 6.1c) whereas IL-2-producing cells were similar in Mock-RV or GATA3-RV in IL-4(-/-) cells in neutral culture conditions (Fig. 6.2b.).

Transduction of GATA-3 in IL-4(-/-) naïve CD4 T cells differentiated under Th1 conditions induced a greater number of IL-10-producing cells than that observed for WT cells (Fig. 6.1d and 6.2c). The IL-10-producers in the GFP-negative/GATA-3-transduced population were also increased (Fig. 6.2c), which was also observed at the mRNA level (data not shown). This probably reflects the loss of GFP expression in a portion of the GATA-3-transduced cells (77). Therefore, as in all the conditions, the most accurate comparison is between the GFP+Mock-RV-transduced cells and GATA3-RV-transduced cells. Over-expression of GATA-3 induced similar changes in the percentage of IL-5-, IFN-γ- and IL-2-producing cells in WT (Fig. 6.1e) or IL-4(-/-) cells (Fig. 6.2d). Together, the IL-4(-/-) RV studies allude to GATA-3 having a possible direct effect on the expression of the *Il-10* gene.

To investigate the effect of GATA-3 on *Il-10* transcription in WT and IL-4(-/-) CD4 T cells, GATA-3- or Mock- transduced cells were isolated by flow cytometry on the basis of their GFP expression on day 6 post-transduction. qPCR was performed on Mock-RV and GATA3-RV transduced cells (unstimulated and stimulated) to investigate cytokine expression and various transcription factors implicated in *Il-10* gene regulation (Fig. 6.3).

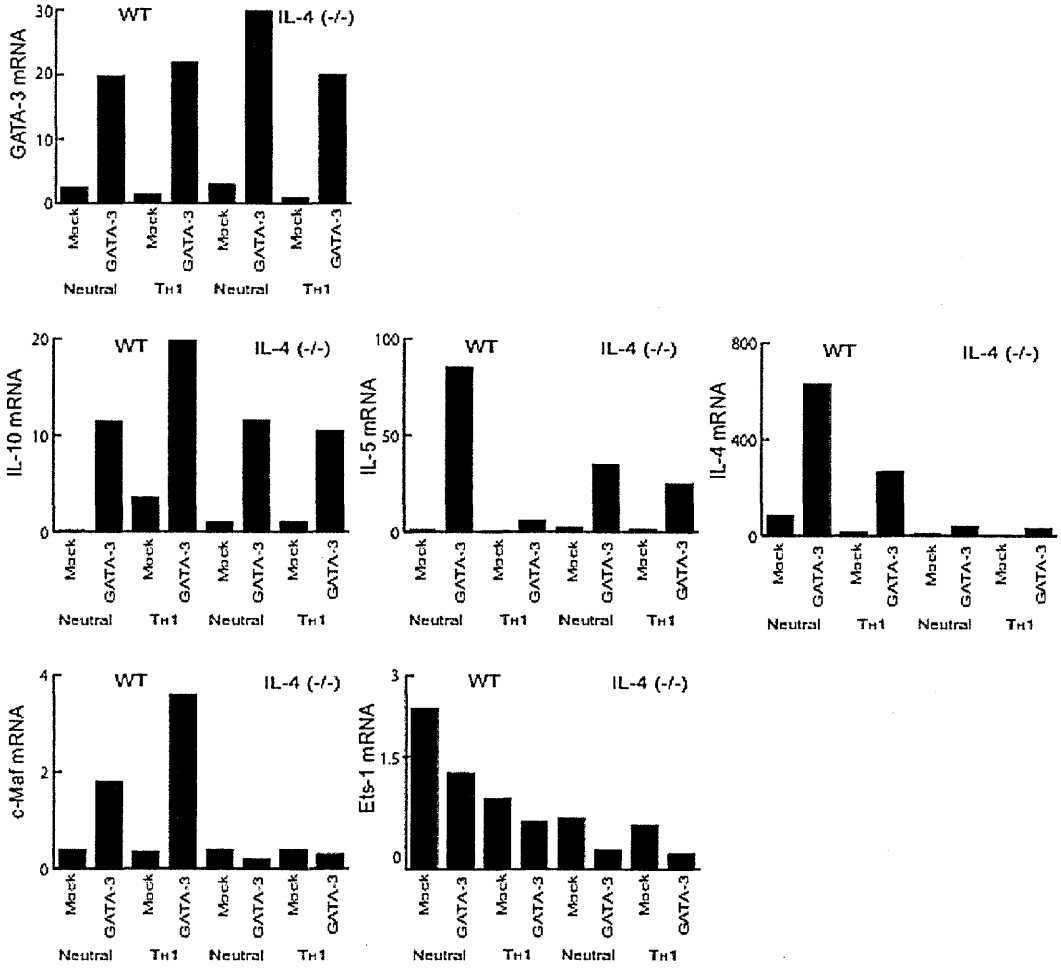


Figure 6.3. GATA-3 enhances IL-10 at the transcriptional level.
CD4 T cell populations described in Figures 6.1. and 6.2. were purified by flow-cytometry into GFP-positive populations and mRNA was collected at unstimulated time-point and after activation for 3 hr. with PMA/Ionomycin. Real-time PCR was performed to detect cytokine and transcription factor expression. Data shown is an accurate representation of three biological replicates.

As expected, levels of GATA-3 mRNA (Fig. 6.3) was higher in the GATA-3-transduced GFP-positive T cells than in the Mock-transduced GFP-positive T cells. Independent of the polarizing conditions used and of the presence of IL-4, IL-10 transcription was significantly and consistently up-regulated by GATA-3 in keeping with the ICS FACS. IL-5 mRNA was also consistently up-regulated in the presence of GATA-3, to a maximum amount in WT cells under neutral conditions (Fig. 6.3).

Transcription factor profiling was investigated to determine whether GATA-3 presence was directly influencing other possible direct regulators of IL-10 expression. One clear observation was that in IL-4(-/-) cells as opposed to WT cells, transduction of GATA-3 did not induce the transcription of c-Maf (Fig. 6.3), confirming that c-Maf expression requires IL-4 and strongly correlates with IL-4 signaling (74). The expression of Ets-1 was clearly down-regulated in the presence of GATA-3, independently of IL-4 production and polarizing condition (Fig. 6.3). Other IL-10-associated transcription factors were analyzed and no other expression profiles indicated differential regulation in GATA-3- versus Mock-transduced cells in either polarizing condition at both unstimulated and stimulated time-points.

6.3 GATA-3 binds to the *Il-10* locus

The results indicating that GATA-3 induced IL-10 mRNA and protein, in the absence of IL-4, suggested a direct, yet unknown effect of this transcription factor on the *Il-10* locus. The molecular mechanism to which GATA-3 may regulate IL-10 expression was an open question. From the *in silico* analysis of the *Il-10* gene I have described the comparison of the DNA sequence of the murine *Il-10* gene to that of other species (human) revealed one conserved site for conserved putative GATA binding motifs in exon 4 within the *Il-10* gene (re-visited in Fig. 6.4a). Since the *in silico* analysis performed in the last chapter was unable to reveal the c-Maf and Sp1/Sp3 functionally described putative binding sites in the IL-10 promoter, a intensive search of the mouse and human 5' region was undertaken for other potential GATA binding motifs. Further analysis of the IL-10

promoter revealed a double conserved (mouse and human) "core" putative GATA binding site. This double GATA-binding site was located -0.865 bp in the 5' proximal region of the mouse *Il-10* gene. Together, the discovery of the 5' putative binding site and the conserved putative GATA binding site in intron 4 (both of which contain described HSS) lead us to speculate that GATA-3 might bind to these potential regulatory regions *in vivo*.

To assess whether GATA-3 bound to these sites *in vivo* primary IL-10-producing T cells (Th2 and IL-10-T_{reg}) were generated and chromatin immunoprecipitation (ChIP) studies performed. Briefly, crosslinked DNA from polarized IL-10-producing T cells were immunoprecipitated with a specific antibody for GATA-3 or with a control antibody. The immunoprecipitated DNA was subsequently purified and amplified by real-time PCR using specific oligonucleotides for the sequence near or spanning the GATA-3 conserved putative binding motif on the *Il-10* gene (Fig. 6.4a). Oligonucleotides specific for the *Il-5* gene 5' region, where GATA-3 is described to bind (83), were included as a control.

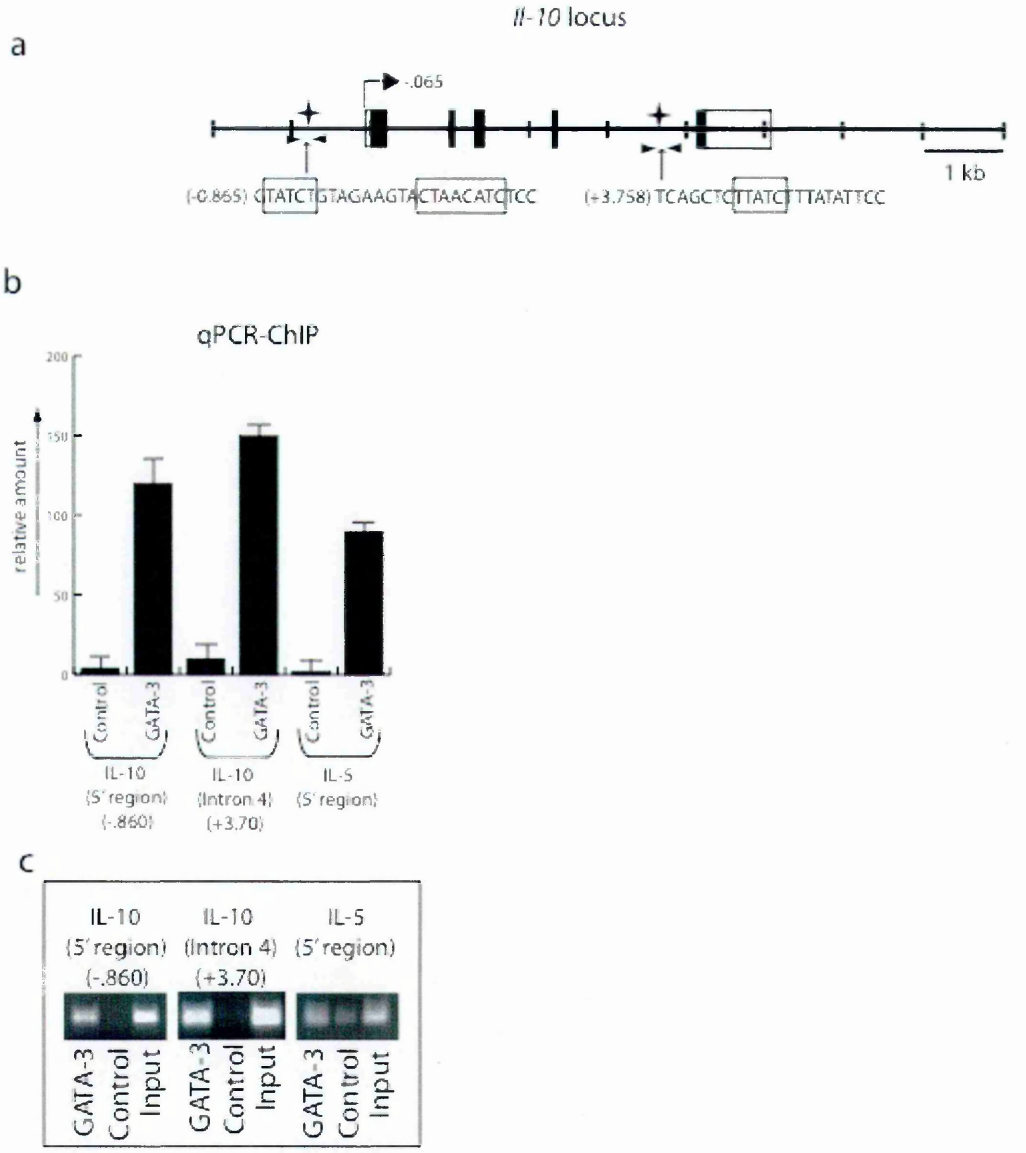


Figure 6.4. GATA-3 binds to the IL-10 promoter *in vivo*.
(a) Conserved GATA-3 transcription factor binding sites in the mouse *Il-10* gene (+1 represents the start site). Indicated are the IL-10 exons (black boxes), the 5' and 3' UTR's (white boxes) and the position of the oligonucleotides used in (b) and the location of the conserved GATA-3 binding sites (✦). (b) Crosslinked chromatin complexes from resting Th2 cells were immunoprecipitated with a GATA-3 or a control antibody. Specific oligonucleotides were used to amplify by real-time PCR the GATA-3 binding sites in the *Il-10* gene or the IL-5 promoter, using the immunoprecipitated or untreated (Input) chromatin as template. Represented is the amount of PCR product obtained upon GATA-3 immunoprecipitation or control antibody immunoprecipitation, normalized to the amount of PCR product obtained for the Input chromatin. The error bars for each condition represent the standard deviation from 3 biological replicates. (c) The PCR products obtained as described in (b) were separated in a 2.5% agarose gel and stained with ethidium bromide. Data shown is an accurate representation of three biological replicates.

ChIP studies verify the presence of GATA-3 at both consensus putative binding sites in the *Il-10* locus in both IL-10-producing T cells (Fig. 6.4b (qPCR) and 6.4c. (gel)) (IL-10-T_{reg} data not shown), strongly supporting a direct role of GATA-3 in the expression of the *Il-10* gene. As controls, binding of GATA-3 as expected was observed at the *Il-5* locus (Fig. 6.4b and 6.4c), but was not observed in the region coding for the *Il-10* exon 5 (data not shown).

6.4 GATA-3 induces changes in the chromatin structure at the *Il-10* locus

The presence of GATA-3 at regulatory sites within the *Il-10* locus suggested that GATA-3 might provide an instructive role in either *trans*-activating the *Il-10* gene (similar to its effect of the *Il-5* and *Il-13* gene) or re-modeling the *Il-10* locus (similar to its effects on the *Il-4* gene). *Trans*-activation studies revealed GATA-3 did not *trans*-activate the full-length IL-10 promoter (data not shown). Therefore, whether GATA-3 was able to induce changes in the chromatin structure at the *Il-10* locus was investigated. For this, CD4 naive T cells from IL-4(-/-) mice were purified and transduced with a GATA3-RV or with a Mock-RV and ChART-PCR was performed in unstimulated cells to investigate the structure of the *Il-10* gene under the differential presence of GATA-3, independently of IL-4. This assay was performed at the earliest possible time (owing to transduction rates), which was day 3 post-transduction with IL-4(-/-) cells. Three separate biological replicates were assayed three days post-transduction and nuclei was isolated from unstimulated GATA-3- or Mock-transduced cells untreated or treated with low-concentration DNase I (225, 240). Based on the previous Chapters results and the study by Saravia *et al.*, specific oligonucleotide pairs that span HSS of interest across the *Il-10*

locus were used. In these studies I have included primer pairs that span described HSS detected upstream of the *Il-10* start site (HSS-2.0), two primer pairs that cover the IL-10 promoter region, intron 3 (HSS+1.65), intron 4 (HSS+2.98) and the 3' non-coding region (HSS+6.40). Of particular interest were the HSSs detected in the 5' proximal region of the *Il-10* gene and in intron 4 since where *in vivo* recruitment of GATA-3 is shown by ChIP. As a control for the quality and quantity of the template DNA used, the chromatin accessibility at exon 5 of the *Il-10* gene is described. The results are shown below in Figure 6.5.

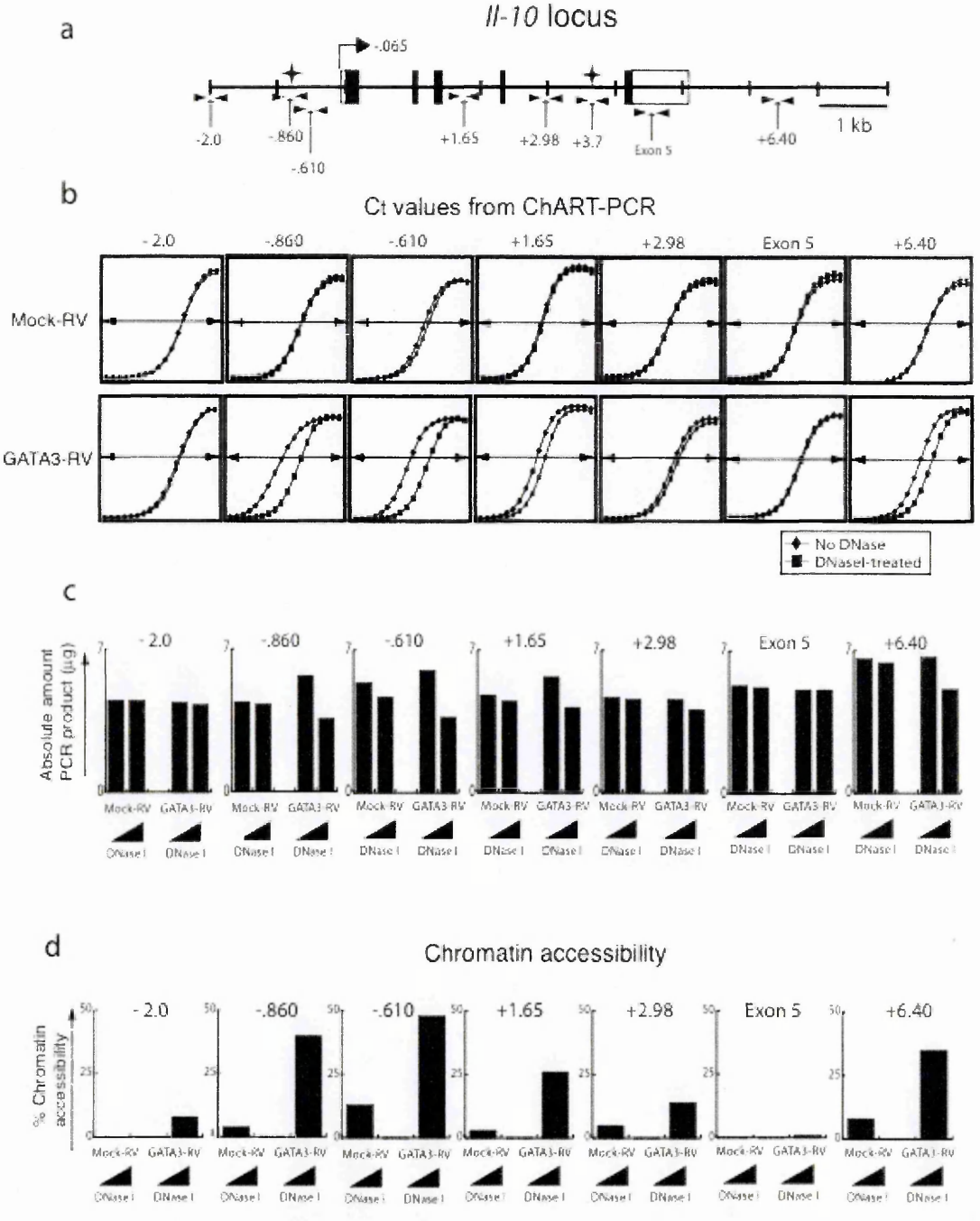


Figure 6.5. GATA-3 induces changes on the chromatin structure at the *Il-10* locus.
(a) Schematic of the fragment of the *Il-10* genomic locus analysed in this study (b) IL-4(-/-) naïve T cells were transduced with a Mock-(Mock-RV) or a GATA-3-expressing retrovirus (GATA3-RV) and differentiated under neutral conditions. Three days after infection, cells were lysed the nuclei isolated and left untreated (No DNase I) or treated with DNase I. The resulting DNA was purified and 10 ng used as template for SYBR Green real-time PCR amplification using specific oligonucleotides for the indicated sites. (c) The Ct values obtained were converted to quantitative DNA amounts using a standard curve generated for each of the primer sets using genomic DNA as template (data not shown). (d) The chromatin accessibility was calculated as a percentage of the untreated (No DNase I) DNA.

In the presence of ectopic GATA-3 the chromatin accessibility at the *Il-10* locus was increased (Fig. 6.5d). This effect was most pronounced at sites located in the proximal 5' region of the *Il-10* gene (HSS-0.860 and HSS-0.610) and described intronic HSSs, including sites that are near the putative GATA-3 binding site in intron 4 (Fig. 6.5d). Chromatin re-modeling was also observed at HSS+6.40 suggesting that GATA-3 may also induce long-range re-modeling of known positive regulatory regions. No significant differences in PCR product amplified (Fig. 6.5b and 6.5c) and on the percentage of DNase I digestion (Fig. 6.5d) was observed at exon 5, suggesting that the observed differences were specific and due to real changes in the chromatin accessibility at sites containing regulatory elements. GATA-3 induced chromatin re-modeling at specific sites of the *Il-10* locus, even in the absence of IL-4. This re-modeling not only occurred at sites shown here to bind GATA-3 but also across the *Il-10* locus at sites known to positively regulate the transcription of the *Il-10* gene, as is the case for HSS+6.40. In summary, the ChART-PCR technique - which reveals potential regulatory regions (HSSs) - demonstrates that GATA-3 induces re-modeling of key enhancing regulatory regions (5' proximal region) of the *Il-10* gene before TCR activation and independently of IL-4.

6.5 GATA-3 induces acetylation of histone H3 and H4 to the *Il-10* locus

The acetylation of histone (H3 and H4) at specific genomic regions has been demonstrated to forecast enhancement of gene expression. With this in mind, the acetylation status of histones H3 and H4 in primary CD4 T cells in the absence or

presence of ectopic GATA-3 was investigated at the *Il-10* gene. For this, naive CD4 T cells were transduced with GATA3-RV or Mock-RV, under neutral conditions and FACS purified based on GFP-positive expression at day 6 post-transduction. Crosslinked chromatin was isolated and immunoprecipitated with antibodies that specifically recognize acetylated residues of histones H3 and H4 versus control antibodies. Immunocomplexes were further purified and DNA was used as template for qPCR with oligonucleotide sets that cover the predicted GATA-3 binding sites in the *Il-10* locus (HSS-0.860 and HSS+3.7) and the previously described AP-1 binding site (HSS+6.40) (110).

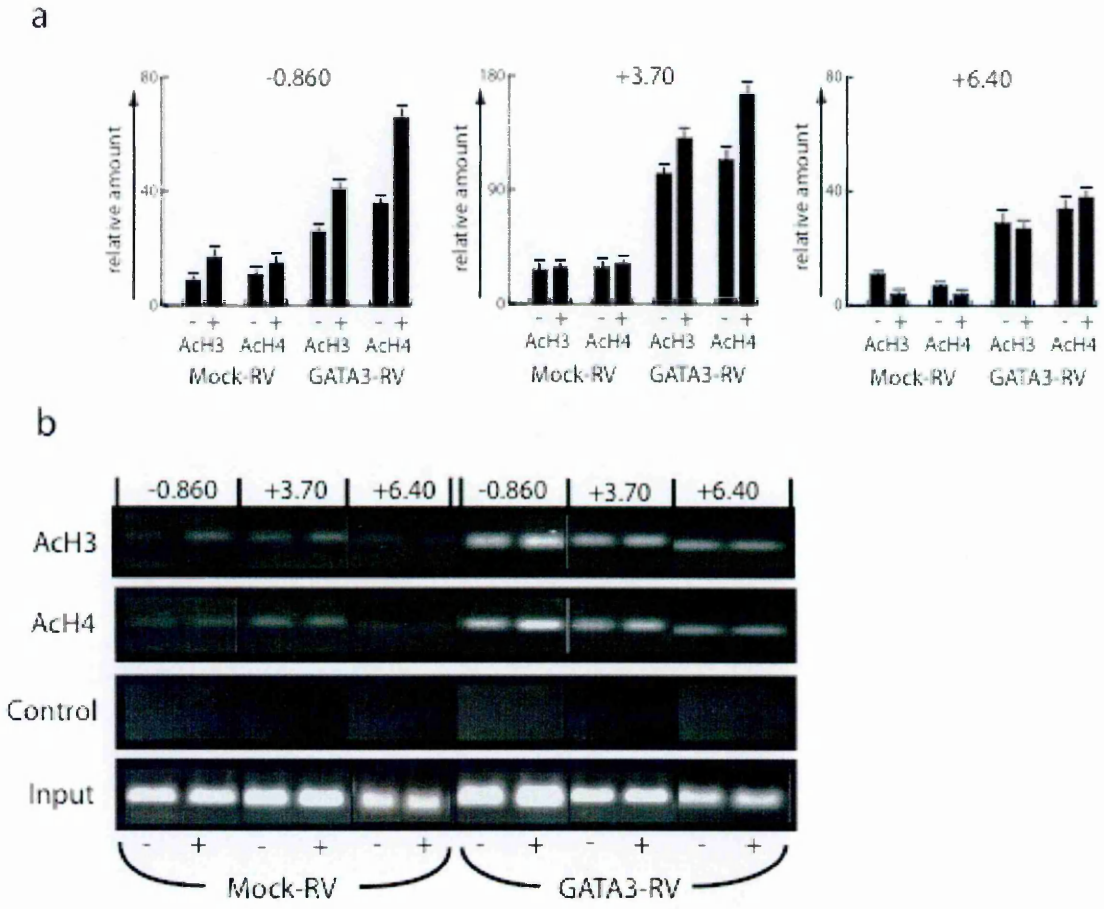


Figure 6.6. GATA-3 induces acetylation of histones H3 and H4 across the *Il-10* locus.
(a) Crosslinked chromatin complexes from resting and activated neutral cultured Mock-RV and GATA3-RV cells were immunoprecipitated with anti-acetyl-histoneH3, anti-acetyl-histoneH4, and control antibodies. Specific oligonucleotides were used to amplify by real-time PCR the GATA-3 binding sites in the *Il-10* locus (-0.86 and +3.70 kbp) and HSS+6.70, using the immunoprecipitated or untreated (Input) chromatin as template. Represented is the amount of PCR product immunoprecipitated with specified antibodies normalized to the amount of PCR product obtained for the Input chromatin. The error bars for each condition represent the standard deviation from 3 replicates. (b) The PCR products obtained as described in (a) were separated in a 2.5% agarose gel and stained with ethidium bromide.

Clearly, both histones H3 and H4 were acetylated to a significantly higher amount when cells had been transduced with GATA-3 (Fig. 6.6a and 6.6b). The acetylation of histones H3 and H4 was readily observed in unstimulated GATA-RV cells, but was further increased upon activation at HSS-0.860 and HSS+3.70 (Fig. 6.6a and 6.6b).

The finding that GATA-3 induced histone acetylation at the *Il-10* locus further supports the ChART-PCR data and GATA-3 role in instructing direct modifications of the *Il-10* locus that lead to IL-10 expression. However, it should be noted that the acetylation studies demonstrate an additional level of positive *Il-10* gene regulation mediated by GATA3-RV transduction, because ChART-PCR indicates exposed HSS, but additional information about histone acetylation of H3 and H4 indicates that these HSSs are indeed positive regulatory regions. In a previous study on chromatin-level *Il-10* gene regulation (114), intron 3 and intron 4 was described to be re-modeled in Th1 cells, but subsequent ChIP studies showed an increased amount of histone deacetylase HDAC1 (and not acetylation of histone H3 and H4) at these regions indicating a "silenced" chromatin structure. This section demonstrates that GATA3-RV induces "active" chromatin structure in three potential *Il-10* gene regions (HSS-.860, HSS+3.7, HSS+6.4). This molecular mechanism is similar to, but independent of, the effects of GATA-3 in re-modeling the chromatin at the *Il-4/Il-5/Il-13* locus and regulation of the Th2-associated cytokines (58, 74, 78). Taken together, our results suggest that GATA-3 acts as a direct regulator of the *Il-10* locus inducing changes in chromatin structure as shown by DNase I digestion and histone acetylation.

6.6 Discussion

To investigate the regulation of *Il-10* gene expression, the transcript-ome of several CD4 T cell populations, including two distinctly regulated T cell populations (Th2 and IL-10-T_{reg}) that expressed high amounts of IL-10. The expression of GATA-3 was up-regulated

in Th2 and IL-10-T_{reg} cells, but not in CD4 T cells that produced low amounts of IL-10, which only expressed little, but detectable GATA-3. In keeping with this, recent studies showed that reduction of GATA-3 expression, by antisense or gene deletion in differentiated Th2 cells decreased the production of IL-10 in addition to a decrease in IL-4 (73, 85). Further, using a transgenic knock-in model in which GATA-3 is ectopically expressed in CD4 T cells, induction of Th2 differentiation and subsequent IL-4, IL-5, and IL-10 production was observed (73, 238). In these studies it was unclear whether GATA-3 was directly exerting its effect on the *Il-10* gene or whether the effects on IL-10 were mediated by variation of IL-4 levels, which has an instructive role in directing Th2 cell differentiation that is accompanied by IL-10 production. Evidence provided in this thesis clearly demonstrates that ectopic expression of GATA-3 increased the transcription and secretion of IL-10 in primary naive CD4 T cells in the complete absence of IL-4.

The molecular mechanism whereby GATA-3 affected the *Il-10* gene remained elusive, the questions being, was GATA-3 recruited to the *Il-10* genomic locus and, if so could it *trans*-activate the IL-10 promoter and/or directly induce re-modeling of the chromatin? By comparing the DNA sequence of the *Il-10* locus between different species two conserved putative GATA-3 binding sites were located at the 5' proximal region (-0.865) and intron 4 (+3.785). The presence of GATA-3 was then shown at these sites *in vivo* in primary Th2 cells. However, unlike its effects on the IL-5 promoter, GATA-3 did not enhance the activity of the IL-10 promoter in *in vitro* reporter assays (data not shown). *Trans*-activation of the *Il-10* locus by GATA-3 via regions other than the IL-10 promoter is currently under study.

ChART-PCR was used to measure and quantify chromatin accessibility at the *Il-10* genomic locus, and showed that GATA-3 induced changes in the chromatin structure at the *Il-10* locus, independently of IL-4. These changes were most pronounced in the vicinity of conserved GATA-3 binding sites, particularly at positions -0.860 and -0.610 bp from the *Il-10* transcription start site. This suggests that the presence of GATA-3 to this region enhanced its chromatin accessibility, possibly by stabilizing the *Il-10* locus in an open, euchromatic form. Furthermore, the accessibility of a recently described positive regulatory region located in the 3' region of the *Il-10* gene (HSS+6.40) (112) was significantly re-modeled under the influence of GATA-3. These observations suggest that GATA-3 may also contribute to long-range changes of the chromatin structure at the *Il-10* locus and therefore to the stabilization of the chromatin conformation needed for the accessibility of transcription factors required for IL-10 expression. Positive regulatory transcription factors likely include the recently described Jun family members, c-Jun and JunB (112), which are shown to enhance IL-10 expression at these long-range sites (CNS-3) (112). However, as no GATA-3 binding was detected by ChIP at HSS+6.4 (data not shown), direct interaction between GATA-3 and this site is unlikely, although GATA-3 may induce changes in chromatin to allow its accessibility to Jun transcription factors.

In further support for an instructive role for GATA-3 in regulating the *Il-10* gene, we found that in the presence of ectopic GATA-3 the histones located in the vicinity of the GATA-3 conserved sites become acetylated, in cells that normally do not express IL-10.

Our data suggest that in the presence of GATA-3, regulatory regions across the *Il-10* locus become accessible (DNase I-sensitive) and "active" (acetylation of histone H3 and H4) to transcription factors.

Whereas changes in the chromatin structure at the *Il-10* locus were induced by GATA-3 prior to TCR stimulation (in unstimulated cells), IL-10 expression and secretion required secondary stimulation, even when ectopic GATA-3 was present. It is possible that GATA-3 instructs the changes at the *Il-10* locus necessary for the action of other transcription factors, such as Sp1, Sp3, SMAD-4, c-Jun, JunB and c-Maf, which have been described in different contexts to enhance IL-10 secretion. Similarly, the presence of GATA-3 may also expose the *Il-10* locus to the function of possible repressors, such as the transcription factor Ets-1 (72).

We also showed that GATA-3-transduced neutral or Th1 polarized T cells have a significant reduction in Ets-1 mRNA compared to the corresponding Mock-transduced cells. This is of interest as in Th1 cells derived from the Ets-1 deficient mice a marked increase of IL-10 production was observed (72), suggesting that the transcription factor Ets-1 may act as a repressor for *Il-10* gene expression. Since it is possible that Ets-1 acts directly on the re-modeled *Il-10* locus to repress *Il-10* transcription, we used crosslinked chromatin from WT neutrally polarized T cells (day 5 post-transduction) GATA-3- or Mock-transduced cells and immunoprecipitated with antibodies that specifically recognize Ets-1 motifs. Since GATA3-RV cells have potential regulatory *Il-10* gene regions re-modeled as opposed to Mock-RV controls, we accessed whether Ets-1 was

differentially present at the *Il-10* locus in either GATA-3- or Mock-transduced cells. We detected Ets-1 was present with primers spanning intron 4 (HSS+3.70) in GATA-3-transduced unstimulated cells, but not in the corresponding Mock-RV cells (data not shown). Furthermore, we detected a consensus putative Ets-1 biding site in the murine *Il-10* gene within intron 4. We did not detect the presence of Ets-1 in GATA-3 or Mock-transduced cells with primers spanning HSS+6.40 (even though both conditions have an accessible HSS here), which contain a conserved putative Ets-1 binding site from our *in silico Il-10* locus analysis. These findings suggest that Ets-1 is able to directly bind to the re-modeled *Il-10* gene (intron 4) and may act to repress *Il-10* expression as indicated by the Ets-1(-/-) results. Additionally, our data suggests that Ets-1 binds only to previously re-modeled regions of the *Il-10* gene. Together, we show that Ets-1 mRNA is down-regulated by GATA-3-RV transduced CD4 T cells when compared to Mock-RV controls; however Ets-1 protein is also exclusively present in GATA3-RV cells within a potential regulatory region of the *Il-10* gene (intron 4). This could partly explain why in GATA-3-transduced neutral or Th1 cells (72) the production of IL-10 is increased, but never to the extent observed in Th2 and IL-10-T_{reg} cells (which have low-abundance of Ets-1 expression).

The possible interactions between several transcription factors may allude to multiple layers of combinatorial effects of transcriptional enhancers and repressors acting directly on the *Il-10* gene, GATA-3 being a key transcription factor that exposes the *Il-10* locus to all these putative regulators. This hypothesis is very attractive and could explain why we detected a basal level of GATA-3 expressed in all cells that produce IL-10 despite their

variation on quantitative amounts of IL-10 production. In this chapter, we systematically found that the expression levels of GATA-3 correlated with IL-10 secretion by different populations of primary CD4 T cells. GATA-3 induced IL-10 expression in primary CD4 T cells under various differentiation conditions, even in the absence of IL-4. We also showed that GATA-3 was present at the *Il-10* locus and induced changes to the *Il-10* gene chromatin structure, and further induces an "active" transcriptional status (acetylation status of histone H3 and H4). Taken together our data suggests an instructive role for GATA-3 in the regulation of *Il-10* expression by directly re-modeling the *Il-10* gene.

7. Potential role of additional transcription factors in *Il-10* gene regulation

In this Chapter I will analyze other transcription factors potentially involved in *Il-10* gene regulation. Transcription factors, c-Maf, PPAR-γ, were functionally investigated in the RV system based upon their unique expression profiles isolated from the GeneChip studies (Fig. 4.2). c-Maf and PPAR-γ were both expressed in IL-10-producing cells (Fig. 4.2). The validated mRNA expression profiles for both c-Maf and PPAR-γ are shown below in Figure 7.1.

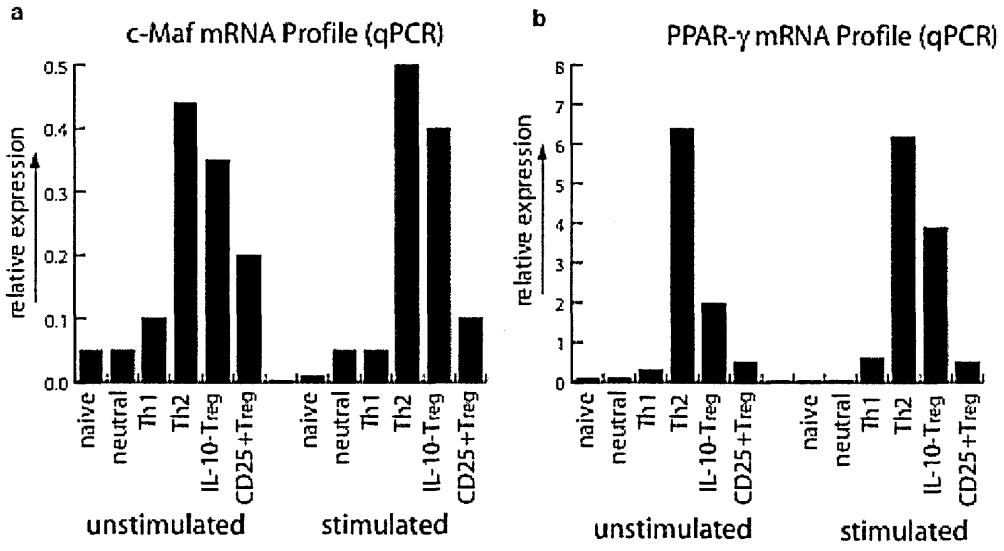


Figure 7.1. c-Maf and PPAR-γ are differentially expressed in IL-10-producing CD4 T cells
(a) Real-time PCR was performed to validate the results from the GeneChip for c-Maf and PPAR-γ expression in unstimulated or stimulated CD4 T cell populations.

These two transcription factors were functionally studied for effects on *Il-10* gene regulation primarily via RV-mediated transduction into naïve CD4 T cells and subsequent polarizing conditions: neutral (no antibodies) and Th1 driven culture conditions. Functional outputs were measured at the secondary re-stimulus time-point via cytokine profiles (ICS FACS) and proliferation numbers comparing (gene-of-interest)-RV versus Mock-RV transduced cells.

This chapter briefly summarizes the findings from the RV system with these transcription factors, performed in primary naïve CD4 T cell populations driven under various culture conditions in the presence of c-Maf-RV or PPARγ-RV and Mock-RV as controls.

7.1 c-Maf weakly enhances IL-10 production in Th1 culture conditions

In terms of CD4 T cell cytokine gene regulation, c-Maf is classically described to *trans*-activate the re-modeled IL-4 promoter (87). However three observations have lead to c-Maf being functionally tested in the RV system for its effects on IL-10 gene regulation. Cao *et al.* demonstrated that c-Maf can also *trans*-activate the proximal IL-10 promoter and induce IL-10 production in TLR stimulated BM-macrophages. Secondly, we showed differential expression of c-Maf in both IL-10-producing T cell populations (Th2 and IL-10-T_{reg} cells). Thirdly, *in silico* analysis defined two putative conserved AP-1 binding sites and one putative c-Maf binding site within intron 2 of the *Il-10* gene as described in Materials and Methods. Therefore, the function of c-Maf-RV transduced cells in terms of IL-10 production in both neutral and Th1 driven culture conditions (two conditions which normally do not express c-Maf) was tested.

Primary WT naive CD4 T cells were isolated and transduced with c-Maf-RV or Mock-RV and polarized in neutral or Th1 conditions. CD4 T cells were re-stimulated on Day 6 post-transduction to reveal effects of the transcription factor on subsequent cytokine production by T cells, and ICS is shown below in Figure 7.2.

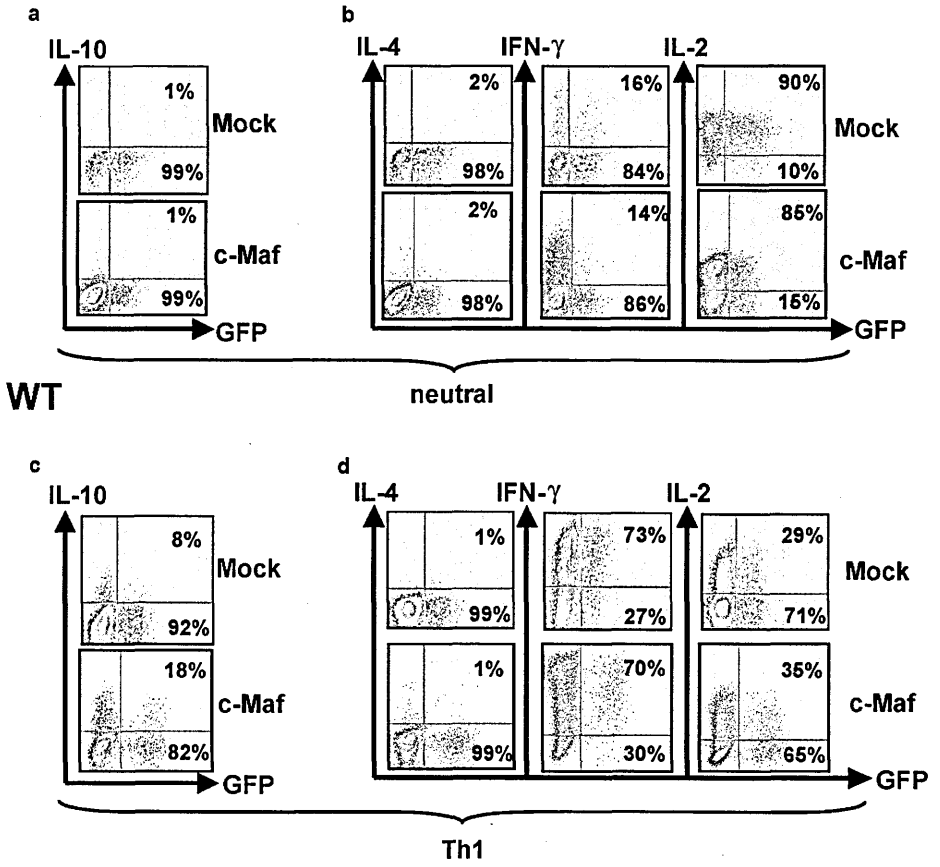


Figure 7.2. c-Maf weakly enhances IL-10 production Th1 driven cell conditions, but not neutral conditions.
WT naïve (CD4+CD62L+CD45RB^{high}) T cells were infected with a Mock or a c-Maf-expressing retrovirus and differentiated under neutral (a, b) and Th1 (c, d) conditions. Six days after infection, cells were stimulated with PMA/Ionomycin for 4 h, intracellular stained for IL-10 (a, c) or for IL-4, IFN-γ, and IL-2 (b, d) and analyzed by FACS. Cells were gated on GFP expression (x-axis) and the percentage of GFP-positive cells expressing the respective cytokines is shown. Data shown is an accurate representation of three experimental replicates.

Under neutral conditions c-Maf-RV and Mock-RV transduced cells showed no significant difference in cytokine expression as shown by ICS for IL-10-, IFN-γ-, IL-4- and IL-2-producers (Fig. 7.2a and 7.2b). However, under Th1 driven culture conditions c-Maf-RV showed a higher number of IL-10 producers (18%) when compared to Mock-RV IL-10 producers (7%) (Fig. 7.2c). IFN-γ-, IL-4- and IL-2-producers had similar profiles when compared to c-Maf-RV and Mock-RV cells (Fig. 7.2d). IL-4-producers, a known target of c-Maf *trans*-activation (87), were not significantly increased in either c-

Maf-RV neutral or Th1 culture conditions as compared to Mock-RV cells (Fig. 7.2b and 7.2d). This suggests that the IL-4 promoter is not accessible and therefore *trans*-activation by c-Maf is not achieved.

In CD4 T cells, c-Maf has been shown to directly enhance IL-4 expression by binding to the re-modeled proximal IL-4 promoter (87). Other immune cells were shown to increase IL-10 expression via c-Maf enhancement within the IL-10 promoter (116). In the above RV studies we show that c-Maf transduced cells are not able to enhance IL-10 production in neutrally driven conditions, but are able to moderately enhance IL-10 production in Th1 conditions. No IL-4 production is achieved in either neutral or Th1-driven c-Maf transduced populations, supporting previous studies where c-Maf could only enhance IL-4 production under neutral conditions (but only when IL-4 was not neutralized, i.e. could not achieve this effect in the complete absence of IL-4, in contrast to GATA-3 which could instruct re-modeling of IL-4 locus under these conditions (74, 78, 87)). The fact that c-Maf-RV does not enhance IL-4-producers when compared to the Mock-RV cells is probably because both neutral and Th1-driven culture conditions did not induce re-modeling of the *Il-4* locus and allow access for the described c-Maf-mediated *trans*-activation of the *Il-4* gene (87). Unlike the *Il-4* locus, the *Il-10* locus is partially re-modeled in Th1-driven conditions (ChART-PCR) and therefore the data presented here suggests c-Maf could act, through a similar mechanism as described for the *Il-4* gene, and enhance IL-10 production once the locus is re-modeled. Cao *et al.* have reported c-Maf mediated *trans*-activation of the minimal IL-10 promoter in the RAW264.7 murine macrophage cell-line. Additionally they showed through deletion studies that a putative

basic leucine transcription factor (bZip) binding site at the -.190 bp position in the IL-10 murine promoter acts as the location for c-Maf-mediated *trans*-activation. This group showed that transfection of c-Maf into primary human monocytes increased their ability to express IL-10 upon TLR activation. The combination of the Cao *et al.* study and our results involving increased IL-10-producers in Th1-driven culture conditions suggests a possible role for c-Maf in enhancing IL-10 expression.

In our studies it is interesting to note that under neutral conditions c-Maf-RV was unable to increase IL-10 production upon re-stimulus. A mechanism for this may be via the partial re-modeling of the *Il-10* gene that we have shown in Th1-driven conditions, allowing for enhancing factors such as, c-Maf, to access sites within the *Il-10* locus. The location whereby c-Maf may directly act to enhance *Il-10* transcription is not clear from our studies. It should be noted that AP-1 sites in both the 3' and intronic regions of the *Il-10* gene could be sites for c-Maf-mediated co-operation, as AP-1 complexes can include JunB:Maf (113). Further, we have shown using an *in silico* generated analysis of the *Il-10* locus that there is a v-Maf conserved putative binding site within intron 2 of the *Il-10* gene. Lastly, the minimal IL-10 promoter region has been described to be *trans*-activated by c-Maf (116); however whether the IL-10 promoter region is accessible in our c-Maf-RV cells polarized under Th1 conditions is not known (however, Th1 cells in our highly sensitive ChART-PCR do not re-model the IL-10 promoter during TCR-unstimulated conditions). In summary, c-Maf-RV weakly enhances IL-10 expression in CD4 T cells under certain conditions but the exact mechanism by which may function at the molecular level is not yet clear.

7.2 PPAR-γ

PPAR-γ, a nuclear receptor transcription factor, was selected for functional studies based upon its IL-10-associated expression profiles described previously (Fig.7.1). PPAR-γ is activated by ligand/receptor interactions thereby becoming transcriptionally active and repressing or enhancing target genes (229). The regulation of PPAR-γ expression is not clear; however PPAR-γ expression and activation in other developmental cell lineages has proven that PPAR-γ can act as a repressive nuclear factor to influence subsequent cell differentiation (229, 230). Moreover, our expression profiles suggested PPAR-γ could be involved in *Il-10* gene regulation and its function was tested in the RV system under neutral and Th1 conditions (two populations which did not express PPAR-γ). Alternatively it is possible that PPAR-γ could repress the expression of other cytokine genes or other functions in IL-10-producing cells.

In both culture conditions (neutral or Th1) there was no difference in differential cytokine producers or proliferation between PPARγ-RV and Mock-RV transduced cells (data not shown, day 7). This suggested that PPAR-γ did not play a role in neutral or Th1 driven culture conditions for CD4 T cell cytokine production (IL-10, IFN-γ, IL-4 and IL-2) or proliferation.

The fact that transduction of PPAR-γ into neutral or Th1 driven cells did not induce differential cytokine production might arise from the absence of PPAR-γ ligands expressed in either neutral or Th1 cells and therefore PPAR-γ - a nuclear receptor - was

not transcriptionally active. A specific-ligand for PPAR-γ activation was not available so these studies were not pursued. It may be likely that neutral or Th1 cultured cells, which do not normally express PPAR-γ, might not express an intrinsic ligand to activate PPAR-γ. In summary, PPAR-γ was selectively up-regulated in IL-10-producing CD4 T cells; however its RV-mediated transduction into differentiating neutral or Th1 populations did not induce cytokine production or affect proliferation related effects when compared to Mock-RV cells.

8. Molecular gene expression profiles in IL-10-Treg vs. CD25+T_{reg}

CD4 T_{reg}-mediated suppression in either the initiation or effector activation of peripheral immune response is currently of great interest (127). The major hypothesis-driven area of research initially set out to understand in this thesis was the regulation of the *Il-10* gene. However, in developing the panel of CD4 T cell populations the realization that two phenotypically different CD4 T_{reg} populations presented a unique opportunity to investigate the mechanisms differing or shared between both CD4 T_{reg} populations. Of specific interest was of the CD4 T_{reg} were the naturally occurring CD4+CD25+ T_{reg} and the antigen-driven IL-10-T_{reg} which share the inability to produce proliferative and effector cytokines upon TCR triggering, and both are anergic when triggered through the TCR (21, 125, 174-177). In this Chapter, the investigation of shared intrinsic mechanisms within CD4 T_{reg} populations suggests that each of these regulatory populations may use independent mechanisms to restrict pro-inflammatory expression of genes upon TCR activation, and to inhibit proliferation induced through TCR-signaling, but also these mechanisms may have some similarities with respect to the targeted pathway.

The classification of "natural occurring" CD4 T_{reg} (CD25+T_{reg} in our panel) is defined by their expression of the lineage specific transcription factor, FoxP3 (129). One central role for FoxP3 expression lies in its ability to functionally abrogate transcription of pro-inflammatory cytokine genes upon TCR activation. In CD4 T_{reg} populations, both IL-10-T_{reg} and CD25+T_{reg} are similar in that they do not express pro-inflammatory cytokines upon TCR activation (21, 174 Shevach, 2001 #221, 175-177). In CD25+T_{reg} this is likely

due to the fact that FoxP3 is expressed, however a FoxP3-equivalent in IL-10-T_{reg} (which do not express FoxP3 (174)), or if IL-10-T_{reg} have a FoxP3 equivalent, has not been described.

FoxP3 interacts with NF-κB and NFAT complexes to regulate their ability to bind DNA and activate gene transcription (137, 140). An alternative mechanism to abrogate NF-κB and NFAT transcriptional activity in IL-10-T_{reg} is suggested by our findings and presented here. In this chapter, one potential transcription factor speculated to have some functional potential to induce an anergic phenotype was GILZ, which was expressed in IL-10-T_{reg} (as I show in this Chapter). GILZ was tested in the RV system in neutral, Th1, and Th2 culture driven conditions to determine whether GILZ would interfere with cytokine production and/or proliferation as described in previous studies with transduction of FoxP3 (122, 124, 128, 129). In summary, the investigation into the potential mechanism whereby IL-10-T_{reg} are inhibited in their ability to produce significant amounts of pro-inflammatory cytokines and to proliferate upon TCR activation, without the expression of FoxP3, will be the focus of this Chapter.

8.1 NF-κB family members

The activation of the NF-κB pathway is closely associated with targeting pro-inflammatory immune response genes after receiving external stimuli (90). In terms of mechanisms after TCR triggering, NF-κB complexes are well-described to undergo modifications that subsequently lead to activation of target gene expression (90). A central role for FoxP3 in CD4+CD25+ is suggested to involve its interaction with NF-κB

complexes to abrogate their downstream activation of target gene expression (140). This mechanism fits with subsequent down-regulation of key NF-κB target pro-inflammatory genes in FoxP3-expressing cells (CD25+T_{reg}). IL-10-T_{reg} exhibits similar down-regulation of NF-κB target genes after TCR activation; however the mechanism is independent of FoxP3 as IL-10-T_{reg} but do not express FoxP3. The regulation and expression of individual NF-κB genes may play a role in subsequent down-regulation of NF-κB target gene activation in IL-10-T_{reg}. Below the GeneChip profiles (Fig. 8.1a) and qPCR (Fig. 8.1b) suggest that NF-κB family members, which are classically described to enhance pro-inflammatory and effector cytokine genes are down-regulated upon TCR activation in IL-10-T_{reg} (Fig. 8.1).

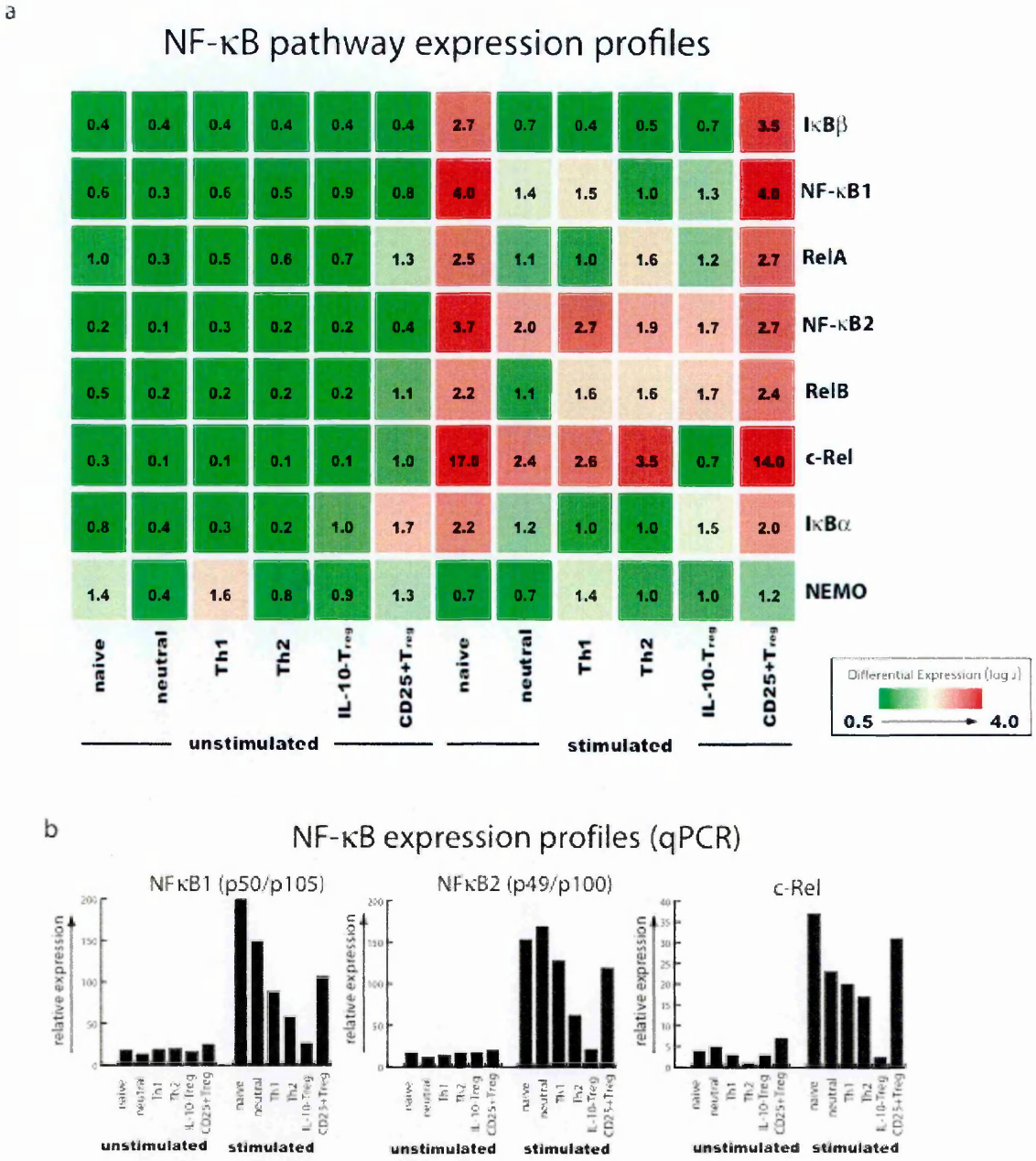


Figure 8.1. Profiling NF-κB pathway reveals down-regulated signature in IL-10-T_{reg}
(a) GeneChip profiles for selected NF-κB genes in CD4 T cells. The GeneChip image was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. (b) mRNA was isolated from unstimulated and stimulated cells (6 hr.) (α-CD3/CD28), converted to cDNA and qPCR was performed with primer/probes acquired from ABI (inventoried: assay-on-demand) and mastermix (ABGene) was used to measure relative expression of NF-κB genes on the ABI Prism 7900. Data shown is an accurate representation of three biological replicates.

Rel/NF-κB family genes are differentially expressed upon activation in CD4 T cell populations by Affymetrix Gene Chip analysis (Fig. 8.1a). To further validate and

quantitate the expression of these transcription factors, qPCR was used (Fig. 8.1b). Within all the CD4 T cell populations the expression of NF-κB1, NF-κB2, c-Rel, and RelA are significantly up-regulated upon TCR-triggering in both naïve and CD25+T_{reg} populations, and to a lesser extent in Th1 and Th2 effector cells (Fig. 8.1). In IL-10-T_{reg} NF-κB1, NF-κB2, and c-Rel are expressed to a lower relative (GeneChip) (Fig. 8.1a) and quantitative amount (qPCR) (Fig. 8.1b) when compared to other CD4 T cell populations in the stimulated condition. However, most dramatically, the relative expression of c-Rel is minimal after activation in IL-10-T_{reg} when compared to CD25+T_{reg} and other CD4 T cell populations (Fig. 8.1). Since NF-κB proteins, especially c-Rel, are necessary for expression of either the *Il-2* (95) or *Ifn*-γ (98) gene upon TCR-pathway activation, the fact that IL-10-T_{reg} express relatively low-levels of these genes is of significance for the potential phenotype and function of these cells.

8.2 NFAT and AP-1 family members

AP-1-mediated target gene expression is strongly dependent on the interactive combinations, between AP-1 family members and on AP-1:NFAT interactions (241). The differential combinations of AP-1 family members, Jun:Fos (c-Jun:c-Fos) versus Jun:Jun or Jun:Maf (c-Jun:JunB or JunB:JunB or JunB:c-Maf), can dictate differential downstream target gene expression (241). For example in CD4 T cells, Fos:Jun heterodimers are associated with activation of pro-inflammatory gene expression, whereas Jun:Jun or Jun:Maf dimers are loosely associated with a subsequent anti-inflammatory gene expression profile (113, 241). Another major regulation step in the AP-1 families ability to activate target gene expression lies within their ability to form

ternary complexes with NFAT family members (59). Jun:Fos dimers are suggested to be the key AP-1-associated dimer in ternary AP-1:NFAT binding (241). Cytokine genes are highly-concentrated with composite putative NFAT:AP-1 binding sites in their respective potential regulatory regions. In this respect, FoxP3 is suggested to interact with NFAT (137, 140), and interfere with NFATs ability to form NFAT:AP-1 ternary pairs (140). The NFAT:FoxP3 interaction is suggested to interfere with subsequent NFAT:AP-1 target gene activation (137, 140). This is in agreement with NFAT:AP-1 ternary pairs being required for IL-2, IL-3, IL-4, IL-5, and IFN-γ expression (59), and FoxP3-expressing cells do not produce these cytokines upon TCR activation.

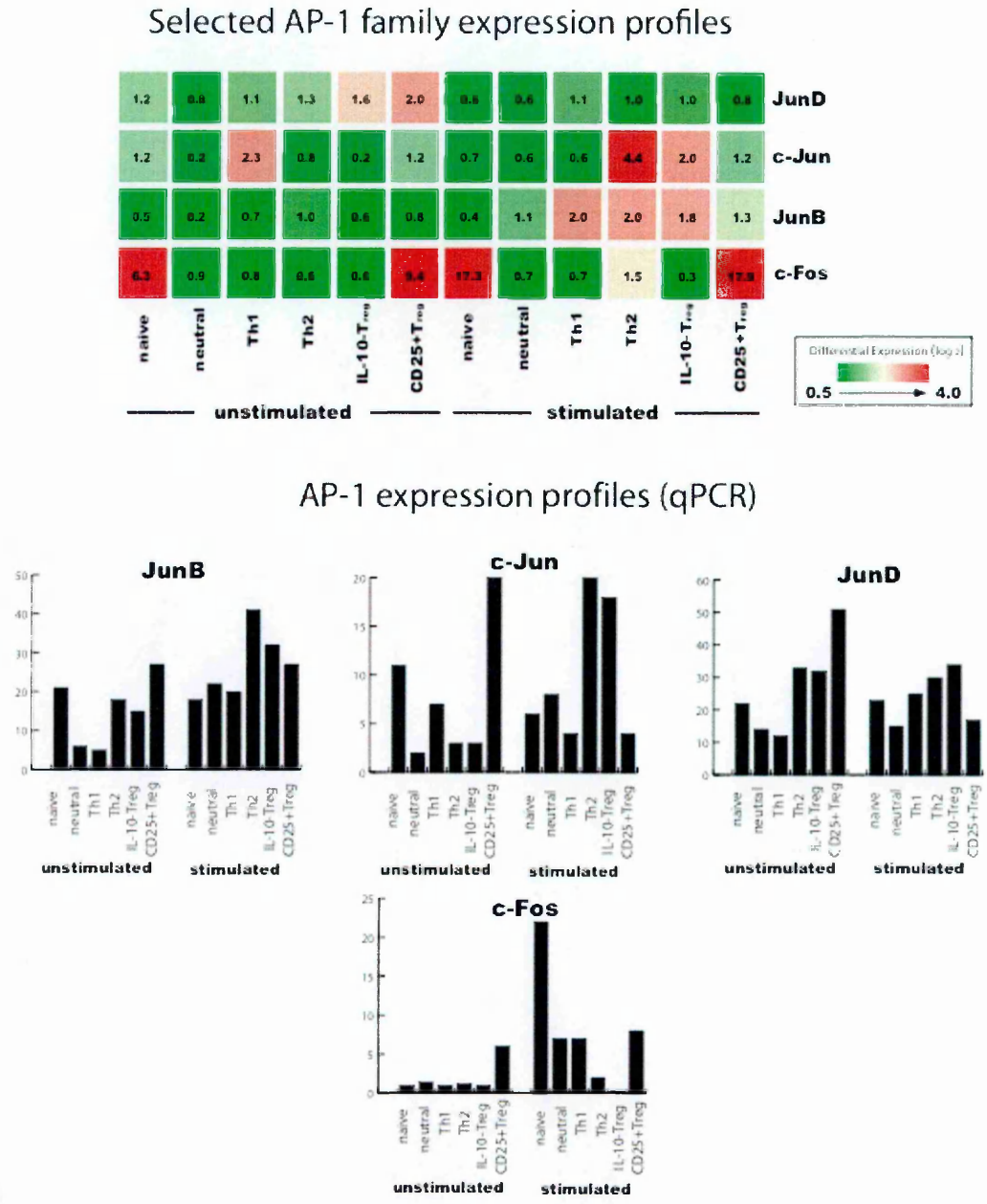


Figure 8.2. Profiling AP-1 genes reveals differential signature in IL-10-T_{reg}
(a) GeneChip profile for AP-1 in CD4 T cells. (b) qPCR profiles for selected AP-1 genes in CD4 T cells. The GeneChip image for both AP-1 genes was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. For qPCR data, mRNA was isolated from unstimulated and stimulated cells (6 hr.) (α-CD3/CD28), converted to cDNA and qPCR was performed with primer/probes acquired from ABI (inventoried: assay-on-demand) and mastermix (ABGene) was used to measure relative expression of both NFAT and AP-1 genes on the ABI Prism 7900. Data shown is an accurate representation of three biological replicates.

In the IL-10-T_{reg} or Th2 populations the expression of the AP-1 family member, c-Fos, is not up-regulated upon TCR-triggering, whereas Jun family members, c-Jun, JunB and c-

Maf are up-regulated as compared to other CD4 T cell populations, although to c-Jun is up-regulated to a lesser extent in CD25+ Treg and Th1 cells (Fig. 8.2a). This suggests that IL-10-T_{reg} and Th2 cells may have higher relative amounts of Jun:Jun and Jun:Maf as opposed to Fos:Jun dimers, which is suggested to correlate with a subsequent anti-inflammatory gene expression profile after TCR activation (113, 241). Figure 8.2b demonstrates that c-Fos is up-regulated in stimulated naïve CD4 T cells. Alternatively, c-Fos expression is down-regulated in both unstimulated and stimulated IL-10-T_{reg} conditions (GeneChip) (Fig. 8.2b). JunD is not up-regulated or down-regulated (>2.0 log or <0.5 log) in any CD4 T cell population or condition as shown by GeneChip (Fig. 8.2b). c-Jun is down-regulated in neutral and IL-10-T_{reg} populations in unstimulated conditions and up-regulated in Th1 (unstimulated) and IL-10-producing populations in stimulated conditions (Fig. 8.2b). JunB is up-regulated (2.0 log) in Th1 and Th2 populations and in CD25+T_{reg} and IL-10 T_{reg} upon TCR activation (Fig. 8.2b).

NFAT family genes are profiled below (Fig. 8.3).

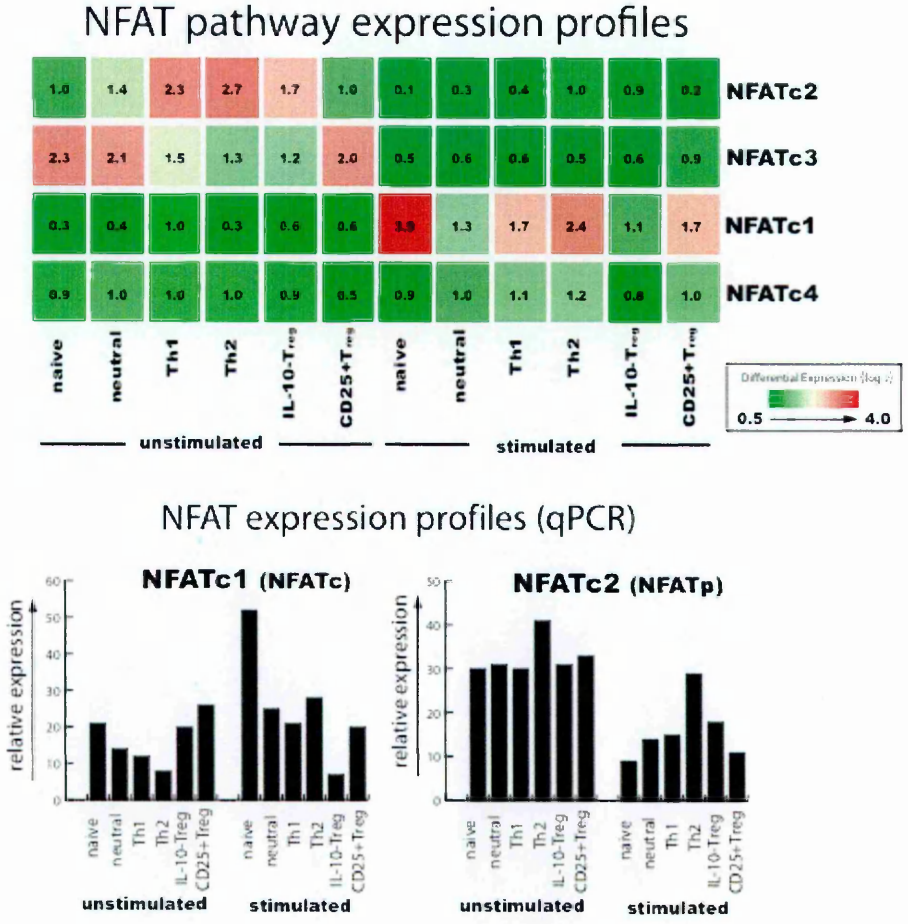


Figure 8.3. Profiling NFAT genes reveals differential signature in IL-10-T_{reg}
(a) GeneChip profile for NFAT in CD4 T cells. (b) qPCR profiles for selected NFAT genes in CD4 T cells. The GeneChip image for both NFAT genes was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. For qPCR data, mRNA was isolated from unstimulated and stimulated cells (6 hr.) (α-CD3/CD28), converted to cDNA and qPCR was performed with primer/probes acquired from ABI (inventoried: assay-on-demand) and mastermix (ABGene) was used to measure relative expression of both NFAT and AP-1 genes on the ABI Prism 7900. Data shown is an accurate representation of three biological replicates.

NFATc1 is interesting in that it is not up-regulated from the baseline value (1.1 log_2) in IL-10-T_{reg} in the stimulated condition, while Th1, Th2 populations and CD25+ T_{reg} up-regulate NFATc1 to similar degrees although less so than naïve T cell populations (quantitatively (qPCR) and differentially (GeneChip)) (Fig. 8.3). Other NFAT family transcripts (NFATc3 and NFATc4) do not exhibit any drastic differences in the degree of up- or down- regulation between CD4 T_{reg} and effector or naïve populations (Fig. 8.3a).

In summary, c-Fos is not significantly up-regulated in IL-10-T_{reg} or Th2 cells (TCR-stimulated) but Jun family members (c-Jun and JunB) are up-regulated in stimulated IL-10-producing populations similarly to in other T cell populations.

8.3 Transduction of GILZ into different effector populations results in differential effects on T cell proliferation

GILZ was initially described as a glucocorticoid induced transcription factor, which (1) enhances CD4 T cell survival upon TCR-mediated stimulus (206) and (2) over-expression of GILZ in the CD4 T cell lineage skews *ex vivo* cytokine production by CD4 T cells towards Th2-associated cytokine profiles (209). The GeneChip profiles revealed high-level expression of GILZ in unstimulated naïve and in both CD25+ and IL-10-T_{reg} populations (data not shown, qPCR profiles are shown in Figure 8.4), although this expression is abrogated upon activation.

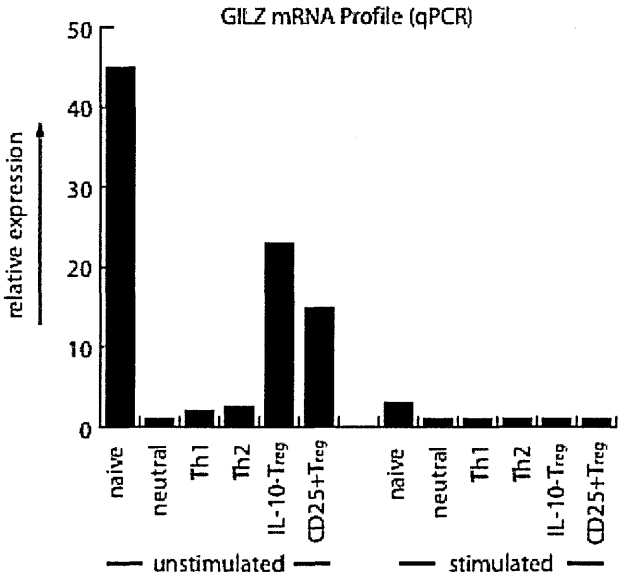


Figure 8.4. GILZ is differentially expressed in unstimulated IL-10-T_{reg}
Real-time PCR was performed to validate the results from the GeneChip for GILZ expression in unstimulated or stimulated CD4 T cell populations.

As previously reported, however, GILZ expression was significantly down-regulated in all GILZ-expressing populations after stimulation (206). Based on previous reports it can be speculated that the ability of the three GILZ-expressing populations (naïve, IL-10-T_{reg}, CD25+T_{reg}) to survive after re-stimulus (α-CD3/CD28) may be enhanced by their expression of GILZ, and therefore the questions addressed were: would GILZ-RV transduced into neutral, Th1 or Th2 populations affect the cell recoveries after stimulation? Secondly, would GILZ-RV transduced cells in neutral, Th1 and Th2 conditions affect cytokine production?

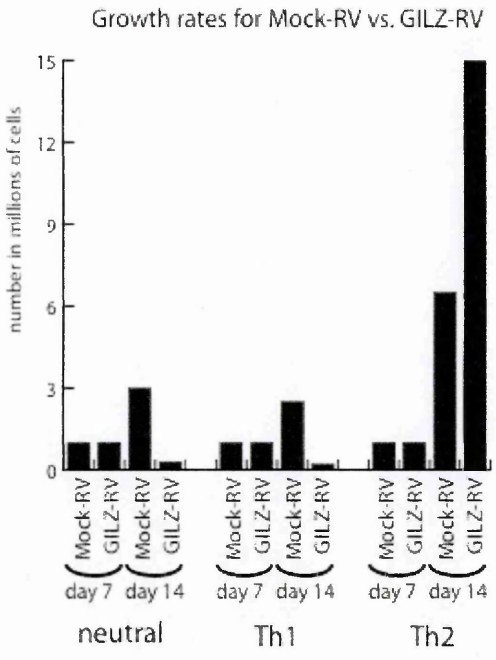


Figure 8.5. GILZ induces reduced cell recoveries in neutral and Th1 driven culture conditions and enhances proliferation in Th2 driven culture conditions upon secondary TCR stimulation.
WT naïve (CD4+CD62L+CD45RB^{high}) T cells were infected with a Mock or a GILZ-expressing retrovirus and differentiated under neutral, Th1 and Th2 conditions. GFP+ Mock or GILZ-expressing cells were purified by flow-cytometry into GFP-positive populations and re-stimulated day 6 post-transduction. Growth values were calculated at Day 14, 7 days after secondary TCR stimulus. Cell values are reported in millions of cells counted.

Strikingly, when either GFP-positive Mock-RV and GILZ-RV populations (MoFlo-isolated day 4 post transduction) were stimulated (α-CD3/CD28), then re-stimulated with

anti-CD3/CD28 and grown for another 7 days to assess effects on cell growth/recovery. GILZ-RV transduced cells in neutral and Th1 driven culture conditions differentially showed extremely low recoveries as compared to Mock-RV cells (Fig. 8.5). Conversely, GILZ-RV transduced cells in Th2 driven culture conditions expanded at a rate ~2.5 times greater than Mock-RV transduced cells (Fig. 8.5) after re-activation on day 7. The proliferation of Mock-RV transduced cells in Th2 driven culture conditions was similar to normal (non-RV-transduced cells) Th2 driven culture conditions (data not shown). The hyper-proliferation of GILZ-RV cells versus Mock-RV cells in Th2 driven culture conditions continued after Day 14 α-CD3/CD28 re-activation (data not shown). Importantly, before the initial re-activation (day 7), GILZ-RV versus Mock-RV showed no difference in proliferation rates under any condition (neutral, Th1, Th2) studied here.

All GILZ-RV transduced culture conditions (neutral, Th1, and Th2) exhibited a similar cytokine profile (Mock-RV versus GILZ-RV) (data not shown). This was partially in disagreement with recent results from *ex vivo* isolated CD4 T cells from transgenic mice with CD4 T cell-specific GILZ over-expression, which were reported to preferentially produce Th2-associated cytokines upon TCR activation (209). However this discrepancy may be a result of our proliferation results. The results shown here were interesting with respect to the lack of a differential GILZ-mediated cytokine production in either neutral, Th1 or Th2 conditions; however, GILZ-RV populations dramatically induced proliferation in Th2 culture conditions while alternatively inhibiting TCR-mediated growth in neutral and Th1 culture conditions, possibly by inducing apoptosis. Taken together, the data presented here is in agreement with certain aspects of the recent reports

from the GILZ TG over-expression mouse model with respect to enhanced survival of T cells (209), but not the conclusion that GILZ induces intrinsic Th2-associated cytokine production. Our data suggest that the apparent increase in Th2-assocaited cytokine production may actually result from increased proliferation/survival of Th2 cells, rather than by up-regulation of Th2-specific cytokine genes, and can only be achieved when IL-4 is already produced (Fig. 8.5). The mechanism by which GILZ mediates survival in Th2 conditions (a CD4 T cell population which normally does not express GILZ) after TCR triggering is not known.

8.4 Discussion

Transcription factor regulation was our primary goal in undertaking this study with the global gene expression platform. We report that the known signature transcription factors driving differentiation in effector and CD25+ T_{reg} population are clearly shown with the GeneChip and further validation with qPCR. Secondly, we were able to decipher TCR triggered gene regulation in nuclear factors that influence regulation of key cytokine genes particularly NF-κB and AP-1:NFAT gene expression signatures. With respect to the balance between the effector populations (Th1 and Th2) we show that Th1 cells differentially express higher levels of Rel family members (NF-κB1, NF-κB2) upon activation; whereas the Th2 population expresses higher levels of expression between NFAT family members (NFATc1, NFATc2). Secondly, we postulate that the inability of IL-10-T_{reg} and Th2 cells to up-regulate c-Fos transcripts explains the inability of both these populations to produce IL-2, however, since Th2 cells can use IL-4 as an alternative growth factor, whereas IL-10-T_{reg} do not produce this cytokine, this would explain the anergic phenotype of IL-10-Treg that we have described. Furthermore, since IL-10-T_{reg} also fail to up-regulate NFATc1 and c-Rel this could suggest a mechanism as to why IL-10-T_{reg} do not produce the array of Th1 or Th2 cytokine genes that require c-Rel and AP-1:NFAT signaling in order to initiate their expression after activation.

One hypothesis that we set out to understand in this study was the relationship between both regulatory populations ability to not produce proliferative and effector cytokines upon stimuli. Our data suggests that the mechanism for each regulatory population to achieve this may be independent, but also similar. Specifically, IL-10-T_{reg} are

significantly depressed in their ability to express some NF-κB (c-Rel) and AP-1 (c-Fos) and NFAT (NFATc1) family transcripts after activation. In contrast, CD25+ T_{reg} produce high levels of NF-κB and AP-1 family transcripts after activation, but also produce high levels of FoxP3, a known inhibitory molecule for efficient NF-κB and AP-1:NFAT transcriptional activity (137, 140). Taken together, we postulate that both regulatory populations are able to similarly inhibit NF-κB and NFAT activation of cytokine genes, but accomplish this by different means. This part of the study in my thesis is no means complete but merely suggestive of the possible mechanisms whereby IL-10-Treg shut off effector cytokines and T cell proliferation. Furthermore, it remains to be seen whether this phenotype of IL-10-T_{reg} driven in culture with vitD_3/DEX represents the equivalent in vivo population of anergic IL-10-producing cells as previously reported (21, 174 Shevach, 2001 #221, 175-177), driven by repeated administration of high amounts of peptide or protein antigen is the scope of future studies. Regardless of whether the effects that we report on the diminished transcription of particular transcription factors is achieved by an immunosuppressive drug mediated effect only, this is of importance with respect to their mechanism of action which thus far is unclear.

Future Perspectives

9.1 *Il-10* gene regulation

The transcription factor, GATA-3, is described in this thesis as a potent, instructive regulator of *Il-10* gene regulation in CD4 T cells. Differential GATA-3 expression (Fig. 6.1a) correlates with levels of IL-10 production by CD4 T cells (Fig. 3.1). Through utilization of a RV system (over-expression studies) it became clear that GATA-3 induced production of IL-10 and this effect was independent of IL-4 (Fig. 6.2 and 6.3). In fact, the effect of GATA-3 expression (RV system) at early time-points in CD4 differentiation, (up to day 4 after primary TCR stimulus, day 3 after GATA-3 transduction) showed GATA-3 induced significantly higher amounts of IL-10 production versus IL-4 and IL-5 production upon TCR activation (data not shown). This suggests that GATA-3 plays a crucial role inducing IL-10 production at early time-points, possibly by allowing access or inducing "active" chromatin modifications at the *Il-10* locus to nuclear factors - that are not only exclusively available in high-IL-10-producing cells but also other non-IL-10-producing CD4 T cell populations - upon TCR activation. To further support the early GATA-3 effect on the *Il-10* locus, GATA-3 re-models the *Il-10* locus (ChART-PCR) (day 6 post transduction), in IL-4 independent neutral culture conditions (Fig. 6.5), to a similar chromatin-level profile (Southern blot) seen in high-IL-10-producing CD4 T cell populations (Th2 and IL-10-T_{reg}) (110, 115). Furthermore, the "strong" re-modeling of a high-IL-10-producing CD4 T cell specific HSS (IL-10 promoter, HSS-0.860 and HSS-0.610) is achieved by GATA-3 over-expression (Fig. 6.5). GATA-3s direct effect is further supported by GATA-3 presence (ChIP) at the IL-10 promoter and intron 4 before TCR triggering in high-IL-10-producing populations (Th2) (Fig. 6.4). Together, this suggests that the re-modeling of the *Il-10* locus, but in

particular the IL-10 promoter (classified as a high-IL-10-producing CD4 T cell specific HSS site), directly by GATA-3, allows access for certain non-cell specific TCR-triggered enhancing nuclear factors on the *Il-10* gene. Conversely, IL-4 and IL-5 expression may be dependent on additional CD4 T cell differentiation and epigenetic imprinting (further chromatin re-modeling and/or transcription factor enhancers) to allow optimal production of these Th2-associated cytokines, as their expression is lower than IL-10 at earlier time-points in neutral and Th1 GATA-RV culture conditions. It is well described that IL-4 production by CD4 T cells acts in an autocrine fashion to drive additional IL-4 expression and terminal Th2 differentiation (5). IL-4 driven culture conditions in turn drive GATA-3 expression and GATA-3 drives its own regulation through an autocrine-signaling pathway (74). Therefore, previous to these studies the role of GATA-3 in CD4 T cells, independent of IL-4, has never been systemically addressed at the *Il-10* gene. Figure 9.1 represents a cartoon describing the systematic regulation collectively at play on the *Il-10* gene from a naive CD4 T cell in to either a low-IL-10-producuer or a high-IL-10 producing CD4 T cell.

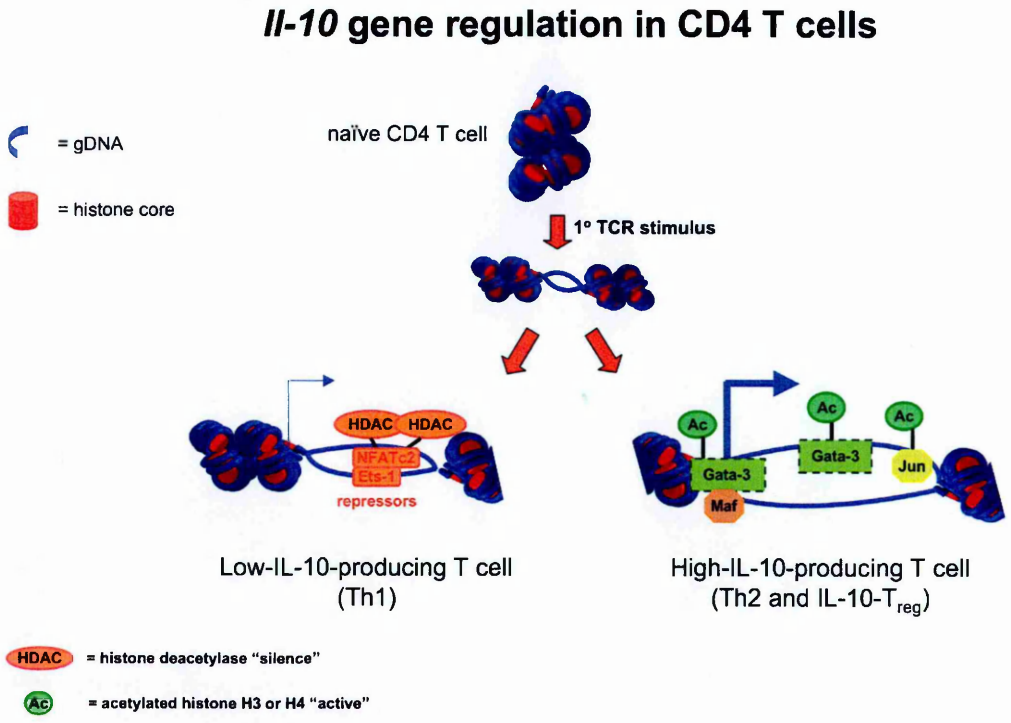


Figure 9.1. ***Il-10*** **gene regulation in low and high IL-10-producing CD4 T cells**
A theoretical model for the key molecular mechanisms described in this thesis and the relevant literature regulating differential *Il-10* gene transcription in CD4 T cells.

Why does GATA-3 over-expression (neutral and Th1 culture conditions) not induce high-level IL-10 producers similar to profiles (ICS) from high-IL-10-producing CD4 T populations (Th2 and IL-10-T_{reg})? I propose this may be dependent on at least three separate molecular mechanisms. Firstly, critical enhancers of the *Il-10* gene may not be available in neutral or Th1 cultured CD4 T cells. GATA-3 induces accessibility to the *Il-10* locus (ChART-PCR) (Fig. 6.5.); however the effect of potential enhancers and compositions of key combinatorial factors upon TCR activation (AP-1 family members) are a likely critical regulator for high-level IL-10 production. The absence of an enhancing transcription factor up-regulated in high-IL-10-producing CD4 T cell

populations (Th2 and IL-10-T_{reg}) (GeneChip data, Figures 4.1 - 4.4) may be necessary to achieve high-level IL-10 production in addition to the GATA-3 over-expression (which induces chromatin accessibility at the *Il-10* locus (Fig. 6.5)) in neutral and Th1 culture conditions. An example of this may be, c-Maf, which is not up-regulated or quantitatively expressed in neutral or Th1 conditions; however it is up-regulated in high IL-10-producing CD4 T cell populations (Th2 and IL-10-T_{reg}) (Fig. 7.1a). Secondly, the accessibility for transcription factors at the *Il-10* locus may be dependent on cell-specific cycling of acetylation/deacetylation of histones at site-specific locations throughout the potential regulatory regions on the *Il-10* locus. Histone modifications represent another layer of chromatin-level regulation for cytokine gene expression (55), in addition to DNase I HSSs. DNase I HSSs within the *Il-10* gene are defined in previous studies and outlined with respect to the highly sensitive chromatin accessibility (using ChART-PCR) in differential IL-10-producing CD4 T cell populations within this thesis (Fig. 5.3). However, the additional investigation of cell-specific, site-specific, activation-specific histone modifications within the *Il-10* gene, supplemented with *in silico* and gene expression profiling may lead to identification of additional molecular mechanisms leading to increased *Il-10* gene transcription. Lastly, the recruitment and presence of "repressor" transcription factors at the *Il-10* locus are likely factors in determining optimal IL-10 production in CD4 T cells. The presence of repressors at regulatory regions is described for other genes to be influenced by "silencing" histone modification (55, 242). The mechanism that comes first; a "silencing" histone modification or presence of a repressing transcription factor, are presently understood to be dynamic processes and not sequential (55). Therefore, combining both an *Il-10* gene site-specific

histone modification profile (ChIP) and site-specific repressor transcription factor analysis (ChIP) throughout the *Il-10* locus in differential IL-10-producing CD4 T cell populations could lead to clues involved in an additional layer of *Il-10* gene regulation. Together, enhancing factors, histone modifications, and repressor transcription factors represent independent molecular mechanisms for *Il-10* gene regulation and they should be treated as separate in their initial investigation with regards to the collective molecular mechanisms at play in the *Il-10* locus.

Does the *Il-10* gene have an important regulatory region within intron 4? Three observations allude to a possible regulatory site within intron 4 of the *Il-10* gene. First, the *in silico* analysis highlights a highly conserved (human) region (~500 bp) of putative transcription factor binding sites within intron 4 (Fig. 5.1b). The "predicted" transcription factor binding sites identifies two adjacent intronic TATA boxes located in the proximal 5' position of exon 5 within the murine *Il-10* gene (Fig. 5.1b). Putative binding sites for IL-10-associated transcription factors, Ets-1(72), GATA-3 (Figure 6.4) and NFATc2 (presence is also shown here (ChIP) (114)) are located in this regulatory region (murine). The combination of the significant homology within this location (intron 4), potential transcription factor binding sites (two of which are functionally shown (ChIP)) and dual conserved TATA boxes (located at the proximal 5' edge of exon 5) suggest strong potential for regulation at this site within the *Il-10* locus (Fig. 5.1b). Secondly, DNase I digestion studies have described this region as a constitutive HSS in effector CD4 Th cells (ChART-PCR) (Fig. 5.3) (110, 114, 115). And further, histone modifications within the region vary between low-IL-10-producing populations (Th1)

and high-IL-10 producing populations (Th2) (114). The fact that deacetylated histone modifications "silencing" are shown in Th1 (D5 cell-line) chromatin at intron 4 (unstimulated and stimulated conditions) (114); whereas acetylated histone modifications "active" are achieved in Th2 (D10 cell-line) chromatin at intron 4 (unstimulated and stimulated conditions) represents a histone modification profile usually reported at "highly" regulatory regions of DNA (114). Thirdly, the observation that the transfection of the IL-10 promoter (1.5 kbp 5' proximal region of the *Il-10* gene) and TCR activation does not enhance reporter assays with the minimal promoter in IL-10-producing cell-lines (D10) (110), suggesting that other regions may provide enhancing features upon TCR activation. The idea that the murine *Il-10* gene has a regulatory region located at intron 4 is based on multiple assumptions; however given the recent reports that cytokine gene regulation can physically interact over long distances (52) suggests that scenarios such as the one presented here.

Ultimately the regulation of potential key non-coding regions of the *Il-10* locus should be verified with coordinate-region deletion studies performed with *in vivo* animal models. These studies would allow for developmental study and various immunological challenges where the optimal production of the gene of interest would be necessary for control or clearance of a pathogen. In the case of IL-10 production, the balance of anti-inflammatory and pro-inflammatory signals during the course of an infection could be tested. Careful design of these types of studies might lead to insight into the cell-specific mechanisms in which the *Il-10* gene is regulated. For example, consider the investigation of the dysregulation of IL-10 production from modified animals with the deletion or

mutation of the GATA binding motifs at either or both positions (-.865 (*Il-10* gene 5' region) or +3.750 (intron 4)) within the *Il-10* locus. Different immunological challenges of these animals with *a priori* essential IL-10-dependent mechanisms in immune cell-specific compartments would give investigators definitive *in vivo* proof for suggested potentially critical regulatory regions. Furthermore, different immune cell-types may produce normal amounts or significantly low amounts of IL-10 production based on the requirement for the modified regulatory region. In summary, a time-intensive, whole animal, gene modification study would serve as an *in vivo* "gold standard" to verify non-redundant *Il-10* gene regulation at a specific-DNA regulatory region.

9.2 IL-10-T_{reg}

IL-10-T_{reg} are suggested to have potential for clinical applications (examples include: autoimmune disease (11) and immunotherapy (27)). In order to further explore the opportunity to use a homogeneous population of *in vitro*- or *ex vivo*-generated antigen-specific IL-10-T_{reg}, for therapeutic intervention there has to be, among numerous other things, a clear understanding for their specific molecular and cellular mechanisms. In this thesis I have outlined some principles for efficient *Il-10* gene regulation in CD4 T cells (summarized in Fig. 9.1). The functional value for IL-10 in the course of an immune response or in regulating autoimmune and allergic/atopic pathology has been described by other groups (including the lab were this data was generated) and only reviewed here. Secondly, the functional value of CD4 T_{reg} in the course of homeostasis and regulating inflammatory immune responses has been described by other groups (including the lab where this data was generated) and only reviewed here. Therefore, I

propose that the generation of any CD4 "IL-10-T_{reg}" population, may need the following conditions for their differentiation or generation.

The molecular mechanisms that regulate both CD4 T_{reg} (CD25+T_{reg} and IL-10-T_{reg}) populations seem to be similar with respect to the molecular targets de-activated during TCR activation. The molecular mechanism from the previously described naturally occurring CD25+T_{reg} studies demonstrates an essential role for FoxP3 in its interactions with NF-κB and NFAT activity (137, 140). The interaction of FoxP3 with components of either NF-κB or NFAT proteins is likely a key element in the regulation of cytokine gene expression by CD25+T_{reg} upon TCR activation (137, 140). This translates into what was classically considered a suppressive or "anergic" (no IL-2 production) CD4 T cell. Now, IL-10-T_{reg} do not produce IL-2 and produce little to no effector cytokines upon TCR activation, in the absence of FoxP3 (174). One conclusion that can be drawn from this comparison is, maybe IL-10-T_{reg} express a functional equivalent to FoxP3? The GeneChip analysis and RV studies (GILZ) are not suggestive of a strong functional FoxP3 equivalent in IL-10-T_{reg}. What seems to be occurring is IL-10-T_{reg} down-regulate the transcripts of functional targets implicated in FoxP3 essential activity.

NF-κB family member, c-Rel plays a non-redundant role in enhancing IL-2 (96) and early IFN-γ expression (98) upon TCR activation. Similarly, c-Fos is an essential molecule in the composition of competent AP-1 complexes, which target pro-inflammatory downstream gene activation (109). Furthermore, the inhibition of Fos:Jun dimers and subsequent skew in the AP-1 balance towards Jun:Jun or Jun:Maf dimers has

shown to induce anti-inflammatory gene expression profiles (109, 113). Both c-Rel and c-Fos transcripts are significantly quantitatively (qPCR) and differentially down-regulated (GeneChip) in IL-10-T_{reg} and Th2 as compared to other CD4 T cell populations (including CD25+T_{reg}) (Fig. 8.3 and 8.4). Finally, NFAT transcriptional activity is inhibited by either DEX and $vitD_3$ and this direct effect is seen here with respect to target NFAT gene expression upon TCR activation (TNF) (226). Other studies, not shown here, demonstrate that DEX-treatment (20 hrs. before TCR activation) of effector Th2 cells, that usually produce high amounts of TNF, are significantly impaired in TNF production (ICS FACS) upon TCR activation. This suggests that the NFAT target for gene activation, TNF (226), is directly inhibited by DEX upon TCR activation. Interestingly, DEX-induced GILZ did not effect TNF production (or any other cytokine) in RV studies. Therefore, a simplistic model for the IL-10-T_{reg} population might be the down-regulation of key NF-κB and AP-1 transcripts as well as inhibition of NFAT transcriptional activity thereby compensating for necessary FoxP3 expression seen with CD25+T_{reg}. Whether the same situation arises in soluble antigen induced IL-10-T_{reg} *in vivo* remains to be seen, but regardless this offers a plausible mechanism for $vitD_3$ and DEX action.

The repeated TCR triggering and differentiation of IL-10-T_{reg} under the presence of DEX and $vitD_3$ is a key component in the generation of homogeneous IL-10-T_{reg} population from a naïve CD4 T precursor cell. GILZ over-expression (RV data), without the influence of other DEX-induced properties, induces apoptosis in neutral or Th1 driven culture conditions, but promotes expanded proliferation in Th2 driven culture conditions

upon secondary TCR-activation. This is in agreement with the recently reported *in vivo* generated data from unmanipulated GILZ TG mice, where IFN-γ production was down-regulated and Th2-associated cytokines were all up-regulated in comparison to WT *ex vivo* stimulated splenic CD4 T cells (ELISA) (209). In the context of an immune response, GILZ TG mice were significantly inhibited in a Th1 immune response, as shown by their inhibition to respond to a delayed-type hypersensitivity model, which is driven by a Th1 dependent response (209). Alternatively, in a Th2-driven immune response (bleomycin-induced pulmonary fibrosis) GILZ TG mice elicit a stronger immune response than WT mice and thereby induce severe pathology within the corresponding target organ (lungs) (209). GILZ is not a FoxP3 functional equivalent, shown by the fact transduction of GILZ does not affect the differential cytokine profile, as compared to control populations (neutral, Th1, Th2) revealed upon secondary TCR-activation (data not shown). The data in this thesis suggests that GILZ-expressing CD4 T cells prevent expansion of both IL-2-producing (neutral) and Th1 populations after secondary TCR activation (Fig. 8.5). Alternatively, GILZ expression in Th2-driven culture conditions promotes increased expansion as compared to Th2 controls after secondary TCR activation (Fig. 8.5). The molecular mechanism for proliferation and survival of the IL-10-T_{reg} population in the absence of cell-autonomous IL-2-production or effector cytokine intrinsic signaling is not clear. GILZ expression may play a central role as a "survival" factor in IL-10-T_{reg}, which exhibit cytokine profiles outside of the typical IL-2 and key effector cytokine (IFN-γ and IL-4) repertoire. In summary, the DEX-induced transcription factor, GILZ, is both complex and unique in terms of its functional role in CD4 T cells and our data suggests it may provide an essential but

possibly indirect role in the survival of IL-10-T_{reg} by inhibiting IL-2- and IFN-γ-producing cell survival but with no direct effects on immediate cytokine expression.

Finally, the key question remains: what are the general molecular mechanisms that guide IL-10-T_{reg} to produce IL-10 in the absence of other cytokines? These molecular mechanisms are not trivial, and definitely not likely to be completely described here, but there seem to be some clear themes. Firstly, NF-κB, NFAT, NFAT:AP-1 transcriptional activity are abrogated in IL-10-T_{reg}. This is observed by the loss of non-redundant NF-κB- and NFAT- and NFAT:AP-1-target gene expression (described through the GeneChip data and validated via qPCR). The mechanism for this is likely from both physical, DEX- and vitD_3-induced factors and the down-regulation of key transcripts within these pathways (ex.: c-Rel, c-Fos, NFATc1). Secondly, IL-10 is a unique cytokine, which is described to be enhanced - at a regulatory site shown here to be universally re-modeled in Th cells - by Jun family members (110). Therefore, optimal IL-10 expression is likely dependent on the skewing of AP-1 signaling from their "strong" interacting partners, Jun:Fos to "weaker" interacting partners Jun:Jun or possibly Jun:Maf (which have been shown to dimerize in the absence of functional c-Fos) (59, 109). The AP-1 gene expression profiling in IL-10-T_{reg} fits this scenario (down-regulated c-Fos and up-regulated JunB and c-Maf expression) (Fig. 8.3.). The presence of GATA-3 is essential for *Il-10* gene regulation (induction of "active" acetylation of histone H3 and H4 along the *Il-10* gene and re-modeling of the *Il-10* gene especially seen with respect to "strong" re-modeling at the 5' region which contains enhancing regulatory regions (Chapter 6)). In keeping with this it is shown here that IL-10-T_{reg} up-regulate

GATA-3 expression (Fig. 6.1a.). Together, these three components, along with GILZ-mediated protection from apoptosis during differentiation, provide the necessary cell-specific environment for the generation of a high-IL-10-producing CD4 T_{reg}. This is summarized in Figure 9.2.

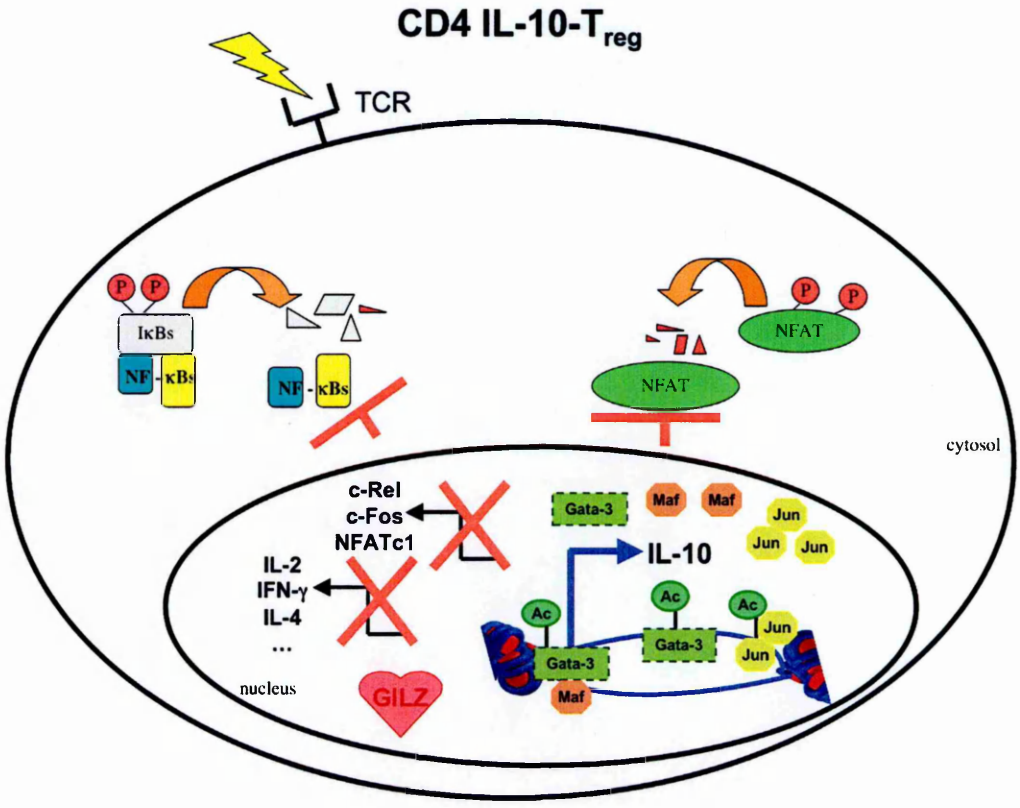


Figure 9.2. Essential molecular properties at play in IL-10-T_{reg}
A cartoon describing the mechanisms involved in IL-10-T_{reg} upon TCR stimulus, as described from work in this thesis.

10. Comparison of associated cellular markers for potential suppressor function in IL-10-T_{reg} vs. CD25+T_{reg}

The ability for CD4 T_{reg} to regulate naïve and effector CD4 T cell - proliferation and downstream pro-inflammatory cytokine production - is suggested to provide one perspective for clinical potential with CD4 T_{reg}. However, the mechanism by which CD4 T_{reg} directly suppress the adaptive immune response is likely via multi-faceted mechanisms under condition-specific microenvironments and a clear understanding of differential CD4 T_{reg}-mediated mechanisms is an ongoing area of research. One validated mechanism by which both of these profiled CD4 T_{reg} populations (IL-10-T_{reg} and CD25+T_{reg}) can suppress CD4 T cell proliferation is via IL-10 production on APC (*in vivo*); however both CD4 T_{reg} populations have been shown to suppress CD4 T cell proliferation independently of IL-10 expression (*in vitro* and *in vivo*) (8). Another clear mechanism of CD4 T_{reg} suppression is via cell-cell contact achieved via expression of specific families of cellular receptors on CD4 T_{reg} (127). In this Chapter I will begin by investigating the similarities between both CD4 T_{reg} populations profiled here with a specific interest in the cellular receptor families implicated in cell-to-cell contact suppression, suggest leave out here and introduce later further down (127). GeneChip generated mRNA profiles and subsequent qPCR validation will serve as a basis for our comparison of differential suppressive mechanisms with an emphasis on the CD28 and TNF families of cellular receptors.

10.1.1 CD4 T_{reg}-associated cellular receptors in CD4 T cell populations

The CD28 receptor family regulates both activation and inhibition within CD4 T cells (142). Ligation of the B7:CD28 pathway can serve as a required secondary signal to

initiate and sustain T cell responses; however some CD28 family members serve as essential secondary signals to down-regulate T cell responses (142). Functional roles of CD28 family members are dependent on multiple variables including: the individual CD28 family member that is engaged, the state of differentiation of the engaged T cell, the signaling cell-type, and the location of the T cell during the engagement (thymus, lymph node, and periphery) (142). CD28 family members include CTLA-4, ICOS, Pcd-1 (142). In this chapter, expression profiles generated between the CD4 T cell panel will allowed comparison of the these proposed cellular inhibitory receptors in CD25+ T_{reg} versus IL-10-Treg and other effector populations.

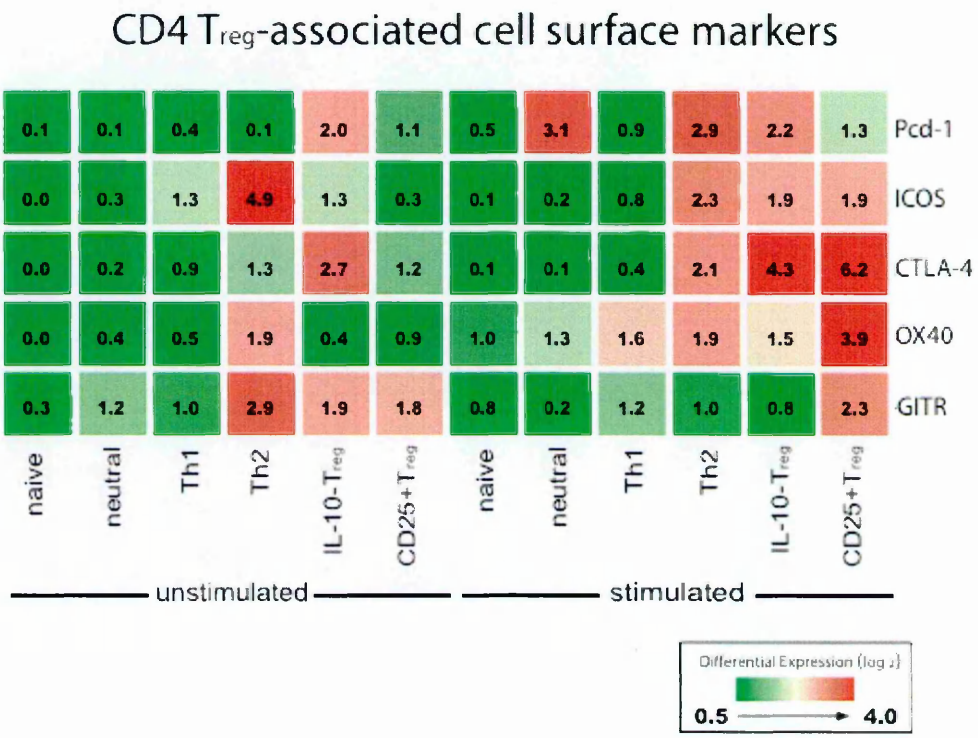


Figure 10.1 Profiling CD4 T_{reg}-associated cellular markers
GeneChip profiles for CD4 T_{reg}-associated cellular markers in CD4 T cells. The GeneChip image was compressed with the GC-RMA algorithm and normalized values (log_2) are shown within each rectangle. More information is available in Materials and Methods for the computational details.

CD4 T_{reg}-associated cellular receptors in the CD28 family (Pcd-1, ICOS, CTLA-4) and TNFR family (OX40 and GITR) are up-regulated in unstimulated or stimulated conditions within CD4 T_{reg}; however they are not exclusively up-regulated when compared to other CD4 populations (Fig. 10.1). Alternatively, both families are strongly down-regulated in naïve CD4 T cells in both conditions (Fig. 10.1). CD28 family members, Pcd-1 and CTLA-4, are up-regulated in IL-10-T_{reg} and in Th2 cells in both conditions, whereas ICOS is up-regulated in IL-10-T_{reg} and Th2 cells in stimulated conditions (Fig. 10.1). CD25+T_{reg} have a different profile in that they up-regulate CD28 family members ICOS and CTLA-4 mainly under re-stimulated conditions (Fig. 10.1). Pcd-1 is not strongly up-regulated in either condition for the CD25+T_{reg} population (Fig. 10.1) but is up-regulated in other T cell populations upon re-stimulation through the TCR. Other populations up-regulate CD28 family members; for example, Pcd-1 in the stimulated neutral population, ICOS in both Th2 conditions, and CTLA-4 in the stimulated Th2 population (Fig. 10.1).

TNF receptors family members OX40 and GITR are implicated to mediate CD4 T_{reg} suppression (127). OX40 is up-regulated in stimulated conditions for both CD4 T_{reg} populations; however it is also up-regulated in both effector populations (Th1 and Th2) within stimulated conditions (Fig. 10.1). GITR is expressed in both CD4 T_{reg} populations in unstimulated conditions (Fig. 10.1). In stimulated conditions GITR is up-regulated exclusively in the CD25+T_{reg} population (Fig. 10.1). Both GITR and OX40 are up-regulated in unstimulated conditions in the Th2 population (Fig. 10.1). TNF receptor family members OX40 and GITR are up-regulated in various conditions within CD4 T_{reg}

populations, although the up-regulated expression-signature within the Th2 population (and to a lesser degree Th1) provides evidence for up-regulation outside of CD4 T_{reg} populations for the TNFR genes, OX40 and GITR. (Fig. 10.1) Both CD28 and TNFR family members shown here were validated with qPCR across all CD4 populations and conditions under both stimuli (α-CD3/-CD28 and PMA/ionomycin) and all transcripts exhibit similar gene expression profiles as reported here with the GeneChip (data not shown).

We have not verified the expression by Flow cytometry of all these markers as yet, although our laboratory has previously shown that GITR and CTLA-4 are expressed on activated effector T cell populations as well as CD4+ T_{reg} by Flow cytometry (data not shown). In summary, we report here that there is no clear correlation between the expression of these markers and the anergic phenotype and suppressive activity of the CD25+T_{reg} or the IL-10-T_{reg}, and that other additional properties (possibly functioning together with these molecules, contribute to the anergic and suppressive phenotype of CD4+ T_{reg}.

10.1.2 CD4 T_{reg}-associated cellular receptors induced by either $vitD_3$ or DEX

CD4 T_{reg}-associated cellular receptors were further investigated within respect to understanding their individual expression profiles under culture conditions with immunosuppressive drugs, $vitD_3$ or DEX. IL-10-T_{reg} were generated with both $vitD_3$ and DEX, and DEX is prescribed for neutralizing pro-inflammatory clinical conditions in humans. Therefore, this suggested that individually, each immunosuppressive drug

might induce expression of either CD28 or TNFR family member, with direct implications to the clinical setting. I have used qPCR to profile the CD4 T_{reg}-associated cellular receptors with populations ($vitD_3$, DEX, and IL-10-T_{reg}) generated under *in vitro* culture conditions and the *ex vivo* isolated CD25+T_{reg}. TCR activation was performed with α-CD3/-CD28 to represent stimulated conditions.

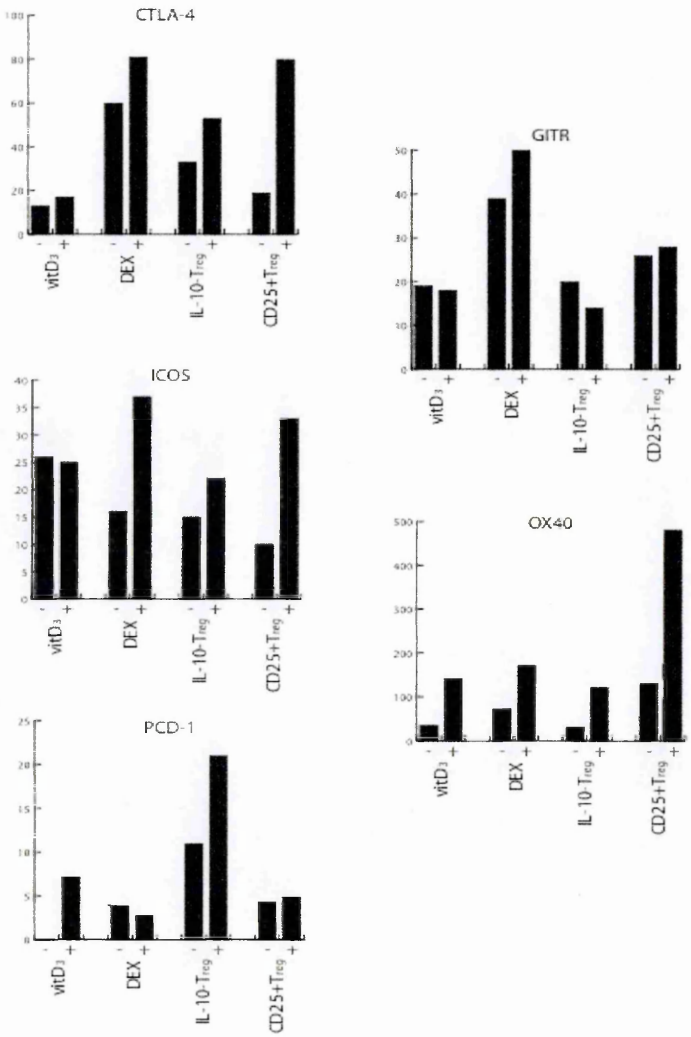
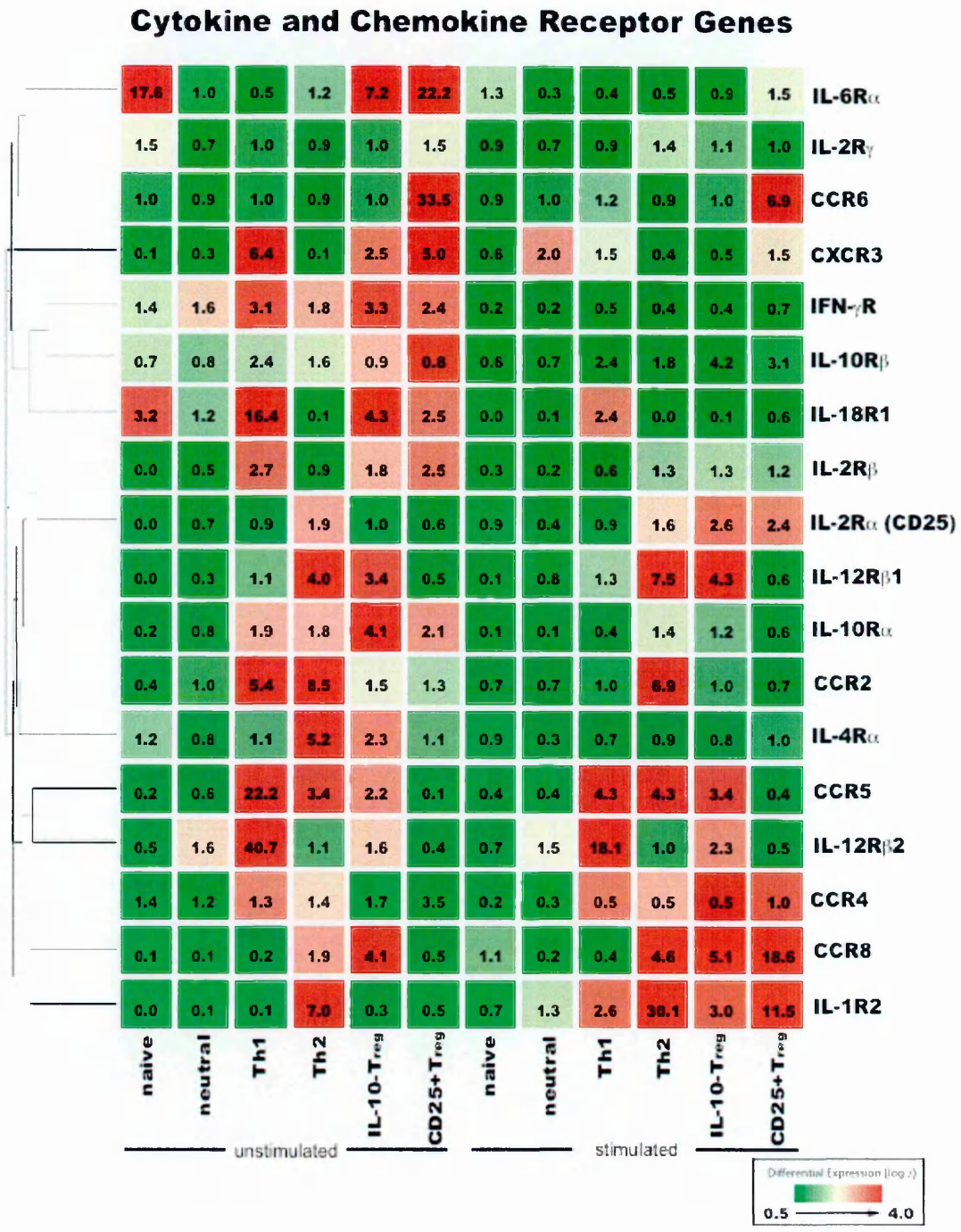


Figure 10.2. qPCR of CD4 T_{reg}-associated cellular markers in $vitD_3$, DEX, IL-10-T_{reg}, and CD25+T_{reg}
Real-time reverse transcriptase PCR (qPCR) of CD4 T_{reg}-associated cellular markers in $vitD_3$, DEX, IL-10-T_{reg} and CD25+T_{reg} (same transcripts as Figure 10.1). mRNA was isolated from unstimulated and stimulated cells (6 hr.) (α-CD3/CD28), converted to cDNA and qPCR was performed with primer/probes acquired from ABI (inventoried: assay-on-demand) and mastermix (ABGene) was used to measure relative expression of CD4 T_{reg}-associated cellular markers on the ABI Prism 7900. Data shown is an accurate representation of three experimental replicates.

CD4 T cells generated under DEX culture conditions up-regulated CD4 T_{reg}-associated cellular receptors when compared to $vitD_3$, IL-10-T_{reg}, and CD25+T_{reg} (Fig. 10.2). Upon TCR activation, DEX populations expressed relative amounts of ICOS, CTLA-4, and GITR at levels equal to or higher than CD25+T_{reg} (Fig. 10.2). DEX populations also expressed equal or higher-relative amounts of ICOS, CTLA-4, OX40 and GITR as compared to $vitD_3$ or IL-10-T_{reg} generated conditions (Fig. 10.2). Interestingly, Pcd-1 was the only CD4 T_{reg}-associated cellular receptor up-regulated with the combination of $vitD_3$ and DEX (IL-10-T_{reg}) (Fig. 10.2). The expression profiles of CD4 T_{reg}-associated cellular receptors (Fig. 10.2) demonstrates the ability of DEX to influence the expression of these gene in DEX generated CD4 T cells as well as suggest DEX is important in inducing expression of these cellular receptors within the IL-10-T_{reg} population. Again, regardless of whether the effects that we report on the expression of particular cell surface molecules is achieved by an immunosuppressive drug mediated effect only, this is of importance with respect to their mechanism of action which thus far is unclear (127).

10.2 Hierarchical clustering of cytokines and chemokines in CD4 T cells

10.3 Hierarchical clustering of cytokine and chemokine receptors in CD4 T cells



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