MOLECULAR DETERMINANTS OF HUMAN T CELL DIFFERENTIATION AND HOST-PATHOGEN INTERACTION

By

Mark S. Sundrud

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Approved:

Professor Derya Unutmaz

Professor Wasif Khan

Professor Eugene Oltz

Professor Luc Van Kaer

Professor Mark DeCaestecker

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DEDICATION

To my amazing wife Erika, for her infinite support and inspiration and to my family for instilling drive, courage and curiosity to always question what is possible and never stop pursuing my goals.

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CHAPTER I

INTRODUCTION

The evolution of immune systems within complex, multi-cellular biological organisms has been fueled by the necessity of self-protection from parasitism in order to better ensure species propagation and survival. Given the sheer biomass of human pathogens (namely bacteria, viruses, fungi and helminths) and their extraordinary capacity to adapt to and thrive under intense selective environmental pressures, multi-cellular organisms have co-evolved a complex and intricate series of defense mechanisms in order to protect themselves from parasitic insults. Factors such as physical barriers (i.e. skin and mucosal surfaces), the fever response, soluble factors including anti-microbial enzymes secreted in saliva and liver-derived complement molecules as well as phagocytic and cytolytic cells such as polymorphonuclear (PMN) leukocytes and natural killer (NK) cells present within tissues provide humans with broad-ranging, immediate, yet transient protection from microbial pathogens. As a whole these components comprise a part of the human immune system termed natural or innate immunity. The innate immune system is referred to as such due to the fact that, 1) innate immunity is naturally present in humans at birth and in aseptic environments, and 2) its components are not selected for or enhanced by exposure to infectious microbes. The evolutionary conservation of innate immunity between humans and lower invertebrates underscores the critical role

for this system in providing protection from microbes. However, in addition to these lines of defense, higher vertebrates have uniquely developed additional measures to ward off microbial pathogens, namely the adaptive immune system.

The human adaptive immune system tailors protective immune responses in a pathogen-specific manner

The human adaptive immune system forms a unique and integral part of human immunity. The significance of the system in providing immunity to infectious diseases is underscored by a rare set of individuals carrying genetic deficiencies that clinically manifest as a disorder called severe combined immunodeficiency syndrome (SCID), which is characterized by the absence of most adaptive immune cells (1-7). These patients suffer from chronic and opportunistic infections and typically succumb to these onslaughts shortly (typically ~1 year) after birth. The cellular (B and T lymphocytes) and acellular (molecules secreted by lymphocytes called cytokines and antibodies) components of the adaptive immune system work in synergy with factions of the innate immune system to recognize, respond to and clear invading microorganisms. In this context, the adaptive immune system, once activated by invasive pathogens, feeds back to innate immune cells and tissues to activate phagocytosis (8) and complement-mediated cytolysis of bacteria (9). Furthermore, adaptive immunity can mobilize innate immune cells to concentrate within tissues at sites of infection or at mucosal barriers that serve as entry portals for invading microbes (10, 11). In all respects, the adaptive and innate immune systems form a co-operative and non-

redundant symbiosis within humans to guard against microbial insults. Yet while synergistic in its collaboration with innate immunity, the adaptive immune system has, in and of itself, evolved three hallmark features that differentiate it from innate immunity.

The first such distinguishing characteristic of adaptive immunity is the mode of pathogen recognition. Adaptive immune cells recognize pathogen-specific molecules as presented by components of innate immunity, whereas the innate immune system senses invading pathogens in a more indiscriminant manner. Specifically, innate immune surveillance involves the use of germline-encoded pattern recognition receptors (PRRs), one class of which is called toll-like receptors (TLR). These receptors recognize broadly conserved components of bacterial and yeast cell walls, bacterial DNA motifs and double-stranded RNA from viruses (12-19). By contrast, the adaptive immune system has evolved more exquisite mechanisms of pathogen recognition. B and T lymphocytes recognize microbial antigens with remarkable specificity through the B cell receptor/antibody and T cell receptor (TCR), respectively (20, 21). However, size constraints within the human genome preclude encoding enough diversity within antigen receptors to specifically recognize the vast multitude of human pathogens. Moreover, such a strategy of immuno-recognition by lymphocytes would render host cells incapable of recognizing microbes that can modify immunogenic epitopes when exposed to selective immune pressures. Thus, In order to generate a sufficiently broad-ranging protective repertoire of antigen receptors, B and T lymphocytes have evolved to somatically recombine a limited

set of germline-encoded antigen receptor gene segments through the activity of the recombinase activating genes (RAG) 1 and 2 (22-24). Indeed, antigen receptor gene rearrangement, along with other sources of diversification intrinsic within the process of V(D)J recombination (combinatorial and junctional) can generate greater than 10^9 possible receptors clonally expressed on the surface of human lymphocytes. Yet as with most facets of biology, the random generation of antigen receptors on lymphocytes is not without its faults, as it can give rise to self-reactivity and subsequent autoimmunity.

The adaptive immune system is further unlike the innate, in that it is highly dynamic in nature, capable of generating qualitatively distinct immune responses as shaped by the type of pathogen encountered. Two major types of adaptive immune responses that have been historically classified based on the cells and primary soluble mediators involved are humoral and cell-mediated immune responses. Humoral immunity involves the activation of B-lymphocytes (i.e. B cells) by microbial products present throughout the extracellular environment and thus, is typically generated against extracellular bacteria, bacterial toxins, fungi, parasitic helminths and surface-exposed portions of circulating viral particles. Once activated, B cells secrete antigen-specific antibodies into circulating body fluids or humor. Secreted antibodies can provide immune protection by opsonizing microbial cell-surfaces, thereby activating complement-mediated destruction (9, 25) and/or enhancing phagocytosis of the microbe via cellular recognition of antibody Fc regions (26, 27). Antibodies present on the surface of bacteria or pathogenic fungi can also inhibit adhesion to and transcytosis across

mucosal barriers (28) and can block viral or intracellular bacterial entry into and subsequent infection of host cells (28-31). Other antibodies, specifically of the IgE isotype, also serve as central mediators in protection against parasitic helminthes by promoting the release of vesicular elements stored within PMN basophils and eosinophils such as reactive oxygen/nitrogen intermediates and cytotoxic enzymes (32, 33). As with all types of immune responses, however, dysregulated production of antibodies can also cause pathophysiologies, such as atopic and allergic disorders (34). Moreover, antibody production against self-antigens can lead to the pathogenesis of autoimmune systemic lupus erythematosis (SLE) and many other autoimmune disorders.

While B cells are responsible for the direct production of antibodies, a specific class of T-lymphocytes (i.e. T cells) called T helper (Th) cells, classified by the cell-surface expression of CD4, play a critical role in the mobilization and regulation of humoral responses. Effector Th cells are activated and secrete soluble cytokines upon interaction with B cells displaying microbial peptide antigens in the context of majorhistocompatability complex (MHC) class II molecules. The cytokines secreted by Th cells in turn instruct antibody class-switch recombination in antigen-specific B cells (Table 1-1). The resultant plasma cells, which differentiate from activated B cells upon Th cell interaction, secrete antibodies of the IgG, IgA and IgE isotypes that most appropriately activate protective immune responses in a pathogen-specific fashion.

In addition to humoral immune responses, the adaptive immune system can also generate cell-mediated effector functions against pathogenic microbes.

Cell-mediated responses are primarily constituted by the activation of antigen-specific $CD8^+$ cytolytic T lymphocytes (CTLs) and IFNγ-producing Th cells in response to invasion by intracellular bacteria and viruses. CTLs receive activation signals in the context of microbial antigens presented on MHC class I molecules. MHC class I is expressed on nearly every human cell type in all tissues throughout the body and presents intracellular peptides proteolytically generated by cytosolic proteases, thus allowing CTLs to survey for and specifically identify infected host cells. Upon antigen recognition by CTLs, cytolytic enzymes including perforin and granzymes are released, directly killing infected cells as to limit the replication and systemic spread of intracellular pathogens (35). As with humoral immune responses, Th cells also play a pivotal role in the activation of CTLs as well as cytolytic natural killer (NK) cells through the production of IFNγ by effector and memory Th cells (Table 1-1).

The final distinguishing feature of adaptive immunity is the capacity to amplify pathogen-specific immune responses such that secondary responses to identical microbial antigens are more rapid and greater in magnitude than the primary response. This feature is termed immunological memory and is the fundamental underpinning of vaccinations and naturally acquired long-term immunity. Immunological memory is achieved through the generation and long-term maintenance of antigen-specific plasma B cells and memory T cells, even in the absence of antigen (36-40). In the context of memory, appropriate development of memory Th cells is of keen importance given their vital roles in mobilizing and regulating both humoral and cell-mediated facets of adaptive

immunity. Thus, the development, function and interaction of Th cells with microbial pathogens are critical steps in determining the outcome of pathogenic infections.

Collectively, these hallmark features of adaptive immunity are essential to orchestrating successful defense strategies against diverse sets of human pathogens. However, a highly malleable system if not tightly regulated, can lose self-discriminatory capacities and thus instigate autoimmunity. Simply stated, autoimmune responses are a failure of the cardinal purpose of immune systems: to react to and protect against foreign parasites (non-self) without damaging host cells or tissues (self). And while entire anatomical organs, namely long bone marrow and the thymus, facilitate the selection of non-self reactive mature B and T cells, respectively (41-43), autoreactivity remains a common cause of human morbidity and mortality. Interestingly, autoimmunity is, without exception, a manifestation of misguided adaptive, not innate, immune activation. This is likely due to the fact that innate immunity is “older” in evolutionary terms than the adaptive immune system and that innate immunity is strictly encoded within the germline of the organism, which is inherently non-autoreactive. Nonetheless, evolution of the adaptive immune system in the face of possible autoimmunity speaks to the immense selective pressure exerted on higher vertebrates by pathogenic microbes and the premium placed on a dynamic adaptive immune system.

T helper cells differentiate into heterogeneous effector and memory cells to regulate adaptive immunity

The adaptive immune system is a hierarchy of individual cell types that sequentially communicate in order to organize and concentrate protective responses against invading pathogens. $CD4^+$ Th cells play a central role in orchestrating the quality and efficacy of immune responses through their secretion of effector cytokines. Thus, elucidating the molecular mechanisms involved in Th cell differentiation to effector and memory Th cell subsets and the resultant acquisition of effector function has broad-reaching implications in understanding how qualitatively distinct immune responses are generated and modulated. Such insight also provides an important framework for vaccine design and the development of novel therapeutic strategies to enhance anti-microbial immunity and pacify immunopathologies.

Table 1-1: Cytokine production by Th cells regulates immunity.

Cytokine	Source	Targets and Activities
IL-4	Th2 cells	B cell proliferation and IgE production Augment Th2, inhibit Th1 differentiaton Inhibits macrophage activation Mast cell proliferation
IL-5	Th2 cells	B cell proliferation and IgE, IgA production Eosinophil differentiation and growth factor
IL-10	Th2 cells	Inhibits T cell growth and cytokine production Inhibits cytokine production by activated macrophages
IL-13	Th2 cells	B cell IgE production Inhibits macrophage activation
IFNγ	Th1 cells	Macrophage activation NK cell activation CTL differentiation Augment Th1, inhibit Th2 differentiation Increase cellular MHC I and MHC II expression

Generation and function of central and effector memory Th cells

Antigen-specific effector and memory Th cells differentiate from clonal naïve precursor (T_N) cells in the periphery via antigen-dependent activation during pathogenic infection. Yet not all memory Th cells are created equal. Rather, memory cells are a complex and heterogeneous population, whose constituents form a dynamic network ready to adapt and strike against any external threats to the organism. T_N cells activated by cognate antigen differentiate into compartmentalized pools of antigen-specific Th cell subsets, termed central and effector memory cells that are both functionally and phenotypically distinguishable (38, 44). It is ultimately the type and quality of the antigen-specific memory Th cell repository generated during a primary immune response, which in turn shapes anti-microbial effector responses and forms the basis of immunological memory. It is noteworthy that antigen-experienced $CD8^+$ CTLs also differentiate into central and effector memory T cell subsets (40, 45). However, per the focus of the research detailed herein the following overview focuses on the $CD4^+$ subset of T cells.

Central memory (T_{CM}), and effector memory (T_{EM}) T cells were first identified and are canonically differentiated based on their distinct effector functions and migratory properties (44, 46). T_{CM} cells are highly similar to T_N cells in that they express receptors necessary to circulate through draining peripheral lymph nodes ($CCR7^+$, $CD62L^+$), display high levels of T cell receptor excision circles (TRECs) and exhibit weak effector function upon antigenic stimulation (44, 47). Nonetheless, T_{CM} cells represent an antigen-primed population of memory

Th cells functionally distinct from T_N cells. Specifically, T_{CM} cells more rapidly respond to antigenic stimulation than naïve cells, are more sensitive to limiting concentrations of antigen and co-stimulatory molecules and produce greater quantities of IL-2 and up-regulate CD40L to a greater extent upon activation as to provide efficient help to B cells (48-51). T_{CM} cells also survive, proliferate and acquire the ability to produce effector cytokines in response to stimulation with homeostatic cytokines (IL-2, IL-7, IL-15) unlike T_N cells (46). The molecular mechanism underlying antigen-independent T_{CM} differentiation to T_{EM} cells remains poorly understood.

Recently it has been discovered that the T_{CM} cell repertoire can be further sub-divided into non-polarized or pre-effector T_{CM} cell populations based on chemokine receptor expression profiles. Truly non-polarized T_{CM} cells that completely lack effector function express CXCR5, which serves to draw the cells to B cell follicles via its chemokine ligand CXCL13 and provide help to B cells during humoral activation (52, 53). Conversely, $CXCR5^-$ T_{CM} cells that attain expression of the inflammatory chemokine receptors CXCR3 or CCR4 represent a pre-effector lineage of cells that, while lacking immediate effector function can differentiate in antigen-dependent and independent mechanisms to Th1 or Th2 T_{EM} cells in response to TCR and cytokine stimulation, respectively (47).

In contrast to T_{CM} cells, T_{EM} cells lose the ability to traffic to secondary lymphoid organs ($CCR7^-$, $CD62L^-$) and up-regulate pro-inflammatory chemokine receptors (44), allowing them to extravasate to inflamed peripheral tissues and secrete robust levels of polarized effector cytokines upon antigen recognition (54,

55). T_{EM} cells respond to activation signals by rapidly synthesizing effector cytokines, but produce little IL-2, proliferate poorly and are highly susceptible to activation-induced cell death (AICD) compared to T_N and T_{CM} cells (49-51, 56).

The physiologic function of and relationship between T_{CM} and T_{EM} cells remains an area of intense investigation. While the strong effector functions of T_{EM} cells suggest a more central role for the subset during immune activation, both T_{CM} and T_{EM} cell populations contain antigen-specific clones in roughly equivalent ratios across a wide variety of pathogens (47). Moreover, T_{CM} cells have longer half-lives than their T_{EM} counterparts (56-58), and more actively re-populate immunocompromised mice in adoptive transfer experiments (45, 59), suggesting T_{CM} cells are indeed a vital component of long-lived immunologic memory. The remarkable capacity of T_{CM} cells to further develop into T_{EM} cells upon re-stimulation through TCR or cytokine receptors, taken together with studies showing that T_{CM} cells are phenotypically more “naïve” than T_{EM} cells, have higher levels of TRECs and display greater functional plasticity, all strongly suggest that T_{CM} cells represent an earlier stage of T cell differentiation than T_{EM} cells and implies a precursor-product relationship (44, 46, 47, 60). Such a relationship, however teleologically intuitive, is not without controversy. Longitudinal clonotypic analyses of the TCR repertoires of murine and human T_{CM} and T_{EM} subsets have suggested it may also be possible for T_{EM} cells to subsequently populate the T_{CM} compartment. Regardless, a scenario involving the progressive differentiation of T_N – T_{CM} – T_{EM} cells would allow for the rapid

generation and maintenance of a diverse repertoire of antigen-specific memory T cells in the face of constantly evolving human pathogens (40, 44, 46) (Fig. 1-1).

The Th1/Th2 dichotomy

In addition to yielding diverse antigen-specific memory cell populations that either lack or acquire effector activity, T_N cell differentiation also generates distinct sets of T_{EM} cells that display polarized effector function and lymphoid homing propensities, termed T helper type 1 (Th1) or type 2 (Th2) cells. On one hand, Th1 cells secrete IFNγ, thereby activating macrophages as well as CTL and NK cell cytolysis to eliminate intracellular pathogens. In contrast, Th2 cells secrete IL-4, IL-5 and IL-13, which orchestrate humoral responses and activate PMN eosinophils against helminthic parasites and extracellular microbes (61-63). Importantly, negative cross-regulation between the two effector subsets is a critical feature for mounting effective immune responses while preventing immunopathologies that stem from skewed effector Th cell responses, such as autoimmune diseases and allergic inflammation.

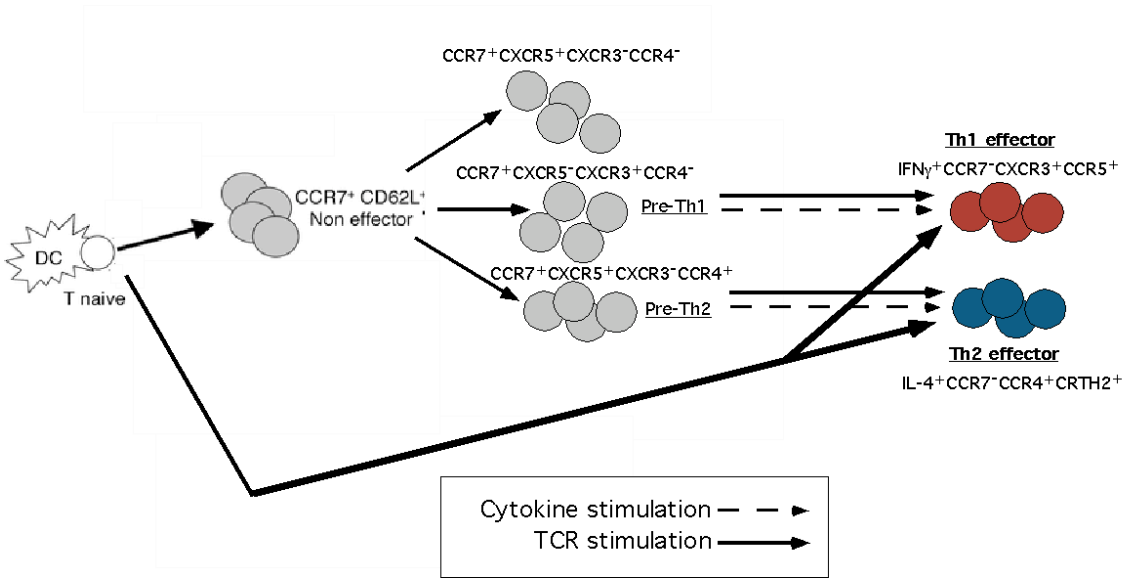

Figure 1-1: Development of central and effector memory Th cell subsets.
T_N cells activated by antigen presenting cells (APCs) integrate cytokine and TCR/co-stimulatory signals, undergo clonal expansion and are programmed to differentiate to either central or effector memory subsets. T_{CM} cells are further sub-divided into follicular Th cells ($CXCR5^+$), pre-Th1 ($CXCR5^-$ $CXCR3^+$) or pre-Th2 ($CXCR5^-$ $CCR4^+$) T_{CM} cells. Pre-Th1 and –Th2 differentiate to Th1 and Th2 T_{EM} cells upon stimulation with homeostatic cytokines. T_{EM} cell populations are comprised of highly polarized Th1 and Th2 cell subsets. Differentiation of T_N cells to T_{CM} cells is driven by short exposure to antigenic stimulation in the absence of polarizing cytokines whereas T_{EM} cell differentiation occurs under optimal TCR stimulation in the presence of influential cytokine signals.

T_N cell differentiation to effector subsets involves the integration of a complex series of extracellular stimuli delivered from APC during antigen recognition and activation. The dose of antigen (48, 64, 65), nature of the peptide ligand (66-68), and source of co-stimulation (69, 70) have all been reported to influence T_N cell differentiation. However, cytokine signals present during T cell activation are paramount in driving T_N cell polarization to effector subsets (63, 71, 72). Of importance, IL-12, IL-18, IL-27 and IFNγ can all promote Th1 differentiation, whereas IL-4 and IL-13 promotes Th2 cell development. As such, mice deficient in these cytokines, their receptors and downstream signaling components display perturbations in T_N cell differentiation (73-78). Ultimately, the combinatorial signals emanating from the T cell and cytokine receptors culminate in the induction of the master transcription factors T-bet and GATA-3, which in turn specify the fate of T_N cell differentiation to Th1 or Th2, respectively (79-81) (Figure 1-3).

There are two phases of T_N cell differentiation that are both spatially and temporally distinct. The first phase takes place in peripheral lymph nodes as T_N cells recognize antigen-pulsed APC, receive instructive activation signals, and commence differentiation. Subsequent clonal expansion and migration to areas of tissue inflammation ensues. The resultant lineage-committed Th1 or Th2 cells respond to secondary challenge of antigen by secreting lineage-specific cytokines independent of polarizing signals, a process referred to as cytokine memory that involves the lineage-specific programmed alteration of chromatin structure at specific cytokine gene loci. Th1 cells stabilize the *IFNγ* gene locus in

a transcriptionally active state, as opposed to Th2 cytokine gene loci, which remain silent (82-85). Paradoxically, Th2 cells maintain *IL-4*, *IL-5* and *IL-13* gene loci in states competent for stable expression, with the *IFNγ* locus being set in heterochromatin (82-85). Such epigenetic changes resulting in cytokine memory implies stability within the differentiation programs of Th cells, yet more recent evidence demonstrates human Th1 and Th2 cells are capable of modifying their effector functions during secondary encounters with antigen. Th2 cells have been shown to retain responsiveness to IL-12, producing IFNγ upon TCR re-stimulation in its presence (83, 86). Moreover, a TCR-transgenic $CD4^+$ memory T cell population has been shown capable of secreting distinct patterns of effector cytokines depending on the nature of the TCR re-triggering (68). While precipitated by extracellular cues, the reprogramming of entrenched Th1 and Th2 cells involves the induction of master transcription factors from the opposing lineage. Work from our lab detailed herein has demonstrated that lineage-committed Th1 or Th2 cells can be directly reprogrammed by ectopic expression of GATA-3 or T-bet, respectively, irrespective of exogenous cytokine signals (60). Indeed, an adaptable memory T cell response, exhibiting plasticity in the context of antigen re-exposure, could be a highly beneficial strategy against constantly evolving pathogens and suggest that long-term human T cell memory could be modulated by either novel vaccine or gene therapy approaches.

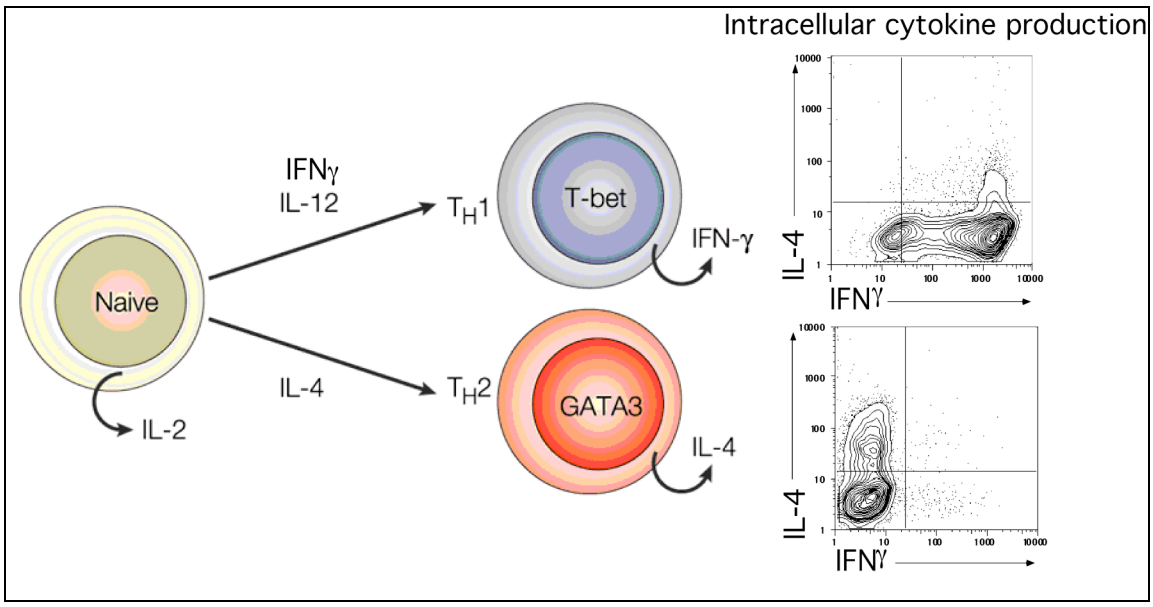

Figure 1-2: Molecular regulation of Th1/Th2 differentiation.
Activation of T_N cells by TCR stimulation in the presence of IL-12 or IFNγ signals promotes the specific up-regulation of T-bet and subsequent Th1 differentiation. Contrarily, IL-4 presence during TCR stimulation of T_N cells activates GATA-3, thereby orchestrating differentiation to the Th2 lineage. Lineage-committed Th1 and Th2 cells reveal their polarized effector functions upon re-stimulation in the absence of exogenous cytokines (see methods, chapter II, cytokine detection). Th1 cells are $IFN\gamma^{+}IL\text{-}4^{-}$ whereas Th2 cells produce IL-4 but not IFNγ.

Polarized Th1 and Th2 cells were originally identified and are canonically characterized based on polarized cytokine production. In addition to cytokines, however, chemokines and their cognate receptors play pivotal roles in adaptive immunity by directing lymphoid trafficking to various immune organs and tissues (11). Indeed, more recent work has uncovered that Th1 and Th2 cells also express distinct combinations of chemokine receptors on their cell surface. Specifically, CXCR3 and CCR5 are expressed at higher levels on Th1 cells (87-90), whereas CCR3, CCR4, and CCR8 are preferentially expressed on Th2 cells (87, 91). The recently identified prostaglandin D_2 receptor (CRTH2) (92), although not a member of the chemokine receptor family, is also highly Th2-specific and has been shown to hold chemotactic activity for this subset (93). Differential expression of chemokines by activated phagocytes, stromal cells, endothelial and epithelial cells within inflamed tissues and lymphoid organs can thus direct tissue-specific trafficking of Th1 and Th2 cells to areas where their effector functions would be most effective (11). Whereas much is now known regarding the differential function of human Th1 and Th2 cells, the mechanisms governing their differentiation remain poorly resolved.

T-bet and GATA-3: masters of their domains

During the initial activation of T_N cells by APC, both type 1 (IFNγ) and type 2 (IL-4) cytokines are transiently produced within the first 24-48 hours. Subsequently, instructive antigen receptor and cytokine signals induce and stabilize the expression of either T-bet or GATA-3, which in turns specifies the fate of T_N cell

differentiation to Th1 or Th2 cells, respectively. TBX21, or T-box expressed in T cells (T-bet) is a member of the T-box family of transcription factors and a 535 amino acid protein. Structurally, T-bet contains a prominent 189 amino acid stretch that makes up a highly conserved T-box DNA-binding domain. In T cells, T-bet is induced in a STAT-1-dependent mechanism upon IFNγ signaling during Th1 differentiation (94) and strongly promotes IFNγ production and expression of the signaling β-2 subunit of the IL-12 receptor (IL-12Rβ2), while repressing Th2 differentiation (80, 94, 95). Indeed, T-bet-deficient mice exhibit a profound defect in mounting Th1 immune responses and spontaneously develop airway hypersensitivity, a pathologic feature consistent with enhanced Th2 development and onset of asthma (96). Likewise, human asthmatic patients carrying a naturally occurring T-bet variant protein (H33G), which confers increased Th1 and decreased Th2 cytokine production in vitro, respond significantly better to corticosteroid treatment (97). Moreover, short-interfering RNA (siRNA)-mediated silencing of T-bet in murine Th cells inhibits Th1 cell development and suppresses Th1 cell-mediated immunopathologies in an autoimmune mouse model of multiple sclerosis (MS) (98). Interestingly, knockout studies demonstrate that whereas T-bet is required for IFNγ production in Th and NK cells, it is largely dispensable in other IFNγ-producing cell types such as CTLs (99).

By contrast to T-bet, GATA-3 is a zinc finger protein and member of the GATA family of transcription factors (GATA-1-6), which all play critical roles in the development, differentiation and function of multiple cell types and tissues (100).

GATA-3 was originally identified based on its capacity to bind the promoter of all 4 *TCR* genes (*alpha*, *beta*, *gamma* and *delta*) and enhance their expression during thymocyte development (101, 102). More recently, however, GATA-3 has been shown to be instrumental in the development and function of Th2 cells in the periphery. GATA-3 is constitutively expressed at low, basal levels within mature, antigen-inexperienced T_N cells and is specifically induced in response to IL-4/STAT-6 signaling during TCR-activation. In turn, GATA-3 undergoes STAT6-independent auto-amplification, *trans*-activates the expression of *IL-5*, directs chromatin remodeling to stabilize transcription of *IL-4* and *IL-13* and renders developing Th cells refractory to Th1 polarizing cytokines (103). Moreover, ectopic expression of GATA-3, even in developing or fully differentiated Th1 cells, induces Th2 cytokine production, reduces IFNγ secretion and promotes chromatin remodeling of the *IL-4* promoter locus (104-107). Conversely, gene deletion or siRNA-mediated silencing of GATA-3 in mature Th cells leads to a profound disruption in Th2 differentiation and effector function (108, 109).

Structurally, GATA-3 contains 443 amino acids and is comprised of two centralized C4-type zinc-finger motifs that mediate DNA binding (100, 110, 111). The zinc-finger domain of GATA-3, along with an adjacent C-terminal stretch of basic residues, serve to mediate DNA binding to the consensus element (A/T)GATA(A/G), a highly conserved property amongst all GATA transcription factors (110-113). The GATA zinc-fingers also mediate protein-protein interactions with other transcription factors, most notably AP-1, FOG, CBP/p300 and NFAT (114-122). Outside its DNA-binding zinc-fingers, GATA-3 has putative

trans-activation domains that contain multiple acceptor sties for post-translational modifications. Indeed, GATA-3 has been shown to be targeted for phosphorylation and acetylation (123-125). However, the manner in which post-translational modifications modulate GATA-3 activity during Th cell differentiation remains uncertain.

While definitive master regulators of Th cell differentiation, it has become increasingly evident that T-bet and GATA-3 do not act in isolation to carry out the Th1 and Th2 differentiation programs. Indeed, activation of Th cells through the TCR and cytokine signals elicits an extraordinarily complex series of signal transduction events that result in the activation of many other transcription factors (aside from T-bet and GATA-3) important for the activation, proliferation, and differentiation of T cells. Thus, the unique combination of transcription factors activated during the primary antigenic stimulation of T_N cells synergistically co-ordinates a specialized program of gene expression that determines if, and what type of effector functions will be acquired.

Specific aims

The differentiation of antigen-specific effector and memory Th cells is a critical step in the mobilization and regulation of human immune responses to invading pathogens. Yet the molecular mechanisms that govern Th cell differentiation remain poorly defined. As such, the central goals of this thesis are 1) to further define the molecular mechanisms whereby T-bet and GATA-3 program T_N cell differentiation to Th1 and Th2 subsets, 2) to characterize the roles of T-bet and

GATA-3 in maintaining the effector function and lymphoid homing potentials in differentiated memory Th cells, and 3) to understand how key human pathogens interact with, and manipulate Th cell function to evade the human immune response.

With respect to how human T_N cells differentiate into functionally and phenotypically distinct effector/memory cells, we favor a model wherein the central goal of Th cell differentiation is to acquire a potent, and specialized set of effector functions. Therefore, we hypothesize that cells within the T_N, T_{CM} and T_{EM} subsets all represent unique stages of Th cell differentiation, with T_{EM} cells signifying the end point of Th cell differentiation. Thus, in order to gain further insight into how T-bet and GATA-3 regulate human Th cell differentiation and to further define the relationship between naïve and memory subsets, in chapter II we determine the capacities of the master transcription factors to reprogram the functional properties of human memory Th cell subsets.

While the pivotal role of T-bet in Th1 cell development is clear, the requirement for T-bet in maintaining the effector function of lineage-committed Th1 cells has yet to be determined. To this end, in chapter III we develop a stable form of RNA interference (RNAi) using a lentiviral-based system to ask whether silencing of T-bet expression in fully differentiated Th1 cells perturbs their pre-existing effector function. We posit that the key role of T-bet in effector and memory Th cells is not to directly regulate *IFNγ* transcription, but is rather to maintain Th1 effector function by suppressing Th2 reprogramming.

We also aim to further define how GATA-3 regulates gene expression during Th2 differentiation. GATA-3 binds to DNA via its conserved dual zinc-finger domain in order to program Th2 differentiation. Yet little is known as to whether this domain is sufficient to direct Th2 cell development. As such, in chapter IV we invoke the use of a heterologous GATA transcription factor, GATA-1, which shares conserved DNA-binding propensities with GATA-3, yet lacks significant sequence similarity in N- and C-terminal regions of the gene/protein. Using this approach, we design experiments to determine if the highly conserved zinc-finger domain of GATA-1 is sufficient to redundantly regulate Th2 cell polarization, akin to GATA-3.

The expression of T-bet and GATA-3 during Th cell differentiation ultimately determines the acquisition of effector function as well as the expression of chemokine receptors that mediate Th cell recruitment to inflamed tissues. Intriguingly, chemokine receptor expression can also influence Th cell susceptibility to human immunodeficiency virus (HIV)-1. Specifically, this is achieved through the differential expression of the major HIV-1 co-receptor CCR5 (126-128), which is expressed at higher levels on Th1 than Th2 cells. Moreover, *CCR5* promoter regions contain several GATA-binding sites (129, 130), suggesting a potential CCR5 regulatory activity by GATA-3. However, this potential role of GATA transcription factors in the regulation of *CCR5* gene expression in primary human Th cells has yet to be explored. In chapter IV, we seek to directly link the expression of GATA transcription factors in human Th cells to the expression of CCR5 and, in turn, to the susceptibility of Th cells to

HIV-1 infection. Moreover, utilizing GATA-1 and GATA-3 we dissect the fundamental regulatory features of GATA transcription factors that determine the expression of CCR5. Indeed, the identification of transcription factors that directly regulate the expression of CCR5 could lead to new anti-retroviral therapies aimed at down-regulating CCR5 expression in individuals afflicted with HIV-1.

A second important human pathogen that evades the immune system is *Helicobacter pylori* (*H. pylori*), a bacterium that establishes a persistent infection in the gastric mucosa of more than half of the human population (131, 132). Clearance of *H. pylori* has been suggested to be dependent on the successful generation of *H. pylori*-specific effector and memory Th cells (133-135), which in turn requires efficient activation-dependent clonal expansion of developing Th cells via the autocrine production of IL-2. Perhaps not surprisingly, *H. pylori* has been recently shown to inhibit T cell activation through the activity of its vacuolating cytotoxin (VacA) (136, 137). To better understand this mechanism of VacA-mediated inhibition of human Th cell activation, in chapter V we more specifically determine the cellular effects of VacA intoxication and perform valuable structure/function analyses to characterize the manner in which VacA inhibits Th cell activation. These key studies provide the fundamental framework to understanding how *H. pylori* evades the immune response and persists within its human host. Moreover, as *H. pylori* infection is associated with peptic ulcer disease and gastric cancers, this work may aid the development of new therapies against *H. pylori*.

The transcriptional regulation of Th cell differentiation by T-bet and GATA-3 ultimately determines the quality of adaptive immune responses that determines if the host response will be protective, ineffective or pathologic in nature. Thus, the key questions addressed herein are of keen importance to decode the differentiation programs of human Th cells and have broad-reaching therapeutic implications. The knowledge gained from these studies provides novel insight into the roles and function of T-bet and GATA-3 during human Th cell differentiation. Moreover, these findings may aid in the development of novel vaccine-based or therapeutic strategies to manipulate Th cell differentiation and function in order to fine-tune human immune responses against pathogenic microbes or pacify immunopathologies stemming from skewed effector and memory Th cell function.

CHAPTER II

GENETIC REPROGRAMMING OF HUMAN T CELLS REVEALS FUNCTIONAL PLASTICITY IN HUMAN T CELL DIFFERENTION

Abstract

Activation of naïve T cells (T_N) through the TCR and cytokine signals directs their differentiation into effector or memory subsets with different cytokine profiles. Here, we tested the flexibility of human Th1 or Th2 differentiation by forced expression of transcription factors T-bet and GATA-3. Ectopic expression of T-bet and GATA-3 in freshly isolated human T_N cells resulted in their differentiation to a Th1 and Th2 phenotype, respectively, in the absence of polarizing cytokines. Introduction of GATA-3 into lineage-committed Th1 cells induced the expression of Th2-specific cytokines (IL-4 and IL-5) and chemotactic receptors (CCR4, CRTH2). However, these cells partially maintained their Th1-specific profile (IFNγ and IL-12Rβ2 expression). Conversely, expression of T-bet in differentiated Th2 cells caused a more profound switch to the Th1 phenotype, including up-regulation of CXCR3 and down-regulation of CCR4 and CRTH2. Interestingly, similar to the T_N subset, T_{CM} cells were also largely programmed towards Th1 or Th2 effector cells upon expression of T-bet and GATA-3, respectively. However, expression of these transcription factors in T_{EM} cells was much less influential on cytokine and chemokine receptor expression profiles. Our results reveal remarkable plasticity in the differentiation programs of human memory T cells

and have important implications in understanding the molecular mechanisms of human T cell differentiation and for devising novel therapeutic strategies aimed at immunomodulation of skewed effector T cell responses.

Introduction

Protective immune responses against pathogens are mediated by functionally distinct subsets of antigen-specific effector T cells, termed Th1 or Th2 cells, which display distinct cytokine profiles. The Th1 subset secretes IFNγ and is instrumental in mobilizing cell-mediated immunity to eliminate intracellular pathogens. In contrast, Th2 cells secrete IL-4, IL-5 and IL-13, which orchestrate humoral responses against helminthic parasites and extracellular microbes (61-63). Cross-regulation between the Th1 and Th2 subsets is critical for mounting effective immune responses and for preventing immunopathologies, such as those associated with allergy and autoimmune diseases. Cytokine signals are paramount in driving the effector fate of naïve T lymphocytes (63, 71). Of key importance, IL-12 or IFNγ signals drive Th1 differentiation, whereas IL-4 promotes Th2 cell development (61, 63, 71). Signals emanating from the T cell and cytokine receptors culminate in the activation of transcription factors, which in turn specify the fate of naïve T cell differentiation.

Induction of the transcription factors GATA-3 and T-bet are critical for differentiation of naïve T cells into Th2 and Th1 cells, respectively (79-81). GATA-3 is a zinc finger protein that is preferentially expressed during the course

of Th2 differentiation in response to IL-4 signals. In turn, GATA-3 *trans*-activates the expression of IL-5 and directs chromatin remodeling to stabilize the production of IL-4 and IL-13 (79, 104, 138-141). Several studies have demonstrated that GATA-3 expression is sufficient to direct differentiation into Th2 cells (96, 105-107, 142-144). GATA-3 over-expression in murine Th1 cells has been shown to result in the production of Th2 cytokines, as well as a decrease in IFNγ secretion and chromatin remodeling of the IL-4 locus (105-107, 145). GATA-3 expression in Th2-lineage committed cells is followed by induction of the transcription factor c-maf which synergistically controls the expression of IL-4 (146-148). In addition, GATA-3 also potently *trans*-activates IL-5 and IL-13 expression (140, 149, 150). In contrast, T-bet, a member of the T-box family of transcription factors, is a master regulator of Th1 lineage commitment (80, 99). T-bet is induced by STAT-1 mediated signals and strongly promotes IFNγ and IL-12 receptor-β2 (IL-12Rβ2) expression during Th1 cell differentiation while repressing Th2 differentiation (80, 94, 95, 151). Indeed, T-bet deficient mice exhibit a profound defect in mounting Th1 immune responses (99) and ectopic expression of T-bet in murine Th2 cells directs trans-activation of IFNγ, as well as the up-regulation of IL-12Rβ2 (80, 94, 95).

Polarized Th1-and Th2-subtypes also express distinct chemokine receptor profiles. Specifically, CCR3, CCR4, and CCR8 are preferentially expressed on Th2 cells (87). In contrast, CXCR3 and to a lesser extent CCR5 are expressed at higher levels on Th1 cells (87). The recently identified prostaglandin D_2 receptor (CRTH2) (92), although not a member of the chemokine receptor family, is also

highly Th2 cell specific and has been shown to have chemotactic activity for this subset (93). Differential expression of chemokines and their receptors within inflamed tissues and lymphoid organs could direct tissue specific trafficking of Th1 and Th2 cells to areas where their effector functions would be most effective. Little is known as to how human T cell differentiation into Th1 or Th2 subsets is regulated. The stability of cytokine profiles in differentiated effector and memory T cell subsets are also not well understood. While a recent study demonstrated that lineage-committed memory T cell subsets are responsive to cytokine signals of the opposing lineage (83), the direct roles of T-bet and GATA-3 in these reprogramming processes remain unclear. The molecular mechanisms that coordinately regulate chemokine receptor expression during T cell differentiation also remain poorly understood. To address these key questions we utilized a lentiviral transduction system (152) to express the Th1- or Th2-specific transcription factors T-bet or GATA-3 in primary human T cells at different stages of differentiation. We show that T_N and T_{CM} human T cells display a high level of flexibility in their cytokine and chemotactic receptor expression profiles as directed by GATA-3 or T-bet. In contrast, effector T cells are more limited in their ability to reprogram their cytokine and chemokine receptor expression. This multi-faceted plasticity of human T cells suggests potential strategies for manipulating immune responses during immunological diseases or vaccine development.

Materials and Methods

Lentiviral vectors

Construction of HIV-derived vectors (HDV) was previously described (152). To create a bicistronic vector that expressed a gene-of-interest and a marker gene we sub-cloned the murine CD24 (HSA) gene that also contained an upstream internal ribosome entry site (IRES, obtained from Clontech) into the HDV vector. Briefly, both IRES and HSA sequences were PCR-amplified and triple ligated into NotI and XhoI sites of HDV in place of the nef gene. Full length GATA-3 cDNA was obtained from the Incyte Genomics clone repository. The T-bet gene was PCR-amplified from a human Th1 specific cDNA library using T-bet specific primers and subcloned into the HDV vector. All constructs were confirmed by sequencing of the insert regions.

Purification of resting human T cells

PBMC were separated from neonatal placental cord blood or from adult blood by Ficoll (Pharmacia) centrifugation. Resting $CD4^+$ T cells were purified as previously described (152). Briefly, $CD4^+$ T cells were positively sorted using anti-CD4 Dynabeads followed by detachabead removal of the beads (Dynal). Purified $CD4^+$ cells were then incubated with anti-CD8, anti-HLA-DR, anti-CD14, and anti-CD45RO (BD Biosciences), followed by Dynabeads conjugated with goat-anti-mouse IgG (Dynal) to deplete bead-bound pre-activated or memory T cells as well as residual $CD8^+$ T cells, monocytes, or dendritic cells. After final

purification, the cells were 99.5% $CD4^+CD45RA^+RO^-$ as determined by FACS analysis. To purify cells that were infected with HDV constructs expressing the reporter protein mouse CD24 (HSA), T cells were incubated with biotin-conjugated anti-mouse-HSA (1 ug/ml) for 30 minutes on ice. Cells were washed twice with PBS, and subsequently incubated with streptavadin-conjugated MACS beads (Miltenyi biotech) for 20 minutes on ice. After washing, HSA^+ cells were sorted using a magnetic sorter (MACS system from Miltenyi biotech). To purify central and effector memory T cells, purified $CD4^+$ cells were first stained with anti-CD45RA antibodies followed by goat-anti-mouse IgG conjugated to magnetic beads (Dynal) and $CD45RA^+$ naïve T cells were removed. The negative portion, which typically is 98% $CD45RO^+RA^-$, was stained with an anti-CCR7 antibody (R&D systems) and sorted into $CD45RO^+CCR7^+$ (central memory) and $CD45RO^+CCR7^-$ (effector memory) T cells using a flow cytometer sorter (Vanderbilt University flow cytometry facility).

T cell activation and differentiation

Differentiation of naïve T cells was accomplished via TCR stimulation using anti-CD3 (OKT3, ATCC) and anti-CD28 (BD Biosciences) antibodies in the presence or absence of polarizing cytokines. Flat-bottom 96 well plates were coated with 10 ug/ml goat-anti-mouse IgG (Caltag) for one hour at 37˚C. Wells were washed twice with PBS and coated with 1 ug/ml anti-CD3 for an additional hour at 37˚C. After washing to remove unbound CD3 antibodies, T cells were added to antibody coated wells with soluble anti-CD28 (1 ug/ml) and the following cytokine

and blocking antibody combinations: human recombinant IL-4 (R&D Systems, 20 ng/ml) and neutralizing anti-IFNγ (R & D Systems, 2.5 ug/ml) antibody, or human recombinant IL-12 (R & D Systems, 30 ng/ml) and neutralizing anti-IL-4 (R & D Systems, 0.5 ug/ml) antibody. Cells were removed from activation signals after 48-72 hours and expanded in recombinant IL-2 (Chiron, 200 U/ml) containing media. The culture media used in all experiments was RPMI supplemented with 10% fetal calf serum, as described before (152).

Virus production and infections

VSV-G pseudotyped, replication incompetent HIV-1 particles were generated as previously described (152). Briefly, HEK-293T cells were transfected with HDV and VSV-G plasmids. Supernatants were collected at 48 hours post transfection, filtered through 0.45 um filters, and stored at –80˚C. Cytokine or TCR-activated T cells were infected in flat-bottom 96-well plates at a 3-5 multiplicity of infection (MOI). In some experiments, cells inoculated with virus were centrifuged for 1 hour at 2000 rpm to enhance infections as described (153).

FACS Analysis

T cells were stained with the relevant antibody on ice for 45 min in PBS buffer containing 2% FCS and 0.1% sodium azide. Cells were then washed twice, fixed with 1% paraformaldeyde, and analyzed with a FACSCalibur® four color cytometer, using the CellQuest program. Live cells were gated based on forward and side scatter properties and analysis was performed using FlowJo software (Tree Star). The following anti-human antibodies were used for staining: CD3, CD4, CD8, CD45RO, CD45RA, CD14, CCR4, CXCR3**,** IL-12Rβ2 (all from BD Biosciences), CCR7 (R&D sciences) and a murine antibody against CD24 (HSA) cell surface antigen (Pharmingen). The CRTH2 antibody used for these experiments has been previously described (92)**.**

Real time PCR

Total RNA from resting and activated Th cells was isolated using Qiagen RNeasy Midi Kit (Qiagen) following the manufacturer's instructions. RNA samples were treated with Qiagen DNase (Qiagen) to remove any contaminating DNA and subjected to reverse transcription using Life Technologies SuperScript First-Strand Synthesis System (Invitrogen) following the manufacturer's instructions. The cDNA was then used to perform real-time PCR with the TaqMan universal PCR Master Mix (Roche) in a Model 7900 ABI Sequence Detection System. RNA samples were normalized using GAPDH primers and probe (Applied Biosystems). The sequences of the primers and probes are as follows:

GATA-3 forward: GGACGAGAAAGAGTGCCTC

GATA-3 reverse: TGGGACGACTCCAGCTTCA

GATA-3 probe: FAM-AGGTGCCCCTGCCCGACAGC-BHQ

T-bet forward: GCTGAGTTTCGAGCAGTCAGC

T-bet reverse: AGTAGGACATGGTGGGCCC

T-bet probe: FAM-TGAAGCCTGCATTCTTGCCCTCTGC-BHQ

c-maf forward: AAGAGGCGGACCCTGAAAA

c-maf reverse: CGTGTCTCTGCTGCACCCT

c-maf probe: FAM-CGGCTATGCCCAGTCCTGCCG

Cytokine detection

For intracellular cytokine analysis, T cells were stimulated with PMA (Sigma, 50 ng/ml) and Ionomycin (Sigma, 0.5 ug/ml) in the presence of .67ml/ml GolgiStop (Pharmingen). The cells were incubated for 4-6 hours at 37˚C followed by fixation and permeabilization using a commercial kit (Pharmingen) according to manufactures instructions. Subsequently, cells were stained with the following antibodies against human cytokines: APC-conjugated anti-IFNγ, PE-conjugated anti-IL-4 and, in some experiments, with PE-conjugated anti-IL-13 and APC conjugated anti-IL5 (all from Pharmingen). For detection of secreted cytokines, T cells were stimulated with anti-CD3 (OKT-3, ATCC) coated plates in the presence of soluble anti-CD28 (Pharmingen, 1 ug/ml) for 18 hours. Supernatants were assayed using a cytometric bead array (CBA) according to the

manufacturer's instructions (BD Biosciences) (154), and analyzed using CBA 6-bead analysis software (BD Biosciences).

Western blot analysis of T-bet and GATA-3 expression

$CD4^+$ T cells transduced with either the control HDV, HDV.GATA-3, or HDV.T-bet were lysed at $5x10^7$ cells/ml in SDS lysis buffer containing: 0.25 M Tris base (Sigma), 8% SDS, 4% β-mercapthoethanol (EM Science) and protease inhibitor cocktail (Boehringer Mannheim). About 30ug of protein lysates were fractionated on 10% SDS-PAGE gels (Invitrogen) and transferred onto PVDF membranes (Bio-rad). Blots were probed with anti-T-bet (Santa Cruz, N-15) and anti-GATA-3 (Santa Cruz, HG3-31) antibodies followed by HRP-conjugated anti-goat IgG (for T-bet antibody) or anti-mouse IgG (for GATA-3 antibody) (Jackson laboratories). Blots developed using west pico luminal/peroxide solutions (Bio-Rad) and autoradiographed (Amersham). To normalize for protein content, the membranes were stripped using a commercially available stripping solution (Bio-rad) for 5 min at 37 C and 7 min at 25 C. Stripped membranes were then probed with anti-β-actin (Santa Cruz, I-19) antibody followed by anti-mouse IgG-HRP (Jackson laboratories).

Results

Differentiation of T_N cells by the ectopic expression of GATA-3 and T-bet.

Human naive T cells can be polarized to differentiate into Th1 or Th2 cells upon stimulation through the TCR and by either IL-12 or IL-4, respectively. To establish Th1 and Th2 lineage committed cells from neonatal cord blood, we isolated $CD4^+$ T_N cells and activated with anti-CD3 and anti-CD28 antibodies in the presence IL-12 or IL-4. Activated T_N cells were expanded in IL-2-supplemented media for 8-10 days, and effector populations were either probed for expression of the Th2-specific cell-surface molecule, CRTH2, or re-stimulated through the TCR for determination of cytokine profiles. T_N cells differentiated into Th1 ($IFN\gamma^{high}IL\text{-}4^{low}CRTH2^{neg}$) or Th2 ($IFN\gamma^{neg}IL\text{-}4^{high}CRTH2^{high}$) effector cells in the presence of the appropriate cytokine milieu (Fig. 2-1A).

We next asked whether stable expression of T-bet or GATA-3 in highly purified human T_N cells could override the requirement for Th1 and Th2 polarizing cytokine signals during Th1 and Th2 cell differentiation, respectively. In order to ectopically introduce these transcription factors into T_N cells, we used an HIV-derived lentiviral vector (HDV) system that allows for highly efficient and stable gene transduction in primary human T cells (152). To monitor the functional effects of T-bet or GATA-3 expression in primary human T cells, we utilized HDV constructs that bicistronically express a marker gene (HSA). T_N cells were infected with HDV, HDV.GATA-3, or HDV.T-bet viruses at the time of CD3 and CD28 stimulation. Activated and transduced cells were expanded for 8 days

and HSA^+ cells were purified. Upon reactivation, cells expressing T-bet or GATA-3 displayed typical Th1 or Th2 cytokine profiles, respectively, similar to T_N cells polarized with cytokines (Fig. 2-1B). Furthermore, GATA-3 transduced cells expressed high levels of CRTH2, similar to Th2 cells polarized by IL-4 signals (Fig. 2-1A and 2-1B). These experiments establish a genetic system that can be effectively used to program human T cell lineage commitment.

To directly assess the level of expression of GATA-3 and T-bet in HDV-transduced primary T cells, we conducted western blot experiments. We detected high levels of GATA-3 or T-bet in T cells transduced with lentiviral vectors expressing these genes as compared to cells transduced with HDV alone (Fig. 2-2A and 2-2B). In addition, we also performed real time PCR analysis, which demonstrated at least 10-fold increase in transcript levels both for T-bet and GATA-3 in cells transduced with these genes (data not shown).

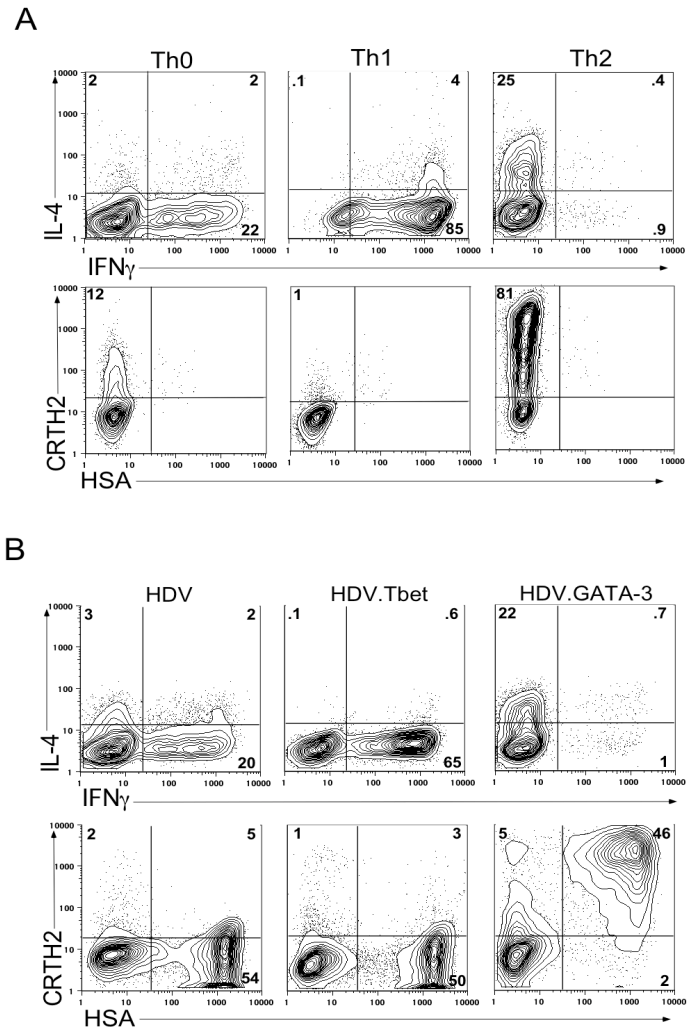

Figure 2-1: Differentiation of T_N cells expressing T-bet or GATA-3.
(A) Human T_N cells purified from neonatal cord blood or adult PBMC were activated through the TCR in the following conditions: 1) Th0: media alone 2) Th1: IL-12 and neutralizing α-IL-4, or 3) Th2: IL-4 and neutralizing α-IFNγ. Cells were activated with α-CD3 and α-CD28 antibodies and expanded in IL-2-containing media for 8-10 days. These cells were then re-stimulated with PMA and Ionomycin in the presence of monensin (4 hours) for intracellular cytokine staining. CRTH2 cell surface expression was determined via FACS analysis. (B) Human T_N cells isolated as described above were again activated through the TCR in media without polarizing cytokines, but containing the following viral supernatants HDV, HDV.GATA-3, or HDV.T-bet. Cells were expanded for 8-10 days and cytokine and CRTH2 expression profiles were assessed as detailed in (A). The transduced cells were identified by co-staining for HSA expression. These data represent one out of four experiments with naïve T cells purified from different adult and neonatal blood samples.

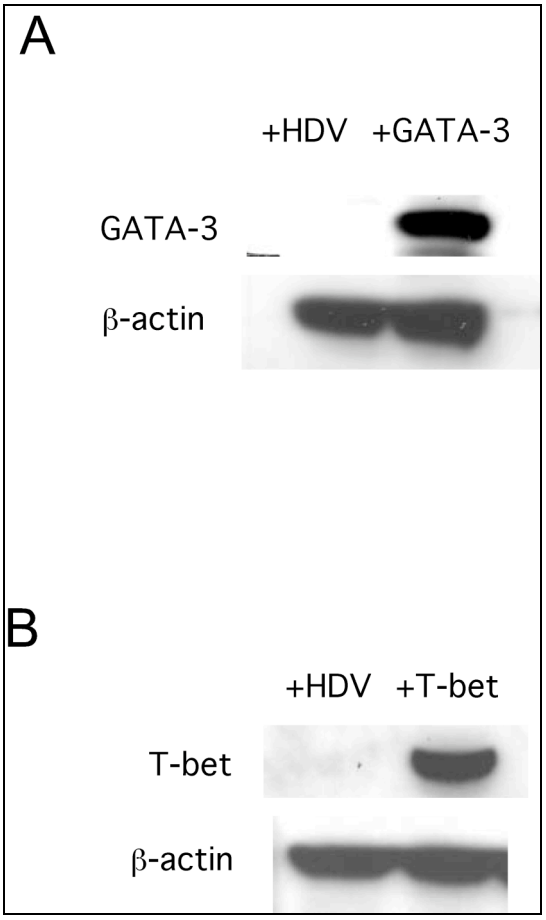

Figure 2-2. Expression of T-bet and GATA-3 in transduced cells.
(A) Purified $CD4^+$ T cells were activated and transduced as detailed in the figure 1B legend, either with HDV, HDV.GATA-3 or HDV.T-bet viruses. Cells were expanded for 8 days in IL-2-suplemented media and sorted for HSA expression. These cells were then lysed and western blot analysis was performed for: A) GATA-3 and B) T-bet expression using specific antibodies. The membranes were stripped and subsequently probed for β-actin (lower panels). The data are representative of two separate western blot experiments using transduced cells obtained from different adult blood donors.

Reprogramming of lineage-committed Th1 and Th2 human T cells by ectopic expression of GATA-3 or T-bet

We next designed experiments to ask whether the phenotype of activated human T_N cells that have already committed to Th1 or Th2 effector states can be reversed upon ectopic expression of GATA-3 or T-bet, respectively. The Th1 and Th2 cells generated from T_N cells (Fig. 2-1) were transduced with HDV.GATA-3, HDV.T-bet, or the control HDV (Fig. 2-3). After three days post-infection, transduced Th1 and Th2 cells were either analyzed for cell surface expression or stimulated through TCR to determine their cytokine profile. While expression of GATA-3 in committed Th1 cells induced IL-4 and IL-5 to varying degrees and induced CRTH2 expression, it had little effect on IFNγ production and IL-12Rβ2 cell-surface expression (Fig. 2-3). In contrast, expression of the Th1-specific transcription factor T-bet in committed Th2 effector cells greatly increased IFNγ production and IL-12Rβ2 (Fig. 2-3). T-bet expression also almost completely extinguished IL-5 expression and resulted in a 10-fold reduction of IL-4 production by Th2 cells (Fig. 2-3). In addition, expression of T-bet in Th2 cells abrogated CRTH2 cell-surface expression (Fig. 2-3). These data suggest early-lineage-committed human T cells retain flexibility in reprogramming their effector functions.

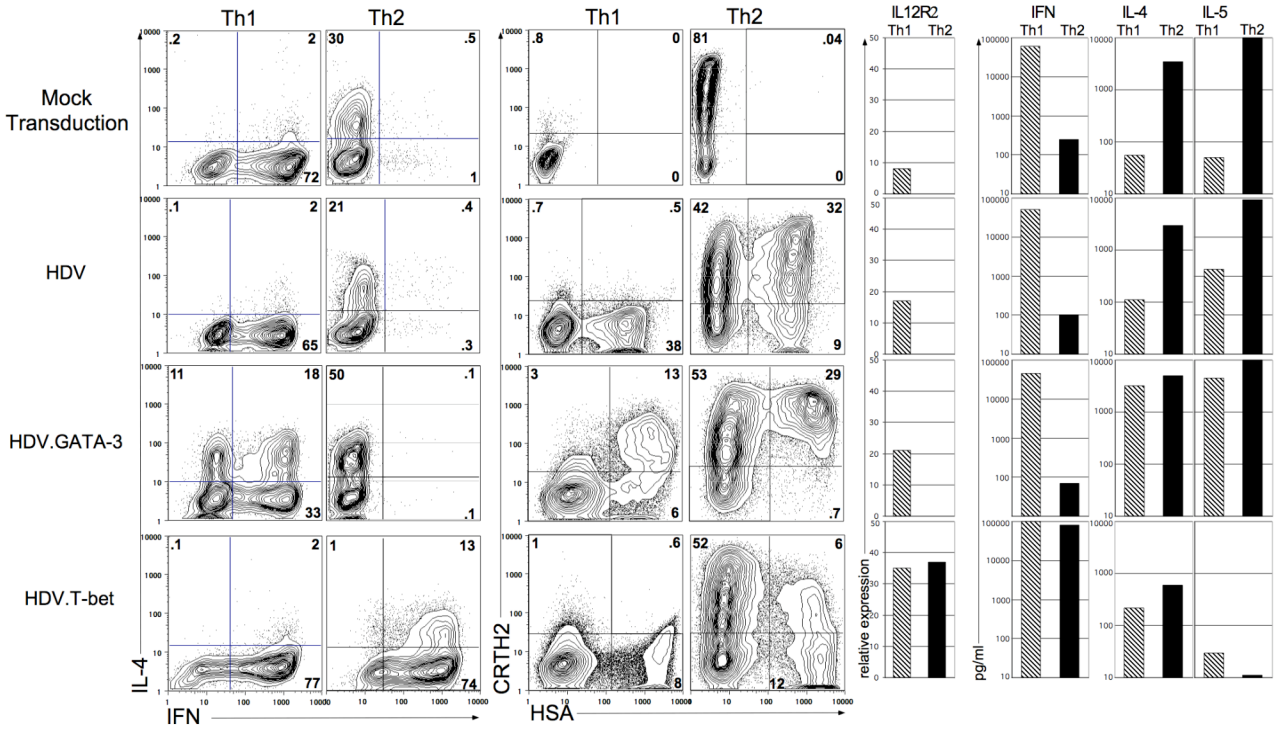

Figure 2-3. Ectopic expression of GATA-3 or T-bet in committed Th1 or Th2 cells directs the reprogramming of effector functions.

Freshly purified human naïve $CD4^+$ T cells were activated through the TCR in either Th1 or Th2 polarizing cytokine conditions as described in the Figure 1 legend. Cells were expanded for 13 days in IL-2 supplemented media and subsequently transduced with the control HDV, HDV.GATA-3, or HDV.T-bet. Cells were sorted 3 days post-transduction based on HSA expression (for intracellular cytokine analyses and CBA experiments). Cell-surface CRTH2 expression was determined as described in the Figure 1 legend using unsorted cells. These data are representative of three experiments.

We then asked whether this flexibility in the programs of cytokine production during Th cell differentiation extends to the regulation of chemokine receptor expression. Committed Th1 and Th2 cells transduced with either the control HDV or an HDV expressing the opposing transcription factor were harvested and cell surface chemokine receptor expression was ascertained through flow cytometric analyses. Genetically unaltered Th1 cells, as well as those transduced with the control HDV expressed a chemokine receptor profile characteristic of Th1 cells ($CCR4^-$, $CXCR3^+$). Indeed, Th1 cells in which GATA-3 was ectopically expressed significantly down-regulated CXCR3 expression while inducing high-level CCR4 expression (Fig. 2-4). On the contrary, lineage-committed Th2 cells not transduced or those transduced with HDV displayed the expected Th2-specific chemokine receptor expression profile ($CCR4^+$, $CXCR3^-$) (Fig. 2-4). Whereas, Th2 cells transduced with HDV.T-bet up-regulated CXCR3 expression, and down-regulated CCR4 expression (Fig. 2-4). These data imply that lineage-committed effector T cells can be directly reprogrammed by T-bet or GATA-3 to change their chemokine receptor pattern and thus their migratory preferences.

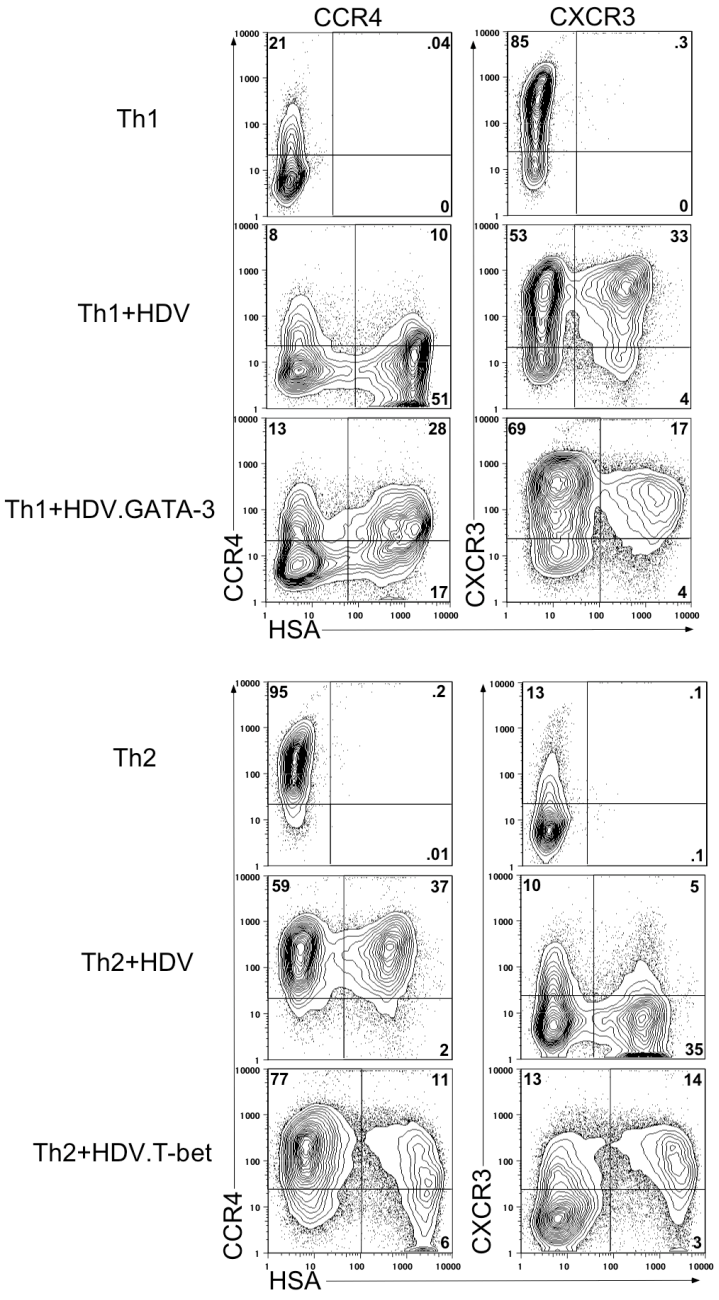

Figure 2-4. Lineage-committed Th1 and Th2 cells modify their chemokine receptor expression profiles upon GATA-3 and T-bet ectopic expression. Similar primary human Th1 and Th2 cell lines were generated via TCR activation as described in the figure 3 legend. Transduced cells for these experiments were left unsorted and chemokine receptor expression profiles were determined via flow cytometric analyses 3 days post transduction, with transduced cells being separated by co-staining for HSA. These results are representative of three experiments using cells from different donors.

Flexibility in effector functions of human memory T cell subsets

T_N cells activated through TCR-signaling acquire the ability to produce cytokines and gain other effector functions (155). A portion of effector T cells become long-lived antigen-specific memory T cells. Recently, human memory T cells I have been shown to consist of two subsets, termed central memory (T_{CM}) and effector memory (T_{EM}) T cells (44). T_{CM} cells, defined by the expression of CCR7, express lymphoid homing receptors and lack robust effector functions. However, T_{CM} cells can rapidly respond to pathogens or cytokines and mature into T_{EM} cells, which lose the expression of CCR7, acquire tissue-homing receptors, and secrete large quantities of cytokines (44). The ability of GATA-3 and T-bet to change the cytokine profile of lineage-committed T cells led us to investigate whether the effector functions of T_{CM} and T_{EM} cells could be modified by expression of these transcription factors. The T_{CM} and T_{EM} cells were purified based on expression of CCR7 and CD45RO as previously described (44). To confirm that the $CD45RO^+CCR7^+$ and $CD45RO^+CCR7^-$ subsets represent T_{CM} and T_{EM} cells respectively, we stimulated these cells through the TCR and determined their cytokine production. As previously reported (44), we found that T_{EM} cells secreted higher quantity of cytokines (TNFα, IFNγ, IL-4 and IL-5) as compared T_{CM} cells (data not shown). Highly purified T_{CM} and T_{EM} cells were then transduced with GATA-3 and T-bet expressing HDV and activated with anti-CD3 and anti-CD28. Cells were expanded for 8 days in IL-2 and re-stimulated through the TCR to evaluate their effector functions. Expression of GATA-3 in T_{CM} cells resulted in a 5-fold increase in cells that were $IL\text{-}4^+IFN\gamma^-$, typical of a Th2

phenotype, whereas T-bet expression greatly increased the number of IFNγ^+IL-4$^-$ T_{CM} cells as compared to non-transduced or control HDV-transduced T_{CM} cells (Fig. 2-5A). Transduction of GATA-3 into T_{EM} cells also resulted in an increase of IL-4 producing cells (2-fold), albeit this was at a lower magnitude than in T_{CM} cells (Fig. 2-5A). Ectopic expression of T-bet in T_{EM} cells caused most to express IFNγ, similar to that observed for T_{CM} subset. However transduction of T-bet into T_{EM} cells was less effective in suppressing IL-4 expression (Fig. 2-5A). We also examined the intracellular expression patterns of two other Th2-specific cytokines, IL-5 and IL-13, in GATA-3 or T-bet expressing T_{CM} and T_{EM} cells (Fig 2-5B). GATA-3 over-expression resulted in a 2-fold increase in IL-5$^+$ and IL-13$^+$ T_{CM} and T_{EM} cells (Fig. 2-5B). In contrast, expression of T-bet greatly reduced the number of IL-5 producing cells, and resulted in a two-fold decline in IL-13$^+$ T_{CM} and T_{EM} T cells (Fig. 2-5B).

Concomitant analysis of CRTH2, revealed that GATA-3 expression in T_{CM} cells, mimicked the effects observed in T_N cells, by resulting in an approximate 10-fold induction of CRTH2$^+$ cells, whereas GATA-3 transduction into T_{EM} cells only resulted in a 2-fold increase in CRTH2 expression (Fig. 2-6A). In contrast, T-bet expression in both human memory T cell subsets resulted in near abrogation of CRTH2 cell-surface expression (Fig. 2-6A).

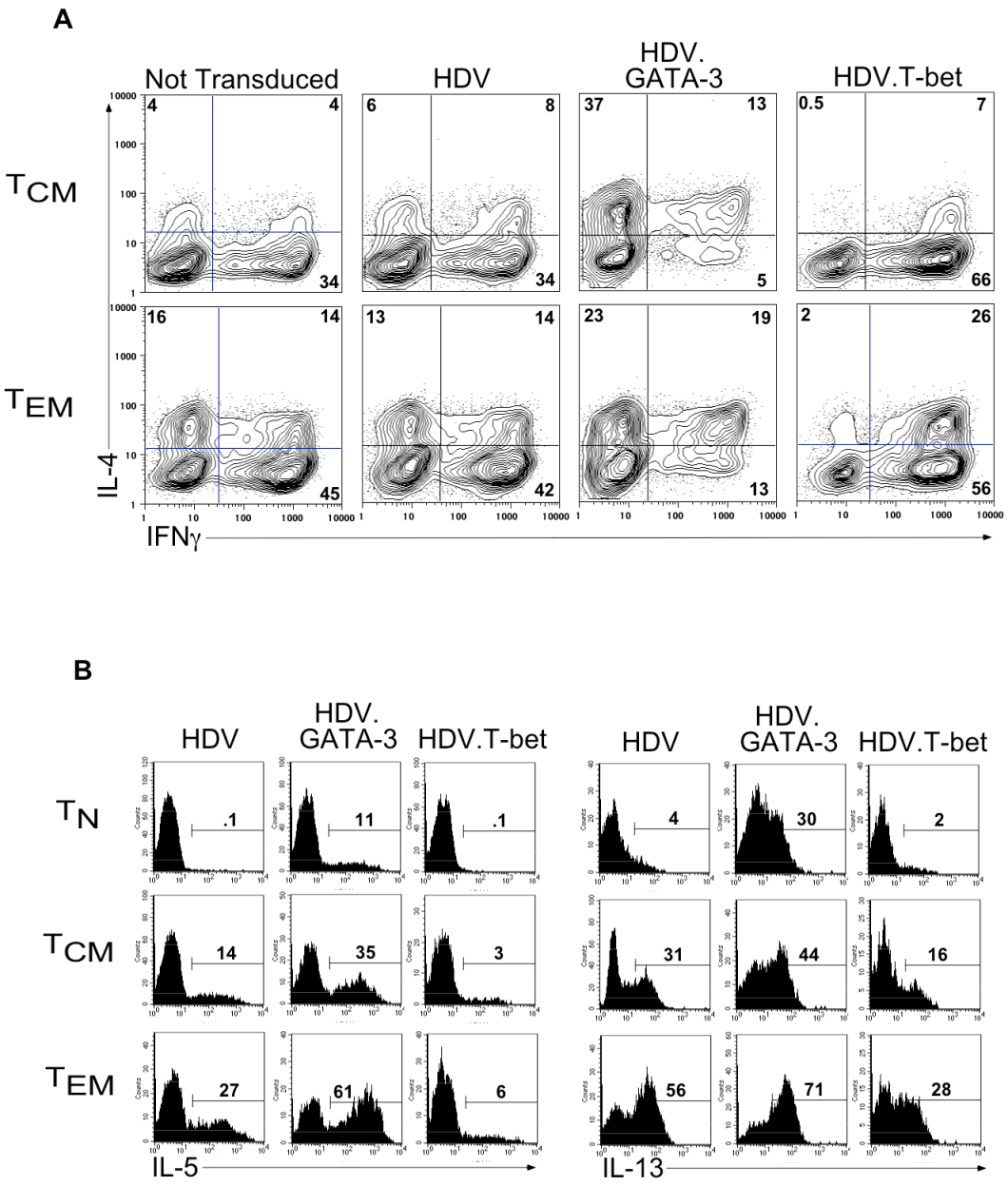

Figure 2-5. Human central and effector memory T cells transduced with T-bet and GATA-3 display flexible cytokine profiles.
CD45RO$^+$RA$^-$ memory T cells were sorted via FACS into T_{CM} (CCR7$^+$) and T_{EM} (CCR7$^-$) cells. The two memory T cell populations were then activated for 24 hours through the TCR via antibody stimulation (see methods), and infected with viruses containing either the control HDV, HDV.GATA-3, or HDV.T-bet. After 8 days of expansion, intracellular expression of the cytokines A) IL-4 and IFNγ, B) IL-5 or IL-13, were determined by flow cytometry as described in the methods. For these experiments transduced cells were left unsorted, but were co-stained with an anti-HSA antibody and the HSA positive populations were gated on for analysis of intracellular cytokine production.

We next determined whether the chemokine receptor profiles of T_{CM} and T_{EM} cells could also be modified by the ectopic expression of GATA-3 and T-bet, similar to lineage-committed effector Th1 and Th2 cells (Fig 2-3). Similar to T_N cells, in the presence of GATA-3, CCR4 levels were highly induced in T_{CM} cells while CXCR3 expression was lower (Fig. 2-6B). T-bet expression in T_{CM} cells resulted in the up-regulation of CXCR3, while conversely down-regulating CCR4 expression (Fig. 2-6B). Introduction of GATA-3 and T-bet into T_{EM} cells also had an effect on chemokine receptor expression, however these effects were much more modest as compared to T_N and T_{CM} subsets (Fig. 2-6B). Taken together, these results show that the effector functions and chemokine receptor expression profiles of human memory T cells can be genetically reprogrammed through the expression of master transcription factors. However, the functional plasticity observed in effector/memory T cells progressively diminishes as cells move through their differentiation.

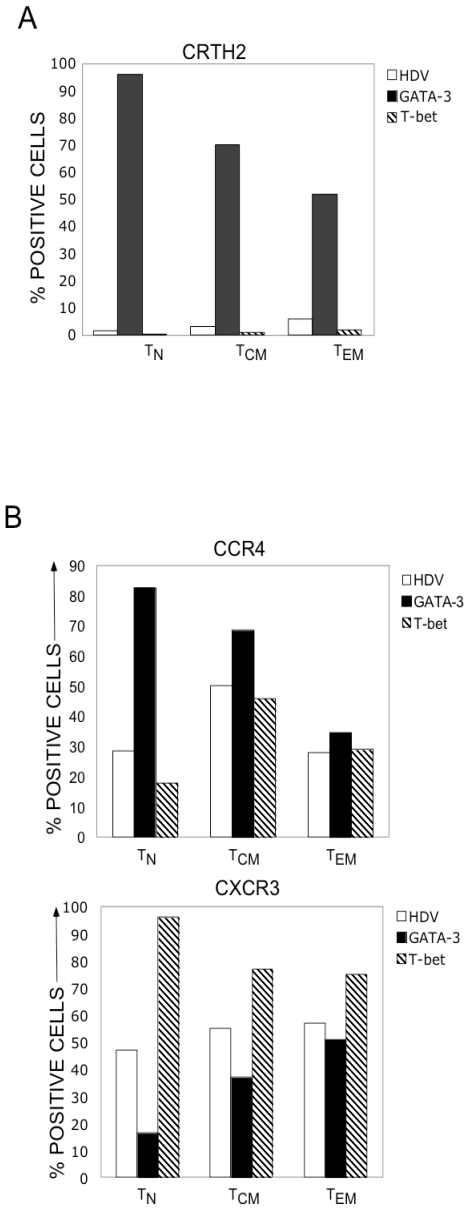

Figure 2-6. Chemokine receptor expression profiles in human T_{CM} and T_{EM} subsets are modified by ectopic expression of T-bet and GATA-3.
Human central and effector memory cells were purified, activated, and transduced with HDV alone, HDV.GATA-3, or HDV.T-bet as in the figure 5 legend. Following expansion, unsorted cells were stained with antibodies against either: A) CRTH2, B) CCR4 or CXCR3 in conjunction with an anti-HSA antibody to gate on transduced populations. The percent of positive cells for the appropriate chemokine receptor is shown. Results are representative of one of four experiments using T cell subsets from distinct adult donors.

Discussion

We demonstrate that lineage-committed and primary human memory T cells, can be genetically reprogrammed towards Th2 or Th1 type cells by the transcription factors GATA-3 and T-bet, respectively. Transduction of GATA-3 in T_N and T_{CM} cells induce cytokine and chemokine receptor expression patterns ascribed to Th2 cells. However, GATA-3 fails to significantly down-regulate IFNγ production and IL-12Rβ2 expression in cells already committed to the Th1 lineage, and in the T_{EM} subset. Conversely, T-bet directs T_N and T_{CM} human T cells to a Th1 phenotype, while it was less effective upon expression in the T_{EM} subset. These data suggest a progressive loss of flexibility as T helper cells proceed from naïve to effector memory stages of their differentiation.

The acquisition of effector functions and more rapid recall responses to antigens during T cell differentiation form the basis of immunological memory. The T_{CM} and T_{EM} subsets of human memory T cells have distinct effector functions and migratory properties (44, 156). T_{CM} cells express receptors necessary for lymphoid organ trafficking, and display only moderate effector function upon antigenic stimulation. Conversely, T_{EM} cells lose the ability to traffic to lymphoid organs and up-regulate pro-inflammatory chemokine receptors, while secreting robust levels of effector cytokines. T_{CM} cells can further develop into T_{EM} cells upon re-stimulation through TCR or cytokine receptors, thus maintaining a diverse repertoire of antigen-specific memory cells (44, 156). We have shown that the cytokine profiles of T_{CM} and, to a lesser extent, T_{EM} cells can

be reprogrammed by constitutive expression of T-bet or GATA-3. These findings support our hypothesis that T_{CM} cells represent an antigen-primed subset, which is capable of modifying its lineage commitment upon antigenic stimulation to generate new waves of T_{EM} cells. On the other hand, one would expect differentiated T_{EM} cells, which have already been programmed to mount specific responses to an invading pathogen, to lack the adaptability to environmental cues that T_{CM} cells exhibit. However, our findings demonstrate that even the cytokine secretion in T_{EM} cells can be partially modified, particularly in response to forced T-bet expression. Indeed, an adaptable memory T cell response, exhibiting plasticity in the context of antigen re-exposure, could be a highly beneficial strategy against constantly evolving pathogens. This hypothesis is also supported by a previous report which demonstrated distinct patterns of cytokine production were elicited in a TCR-transgenic mouse model (68). Our results also suggest that long-term human T cell memory could be modulated by either novel vaccine approaches or genetic manipulation of T cell differentiation programs.

Signaling from IL-12Rβ2 or the IL-4R is thought to be a primary mode of induction of T-bet or GATA-3, respectively, during TCR-stimulation of naïve T cells (157). In our studies we bypassed these receptor signals by over-expressing transcription factors. Our observations extend recent findings that the effector functions of human memory T cell subsets exhibit malleability in response to IL-4 signals (83). Although, IL-12Rβ2 expression is not detectable on Th2 cells and was found to be non-functional in murine studies (Fig. 2-3 and refs (158-161)), recent results suggest that Th2 committed human T cells can

respond to IL-12 signals ((86) and unpublished data)). In addition, it is possible that, independent of IL-4 or IL-12 signals, either the strength of antigen-receptor activation or other co-stimulatory signals could act to induce GATA-3 or T-bet in Th1 or Th2 polarized memory T cells, respectively (48, 66, 69, 70).

Consistent with our conclusions, a recent report by Messi et al. Demonstrated that cytokine signals are capable of re-directing cytokine profiles in human effector and memory T cell subsets (33), suggesting functional cytokine receptors are maintained on lineage-committed T cells to some extent. In this study a subset of memory T cells did not change their effector status in the presence of opposing cytokines. It was not clear whether this resistance to re-commit was maintained through the regulation of cytokine receptor levels, downstream signaling modules, or the induction or activity of opposing transcription factors. Indeed, our results show that ectopic expression of T-bet and GATA-3 can modify effector function and lymphoid homing receptor expression in lineage-committed effector and memory T cells. However, similar to findings by Messi et al., we observed a population of memory T cells were resistant to T-bet or GATA-3-mediated re-programming. This resistant subset grew in size as cells transitioned from T_{CM} to T_{EM} cells. Because we bypassed receptor signals by ectopically expressing programming transcription factors, the likely mechanism for the progressive loss of flexibility within the memory population are heritable epigenetic changes at effector gene (cytokine, chemokine receptor) loci. Messi et al.'s study supports this hypothesis by

demonstrating that lineage-committed memory cells failed to modify their histone acetylation patterns of cytokine genes, as compared to T_N and T_{CM} cells (33).

Our results also show that ectopic expression of T-bet not only induces IFNγ and IL-12Rβ2 expression, but can also function to repress expression of Th2 cytokines in lineage committed Th2 cells, whereas GATA-3 was not effective in suppressing IFNγ secretion. One possible explanation for this observation could be due to epigenetic changes such as demethylation of cytosine nucleotides in CpG pairs (162) and/or histone-tail modifications (82) which allow for structural rearrangements at appropriate cytokine gene loci. T-bet was shown to be critical for robust histone acetylation of the *IFNγ* promoter (163). It is possible that T-bet may also help in recruitment of histone deacetylases to Th2 cytokine gene loci thereby suppressing transcription. In support of this, it was found that the *IL-4* promoter and enhancer were hyperacetylated in T-bet–deficient Th1 cells (164). Another possibility could be due to the down-regulation of other Th2-specific transcription factors such as *c-maf* (120, 146, 165, 166). Indeed transduction of T-bet into resting or activated Th2 cells also resulted in a 3 to 6-fold reduction in *c-maf* gene expression as assessed by real-time PCR analysis (data not shown). In contrast to the potency of T-bet, the failure to fully reprogram lineage committed Th1 cells into Th2 cells by GATA-3 may be due to a requirement for c-maf and perhaps other as yet-to-be-discovered transcription factors (81).

In addition to cytokine production by effector and memory human T cells, chemokines and their cognate receptors play a pivotal role in adaptive immunity.

We have shown that primary human T_N, T_{CM} and Th1 lineage-committed T cell subsets transduced to express GATA-3 greatly up-regulated CCR4, while partially down-regulating CXCR3 expression. T-bet over-expression, on the other hand, resulted in an opposite expression pattern. However, T_{EM} cells were largely resistant to modulation of CCR4 and CXCR3, consistent with the notion of less flexibility within this subset. Our data show that functional plasticity of early lineage-committed effector and T_{CM} cells extends to the control of lymphoid homing receptors. These findings are significant in understanding how the selective expression of chemokine receptors by Th1 and Th2 cells allows for their preferential recruitment to peripheral tissues and sites of inflammation (167-169). Indeed, CXCR3 ligands, such as IP-10, MIG, and I-TAC, as well as the CCR4 ligands MDC, and TARC are expressed by a number of cell types and are produced in peripheral tissues during inflammatory reactions (170-172), Hence, we propose that functional modifications in T_{CM} subset upon induction of T-bet and GATA-3 could lead to a re-structuring of the lymphoid trafficking framework during recall responses to antigens.

Another Th2-specific receptor, CRTH2, upon binding to its ligand, PGD_2, serves in the selective recruitment of Th2 cells (92, 93). Our results clearly show that GATA-3 greatly up-regulates cell-surface CRTH2 expression, whereas T-bet expression promotes its down-regulation in cells at all stages of differentiation. Interestingly, some cells, such as mast cells, that are potent producers of prostaglandins, including PGD_2, also express high levels of IL-4 when stimulated (173). Thus, CRTH2 may play an as yet unidentified role in the amplification of

Th2 differentiation by bringing Th2 cells into proximity with high levels of IL-4 in the periphery. It is also possible that prostaglandins themselves may signal through GATA-3 induced CRTH2 to serve as an amplifier, facilitating differentiation into the Th2 lineage. Additionally, CRTH2 expression may play a role in quenching Th1 responses by helping to recruit IL-4 producing Th2 cells to the site of inflammation.

In conclusion, our findings reveal a remarkable degree of plasticity in the differentiation programs of human T cells. In particular, we show that lineage-committed effector T cells, as well as antigen-specific central memory T cell subsets, retain the ability to modify their cytokine and chemokine receptor profiles in response to expression of opposing transcription factors. These findings suggest that during the course of an immune response antigen-specific memory T cells can modulate their effector functions and lymphoid trafficking properties according to the cytokine milieu or other signals provided by antigen presenting cells. Our results may also have implications in designing therapeutic interventions aimed at ameliorating immunopathologies stemming from misbalanced memory T cell responses, such as allergic asthma or autoimmune diseases. In addition, the feasibility of genetically modifying naïve human T cells can be utilized for the rapid functional validation of genes involved in T helper cell differentiation.

CHAPTER III

RNA INTERFERENCE IN HUMAN T HELPER CELLS REVEALS T-BET EXPRESSION IS REQUIRED FOR TH1 DEVELOPMENT BUT DISPENSABLE FOR MAINTAINING LINEAGE-COMMITTED EFFECTOR FUNCTION

Abstract

T-bet is a master transcription factor required for the differentiation of Th1 effector and memory cells from naïve T (T_N) cell precursors. Upon differentiation, Th1 cells display high-level production of the type 1 cytokine IFNγ independent of polarizing cytokine signals when re-exposed to antigen. Whether T-bet expression in lineage-committed Th1 cells is required for the maintenance of effector function remains uncertain. To address this fundamental question of T cell immunobiology we developed a lentiviral-based system allowing for the stable introduction of T-bet short hairpin RNAs (shRNA) into primary human T helper (Th) cells that specifically silences T-bet expression. Here we detail the generation of a RNA interference (RNAi) vector and selection of functionally active T-bet shRNAs. Utilizing this unique genetic tool, we demonstrate that the expression of T-bet is required in developing Th1 cells for optimal acquisition of IFNγ-producing capacity (i.e. Th1 differentiation), but is dispensable for maintaining effector function in differentiated Th1 effector cells. These results demonstrate the utility of RNAi as a tool to decode the differentiation programs of

human Th cells and lends further insight into the role of T-bet during Th cell differentiation.

Introduction

T-bet is a member of the T-box family of transcription factors and is the master transcriptional regulator of Th1 differentiation (80, 99). In chapter II we demonstrate that T-bet expression, even in lineage-committed Th2 cells or human memory Th cell subsets, reprograms their effector function and chemokine receptor expression patterns to a Th1 profile (60). In contrast to T-bet over-expression, genetic deficiency or siRNA-mediated silencing of T-bet in mice causes a profound defect in mounting Th1 immune responses and attenuates the pathogenesis of a Th1 cell-mediated immunopathology in an autoimmune mouse model of multiple sclerosis (98, 99). Indeed, the critical and instructive role of T-bet during Th1 cell development is underscored by previous reports demonstrating that ectopic expression of T-bet can program Th1 differentiation even in T cells genetically deficient in IL-12 signaling (95).

However important in the differentiation of and IFNγ production by Th1 cells, T-bet does not appear to be required in other IFNγ-producing cell lineages, including $CD8^+$ CTLs (99, 174). These key findings suggest that IFNγ can be regulated in both T-bet-dependent and independent manners. However, whether lineage-committed Th1 cells that produce IFNγ upon re-exposure to antigen in the absence of polarizing cytokines require maintained T-bet expression has yet

to be determined. Here we utilize RNA interference (RNAi) of T-bet expression to further elucidate the role of this master transcription factor in maintaining Th1 cell effector function in differentiated effector cells.

The phenomenon of RNA interference (RNAi) was originally discovered in studies using transgenic plants wherein the introduction of specific DNA transgenes was capable of inactivating expression of the endogenous homolog and was originally called co-suppression (175-177). Subsequent pioneering studies in plants, as well as in the nematode *C. elegans* and *drosophila* more faithfully characterized RNAi as the sequence-specific, post-translational inhibition of mRNA expression mediated by 19 to 25 base-pair oligonucleotides (oligos) termed short interfering RNA (siRNA) (178-183). More recently, it has become clear that RNAi is an evolutionarily conserved mechanism of regulating gene expression during cell development and embryogenesis and also plays an important role in immune protection (184-188).

Mechanistically, siRNAs mediate mRNA degradation through the activation of the cellular RNAi machinery without triggering intrinsic inflammatory reactions to double-stranded RNA (dsRNA) as mediated by type I interferons (177). siRNAs are generated from dsRNA molecules that are derived from endogenously expressed cellular microRNAs (188), vector derived short-hairpin RNAs (shRNAs) (189, 190) or from infectious viruses during replication (184, 191). dsRNA precursors are approximately 70-nucleotides in length and are enzymatically processed into siRNAs (19 to 25 base pairs). The enzyme responsible for siRNA production was originally discovered in cell-free *drosophila*

extracts to be an RNase III enzyme that is now called Dicer (183, 192, 193). Once produced in cells siRNAs bind to cognate cellular or viral mRNA molecules in a sequence-specific manner and target their enzymatic degradation by an effector nuclease called RNA-induced silencing complex (RISC) (194-196). One remarkable feature of RNAi is that only a few molecules of locally-administered dsRNA in plants, nematodes and *drosophila* can be exponentially amplified, become systemic and can even be passed on to progeny (177, 178, 197). However, it has become increasingly evident that this process, termed systemic interference, is not conserved amongst higher vertebrates and mammals. Thus, dsRNA transfection into human cells only results in the transient expression of siRNAs and subsequent gene silencing, thereby limiting the utility of RNAi in mammalian cells (198, 199). To circumvent this obstacle, we developed a lentiviral-based method allowing for the stable expression of siRNA in primary human Th cells.

Here we describe the successful development and expression of T-bet-specific siRNA in primary human Th cells. Utilizing this important genetic tool, we show that siRNA-mediated silencing of T-bet expression in T_N cells driven to differentiate to the Th1 lineage severely impairs acquisition of IFNγ production and expression of Th1-associated chemokine receptors. In contrast, however, lineage-committed Th1 effector cells were not altered in their IFNγ-producing potentials upon T-bet silencing. These novel findings suggest that whereas maintained T-bet expression is obligatory for Th1 development from naïve precursors, it is dispensable for the maintenance of Th1 effector function upon-

lineage commitment. Our results further define the role of T-bet in Th1 development and provide novel insight into the genetic regulation of IFNγ in human Th cells.

Materials and Methods

Generation of lentiviral vectors for RNAi

The HIV-derived self-inactivating lentiviral vector FUGW (200) was modified for siRNA expression by blunt-end ligation of a Gateway cloning cassette (Invitrogen) at the unique Pac I site upstream of the human Ubiquitin C promoter. DNA oligonucleotide duplexes encoding shRNA cassettes were cloned into pENTR/U6 (Invitrogen) downstream of the U6 promoter site according to the manufacturer's instructions. The U6-shRNA cassettes were then shuttled into FUGW by LR recombination (Invitrogen). The fidelity of shRNA sequences was confirmed after the final cloning reaction via sequencing. All shRNA cassettes contain stems of 21-bp organized in a 5'-sense-loop-antisense-3' orientation with the following loop sequence: 5'-AGAGCTTG-3'. The target sequences for the constructs used in this study are as follows:

T-bet (#2 – Figure 3-2)	5'-GTTGTGGTCCAAGTTTAATCA-3'
NegControl	5'-GTTATTCGCGCGAATAACGTT -3'

Purification of human Th cells

PBMC were separated from neonatal placental cord blood or from adult blood by Ficoll (Pharmacia) centrifugation. Resting $CD4^+$ T cells were purified as previously described (152) via anti-CD4 Dynabeads followed by detachabead removal of the beads (Dynal). After final purification, the cells were 99.5% $CD4^+CD3^+$ as determined by FACS analysis. GFP^+ siLENT-transduced cells were sorted via flow cytometric cell sorting (FACSAria - Vanderbilt University flow cytometry facility).

T cell activation and differentiation

Activation and differentiation of purified human $CD4^+$ Th cells was accomplished via TCR stimulation using anti-CD3 (OKT3, ATCC) and anti-CD28 (BD Biosciences) antibodies in the presence or absence of polarizing cytokines as described (60). Briefly, 96 well plates were coated with 10 ug/ml goat-anti-mouse IgG (Caltag) for one hour at 37˚C. Wells were washed twice with PBS and coated with 1 ug/ml anti-CD3 for an additional hour at 37˚C. After washing to remove unbound CD3 antibodies, T cells were added to antibody-coated wells with soluble anti-CD28 (1 ug/ml) in either non-polarizing or Th1-polarizing cytokine conditions as previously described (60). Cells were removed from activation signals after 48 hours and expanded in recombinant human IL-2 (Chiron, 200 U/ml) supplemented media. The culture media used in all experiments was RPMI supplemented with 10% fetal calf serum as described before (152).

Virus production and infections

VSV-G pseudotyped, replication-incompetent HIV-1 particles were generated as previously described (152). Briefly, HEK-293T cells were triply transfected with siLENT, gag/pol and VSV-G plasmids. Supernatants harvested 48 hours post transfection time points were pooled and subjected to 0.45 micron PVDF filtration (Millipore) followed by ultracentrifugation at 25,000 rpm for 2.5 h at 4C in a 70Ti fixed-angle rotor (Beckman). Viral pellets were resuspended in 1mM EDTA/ PBS by overnight shaking. Titers were determined by serial infections of Jurkat T cells with newly made virus and resolving the percent GFP-positive (transduced) cells 48 hour after infection via FACS analysis. For siLENT vector transduction experiments, TCR-activated Th cells were infected in flat-bottom 96- or 24-well plates at a 3-8 multiplicity of infection (MOI) and cells inoculated with virus were centrifuged for 1 h at 2000 rpm to enhance infections as described (153).

FACS analysis

T cells were stained with the relevant antibody on ice for 30-45 min in PBS buffer containing 2% FCS and 0.1% sodium azide. For monitoring cell-surface expression of chemokine receptors, T cells were incubated with un-conjugated antibodies for 20 minutes at room temperature, washed twice and incubated with PE-conjugated secondary antibodies on ice for 30-45 minutes. Cells were then washed twice, fixed with 1% paraformaldeyde, and data was acquired with a FACSCalibur® four color cytometer, using the CellQuest program. Live cells were gated based on forward and side scatter properties and analysis was performed

using FlowJo software (Tree Star). The following anti-human antibodies were used for staining: CD3, CD4, CCR4, CCR5, CXCR3 and CCR4 (all from BD Biosciences).

Real time PCR

Total RNA from resting and activated Th cells was isolated using Qiagen RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA samples were treated with DNase (Qiagen) to remove contaminating DNA and were then subjected to reverse transcription using Life Technologies SuperScript First-Strand Synthesis System (Invitrogen) following the manufacturer's instructions. The cDNA was then used to perform real-time PCR with the TaqMan universal PCR Master Mix (Roche) in a Model 7900 ABI Sequence Detection System. RNA samples were normalized using GAPDH primers and probe. The sequences of the T-bet primers and probe are as follows:

T-bet forward: GCTGAGTTTCGAGCAGTCAGC

T-bet reverse: AGTAGGACATGGTGGGCCC

T-bet probe: FAM-TGAAGCCTGCATTCTTGCCCTCTGC-BHQ

The human real-time PCR primers/probe set for GAPDH (assay ID# Hs99999905_m1) was purchased from applied biosystems (Foster City, CA).

Cytokine detection

Intracellular cytokine analysis of human Th cells stimulated with PMA and Ionomycin was performed as previously detailed (60). Cells were stained with the following antibodies against human cytokines: APC-conjugated anti-IFNγ and PE-conjugated anti-IL-4 (Pharmingen). For detection of secreted cytokines, T cells were stimulated via antibody cross-linking for 18 hours. Supernatants were assayed using a cytometric bead array (CBA) according to the manufacturer's instructions (BD Biosciences) (154), and analyzed using CBA 6-bead analysis software (BD Biosciences).

Results

Development of a lentiviral RNAi vector and selection for active T-bet shRNAs

Gene-specific silencing by RNAi is an important, evolutionarily conserved mechanism of regulating gene expression and also provides a powerful genetic tool that can be utilized to identify and functionally characterize genes involved in a host of biological processes (201, 202). To date, however, wide spread usage of RNAi in mammalian cells has been relatively limited due to the lack of siRNA amplification and systemic spread that is seen in nematodes, *drosophila* and plants (198, 199). We bypassed the inherently transient nature of siRNA expression in mammalian cells by developing a lentiviral-based transduction system that confers stable siRNA expression. The salient features of the newly generated HIV-derived lentiviral vector used to express siRNA in human Th cells

(siLENT) include: 1) the vector is genetically modified as to be replication-defective, 2) does not express any HIV accessory genes, enzymes or structural components, 3) contains the human U6 promoter upstream of shRNA oligonucleotides that activates the cellular RNAi machinery, and 4) carries a marker gene expression cassette (GFP) that is independently expressed off of the human ubiquitin C promoter, allowing for the identification and isolation of transduced cells via flow cytometry (Fig. 3-1).

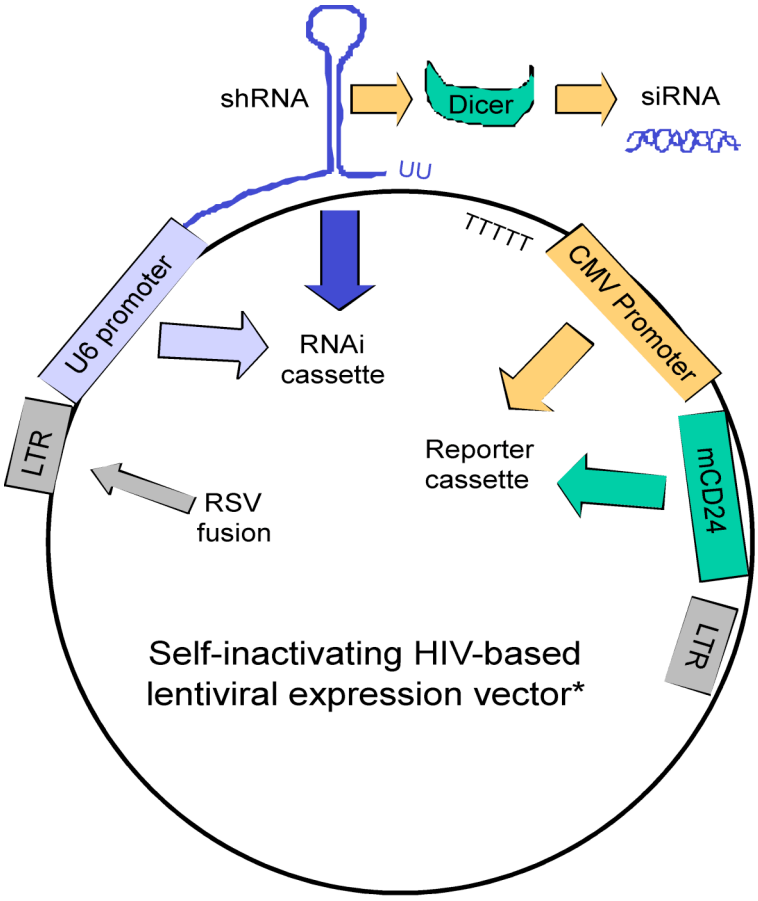

Figure 3-1: Schematic diagram of lentiviral RNAi vector (siLENT).
The HIV-derived vector (siLENT) developed for stable expression of siRNA in primary human Th cells is genetically modified as noted in the text. Importantly, the vector's 5' LTR is self-inactivating as to maintain transcriptional control of the shRNA oligo by the U6 promoter.

In order to further elucidate the role of T-bet in human Th cell differentiation we sought to generate and functionally validate T-bet-specific shRNAs. To this end, cDNA encoding the T-bet ORF was cloned into a reporter vector 3' of firefly luciferase (Fluc) containing a stop codon between Fluc and T-bet mRNA. Thus, chimeric mRNA molecules containing T-bet-tagged Fluc are produced (Fig. 3-2A) and T-bet-specific siRNA activity can be assessed upon triple-transfections of HEK-293T cells with the chimeric Fluc reporter vector, a control reporter vector (Renilla luciferase (Rluc) alone) and vectors containing the human U6 promoter driving expression of candidate T-bet shRNAs (Fig. 3-2B). Indeed, three of the 12 candidate shRNA oligos demonstrated strong T-bet-specific siRNA activity as measured by the targeted down-regulation of the Fluc-T-bet chimeric mRNA by approximately 80% without significantly altering expression of Rluc (Fig. 3-2C). Taken together, these findings demonstrate the generation and functional activity of T-bet shRNAs that potently and specifically inhibits T-bet mRNA expression.

siRNA-mediated stifling of T-bet expression in developing Th1 cells inhibits acquisition of effector functions

T-bet is a master transcription factor that orchestrates T_N cell differentiation to Th1 effector cells (80, 81, 95, 122). In order to functionally validate the efficacy of T-bet-specific shRNAs in primary human Th cells we cloned a T-bet shRNA oligo (#2-Fig. 3-2C) into the siLENT vector and, as a proof of principle, first asked if expression of T-bet shRNAs inhibits human Th1 differentiation.

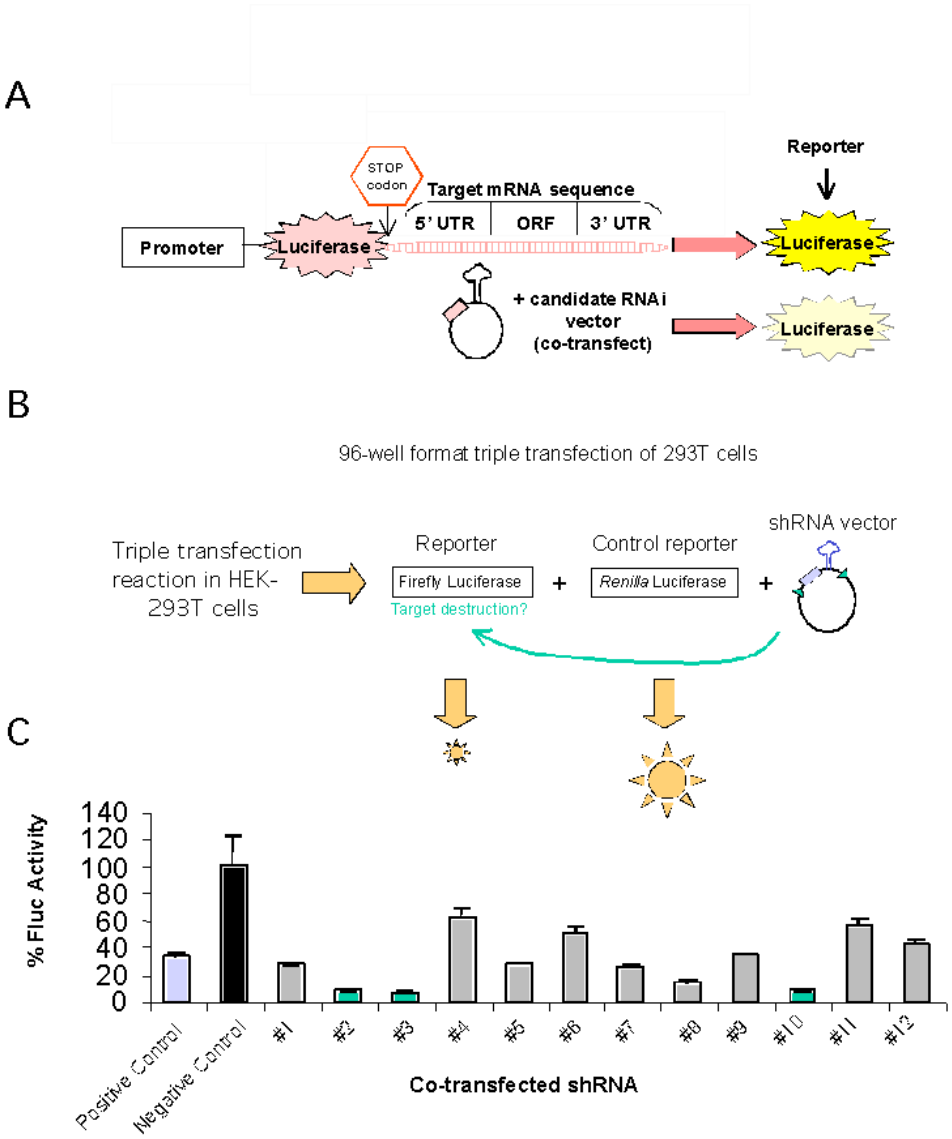

Figure 3-2: Development of and selection for T-bet siRNA.
(A) Screening for active T-bet shRNAs were performed by triple transfection of HEK-293T cells with a Fluc reporter containing the T-bet mRNA sequence, a control Rluc vector and vectors expressing T-bet shRNA oligos. SiRNA activity was scored by specific down-regulation of Fluc signal, without perturbing Rluc. (B) The Fluc reporter vector using in the T-bet shRNA screening reactions was generated by cloning the mRNA sequence of human T-bet down-stream of Fluc, separated by a stop codon as to generate chimeric Fluc-T-bet mRNA molecules. (C) Screening reaction of 12 candidate T-bet shRNA oligos logically design using available algorithms. Fluc activity in each sample was normalized for Rluc activity. The positive control used is a Fluc-specific shRNA and the negative control is an irrelevantly designed shRNA that doesn't affect T-bet expression. These screens were performed in triplicate reactions.

For these experiments T_N cells purified from PBMC of healthy adult donors were TCR-activated via cross-linking antibodies under either non-polarizing (Th0) or Th1 polarizing cytokine conditions (see methods). T_N cells driven to Th1 differentiation were left uninfected, or were infected with lentiviruses containing either control (siLENT.control_shRNA) or T-bet-specific shRNA (siLENT.T-bet_shRNA) at the time of activation. Transduced Th cells were expanded for 8-10 days and T-bet expression was assessed via quantitative real-time PCR. T-bet transcript levels were reduced nearly 5-fold in Th1 cells expressing siLENT.T-bet_shRNA as compared to those expressing control shRNA (Fig. 3-3A), confirming our previous screening data where ~80% reduction was seen in T-bet mRNA levels (Fig. 3-2C). We next determined cytokine production and chemokine receptor expression in the transduced Th cells. As expected, T_N cells activated under Th1 polarizing conditions produced more IFNγ and expressed higher levels of the Th1 chemokine receptor CXCR3 than non-polarized Th0 cells (Fig. 3-3B). Importantly, the observed siRNA-mediated inhibition of T-bet expression levels in T cells programmed to the Th1 lineage was in parallel with reduced IFNγ production and less cell-surface expression of CXCR3 (Fig. 3-3B). Additionally, expression of siLENT.T-bet_shRNA enhanced the secretion of Th2 cytokines (IL-4, IL-5 and IL-10) and expression of the Th2 chemokine receptor CCR4 compared to both genetically unmodified Th1 cells and those expressing control shRNA (Fig. 3-3B, 3-3C). These key findings demonstrate that siRNA-mediated silencing of T-bet expression in primary human T_N cells inhibits their differentiation into Th1 effector cells.

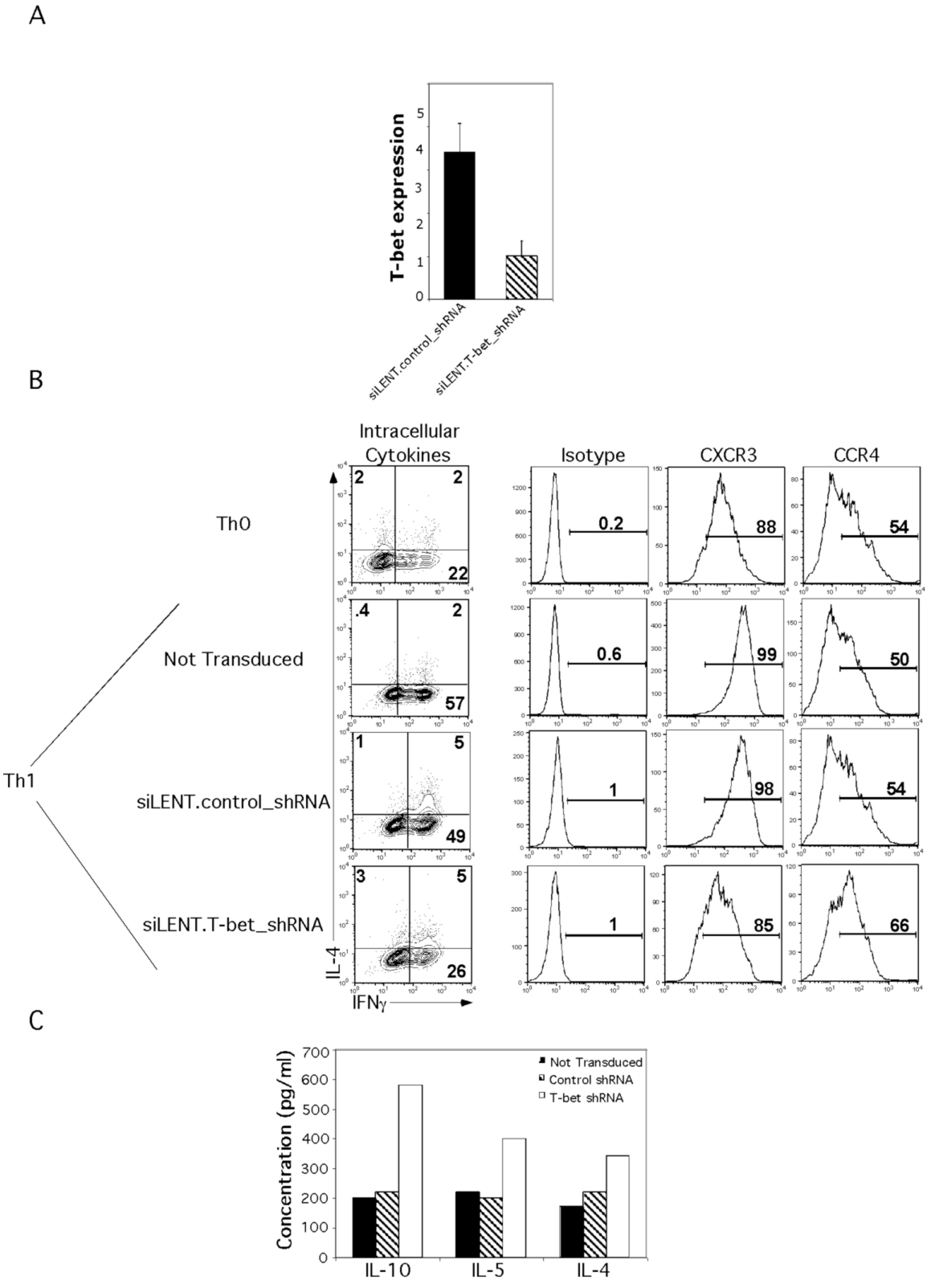

Figure 3-3: T-bet siRNA inhibits T_N cell differentiation to Th1 effector cells.

Figure 3-3 continued.
(A) T_N cells purified from healthy adult donors were activated under Th1 polarizing cytokine conditions and transduced to express control or T-bet shRNA. Activated and transduced cells were expanded for 10 days in IL-2-supplemented media and FACS-sorted based on marker GFP expression to obtain pure populations. Sorted cells were then re-stimulated for 18 hours via α-CD3/α-CD28 stimulation, lysed and T-bet mRNA expression was determined via quantitative real-time PCR. (B) T_N cells were activated under either non-polarizing (Th0) or Th1 polarizing conditions. Those polarized to Th1 differentiation were simultaneously transduced with siLENT.control_shRNA, siLENT.T-bet_shRNA or left untransduced. All cells were expanded as in (A) for 8-10 days and unsorted cell populations were either re-stimulated with PMA/Ionomycin and probed for intracellular cytokine production or analyzed for cell-surface expression of Th1- and Th2-associated chemokines receptors as indicated. (C) Activated and transduced developing Th1 cells as in (B) were expanded for 8-10 days as above, sorted for marker GFP gene expression, re-stimulated for 18 hours via α-CD3/α-CD28 stimulation and Th2 cytokine secretion was determined by CBA and FACS analysis. These data represent two sets of experiments using cells from individual adult donors.

T-bet expression in lineage-committed Th1 cells is dispensable for the maintenance of IFNγ production

We have observed that repressing T-bet expression in T_N cell programmed to differentiate into Th1 cells inhibits the acquisition of Th1 effector function and lymphoid trafficking potentials. However, the role of T-bet in maintaining Th1 effector function upon lineage-commitment remains poorly elucidated. Therefore, we next determined if siRNA-mediated T-bet silencing affects the IFNγ-producing potential of Th1 effector cells.

For these experiments, Th1 cells were generated in vitro from T_N cells as detailed above and were either left unmodified, or transduced with siLENT.control_shRNA or siLENT.T-bet_shRNA subsequent to lineage-commitment. To account for the half-life of previously expressed T-bet protein within the cells and the fact that lentiviral-delivery of transgenes takes approximately 36-48 hours for high-level expression to ensue, transduced Th1 cells were maintained in culture for 5 days prior to harvesting and intracellular cytokine analyses. Th1 cells expressing siLENT.T-bet_shRNA maintained high-level, polarized IFNγ production that was indistinguishable from that of siLENT.control_shRNA-expressing cells and those Th1 cells left untransduced (Fig. 3-4). These data indicate that T-bet expression in lineage-committed Th1 cells is dispensable for IFNγ production.

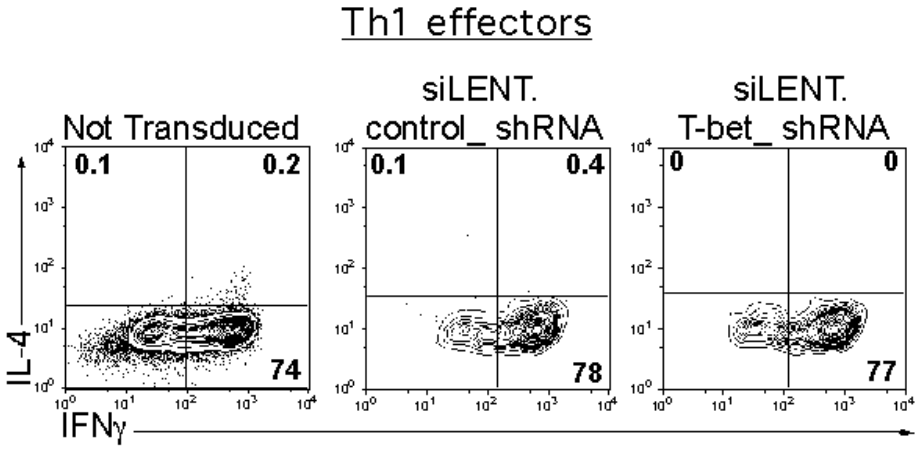

Figure 3-4: T-bet-independent maintenance of IFNγ production in lineage-committed Th1 cells.

Adult blood human Th1 effector cells were generated via TCR-activation as in Figure 3-3 legend. 12 days post initial activation, differentiated Th1 cells were transduced with siLENT.control_shRNA or siLENT.T-bet_shRNA or left untransduced. To allow for siRNA synthesis and T-bet protein and transcript half-life, cells were harvested 5 days post transduction and re-stimulated with PMA/Ionomycin to determine intracellular production of IL-4 and IFNγ. These data represent two independent experiments using cells from different donors.

Discussion

Here we adapt a lentiviral-based gene delivery strategy to stably express T-bet siRNA in primary human Th cells. We demonstrate that T-bet expression is required for the differentiation of primary human T_N cells to the Th1 effector subset but is expendable in maintaining high-level IFNγ production following lineage-commitment. These findings further elucidate the role of T-bet in the regulation of Th1 differentiation and regulation of effector function and also sheds new light into the complex molecular regulation of IFNγ within human Th cells.

The production and regulation of IFNγ by Th cells is of critical importance to the activation of both innate and adaptive immunity against intracellular pathogens. Genetic deficiencies leading to weak IFNγ production by Th cells can lead to heightened susceptibility to acute and chronic intracellular parasitism in animal models (203-205), whereas dysregulated production of the cytokine can be pathogenic and is associated with severe auto-immune disorders (206, 207). Interestingly, T-bet knockout studies clearly suggest that the regulation of IFNγ is cell-type dependent. Th cells and innate NK cells lacking T-bet display severe impairment in IFNγ production, whereas CTLs do not require T-bet expression for high-level IFNγ production, suggesting both T-bet-dependent and independent IFNγ regulatory mechanisms may physiologically occur during immune activation (99). Yet whether impaired IFNγ production in Th cells lacking T-bet is due to a perturbation in Th1 effector cell development, or is the result of diminished IFNγ production by Th1 effector and memory T cells remains unresolved (98, 99). Our

results favor a model of bi-phasic model of IFNγ regulation during human Th cell differentiation wherein optimal acquisition of polarized, high-level IFNγ production in recently programmed Th1 cells is dependent upon T-bet expression. In contrast, our findings imply that lineage-committed Th1 effector cells can maintain high-level IFNγ-producing potential in a T-bet-independent fashion, analogous to CTLs.

The T-bet independent IFNγ-production displayed by CTLs has prompted further investigation into the regulation of IFNγ in the absence of T-bet. Recently, a paralog of T-bet, called eomesodermin (eomes), was identified that is specifically induced upon activation of naïve $CD8^+$ T cells and is sufficient to program their differentiation to IFNγ-producing CTLs, even in the absence of T-bet (174). Indeed, ectopic expression of eomes in T-bet-deficient Th cells can also rescue Th1 differentiation and promote high-level IFNγ production upon antigen re-stimulation (174). Moreover, *eomes*$^{+/-}$ CTLs displayed significant impairment in both eomes expression and IFNγ production (174). However, while the identification of eomes provides novel insight into T-bet-independent mechanisms of IFNγ regulation, it is not expressed in Th cells, and thus cannot account for IFNγ maintenance in the absence of T-bet in this study.

Another transcription factor, H2 O-like homeobox protein 1 (Hlx1), has been recently characterized to regulate IFNγ expression (163). Hlx1 is expressed in Th cells and appears to be important for stabilizing Th1 effector function and IFNγ expression. Hlx1 is induced in T_N cells during Th1 development, and subsequently acts in concert with T-bet to potentiate IFNγ expression (163).

Extensive demethylation of the IFNγ locus ensues only after T-bet/Hlx1-co-ordinated IFNγ expression, and thus Hlx1 may confer the stable T-bet-independent IFNγ production displayed by Th1 effector T cells observed in the present study (163). Alternatively, as the siRNA-mediated silencing of T-bet observed is not 100%, it is also possible that lineage-committed Th1 effector cells require less T-bet to maintain high-level IFNγ production, analogous to the manner in which effector and memory Th cells are more sensitive to antigenic re-stimulation (38, 155).

In summary, through the development and expression of T-bet shRNA in primary human Th cells we show that T-bet is an obligate transcription factor during Th1 cell development that confers optimal acquisition of effector function and lymphoid trafficking tendencies. Upon lineage-commitment, however, T-bet expression appears to be dispensable for maintaining polarized IFNγ production. These findings provide novel insight into the role of T-bet in the regulation of Th1 cell development and function and uncover a previously unappreciated mechanism of IFNγ regulation in Th cells. Moreover, the feasibility of expressing siRNA in primary human Th cells can be utilized to rapidly identify and functional characterize genes involved in T cell differentiation.

CHAPTER IV

THE TRANSCRIPTION FACTOR GATA-1 POTENTLY REPRESSES THE EXPRESSION OF THE HIV-1 CO-RECEPTOR CCR5 IN HUMAN T CELLS AND DENDRITIC CELLS

Abstract

The major transmissible HIV-1 strains require expression of the chemokine receptor CCR5 for entry into $CD4^+$ cells. The molecular mechanisms regulating CCR5 expression in the primary targets of HIV-1, human T cells, is poorly understood. Because the CCR5 promoter contains DNA-binding sites for GATA transcription factors, we investigated whether GATA transcription factors can regulate CCR5 expression. We show that expression of GATA-1 in primary human T cells, but not GATA-3, potently inhibits CCR5 expression, rendering cells refractory to infection by CCR5-tropic HIV-1. GATA-1 expression in other HIV-1 target cells, notably iNKT cells and dendritic cells, also suppresses CCR5 expression. Mechanistically, GATA-1 inhibits CCR5 promoter activity and gene expression in human T cells, suggesting direct transcriptional repression. Despite the differential inhibition of CCR5 and other Th1-associated effector molecules (CXCR3 and IFNγ) exhibited by these GATA factors, we show that GATA-1 can functionally replace GATA-3 in programming Th2 gene expression. These findings indicate the DNA-binding domain shared between GATA-1 and GATA-3 is sufficient to program Th2 cell differentiation but not for CCR5 repression.

These results lend novel insight into CCR5 gene regulation and T cell differentiation, which together can profoundly influence HIV-1 transmission and disease progression.

Introduction

CC chemokine receptor 5 (CCR5) is a receptor for β-chemokines that regulate the migration of leukocytes into inflamed peripheral tissues (208). CCR5 also serves as the primary co-receptor on $CD4^+$ cells for viral entry of macrophage-tropic strains of HIV-1, HIV-2 and SIV (126-128). As such, β-chemokines secreted by cytolytic T cells or bioactive small molecule inhibitors that compete with HIV-1 gp120 for CCR5 binding and facilitate receptor internalization inhibit HIV-1 infection and replication (209-211). Moreover, individuals carrying a naturally occurring homozygous 32bp-deletion within the CCR5 ORF (CCR5Δ32) that precludes cell-surface expression, display almost complete resistance to HIV-1 transmission, infection and progression to AIDS (212-217). More recent reports have demonstrated that inhibition of CCR5 expression via intracellular immobilization (218) or siRNA (219) also renders T cells resistant to R5-tropic HIV-1 infection. Together these findings corroborate the pivotal role of CCR5 expression during HIV-1 infection and highlight the importance of understanding how it is regulated.

CCR5 is expressed within the immune system by $CD4^+$ effector or memory T helper (Th) cells, invariant natural killer T (iNKT) cells, macrophages,

immature dendritic cells (DCs) and in bone marrow precursor cells during hematopoiesis (208, 220). While CCR5 expression in most cell types is uniform, its expression in human Th cells is highly variable and tightly linked to the type of effector function acquired during naïve T (T_N) cell differentiation to effector and memory subsets. More specifically, CCR5 is prominently expressed on IFNγ-secreting Th1 cells as compared to IL-4-producing Th2 cells (38, 88, 221). In addition to the differential expression of CCR5, Th1 cells also prominently express CXCR3 (88-90), whereas Th2 cells preferentially express CCR4 (88, 91) and the prostaglandin D_2 (PGD_2) receptor (CRTH2) (60, 92, 93). Unique chemotactic receptor expression profiles on Th1 and Th2 cells, including CCR5, are critical for regulating their migratory propensities to sites of inflammation and may also determine cell susceptibility to HIV-1 infection.

Promoter analyses of CCR5 have identified putative DNA binding sites for several transcription factors including members of the GATA family (129, 130, 222). Moreover, a previous report has shown that GATA transcription factors can bind to CCR5 promoter elements and that GATA-1 expression in a T-lymphoid cell line *trans*-activates CCR5 expression (223). However, the potential regulation of CCR5 gene expression by GATA transcription factors within any physiologic cellular targets of HIV-1 has not been determined.

Structurally, GATA transcription factors are comprised of either one or two centralized C4-type zinc-finger motifs that mediate DNA binding and protein-protein interactions (110, 113, 224-227). Based on tissue expression profiles and the degree of sequence conservation within the zinc-finger domain of GATA

family members, GATA-1, GATA-2 and GATA-3 are most closely related, all of whom serve as key regulators of hematopoiesis in mammals (101, 138, 220, 228-231). These GATA family members share significant sequence similarity within the zinc-finger domain, thus displaying similar DNA binding preferences for the consensus DNA element (A/T)GATA(A/G) (110-112, 232). However, homology in amino- and carboxy-terminal regions of GATA-1, -2, and –3 located outside the zinc-fingers is minimal.

GATA-3 is a master regulator of T_N cell differentiation into Th2 effector cells that predominantly produce the cytokines IL-4, IL-5 but not IFNγ and express the chemotactic receptors CCR4 and CRTH2 [25,](79, 81, 103, 104, 157, 233, 234). Indeed, expression of GATA-3 enhances Th2 cytokine production, decreases IFNγ secretion and promotes chromatin remodeling of the IL-4 locus (105-107). On the other hand, GATA-1 is expressed in HSCs, erythroblasts, megakaryocytes, eosinophils and mast cells, serving to regulate megakaryopoiesis and erythropoiesis in mice and humans (220) (235-238). Mice lacking GATA-1 display severe hematopoietic dysfunction and do not survive gestation due to lethal anemia (239). In humans, mutations that interfere with GATA-1 expression or function result in lethal anemic disorders or hematopoietic cancers (240-242). Interestingly, studies using GATA-1-deficient embryonic stem cells or knock-out mice demonstrate that GATA-3 expression can rescue hematopoietic deficiencies and embryonic lethality in the place of GATA-1 (243-245). However, adult animals that have GATA-3 substituted for GATA-1 generate reduced levels of improperly functioning platelets and erythrocytes suggesting

GATA-3 can not completely reconstitute GATA-1 function (244). Nevertheless, these findings suggest that the DNA binding zinc-finger domain of GATA-3, which is nearly 95% identical to that of GATA-1, is largely sufficient to direct hematopoiesis.

Here we demonstrate that expression of GATA-1, but not GATA-3, potently inhibits CCR5 gene expression in primary human Th cell subsets, iNKT cells and DCs. As such, GATA-1-expressing human Th cells are resistant to infection by CCR5-tropic HIV-1. Similar to GATA-3, however, ectopic expression of GATA-1 in human naïve and memory Th cells programs Th2 cytokine and chemotactic receptor expression profiles. These findings identify GATA-1 as a potent transcriptional silencer of CCR5 expression and could have implications in regulating its expression in the context of HIV-1 infection. Moreover, our results highlight functional redundancies and divergences between heterologous GATA transcription factors, thus providing novel insight into the molecular regulation of Th cell differentiation by GATA-3.

Materials and Methods

Lentiviral vectors

Construction and use of HIV-derived vectors (HDV) that contain bicistronic marker genes have been previously described (152). HDV bicistronically expressing GATA-3 and a marker gene (murine CD24 or GFP) has also been described (60). To generate HDV expressing GATA-1, murine GATA-1 was sub-

cloned into the PmeI site of the HDV vector and confirmed by sequencing of the insert regions. Murine and human GATA-1 share 90% and 100% sequence identity within the open reading frame and zinc finger domains, respectively.

Purification of T cell subsets

PBMCs were isolated from neonatal placental cord blood or from adult blood by Ficoll (Pharmacia) density centrifugation. Resting $CD4^+$ Th cells were purified as previously described using CD4-Dynabeads (60, 152) and were 99.5% $CD4^+CD3^+$ as determined by FACS analysis. Primary Th cells transduced with HDV express the reporter protein mCD24 on the cell surface and were enriched for by sorting for $mCD24^+$ cells as described (60). In some experiments purified $CD4^+$ T cells were further subdivided into central ($CD45RO^+CCR7^+$) and effector ($CD45RO^+CCR7^-$) memory and naïve ($CD45RO^-CCR7^+$) T cells from adult blood, using magnetic bead and flow cytometry (FACSAria) sorting as previously described (60). Human NKT cells were isolated from adult blood PBMC as previously described (246).

$CD34^+$ hematopoietic stem cell isolation, culture and DC differentiation

Human $CD34^+$ HSCs were isolated from neonatal umbilical cord blood. Briefly, total mononuclear cells recovered from cord blood (see purification of resting human T cells section) were positively sorted for stem cells via automated magnetic sorting using anti-CD34 antibodies directly conjugated to MACS beads

(Miltenyi). Purified stem cells were cultured in serum free media (Stem Cell technologies, serum free expansion media (SFEM) – catalogue #: 09600) supplemented with stem cell factor (SCF, 50 ng/ml), FMS-related tyrosine kinase 3 ligand (Flt3L, 100 ng/ml), IL-3 (20 ng/ml), thrombopoietin (200 ng/ml) (all from R&D systems) and penicillin/streptomycin (Cellgro) for the first 3 days following purification. HSCs were subsequently expanded for an additional 6-8 days in serum free media containing SCF. Stem cells were then harvested and either kept in SCF-containing serum free media to maintain pluripotency or washed and cultured in DC-differentiating media: RPMI plus 10% FCS supplemented with human IL-4 (50 ng/ml) and GM-CSF (20 ng/ml) (R&D systems). DC differentiation from HSCs was confirmed via staining for CCR5 expression along with anti-CD1a (BD biosciences) or anti-CD1c (Miltenyi) antibodies and FACS analysis 4-6 days post differentiation.

T cell activation and differentiation

Activation and differentiation of purified human $CD4^+$ Th cells was accomplished via TCR stimulation using anti-CD3 (OKT3, ATCC) and anti-CD28 (BD Biosciences) antibodies in the presence or absence of polarizing cytokines as described (60). Briefly, 96 well plates were coated with 10 μg/ml goat-anti-mouse IgG (Caltag) for one hour at 37˚C. Wells were washed twice with PBS and coated with 0.5 μg/ml anti-CD3 for an additional hour at 37˚C. After washing to remove unbound CD3 antibodies, T cells were added to antibody-coated wells with

soluble anti-CD28 (1 μg/ml) in the presence of Th1 or Th2 polarizing cytokine conditions as previously described (60). Cells were removed from activation signals after 48 hours and expanded in recombinant human IL-2 (Chiron, 200 U/ml) supplemented media. iNKT cells were purified cells, activated and expanded as previously described (246). The culture media used in all experiments was RPMI supplemented with 10% fetal calf serum as described previously (152).

Virus production and infections

VSV-G pseudotyped, replication incompetent HIV-derived viruses (HDV) were generated as previously described (152). For HDV, HDV.GATA-3 or HDV.GATA-1 transduction experiments, TCR-activated Th cells or NKT cells were infected in flat-bottom 96- or 24-well plates at a multiplicity of infection (MOI) of 3-8. Stem cell-derived DCs were infected after 4 days in DC differentiating media at 5 MOI. For some experiments, cells inoculated with virus were centrifuged for 1 h at 2,000 rpm to enhance infections as described (153). Replication-competent HIV-1 infections of purified HDV-, HDV.GATA-3- or HDV.GATA-1-expressing Th cells, were conducted by inoculating T cells with various MOIs of CCR5-tropic replication-competent strain of HIV (R5.HIV) or with VSV-G-pseudotyped, replication-incompetent HIV (VSV-G.HIV). The latter two viruses were engineered to express GFP in the place of the *nef* gene. HIV-1 infection of Th cells was monitored by FACS analysis of GFP expression and viral replication

was determined by quantifying the HIV-1 core protein p24 in culture supernatants by ELISA.

Antibodies and FACS Analysis

Cells were stained with the relevant antibodies and analyzed using a flow cytometer (FACSCalibur®) as previously described (60) Analysis was performed using FlowJo software (Tree Star). The following anti-human antibodies were used for staining: CD3, CD4, CD45RO, CD45RA, CCR4, CCR5, CXCR3**,** CCR4, CD1a and CD1c (all from BD Biosciences), CCR7 (R&D sciences), anti-Vβ11 (Coulter) and a murine antibody against CD24 (HSA)(Pharmingen). The CRTH2 antibody used for these experiments has been described previously (92)

Real time PCR

Total RNA and cDNA synthesis from primary cells was performed as described (247). The cDNA was used to perform real-time PCR to determine expression levels of GATA-3, GATA-1 and CCR5, with GAPDH levels being concurrently assessed as to normalize samples for differences in RNA content. Real-time PCR was conducted using the TaqMan universal PCR Master Mix (Roche) in a Model 7900 ABI Sequence Detection System. The sequences of the primers and probes are as follows:

GATA-3 forward: GGACGAGAAAGAGTGCCTC

GATA-3 reverse: TGGGACGACTCCAGCTTCA

GATA-3 probe: FAM-AGGTGCCCCTGCCCGACAGC-BHQ

Human real-time PCR primers/probe sets for CCR5 (assay ID# Hs00152917_m1), GAPDH (assay ID# Hs99999905_m1) and GATA-1 (assay ID# Hs00231112_m1) were purchased from Applied Biosystems (Foster City, CA).

CCR5 promoter analysis in T cells

To study the regulation of CCR5 promoter activity, TCR-activated primary human Th cells expressing HDV, HDV.GATA-3 or HDV.GATA-1 were re-stimulated via anti-CD3 and anti-CD28 antibodies for 24 hours as described above. 1-2 x 10^6 cells were then harvested, washed twice with PBS and resuspended in 160μl human T cell nucleofection buffer (Amaxa). Cells were transfected with 2 μg of either the promoterless pGL3-Basic vector (Promega) or a vector that contains a CCR5 promoter construct that spans both promoters 1 and 2. The design of this CCR5 promoter construct were described previously and are shown in Fig. 5 (130). The CCR5 promoter construct was cloned upstream of the luciferase gene in this vector. 0.3 μg of a *renilla* gene-containing vector, (pHRL-CMV vector-Promega) DNA was added to cells to normalize for transfection efficiency. The cell/DNA mixtures were transfected via a nucleofection device (Amaxa) per manufacturers' instructions. Following transfection, cells were immediately diluted into fresh IL-2-supplemented media and cultured for 18 hours. Cells were

subsequently lysed and both firefly and *Renilla* luciferase activities were determined using a Dual-Luciferase assay kit per recommendations of the manufacturer (Promega) and analyzed using a luminometer (Mediators PhL).

Cytokine detection

Intracellular cytokine analysis of human Th cells stimulated with PMA and ionomycin was performed as described previously (60). Cells were stained with antibodies for the following human cytokines: APC-conjugated anti-IFNγ, PE-conjugated anti-IL-4, PE-conjugated anti-IL-13 and APC conjugated anti-IL-5 (all from Pharmingen). For detection of secreted cytokines, Th cells were stimulated via antibody cross-linking for 18 hours. Supernatants were assayed using a cytometric bead array (CBA) per manufacturer's instructions (BD Biosciences) and analyzed using CBA 6-bead analysis software (BD Biosciences) using flow cytometry.

Western blot analysis of GATA-3 and GATA-1 expression

Western blot analyses on primary human Th cells transduced with either the control HDV, HDV.GATA-3, or HDV.GATA-1 was performed as described previously (60). Blots were performed first by probing with anti-GATA-3 (Santa Cruz, HG3-31) antibodies followed by HRP-conjugated anti-mouse IgG. Blots were developed using West Pico luminol/peroxide solutions (Bio-Rad) and

autoradiographed (Amersham). The membranes were stripped using a commercially available stripping solution (Bio-rad) for 5 min at 37^{0}C and 7 min at room temperature. Stripped membranes were re-probed with anti-GATA-1 (Abcam) antibody followed by HRP-conjugated anti-rabbit antibody. To normalize for protein content, the membranes were stripped again and probed with anti-β-actin (Santa Cruz, I-19) antibody followed by anti-mouse IgG-HRP. All HRP-conjugated secondary antibodies were obtained from Jackson Laboratories.

Results

Expression of GATA-1 is down-regulated as human CD34$^+$ stem cells differentiate into DCs and up-regulate CCR5.

GATA transcription factors have been implicated in the regulation of CCR5 expression. CCR5 promoter regions contain multiple DNA-binding sites for GATA transcription factors (129, 130) and expression of GATA-3 during Th cell differentiation promotes development of Th2 effector cells that express low levels of CCR5 (79, 88). However, ectopic expression of GATA-3 in CCR5 expressing T cells does not markedly reduce its expression (our unpublished data and Figure 4-2). Therefore, we sought to determine if other GATA transcription factors may be capable of regulating CCR5 expression. As such, we chose to investigate the potential role of GATA-1 in CCR5 regulation. GATA-1 has previously been shown to bind to the CCR5 promoter and *trans*-activate CCR5 gene expression in a transformed T-lymphoid cell line (223). While, GATA-1 is not expressed on T cells, it is expressed in CD34$^+$ hematopoietic stem cells (HSCs) (220) ,(248).

HSCs can differentiate into immature dendritic cells (DCs) that express robust levels of CCR5 (249) and are primary targets of initial HIV-1 infection upon viral transmission (250-252). We therefore asked if there was a relationship between GATA-1 and CCR5 expression during HSC differentiation into DCs.

Primary human HSCs were isolated from neonatal umbilical cord blood and expanded in SCF-supplemented media for 8-10 days to pluripotency. Cell-surface CCR5 and endogenous GATA-1 expression were assessed via flow cytometric and quantitative real-time PCR analyses, respectively (Fig. 4-1). HSCs were either maintained in SCF-supplemented media or were washed and cultured in DC-differentiating media. CCR5 and GATA-1 expression levels were evaluated throughout 6-day culture periods. Human HSCs express high levels of GATA-1 while only minimally expressing CCR5 (Fig. 4-1). Intriguingly, as HSCs were driven to differentiated into DCs, GATA-1 expression was decreased while CCR5 expression levels was greatly increased (Fig. 4-1). These findings suggest that GATA-1 may physiologically repress CCR5 gene expression in bone marrow precursor cells that are potential targets of HIV-1 infection.

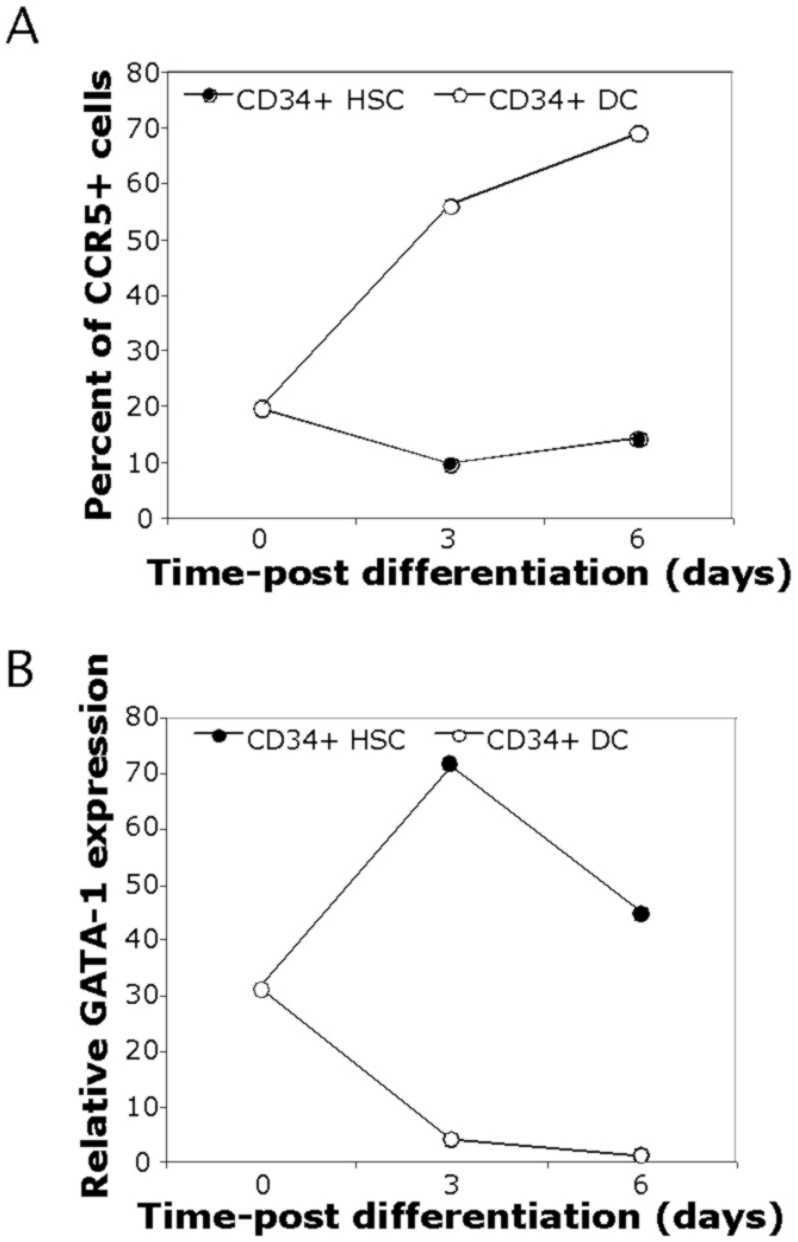

Figure 4-1. Endogenous GATA-1 expression is inversely related with CCR5 expression in human CD34$^+$ hematopoietic stem cells upon DC differentiation.
Purified and expanded primary human HSCs were cultured in stem cell media or DC differentiating media (see methods) for 6 days and (A) cell surface CCR5 expression was assessed every 3 days via flow cytometry or (B) endogenous GATA-1 mRNA levels were determined via quantitative real-time PCR. These data represent 2 experiments performed using stem cells isolated from independent umbilical cord blood samples.

Expression of GATA-1 in primary cellular targets of HIV potently reduces CCR5 expression

In order to directly test if GATA-1 can regulate CCR5 expression we ectopically expressed either GATA-1 or GATA-3 in either stem cell-derived dendritic cells or T cells. For these experiments, we first generated stem cell-derived DCs as described above and transduced with lentiviral vectors encoding GATA-1 (HDV.GATA-1) or GATA-3 (HDV.GATA-3). To express GATA transcription factors in T cells subsets, $CD4^+$ T cells were first isolated from healthy individuals. These cells were further fractionated into naïve (T_N - $CD45RO^-$ $CCR7^+$), central memory (T_{CM} - $CD45RO^+$ $CCR7^+$) and effector memory (T_{EM} - $CD45RO^+$ $CCR7^-$) subsets, since these cells differ in their CCR5 expression and effector functions (44). In addition, iNKT cells, which have been shown to express high levels of CCR5 and be highly susceptible to HIV-1 infection (246, 253-255) were also prepared as previously described (246). Once isolated, all T cell subsets were TCR-activated and simultaneously transduced to express HDV, HDV GATA-3 or HDV.GATA-1 that bicistronically expresses a reporter gene, mCD24, which is expressed on the cell surface of transduced cells. Transduced T cells were expanded for 8-10 days in IL-2-supplemented media and CCR5 expression on $mCD24^+$ T cells and DCs was determined via flow cytometry.

Ectopic expression of GATA-1 or GATA-3 in transduced DCs and T cells was confirmed at the mRNA level with real-time PCR analysis (data not shown) and protein expression levels were determined by western blot analyses on primary human T cells transduced with HDV.GATA-1, HDV.GATA-3 or the control HDV (Fig. 4-2). Expression of GATA-1, but not GATA-3 profoundly

suppressed CCR5 expression in both dendritic cells (Fig. 4-3A) and effector T cell subsets (Fig. 4-3B). Furthermore, GATA-1-mediated CCR5 repression was stable as it was maintained 7-12 days post transduction (data not shown). On the other hand, GATA-3-expressing cells only displayed modest reductions in CCR5 levels (Fig. 4-3). Together these key findings demonstrate GATA-1 is a potent repressor of CCR5 expression in DCs, conventional Th cell subsets and iNKT cells, all of which are targets of HIV-1 infection in vivo. Moreover, the differential regulation of CCR5 by GATA-1 and GATA-3 strongly suggest that the highly conserved zinc-finger domain is not sufficient to suppress CCR5 expression.

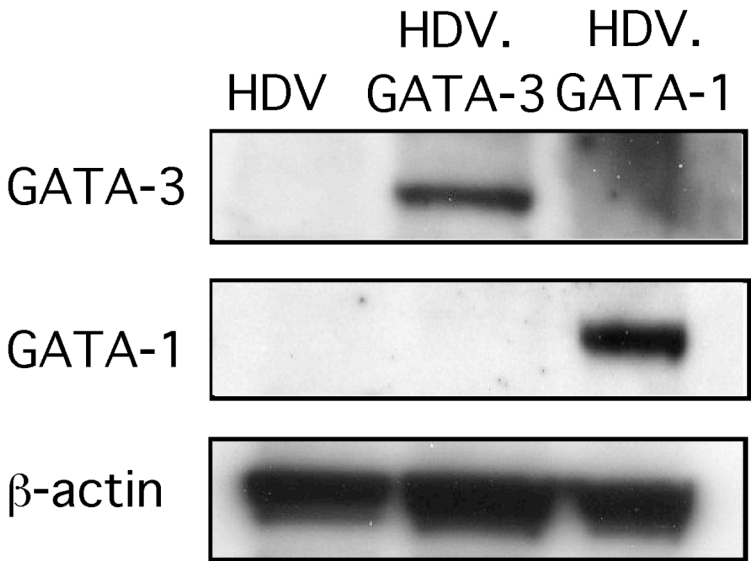

Figure 4-2. Expression of GATA-3 and GATA-1 in transduced cells.
Purified human Th cells were activated and transduced with either HDV, HDV.GATA-3 or HDV.GATA-1 viruses (see methods). Cells were expanded for 8 days in IL-2-suplemented media and magnetically sorted for mCD24 expression. Sorted cells were lysed and western blot analyses were performed for GATA-3 (upper panel), GATA-1 (middle panel) and β-actin (lower panel) expression using specific antibodies. The membranes were stripped following each blot and re-probed in the order indicated above. These data are representative of two separate western blot experiments using transduced cells obtained from different donors.

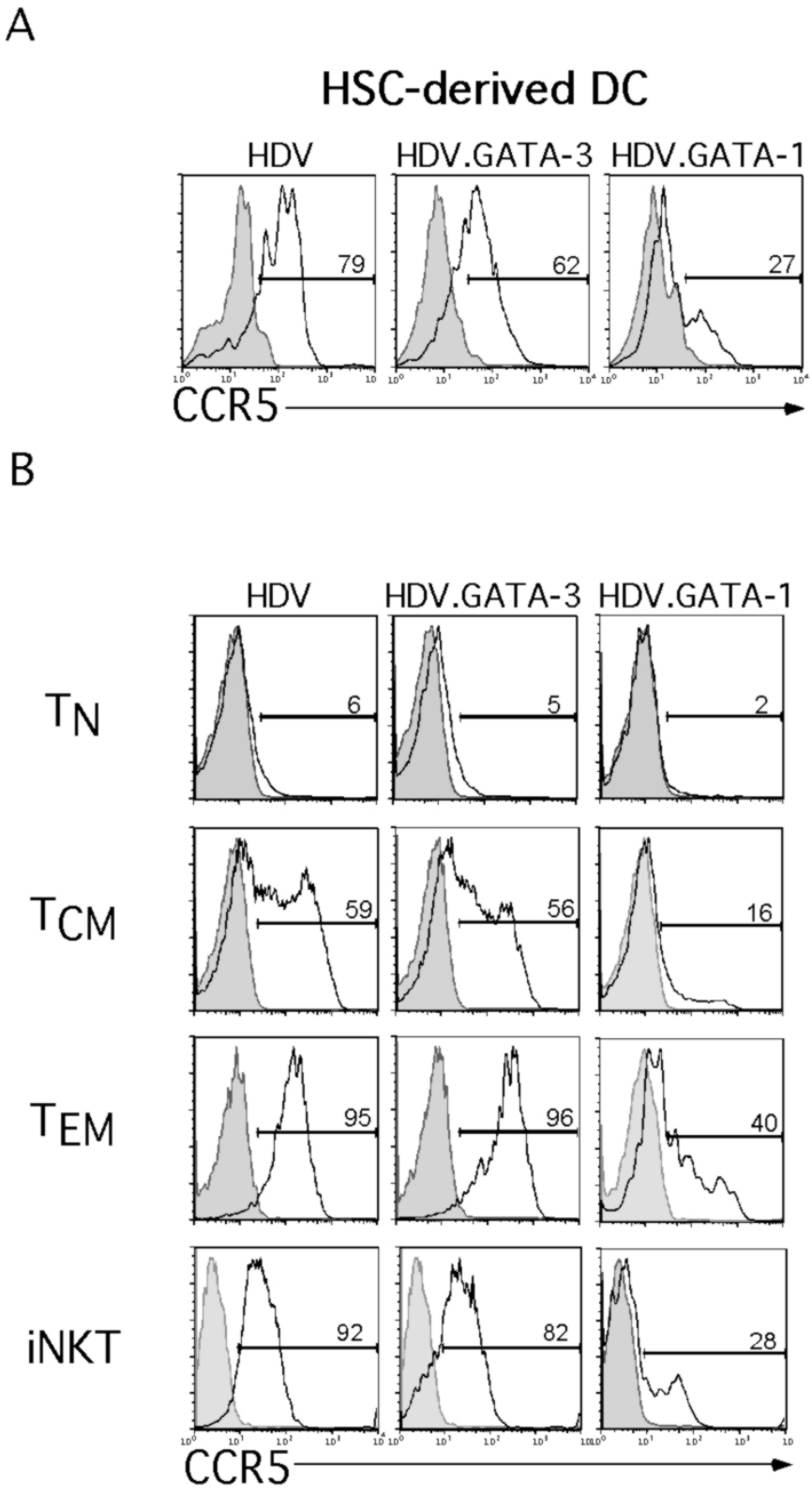

Figure 4-3. Ectopic expression of GATA-1 inhibits CCR5 expression in human target cells of HIV-1.

Figure 4-3 continued.
(A) Primary human HSCs were cultured in DC media for 4 days to drive differentiation (see methods). At day 4, DCs were transduced with HDV, HDV.GATA-1 or HDV.GATA-3 and cultured for an additional 4 days. CCR5 expression on stem cell-derived DCs was determined via flow cytometric analyses by co-staining cells with anti-CCR5, a PE-conjugated anti-human CD1c antibody (Miltenyi Biotech) to monitor DC differentiation and anti-mCD24 antibodies to resolve DCs expressing the lentiviral transgene. (B) Primary human conventional Th cell subsets (upper 3 panels) or iNKT cells (bottom panel) were TCR-activated and transduced to express GATA-1, GATA-3 or the control HDV (see methods). T cell subsets were all expanded in the presence of IL-2 and cell-surface CCR5 expression was determined on unsorted populations of transduced cells and co-stained with anti-mCD24 antibodies as above. Additionally, CCR5 expression on iNKT cells were further stained with a FITC-conjugated anti-human Vβ11 antibody (Coulter), to exclude any contaminating non-iNKT cells. These results are representative of at least 3 experiments performed on each cell type from unique adult or umbilical cord blood preparations.

GATA-1 expression in human T cells inhibits R5-tropic HIV-1 infection.

CCR5 cell-surface expression levels closely correlates with susceptibility to HIV-1 infection in vitro (256). In vivo, allelic or mutational variants of CCR5 that alter cell-surface expression of the chemokine receptor can influence the rate of HIV-1 pathogenesis (213-217). In light of our finding that GATA-1 mediates potent CCR5 down-regulation in HIV-1 targets, we assessed if GATA-1-mediated down-regulation of CCR5 renders Th cells less susceptible to HIV-1 infection. To test this, TCR-activated primary human Th cells were transduced to express GATA-1, GATA-3 or the control HDV and expanded for 7-8 days. The transduced cells were then purified by sorting for mCD24-expressing cells, and infected with either replication-competent HIV-1 that use CCR5 as a co-receptor (R5.HIV) or replication-defective HIV-1 pseudo-typed with the envelope glycoprotein of the vesicular stomatitis virus (VSV-G.HIV) that bypasses the CCR5 requirement for viral entry. HIV-1 infection and replication within Th cells were assessed over the course of nine-day cultures as described (246). Th cells expressing the control HDV and HDV.GATA-3 were readily susceptible to R5.HIV infection, thus virus replicated and spread efficiently within the culture (Fig. 4-4A). In contrast, GATA-1-expressing Th cells displayed reduced initial R5.HIV infection and subsequent spread of infection and viral replication was markedly diminished in these cultures (Fig. 4-4A). GATA-1-expressing cells were, however, highly susceptible to infection with VSV-G.HIV (Fig. 4-4B), suggesting the limitation in R5.HIV infection of Th cell expressing GATA-1 is specifically due to reduced expression of CCR5 levels.

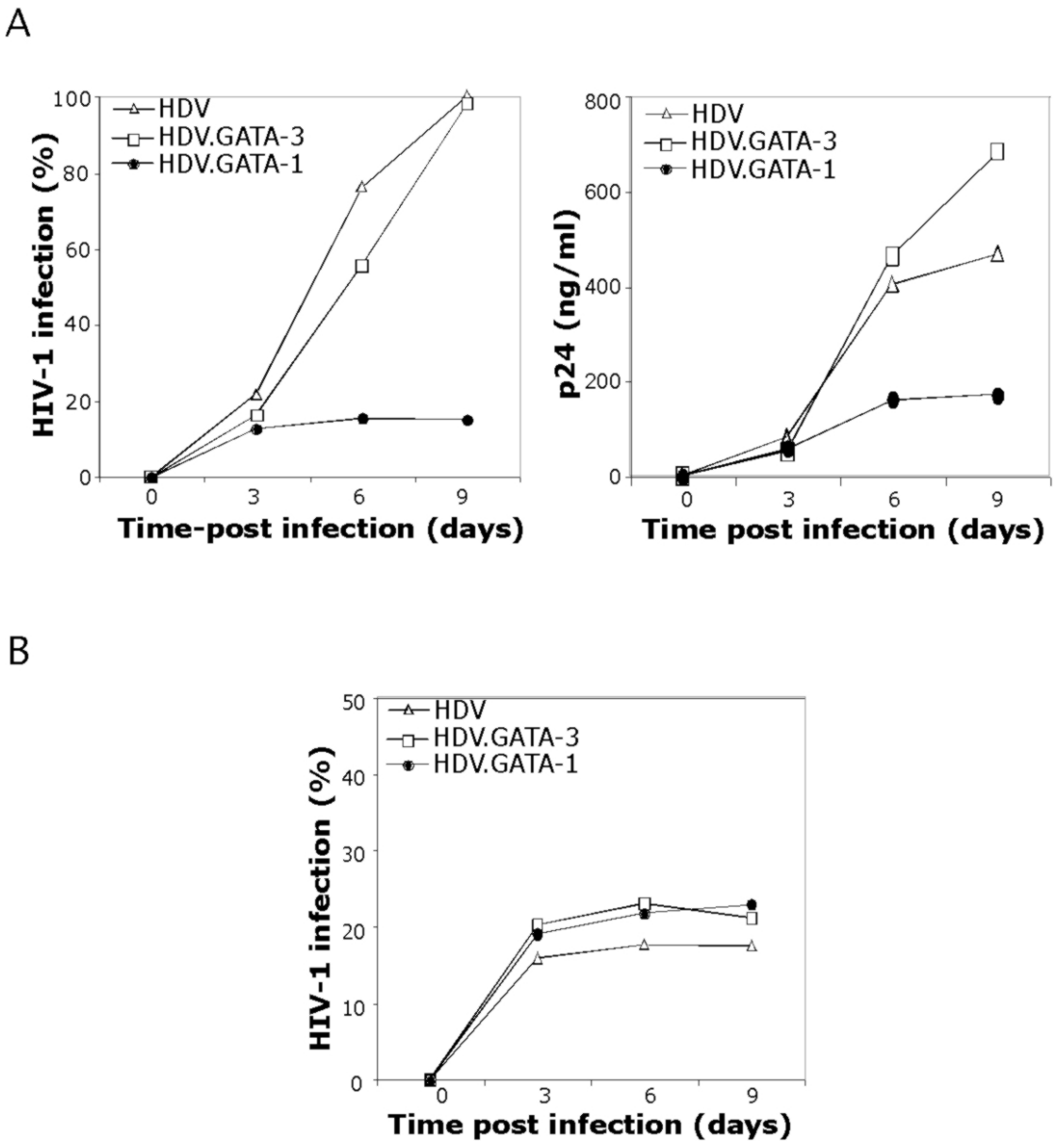

Figure 4-4. GATA-1-induced CCR5 down-regulation inhibits R5-tropic HIV-1 infection of human Th cells.

Th cells expressing GATA-3 or GATA-1 were prepared as described in the figure 1 legend. $mCD24^{+}$ cells were sorted and super-infected with either (A) replication-competent CCR5-tropic HIV-1 (R5.HIV) or with (B) replication-incompetent VSV-G-pseudotyped HIV-1 (VSV-G.HIV) at MOI of 1. Cultures were maintained for 9 days and infected cells were analyzed every 3 days for GFP expression (left panel). Infections were normalized to the maximum infection of HDV-expressing T cells at day 9 (~8%). Culture supernatants were also collected at the indicated times post infection with R5.HIV and viral replication was quantified via p24 ELISA (right panel). These data are representative of three independent experiments performed using T cells isolated from individual donors.

Expression of GATA-1 in primary human T cells potently suppress CCR5 promoter activity

Because GATA-1-mediated CCR5 repression was remarkably similar across multiple cell types, we next asked how GATA-1 inhibits CCR5 cell-surface expression. We hypothesized that GATA-1 negatively regulates CCR5 gene expression. To test this possibility, we determined CCR5 mRNA levels by quantitative real-time PCR in human Th cells isolated from healthy donors. Th cells were TCR-activated via cross-linking antibody stimulation and transduced with HDV, HDV.GATA-3 or HDV.GATA-1. Cells were expanded and cell-surface CCR5 down-regulation in GATA-1-expressing Th cells was confirmed by flow cytometric analysis (Fig. 4-5A). Transduced cells were then sorted based on mCD24 expression and either left un-stimulated in IL-2 media or were re-stimulated through the TCR for 18-24 hours. Cells were lysed and real-time PCR analysis of CCR5 mRNA levels was performed. GATA-1-expressing Th cells displayed approximately 10-fold less CCR5 transcript levels compared to those expressing the control HDV (Fig. 4-5B). By contrast, the reduction in CCR5 mRNA was only 2-fold lower in GATA-3-expressing cells (Fig. 4-5B), findings that were concordant with our previous observations that GATA-3 was much less efficient compared to GATA-1 in reducing CCR5 cell surface expression level.

Because GATA-1-expressing cells had lower RNA and protein expression of CCR5, we next asked if this effect was, in part, mediated by repression of CCR5 promoter activity. To evaluate activity of the CCR5 promoter in primary human Th cells expressing GATA-1 or GATA-3, we generated and sorted cells

transduced with these transcription factors as described above. The cells were re-stimulated for 18-24 hours and transfected with either a construct containing the full-length CCR5 promoter 1 and 2 (Fig. 4-5C, upper panel) driving firefly luciferase expression or a promoterless firefly luciferase construct. Cells were harvested 24 hours post transfection and luciferase activity was analyzed. GATA-1 expression in primary human Th cells significantly reduced CCR5 promoter strength compared to both control and GATA-3-expressing Th cells (Fig. 4-5C, lower panel), with promoter activity displaying levels similar to background. A slight decline in CCR5 promoter activity was observed in the cells expressing GATA-3 compared to those expressing the control HDV (Fig. 4-5C, lower panel), mirroring the modest inhibition of CCR5 message and cell-surface protein levels. These findings indicate that CCR5 promoter activity in primary human Th cells is potently silenced in the presence of GATA-1, whereas only a slight reduction is seen with GATA-3.

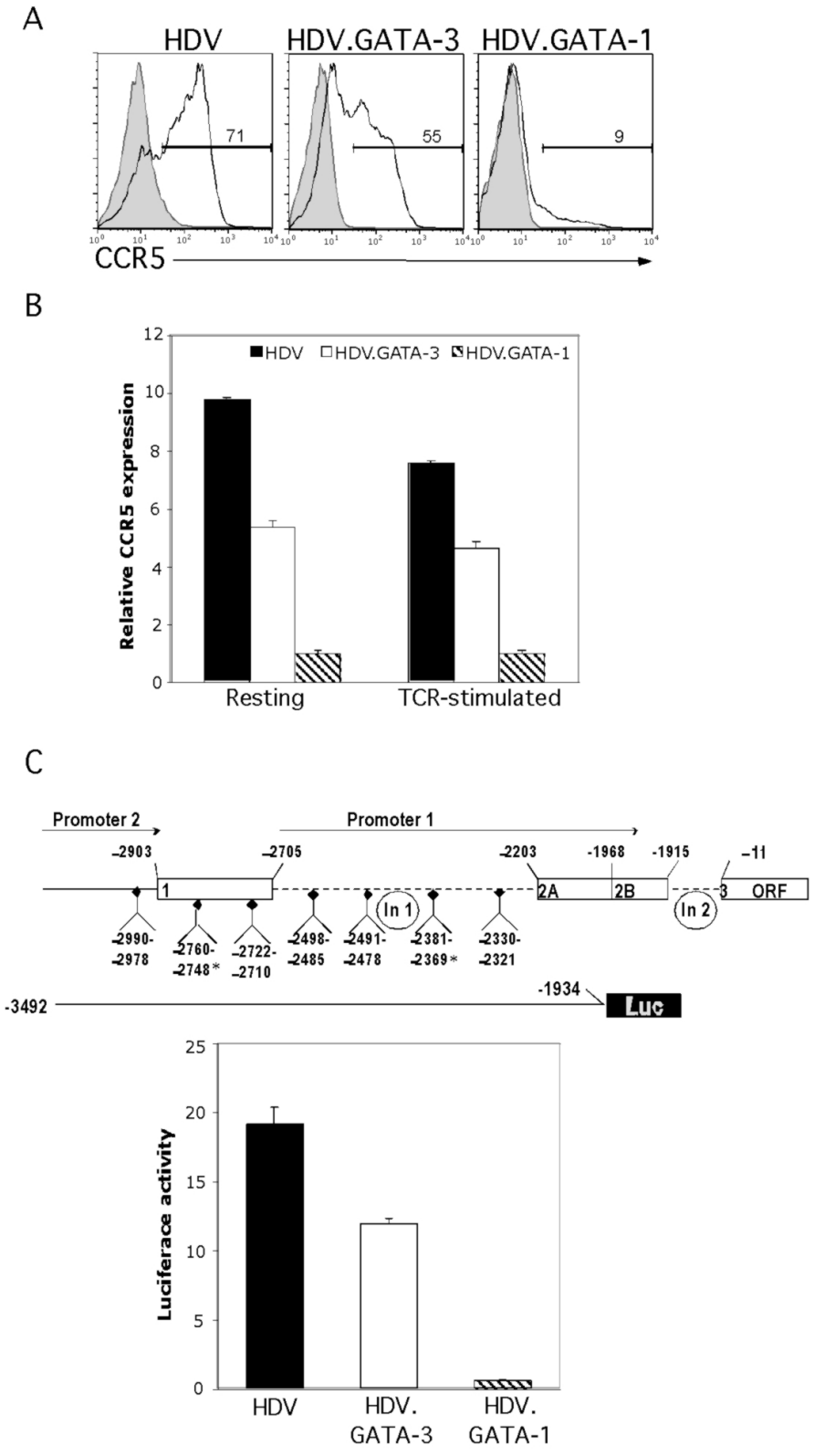

Figure 4-5. GATA-1 expression in primary human Th cells suppresses CCR5 promoter activity.

Figure 4-5 continued.
(A) Total resting $CD4^+$ T cells were TCR-activated and transduced with HDV, HDV.GATA-3 or HDV.GATA-1 as in the figure 1 legend. Transduced cells were expanded for 8-10 days and cell-surface expression of CCR5 was assessed on unsorted cells by staining with either isotype control (gray peaks) or anti-CCR5 (white peaks) antibodies in conjunction with an anti-mCD24 antibody. The histograms are gated on the $mCD24^+$ populations and the percent of CCR5 positive T cells are displayed. The displayed histograms are representative of 6 independent stainings performed on cells isolated from 6 different blood donors. (B) Total $CD4^+$ human T cells expressing HDV, HDV.GATA-1 or HDV.GATA-3 as in the figure 5A were sorted based on mCD24 expression (see methods) and either left un-stimulated (resting) or re-stimulated via anti-CD3 and anti-CD28 cross-linking for 18 hours (re-stimulated). Cells were lysed, cDNA was generated and quantitative real-time PCR was performed using gene-specific primers. Data was analyzed by normalizing CCR5 expression levels to GAPDH levels and are displayed as fold differences in relative expression. These data represent three independent real-time PCR reactions run from cDNA generated from separate sets of cells from different donors. (C) *Top panel* - Genomic organization of CCR5. Exons are represented as numbered white boxes, with exons signified by areas above numbered black boxes. +1 represents the start site of the open reading frame and every number is relative to it. Promoters 1 and 2 are as indicated, with GATA binding sites indicated and numbers represent the start of the binding sites. *Bottom panel* - Human Th cells positively sorted for mCD24 expressing HDV, HDV.GATA-1 or HDV.GATA-3 were re-stimulated through the TCR as in the figure 1B legend. The cells were transfected with either a plasmid containing the full-length CCR5 promoter driving firefly luciferase expression or a promoterless-firefly luciferase plasmid. Cells were co-transfected with a CMV-driven renilla luciferase expression plasmid (see methods). Transfected cells were lysed and luciferase expression was quantified. Firefly luciferase levels were normalized to renilla levels within samples and the data are displayed as relative fold induction of firefly luciferase as driven by CCR5 promoter over the promoterless control vector. These data represent three independent sets of transfections performed on cells from separate donors.

GATA-1 expression in naïve and memory Th cell subsets induces Th2 effector cytokine profiles.

The above results demonstrated remarkably distinct regulation of CCR5 gene expression by GATA-1 and GATA-3. However, GATA-1 and GATA-3 contain a highly conserved zinc-finger domain and that they likely bind to very similar DNA sequences (110, 111). Moreover, previous studies have demonstrated that GATA-3 expression in GATA-1-deficient embryonic stem cells or knock-out mice can rescue hematopoietic deficiencies and embryonic lethality in the place of GATA-1 (243-245), suggesting a high degree of functional redundancy may exist between GATA-1 and GATA-3. Therefore, we sought to determine if the reciprocal was also true. We asked whether GATA-1 could redundantly program Th2 cytokine gene expression in human naïve and memory Th cell subsets similar to GATA-3 (60).

For these experiments, T_N, T_{CM} and T_{EM} cells were isolated as before. These Th cell subsets were then TCR-activated and transduced with HDV.GATA-1, HDV.GATA-3 or the control HDV. The cells were expanded in IL-2-supplemented media for 8-10 days and were probed for intracellular expression of Th1 and Th2 cytokines. Similar to GATA-3, ectopic expression of GATA-1 in both T_{CM} and T_{EM} subsets resulted in a marked up-regulation of IL-4 and IL-13, and to a lesser extent IL-5, compared to memory T cells that only expressed HDV (Fig. 4-6). Interestingly, T_{CM} and T_{EM} cells ectopically expressing GATA-1 produced less IFNγ compared to either control- or GATA-3-expressing memory T cells (Fig. 4-6). Given the similar expression levels of GATA-1 and GATA-3 observed in transduced cells (Fig. 4-2), these data suggest the

quantitative differences in cytokine production are not due to differential expression of the transcription factors. Collectively, these findings indicate that expression of GATA-1 in primary human naïve and memory Th cells promotes the expression of Th2 cytokines and down-regulates IFNγ more effectively than GATA-3.

We further determined if GATA-1-directed Th2 programming was occurring indirectly, through the induction GATA-3 expression. Indeed, a previous report demonstrated that expression of heterologous GATA transcription factors in murine T cells activates GATA-3 expression, thereby promoting Th2 differentiation (257). However, in our studies we did not detect GATA-3 protein expression in human Th cells ectopically expressing GATA-1 (Fig. 4-2 and data not shown). Quantitative real-time PCR analyses comparing GATA-3 expression levels in primary human Th cells expressing GATA-1 to Th1 and Th2 cells corroborated these results. We found high-level GATA-3 mRNA expression was tightly restricted to Th2 cells and GATA-1-expressing cells did not display elevated GATA-3 levels compared to those expressing the control HDV (Fig. 4-7). Moreover, GATA-3 expression levels in cells expressing GATA-1 mirrored those found in Th1 cells and were between 10- to 17-fold lower than those observed in Th2 cells (Fig. 4-7). The failure to detect expression of GATA-3 in GATA-1-expressing cells strongly suggests that GATA-1 directly and redundantly programs Th cell cytokine gene expression similar to GATA-3.

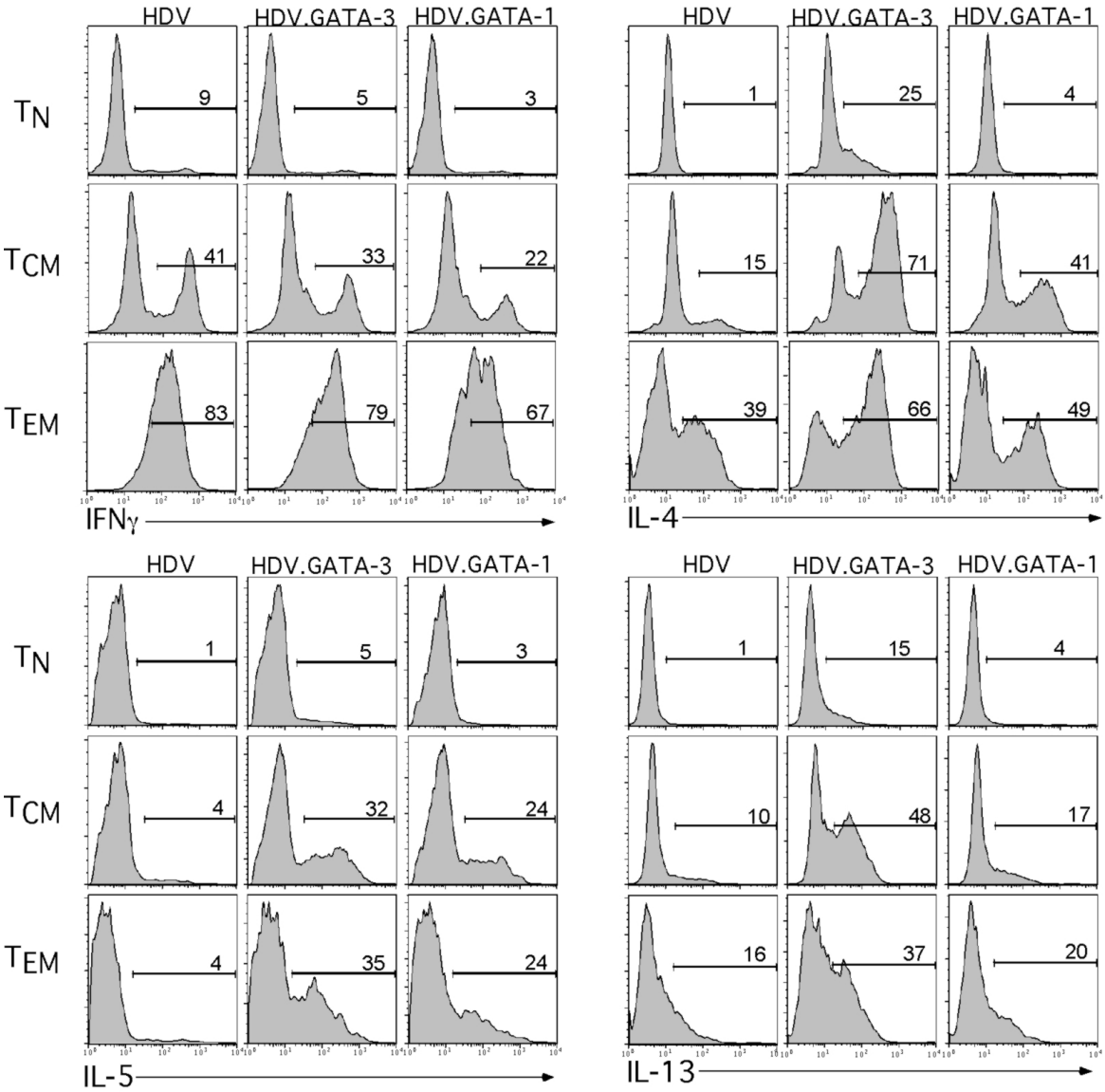

Figure 4-6. Ectopic GATA-1 expression in human T_N, T_{CM} and T_{EM} cells leads to Th2 cytokine profiles.

$CD45RO^-RA^+$ naïve and $CD45RO^+RA^-$ memory T cells were purified from adult blood PBMC of healthy donors via magnetic bead cell sorting (see methods) and were sorted by FACS for expression of CCR7 into T_{CM} ($CCR7^+$) and T_{EM} ($CCR7^-$) cells. The Th cell populations were activated for 48 hours through the TCR via antibody stimulation and infected with viruses containing either HDV, HDV.GATA-1, or HDV.GATA-3 at the time of TCR-activation as described in the materials and methods. After 8-10 days of expansion, intracellular expression of the cytokines IFNγ, IL-4, IL-5 or IL-13 were determined by flow cytometry (see methods). For these experiments transduced cells were left unsorted, but $mCD24^+$ cells were identified and gated on by co-staining with an anti-mCD24 antibody. These data are representative of three separate experiments using memory T cell subsets purified from different adult donors.

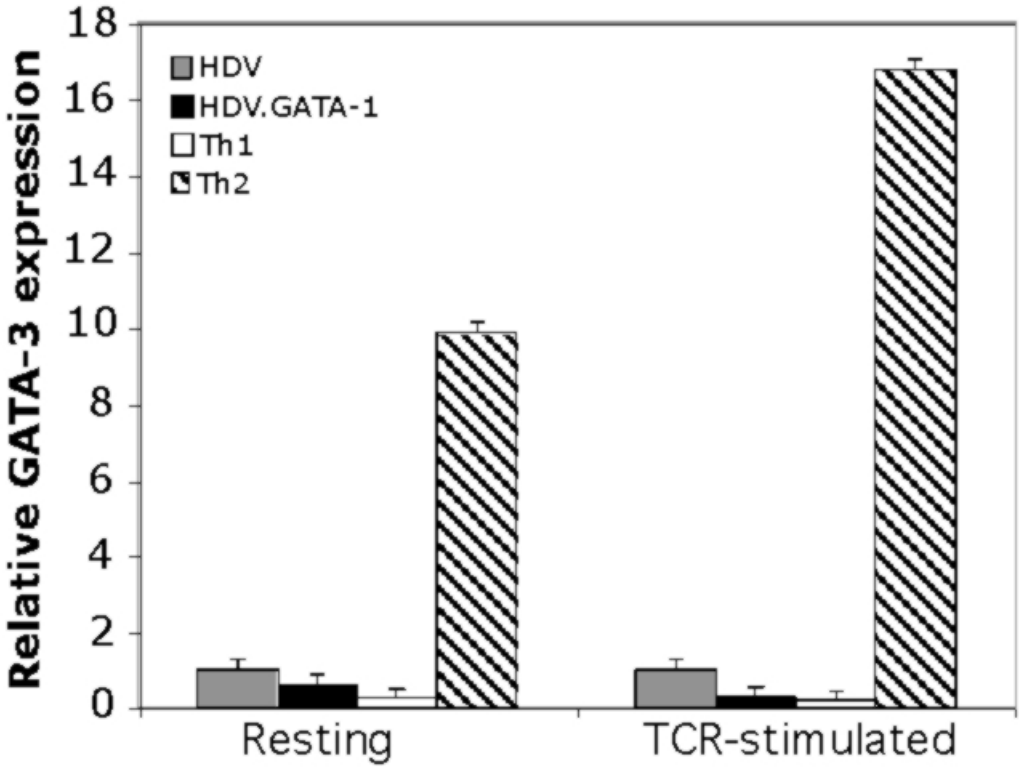

Figure 4-7. Expression of GATA-1 in human Th cells does not induce GATA-3 expression.
Human T_N cells purified from adult blood or neonatal umbilical cord blood were activated through the TCR in Th1 or Th2 cytokine conditions (see methods). Additionally, T_N cells were also activated without polarizing cytokines and infected with viruses containing HDV or HDV.GATA-1 and all cells were expanded for 8-10 days in the presence of IL-2-supplemented media. Transduced cells were sorted for mCD24 expression and cells were then either left un-stimulated (resting) or re-stimulated with anti-CD3 and anti-CD28 antibodies (re-stimulated) for 8 hours. Resting and re-stimulated T cells were harvested and GATA-3 expression was evaluated by quantitative real-time PCR. These data were analyzed by normalizing GATA-3 expression levels to GAPDH levels and are displayed as relative GATA-3 expression (fold differences). These data represent 3 experiments using T_N cells from three independent adult or umbilical cord blood donors.

Expression of GATA-1 in primary human naïve and memory T cells reprograms chemotactic receptor expression patterns to a Th2 profile

We found that expression of GATA-1 in human Th cells could functionally replace GATA-3 to program Th2 cytokine gene expression. However, given the distinct regulation of CCR5 observed between GATA-1 and GATA-3, we next determined if GATA-1 expression in human naïve and memory Th cells also influences the cell-surface expression of Th2-associated chemotactic receptors analogous to GATA-3 (60).

Human Th cell subsets were TCR-activated and transduced with HDV.GATA-1, HDV.GATA-3 or the control HDV and chemotactic receptor expression profiles were determined. Similar to GATA-3 expression in all three Th cell subsets, expression of GATA-1 was associated with induction of the Th2 chemotactic receptors CCR4 and CRTH2 (Fig 4-8). However, expression of GATA-3 in memory T cell subsets failed to suppress expression of the Th1-biased chemokine receptor CXCR3, whereas GATA-1 expression reduced CXCR3 cell-surface expression in T_{CM} and T_{EM} cells (Fig. 4-8). These results provide further evidence that GATA-1 and GATA-3 can induce both Th2 effector function and cell migration patterns by influencing the expression of chemotactic receptors. However, the effects of GATA-3 and GATA-1 are not completely redundant as GATA-1 is a significantly more potent repressor of Th1 chemokine receptors and effector molecules relative to GATA-3 (Table 4-1).

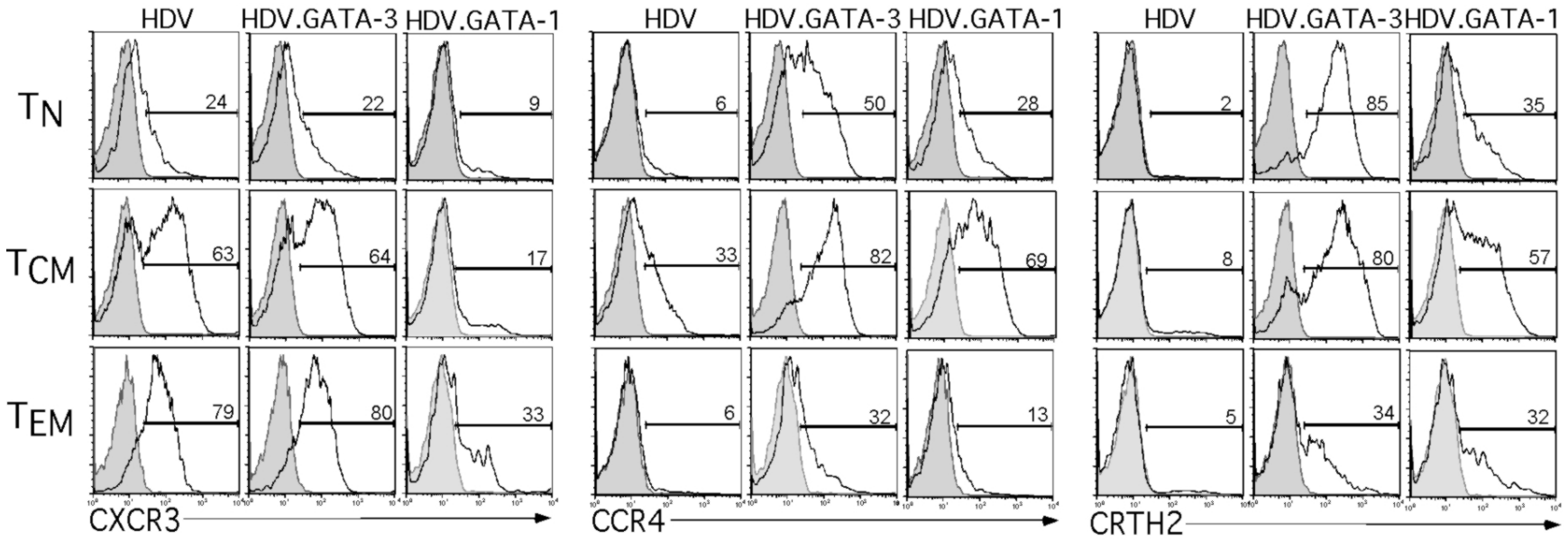

Figure 4-8. Expression of GATA-1 in human T_N, T_{CM} and T_{EM} cell subsets induces Th2- and potently inhibits Th1-associated chemokine receptors.

Human T_N, T_{CM} and T_{EM} cells were purified, activated, and transduced with HDV alone, HDV.GATA-1, or HDV.GATA-3 (see methods). Following expansion, unsorted cells were stained with antibodies against CXCR3, CCR5, CCR4, CRTH2 or the appropriate isotype control in conjunction with an anti-mCD24 antibody to gate on transduced populations. The percent of cells positive for the appropriate chemokine receptor is shown, with the gray, tinted peak representing isotype control staining. Results are representative of one of three experiments using T cell subsets from distinct adult donors.

Table 4-1: GATA-1-mediated repression of Th1 effector molecules.

Th1 cells expressing	Effector molecules		
	IFNγ*	CXCR3**	CCR5***
HDV	64.7 +/- 38.9	280.6 +/- 49.8	314.2 +/- 95.2
HDV.GATA-3	55.0 +/- 36.3	214.1 +/- 10.9	321.5 +/- 64.5
HDV.GATA-1	30.6 +/- 21.9+	118.6 +/- 5.3+	126.9 +/- 35.6+

* Concentration of IFNγ (pg/ml) secreted 18 hours-post TCR stimulation as determined by CBA

** Mean fluorescent intensity (MFI) of CXCR3 cell surface expression as determined by FACS analysis

*** Mean fluorescent intensity (MFI) of CCR5 cell surface expression as determined by FACS analysis

\+ Significantly lower than Th1-HDV (p=<.05) and Th1-HDV.GATA-3 (p=<.05)

Table 4-2: Summary of GATA-1 and GATA-3 effects on gene expression in T cells.

	INDUCTION OF Th2 GENES					SUPPRESSION OF Th1 GENES		
	IL-4	IL-5	IL-13	CCR4	CRTH2	IFNγ	CXCR3	CCR5
GATA-3	++++	++++	+++	+++	+++++	+	+/-	+/-
GATA-1	++	+++	++	+++	++++	+++	++++	++++

Each + represents 20% change in T cell subsets expressing the indicated protein as monitored by flow cytometry compared to HDV-expressing T cells.

Discussion

Despite its critical importance in the pathogenesis of HIV-1, there is a significant knowledge gap in our understanding of the molecular determinants that control CCR5 expression in the physiologic cellular targets of HIV-1. By concurrently comparing the effects of GATA-3 and GATA-1 on CCR5 expression, we demonstrate that these two TFs mediate markedly distinct regulatory effects on CCR5 expression, but also display overlapping functions in the regulation of Th1 and Th2 gene expression during T cell differentiation (summarized in Table 4-2).

Given that GATA-3 is highly expressed on Th2 cells, and that these cells display lower levels of CCR5, we had hypothesized that ectopic expression of GATA-3 would suppress CCR5 expression levels. However, this was not the case, and by contrast, GATA-1 potently repressed CCR5 expression at both the protein and transcript levels in primary human T cell subsets and DCs. These effects are, in part, mediated by the inhibition of *CCR5* transcription, and support the notion that GATA TF binding to their cognate *cis*-elements within the *CCR5* promoter is functional (Fig. 4C and refs (129, 130)). Interestingly, GATA-1 was previously shown to *trans*-activate *CCR5* promoter activity in a transformed T-lymphoid cell line (223). However, our results clearly demonstrate that GATA-1 is a potent repressor of CCR5 expression in primary human T cells and dendritic cells. This discrepancy in *CCR5* regulation by GATA-1 in primary versus transformed cell lines may reflect unique combinations or levels of TFs present within constitutively proliferating cells that do not faithfully mimic those in

physiological primary cells. Substantiating this possibility, we recently found that the *CCR5* promoter 2 (258) is highly active in primary cells compared to cell lines that serve as surrogates for T cells (e.g. Jurkat) (Mummidi et al, manuscript in preparation). Moreover, the differential effects of GATA-1 in T cell lines and primary T cells may also be attributed to other differences in these cell types, such as cell-type specific differences in the epigenetic architecture of the *CCR5* locus.

The precise mechanism by which GATA-1 represses *CCR5* gene transcription is not yet clear, and two possible mechanisms can be envisaged. First, GATA-1 might influence *CCR5* transcription independently or, as reported previously in other gene systems, in concert with other TFs that bind to *cis*-elements within the *CCR5* promoter (129, 130, 236, 259). Second, GATA-1 may epigenetically remodel the *CCR5* promoter. This possibility is based on the finding that GATA-1-responsive loci are epigenetically modified during hematopoiesis (220). If, in fact, GATA-1 restructures chromatin within the *CCR5* locus, this could cause a transcriptionally silent imprint in HSCs during hematopoiesis that may be maintained in developing T cells. Indeed, T_N cells that develop in the thymus and are maintained in the periphery do not express CCR5 and require extracellular activation signals to induce its expression (208). Whether GATA-1 can regulate an epigenetically silent imprint at the *CCR5* promoter in hematopoietic cell lineages warrants further investigation.

It is likely that the functional correlate of reduced CCR5 expression mediated by GATA-1, namely the inhibition of R5-tropic HIV-1 infection and viral

replication in T cells, will be evident in other HIV-1 target cells. However, the expression of GATA-1 is highly restricted during hematopoiesis and is not physiologically expressed in peripheral T cells, iNKT cells or DCs (data not shown). It is conceivable, however, that GATA-1 expression may be transiently induced in these cell types by specific activation signals, which could then influence the expression of CCR5. In a more likely scenario, GATA-1, which is clearly expressed in HSCs and is silenced upon their differentiation to CCR5-expressing DCs (Fig. 1), may play a physiologic role in modulating HIV-1 susceptibility of these cell types during hematopoietic development, as well as mast cell progenitors, which are all potential targets of viral infection in vivo (248, 260, 261). A better understanding of GATA-1-mediated CCR5 repression opens the door to exciting new therapeutic approaches to down-regulate CCR5 in HIV-1 infected individuals.

In addition to the CCR5 inhibitory activity, ectopic expression of GATA-1 was more potent at down-regulating IFNγ and CXCR3 expression compared to GATA-3, even in lineage-committed T_{EM} cells. These findings highlight a remarkable ability to reprogram the expression of Th1 cytokines and chemokine receptors in human T_{EM} cells, which are relevant in the context of T cell-mediated pathologies such as in autoimmune diseases and allograft rejection (87). Mechanistically, these findings suggest that the divergent N- and C-terminal portions of GATA-1 and GATA-3 differentially influence the expression of Th1-associated cytokines and chemokine receptors. We speculate that three potential mechanisms may explain the differential activity of GATA-1 as compared to

GATA-3. First, the non-conserved *trans*-activation domains of GATA-1 may serve as additional protein interaction interfaces (262), which could allow it to selectively recruit repressive co-factors to the *IFNγ* and *CXCR3* promoters. Second, the TF friend of GATA (FOG-1), which can interact with both GATA-1 and GATA-3, might differentially regulate their function. Whereas FOG-1 interaction with GATA-1 is required for its activity during erythropoiesis and megakaryopoiesis (117), it has been shown to inhibit the function of GATA-3 during T cell differentiation (120). Therefore, the interaction of these GATA TFs with FOG-1 may impart differential regulation of Th1-associated gene expression. Finally, It is also plausible that GATA-1 and GATA-3 may be differentially phosphorylated, acetylated or methylated within these *trans*-activation domains, resulting in unique regulation of Th1 gene expression (123, 263, 264). Future structure/function studies utilizing chimeric GATA TFs may be useful to uncover specific GATA regulatory domains and auxiliary transcription factors that determine the expression of Th1 chemokine receptors and cytokines.

While ectopic expression of GATA-1 is much more potent than GATA-3 in repressing the expression of CCR5 and other Th1 effector molecules, it is highly similar to GATA-3 in inducing Th2 cytokines (IL-4, IL-5, IL-13) and chemotactic receptors (CCR4 and CRTH2) (Table 4-2). In contrast to a previous study performed in murine T cells (257), we found that the regulation of Th2 cytokines and chemokine receptors was a direct function of GATA-1, occurring without *trans*-activation of GATA-3 expression. Thus, it seems likely that GATA-3 expression is differentially regulated in mouse and human T cells. Our findings

strongly suggest that the conserved DNA binding zinc-finger domain shared between these GATA TFs is largely sufficient to program Th2 effector functions and lymphoid homing propensities.

In summary, we have discovered that GATA-1 is a potent repressor of CCR5 expression in multiple human cell types that are physiologically targets of HIV-1 in vivo. Decoding the mechanisms underlying the functional similarities and differences between GATA-1 and GATA-3 in programming human Th2 differentiation and repressing CCR5 expression have implications in understanding the molecular regulation of *CCR5* expression, as well as other loci that contain GATA-responsive *cis*-sites. Importantly, these findings raise the unique possibility of harnessing the mechanisms by which GATA-1 mediates the repression of CCR5 as a therapeutic modality to render human T cells refractory to HIV-1 infection.

CHAPTER V

INHIBITION OF ACTIVATION-INDUCED T CELL PROLIFERATION BY HELICOBACTER PYLORI VACUOLATING TOXIN IS INDEPENDENT OF EFFECTS ON IL-2 SECRETION

Abstract

Recent evidence indicates that the secreted *Helicobacter pylori* VacA toxin inhibits the activation of T cells. VacA blocks IL-2 secretion in transformed T cell lines through the suppression of NFAT activation. In this study we investigated the effects of VacA on primary human $CD4^+$ T cells. VacA treatment of primary human T cells activated through the T cell receptor and CD28 resulted in potent inhibition of proliferation, without inhibiting IL-2 secretion or NFAT activation. VacA suppressed IL-2-induced cell-cycle progression and proliferation of primary human T cells, but did not affect IL-2-dependent survival. Through the analysis of a panel of mutant VacA proteins, we demonstrate that VacA-mediated inhibition of T cell proliferation requires an intact amino-terminal hydrophobic region necessary for the formation of anion-selective membrane channels. Remarkably, we demonstrate that one of these mutant VacA proteins (VacA-Δ6-27) abrogates the immunosuppressive actions of wild-type VacA in a dominant negative fashion. These results reveal that VacA inhibits the proliferation of primary human T cells by a mechanism distinct from that described in transformed T cell lines. Our findings suggest that *H. pylori* may utilize this mechanism to inhibit

clonal expansion of T cells that have already been activated by *H. pylori* antigens, thereby evading the adaptive immune response and establishing chronic infection.

Introduction

Helicobacter pylori is a gram-negative, spiral-shaped, microaerophilic bacterium that colonizes the gastric mucosa of more than 50% of the human population (131, 132). *H. pylori* colonization can persist for decades in the absence of antibiotic treatment. Furthermore, infection with this bacterium is consistently associated with gastric mucosal inflammation, and is a risk factor for the development of peptic ulcer disease, distal gastric adenocarcinoma, and gastric lymphoma (131, 132).

Most *H. pylori* strains secrete a vacuolating cytotoxin (VacA) into the extracellular space (265, 266). Epidemiological studies and experiments using animal models have suggested that VacA is an important *H. pylori* virulence factor in the pathogenesis of peptic ulceration and gastric cancer (267-271). Incubation of VacA with cultured mammalian cells has been shown to induce formation of intracellular vacuoles, depolarization of the cellular membrane potential, permeabilization of epithelial monolayers, apoptosis, detachment of epithelial cells from the basement membrane, and interference with the process of class II antigen presentation (265, 266). Many of these effects are dependent on the capacity of VacA to form anion-selective membrane channels (272-276).

VacA has also been reported to alter the expression of syntaxin 7 (277), and to induce the activation of p38-mediated signaling pathways (137, 278).

H. pylori persists in the human gastric mucosa for decades despite the development of gastric mucosal inflammation and specific antibody production. Several lines of evidence indicate that $CD4^+$ T cells are critical for protection against *H. pylori* colonization (133, 135, 279, 280). Thus, it seems possible that immune evasion strategies of *H. pylori* may involve the inhibition or modulation of T cell immunity. Indeed, two reports have recently demonstrated that VacA inhibits activation of Jurkat T cells (a human T cell lymphoma/leukemia cell line) as well as human peripheral blood lymphocytes (136, 137). Studies of Jurkat T cells indicate that VacA blocks TCR-mediated activation of the nuclear factor of activated T cells (NFAT), a key transcription factor required for optimal T cell activation (136, 137). The process by which VacA inhibits NFAT activation in Jurkat T cells is reportedly similar to the actions of the immunosuppressive drugs cyclosporine A and FK-506, which inactivate the NFAT phosphatase calcineurin (136). However, the process by which VacA inhibits activation of primary human $CD4^+$ T cells has not yet been studied in any detail.

In this report, we show that VacA inhibits the proliferation of primary human $CD4^+$ T cells without affecting IL-2 expression or NFAT activation. In addition, we show that VacA suppresses IL-2-induced cell cycle progression without affecting IL-2-dependent survival. We also show that VacA-mediated inhibition of primary T cell proliferation is dependent on an intact VacA amino-terminal hydrophobic domain required for membrane channel formation, and that

a mutant toxin lacking this domain blocks the T cell-suppressive action of wild-type VacA in a dominant negative manner. These findings indicate that VacA can inhibit proliferation of primary human T cells by a mechanism distinct from that described in transformed T cell lines.

Materials and Methods

Purification of VacA

H. pylori strains (wild-type strain 60190 and isogenic mutant strains) were grown as described (273, 275). Oligomeric forms of VacA were purified from broth culture supernatants of *H. pylori* as described (281). All experiments were performed using acid-activated preparations of VacA or acidified buffer control (PBS), unless stated otherwise. The final VacA concentration was 10 μg/ml for all the experiments, unless stated otherwise. For the dominant-negative assays, wild-type VacA was mixed with varying concentrations of VacA mutant toxins and the mixtures were acid-activated prior to addition of these samples to cells (275).

Primary human T cell purification and Carboxy Fluorescein Diacetate Succinimide Ester (CFSE) labeling

Resting $CD4^+$ human T cells were purified from healthy adult donors as previously described (60). The purified cells were 99% $CD3^+CD4^+$ as assessed by staining and flow cytometric analysis. Cell proliferation was monitored by labeling T cells with 5 μM CFSE (Molecular Probes) before stimulation with α-CD3/α-CD28 antibodies.

Primary human T cell purification and activation

Activation of T cells was accomplished using anti-CD3 (OKT3, ATCC) and anti-CD28 antibodies (BD Biosciences) (hereafter termed TCR stimulation) as described (60). Cells were removed from the activation signals after 48 hours and expanded in media supplemented with recombinant human IL-2 (Chiron, 200 U/ml). Activated T cells were cultured as described previously (60). Jurkat T cells were TCR/CD28-stimulated as described above, or with phorbol myristate acetate (PMA, 50 ng/ml; Sigma) and ionomycin (500 ng/ml; Sigma), and maintained in RPMI media containing 10% FCS. To inhibit TCR/CD28 stimulation, T cells were treated with the following immunosuppressive drugs: cyclosporine A (50 nM, Alexis Biochemicals), FK506 (100 nM, Alexis Biochemicals), or rapamycin (200 ng/ml, Alexis Biochemicals).

FACS Analysis and IL-2 detection

IL-2 receptor (CD25) surface expression was detected by staining with phycoerythrin (PE)-conjugated anti-human CD25 (BD Biosciences) as described previously (60). IL-2 secretion into culture supernatants was determined using cytometric bead array (CBA) according to the manufacturer's instructions (BD Biosciences), and analyzed using CBA 6-bead analysis software (BD Biosciences). Samples were analyzed on a FACSCalibur® four color cytometer, using the CellQuest program. Live cells were gated based on forward and side scatter properties and analysis was performed using Cellquest software (BD Biosciences) as described previously (60).

Generation and use of NFAT reporter primary human T cells

To generate primary human $CD4^+$ T cells that expressed an NFAT transcriptional reporter, three tandem copies of the NFAT binding site of the IL-2 promoter were sub-cloned upstream of the EGFP gene (Clontech) to direct its transcription (282). This NFAT-GFP expression cassette was cloned into a lentiviral vector in reverse orientation and VSV-G pseudotyped viruses were generated via co-transfection of 293 T cells as described previously (60). Activated primary $CD4^+$ T cells were transduced with these pseudotyped viruses at a suboptimal MOI. After 7 days, cells constitutively expressing GFP were removed by FACS sorting and GFP-negative cells were stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours. NFAT-GFP T cells that up-regulated GFP upon activation were positively sorted by flow cytometry and further expanded in IL-2-supplemented media. Following enrichment, ~10% of the NFAT-GFP T cell population displayed GFP-inducible expression upon TCR stimulation.

Cell cycle analysis of T cell proliferation

Activated T cells were washed to remove exogenous IL-2 and maintained in IL-2 free medium for 24 hours to synchronize cells at phase G1 of the cell cycle. During this period, the cells were treated with VacA or other additives. Following incubation in IL-2-containing medium for the indicated time, cells were subjected to propidium iodide (PI) staining and analyzed by flow cytometry as described previously (283). To evaluate DNA replication, T cells were washed to remove IL-2, and treated with VacA. Following VacA treatment, cells were incubated for 24

hours in IL-2 medium. 10μM 5-bromo-2-deoxyuridine (BrdU) was added to the cultures, and cells were harvested at different time points for BrdU antibody staining with a commercially available kit (BD biosciences).

Results

VacA inhibits activation-induced proliferation of primary human $CD4^+$ T cells

Previous reports have indicated that VacA inhibits T cell activation (136, 137). These studies clearly demonstrated that the suppressive effect of VacA on a transformed T cell line (Jurkat T cells) is due to interference with NFAT activation, resulting in the inhibition of IL-2 secretion. While several experiments were performed on unfractionated peripheral blood leukocytes, the mode of VacA action on purified primary T cells was not assessed (136, 137). To investigate whether VacA inhibits the activation of primary T cells in a manner similar to its effect on transformed cells, we purified $CD4^+$ primary human T helper (Th) cells from peripheral blood mononuclear cells (PBMC) of healthy individuals and labeled them with CFSE, a cell-permeable dye that allows for the quantification of cell division within a population. CFSE-labeled Th cells were pre-treated with media, PBS, or VacA, followed by TCR stimulation using anti-CD3 and anti-CD28 antibodies for 48 hours. After TCR stimulation, cells were expanded in IL-2-containing media for an additional 3 days. As expected, Th cells pre-treated with media or PBS rapidly proliferated, resulting in up to 7 divisions when analyzed at 5 days post TCR-activation (Fig.5-1A and 5-1B). In contrast, treatment with VacA

potently inhibited the proliferation of Th cells (Fig. 5-1A and 5-1B) in a dose-dependent manner (Fig. 5-1C). Incubation of purified VacA at acid pH (pH <4.5; a process termed acid-activation) markedly enhances the capacity of the toxin to undergo internalization and cause vacuolating cytotoxic effects in HeLa or gastric epithelial cells (284, 285). Accordingly, the pH of VacA preparations were adjusted to either pH 3 or pH 7.5 (with HCl or PBS) prior to treatment of Th cells as described above. As shown in figure 5-1D, acid-activation markedly enhanced the capacity of VacA to inhibit Th cell proliferation.

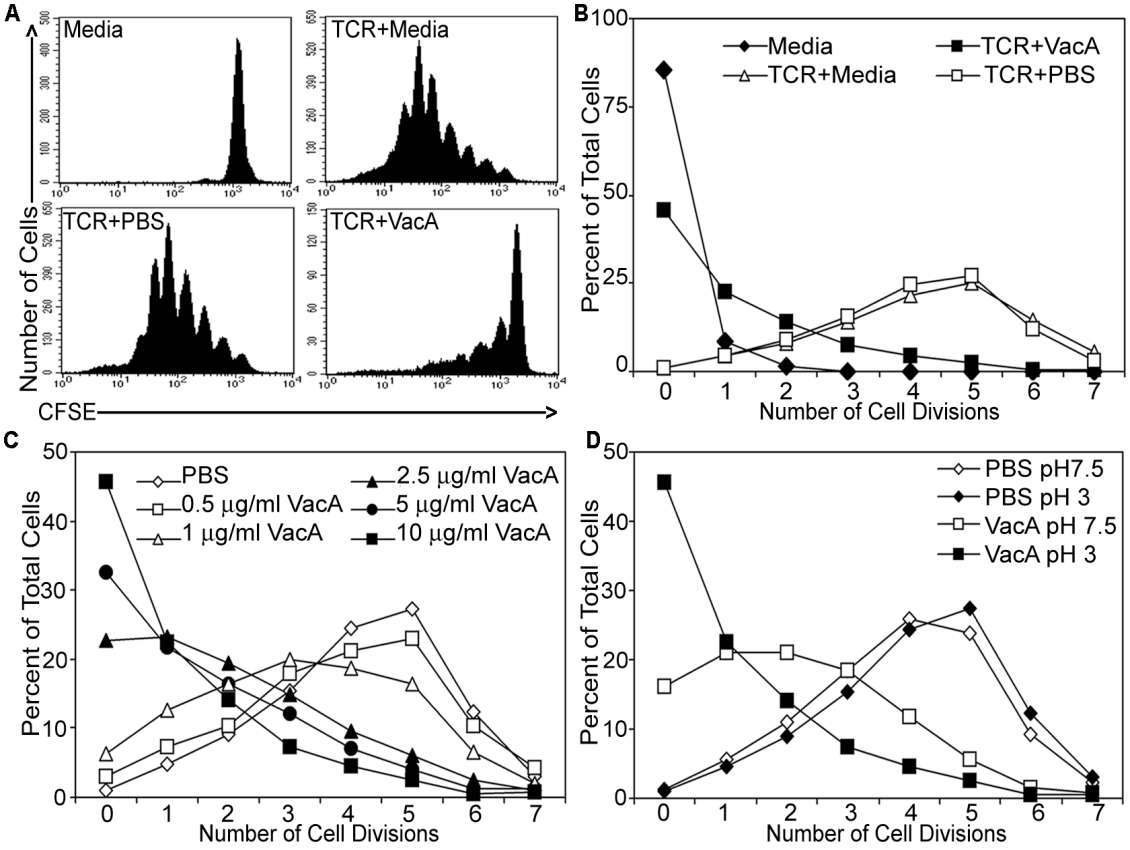

Figure 5-1. VacA inhibits activation-induced proliferation of primary human CD4$^+$ Th cells.

A) Purified primary human Th cells were labeled with CFSE and treated with acid-activated VacA (10 μg/ml), acidified-PBS (PBS), or medium alone for 1 hour, followed by TCR stimulation with α-CD3/ α-CD28 antibodies for 48 hours as described in Materials and Methods. Activated T cells were expanded in IL-2-containing media and T cell proliferation was analyzed at day 5 post-activation by flow cytometry. *B*) Graphic representation of the histograms shown in panel *A*. *C*) Dose-response analysis of VacA effects on primary human CD4$^+$ T cell proliferation. Th cells were CFSE-labeled and treated with different concentrations of acid-activated (pH 3) VacA for 1 hour. Cells were then stimulated and analyzed as in panel *A*. *D*) Effects of acid-activated VacA (pH 3) and non-acid-activated VacA (pH 7.5) on T cell proliferation. Th cells were CFSE-labeled and treated with acid-activated or non-activated VacA (10 μg/ml) as described above. All the results are representative of three experiments using cells from different donors and different toxin preparations.

VacA potently suppresses IL-2 secretion in Jurkat T cells, but not in primary human Th cells

It has been reported recently that VacA blocks the secretion of interleukin-2 (IL-2) by mitogen-stimulated Jurkat T cells (136). Therefore, we investigated whether VacA also inhibits IL-2 secretion in primary Th cells. In agreement with a previous report (136), we found that wild-type VacA potently suppressed IL-2 secretion in Jurkat T cells, very similar to the immunosuppressive drugs cyclosporine A and FK506, regardless whether the cells were stimulated with anti-CD3 and anti-CD28 antibodies or stimulated with PMA and ionomycin (Fig. 5-2A and data not shown). Notably, a VacA mutant toxin deficient in vacuolating cytotoxic activity (VacA ∆6-27) (275) (Table 5-1) did not inhibit IL-2 secretion in Jurkat T cells (Fig. 5-2A). In contrast to the strong effects of wild-type VacA on IL-2 secretion by Jurkat T cells, VacA had a very modest effect on IL-2 secretion by primary human Th cells (Fig. 5-2A). TCR stimulation of T cells induces the expression of both IL-2 and the high affinity IL-2 receptor anti-chain (CD25). Therefore, we next investigated whether VacA treatment inhibited the surface-expression of CD25. Primary human Th cells were pre-treated as described above and CD25 expression was analyzed both at 24 hours and 5 days post TCR stimulation by flow cytometric analysis. No significant difference was observed in CD25 expression between VacA-treated primary human T cells and those treated with PBS or media (data not shown). Although VacA treatment did not cause a biologically significant reduction in the amount of IL-2 produced, we further supplemented these cultures with saturating concentrations of exogenous recombinant IL-2 (50 ng/ml) during the activation. Resting Th cells were pre-

treated with PBS, wild-type VacA, or VacA-Δ(6-27) in the presence of exogenous IL-2 for 1 hour prior to TCR-activation. These pretreated cells were then TCR stimulated with anti-CD3 and anti-CD28 antibodies in the presence of IL-2-containing media, and proliferation was analyzed 5 days after stimulation. VacA potently inhibited the proliferation of Th cells, even in the presence of excess exogenous IL-2 (Fig. 5-2B). Thus, we conclude that the VacA-mediated inhibition of primary human Th cell proliferation cannot be attributed to a VacA-induced reduction in IL-2 secretion.

Table 5-1: Functional characterization of VacA mutant toxins.

VacA Toxins	Oligomer formation [a]	Binding / internalization [b]	Channel formation [c]	Cytotoxic Activity [d]
WT [e]	+	+	+	+
Δ6-27	+	+	-	-
P9A	+	+	-	-
G14A	+	+	-	-

[a] Oligomer formation was analyzed by determining whether these proteins eluted as large oligomeric structures (~1,000 kDa) from a gel filtration chromatography column (11, 12).
[b] Binding and internalization of VacA toxins was assessed by indirect immunofluorescence analysis using HeLa cells (10, 45).
[c] VacA channel activity was assessed using planar lipid bilayers (11-13).
[d] Toxins (20 μg/ml) that induced vacuolation in more than 50% of HeLa cells were scored positive for cytotoxic activity (11, 12).
[e] WT, wild-type VacA from *H. pylori* strain 60190.

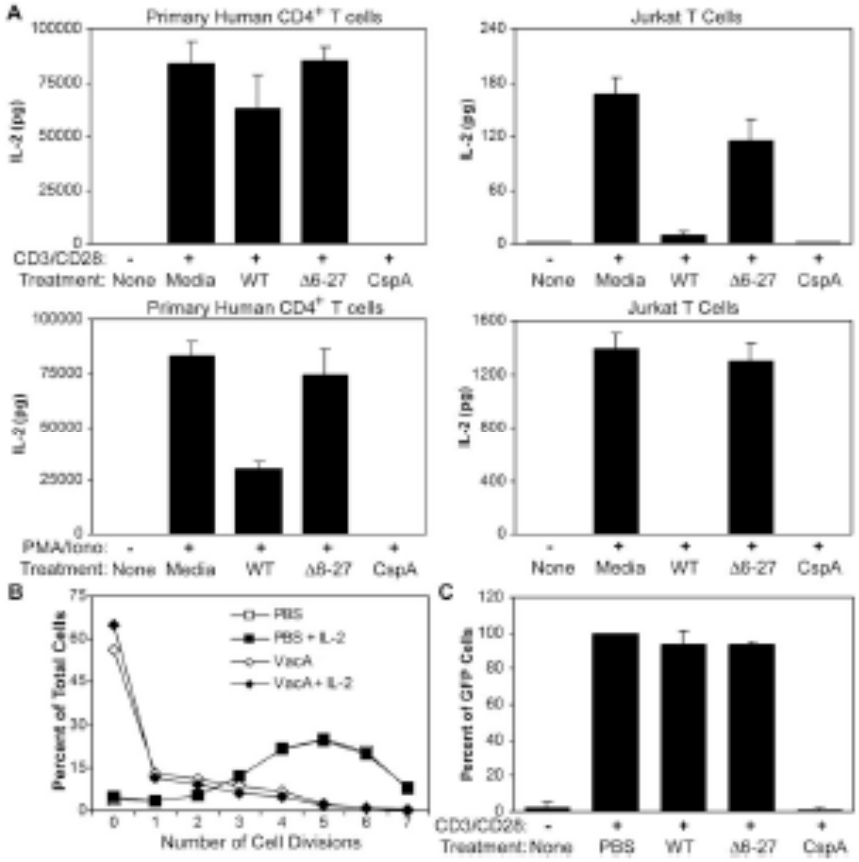

Figure 5-2. VacA inhibits activation-induced proliferation of primary human Th cells independent of effects on IL-2 secretion and NFAT activation.
A) Purified primary human Th cells or Jurkat T cells were pre-treated with media, wild-type (WT) VacA, VacA-Δ(6-27), cyclosporine A (CspA), or no additive for 1 hour, followed by TCR stimulation with a-CD3/a-CD28 antibodies (top panels) or with PMA (50 ng/ml) and ionomycin (500 ng/ml) (middle panels), as indicated. IL-2 secretion was measured at 24 hours after stimulation using a cytometric bead array (CBA) as described in Materials and Methods. Results represent the mean + S.D. from triplicate samples. *B*) Purified primary human Th cells were CFSE-labeled and pretreated with wild-type VacA or PBS in the presence or absence of supplemental IL-2 for 1 hour as indicated. Cells were then TCR stimulated with antibodies for 48 hours, expanded in IL-2 supplemented media, and subjected to flow cytometric analysis at day 5 post-treatment. *C*) Primary human Th cells stably transduced with a GFP reporter under the control of NFAT (NFAT-GFP Th cells; see Materials and Methods) were pre-treated with the different conditions as in panel *A* for 1 hour prior to TCR stimulation with cross-linking antibodies. GFP expression was assessed by flow cytometric analysis 24 hours after stimulation. Results represent the mean + S.D. from triplicate samples and are expressed as the percentage of cells demonstrating inducible expression of GFP, relative to the PBS-treated cells. CspA, cyclosporine A (50 nM); WT, wild-type VacA toxin (10 μg/ml); and Δ6-27, VacA-Δ(6-27) mutant toxin (10 μg/ml).

VacA inhibits primary human T cell proliferation through an NFAT independent mechanism

VacA has been shown to inhibit NFAT activation in Jurkat T cells, resulting in a loss of IL-2 secretion (136, 137). However, since VacA has only minimal effects on IL-2 secretion in primary T cells upon stimulation (Fig 5-2A), we hypothesized that VacA-mediated inhibition of primary human T cell proliferation may occur via an NFAT-independent mechanism. To test this hypothesis, we developed a primary human Th cell stably transduced with a lentiviral vector in which the NFAT binding site of the IL-2 promoter drives the expression of the green fluorescent protein (NFAT-GFP T cells). NFAT-GFP T cells were treated with wild-type VacA, PBS, cyclosporine A, FK506, or VacA-Δ(6-27) for 1 hour prior to stimulation with anti-CD3 and anti-CD28 antibodies. GFP expression was then analyzed 24 hours-post stimulation via flow cytometric analysis. As expected, GFP expression was only induced upon TCR-stimulation of NFAT-GFP T cells (Fig. 5-2C). In the presence of cyclosporine A or FK-506, which are potent inhibitors of NFAT activation, stimulated NFAT-GFP T cells did not express GFP (Fig. 5-2C and data not shown). In contrast, stimulated cells treated with VacA maintained the expression of GFP similar to the PBS-treated cells (Fig. 5-2C). These results, taken together with our findings that VacA causes minimal effects on IL-2 secretion in primary Th cells, suggest that VacA can inhibit proliferation of primary human Th cells via an NFAT-independent mechanism.

VacA inhibits IL-2-driven proliferation of primary human Th cells, but not IL-2-dependent survival

To gain further insight into the mechanism employed by VacA to suppress activation-induced proliferation of primary T cells, we investigated the kinetics of VacA-mediated effects. CFSE-labeled primary human Th cells were stimulated as previously described, and treated with PBS, wild-type VacA or VacA-Δ(6-27) at different time points after TCR stimulation with anti-CD3 and anti-CD28 antibodies. T cell proliferation was assessed 5 days after stimulation by flow cytometric analysis. VacA inhibited proliferation of Th cells even when added 48 hours after stimulation, suggesting that VacA effects are largely independent of early TCR signals (Fig. 5-3A). In contrast, cyclosporin A and FK506 completely inhibited Th cell proliferation when added within the first 24 hours, but had little effect when added 48 hours after TCR stimulation (data not shown).

We next tested whether VacA blocked IL-2 dependent proliferation of primary T cells at later time points (96 hours) after stimulation, a stage in which T cell proliferation and survival are solely dependent on IL-2 signals (286). For these experiments, Th cells were stimulated with anti-CD3 and anti-CD28 antibodies for 48 hours and expanded in the presence of IL-2 for an additional 2 days. At day 4 post-activation, T cells were removed from IL-2, and treated with VacA or other additives for 24 hours. IL-2 was then added back to the media and cells were expanded for an additional 3 days. Cell counts were performed to assess cellular proliferation at day 0- and day 3-post IL-2 stimulation. As expected, activated T cells treated with PBS and stimulated with IL-2 proliferated approximately 6-fold from day 0 to day 3, and no proliferation was observed in

the absence of IL-2 (Fig. 5-3B). In the presence of VacA, however, T cell numbers only increased about 3-fold ($p < 0.001$), similar to cells treated with rapamycin (Fig. 5-3B) that blocks IL-2 driven proliferation of T cells (287, 288). Analogous results were obtained when proliferation was assessed via CFSE-fluorescence (data not shown). These data indicate that VacA inhibits IL-2 driven proliferation of activated primary human Th cells.

IL-2 signals are required not only for activation-induced T cell proliferation, but also for survival of these cells (286). Therefore, we also monitored the viability of the activated Th cells cultured in the presence or absence of VacA. Indeed, the majority of activated T cells (>90%) kept without IL-2 for 3 days underwent apoptosis (Fig. 5-3C). In contrast, VacA treatment did not result in a significant increase in cell death as compared to T cells treated with PBS (Fig. 5-3C), suggesting that VacA inhibits IL-2-driven proliferation without altering IL-2-dependent survival.

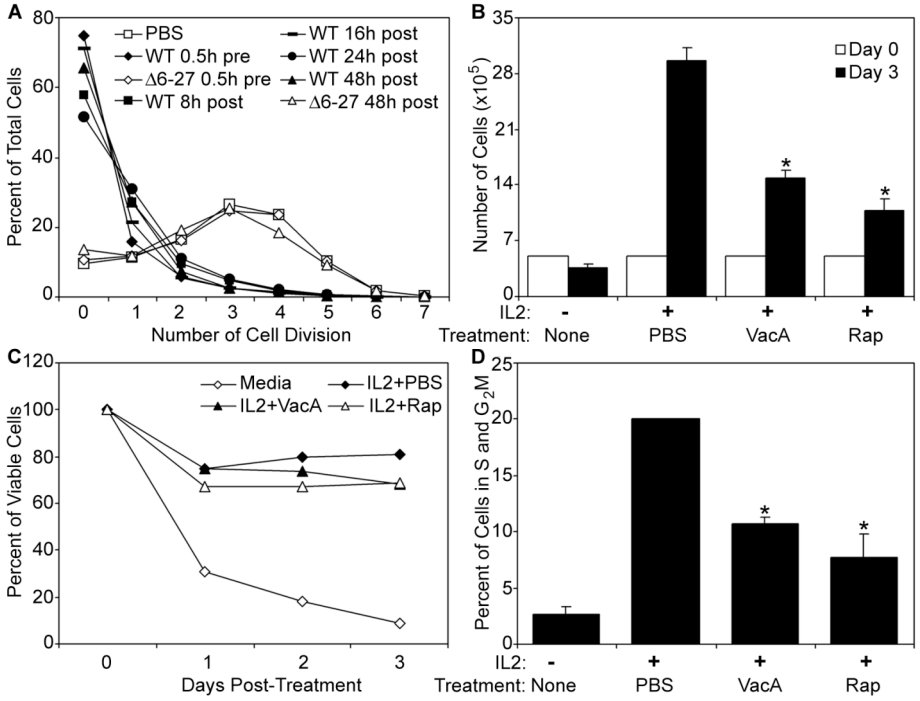

Figure 5-3. VacA inhibits IL-2-driven proliferation of primary human Th cells.

A) Wild-type (WT) VacA (10 μg/ml) and VacA-Δ(6-27) (10 μg/ml) were added to CFSE-labeled purified primary human Th cells at the indicated time points either preceding or following (pre or post) TCR stimulated with α-CD3/ α-CD28 antibodies. Activated cells were expanded in IL-2-containing media and cell proliferation was analyzed by flow cytometry at day 5 after stimulation. *B*) primary human Th cells were TCR stimulated using antibodies as in (A) for 48 hours and expanded in the presence of IL-2 for 2 additional days. At day 4 after stimulation, T cells were removed from IL-2 and treated with PBS, wild-type VacA (10 μg/ml), or rapamycin (Rap; 200 ng/ml) for 24 hours. After 24 hours, IL-2 was added back to the media as indicated and cells were treated again with the different additives and expanded in fresh media containing supplemental IL-2 for 3 days. Cell proliferation was assessed by cell counting with a hemacytometer. Results represent the mean + S.D. from triplicate samples. * = $p < 0.001$ when compared to the PBS treated cells. *C*) Th cell viability of the was determined by gating for viable cells based on forward and side scatter properties via flow cytometric analysis at day 1, 2, and 3 after stimulation with antibodies. *D*) For cell cycle analysis, primary human Th cells were treated as described in panel *B*. Cell-cycle distribution was analyzed at 36 hours following IL-2 stimulation using PI staining and flow cytometry analysis, as described in Materials and Methods. The percentages of total cells in S-phase and G2/M-phase are shown. Results represent the mean + S.D. from triplicate samples. * = $p < 0.01$ when compared to the PBS treated cells. Results are representative of at least two experiments using cells from different donors and different toxin preparations.

VacA attenuates IL-2-dependent cell cycle progression in primary human Th cells

To determine whether VacA-mediated inhibition of IL-2-driven proliferation is due to a cell cycle arrest, we assessed cell cycle progression of activated T cells in the presence of VacA. Primary human Th cells were stimulated with anti-CD3 and anti-CD28 antibodies, and expanded in IL-2- containing media as described above. Day 4-activated Th cells were removed from IL-2 for 24 hours to induce a G1-phase cell-cycle arrest (288), and were then treated with VacA or other additives. IL-2 was then added back to the media, and cell cycle distribution was analyzed at different time points through propidium iodide (PI) staining. The activated Th cells arrested in G1-phase re-entered the cell cycle approximately 24 to 36 hours after IL-2 stimulation, as seen by the increased number of Th cells in S and G2M phases (Fig. 5-3D). In contrast, cells treated with either rapamycin (which inhibits IL-2-induced cell cycle progression by arresting the cells at the G1-phase) (287, 289) or VacA displayed impaired IL-2-induced cell cycle progression (Fig. 5-3D). We also determined the rate of DNA synthesis in VacA-treated Th cells by assessing incorporation of BrdU, a thymidine analog that is incorporated into newly synthesized DNA during S-phase. VacA-treated activated T cells were about 2-fold less efficient in the rate of BrdU uptake when compared to PBS-treated control cells (data not shown), thus corroborating the PI results (Fig. 5-3D). Taken together, these data provide strong evidence that VacA inhibits IL-2-driven proliferation in activated primary human Th cells by inhibiting cell cycle progression.

The N-terminal hydrophobic domain of VacA is required for inhibition of primary human Th cell proliferation

Structure-function analyses have revealed that an intact structure of a hydrophobic domain within the VacA amino-terminal region is required for the formation of anion-selective membrane channels (Table 5-1) (272-276). Because VacA-Δ(6-27), a VacA mutant that lacks the entire hydrophobic domain, did not suppress proliferation of primary Th cells (Fig. 5-3A) or IL-2 secretion in Jurkat T cells (Fig. 5-2A), we hypothesized that the formation of VacA anion-selective channels plays an important role in the VacA-mediated inhibition of T cell activation-induced proliferation. To further test this hypothesis, we examined the effects of two VacA mutant toxins that contain single amino acid substitutions in the hydrophobic domain of VacA (VacA-P9A and VacA-G14A) (273) on proliferation of primary human Th cells. These VacA mutants proteins, including VacA-Δ(6-27), are defective in channel-forming activity, but retain other structural and functional characteristics of the wild-type VacA protein, including the ability to form oligomeric structures and the capacity to bind and enter cells (Table 5-1) (273, 275, 276). In contrast to wild-type VacA, these mutant proteins did not cause any detectable inhibition of T cell proliferation (Fig. 5-4). These data indicate that an intact VacA amino-terminal hydrophobic domain is required for VacA-mediated inhibition of T cell proliferation, and suggest that the formation of VacA anion-selective membrane channels is important for the suppression of activation-induced proliferation of primary Th cells.

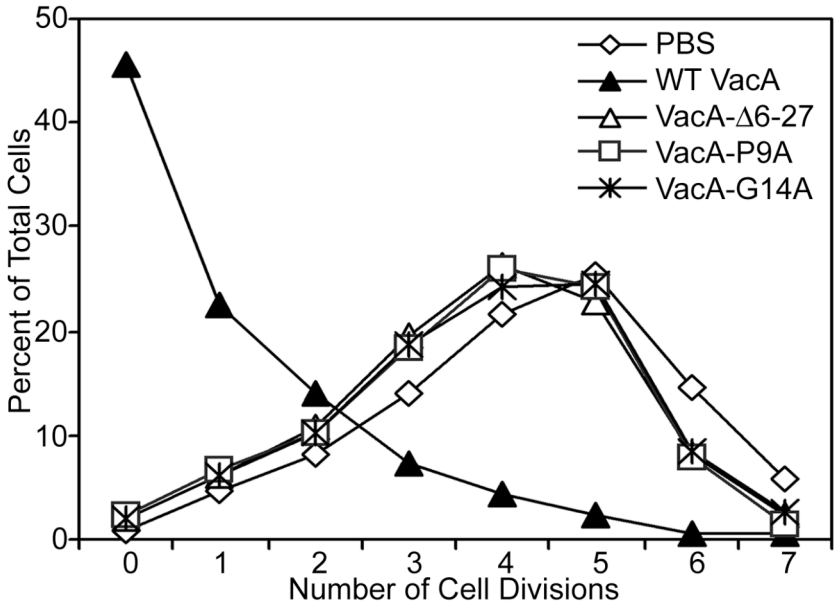

Figure 5-4. Analysis of VacA mutant proteins demonstrates that an intact N-terminal hydrophobic domain is required for VacA-mediated effects on T cells.

Purified primary human Th cells were CFSE-labeled and treated with wild-type VacA (WT; VacA at 10 μg/ml), one of 3 different mutant toxins (each 10 μg/ml), or PBS for 1 hour. Cells were then TCR stimulated with α-CD3/α-CD28 antibodies for 48 hours, expanded in IL-2-containing media, and analyzed by flow cytometry at day 5 after stimulation as described in Materials and Methods. Results are representative of three experiments using cells from different donors and different toxin preparations.

VacA-Δ(6-27) inhibits the immunosuppressive effects of wild-type VacA on T cells

In studies of VacA-induced effects on HeLa cells and AGS cells (a human gastric epithelial cell line), the VacA mutant toxin (VacA-Δ(6-27)) has been reported to exhibit a dominant-negative phenotype (275). When mixed in an equimolar ratio with wild-type VacA, VacA-Δ(6-27) blocks the capacity of wild-type VacA to cause cell vacuolation (275), form anion-selective membrane channels (275), induce cytochrome c release (276), and induce apoptosis (290). The inhibitory actions of VacA-Δ(6-27) are thought to be due to the formation of inactive mixed-oligomeric complexes, comprised of both wild-type and mutant toxin (275, 291, 292). To investigate whether VacA-Δ(6-27) could block the actions of wild-type VacA on Th cells, the two toxins were mixed and added at various stoichiometric ratios to CFSE-labeled, resting Th cells. CFSE-labeled primary human Th cells were subsequently stimulated with anti-CD3 and anti-CD28, and proliferation was evaluated by flow cytometric analysis 5 days post stimulation. VacA-Δ(6-27) potently blocked wild-type VacA-mediated inhibition of T cell proliferation in a dominant-negative fashion, since it was partially effective even in the presence of 20-fold molar excess of wild-type VacA (Fig. 5-5A). Furthermore, VacA-Δ(6-27) also blocked the inhibitory effects of wild-type VacA on IL-2 secretion in Jurkat T cells (Fig. 5-5B). Importantly, the dominant-negative phenotype exhibited by the VacA-Δ(6-27) mutant toxin was specific for the Δ6-27 mutation, since two other mutant toxins containing point mutations within the VacA N-terminal hydrophobic domain (P9A and G14A) did not exhibit a dominant-negative phenotype (data not shown).

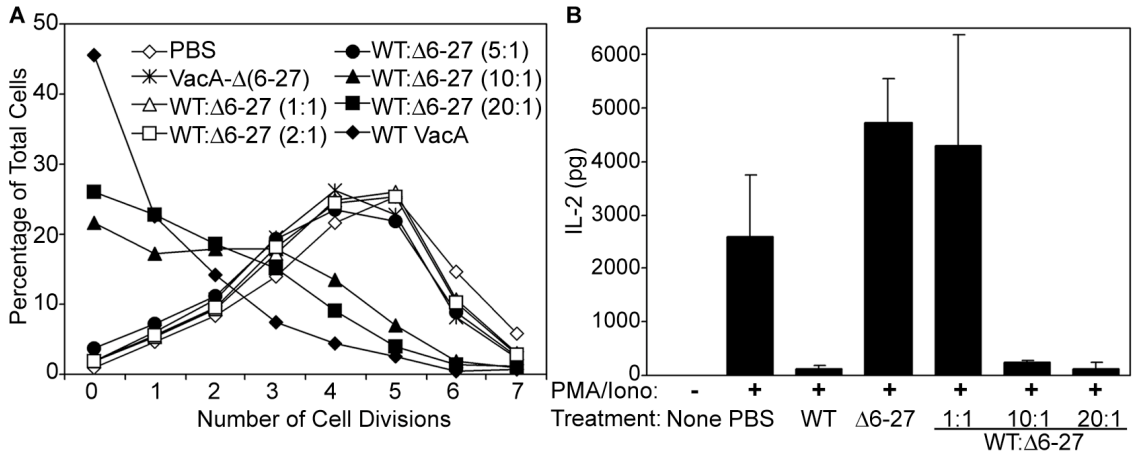

Figure 5-5. Effects of a dominant-negative mutant VacA toxin.
A) Primary human Th cells were CFSE-labeled and then treated for 1 hour with wild-type VacA (10 μg/ml) and VacA-Δ(6-27) in different ratios (WT:Δ6-27) as indicated. Cells were then TCR stimulated with α-CD3/ α-CD28 and expanded in IL-2-supplemented media as described in Materials and Methods. Cell proliferation was analyzed by flow cytometric analysis on day 5 after stimulation. *B*) Jurkat T cells were pre-treated as in panel *A* with the different conditions for 1 hour as indicated, activated with PMA (50 ng/ml) and ionomycin (500 ng/ml), and then incubated for 24 hours. Culture supernatants were assayed for IL-2 secretion using a cytometric bead array (CBA) assay as described in Materials and Methods. Results are representative of three experiments using cells from different donors and different toxin preparations.

Discussion

Here we show that VacA inhibits the proliferation of primary human $CD4^+$ Th cells upon TCR/CD28-stimulation (Fig. 5-1). In addition, our data demonstrate a qualitative difference between VacA-mediated effects on IL-2 secretion by Jurkat T cells and primary human Th cells (Fig. 5-2). In contrast to its potent inhibitory effects on IL-2 production in Jurkat T cells (Fig. 5-2A) (136, 137), VacA-treated primary human T cells retain the capacity to secrete high levels of IL-2 (Fig. 5-2A). The suppressive effect of VacA on Jurkat T cell proliferation has been previously attributed to the suppression of IL-2 expression, occurring at the level of transcriptional regulation due to NFAT inhibition (136, 137). In contrast, our results provide evidence that VacA-mediated inhibition of primary human Th cell proliferation occurs at a later stage of T cell activation, wherein the immunosuppressive drugs cyclosporine A and FK-506 (NFAT activation inhibitors) are no longer inhibitory (Fig. 5-3A, 5-3B and data not shown). Specifically, we show for the first time that VacA impairs IL-2-induced cell-cycle progression in activated primary human Th cells (Fig. 5-3B), resulting in an inhibition of IL-2-driven proliferation. Indeed, the inhibitory activity of VacA on IL-2-driven T cell proliferation appears similar to the actions of the immunosuppressive drugs rapamycin and sanglifehrin A (287, 293), two known inhibitors of IL-2 proliferation.

We also show, based on analysis of single-point mutant toxins, that the inhibitory effect of VacA on primary human Th cell proliferation and IL-2 secretion

in Jurkat T cells is dependent on the integrity of the VacA N-terminal hydrophobic domain. Three different mutant toxins defective in membrane channel formation were each unable to inhibit the proliferation of primary human Th cells (Fig. 5-4), providing strong evidence that VacA-mediated effects on T cell proliferation are dependent on the formation of membrane channels. Further evidence in support of this conclusion is provided by experiments with a dominant-negative mutant VacA protein, VacAΔ(6-27) (275), which potently blocked the effects of wild-type VacA on T cells (Fig. 5-5). This dominant-negative VacA protein interacts with wild-type toxin, resulting in the formation of non-functional mixed-oligomeric structures (273, 275, 291). Inhibition of wild-type VacA activity by a dominant negative mutant protein is consistent with a model in which the formation of oligomeric VacA structures, such as membrane channels, is required for VacA-induced effects on T cells. We hypothesize that formation of anion-selective VacA membrane channels (272, 274) induces membrane-depolarization of T cells, resulting in an altered equilibrium of numerous ions, and that these phenomena are mechanistically important in the inhibition of IL-2-dependent T cell proliferation. Our hypothesis is supported by a previous study demonstrating that activation of glycine-gated chloride channels induces membrane depolarization of T cells, resulting in a decreased open probability of plasma membrane calcium channels and in the inhibition of IL-2-dependent proliferation without affecting IL-2 secretion or NFAT activation (294).

Gastric biopsies from *H. pylori*-infected individuals consistently demonstrate infiltration of $CD4^+$ T helper cells (134, 279, 280), and specific anti-

H. pylori T cells have been detected in the gastric mucosa (134, 295). Nevertheless, *H. pylori* is able to evade the immune response and establish persistent infection. Experiments in mice indicate that the quality of the Th cell response is critically important for eradication of *H. pylori* and prevention of *H. pylori*-induced pathology (133, 135). Based on the results of the current study, we propose that VacA can inhibit the clonal expansion, and thus the acquisition of effector functions of T cells that have already been activated by *H. pylori* antigens. This immunosuppressive activity of VacA is likely to play an important role in the process by which *H. pylori* evades the adaptive immune response.

CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

The human immune system is a complex, hierarchical network of symbiotic cells that coordinate protection against parasitic microorganisms. Defensive barriers, immune surveillance and immediate inflammatory responses at anatomical sites of pathogenic invasion are mediated by the innate immune system. However, a cardinal feature of immunity in higher vertebrates is the ability to mount qualitatively distinct responses to discrete pathogens, an attribute ultimately determined by the differentiation of $CD4^+$ Th cells from naïve to effector and memory subsets. In this respect antigen-specific Th cells are analogous to military field generals, mobilizing and regulating the various arms of the human immune forces. On the other hand, successful pathogens that have remained prevalent throughout the human population have co-evolved an intricate series of immune evasion tactics, which often involve the perturbation or usurpation of Th cell development and function in order to thrive under intense selective immune pressures. As such, further elucidating how human Th cells differentiate, gain effector function and interact with microbial pathogens is important to understand what makes an immune response protective, ineffective or pathogenic and may identify how such responses can be therapeutically manipulated.

Flexible programs of human Th cell differentiation

Our findings detailed in chapter II demonstrate that lineage-committed primary human memory Th cells can be genetically reprogrammed towards Th2 or Th1 type cells by expression of the transcription factors GATA-3 and T-bet, respectively (60). Transduction of GATA-3 in T_N and T_{CM} cells induce cytokine and chemokine receptor expression patterns ascribed to Th2 cells. However, GATA-3 fails to significantly down-regulate IFNγ production and IL-12Rβ2 expression in cells already committed to the Th1 lineage and in the T_{EM} subset. T-bet expression directs T_N and T_{CM} human T cells to a Th1 phenotype, while it was much less effective at modifying the effector functions of T_{EM} cells. These data suggest a progressive loss in functional flexibility as Th cells mature from naïve to effector memory subsets (60).

These findings support a model of Th cell differentiation wherein T_{CM} cells represent an antigen-primed subset, which is capable of modifying its lineage commitment upon antigenic stimulation to generate new waves of T_{EM} cells (44, 47, 60, 156). On the other hand, differentiated T_{EM} cells, which have already been programmed to mount specific responses to an invading pathogen, more significantly lack adaptability to environmental cues that less differentiated T_{CM} cells exhibit. However, we show that effector function and chemokine receptor expression can even be partially modified in T_{EM} cells, most strikingly in response to forced GATA-1 and T-bet expression. These findings suggest that over the course of an immune response antigen-specific memory Th cells can modulate their effector functions and lymphoid trafficking propensities according to the

milieu of differentiating signals present in the extracellular micro-environment (47, 60, 68, 83, 86, 296). Indeed, adaptable memory responses exhibiting plasticity in the context of antigen re-exposure could be a highly beneficial strategy against continually evolving pathogens. Together, these findings lend novel insight into the differentiation programs of human Th cells and may also have implications in the development of new therapeutic strategies to ameliorate immunopathologies stemming from misbalanced memory T cell responses.

Future studies will aim to further dissect the mechanisms underlying the progressive loss of Th cell functional plasticity during their differentiation to effector and memory subsets. However, given the biologically complex nature of Th cell differentiation we posit that Occam's razor is not applicable. Rather, we propose and will test a three-part hypothesis wherein each component contributes to the sum that manifests as the progressive loss of functional plasticity during Th cell differentiation. We theorize one contributing factor is the chromatin remodeling of key cytokine promoter loci that is associated with T_N cell differentiation to effector and memory cells (82, 83, 164, 297). We propose such structural reorganization of these loci either modifies the regulatory activities of T-bet or GATA-3 by changing the interactive partners present within the transcription complexes or simply precludes these factors from binding their cognate *cis*-elements (298). To this end, future biochemical studies will monitor Th1- and Th2-specific chromatin remodeling at the *IL-4* and *IFN*γ cytokine gene promoters as T_N cells differentiate to polarized effector and memory subsets, aiming to identify specific modifications within these loci that are associated with

the loss of functional plasticity exhibited by effector and memory T cells upon expression of the opposing master transcription factor. These experiments will begin to unravel the “histone code” that regulates the acquisition and maintenance of Th cell effector function (299-302).

A second hypothesis that could, in part, account for the progressive inability of T-bet and GATA-3 to fully reprogram antigen-specific memory Th cells is that these master transcription factors require synergistic activities of auxiliary co-factors whose expression is lost or who become non-functional as T_N cells differentiate to effector and memory subsets. Indeed, a number of transcription factors aside from T-bet and GATA-3 have been identified that influence the Th1/Th2 balance (73, 81, 120, 144, 146, 147, 157, 163, 165, 166, 303-308). However, experiments carefully scrutinizing the expression of these factors in naïve and memory Th cell subsets have not been performed. Additionally, given the vast scale of differential gene expression that is associated with Th cell differentiation (309, 310), we consider it likely that additional Th cell transcriptional regulators remain unidentified. Indeed, we have found that T-bet down-regulates c-maf, a Th2-specific transcription factor that synergistically promotes high-level IL-4 production with GATA-3 (our unpublished data and refs ((146-148)). These findings suggest the expression and activity of c-maf and other auxiliary Th1/Th2 transcription factors in naïve and memory T cell subsets may help determine the extent to which their effector functions can be modified. Therefore, to address the relative contribution of accessory transcription factors in this context, future studies will feature an innovative co-transduction approach.

For these experiments, known Th cell regulatory transcription factors such as STAT-4, STAT-6, NFATc2, c-maf, NFκB, FOG, ROG, SKAT-2 and Hlx (73, 81, 120, 144, 146, 147, 157, 163, 165, 166, 303-308) will be expressed in lineage-committed effector and memory Th cells in conjunction with T-bet or GATA-3 to determine if specific combinations of master and auxiliary transcription factors can more completely reprogram the effector functions of lineage-committed effector and memory Th cells. In addition, we have performed gene expression profiling of Th1 and Th2 cells using oligonucleotide micro-arrays and have generated lists of potential Th1 or Th2 transcriptional regulators (unpublished data). Similar to the studies using known Th cell transcription factors, candidate regulatory genes will be co-expressed with T-bet or GATA-3 in effector or memory cells committed to the opposing lineage to identify new auxiliary Th1/Th2 transcription factors.

The third and final component we hypothesize to play a role in the progressive loss of functional plasticity during Th cell differentiation is the co-repressive nature of T-bet and GATA-3. Several studies characterizing the master regulatory roles of T-bet and GATA-3 clearly demonstrate that T-bet expression in Th1 and GATA-3 expression in Th2 effector and memory Th cells actively represses development of the opposing T cell lineage (60, 79, 95, 104, 107-109, 122, 233, 311). Our experiments show that even forced expression of these factors off of lentiviral transgenes cannot completely reprogram lineage-committed Th cells (60), strongly suggesting that cross-inhibition between T-bet and GATA-3 is not solely at the level of inhibiting gene expression. However, it

remains plausible that T-bet and GATA-3 post-translationally inhibit each other either directly through physical interactions (122) or indirectly through the induction of T-bet/GATA-3 inhibitors (120, 166, 312). Indeed, in studies detailed herein, we show that as T_N cells differentiate into Th1 effector cells, loss of T-bet expression results in more pronounced Th2 cytokine production and expression of Th2-associated chemokine receptors. These key results provide further evidence that T-bet, at least in part, functions to inhibit Th2 development and function. Therefore, to further characterize our T-bet/GATA-3 cross-inhibition hypothesis, future studies will utilize our recently developed RNAi system (chapter III) to co-express GATA-3 in conjunction with T-bet siRNA in Th1 effector and memory cells. Likewise, T-bet will be expressed in the presence or absence of GATA-3 siRNA in Th2 lineage-committed cells. We predict that expression of these master transcription factors in lineage-committed T_{EM} cells will result in more complete functional reversion upon silencing of the opposing factor. These experiments will lend further insight into the cross-regulatory roles of T-bet and GATA-3 during Th cell differentiation. Collectively these studies will begin to decode the transcription factor networks that regulate human Th cell differentiation programs and may lead to innovative therapeutic approaches to manipulate memory Th cell function in the context of infection or immunopathologies.

Mechanisms of gene regulation in human Th cells by GATA transcription factors

GATA proteins comprise a six-member family (GATA-1-6) of transcription factors that play integral regulatory roles in cell ontogeny and function within a multitude of cell types and tissues (100, 110, 111). As such, GATA transcription factors are highly conserved across species, yet display only modest, highly modular conservation between individual family members. Specifically, GATA transcription factors are classified based on a highly conserved C4-type zinc-finger motif (either one or two finger motifs) that mediates DNA binding and protein-protein interactions (226, 227, 313). GATA-1, -2, and –3 are closely related and all are key regulators of hematopoiesis in mammals (101, 138, 220, 228-231). Remarkably, while GATA-1, -2, and –3 share significant sequence similarity within the zinc-finger domain that confers conserved DNA binding propensities for the consensus DNA element (A/T)GATA(A/G) (110-112, 232), homology in amino- and carboxy-terminal regions of the gene/protein located outside the zinc-fingers is minimal. In studies detailed in chapter IV, we have utilized the regions of structural conservation and divergence between GATA-1 and GATA-3 to provide insight into the mechanism whereby GATA-3 regulates gene expression during human Th cell differentiation.

We have shown that expression of GATA-1 in human naïve and memory Th cell subsets, similar to GATA-3, induces Th2 cytokine production (IL-4, IL-5, IL-13) and expression of the Th2 chemotactic receptors CCR4 and CRTH2. These findings strongly suggest the DNA binding zinc-finger domain of GATA-3 is pivotal and largely sufficient to induce Th2 gene expression during Th cell

differentiation, as greater than 90% conservation within this domain of GATA-1 confers redundant Th2 regulatory activity. Importantly, regulation of Th2 cytokines and chemokine receptors occurred directly by GATA-1, without *trans*-activating GATA-3 expression. The parallel regulation of Th2 cytokines and chemokine receptors by GATA-3 and GATA-1 indicate conserved DNA binding and interactions with auxiliary transcription factors mediated by the central zinc-finger domain may be important in their trans-activation (236, 259).

While redundant in their capacity to promote the expression of Th2-associated genes, we note remarkably unique regulation of Th1 effector molecules by GATA-1. Expression of GATA-1 inhibits the production of IFNγ more so than GATA-3 and also prominently reduces the expression of the Th1-associated chemokine receptors CXCR3 and CCR5, even in lineage-committed T_{EM} cells. These results imply that domains lying in areas of significant divergence between GATA transcription factors (123, 263, 264, 314) can alter the expression of Th1 effector molecules. Indeed, amino- and carboxy-terminal portions of GATA transcription factors contain putative *trans*-activation domains that presumably function either as additional protein interaction interfaces or as acceptor sites for post-translational modifications (263, 264, 314-316). Thus, distinct *trans*-activation domains of GATA-1 and GATA-3 may impart differential regulation of IFNγ, CXCR3 and CCR5 by mediating unique interactions with transcription complexes within these promoter loci. Alternatively, GATA-1 and GATA-3 may be differentially modified within these domains, resulting in distinct regulatory activities (123, 263, 264, 314). Collectively, these findings indicate that

the domains of GATA transcription factors that control Th1 and Th2 effector loci may be discrete.

Although a biologically useful and important comparison, our findings only yield hints as to the key regulatory domains within GATA-3 that controls gene expression during human Th cell differentiation. Thus, future structure/function studies utilizing chimeric GATA transcription factors will be pursued to uncover specific regulatory regions and potentially important auxiliary transcription factors that can determine the expression of Th1 cytokines and chemokine receptors. We hypothesize that a chimeric GATA-1 transcription factor containing the zinc-finger domain of GATA-3 will be equally capable of down-regulating Th1 effector genes as wild-type GATA-1, whereas a GATA-3 protein with the zinc-finger domain of GATA-1 will remain unable to repress Th1 effector function. If our hypothesis holds true additional follow-up studies will generate a series of chimeras that more finely swap regions of the GATA transcription factors outside of the central zinc-fingers. Together these analyses may divulge the molecular mechanisms whereby GATA-3 controls gene expression in and effector function of human Th cells during immune activation. Such information could lead to the development of small-molecule inhibitors that could effectively target GATA-3 to stem the onset or pathogenesis of Th2-mediated atopic disorders.

Regulating Th cell expression of CCR5 and susceptibility to HIV-1 infection

Given the pivotal role of Th cells in regulating human immunity it is perhaps not surprising that numerous pathogens have counter-evolved immune evasion

strategies focused on disrupting Th cell differentiation and function. This strategy is exemplified by the human immunodeficiency virus (HIV). HIV-1 potently and selectively targets human Th cells for infection and eventual destruction through its keen tropism for biologically important receptors (CD4, CCR5, CXCR4) that are necessary for viral attachment and entry into host cells (126-128, 317-319). While CD4 is required for Th cell development and function and is thus constitutively expressed, the co-receptors CCR5 and CXCR4 are differentially expressed amongst naïve, effector and memory Th cell subsets (208, 317, 318). Of these two chemokine receptors usurped by HIV-1, CCR5 appears to be the most significant as the majority of natural HIV isolates are CCR5 (R5)-topic (320, 321). Indeed, HIV-1-infected individuals carrying mutations within the CCR5 gene that precludes or reduces cell-surface expression are resistant to HIV-1infection or progression to AIDS, respectively (212-217). Moreover, active inhibition of CCR5 expression and function by intracellular immobilization (218), small-molecule inhibitors (211) and siRNA (219) blocks in vitro HIV-1infection of Th cells. Thus, understanding the molecular regulation of CCR5 is paramount to developing effective anti-retroviral therapies.

Multiple consensus GATA-binding elements exist within the CCR5 promoter locus (129, 130) and previous studies have shown GATA transcription factors can bind these sites (223), suggesting a potential regulatory role for GATA family members in CCR5 gene expression. Our studies in chapter IV provides novel evidence that GATA-1 is a potent inhibitor of CCR5 expression, even in highly differentiated $CCR5^{+}$ T_{EM} cells, iNKT cells and terminally

differentiated DCs, resulting in significant Th cell resistance to R5-tropic HIV-1 infection and viral replication. Mechanistically, we demonstrate that GATA-1 suppresses CCR5 cell-surface expression in human Th cells by potently silencing promoter activity, strongly suggesting that GATA-1 may be a master regulator of CCR5 gene expression. These key results expose GATA-1 as a potential therapeutic tool in the fight against HIV-1 and AIDS.

However potent as a CCR5 repressor, the expression of GATA-1 is highly restricted during hematopoiesis and is not expressed in peripheral Th cells, iNKT cells or dendritic cells (our unpublished data and ref (220)). Therefore, the physiologic role of GATA-1 in regulating CCR5 expression and thus, susceptibility to HIV-1 in vivo remains uncertain. However, work from these studies and others have shown that GATA-1 is highly expressed in pluripotent $CD34^+$ hematopoietic stem cells (HSCs), which are reportedly susceptible to HIV-1 infection and are potential targets of viral infection in vivo (220, 237, 248, 260). Indeed, our findings that primary human HSCs silence GATA-1 expression and concomitantly up-regulate CCR5 upon differentiation to DC further supports our hypothesis that GATA-1 may a master regulator of CCR5 expression irrespective of cell type and suggest it may play a key physiologic role in regulating bone marrow precursor cell susceptibility to HIV-1 infection (322).

Analyses of CCR5 promoter regions have revealed a plethora of putative transcription factor binding sites. In addition to GATA transcription factors, other transcription factors have been characterized that bind to *cis*-elements within the CCR5 promoter (129, 130), including octamer transcription factors (Oct-1, Oct-2)

and YY-1 (323, 324). While the overwhelming majority of these studies have been performed using transformed T cell lines and must be validated in the primary cell context, intricate transcriptional complexes of enhancers and repressors likely regulate CCR5 gene expression in human cells. Therefore, future studies will be performed to identify and functionally validate the roles of additional transcription factors in the regulation of CCR5. Complimentary sets of experiments using ectopic expression and siRNA-mediated silencing of candidate transcription factors in human Th cells will be used to evaluate how their expression influences CCR5 expression and susceptibility to HIV-1 infection. Indeed, these experiments will help divulge the molecular regulation of CCR5 within human Th cells that ultimately determines the balance of susceptibility or resistance to HIV-1.

Immunology lessons from a pathogen: using VacA to probe the molecular regulation of Th cell proliferation

Another pervasive human pathogen that has evolved elaborate immune evasion strategies is the microaerophilic bacterium *Helicobacter pylori*. *H. pylori* colonizes the gastric mucosa of more than 50% of the human population (131, 132) and can persist for decades in the absence of antibiotic treatment. During infection, Th cells are critical for protection against *H. pylori* colonization (133-135, 279, 280). Studies detailed here in chapter V demonstrate that a vacuolating cytotoxin produced by *H. pylori*, VacA, inhibits the activation-induced proliferation of primary human Th cells (325). We found that VacA inhibits activation-induced proliferation of human Th cells without affecting early TCR/co-stimulatory signals,

IL-2 production or up-regulation of the high-affinity alpha chain (CD25) of the IL-2 receptor. Our findings were contrary to previous work investigating the immunosuppressive activities of VacA on a transformed T cell line that indicated the toxin blocked early activation signals by inhibiting NFAT activation, which in turn resulted in a diminution of IL-2 secretion and dampened expression of CD25 (136, 137). These findings again highlight the functional discrepancies between transformed and primary T cells and demonstrate the value of investigating biological processes in their appropriate physiologic context. Our data further indicate that the mechanism whereby VacA inhibits growth of activated human Th cells is by attenuating IL-2-driven proliferation (325).

Mechanistically, structure/functional analysis of VacA requirements for its immunosuppressive activity on human Th cells demonstrates that mutant toxins defective in membrane channel formation are unable to inhibit the proliferation of primary human Th cells. Moreover, a dominant-negative mutant VacA protein that interacts with the wild-type toxin, resulting in the formation of non-functional mixed-oligomeric structures incapable of forming membrane channels (273, 275) blocks the inhibitory activity of wild-type VacA (325). Together, these key findings provide strong evidence that VacA-mediated effects on T cell proliferation are dependent on the formation of membrane channels. We hypothesize that formation of anion-selective VacA membrane channels (272, 274, 326) induces membrane-depolarization of T cells, resulting in an altered equilibrium of intracellular ions that are mechanistically important during IL-2-dependent T cell proliferation. Accordingly, a previous report shows that glycine treatment of

activated T cells inhibits IL-2-driven proliferation through the perturbation of chloride channels, causing membrane depolarization (294). Alternatively, the channel-forming domain of VacA may also contain interfaces that mediate host protein interactions that are obligate for the inhibition of Th cell proliferation.

Regardless of the inhibitory mechanism, immunosuppressive activities of *H. pylori* mediated by VacA are likely to be physiologically important during infection. Indeed, gastric biopsies from *H. pylori*-infected individuals consistently demonstrate Th lymphocytic infiltration (134, 279) and antigen-specific Th cells have been detected in the gastric mucosa (134, 295). Nevertheless, *H. pylor*i is still able to evade the immune response and establish persistent infection. Given the importance of Th cells in mobilizing immune responses against extracellular bacteria, including *H. pylori*, our findings indicate that VacA can inhibit the clonal expansion, and thus the acquisition of effector functions of T cells that have already been activated by *H. pylori* antigens (325).

Future studies have been initiated and will continue to use VacA as an immunological tool to further understand the molecular regulation of Th cell clonal expansion as driven by IL-2. Specifically, human Th cells intoxicated with VacA will be further scrutinized for their biochemical signaling responses to IL-2 as compared to normally growing untreated cells or those treated with non-functional VacA mutant toxins. In addition, gene expression profiles of VacA intoxicated Th cells will also be evaluated in efforts to identify changes in cell-cycle-related gene expression that accompanies the VacA-induced attenuation of IL-2-driven cell-cycle progression. Together, these studies will have implications

in understanding the molecular mechanisms governing Th cell clonal expansion in response to antigenic activation and will garner further elucidation of the fundamental processes that regulate cell growth.

In summary, the activation, differentiation and resultant function of Th cells responding to pathogenic microbes specifies the efficacy and long-term viability of human immunity and can also directly influence the manner by which Th cells interact with invading pathogens. By decoding the physiologic programs of human Th cell differentiation and elucidating the mechanisms whereby microbes hijack normal Th cell function to evade host immune responses we may be able to therapeutically manipulate these processes to better protect against infectious human diseases and ameliorate immunopathologies.

APPENDIX A

LIST OF PUBLICATIONS

I. **Mark S. Sundrud**, Scott VanCompernolle, Karla A. Eger, Tullia C. Bruno, Srinivas Mummidi, Sunil Ahujua and Derya Unutmaz. The transcription factor GATA-1 potently represses the expression of the HIV-1 co-receptor CCR5 in human T cells and dendritic cells. *Manuscript submitted-Blood*.

II. Kyra Oswald-Richter, Stacy M. Grill, Nikki Shariat, Mindy Leelawong, **Mark S. Sundrud**, David W. Haas and Derya Unutmaz. HIV Infection of Naturally Occurring and Genetically Reprogrammed Human Regulatory T-cells. *PLoS Biol.* Jul 2004; 2(7): E198.

III. **Mark S. Sundrud**, Victor J. Torres, Derya Unutmaz and Timothy L. Cover. Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc Natl Acad Sci USA*. May 2004 18; 101(20): 7727-32.

IV. **Mark S. Sundrud**, Stacy M. Bohks, Donghui Ni, Sefik S. Alkan, Arun Subramaniam and Derya Unutmaz. Genetic reprogramming of human naïve and memory T cells reveals functional plasticity in T helper cell differentiation. *J. Immunol*. Oct 2003; 171: 3542-3549.

APPENDIX B

LIST OF PRESENTATIONS AT SCIENTIFIC MEETINGS

I. "Genetic reprogramming of human naïve and memory T cells reveals functional plasticity in T helper cell differentiation"

- The Keystone Symposium on regulation of the adaptive immune response. Keystone, CO March 2003

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