UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of Id proteins in T cell immunity

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in

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by

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The dissertation of Laura Ann Shaw is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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EPIGRAPH

I suppose I could have stayed home and baked cookies and had teas,

but what I decided to do was to fulfill my profession.

— Hillary Rodham Clinton

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ABSTRACT OF THE DISSERTATION

The role of Id proteins in T cell immunity

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Doctor of Philosophy in Biomedical Sciences

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Upon infection, naive T lymphocytes proliferate and differentiate into highly specialized cell types to combat the pathogen: $CD4^+$ T cells into specialized helper subsets and $CD8^+$ T cells into armed effectors. Although the majority of the antigen-specific T cells from both lineages will die as the immune response wanes, a few will survive indefinitely to establish memory populations, providing long-lived protection against reinfection. Transcriptional regulators of the E-protein and Id families are important arbiters of both T cell development in the thymus, and differentiation in response to infection. We and others, have shown that E/Id proteins cooperate

to balance expression of genes that control $CD8^+$ T cells throughout their differentiation, however, their role in the differentiation of $CD4^+$ helper subsets has not been studied as extensively. My recent work uncovered a role for Id and E proteins in the differentiation of $CD4^+$ T helper 1 (T_H1) and T follicular helper (T_{FH}) cells following infection. I found that that T_H1 cells showed more robust Id2 expression than that of T_{FH} cells, and depletion of Id2 via RNA-mediated interference increased the frequency of T_{FH} cells. Furthermore, T_H1 differentiation was blocked by Id2 deficiency, which led to E-protein-dependent accumulation of effector cells with mixed characteristics during viral infection and severely impaired the generation of T_H1 cells following infection with *Toxoplasma gondii*. Finally, the T_{FH} cell-defining transcriptional repressor Bcl6 bound the *Id2* locus, which provides a mechanism for the bimodal expression of Id2 and reciprocal development of T_H1 cells and T_{FH} cells. Investigation of Id3 revealed that naive $CD4^+$ T cells expressed high levels of Id3, which, when compared to T_H1 cells, is maintained by T_{FH} cells following LCMV infection. I found that Id3 was required to restrict unchecked differentiation of T_{FH} and GC T_{FH} cells. Lastly, I showed that expression of Id3 marks $CD4^+$ T cells with multipotent recall potential following LCMV infection. These studies inform the functional relevance of E/Id proteins in $CD4^+$ T cells, given the importance of leveraging the recall capabilities of memory T cells to fight reinfection.

Chapter 1

Remembering one's Id/E-ntity: E/Id protein regulation of T cell memory

1.1 Introduction

The immune sytem is a complex balance of coordinated interactions between different types of cells, organs, factors and signals, but in the end, its job can be boiled down to protecting the host by distinguishing self from non-self and harmless from harmful [70]. There are two separate, but complementary, 'arms' to the immune system, termed innate and adaptive, that work together to maintain this balance [8, 70]. Cells of the innate immune system, which include natural killer cells and phagocytic cells such as dendritic cells and macrophages, have germline-encoded recognition receptors and orchestrate the immediate 'front line' immune response to infection [70]. These cells recognize, and are activated by, 'non-self' molecules that are common among classes of pathogens (pathogen associated molecular patterns, PAMPS) [71]. Following PAMP recognition, innate cells

upregulate expression of antimicrobial genes and release inflammatory cytokines to neutralize and/or destroy the microbe; these maturation processes also function to alert and mobilize adaptive immune cells [71].

By comparison with innate immunity, which have pre-encoded receptors to recognize PAMPS, the adaptive immune system is comprised of cells where each individual clone within the population expresses a unique receptor and can recognize different antigen [101]. Rather than a fixed germline-encoded receptor, lymphocytes express a receptor that has undergone random rearragement of germline-encoded gene segments, with additional variability introduced between these segments, generating many millions of diverse receptors with endless specificity [73, 24]. A mechanism for this phenomenon came from the revelation that the genes encoding antibodies were generated by combinations of germline-encoded gene segments, which was later found to be mediated by specific enzymes [42, 97]

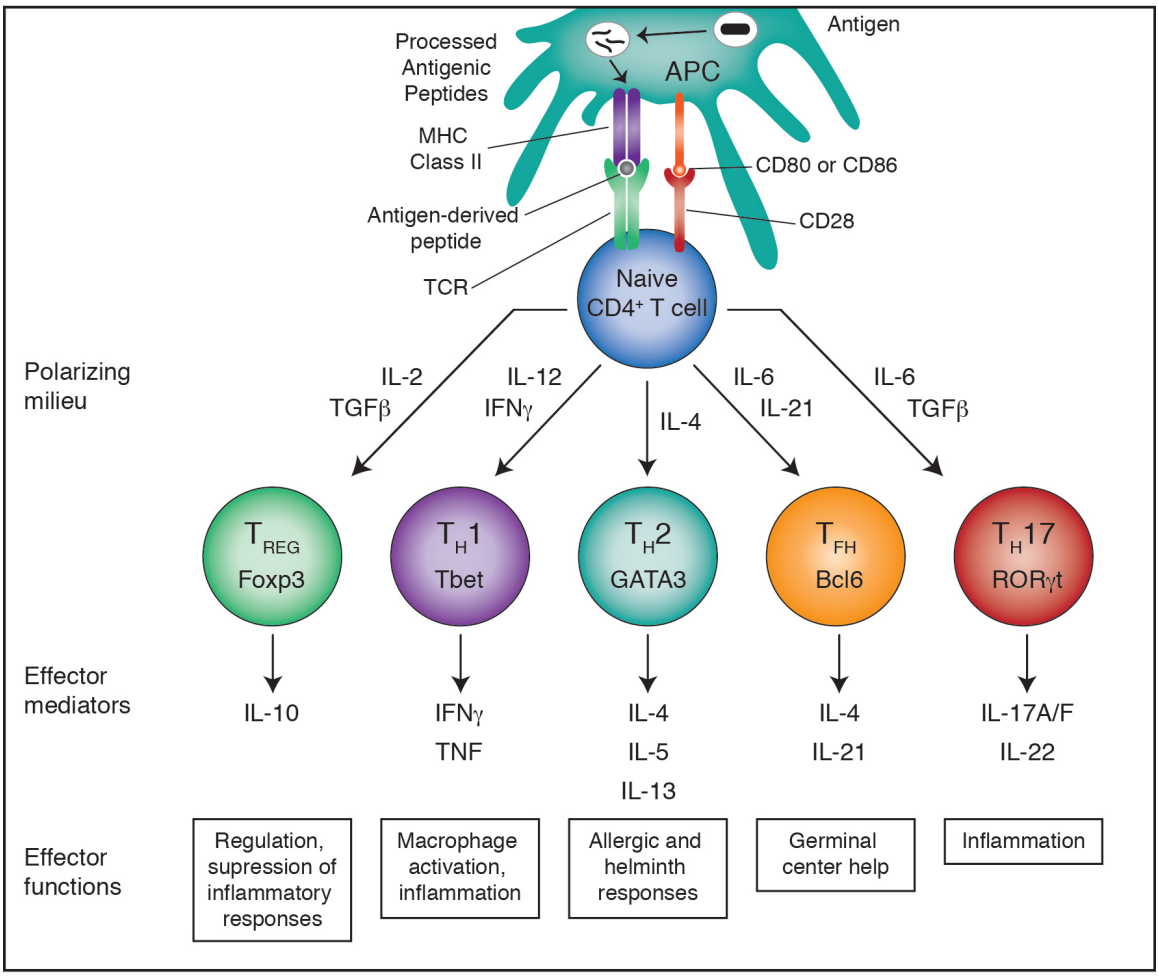
The diversity of antigen receptors is so vast, in fact, that the adaptive immune system was found to respond to many different types of antigens, even synthetic compounds which presumably did not occur naturally [59]. This ensures that there will always be at least one lymphocyte specifically tailored to respond to every possible pathogen. This idea was the foundation for the clonal selection hypothesis of immune responses [101, 9], where individual clones capable of recognizing the pathogen expand many thousand-fold in response to activation [78]. The adaptive immune system is divided into two groups, B cells and T cells, both of which express a randomly generated receptor, but the specificity of these two related receptor types, and by extension the function of these two cell types, are different.

B cell antigen receptors (BCR) have an almost completely unbiased repertoire,

where individual clones have been found to respond to antigens of diverse chemical structures. The B cell binds to the conformational epitope of antigens via the BCR, which will neutralize the antigen, or tag it for destruction by other cells; T cells recognize antigen bound to major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APC). In comparison to B cells, T cells undergo a specific maturation process in the thymus, whereby only cells expressing receptors that are capable of recognizing self are selected to mature [45]. Despite differential recognition of antigen, there were early observations that B cells and T cells could cooperate during an immune response [19, 72]. These experiments addressed the question in a straightforward, but clever way; mice that were given either cells from the bone marrow (B cells) or cells from the thymus (T cells) had a less robust antibody response following immunization than mice receiving B cells and T cells together [19]. Further work identified B cells as the antibody producing cells, and that the presence of T cells 'helped' the B cells generate a more effective antibody response [72]. As groups worked to understand the molecular basis of T cell recognition of cell associated antigen, this led to the concept of altered self, where T cells recognize antigen and self-MHC together as a complex, rather than as individual entities [118, 119]. Recognition of cell-associated antigen required physical closeness of a T cell and APC, which meant that T cells had the potential to effect a function directly to another cell. Add to this that B cells and T cells could recognize different components of the same antigen, and that they migrated to the same area of the lymph node following immunization, and the idea of a T cell helping a B cell generate an antibody response gained further ground [33]. Integral to the understanding of T cell function was the identification of CD4 and CD8 as

coreceptors for the two different types of T cells [88]. In these experiments, antisera directed against the CD4 surface antigen interfered with 'helper' function, and cytolytic 'killer' function was disrupted by antisera against the CD8 surface antigen [11, 88], cementing the theory that $CD4^+$ T cells conferred help upon other cells. We now know that T cells can provide help to B cells in numerous ways, so referring to "B cell help" as a single entity is a slight misnomer [22]. Through direct interaction via binding of CD40/CD40L (on the B cell/T cell, respectively) and the secretion of cytokines such as IL-4, $CD4^+$ T cells can induce the proliferation and survival of B cells [22]. $CD4^+$ T cells also support additional B cell maturation including somatic hypermutation, receptor class switching and differentiation into antibody producing plasma cells, all of which are influenced by the secretion of cytokines and other factors [22].

It has become evident that $CD4^+$ T cells can differentiate into many different 'helper' (T_H) lineages upon activation, depending on at least in part, transcription factor expression and the cytokine milieu of their microenvironment (Figure 1.1) [43]. In turn, $CD4^+$ T_H cells secrete cytokines and express cell-surface markers that give instructions to other cells; they also participate indirectly in host defense by promoting the development of fully functional $CD8^+$ T cells to combat intracellular infection [89]. Originally described by Mosmann and Coffman, elegant studies identified T_H1 and T_H2 cells as the first unique T_H lineages based largely upon the cytokines they produced, and ability to 'help' B cells [77]. Further work by a separate group a few years later showed that this heterogeneity in T_H cell differentiation is influenced by the environment in which the activation takes place; following *Listeria monocytogenes* infection, macrophage IL-12 supported the development of



Adapted from Swain *et al.*,
Nature Reviews Immunology, 2012

Figure 1.1: Activated CD4$^+$ T cell differentiation into distinct T_H subsets is controlled by key transcription factors and cytokines.

T_H1 cells [43]. More recently, the emergence of additional functionally distinct T helper subsets have added significant complexity to the resolution of CD4$^+$ memory populations. Follicular T helper (T_{FH}) cells, for example, are the requisite lineage for 'helping' B cells to produce high-affinity antibodies against extracellular microbes [20]. T_H17 cells produce the cytokine IL-17, and have been shown to confer protection against viruses, but can also drive autoimmunity and immunopathology [87, 69, 23]. Regulatory T cells (T_{REG}), depending on the infectious setting, have

also been implicated in both diminishing and driving pathology [94].

$CD8^+$ T cells acquire effector function following activation, which includes the capacity to secrete the effector cytokines, interferon (IFN) and tumor necrosis factor (TNF), and release cytolytic molecules such as perforin and granzymes [38, 1, 52]. This expanded population of $CD8^+$ T cells is a heterogeneous mixture of effector T cells ((which can be identified by high levels of the surface receptor killer cell lectin-like receptor G1 (KLRG1) and low levels of Interleukin-7 receptor (IL-7Rα), CD127)), as well as memory-precursor cells (contained within the $KLRG1^{lo}CD127^{hi}$ population) [52].

Following pathogen clearance, a majority of the activated $CD4^+$ and $CD8^+$ populations will succumb to programmed cell death. However, about 5% of the effector cells will survive in greater numbers than their naive precursors; these cells are transcriptionally programmed to seed the long-lived memory pool providing protection against re-infection [38, 52, 41, 78]. Several groups have attempted to relate unique phenotypic markers found on specific $CD4^+$ T cells at the peak of infection to memory potential [18, 89, 65]. Two subsets of $CD4^+$ memory cells were originally proposed: effector-memory T cells (TEM) and central-memory T cells (TCM) [89, 95]. TEM were described based on low expression of CD62L and CCR7, residing in non-lymphoid sites and the ability to produce effector cytokines hours following TCR stimulation. TCM cells were characterized by high levels of CD62L and CCR7, they could recirculate through lymph nodes, secrete IL-2 upon reactivation and proliferate considerably to generate secondary effector cells [89]. However, it has become evident that the populations identified to date are heterogeneous; each with a pool of cells ultimately destined to become long-lived

memory cells; illustrating how little is known about the differentiation of $CD4^+$ memory subsets as compared to that for the $CD8^+$ populations.

1.2 E and Id proteins

E proteins are transcription factors in the basic helix-loop-helix (bHLH) family that control many aspects of lymphocyte biology [79].They are well-established regulators of thymocyte development and are required for proper control of progression, survival, proliferation and T cell receptor (TCR) rearrangements by T cell progenitors [79].

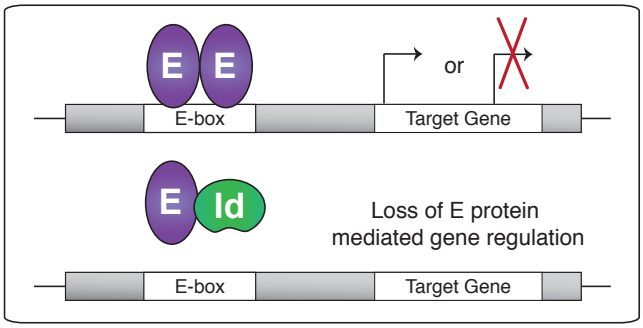


Figure 1.2: E protein activity is regulated by Id proteins. E protein dimers bound to DNA can activate (top) or repress gene transcription. When E proteins heterodimerize with Id proteins, DNA binding is inhibited, also blocking target gene transcription (bottom).

Four different E proteins, E12 and E47 (splice variants of E2A), E2-2 and HEB, are present in mammals. E proteins can interact as homo- and hetero-dimers via their HLH domains and bind specifically to DNA at E-box-consensus sequences acting as transcriptional activators or repressors (Figure 1.2) [79, 55]. The ability of E proteins to bind DNA and regulate gene expression is inhibited by the highly

related Id proteins, which share the HLH domain and thus form heterodimers with E proteins, but lack a DNA-binding domain, preventing E protein function (Figure 1.2) [67, 5]. There are four Id family members (Id1-Id4); with Id2 and Id3 emerging as the players relevant in shaping lymphocyte differentiation [79, 67, 5]. While Id protein-mediated regulation of E proteins is known to be crucial to lymphocyte development, the role of these factors in mature T cells is only beginning to be revealed. Recently it was discovered that E and Id proteins also regulate the differentiation of both the short-lived effector and memory-precursor populations of $CD8^+$ T cells [10, 57, 68, 111, 46]. Importantly, a reciprocal relationship between Id2 and Id3 has been described in the differentiation of mature $CD8^+$ T cells during the response to infection [111], which raises the possibility of an analogous role for these molecules in determining $CD4^+$ T cell fate.

1.2.1 E and Id proteins in $CD8^+$ T cells

$CD8^+$ T cells are crucial to host control of infection by pathogens that reside in the cytoplasm such as viruses, intracellular bacteria and protozoan parasites. In the uninfected state, a diverse repertoire of resting, naive $CD8^+$ T cells populate peripheral lymphoid organs. In response to infection, $CD8^+$ T cells transition from quiescent cells with minimal effector capacity, to proliferating cells with cytolytic function and the capacity for rapid cytokine production. After pathogen clearance, the majority of $CD8^+$ T cells die, leaving a select few with long-term memory capacity to protect from reinfection. Asymmetric T cell division [13], T-box transcription factors driven by inflammatory cytokines, and antigen/inflammation duration have all been proposed to control this differentiation, yet questions remain as to

what factors promote or suppress memory versus effector formation [50]. Useful to our understanding of $CD8^+$ T cell memory potential is cell-surface expression of CD127 and KLRG1 on antigen-specific $CD8^+$ T cells after infection, which can be used to delineate two subsets with distinct long-term memory potential [53]; most cells upregulate KLRG1 and remain $CD127^{lo}$, while fewer cells re-express CD127 and stay $KLRG1^{lo}$. The $KLRG1^{hi}$ cells form a short-lived effector/memory population, able to produce cytokines IFNγ and TNFα but less IL-2 upon TCR stimulation, while the $CD127^{hi}$ population contains long-lived memory precursors [33], which produce IFNγ, TNFα, and IL-2 [96]. Importantly, recent studies have shown that CD127 and KLRG1 are correlative but not deterministic factors in $CD8^+$ T cell memory formation [37]. Thus, the factors that determine and enhance memory formation are not fully understood. Each differentiation state–naive, effector, terminally-differentiated effector, and memory–is thought to be orchestrated by a transcription factor network with key downstream targets which enable and enforce stage-specific cellular traits. Validating this concept, certain transcriptional activators/repressors are well established as essential regulators of gene expression by $CD8^+$ T cells during infection, including: T-bet, Tcf7, Eomes, Id2, Id3, and Blimp-1; yet it is likely that many additional factors that impact $CD8^+$ T cell differentiation are yet to be described. Work from our lab highlighted a clear role for E/Id protein interactions during the $CD8^+$ T cell response to viral infection [10, 57, 26, 28].

Although downregulated early in infection, Id2 expression is upregulated at the peak and maintained in memory T cells, albeit at lower levels (Figure 1.3)[10, 68, 111, 25]. Id2 plays important roles in the $CD8^+$ T cell response to infection by mediating survival and differentiation of effector cells and repressing memory

formation [10, 57, 68]. Id3 is expressed at its highest level in naive CD8$^+$ T cells and is rapidly downregulated upon activation. Expression of Id3 later increases during contraction of the effector response and coincides with the appearance of memory CD8$^+$ T cells, effectively acting as a marker of memory-precursor cells (Figure 1.3) [111].

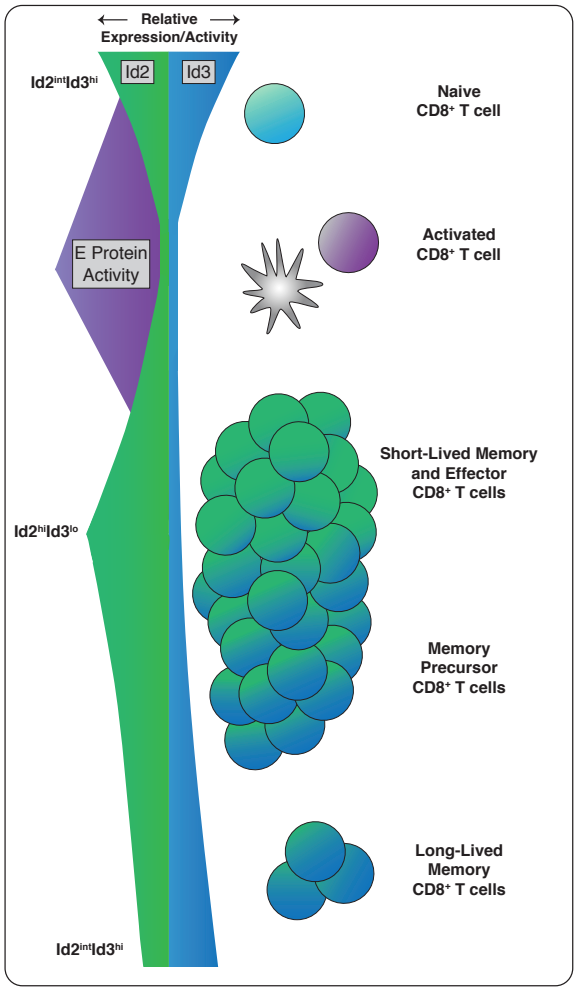


Figure 1.3: E and Id proteins show coordinated expression during CD8$^+$ T cell activation and differentiation. Id2 and Id3 levels are downregulated upon CD8$^+$ T cell stimulation, coincident with an increase in E protein DNA-binding activity. Id2 expression increases at the peak of infection, promoting survival and terminal differentiation. Conversely, Id3 expression is downregulated at the peak of infection, but increases to mark memory precursors and maintain the long-lived memory pool.

Both Id3-deficient and Id2-deficient cells showed defective $CD8^+$ T responses, failing to generate long-lived memory cells when Id3 deficient, or short-lived effector/memory cells when Id2 deficient. Using novel Id2-YFP and Id3-GFP knock-in reporter mouse lines generated in our lab, we found that $CD8^+$ effector cells expressing high levels of Id3-GFP and intermediate levels of Id2-YFP preferentially differentiated into $KLRG1^{lo}CD127^{hi}$ memory precursors, survived longer, and responded better to secondary challenge compared to effector cells that remained Id3-GFP^{lo}Id2-YFP^{hi} [111]. Strikingly, the Id3-GFP^{hi}Id2-YFP^{int} effector population exhibited a similar transcriptional gene-expression profile to long-lived memory cells, prior to surface expression of known markers of $CD8^+$ memory.

E2A expression is upregulated by $CD8^+$ T cells upon activation, and increased E protein DNA-binding activity is observed in antigen-specific $CD8^+$ T cells early during infection (Figure 1.3) [25]. Deletion of E2A, E2-2, or HEB had minimal effects on the expansion and phenotype of $CD8^+$ T cells responding to infection, indicating compensatory functions between E proteins in this context. However, deficiency in both E2A and HEB resulted in an increased frequency of $KLRG1^{hi}$ terminally-differentiated effectors [25]. Activated $CD8^+$ T cells lacking E proteins exhibited altered gene-expression profiles with upregulation of genes linked to early effector populations and activation (CD28, Lymphocyte activation gene 3 (Lag3)) and a downregulation of genes associated with memory formation (*Il7r, Eomes*) [25]. The genes identified to be differentially regulated upon loss of E proteins also possessed E2A-bound E-box sites in close proximity to their transcriptional start site (TSS), strongly suggesting direct regulation by E proteins [61]. Overall, these studies suggest that E proteins regulate transcription factors, cell-surface

markers, and cytokine signaling early during CD8$^+$ T cell activation to support memory-precursor formation [25].

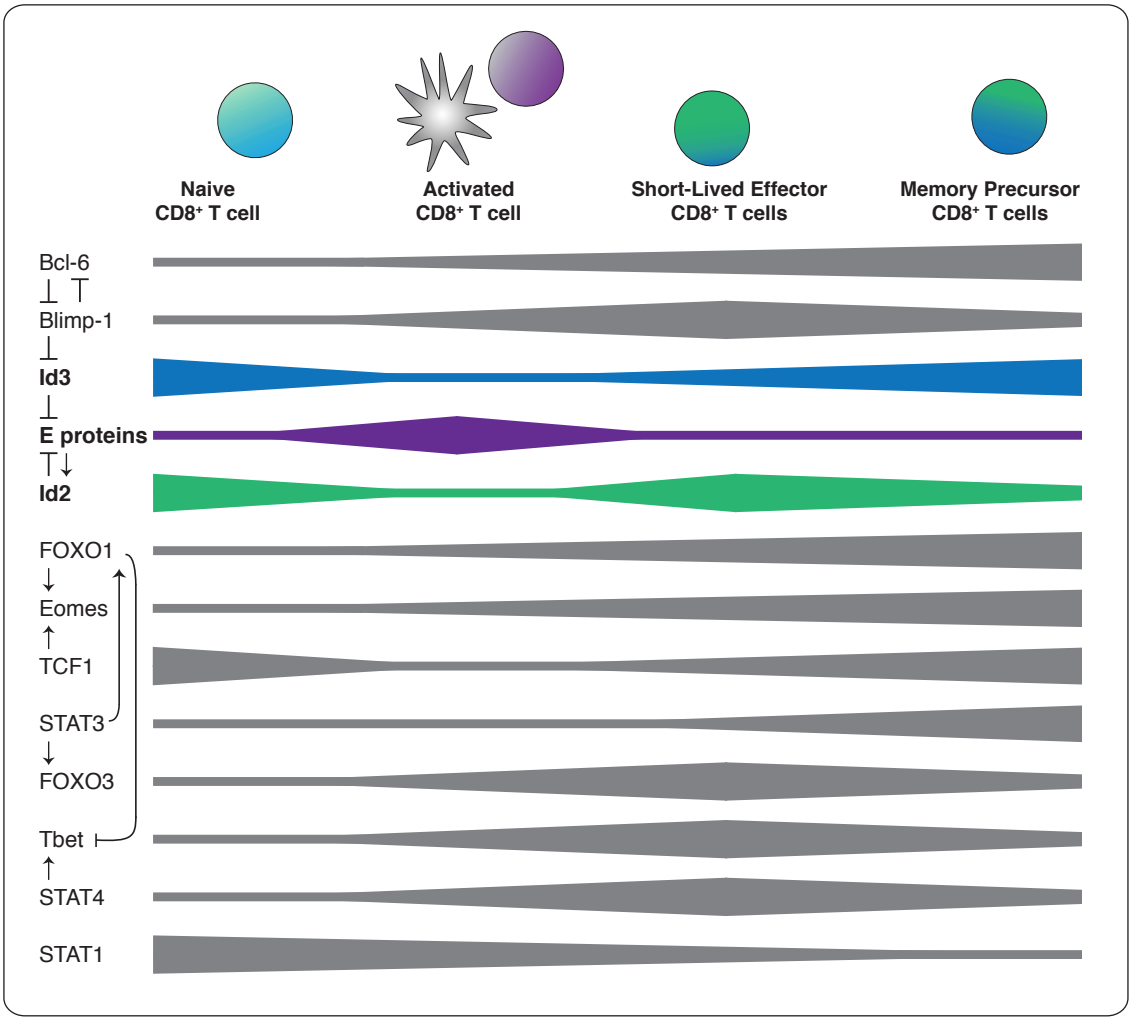


Figure 1.4: Interplay of transcription factor networks during CD8$^+$ T cell activation and differentiation. Width of bar indicates transcription factor activity and/or expression.

Recent studies have provided a further link between E proteins and the regulation of other transcription factors central to cell-fate decisions of CD8$^+$ T cells (Figure 1.4). E proteins are known to directly impact the expression of the Foxo transcription factors [61, 107]. Interestingly, two family members, Foxo1 and Foxo3,

have been recognized as important to memory formation [40, 100, 102]. While Foxo1 appears to be a key mediator of $CD8^+$ T cells differentiating into long-lived memory cells [40], Foxo3 is suggested to function during the contraction phase of the T cell response [100, 102, 2]. E proteins have also been shown to regulate *Tcf-1* expression, a factor central in $CD8^+$ T cell immunity [68, 44]. TCF-1, also a likely E protein target, was shown to control Eomes expression and the differentiation, maintenance and function of $CD8^+$ T cell memory [116]. From these studies, it is clear that E proteins play an important role in orchestrating the transcriptional network necessary for the generation of productive $CD8^+$ T cell memory.

The regulation of Id2 and Id3 expression and their relative levels over time within a responding T cell is likely a major determinant of its fate. Early during infection, E protein expression is upregulated, coinciding with downregulation of Id2 and Id3. This allows E proteins to drive target gene expression, inducing formation of memory precursors [10, 111, 46, 25]. As the T cell response peaks, Id2 protein levels increase, possibly induced through cytokine signaling. E protein activity is then inhibited, permitting the survival and differentiation of late $CD8^+$ effector cells [68, 25]. Reciprocally, cells expressing Id3 and lower levels of Id2 are memory precursors (Figure 1.3). Although both Id2 and Id3 are thought to similarly function by repressing E protein activity, there is a clear distinction in the role they play in $CD8^+$ T cell differentiation.

1.2.2 E and Id proteins in $CD4^+$ T cells

Unlike $CD8^+$ T cells, where memory subsets have been defined in substantial detail, the gene-expression and phenotypic changes that $CD4^+$ effector T cells

undergo during memory formation is less clear. To address this, I performed a microarray analysis of polyclonal antigen-specific $CD4^+$ T cells at days 7 and 30 after LCMV infection (Figure 1.5). I compared my results to $CD8^+$ T cell data generated from the Immunological Genome Project (Immgen) on days 6 (effector) and 45 (memory) of Vesicular stomatitis virus (Vsv)-OVA infection [6]. Interestingly, there was significant similarity in the transcriptional profile of $CD4^+$ and $CD8^+$ effector and memory T cells (Figure 1.5).

This supports the idea that while E and Id proteins play a role in $CD8^+$ T cell differentiation, they may also be important in $CD4^+$ T cell differentiation. Since effector $CD4^+$ T cells can differentiate into multiple T_H populations, additional complexity exists and analysis of memory formation from each effector subset needs to be performed. As in the case of $CD8^+$ T cell responses, relative Id2 and Id3 levels may act as novel markers of early $CD4^+$ T cell memory-precursors, in addition to regulating gene-expression programs that govern effector versus memory cell formation. It was demonstrated that Id2 was highly expressed in the T_H1 population, whereas Id3 transcript was almost exclusively expressed in the T_{FH} population after infection [18]. Recently, studies have indicated roles for Id2, Id3 and E proteins in $CD4^+$ T cell differentiation and maintenance, particularly in regulatory T cells (T_{REG}) and the T_H17 subset of helper T cells [32, 66, 75, 63]. Deletion of E proteins leads to an increase in the differentiation of T_{REG} cells, and Id2 and Id3 are required for $Foxp3^+$ T_{REG} cells to suppress inflammatory disease. However, in the absence of Id2 and Id3, both conventional T_{REG} cells and follicular T_{REG} (T_{FR}) cells have defects in localization and maintenance [75]. Id3 has also been implicated in the TGFβ1-dependent reciprocal regulation of T_{REG} and T_H17

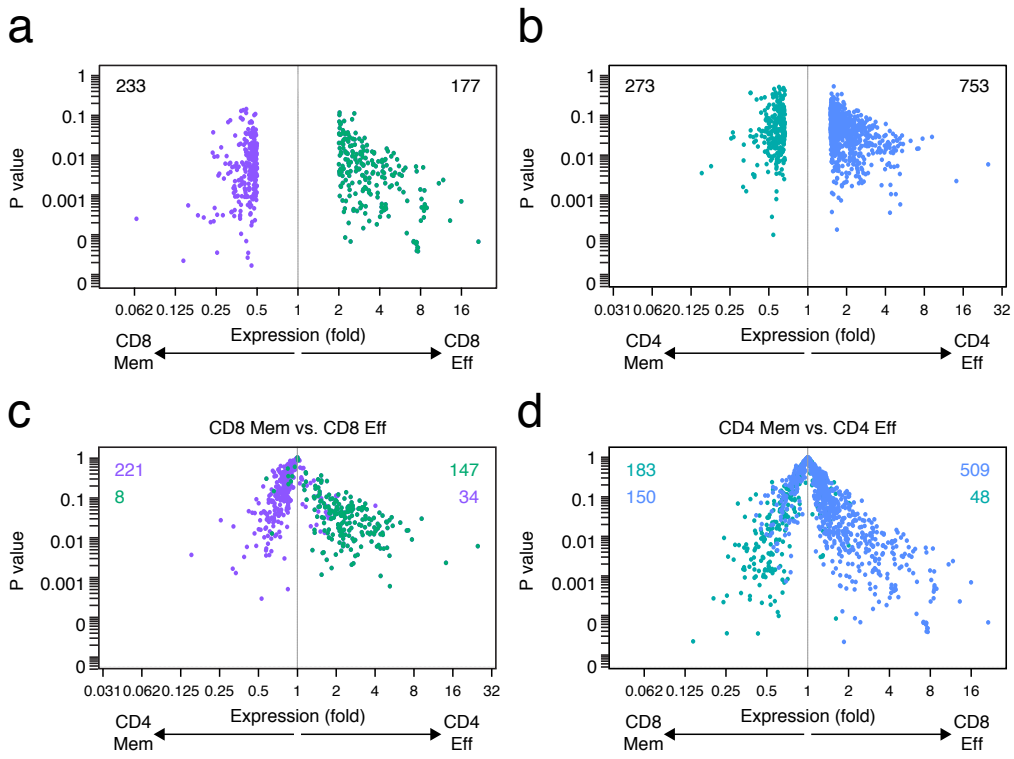


Figure 1.5: Transcriptional profiles of effector and memory T cells. Microarray analysis of gene expression in memory $CD8^+$ T cells versus effector $CD8^+$ T cells following Vsv-OVA infection (**a**) or memory $CD4^+$ T cells cells versus effector $CD4^+$ T cells following LCMV infection (**b**), among genes with a difference in expression of more than 2-fold (CD8) or 1.5-fold (CD4), a coefficient of variation of $\leq$0.10 and an expression value of $\geq$40: colors indicate genes upregulated in memory $CD8^+$ T cells relative to their expression in effector $CD8^+$ T cells (purple) or vice versa (green) (**a**) or genes upregulated in memory $CD4^+$ T cells relative to their expression in effector $CD4^+$ T cells (teal) or vice versa (blue) (**b**). (**c**) Expression of memory $CD8^+$ T cell-associated genes (purple) or effector $CD8^+$ T cell-associated genes (green) (upregulated in (**a**), assessed in memory $CD4^+$ T cells and effector $CD4^+$ T cells (horizontal axis) and plotted against P value (vertical axis). Numbers in corners indicate total number of genes upregulated in memory $CD4^+$ T cells (top left) or effector $CD4^+$ T cells (top right). (**d**) Expression of memory $CD4^+$ T cell-associated genes (teal) or effector $CD4^+$ T cell-associated genes (blue) (upregulated in (**b**), assessed in memory $CD8^+$ T cells and effector $CD8^+$ T cells (horizontal axis) and plotted against *P* value (vertical axis). Numbers in corners indicate total number of genes upregulated in memory $CD8^+$ T cells (top left) or effector CD8+ T cells (top right). Data are representative of two (CD4) or three (CD8) independent experiments with n = 5 mice per group in each.

development [66, 7, 115]. Another study demonstrated that Id3-deficiency resulted in aberrant formation of effector-memory-like $CD4^+$ T cells, suggesting that Id3 is important for the maintenance of the naive state. Furthermore, Id3-deficiency led to the upregulation of T_{FH} markers at the RNA (Bcl6) and protein level (CXCR5, ICOS, and PD-1), as well as elevated IFNγ and IL-4 production following stimulation [76]. Id2 and Id3 have also been examined in models of $CD4^+$ T cell-mediated autoimmunity. Id2 was shown to be an important factor in the development of a murine experimental autoimmune encephalomyelitis (EAE), where the most encephalitogenic $CD4^+$ T cells expressed high levels of Id2, and Id2-deficient $CD4^+$ T cells were unable to mount a functional Th17 response [63]. Id2 also appeared to be important in mediating cytokine production in this system by regulating expression of the repressor SOCS3 [63]. These studies suggest that the balance between Id2 and Id3 will also be important in the fate decisions of $CD4^+$ T cells as they respond to infection and differentiate into distinct effector and memory populations.

1.3 Discussion

Immunological memory mediated by adaptive immunity ensures that, once infected by a particular virus or bacteria, individuals are generally protected from a second encounter with that same pathogen. This ability of lymphocytes to 'remember' is the basis for protection following vaccination. It is known that E protein transcription factors and their inhibitors, Id proteins, operate to balance expression of genes that control $CD8^+$ T cell differentiation during these processes. However, the signaling pathways and molecular mechanisms that regulate the formation and

maintenance of different effector and memory $CD4^+$ T cell lineages are not fully established. In this thesis, I will investigate the role of Id2 and Id3 in promoting the generation and survival of effector and memory populations, as well as their reciprocal roles in shaping the overall $CD4^+$ T cell response to infection.

Chapter 1, in part, is a reprint of the material as it appears in Current Opinion in Immunology. Omilusik KD, **Shaw LA**, Goldrath AW. *Remembering one's ID/E-ntity: E/ID protein regulation of T cell memory*, Current Opin Immunol, Volume 25, Issue 5, 2013. *The thesis author was a primary author of this paper.

Chapter 2

Id2 reinforces T_H1 differentiation and inhibits E2A to repress T_{FH} differentiation

2.1 Introduction

The recognition of a pathogen by the immune system initiates a multi-step transcriptional program that directs the differentiation of $CD4^+$ T cells into distinct helper T cell populations that coordinate the eradication of infection. T_H1 effector cells secrete inflammatory cytokines and activate immune cells [117]. Follicular helper T cells (T_{FH} cells) secrete cytokines and upregulate the expression of ligands that induce B cells to form germinal centers (GCs), undergo class switching and generate high-affinity antibodies [20]. The differentiation of $CD4^+$ T cells is directed by cytokine-induced activation of members of the STAT family of transcription factors and lineage-determining transcription factors such as T-bet and the tran-

scriptional repressor Bcl6 [104]. After being activated, T_H1 cells receive signals that initiate T-bet expression and induce migration of the cells from the lymphoid tissues to infected or inflamed areas of the body [117]. In contrast, for proper differentiation, T_{FH} cells must upregulate expression of Bcl6 and the chemokine receptor CXCR5 to allow their movement from the T cell zone into the B cell follicle [20]. The differentiation of T_H1 cells and T_{FH} cells is interconnected through antagonistic interplay between the transcription factors T-bet and Bcl6, and Bcl6 and Blimp-1 [84, 112, 80, 85, 48].

E-protein transcription factors and their natural repressors, the Id ('inhibitor of DNA binding') proteins, have a crucial role in the differentiation of various lymphocyte populations, such as B cells, innate lymphoid cells, natural killer cells, invariant natural killer T cells, $\alpha\beta$ T cells, $\gamma\delta$ T cells and $CD8^+$ effector and memory T cells [10, 29, 27, 49, 30, 111, 25, 68, 46]. Published studies have highlighted the roles of Id2, Id3 and E proteins in mature $CD4^+$ T cells, particularly in the differentiation and maintenance of regulatory T cells (T_{REG} cells) and the T_H17 subset of helper T cells [32, 66, 75, 63]. Deletion of E proteins leads to an increase in the differentiation of T_{REG} cells; however, deletion of Id2 and Id3 cripples the differentiation and localization of $Foxp3^+$ T_{REG} cells [32, 75]. Additionally, Id2-deficient $CD4^+$ T cells have been shown to be unable to mount a robust T_H17 response in a mouse model of experimental autoimmune encephalomyelitis [63]. Ectopically expressed basic helix-loop-helix transcription factor Ascl2 binds E-box sites to drive upregulation of CXCR5 expression *in vitro*, which results in augmented accumulation of $CD4^+$ T cells in the B cell follicle *in vivo* [64]. However, Ascl2 does not induce Bcl6 expression, which raises the question of how E-protein activity and induction of the expression

of CXCR5 and Bcl6 are interrelated. Furthermore, there is differential expression of Id2 mRNA and Id3 mRNA in T_H1 cells and T_{FH} cells [18]. Thus, we further explored the biology of Id2 and Id3 in the differentiation of T_H1 cells and T_{FH} cells during infection.

2.2 Results

2.2.1 Expression of Id2 defines effector T_H1 cells

We assessed the abundance of Id2 in $CD4^+$ T cell subsets through the use of reporter mice in which cDNA encoding yellow fluorescent protein (YFP) inserted into *Id2* [111] (for the expression of Id2-YFP). We crossed these to SMARTA mice (which have transgenic expression of an MHCII I-Ab-restricted T cell antigen receptor (TCR) specific for lymphocytic choriomeningitis virus (LCMV) glycoprotein (amino acids 66-77) to generate $Id2^{YFP/+}$ SMARTA $CD4^+$ T cells, which we transferred into C57BL/6 (B6) hosts that we then infected with LCMV Armstrong strain. We assessed the differentiation of T_H1 cells and T_{FH} cells among Id2-YFP^{lo} and Id2-YFP^{hi} subsets following infection. In parallel, we infected $Id2^{YFP/+}$ mice with LCMV to monitor the differentiation of polyclonal $CD4^+$ T cells. We observed that Id2-YFP^{lo} cells were almost exclusively T_{FH} cells ($CXCR5^+SLAM^{lo}$ or $CXCR5^+PD\text{-}1^{lo}$) and GC T_{FH} cells ($CXCR5^+PD\text{-}1^+$), while the vast majority of Id2-YFP^{hi} cells displayed a T_H1 phenotype ($SLAM^+CXCR5^-$ or $CXCR5^-PD\text{-}1^-$) (Figure 2.1a and data not shown). Histology revealed that many of the Id2-YFP-expressing $CD4^+$ T cells were excluded from the B cell follicle and GC (Figure 2.1b). Our results demonstrated contrasting expression patterns of Id2 in T_H1 cells and T_{FH} cells

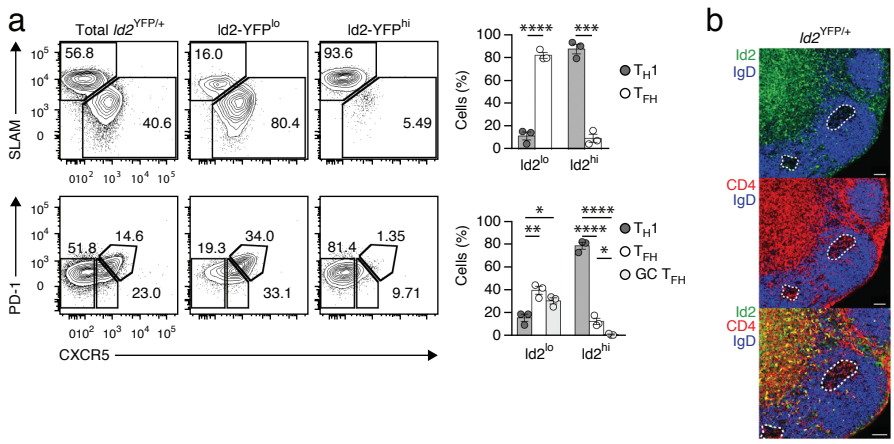


Figure 2.1: Id2 expression defines T_H1 cell subsets.
(**a**) Flow cytometry of donor cells from B6 host mice given *Id2*$^{YFP/+}$ SMARTA CD4$^+$ T cells, followed by infection of the host mice with LCMV and analysis 7 days later. Numbers indicate percent SLAM$^+$CXCR5$^-$ (T_H1) cells (top left) or SLAM^{lo}CXCR5$^+$ (T_{FH}) cells (bottom right) (top row), or CXCR5$^-$PD-1$^-$ (T_H1) cells (bottom left), CXCR5$^+$PD-1$^-$ (T_{FH}) cells (bottom right) or CXCR5$^+$ PD-1$^+$ (GC T_{FH}) cells (top right) (bottom row) among the populations above plots. Right, quantification of results at left. (**b**) Microscopy of sections of draining lymph nodes from *Id2*$^{YFP/+}$ mice 13 days after subcutaneous immunization with phycoerythrin emulsified in immunoadjuvant, showing nodes stained for IgD (blue) and CD4 (red), as well as the Id2-YFP reporter (green). Each symbol represents an individual mouse. **P* <0.05, ***P* <0.01, ****P* <0.001 and *****P* <0.0001 (two-tailed unpaired Student′ s t test). Data are representative of three experiments with n = 3 mice per group (**a**; mean ± s.e.m.) or two experiments with n = 2 mice per group (**b**).

following acute infection with LCMV.

2.2.2 Impaired *Id2* expression enhances T_{FH} differentiation

To determine if differential *Id2* expression in CD4$^+$ T cells influenced the differentiation of CD4$^+$ T cells *in vivo*, we transduced SMARTA CD4$^+$ T cells with retrovirus carrying microRNA-adapted short hairpin RNA (shRNA) specific for *Id2* (sh*Id2*) or a control microRNA-adapted short hairpin RNA (shCtrl), transferred the cells into B6 mice and analyzed T cell differentiation after infection of the host mice with LCMV. Expression of sh*Id2* in SMARTA CD4$^+$ T cells reduced the expression

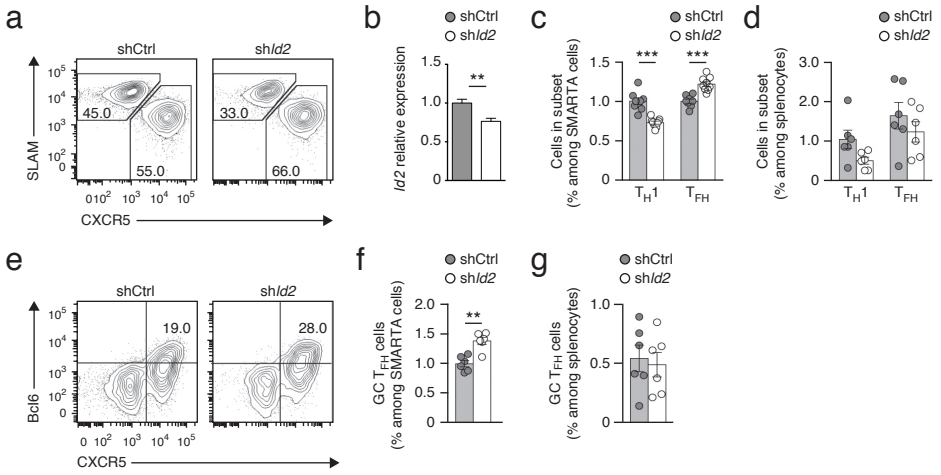


Figure 2.2: Knockdown of Id2 results in enhanced T_{FH} differentiation. (**a**) Flow cytometry of cells from B6 host mice given SMARTA $CD4^+$ T cells transduced with shCtrl or sh*Id2* (above plots), followed by infection of the host mice with LCMV and analysis 6 days after infection. Numbers in outlined areas indicate percent $SLAM^+CXCR5^-$ (T_H1) cells (top left) or $SLAM^{lo}CXCR5^+$ (T_{FH}) cells (bottom right). (**b**) RNA was isolated from shRNAmir-RV^+ $CD4^+$ T cells and *Id2* expression was determined by qRTPCR. (**c,d**) Frequency of T_H1 cells or T_{FH} cells among SMARTA $CD4^+$ T cells (**c**) or total splenocytes (**d**) as in **a**; results in **c** are normalized to the average for mice given $shCtrl^+$ cells. (**e**) Flow cytometry of cells from B6 host mice as in **a**. Numbers indicate percent $CXCR5^+Bcl6^+$ (GC T_{FH}) cells. (**f,g**) Frequency of GC T_{FH} cells among SMARTA $CD4^+$ T cells (**f**) or total splenocytes (**g**) as in **e** (results in **f** normalized as in **c**). Each symbol represents an individual mouse. $^*P$ <0.05, $^{**}P$ <0.001 and $^{***}P$ <0.0001 (two-tailed unpaired Student′ t test). Data are pooled from two (**a**) four (**b-g**) independent experiments with n = 6-14 mice per group (mean ± s.e.m.) Experiments performed in collaboration with Dr. Simon Bélanger.

of Id2 mRNA (Figure 2.2a).

There was a greater frequency of T_{FH} cells and lower frequency of T_H1 cells among cells expressing sh*Id2* (sh$Id2^+$ cells) than among those expressing shCtrl ($shCtrl^+$ cells) (Figure 2.2b-d). The bias was attributed predominantly to GC T_{FH} cells, identified as $CXCR5^+Bcl6^+$ cells (Figure 2.2e-g). We then assessed the differentiation of sh$Id2^+$ cells earlier after infection (Figure 2.3a) and observed a greater frequency of $CXCR5^+Bcl6^+$ T_{FH} cells and a smaller population of $CXCR5^-Bcl6^-$ T_H1

cells among sh*Id2*$^+$ cells than among shCtrl$^+$ cells (Figure 2.3b-d). Analysis of early T_{FH} cells (CXCR5$^+$CD25$^-$)[17, 16] also revealed a greater proportion among sh*Id2*$^+$ cells than among shCtrl$^+$ cells (Figure 2.3e-g). Thus, impaired Id2 expression seemed to favor T_{FH} differentiation. Analysis of early T_{FH} cells (CXCR5$^+$CD25$^-$)[17, 16] also revealed a greater proportion among sh*Id2*$^+$ cells than among shCtrl$^+$ cells (Figure 2.3e-g). Thus, impaired Id2 expression seemed to favor T_{FH} differentiation.

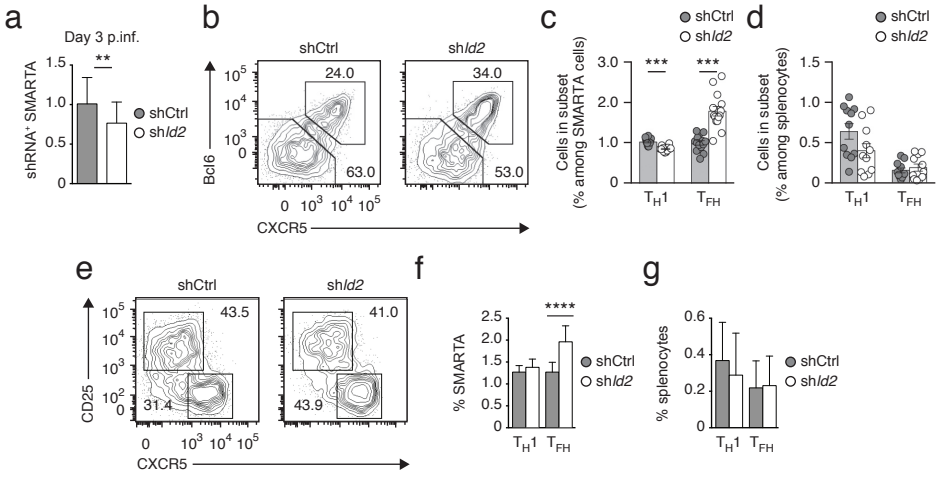


Figure 2.3: Knockdown of Id2 results in enhanced early T_{FH} differentiation. Flow cytometry of cells from B6 host mice given SMARTA CD4$^+$ T cells transduced with shCtrl or sh*Id2*, followed by infection of the host mice with LCMV and analysis 3 days after infection. (**a**) Quantitation of shRNA$^+$ SMARTA CD4$^+$ T cells. (**b**) Numbers indicate percent Bcl6$^+$CXCR5$^+$(T_{FH}) cells (top right) or Bcl6$^-$CXCR5$^-$ (T_H1) cells (bottom left). (**c,d**) Frequency of T_H1 cells or T_{FH} cells among SMARTA CD4$^+$ T cells (**c**) or total splenocytes (**d**) as in **b**. (**e-g**) T_{FH} (CXCR5$^+$CD25$^-$) and T_H1 (CD25$^+$CXCR5$^-$) differentiation was analyzed by flow cytometry (**e**) and quantified as a fraction of SMARTA CD4$^+$ T cells (**f**) or total splenocytes (**g**). Each symbol represents an individual mouse. $^{**}P$ <0.001 and $^{***}P$ <0.0001 (two-tailed unpaired Student′ t test). Data are pooled from two (**a**) or five (**c-g**) independent experiments with n = 6-14 mice per group (mean ± s.e.m.) Experiments performed in collaboration with Dr. Simon Bélanger.

2.2.3 Id2 is needed for T_H1 cell differentiation during infection

We next sought to determine how a total absence of Id2 would affect $CD4^+$ T cell differentiation. We crossed mice with *lox*P-flanked *Id2* alleles ($Id2^{fl/fl}$)[83] to SMARTA mice with transgenic expression of Cre recombinase driven by the promoter of the T cell-specific gene *Cd4* (CD4-Cre) to generate $Id2^{fl/fl}$CD4-Cre^+ SMARTA mice (called ''$Id2^{-/-}$ mice' here), in which *Id2* was deleted in αβ thymocytes. Naive $Id2^{-/-}$ cells were $CD44^{lo}$ and were indistinguishable from cells from naive $Id2^{+/+}$CD4-Cre^+ SMARTA mice (called ''$Id2^{+/+}$ mice' here). We transferred cells from $Id2^{+/+}$ or $Id2^{-/-}$ mice into B6 hosts and monitored their differentiation after infection with LCMV. $Id2^{-/-}$ cells did not form a distinct T_H1 population (Figure 2.4a-c).

The loss of T_H1 differentiation by $Id2^{-/-}$ cells was accompanied by decreased expression of granzyme B, T-bet and interferon-γ (IFN-γ) and increased expression of the transcription factor TCF-1 (Figure 2.5a-c). Notably, a prominent $SLAM^{int}CXCR5^{int}$ population emerged among $Id2^{-/-}$ effector $CD4^+$ T cells that was not observed among their $Id2^{+/+}$ counterparts (Figure2.4a,b). This phenotype was also apparent in a polyclonal $CD4^+$ T cell response (data not shown). These results showed that *Id2* was required for the differentiation of T_H1 cells.

To understand the dysregulation of the $Id2^{-/-}$ T_H1 cells, we further characterized the phenotypes of the donor populations in mice that received $Id2^{+/+}$ or $Id2^{-/-}$ cells and were infected with LCMV. $Id2^{+/+}$ and $Id2^{-/-}$ T_H1 cells maintained high expression of the P-selectin glycoprotein ligand PSGL-1 and the cytokine receptor chain IL-2Rα, which both need to be downregulated for proper T_{FH} differentiation [17, 47, 91, 92] (Figure 2.6a,b).

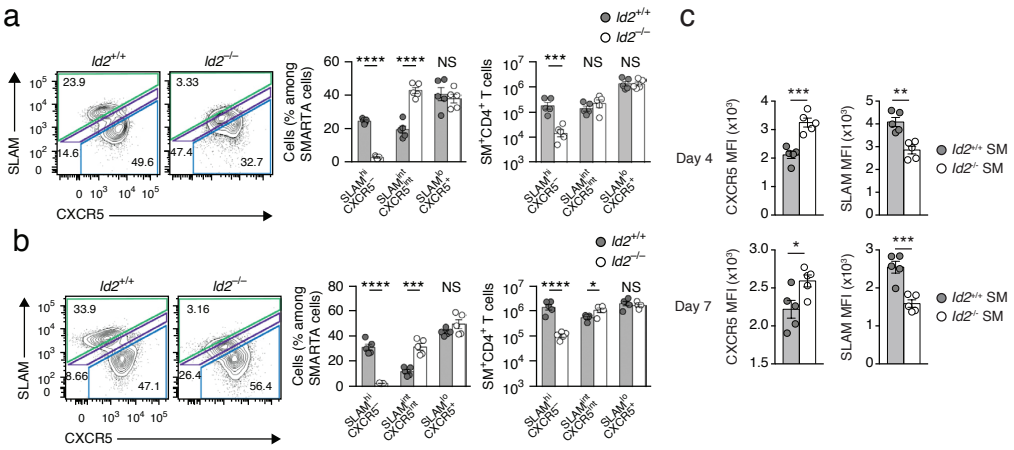


Figure 2.4: Id2 is necessary for the generation of CD4$^+$ T_H1 cells during infection.

(**a,b**) Flow cytometry (left) of donor cells from B6 host mice given *Id2*$^{+/+}$ or *Id2*$^{-/-}$ CD4$^+$ T cells (above plots), followed by infection of the host mice with LCMV and analysis 4 days (**a**) or 7 days (**b**) after infection. Numbers indicate percent SLAM^{hi}CXCR5$^-$ cells (top left; green), SLAM^{int}CXCR5^{int} cells (middle 'slice'; purple) and SLAM^{lo}CXCR5$^+$ cells (bottom right; blue). Right, frequency of SLAM^{hi}CXCR5$^-$, SLAM^{int}CXCR5^{int} and SLAM^{lo}CXCR5$^+$ cells among SMARTA CD4$^+$ T cells (middle right) and total cells of those subsets (far right). (**c**) CXCR5 and SLAM expression quantified as gMFI on days 4 and 7. Each symbol represents an individual mouse. **P* <0.05, ***P* <0.001 and ****P* <0.0001 (two-tailed unpaired Student' t test). Data are pooled from three (**a-c**) independent experiments with n = 10 mice per group (mean ± s.e.m.)

Analysis of the SLAM^{int}CXCR5^{int} population revealed that *Id2*$^{-/-}$ cells shared a partial phenotype with T_H1 cells, including high expression of PSGL-1, Ly6C and IL-2Rα, and were Bcl6^{lo}, in contrast to *Id2*$^{+/+}$ or *Id2*$^{-/-}$ T_{FH} cells, which were Bcl6^{hi} (Figure 2.6a,b). Thus, complete absence of *Id2* affected CD4$^+$ T cells throughout differentiation and permanently disrupted T_H1 cells.

To further characterize the differentiation status of *Id2*$^{-/-}$ CD4$^+$ T cells outside the limitations imposed by two-parameter flow cytometry, and because many of the *Id2*$^{-/-}$ cells could not be unambiguously assigned to either the T_H1 subset or T_{FH} subset on the basis of expression of signaling lymphocytic-activation molecule

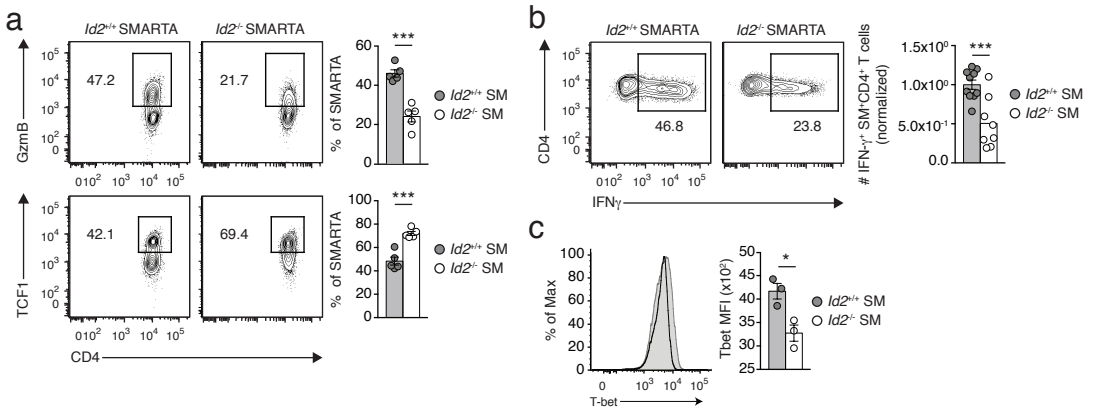


Figure 2.5: Id2 is necessary for the generation of CD4$^+$ T_H1 cells during infection.

(**a-c**) Flow cytometry (left) of donor cells from B6 host mice given *Id2*$^{+/+}$ or *Id2*$^{-/-}$ CD4$^+$ T cells, followed by infection of the host mice with LCMV and analyzed 7 days after infection. (**a**) Flow cytometric analysis of granzyme B and TCF1 expression (left panels), quantification as a frequency of SMARTA CD4$^+$ T cells (right panels). (**b,c**) Flow cytometric analysis of (**b**) IFN-γ and (**c**)T-bet expression in total SMARTA CD4$^+$ T cells. gMFI of T-bet expression and total number of IFN-γ$^+$ SMARTA CD4$^+$ T cells is shown. Each symbol represents an individual mouse. **P* <0.05, *and ****P* <0.0001 (two-tailed unpaired Student′ t test). Data are pooled from three (**a-c**) independent experiments with n = 10 mice per group (mean ± s.e.m.)

(SLAM) or CXCR5, we employed viSNE ('visual interactive stochastic neighbor embedding') multi-parameter clustering, in which the overall position of each cell reflects similarity to neighboring cells or dissimilarity to non-neighboring cells on the basis of expression of the co-receptor CD4, the congenic marker CD45.1, SLAM, CXCR5, Bcl6, TCF-1, the costimulatory molecule PD-1 and T-bet [3]. Among total CD4$^+$ T cells or among SMARTA CD4$^+$ T cells, we observed two 'geographically' distinct populations that uniquely expressed the T_H1 cell marker SLAM or the T_{FH} cell marker Bcl6 (Figure 2.6c). The *Id2*$^{+/+}$ and *Id2*$^{-/-}$ T_{FH} populations were similar in location and appearance (Figure 2.6c). However, *Id2*$^{-/-}$ T_H1 cells were located outside the T_H1 multiparameter gate defined by *Id2*$^{+/+}$ T_H1 cells (Figure 2.6c), which further suggested that Id2 was required for proper T_H1 differentiation.

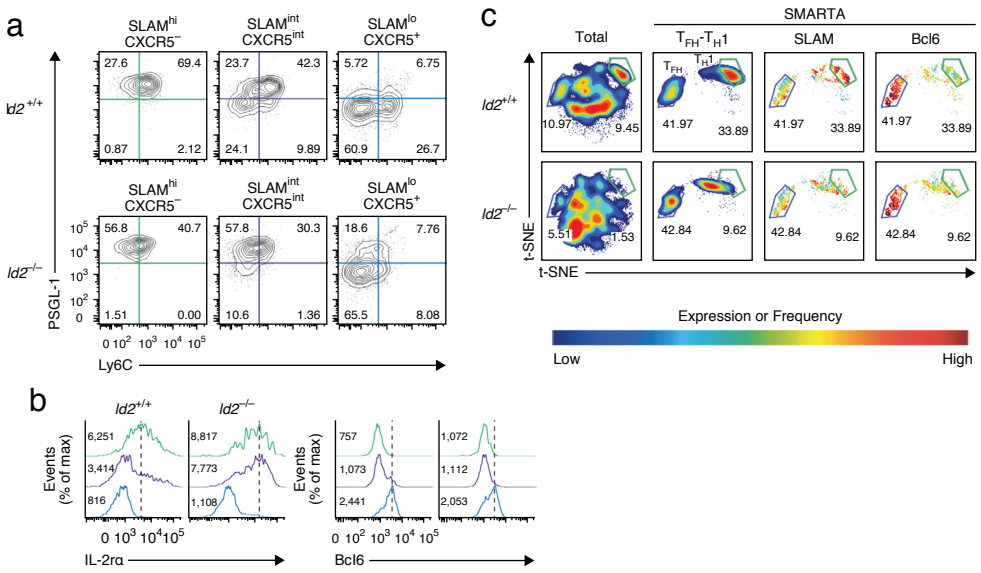


Figure 2.6: Id2 is necessary for the generation of CD4$^+$ T_H1 cells during infection.
(**a,b**) Expression of PSGL-1 and Ly6C (**a**) and IL-2Rα or Bcl6 (**b**) on cell subsets in mice as in **Figure 2.4a**. (**c**) viSNE analysis of the overall similarity of total splenic CD4$^+$ T cells (far left) or SMARTA CD4$^+$ T cells (right) and expression of SLAM and Bcl6 in SMARTA CD4$^+$ T cells (right) in mice as in **Figure 2.4a**. Numbers indicate percent cells in each. Data are representative of three experiments with n = 5-10 mice per group in each (mean ± s.e.m.).

We then assessed the ability of *Id2*$^{-/-}$ cells to support B cell responses. We transferred *Id2*$^{+/+}$ or *Id2*$^{-/-}$ cells into *Bcl6*$^{fl/fl}$CD4-Cre$^+$ mice and infected the host mice with LCMV; at 8 days after infection, we observed a greater frequency of *Id2*$^{-/-}$ T_{FH} cells than *Id2*$^{+/+}$ T_{FH} cells, but the numbers of these cells were similar (Figure 2.7a). The frequency of plasma cells and titers of anti-LCMV IgG in the serum were similar in these groups of mice (Figure 2.7b, data not shown). However, GC B cell development was impaired in the mice that received *Id2*$^{-/-}$ cells (Figure 2.7c), which suggested that the *Id2*$^{-/-}$ T_{FH} cells might have had impaired function.

To assess the defect of T_H1 differentiation in Id2-deficient cells, we employed a model of infection with *Toxoplasma gondii*, as the role of IFN-γ-mediated T_H1

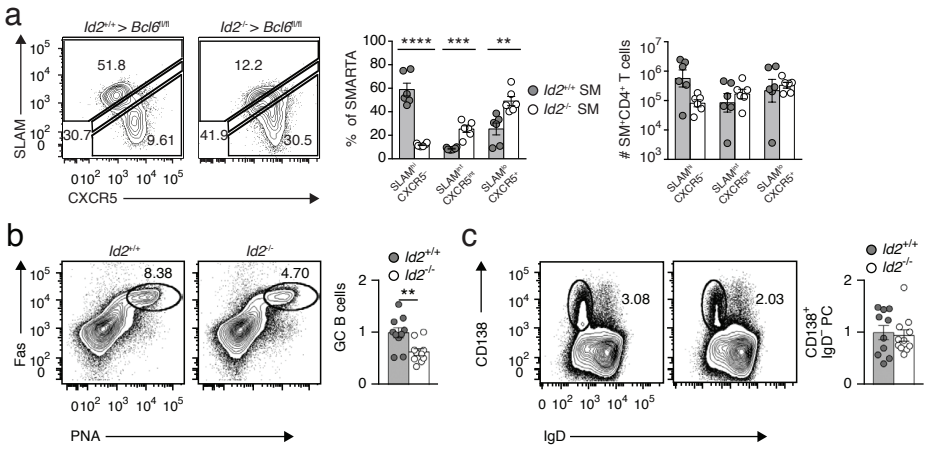


Figure 2.7: Id2-deficient CD4$^+$ T cells are not more proficient at B cell help. (**a**) *Id2*$^{+/+}$CD4-Cre$^+$ or *Id2*$^{fl/fl}$CD4-Cre$^+$ SMARTA CD4$^+$ T cells were transferred into *Bcl6*$^{fl/fl}$ CD4-Cre$^+$ mice and analyzed 8 days after LCMV infection. SLAM^{hi}CXCR5$^-$, SLAM^{int}CXCR5^{int} and SLAM^{lo}CXCR5$^+$ cells were analyzed by flow cytometry (left panels) and quantified as a frequency of SMARTA CD4$^+$ T cells (middle panel) or as total numbers (right panel). **(**b,c) Flow cytometry of cells from *Bcl6*$^{fl/fl}$CD4-Cre$^+$ host mice given *Id2*$^{+/+}$ or *Id2*$^{-/-}$ CD4$^+$ T cells, followed by infection of the host mice with LCMV and analysis 8 days after infection. Numbers indicate percent (**b**) Fas$^+$PNA$^+$ (GC B) cells or (**c**) CD138$^+$IgD$^-$ (plasma) cells. Right, frequency of cells at left (results normalized to those of recipients of *Id2*$^{+/+}$ cells). Each symbol represents an individual mouse. $^{**}P$ <0.01, $^{***}P$ <0.001, $^{****}P$ <0.0001 (two-tailed unpaired Student's t test). Data are representative of three (**a**) independent experiments with n=3-10 mice per group (mean ± s.e.m.), or are pooled from two independent experiments with n = 10 mice per group in each (**b,c**; mean ± s.e.m.).

responses in long-term resistance to this pathogen and control of infection with this pathogen is well established [98]. After infection with *T. gondii*, CD4$^+$ T cells from the lamina propria of the small intestine of *Id2*$^{fl/fl}$CD4-Cre$^+$ mice had much lower expression of both IFN-γ and T-bet than that of their *Id2*$^{+/+}$CD4-Cre$^+$ counterparts (Figure 2.8a,b).

No significant alteration in the frequency of Foxp3$^+$ T_{REG} cells could be detected in *Id2*$^{fl/fl}$CD4-Cre$^+$ mice relative to their frequency in *Id2*$^{fl/fl}$CD4-Cre$^+$ mice (Figure 2.8a,b). Thus, in two distinct models of infection, we observed a

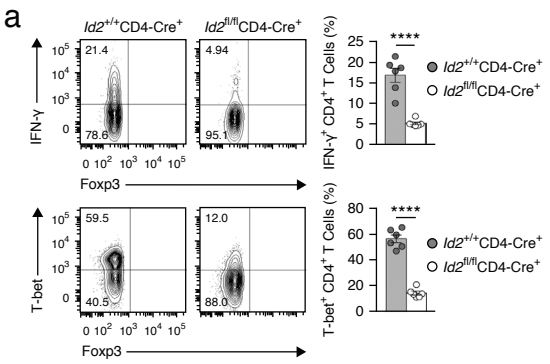


Figure 2.8: Id2 is required to generate a T_H1 response to *Toxoplasma gondii.* (**a**) Flow cytometry of $CD4^+$ T cells from the lamina propria of $Id2^{+/+}$CD4-Cre^+ or $Id2^{fl/fl}$CD4-Cre^+ mice 7 days after infection with *T. gondii*. Numbers in quadrants (left) indicate percent IFN-γ^+Foxp3$^-$ cells or IFN-γ^-Foxp3$^-$ cells (top row), or T-bet$^+$Foxp3$^-$ cells or T-bet$^-$Foxp3$^-$ cells (bottom row). Right, quantification of results at left. Each symbol represents an individual mouse. ****P <0.0001 (two-tailed unpaired Student′s t test). Data are representative of or two experiments with n = 5-10 mice per group in each (mean ± s.e.m.) Experiments performed in collaboration with Dr. Sunglim Cho.

substantial loss of effector T_H1 cells.

2.2.4 Altered expression of key helper T cell genes after loss of Id2

To understand how Id2 affects the differentiation of T_H1 cells and T_{FH} cells, we studied the global transcriptional changes in $CD4^+$ T cells that resulted from *Id2* deficiency. $Id2^{+/+}$ and $Id2^{-/-}$ T_H1 cells (encompassing both SLAM^{hi}CXCR5$^-$ and SLAM^{int}CXCR5^{int} populations) and $Id2^{+/+}$ and $Id2^{-/-}$ CXCR5$^+$SLAM^{lo} T_{FH} cells were used for comparative gene-expression profiling (Figure 2.9a).

Differential expression of genes associated with T_H1 cells and T_{FH} cells was confirmed for $Id2^{+/+}$ T_H1 and T_{FH} cells (Figure 2.9b). We then compared gene-expression profiles of $Id2^{+/+}$ and $Id2^{-/-}$ T_H1 cells and observed downregulation of

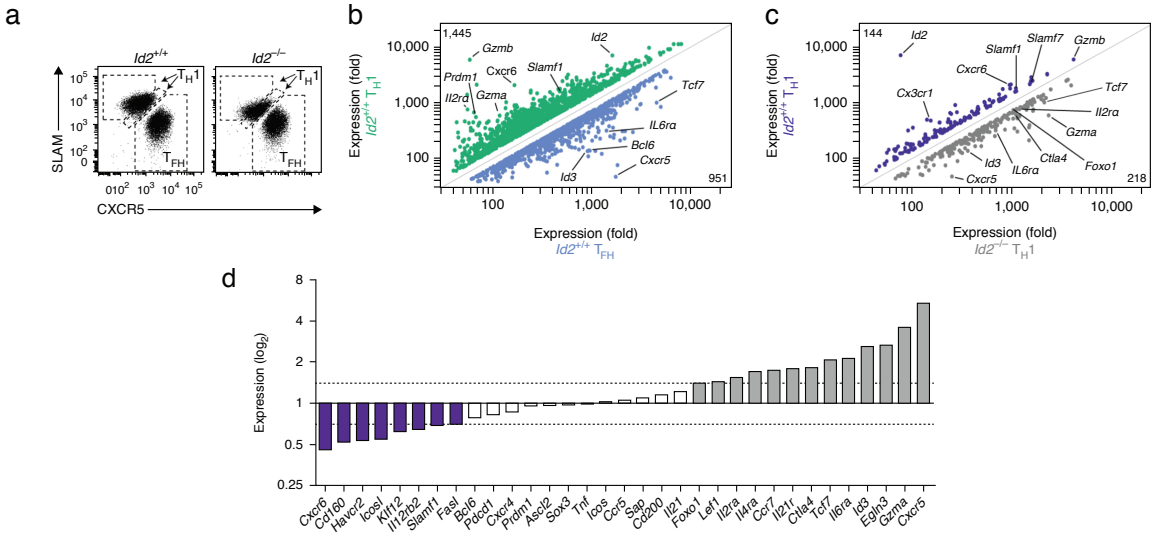


Figure 2.9: Increased E2A binding in the absence of Id2 regulates expression of key helper T cell genes.

(**a**) Flow cytometry of cells from B6 host mice given *Id2*$^{+/+}$ or *Id2*$^{-/-}$ CD4^{+} T cells, followed by infection of the host mice with LCMV and analysis 7 days after infection; outlined areas indicate SLAM^{+}CXCR5^{-} (T_H1) cells or SLAM^{lo}CXCR5^{+} (T_{FH}) cells, sorted for subsequent microarray analysis. (**b,c**) Microarray analysis of gene expression in *Id2*$^{+/+}$ T_H1 cells versus *Id2*$^{+/+}$ T_{FH} cells (**b**) or *Id2*$^{+/+}$ T_H1 cells versus *Id2*$^{-/-}$ T_H1 cells (**c**), among genes with a difference in expression of 1.4-fold or more, a coefficient of variation of $\leq$0.10 and an expression value of $\geq$40: colors indicate genes upregulated 1.4-fold or more in *Id2*$^{+/+}$ T_H1 cells relative to their expression in *Id2*$^{+/+}$ T_{FH} cells (green) or vice versa (blue) (**b**) or genes most downregulated (purple) or upregulated (gray) in *Id2*$^{-/-}$ T_H1 cells relative to their expression in *Id2*$^{+/+}$ T_H1 cells (**c**). Labels in plots indicate genes of general interest in the development of T_H1 cells and T_{FH} cells. (**d**) Microarray analysis of putative E2A-target genes identified by ChIP-Seq; bar colors match dot colors in **c** (white, genes without significantly differential expression). Data are representative of two independent experiments with n = 5 mice per group in each.

T_H1 cell-associated genes (*Gzmb*, *Slamf1* and *Cxcr6*) in the context of Id2 deficiency, while genes associated with the T_{FH} cell program (*Cxcr5*, *Il6ra* and *Tcf7*) were upregulated (Figure 2.9c). However, expression of *Bcl6*, *Ascl2*, *Pdcd1* and *Icos*, which all have high expression by T_{FH} cells and encode products important for T_{FH} differentiation, was not higher in *Id2*$^{-/-}$ T_H1 cells than in *Id2*$^{+/+}$ T_H1 cells (Figure 2.9d), which indicated that E proteins controlled the expression of only a portion of

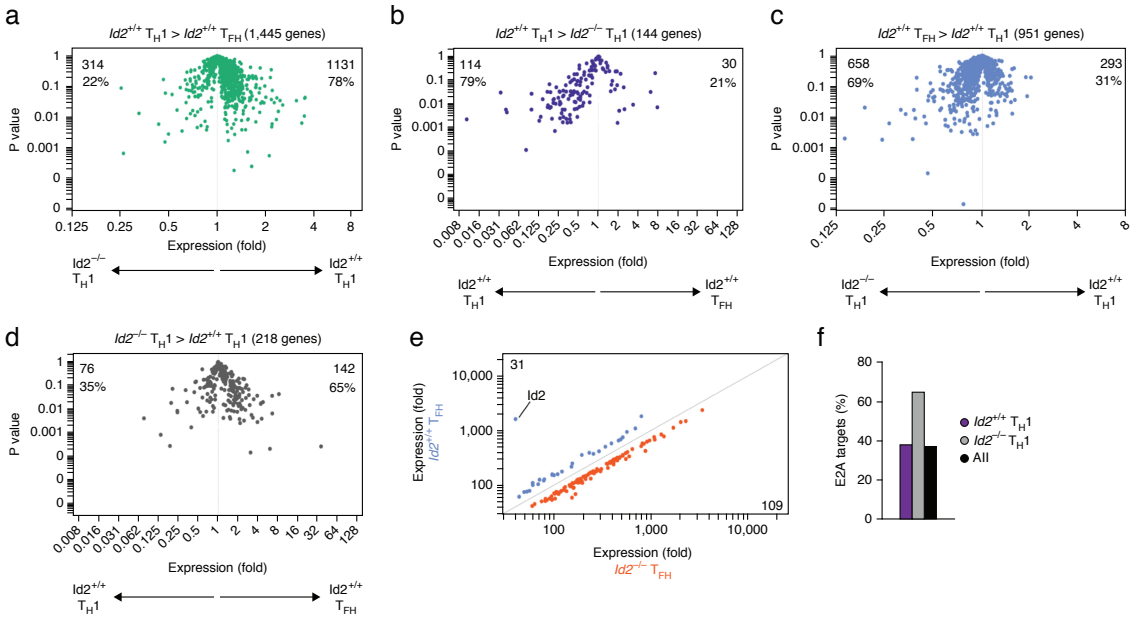


Figure 2.10: Increased E2A binding in the absence of Id2 regulates expression of key helper T cell genes.

(**a**) Expression of 1,445 T_H1 cell-associated genes (upregulated in $Id2^{+/+}$ T_H1 cells versus $Id2^{+/+}$ T_{FH} cells ($Id2^{+/+}$ T_H1 >$Id2^{+/+}$ T_{FH}) in **Figure 2.9b**, assessed in $Id2^{-/-}$ T_H1 cells and $Id2^{+/+}$ T_H1 cells (horizontal axis) and plotted against P value (vertical axis). Numbers in corners indicate total (or percent) of those genes upregulated in $Id2^{+/+}$ T_H1 cells (top left) or $Id2^{-/-}$ T_H1 cells (top right). (**b**) Expression of 144 genes downregulated in $Id2^{-/-}$ T_H1 cells versus $Id2^{+/+}$ T_H1 cells ($Id2^{+/+}$ T_H1 >$Id2^{-/-}$ T_H1) in **Figure 2.9c**, assessed in $Id2^{+/+}$ T_H1 cells and $Id2^{+/+}$ T_{FH} cells and plotted against P value. Numbers in corners indicate total (or percent) of those genes upregulated in $Id2^{+/+}$ T_H1 cells (top left) or $Id2^{+/+}$ T_{FH} cells (top right). (**c**) Expression of 951 T_{FH} cell-associated genes (upregulated in $Id2^{+/+}$ T_{FH} cells versus $Id2^{+/+}$ T_H1 cells ($Id2^{+/+}$ T_{FH} >$Id2^{+/+}$ T_H1) in **Figure 2.9b**, assessed in $Id2^{-/-}$ T_H1 cells and $Id2^{+/+}$ T_H1 cells and plotted against P value; numbers in corners, as in (**a**). (**h**) Expression of 218 genes upregulated in $Id2^{-/-}$ T_H1 cells versus $Id2^{+/+}$ T_H1 cells ($Id2^{-/-}$ T_H1 >$Id2^{+/+}$ T_H1) in **Figure 2.9c**, assessed in $Id2^{+/+}$ T_H1 cells and $Id2^{+/+}$ T_{FH} cells and plotted against P value; numbers in corners, as in **b**. (**e**) Microarray analysis of gene expression in $Id2^{+/+}$ T_{FH} cells versus $Id2^{-/-}$ T_{FH} cells (as in **Figure 2.9b,c**); colors indicate genes upregulated 1.4-fold or more in $Id2^{+/+}$ T_{FH} cells relative to their expression in $Id2^{-/-}$ T_{FH} cells (blue) or vice versa (orange). (**f**) Frequency of genes regulated differentially (change in expression of 1.4-fold or more) in $Id2^{+/+}$ T_H1 cells relative to their expression in $Id2^{-/-}$ T_H1 cells, among genes that are also targets of E2A (as indicated by ChIP-Seq analysis), or 'background' frequency of E2A targets (All). Data are representative of two independent experiments with n = 5 mice per group in each.

the T_{FH} cell signature genes.

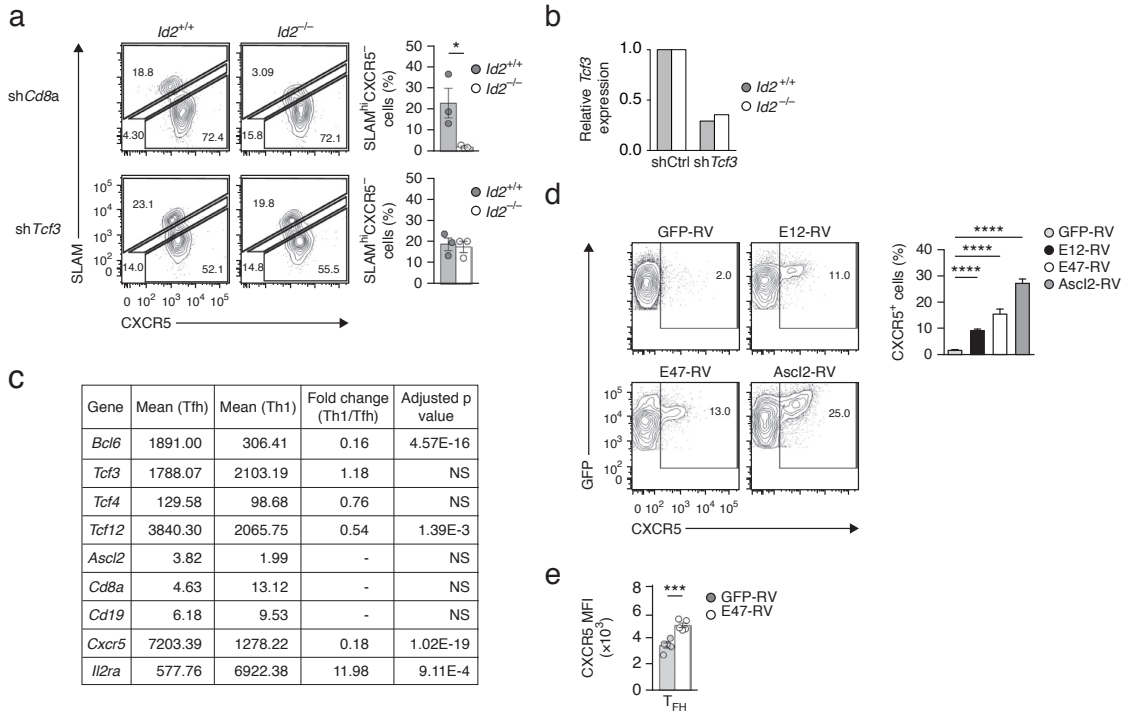
We next characterized the effect of *Id2* deficiency on the expression of T_H1 cell-associated genes. We defined the T_H1 cell gene set as all genes upregulated 1.4-fold or more in *Id2*$^{+/+}$ T_H1 cells relative to their expression in *Id2*$^{+/+}$ T_{FH} cells (Figure 2.9b). *Id2*$^{-/-}$ T_H1 cells had reduced expression of 78% of the T_H1 cell-associated genes (Figure 2.10a). Additionally, of the 144 genes downregulated most substantially in *Id2*$^{-/-}$ T_H1 cells relative to their expression in *Id2*$^{+/+}$ T_H1 cells (Figure 2.9c), 79% had higher expression in *Id2*$^{+/+}$ T_H1 cells than in *Id2*$^{+/+}$ T_{FH} cells (Figure 2.10b). Thus, deletion of *Id2* impaired acquisition of the T_H1 program. We defined the T_{FH} cell gene set as all genes upregulated 1.4-fold or more in *Id2*$^{+/+}$ T_{FH} cells relative to their expression in *Id2*$^{+/+}$ T_H1 cells (Figure 2.9b). *Id2*$^{-/-}$ T_H1 cells uncharacteristically upregulated 69% of the T_{FH} cell-associated genes (Figure 2.10c). Analysis of the genes most upregulated in *Id2*$^{-/-}$ T_H1 cells relative to their expression in *Id2*$^{+/+}$ T_H1 cells (Figure 2.9c) revealed that 65% of these were 'preferentially' expressed in *Id2*$^{+/+}$ T_{FH} cells (Figure 2.10d). These analyses indicated a substantial bias toward the T_{FH} cell gene-expression program in *Id2*$^{-/-}$ T_H1 cells. When the gene expression of *Id2*$^{+/+}$ T_{FH} cells was contrasted with that of *Id2*$^{-/-}$ T_{FH} cells, only 140 genes showed significant differential expression (Figure 2.10e), which indicated that established T_{FH} cells that had lower expression of *Id2* than that of T_H1 cells were moderately affected by *Id2* deficiency. The absence of proper T_H1 development of *Id2*$^{-/-}$ cells suggested that unchecked E2A activity impaired T_H1 differentiation. Analysis of genes expressed differentially in *Id2*$^{+/+}$ T_H1 cells relative to their expression in T_{FH} cells revealed that a larger number of E2A-bound genes were upregulated in *Id2*$^{-/-}$ T_H1 cells than in *Id2*$^{+/+}$ T_H1 cells (Figure 2.9d), consistent

with the inhibition of E2A by Id2. We compared changes in gene expression with a list of genes that are targets of E2A [68] and found that 62% of the genes upregulated in *Id2*$^{-/-}$ T_H1 cells were targets of E2A (Figure 2.10f). These results suggested that Id2 was important for maintenance of the T_H1 cell gene-expression program and that its absence resulted in the acquisition of a partial T_{FH} cell gene-expression program.

2.2.5 E proteins drive CXCR5 expression

Our microarray results suggested that Id2 and E proteins acted together to control CD4$^+$ T cell differentiation in part by regulating CXCR5 and the expression of T_H1 cell effector molecules such as SLAM. We hypothesized that diminished levels of E2A might 'rescue' the defect observed in *Id2*-deficient cells. We transduced *Id2*$^{+/+}$ and *Id2*$^{-/-}$ CD4$^+$ T cells with a retroviral vector encoding shRNA targeting the gene encoding E2A (sh*Tcf3*) or control shRNA (Figure 2.11a,b).

We adoptively transferred the cells into B6 mice infected with LCMV the day before cell transfer and analyzed the differentiation of the transferred cells. As expected, *Id2*$^{-/-}$ cells expressing control shRNA were unable to correctly differentiate into T_H1 cells (Figure 2.11a). However, *Id2*$^{-/-}$ T_H1 cells were 'rescued' by sh*Tcf3* expression, and their defects in the expression of SLAM and that of CXCR5 were both corrected (Figure 2.11a). Thus, the defective T_H1 differentiation we observed in the absence of Id2 was the result of increased activity of E proteins. The E-box-binding transcription factor Ascl2 has been shown to drive robust T_{FH} differentiation by inducing CXCR5 expression when overexpressed in CD4$^+$ T cells [64]. The E-protein-encoding genes *Tcf3* and *Tcf12* (which encodes HEB) both had



Gene	Mean (Tfh)	Mean (Th1)	Fold change (Th1/Tfh)	Adjusted p value
Bcl6	1891.00	306.41	0.16	4.57E-16
Tcf3	1788.07	2103.19	1.18	NS
Tcf4	129.58	98.68	0.76	NS
Tcf12	3840.30	2065.75	0.54	1.39E-3
Ascl2	3.82	1.99	-	NS
Cd8a	4.63	13.12	-	NS
Cd19	6.18	9.53	-	NS
Cxcr5	7203.39	1278.22	0.18	1.02E-19
Il2ra	577.76	6922.38	11.98	9.11E-4

Figure 2.11: E proteins drive CXCR5 expression and inhibit the formation of T_H1 cells.

(**a**) Flow cytometry (left) of donor cells from B6 host mice given *Id2*$^{+/+}$ or *Id2*$^{-/-}$ CD4$^+$ T cells (above plots) transduced with control shRNA targeting the gene encoding CD8α (sh*Cd8*a) or with sh*Tcf3* (left margin) followed by infection of the host mice with LCMV and analysis 7 days after infection. Right, quantification of results at left. (**b**) Graph indicates relative mRNA expression of *Tcf3* by dsRED$^+$ SMARTA CD4$^+$ T cells. (**c**) Gene espression of E proteins and related genes of interest in TH1 and T_{FH} SMARTA at day 3 after LCMV infection measured by RNA-Seq. Data are from GSE67336.(**d**) CXCR5 expression (left) in CD4$^+$ T cells transduced with retroviral vector expressing GFP alone (GFP-RV) or GFP and E12 (E12-RV), E47 (E47-RV) or Ascl2 (Ascl2-RV). Numbers indicate percent CXCR5$^+$ cells among GFP$^+$cells. Right, quantification of results at left. (**d**) Mean fluorescent intensity (MFI) of CXCR5 in SMARTA T_{FH} cells (CXCR5$^+$Bcl6$^+$) from B6 host mice given SMARTA CD4$^+$ T cells transduced with vector expressing GFP alone or GFP and E47, assessed 3 days after infection of the host mice with LCMV. Each symbol represents an individual mouse. **P* <0.05, ***P* <0.01, ****P* <0.001 and *****P* <0.0001 (two-tailed unpaired Student's t-test). Data are representative of two independent experiments with n = 3-8 mice per group in each (mean ± s.e.m.). Experiments performed in collaboration with Dr. Simon Bélanger.

high expression in early T_{FH} cells sorted from mice infected with LCMV 3 days earlier (Figure 2.11c) [16]. In contrast, *Ascl2* expression was essentially undetectable in either T_{FH} cells or T_H1 cells at the same time point (Figure 2.11c). Overexpression of the *Tcf3*-encoded isoforms E12, E47 or Ascl2 induced CXCR5 expression by $CD4^+$ T cells *in vitro* (Figure 2.11d). Ectopic expression of E47 led to enhanced expression of CXCR5 by both early T_H1 cells and early T_{FH} cells relative to its expression by their GFP-RV^+ counterparts *in vivo* (Figure 2.11e and data not shown).

Given that Id2 inhibits the transcriptional activity of E proteins, and E proteins induce CXCR5 expression, we investigated whether Id2 inhibited T_{FH} differentiation by preventing expression of CXCR5. We transduced NIP $CD4^+$ T cells with retrovirus overexpressing Id2 (Id2-RV) or expressing GFP (GFP-RV), transferred Id2-RV^+ or GFP-RV^+ NIP $CD4^+$ T cells into B6 mice and infected the host mice with LCMV. Id2-RV^+ NIP $CD4^+$ T cells underwent less differentiation into early T_{FH} cells than did their GFP-RV^+ counterparts (Figure 2.12a,c) and had impaired CXCR5 expression relative to that of their GFP-RV^+ counterparts (Figure 2.12b).

Next, we constitutively expressed the E proteins E12, E47 or Ascl2 (with a retroviral vector encoding a GFP reporter) together with Id2 (with a retroviral vector encoding an Ametrine reporter) in $CD4^+$ T cells. As expected, the E proteins E12, E47 and Ascl2 drove substantial expression of CXCR5 when $CD4^+$ T cells were co-transduced with an empty retroviral Ametrine vector (Figure 2.12d). When the retroviral vector expressing Id2 was introduced into $CD4^+$ T cells expressing E12 or E47, there was a reduction in CXCR5 expression by GFP^+ $Ametrine^+$ cells (Figure 2.12d). Unexpectedly, Id2 was not able to block the Ascl2-driven induction of CXCR5 expression (Figure 2.12d). These data indicated that Id2 prevented E

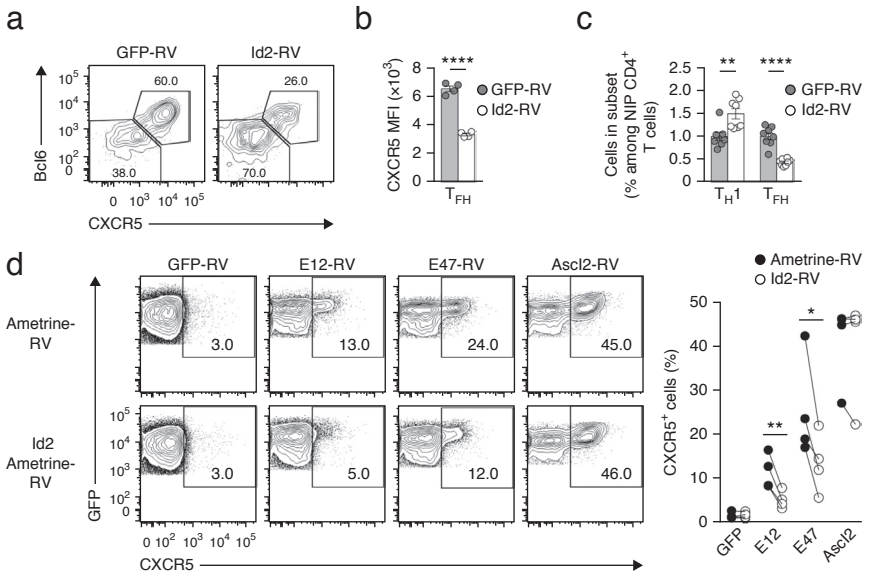


Figure 2.12: E proteins drive CXCR5 expression and inhibit the formation of T_H1 cells.

(**a**) Flow cytometry of cells from B6 host mice given NIP $CD4^+$ T cells transduced with GFP-RV or Id2-RV, followed by infection of the host mice with LCMV and analysis 3 days after infection (numbers in outlined areas, percent T_H1 cells or T_{FH} cells). (**b**) Mean fluorescent intensity of CXCR5 in NIP T_{FH} cells ($CXCR5^+Bcl6^+$) as in **a**. (**c**) Frequency of T_H1 cells or T_{FH} cells as in **a**, among NIP $CD4^+$ T cells (**c**); results in **c** are normalized to the average for mice given cells transduced with GFP-RV. (**d**) Flow cytometry of $GFP^+Ametrine^+$ $CD4^+$ T cells transduced with the reporters in **b** (above plots) and a retroviral Ametrine reporter expressing empty vector Ametrine-RV) or Id2 (Ametrine-Id2) (left margin). Numbers in outlined areas indicate percent $CXCR5^+$ cells among $GFP^+Ametrine^+$ cells. Right, quantification of results at left. Each symbol represents an individual mouse; lines in **d** connect results for the same mouse. $^*P$ <0.05, $^{**}P$ <0.01 and $^{****}P$ <0.0001 (two-tailed unpaired (**a-c**) or paired (**d**) Student's t-test). Data are representative of two (**a-c**), three (**a,b**) independent experiments, or are pooled from two (**c**) or four (**d**) independent experiments with n = 3-8 mice per group in each (mean $\pm$ s.e.m.). Experiments performed in collaboration with Dr. Simon Bélanger.

proteins from inducing CXCR5 expression.

2.2.6 Inhibition of *Id2* expression by Bcl6

Our data showed that the Id2-E protein axis modulated T_H1 and T_{FH} differentiation and that Id2 inhibited *Cxcr5* expression. The transcriptional repressor Bcl6 is essential for T_{FH} differentiation and is important for CXCR5 expression by T_{FH} cells *in vivo* [48, 17, 90] but it does not directly regulate *Cxcr5* [64, 39]. We therefore sought to determine whether Bcl6 induces CXCR5 expression by inhibiting transcription of the gene encoding Id2. We analyzed human primary tonsillar GC T_{FH} cells by chromatin immunoprecipitation (ChIP) followed by deep sequencing (ChIP-Seq) and found recruitment of Bcl6 to the *ID2* locus [39] (Figure 2.13a), a result we confirmed by ChIP followed by quantitative PCR (Figure 2.13b).

To investigate whether Bcl6 represses *Id2* expression, we transduced *Bcl6*$^{fl/fl}$CD4-Cre$^+$ SMARTA CD4$^+$ T cells (called ''*Bcl6*$^{-/-}$'' CD4$^+$ T cells here) with retrovirus expressing Bcl6 or GFP, transferred the transduced *Bcl6*$^{-/-}$CD4$^+$ T cells into B6 mice, infected the host mice with LCMV and assessed expression of Id2 in the transferred *Bcl6*$^{-/-}$CD4$^+$ T cells (Figure 2.14a,b). This re-introduction of Bcl6 into *Bcl6*$^{-/-}$ cells led to significant repression of Id2 expression in IL-2Rα^{hi} T_H1 cells (Figure 2.14b). Published work has demonstrated that separate domains of Bcl6 control T_{FH} differentiation, and replacement of the lysine at position 379 with glutamine (K379Q) substantially hinders Bcl6 activity [81, 82]. Introduction of the Bcl6 K379Q mutant into *Bcl6*$^{-/-}$ cells failed to repress *Id2* expression relative to its expression in *Bcl6*$^{-/-}$ cells given wild-type Bcl6 (Figure 2.14b).

Thus, Bcl6 directly repressed *Id2* in CD4$^+$ T cells. We also sought to de-

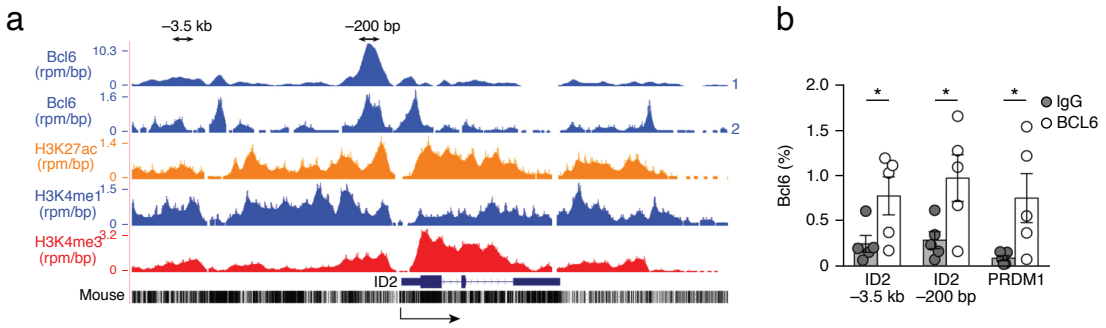


Figure 2.13: Bcl6 inhibits *Id2* expression.
(**a**) ChIP-Seq analysis of Bcl6 or histone H3 acetylated at Lys27 (H3K27ac), monomethylated at Lys4 (H3K4me1) or trimethylated at Lys4 (H3K4me3) at *ID2* in human GC T_{FH} cells (two replicates (1,2) for Bcl6) presented as reads per million per nucleotide (rpm/bp). Top (double-headed arrows), primers at a position 3.5 kilobases (-3.5 kb) or 200 base pairs (-200 bp) upstream of the transcription start site; bottom, sequence conservation (human versus mouse). (**b**) ChIP-quantitative PCR analysis of Bcl6 at *ID2* (primers as in **a**) or *PRDM1* among chromatin prepared from PD-1^{hi} GC T_{FH} cells isolated from human tonsil cells, presented as percent of input. Each symbol represents an individual mouse. *P <0.05 (two-tailed unpaired Student's t test). Data are representative of two experiments (**a**) or are pooled from or five (**b**) independent experiments with n = 5 tonsils per group (**b**; mean ± s.e.m.). Experiments performed in collaboration with Dr. Simon Bélanger.

termine how the copy number of *Bcl6* affected *Id2* expression. We transferred *Bcl6*$^{-/-}$ (*Bcl6*$^{fl/fl}$CD4-Cre^{+} SMARTA), *Bcl6*$^{+/-}$ (*Bcl6*$^{fl/+}$CD4-Cre^{+} SMARTA) or *Bcl6*$^{+/+}$ (*Bcl6*$^{+/+}$ SMARTA) CD4^{+} T cells into B6 mice, infected the host mice with LCMV and sorted IL-2Rα^{hi} (T_H1) and IL-2Rα^{lo} (T_{FH}) SMARTA CD4^{+} T cells from the mice (Figure 2.14c). As expected, the *Bcl6*$^{+/+}$ T_{FH} cells had lower expression of *Id2* than that of the *Bcl6*$^{+/+}$ T_H1 cells (Figure 2.14d). *Id2* expression was significantly higher in *Bcl6*$^{+/-}$ IL-2Rα^{lo} cells than in *Bcl6*$^{+/+}$ IL-2Rα^{lo} cells (Figure 2.14d). Furthermore, complete loss of *Bcl6* (*Bcl6*$^{-/-}$) resulted in significant upregulation of *Id2* expression in IL-2Rα^{lo} cells relative to its expression in *Bcl6*$^{+/-}$ or *Bcl6*$^{+/+}$ IL-2Rα^{lo} cells (Figure 2.14d). Thus, Bcl6 inhibited *Id2*, and *Bcl6* haploinsufficiency resulted in inappropriate *Id2* expression.

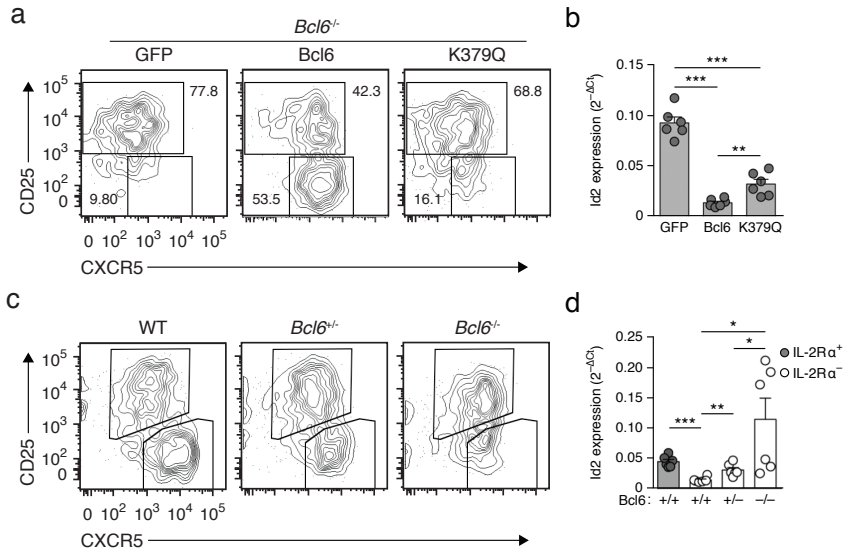


Figure 2.14: Bcl6 inhibits *Id2* expression.
(**a**) *Bcl6*$^{fl/fl}$ CD4-Cre^{+} SMARTA CD4^{+} T cells transduced with the indicated vectors were transferred into B6 mice. (**b**) Quantitative RT-PCR analysis of *Id2* in IL-2Rα^{+} SMARTA CD4^{+} T cells sorted from B6 host mice given *Bcl6*$^{-/-}$ CD4^{+} T cells transduced with retrovirus expression GFP alone, wild-type Bcl6 or the Bcl6 K379Q mutant (horizontal axis), followed by infection of host mice with LCMV, assessed 3 days after infection (results calculated by the change-in-cycling-threshold ($2^{-\Delta Ct}$) method). (**c**) *Bcl6*$^{+/+}$ SMARTA (WT), *Bcl6*$^{fl/+}$CD4-Cre^{+} SMARTA (*Bcl6*$^{+/-}$) or *Bcl6*$^{fl/fl}$ CD4-Cre^{+} SMARTA (*Bcl6*$^{-/-}$)CD4^{+} T cells were transferred into B6 mice. Gates used to sort IL-2Rα^{+} and IL-2Rα^{-} SMARTA CD4^{+} T cells 3 days after LCMV infection are indicated.(d) Quantitative RT-PCR analysis (as in (b) of *Id2* in IL-2Rα^{+} or IL-2Rα^{-} SMARTA cells sorted from B6 host mice given *Bcl6*$^{+/+}$ SMARTA (+/+), *Bcl6*$^{fl/+}$CD4-Cre^{+} SMARTA (+/-) or *Bcl6*$^{fl/fl}$ CD4-Cre^{+} SMARTA (-/-)CD4^{+} T cells (horizontal axis). Each symbol represents an individual mouse. **P* <0.05, ***P* <0.01 and ****P* <0.0001 (two-tailed unpaired Student's t test). Data are pooled from two independent experiments with results pooled from three mice per data point (mean ± s.e.m.). Experiments performed in collaboration with Dr. Simon Bélanger.

2.3 Discussion

Members of the E-protein and Id families are pivotal regulators of lymphocyte development and function. Here we investigated a previously unexplored role for Id2 in the differentiation of helper T cells in response to acute viral infection and found that Id2 controlled the balance of T_H1-versus-T_{FH} differentiation by inhibiting

E-protein activity. Id2 was 'preferentially' expressed in T_H1 cells, and a reduction in the expression of Id2 in $CD4^+$ T cells resulted in a greater proportion of T_{FH} cells. Complete ablation of Id2 hampered the generation of T_H1 cells, which resulted in an abnormal effector population exhibiting mixed traits of the T_H1 cell lineage and T_{FH} cell lineage. Furthermore, Bcl6 specifically inhibited *Id2* to ensure E-protein activity, which drove a portion of the T_{FH} cell program; this established Id2 as a critical enforcer of proper helper T cell differentiation. A reduction in the expression of Id2 shifted the balance of T_H1 cells and T_{FH} cells, which indicated that partial expression of Id2 was able to inhibit E-protein expression enough to maintain both helper T cell populations while still biasing cells toward the T_{FH} cell lineage. Strikingly, $CD4^+$ T cells that completely lacked *Id2* lost the ability to form an effector T_H1 cell population, while they maintained an intact T_{FH} cell population. *Id2*-deficient effector cells exhibited lower expression of T_H1 cell-associated genes and showed simultaneous upregulation of a large portion of the T_{FH} cell gene program (*Cxcr5, Il6ra, Lef1* and *Tcf7*, but not *Bcl6, Icos* or *Pdcd1*). *Id2*-deficient cells might be unable to commit to one lineage for various reasons. While they adopted aspects of the T_{FH} cell transcriptional program, *Id2*-deficient cells also upregulated and maintained high expression of *Id3, Foxo1* and *Il2ra*, which might explain this dichotomy. The transcription factor Foxo1 specifically inhibits the development of T_{FH} cells [109, 99]. Within the first two cell divisions, expression of IL-2Rα is a key factor that drives the 'decision' to commit to the T_H1 cell lineage [17]. Expression of *Foxo1* and *Il2ra* in the absence of Id2 might counterbalance the T_{FH} cell gene program. Thus, Id2 and E proteins were powerful regulators of key T_{FH} cell-associated genes and many T_H1 cell-associated genes, but the unusual phenotype of the *Id2*-deficient effector

$CD4^+$ T cells demonstrated that Id2 and E proteins controlled gene sets that do not themselves result in polarized differentiation of T_H1 cells and T_{FH} cells. Published work has demonstrated a role for Id3 in regulating the T_{FH} cell gene-expression program [75, 64, 114, 74].

Differential expression patterns and unique binding partners are plausible explanations for how Id2 might control distinct helper T cell subsets; Id2 might inhibit DNA binding of different E proteins with differing affinities and also have differentially regulated binding activity and protein stability. In support of that proposal, we observed that Id2 inhibited the induction of *Cxcr5* by E47 but not its induction by Ascl2. Loss of Id2 substantially impaired T_H1 differentiation. These observations support the hypothesis that inhibition of E proteins alters the T_H1 cell-T_{FH} cell balance: Id2 is important in the upregulation of T_H1 cell-associated genes, while Id3 restrains T_{FH} differentiation. Ascl2 has been suggested to act upstream of Bcl6 to regulate early T_{FH} differentiation [64]. However, Ascl2 is generally not detectable in naive $CD4^+$ T cells [56, 35, 113] or early T_{FH} cells [16]. Instead, Ascl2 expression is robust in fully differentiated GC T_{FH} cells in mice and humans [64, 113]. The high expression of E2A and HEB early after infection with LCMV would suggest that these E proteins, not Ascl2, direct early T_{FH} differentiation. Interestingly, Ascl2-induced expression of CXCR5 was not dampened by co-expression of Id2. GC T_{FH} cells had the highest CXCR5 expression, and Ascl2 might be important for amplifying expression at later stages of the differentiation of T_{FH} cells into GC T_{FH} cells. There are various plausible models for the coordination of the expression of genes encoding products that regulate the earliest stages of T_{FH} differentiation *in vivo*. Bcl6 function is critical for T_{FH} differentiation [21, 81, 82] and can be detected as

early as the second cell division *in vivo* [17]. *Tcf7* and *Lef1*, both of which are known targets of E proteins [61], are epistatic to *Bcl6*, and TCF-1 and LEF-1 promote T_{FH} differentiation by enhancing the expression of *Bcl6*, *Il6ra* and *Icos* and repressing the expression of *Prdm1* (which encodes Blimp-1) [16, 108, 110]. Notably, we observed increased expression of *Tcf7*, *Lef1* and *Il6ra* in the absence of Id2, in support of the idea that E proteins such as E2A and HEB normally promote the expression of *Tcf7* and *Lef1*. In this context, Id2 and E proteins act upstream of Bcl6. However, our data also demonstrated that Bcl6 directly repressed *Id2* expression. Together these data suggest that positive feedback mechanisms involving TCF-1, LEF-1, Bcl6, IL-6R, ICOS and E proteins support T_{FH} differentiation under conditions of low Id2 expression and that a feedforward loop could potentially be generated by starting at any of several genes in that gene network.

The relationship among members of the E-protein and Id families and expression of Bcl6 and CXCR5 is of particular interest. Ectopic expression of Bcl6 in human $CD4^+$ T cells results in CXCR5 expression [58]. Coordinated expression of Bcl6 and CXCR5 in early T_{FH} cells is observed in various *in vivo* models [17, 90, 15, 4]. However, Bcl6 does not bind to Cxcr5 [39] and thus must regulate its expression indirectly. One mechanism involves repression of Cxcr5 by Blimp-1 [85]. However, naive T cells do not express Blimp-1, which indicates that this mechanism regulates mainly later expression of CXCR5. Here we have demonstrated a previously unknown mechanism whereby Bcl6 inhibited Id2 expression that yielded enhanced E protein activity to drive Cxcr5 expression. Our data uniquely position Id2, Bcl6 and E proteins in a regulatory triad that controls the balance of T_H1 differentiation and T_{FH} differentiation. Through the inhibition of E proteins, high expression of Id2 in

the T_H1 cell population enforces proper development of the T_H1 cell lineage. Early expression of Bcl6 in T_{FH} cells ensures repression of Id2, which allows E proteins to drive T_{FH} differentiation. Thus, dichotomous expression of Id2 is critical to ensuring the reciprocal development of T_H1 differentiation and T_{FH} differentiation.

Chapter 2, in part, is a reprint of the material as it appears in Nature Immunology. **Shaw LA**, Belanger S, Omilusik KD, Cho S, Scott-Browne JP, Nance JP, Goulding J, Lasorella A, Lu LF, Crotty S and Goldrath A. *Id2 reinforces T_{H1} differentiation and inhibits E2A to repress T_{FH} differentiation*, Nature Immunology, Volume 17, Issue 7, 2016. *The thesis author was the primary author of this paper.

Chapter 3

Id3 as a marker for multipotent potential of $CD4^+$ T cells

3.1 Introduction

It is well established that following activation, T_{FH} cells gain the ability to secrete cytokines and direct a germinal center reaction, promoting class switching and somatic hypermutation in B cells [21]. However, it has recently been appreciated that some T_{FH} cells are able to survive following the contraction phase and can seed the long-lived memory compartment [36]. Generation of a competent $CD4^+$ T cell memory pool is crucial for providing a rapid response following a second encounter with antigen; these $CD4^+$ T cells cells not only re-expand to repopulate the T_{FH} pool following rechallenge, but they also generate the secondary T_H1 effector population.

Id2 reinforces T_H1 differentiation through inhibition of E2A (Chapter 2). However, another Id protein, Id3, is also highly expressed in $CD4^+$ T cells. Previous work has demonstrated a role for Id3 in regulating the TT_{FH} gene-expression

program. Deletion of Id3 during early thymocyte development leads to a number of phenotypes including an expansion of IL-4-dependent $CD8^+$ T cells with innate activation, as well as aberrant differentiation and expansion of T_{FH}-like cells in the thymus [2, 93, 105, 106, 103]. Specific deletion of Id3 in Foxp3-expressing thymocytes results in the acquisition of a regulatory T-follicular helper cell (T_{FR}) specific gene program, suggesting that Id3-mediated inhibition of E protein activity dampens expression of Tfh-related genes in regulatory T cells [75]. Further, Id3-deficient $CD4^+$ T cells favor GC T_{FH} formation compared to WT $CD4^+$ T cells following immunization [64]. Id3 has been suggested to antagonize E-protein repression, allowing expression of E-protein targets such as Foxo1 that prevent premature activation of the T_{FH}-lineage gene signature [74]. What is not known, however, is how Id3 itself shapes the potential for $CD4^+$ T cells to exist as a long lived memory population. Thus, I further explored the role of Id3 during the differentiation and maintenance of memory $CD4^+$ T cells.

3.2 Results

3.2.1 Expression of Id proteins during acute LCMV infection

We previously found that expression of Id2 was biased towards T_H1 cells (Chapter 2), so we first compared the expression of Id2 and Id3 in $CD4^+$ T cell subsets. To achieve this, we generated $Id2^{YFP/+}Id3^{GFP/+}$ dual reporter mice, in which the cDNA encoding yellow fluorescent protein (YFP) or green fluorescent protein (GFP) is inserted into *Id2* [111] or *Id3* [114], respectively (for the expression of Id2-YFP or Id3-GFP). We further crossed the line to SMARTA mice (described in

Section 2.2.1) to generate *Id2*$^{YFP/+}$*Id3*$^{GFP/+}$ SMARTA CD4$^+$ T cells. We transferred these cells into B6 hosts, and analyzed coordinate expression of Id2 and Id3 after infection with LCMV. Naive CD4$^+$ T cells had intermediate expression of Id2 and high expression of Id3 (Figure 3.1a). During the effector phase, T_H1 cells showed higher Id2-YFP expression than that of naive cells (Figure 3.1a). In contrast, T_H1 cells had reduced levels of Id3-GFP, while T_{FH} cells maintained high expression of Id3-GFP (Figure 3.1a). GC T_{FH} cells had expression of Id3-GFP equivalent to that of T_{FH} cells (data not shown). These results demonstrated contrasting expression patterns of Id2 and Id3 in T_H1 cells and T_{FH} cells following acute infection with LCMV.

Interestingly, Id2-expressing T_H1 cells (CXCR5$^-$SLAM^{hi}), which expressed very little Id3 during the effector phase, began to upregulate expression of Id3 following contraction; the point at which the surviving T_H1 cells downregulated effector molecules, such as SLAM, and may begin to adopt the memory cell transcriptional program to become long lived (Figure 3.1b). By day 40, the remaining cells are a mixed population that exhibited high expression of Id2 alone, or concomitant expression of Id2 and Id3 (Figure 3.1b). These results demonstrated that while CXCR5 expressing CD4$^+$ T_{FH} cells have been interrogated as the memory population of CD4$^+$ T cells, Id3 expression could possibly mark CD4$^+$ T cell memory in a more comprehensive way than CXCR5 expression alone.

3.2.2 Expression of Id3 defines the T_{FH} population

In the *Id2*$^{YFP/+}$*Id3*$^{GFP/+}$ line, Id2 is rendered heterozygous, which could be compensated for by upregulation of Id3. Additionally, we wanted to rule out any

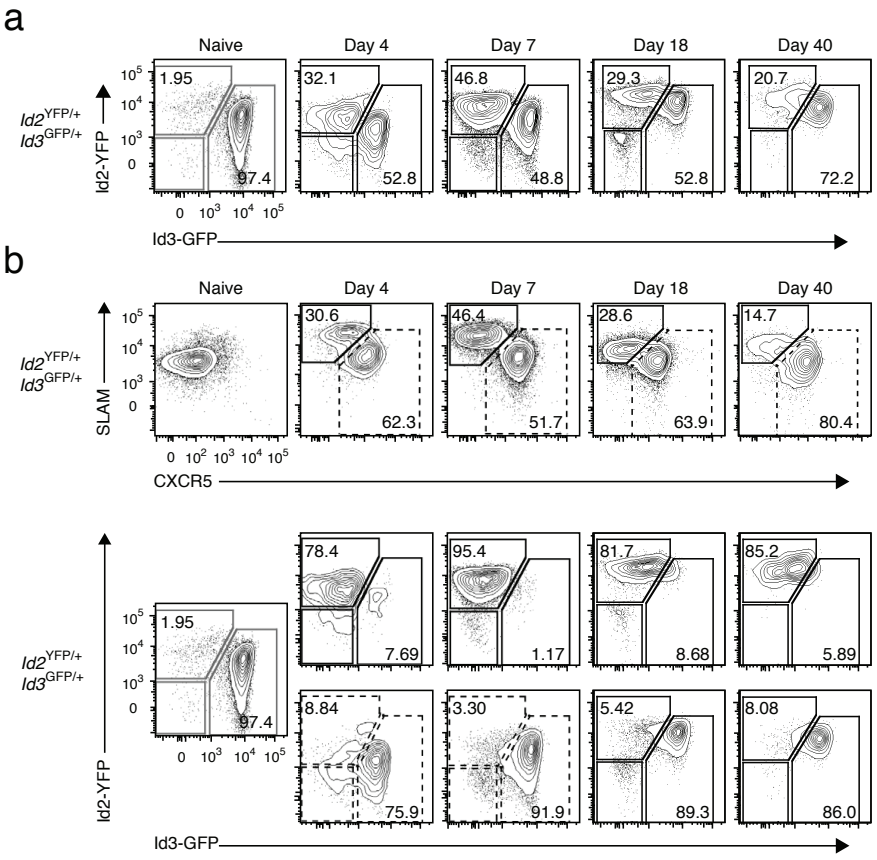


Figure 3.1: Dynamic expression of Id2 and Id3 following LCMV infection. (**a,b**) Flow cytometry of donor cells from B6 host mice given $Id2^{YFP/+}Id3^{GFP/+}$ SMARTA $CD4^+$ T cells, followed by infection of the host mice with LCMV and analysis on indicated days. (**a**) Numbers indicate percent Id2-YFP^+Id3-GFP^- cells (top left) or Id2-YFP^-Id3-GFP^+ cells (bottom right) among the populations. (**b, top**) Numbers in outlined areas indicate percent $SLAM^+CXCR5^-$ (T_H1) cells (top left) or $SLAM^{lo}CXCR5^+$ (T_{FH}) cells (bottom right) among the populations. (**b, bottom**) Numbers indicate percent Id2-YFP and Id3-GFP expression among $SLAM^+CXCR5^-$ cells (solid lines) or $CXCR5^+SLAM^{lo}$ cells (dashed lines) (from above). Data are representative of two experiments with n = 3 mice per group.

potential spillover effects from GFP/YFP. To address both of these concerns, we further confirmed the expression level of Id3 in $CD4^+$ T cells following LCMV infection with Id3-GFP single reporter mice. We again crossed this line to SMARTA mice (described in Section 2.2.1) to generate $Id3^{GFP/+}$ mice. We transferred total $Id3^{GFP/+}$ SMARTA $CD4^+$ T cells into B6 host mice and infected 1 day later with

LCMV Armstrong (LCMV). On day 7 of infection, we found that Id3 expression was highly polarized: Id3-GFP^{lo} cells differentiated into T_H1 cells, while Id3-GFP^{hi} cells became T_{FH} cells (Figure 3.2a).

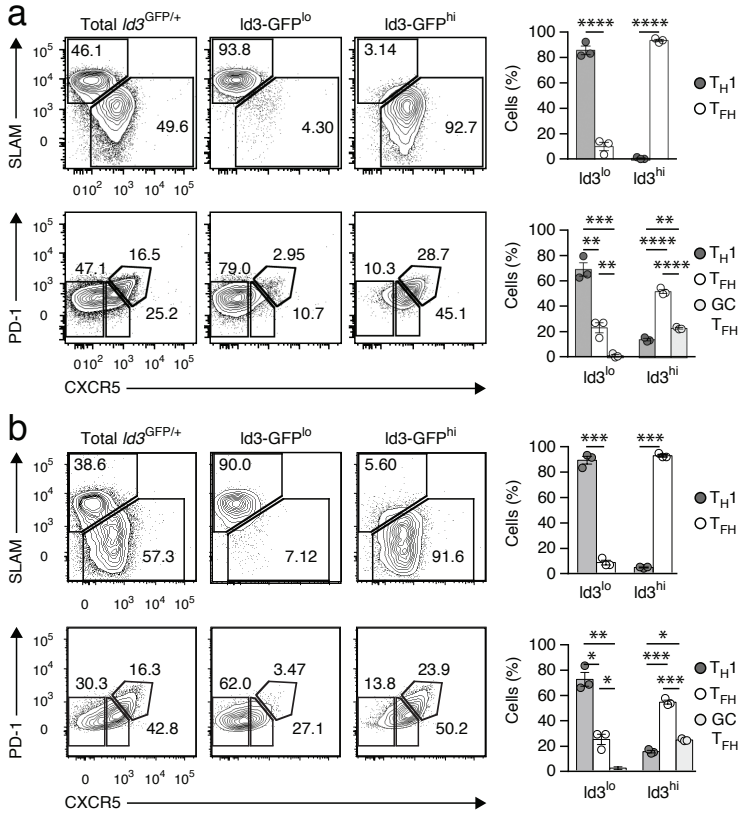


Figure 3.2: Id3 expression defines T_{FH} cell subsets.
(**a**) Flow cytometry of donor cells from B6 host mice given *Id3*$^{GFP/+}$ SMARTA CD4$^+$ T cells, followed by infection of the host mice with LCMV and analysis 7 days later. Numbers indicate percent SLAM$^+$CXCR5$^-$ (T_H1) cells (top left) or SLAM^{lo}CXCR5$^+$ (T_{FH}) cells (bottom right) (top row), or CXCR5$^-$PD-1$^-$ (T_H1) cells (bottom left), CXCR5$^+$PD-1$^-$ (T_{FH}) cells (bottom right) or CXCR5$^+$ PD-1$^+$ (GC T_{FH}) cells (top right) (bottom row) among the populations above plots. Right, quantification of results at left. (**b**) *Id3*$^{GFP/+}$ mice were analyzed 7 days after LCMV infection. T_H1 (SLAM$^+$CXCR5$^-$ or CXCR5$^-$PD-1$^-$), T_{FH} (SLAM^{lo}CXCR5$^+$ or CXCR5$^+$PD-1$^-$) or GC T_{FH} (CXCR5$^+$PD-1$^+$) differentiation for the indicated antigen-experienced (CD49d$^+$CD11a$^+$) CD4$^+$ T cell populations was analyzed by flow cytometry and quantified. *$P < 0.05$, **$P < 0.001$ and ***$P < 0.0001$ (two-tailed unpaired Student′ s t test). Data are representative of three experiments (**a,b**), each with n = 3 mice per group (mean ± s.e.m.).

In parallel, we infected *Id3*$^{GFP/+}$ mice with LCMV to monitor the differentiation of polyclonal CD4$^+$ T cells. We observed that Id3-GFP^{hi} cells were almost exclusively T_{FH} cells (CXCR5$^+$SLAM^{lo} or CXCR5$^+$PD-1^{lo}) and GC T_{FH} cells (CXCR5$^+$PD-1$^+$), while the vast majority of Id3-GFP^{lo} cells displayed a T_H1 phenotype (SLAM$^+$CXCR5$^-$ or CXCR5$^-$PD-1$^-$) (Figure 3.2b).

3.2.3 Restraint of T_{FH} differentiation by Id3

We found that Id3 is expressed chiefly by T_{FH} cells and GC T_{FH} cells following infection, and has been suggested to be an inhibitor of T_{FH} differentiation [64].

However, the role of Id3 in the generation of T_{FH} cells has not been assessed in the context of infection [64]. Thus, we generated *Id3*$^{fl/fl}$ [34] CD4-Cre$^+$ SMARTA mice (called '*Id3*$^{-/-}$ mice' here), transferred cells from those mice into B6 mice and infected the host mice with LCMV. In response to infection, *Id3*$^{-/-}$ cells displayed a greater propensity than did *Id3*$^{+/+}$ cells to become either T_{FH} cells or GC T_{FH} cells (Figure 3.3a). Furthermore, there was a greater frequency of GC T_{FH} cells among polyclonal Id3-deficient CD4$^+$ T cells than among Id3-sufficient CD4$^+$ T cells (Figure 3.3b).

We next investigated whether constitutive expression of Id3 was able to inhibit T_{FH} differentiation. We obtained CD4$^+$ T cells from NIP mice (which have transgenic expression of a TCR specific for LCMV nucleoprotein, amino acids 311-325) [82], transduced the cells with retrovirus (RV) overexpressing Id3 (Id3-RV) or expressing GFP (GFP-RV), transferred Id3-RV$^+$ or GFP-RV$^+$ NIP CD4$^+$ T cells into B6 mice and infected the host mice with LCMV (Figure 3.4a,b). The acquisition of

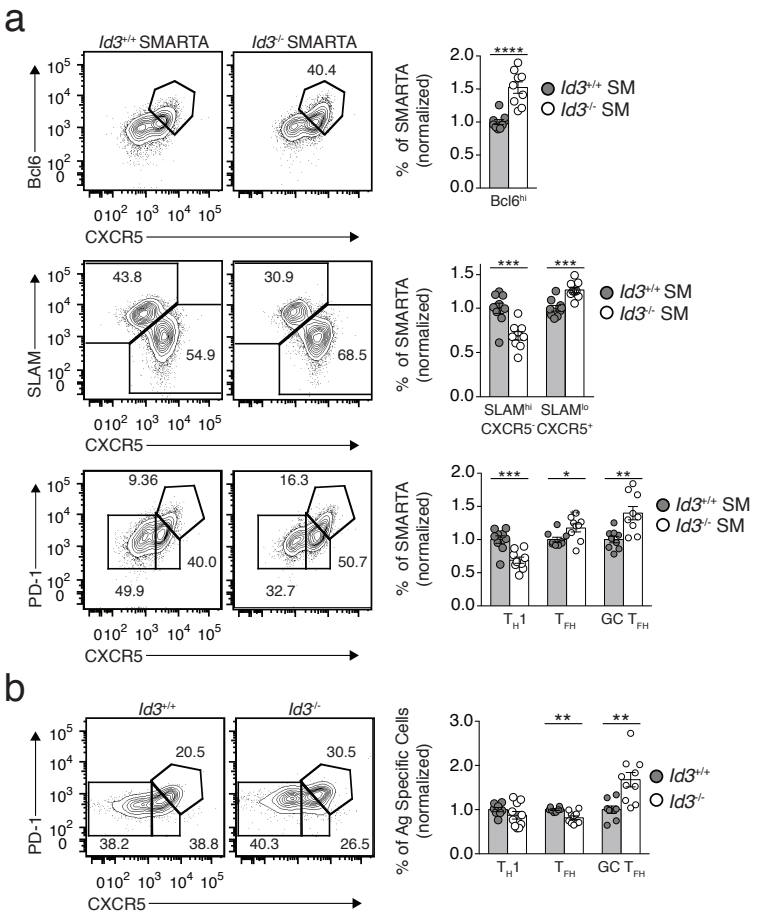


Figure 3.3: Increased frequency of T_{FH} and GC T_{FH} in the absence of Id3. *Id3*$^{+/+}$ CD4-Cre^{+} (*Id3*$^{+/+}$) or *Id3*$^{fl/fl}$ CD4-Cre^{+} (*Id3*$^{fl/fl}$) SMARTA CD4^{+} T cells were transferred into B6 mice and analyzed 7 days after LCMV infection. (**a**) Flow cytometric analysis of CXCR5^{+}Bcl6^{hi} (top), SLAM^{hi}CXCR5^{-} (T_H1) and SLAM^{lo}CXCR5^{+} (T_{FH}) (middle); or PD-1^{-}CXCR5^{-} (T_H1), PD-1^{-}CXCR5^{+} (T_{FH}) and PD-1^{+}CXCR5^{+} (GC T_{FH}) (bottom) populations and quantification as a frequency of SMARTA CD4^{+} T cells (right panels). (**b**) *Id3*$^{+/+}$ CD4-Cre^{+} and *Id3*$^{fl/fl}$ CD4-Cre^{+} mice were analyzed 7 days after LCMV and PD-1^{-}CXCR5^{-} (T_H1), PD-1^{-}CXCR5^{+} (T_{FH}) and PD-1^{+}CXCR5^{+} (GC T_{FH}) expression was analyzed by flow cytometry (left) and quantified as a frequency of antigen-specific (gp66-77) CD4^{+} T cells (right). **P* <0.05, ***P* <0.01, ****P* <0.001, *****P* <0.0001 (two-tailed unpaired Student's t test). Data are representative of two independent experiments with n=8-10 mice per group (mean ± s.e.m.).

characteristics of either T_{FH} cells (Figure 3.4a) or early T_{FH} cells (Figure 3.4b) was abrogated when Id3 was overexpressed. This was consistent with the observation that Id3 inhibits T_{FH} differentiation following immunization with protein [64].

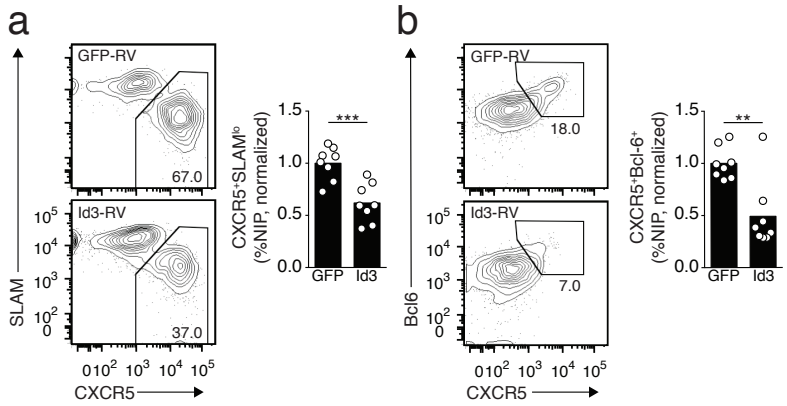


Figure 3.4: Expression of Id3 limits unregulated differentiation of T_{FH} cells and GC T_{FH} cells.
(**a,b**) NIP $CD4^+$ T cells transduced with the indicated RV were transferred into B6 mice and analyzed 6 (**a**) or 3 (**b**) days after LCMV infection. (**a**) T_{FH} ($CXCR5^+SLAM^{lo}$) differentiation was analyzed by flow cytometry and quantified as a frequency of NIP $CD4^+$ T cells. (**b**) Early T_{FH} ($CXCR5^+Bcl6^+$) differentiation was analyzed by flow cytometry and quantified as a frequency of NIP $CD4^+$ T cells. $^{**}P$ <0.01, $^{***}P$ <0.001 (two-tailed unpaired Student′s t test). Data are pooled from two (**a,b**) independent experiments with n=8-10 mice per group (mean ± s.e.m.).

3.2.4 Id3-expressing cells exhibit multipotent potential upon rechallenge

Following LCMV infection, we observed that many of the remaining $CD4^+$ T cells at day 40 expressed high levels of Id3 (Figure 3.2). That led us to investigate whether this expression of Id3 could identify $CD4^+$ T cells that have memory recall potential. To assess this, we transferred *Id3*$^{GFP/+}$ SMARTA $CD4^+$ T cells into a B6 host and infected 1 day later (Day 0) with LCMV (Figure 3.5).

After 30 days, $CD4^+$ T cells were isolated and sorted based on Id3 expression ($Id3^{lo}$ vs $Id3^{hi}$). We then transferred either $Id3^{lo}$ cells or $Id3^{hi}$ cells into a new cohort of B6 hosts, which were infected with LCMV one day later. Following LCMV rechallenge, we found that both populations were able to recall, however,

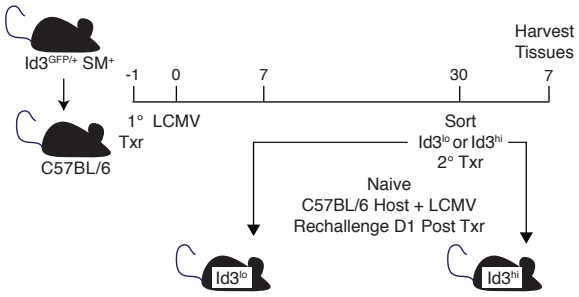


Figure 3.5: Schematic of Id3-GFP^{lo} and Id3-GFP^{hi} transfer and rechallenge. *Id3*$^{GFP/+}$ SMARTA CD4^{+} T cells were transferred into B6 hosts and infected with LCMV. Thirty days after infection, splenocytes were sorted based on GFP expression (Id3^{lo} vs Id3^{hi}) and transferred into naive B6 hosts, which were subsequently infected with LCMV. The memory recall response of the transferred Id3^{lo} and Id3^{hi} cells was analyzed 7 days after secondary infection.

the phenotype of the expanded progeny were different (Figure 3.6). Id3^{lo} cells maintained low expression of Id3, whereas Id3^{hi} cells generated a mixed population of both Id3^{lo} and Id3^{hi} cells (Figure 3.6a).

The resulting Id3^{lo} cells were phenotypically T_H1 cells, with high expression of SLAM and low expression of CXCR5 (SLAM^{+}CXCR5^{-}) (Figure 3.6b). Conversely, the Id3^{hi} cells repopulated the CD4^{+} T cell compartment with both T_H1 (SLAM^{+}CXCR5^{-}) cells and T_{FH} (SLAM^{lo}CXCR5^{+}) cells (Figure 3.6b). Further, the Id3^{hi} cells also generated a higher frequency of PD-1^{+}CXCR5^{+} GC T_{FH} cells when compared to the Id3^{lo} cells (Figure 3.6c). These data suggest that memory cells expressing Id3 retain a multipotent phenotype, capable of differentiating into both T_H1 and T_{FH} cells upon rechallenge.

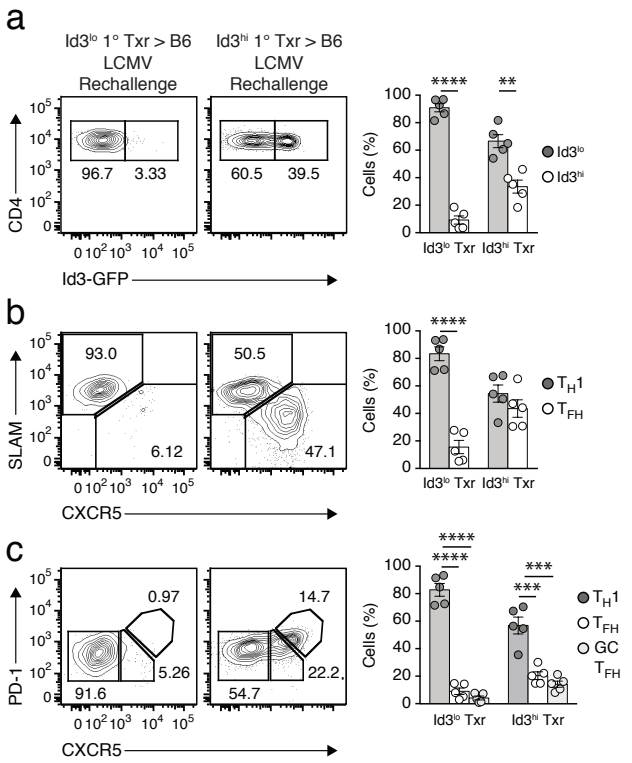


Figure 3.6: Id3-expressing cells generate both T_H1 and T_{FH} populations following rechallenge.
(**a-c**) Flow cytometry of $CD4^+$ memory T cells following LCMV rechallenge. As depicted in Figure 3.2, $Id3^{lo}$ and $Id3^{hi}$ memory cells were sorted and transferred into B6 mice, which were then infected with LCMV and analyzed 7 days later. Numbers indicate percent (**a**) $Id3^{lo}$ (left) or $Id3^{hi}$ (right), (**b**) $SLAM^+CXCR5^-$ (T_H1) cells (top left) or $SLAM^{lo}CXCR5^+$ (T_{FH}) cells (bottom right), (**c**) $CXCR5^-PD\text{-}1^-$ (T_H1) cells (left), $CXCR5^+PD\text{-}1^-$ (T_{FH}) cells (middle) or $CXCR5^+$ $PD\text{-}1^+$ (GC T_{FH}) cells (right) among the populations. (**a-c**) Right, quantification of results at left. Each symbol represents an individual mouse. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ (two-tailed unpaired Student′ s t test). Data are representative of three experiments with n = 3-5 mice per group (**a-c**; mean $\pm$ s.e.m.).

3.3 Discussion

We have described a new role for Id3 in the regulation of $CD4^+$ T cell differentiation and generation of long-lived memory cells following acute LCMV infection. We found that expression of Id3 is largely relegated to T_{FH} and GC T_{FH} cells, and this expression was maintained by cells following contraction of the the T_H1 and

T_{FH} effector populations. Interestingly, we also found that while a small population of Id2-expressing cells remained at day 40, the Id3-expressing memory cells also upregulated expression of Id2 to a higher level than observed in T_H1 effector cells at day 7. Our work further showed that specific deletion of Id3 in helper T cells promoted the formation of T_{FH} cells and GC T_{FH} cells but did not affect T_H1 differentiation following infection. In this way, Id3 deficiency was not a phenocopy of the recently described Id2 deficiency, but instead enhanced the differentiation of T_{FH} cells and GC T_{FH} cells. Forced expression of Id3 inhibited the differentiation of both T_{FH} and GC T_{FH} populations, instead driving cells towards the T_H1 lineage; a similar phenotype to that of forced Id2 expression described in Chapter 2. Finally, following secondary challenge with LCMV, we found that cells which had been partitioned based on $Id3^{lo}$ vs $Id3^{hi}$ expression generated disparate effector recall populations.

Following the resolution of an infection, forever 'remembering' that encounter is the calling card of T cells. This memory of prior exposure allows the remaining antigen specific T cells to respond faster and more robustly, resulting in more efficient elimination of the pathogen. In the past, the specific identity of the memory $CD4^+$ T cell population was somewhat elusive. Contrary to the aspects definining their $CD8^+$ T cell counterparts, memory $CD4^+$ T cells could be hard to find, often didn't exist in large numbers following contraction and could potentially arise from one of the at least 8 (and counting) functionally distinct effector subsets of $CD4^+$ T cells [12]. However, given the role for memory T cells in protecting against reinfection, and in vaccine design, effort has increased towards defining this population. Recently, differential expression of CXCR5 and Ly6C was used to sort

T_H1 ($CXCR5^-Ly6C^{hi}$) or T_{FH} ($CXCR5^+Ly6C^{lo/int}$) cells, which were then subjected to LCMV rechallenge [35]. Following a similar sort/rechallenge strategy as described in this chapter, cells arising from the $CXCR5^-$ population were 80% T_H1 and 20% T_{FH} and those from the $CXCR5^+$ population were 70% T_{FH} 30% TH1 [35]. The resulting $CXCR5^-$ cells that arose from the $CXCR5^+$ population were subpar T_H1 cells in that they exhibited poor expression of IFNγ and Tbet. Our described method of segregating $CD4^+$ T cells based on Id3 expression was considerably more precise at generating a discrete T_H1 population following rechallenge (>90%), allowing visualization of the true ability of $Id3^{hi}$ cells to generate both T_H1 and T_{FH} cells (about 50%/50%). Expression of the T_H1 molecule SLAM was uncompromised, but further work is needed to examine additional T_H1 characteristics. This work suggests that expression of Id3 alone has the potential to identify cells with the potential to seed multiple T-helper lineages following acute viral infection.

Chapter 3, in part, is a reprints of the material as it appears in Nature Immunology. **Shaw LA**, Belanger S, Omilusik KD, Cho S, Scott-Browne JP, Nance JP, Goulding J, Lasorella A, Lu LF, Crotty S and Goldrath A. *Id2 reinforces T_{H1} differentiation and inhibits E2A to repress T_{FH} differentiation*, Nature Immunology, Volume 17, Issue 7, 2016. *The thesis author was the primary author of this paper.

Chapter 4

Conclusions

I have investigated the role of Id2 and Id3 in promoting the generation and survival of $CD4^+$ effector and memory populations, particularly highlighting their reciprocal roles in shaping the $CD4^+$ T cell response. Clearly, a coordinated balance of Id2 and Id3 regulation is necessary for the control of normal $CD4^+$ T cell helper subset differentiation, and their respective expression and function may be temporally controlled as previously observed for effector $CD8^+$ T cell subsets [10, 111, 51]. These data collectively raise the question as to how Id2 and Id3 may serve such distinct functions: both differential expression patterns and unique binding partners are plausible explanations.

Our previous work showed that inflammatory cytokines differentially impact Id2 and Id3 expression: STAT4 and STAT5 bind regulatory regions of the Id2 locus and numerous cytokines known to induce their activity enhance Id2 reporter expression and indirectly repress Id3 expression [111, 60]. Notably, we observe in multiple contexts that Id2-deficient cells upregulate Id3 mRNA [111], including a 3-fold upregulation compared to WT cells in our work discussed in Chapter 2,

which may reflect that E2A-binding sites are found in in the Id3 locus and are bound by E2A in developing thymocytes [76, 62, 68]. Thus, loss of Id2-mediated inhibition of E protein activity may induce Id3 and invoke a partial negative feedback on E protein activity. We show that the Id2 reporter is highly expressed in T_H1 cells to inhibit E-protein driven T_{FH} differentiation and this is important for the formation of the T_H1 effector subset. T_{FH} cells, on the other hand, showed higher levels of the Id3 reporter and lower levels of the Id2 reporter, which may be necessary to restrain exuberant T_{FH}/GC T_{FH} differentiation. In this way, Id2-deficiency allows E proteins to push the acquisition of the T_{FH} program; however, compensatory Id3 expression could play a part in restricting complete lineage commitment, resulting in a population of $CD4^+$ T cells with a partial T_{FH} program. In contrast, Id3-deficiency does not result in increased Id2 expression and thus unchecked growth of T_{FH}/GC T_{FH} cells occurs due to unrestrained E protein activity. I also identified expression of Id3 as a determinant of memory potential, which could streamline the way we identify memory $CD4^+$ T cells, and allow for manipulation of these cells for use in vaccines. While these preliminary experiments are promising, additional work still needs to be done to address the possible mechanism for this phenotype. Ultimately, Id2 and Id3 activity are likely even more complex than distinct expression patterns. It is likely that Id3 and Id2 interact with different members of the E-protein family with differing affinities, and that their binding activity and protein stability may also be differentially regulated.

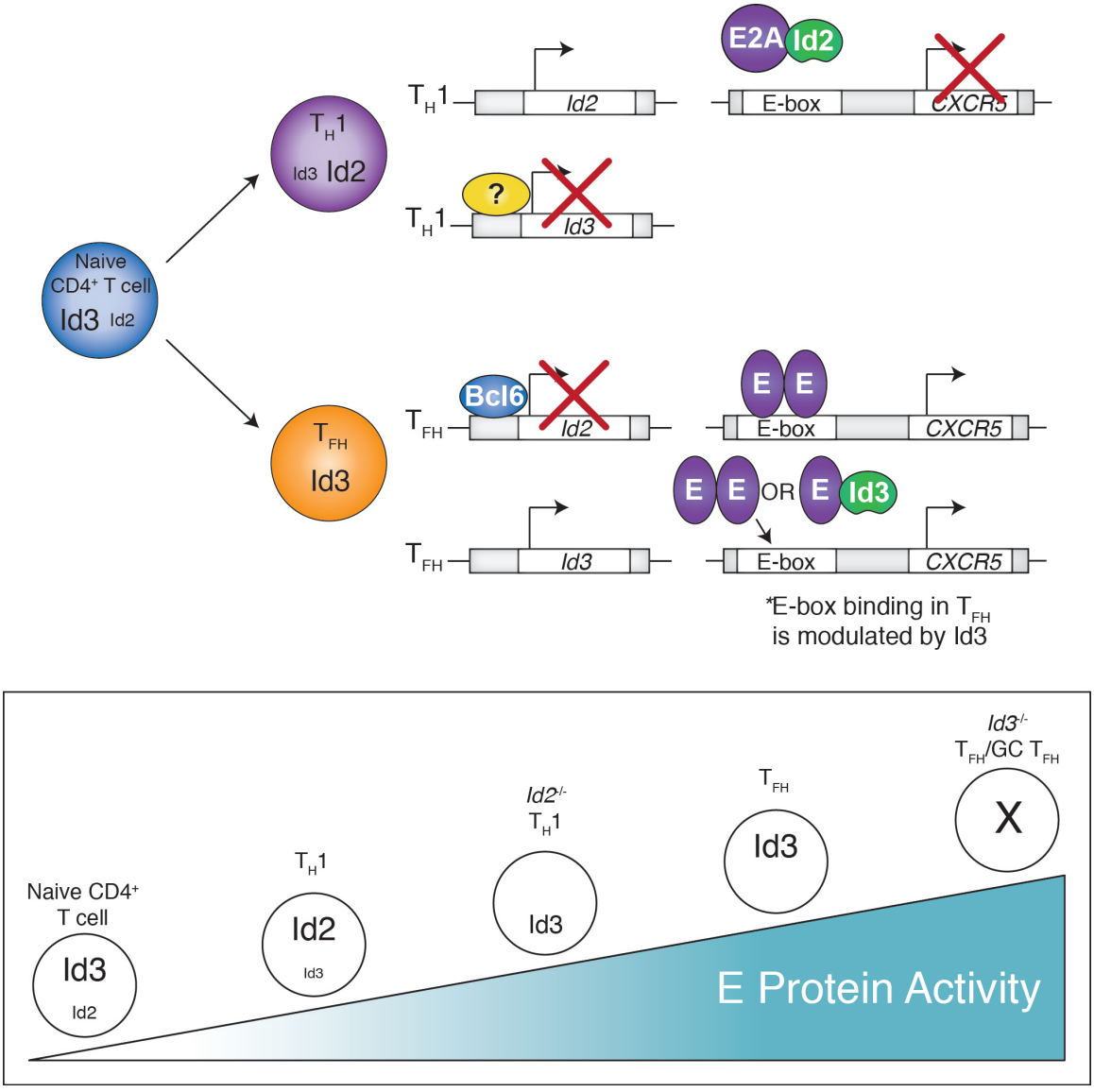


Figure 4.1: Modeling how Id proteins control T cell immunity.

Appendix A

Methods

Mice.

CD4-Cre^{+} mice were from Jackson Laboratory. Mouse strains described below were bred and housed in specific pathogen-free conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of California San Diego or the La Jolla Institute. *Id2*-YFP mice [111], *Id3*-GFP mice [76], *Id2*$^{fl/fl}$ mice [83], *Id3*$^{fl/fl}$ mice [34], SMARTA mice (with transgenic expression of an I-Ab-restricted TCR specific for LCMV glycoprotein amino acids 66-77) [86], *Bcl6*$^{fl/fl}$ [54], CD45.1^{+} congenic mice, NIP mice (with transgenic expression of a TCR specific for LCMV nucleoprotein, amino acids 311-325) [82] and OT-II mice (with transgenic expression of a TCR specific for ovalbumin amino acids 323-339) mice were on a fully B6 background. Recipient C57BL/6J mice were either bred at UCSD or received from The Jackson Laboratory. Both male and female mice were used throughout the study, with sex and age matched T cell donors and recipients.

LCMV infections and protein immunizations.

Recipient mice were infected by intraperitoneal injection of 2 x 10^5 or 5 x

10^5 plaque-forming units of LCMV-Armstrong for analysis at days 6 and 7 or at day 3, respectively. In adoptive transfer experiments of naive $CD4^+$ T cells, LCMV infection was performed 1 d after cell transfer. A total of 20 μg of 4-hydroxy-3-nitrophenylacetyl-OVA (NP16-OVA; Biosearch Technologies) was prepared in 5% alum (aluminum potassium sulfate, Sigma) in a total volume of 20 μl and injected into each footpad.

***T. gondii* infection and lymphocyte isolation from small intestine.**

The ME49 strain of *T. gondii* was maintained in CBA/CaJ mice by intraperitoneal injection of 20 cysts, and cysts were obtained from brain homogenates after 5-6 weeks. Mice were infected with 40 cysts of ME49 by gavage. Small intestine was harvested on day 7 post infection to analyze T_H1 response. To isolate lamina propria lymphocytes, small intestine were cut and washed with plain RPMI-1640, and epithelial cells were removed by incubation with 5 mM EDTA and 1 mM DTT for 20 min at 37°C, followed by enzyme digestion with 0.16 U/ml liberase TL (Roche) for 30 min at 37°C. Lymphocytes were enriched by centrifugation with 47% Percoll.

Flow cytometry and histology.

Single-cell suspensions of spleen or draining popliteal lymph nodes were prepared by standard gentle mechanical disruption. Surface staining for flow cytometry was done with monoclonal antibodies to CD4 (RM4-5, 1:400), CD8 (53-6.7, 1:400), CD45.1 (A20, 1:400), CD25 (PC61.5, 1:400), B220 (RA3-6B2, 1:400), IgD (11-26, 1:400) and PD-1 (J43, 1:400) (eBiosciences, 1:500); PSGL-1 (2PH1, 1:800), CD138 (281-2, 1:500), Fas (Jo2, 1:400) (from BD Biosciences); SLAM (TC15-12F12.2, 1:400), CD25 (PC61, 1:400), CD4 (GK1.5, 1:400), Ly6C (AK1.4, 1:800) (BioLegend) and

PNA (cat # FL-1071, 1:5,000) (Vector Laboratories). Stains were done for 30 min at 4°C in PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide, unless specified otherwise. Phycoerythrin (PE)-labeled I-Ab-gp(66-77) tetramer was supplied by the National Institute of Health (NIH) tetramer core facility. Single-cell suspensions were stained with tetramer at 37°C for 2 h. CXCR5 staining was done as described52, using purified anti-CXCR5 (2G8; BD Pharmingen) for 1 h, followed by biotinylated anti-rat IgG (cat # 112-065-167, Jackson Immunoresearch), and then by PE-, PE-Cy7- or APC-labeled streptavidin (eBioscience) at 4°C in PBS supplemented with 0.5% bovine serum albumin, 2% FCS, and 2% normal mouse serum. Intracellular staining was performed with an Alexa 647- or PE-conjugated monoclonal antibody to Bcl6 (clone K112-91; BD Pharmingen, 1:20), TCF1 (clone C63D9; Cell Signaling, 1:200), IFN-γ (clone XMG1.2; eBioscience, 1:200), T-bet (clone 4B10; eBioscience, 1:200), FoxP3 (clone FJK-16s; eBioscience, 1:200) and the Foxp3 ICS kit buffers and protocol (eBioscience). Stained cells were analyzed using LSRII, LSRFortessa or LSRFortessa X-20 (BD) and FlowJo software (TreeStar). All sorting was done on a FACSAria (BD Biosciences). For RT-PCR analyses, early T_H1 cells (IL-2Rα$^+$) and TFH cells (IL-2Rα$^-$) among total or RV$^+$ SMARTA CD4$^+$ T cells were sorted 3 d after infection with LCMV. Histology was performed as previously described53.

ELISA.

Nunc MaxiSorp plates (Thermo Fisher Scientific) were coated overnight at 4°C with 1 μ/ml NP23-BSA (Biosearch Technologies) or with a 1:60 dilution of LCMV lysate (prepared from LCMV-infected BHK cells) in PBS. Plates were blocked with PBS $^+$ 0.2% Tween-20 $^+$ 1% BSA for 90 min at 25 ÂřC. After washing,

mouse serum was added in a dilution series in PBS + 0.2% Tween-20 + 1% BSA and incubated for 90 min. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (cat # M30107, Thermo Fisher Scientific) was added at 1:5,000 in PBS + 0.2% Tween-20 + 1% BSA for 90 min at 25 ÂřC. Colorimetric detection was performed using a TMB substrate kit (Thermo Fisher Scientific). Color development was stopped after approximately 10 min with 2 N H2SO4, and absorption was measured at 450 nm.

Retroviral vectors, transductions and cell transfer.

MicroRNA-adapted short hairpin RNA and pMIG, Bcl6 MIG and middle domain mutant Bcl6 (K379Q) vectors were described previously [81, 14]. E12, E47, Ascl2, Id2 and Id3 were cloned into the pMIG or pMIA vectors, which contain an IRES-GFP or IRES-mAmetrine, respectively. Virions were produced by transfection of the PLAT-E cell line, as described previously8. Culture supernatants were collected 24 and 48 h after transfection, filtered through a 0.45 μm syringe filter and stored at 4°C until transduction. $CD4^+$ T cells were isolated from whole splenocytes by negative selection (Stemcell Technologies) and resuspended in D-10 (DMEM + 10% FCS, supplemented with 2 mM Glutamax (Gibco) and 100 U/ml Penicillin/Streptomycin (Gibco) with 2 ng/ml recombinant human IL-7 (Peprotech) and 50 μM β-mercaptoethanol (BME). $2x10^6$ cells were stimulated in 24-well plates pre-coated with 8 μg/ml anti-CD3 (17A2; BioXcell) and anti-CD28 (37.51; BioXcell). At 24 and 36 h after stimulation, cells were transduced by adding RV supernatants supplemented with 50 μM BME and 8 μg/ml polybrene (Millipore), followed by centrifugation for 90 min at 524 x g at 37°C. Following each transduction, the RV-containing medium was replaced with D-10 + 50 μM BME + 10 ng/ml human

IL-2. After 72 h of *in vitro* stimulation, $CD4^+$T cells were transferred into six-well plates in D-10 + 50 μM BME + 10 ng/ml human IL-2, followed by incubation for 2.5 days. One day before transfer, the culture medium was replaced with D-10 + 50 μM BME + 2 ng/ml human IL-7. Transduced cells were sorted based on reporter expression (FACSAria; BD Biosciences). Transfer of sorted cells into recipient mice was performed by intravenous injection via the retro-orbital sinus. Transferred cells were allowed to rest in host mice for 3-4 d before infection or immunization. $2x10^4$ or $4x10^5$ transduced $CD4^+$T cells were transferred into each mouse for day 6 or 3 analysis, respectively. For protein immunization, $1x10^5$ transduced $CD4^+$ T cells were transferred into each mouse. DNA fragments encoding shRNA targeting mouse *Tcf3* or *Cd8a* were subcloned into a custom retroviral vector containing the miR30 backbone plus the murine PGK promoter and dsRED as a reporter. $1x10^6$ naive *Id2*$^{+/+}$CD4-Cre$^+$ and *Id2*$^{fl/fl}$CD4-Cre$^+$ SMARTA $CD4^+$ T cells were stimulated for 18 h in 24-well plates pre-coated with anti-CD3 and anti-CD28. Following stimulation, cells were transduced by adding RV supernatants supplemented with 100 U/ml human IL-2 and 8 μg/ml polybrene, followed by centrifugation for 90 min at 2000xg at 37°C. Following transduction, cells were incubated for 3 h at 37°C. $5x10^4$ cells were transferred into day -1 LCMV infected hosts and remaining cells were cultured in vitro with D-10 + 50 U/ml human IL-2 in a parallel time course to assess for knockdown efficiency.

Microarray and ChIP-seq.

Id2$^{+/+}$CD4-Cre$^+$ and *Id2*$^{fl/fl}$CD4-Cre$^+$ SMARTA $CD4^+$ T cells (pooled from five mice) were isolated via flow cytometry on day 7 of LCMV infection (FacsARIA, BD). For microarray analysis, RNA was extracted with TRIzol reagent, amplified

and hybridized to the Affymetrix Mouse Gene 1.0 ST Array. C57BL/6 $CD4^+$ T cells (pooled from five mice) were isolated via flow cytometry on days 7 and 30 of LCMV infection (FacsARIA, BD). For microarray analysis, RNA was extracted with TRIzol reagent, amplified and hybridized to the Affymetrix Mouse Gene 1.0 ST Array. Data were normalized and analyzed with the GenePattern software suite. All data were normalized and analyzed with the GenePattern software suite. E2A Bio-Chip was performed as previously described on total thymocytes from *Tcfe2a*$^{Bio/Bio}$*Rosa26*$^{BirA/BirA}$ mice [68, 31]. Bcl6 ChIP-Seq analyses were of human GC TFH cell Bcl6, H3K4me1, H3K4me3 and H3K27Ac data deposited from ref. 26 (GEO accession code GSE59933), analyzed in the UCSC genome browser. ChIP primers: *ID2* -3.5 kb forward-TTC TGG CCT CTT GAT GTT CTC, reverse-ATT CGC GCC CTC ATT ACT AC; *ID2* -200 bp forward-CTC CTC TAG GTG TTG GAA TGT G, reverse-CCG TGT AGG TGG CAA AGT AA; *PRDM1* forward-CCA GTA GGC CTT TCA TGG CT, reverse-TGC TCA GGT TGA GAA AGC AGT; *CD8*β forward-GTG ACA ACG TAG GCA TCT CA, reverse-AGC GAC AAA CAC CTC ATA CTC; *FOXP3* forward-ACT ATG TTG CCC AGG CTT AC, reverse-CTG TCC TGG TGA CGC TAA AG.

Quantitative RT-PCR and ChIP followed by quantitative PCR.

Total RNA from the sorted cells was extracted and reverse-transcribed, and quantitative PCR was performed using SYBR Select MasterMix (Thermo Fisher Scientific). Results were normalized to the expression of *Gapdh* transcripts. Primary GC T_{FH} were isolated from human tonsil by staining with biotin-conjugated PD-1 (J105, eBioscience) followed by isolation using Streptavidin microbeads (Miltenyi). GC T_{FH} were crosslinked with 1% formaldehyde and then quenched with 125

mM glycine. Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris and 5 mM EDTA) supplemented with protease inhibitors (Thermo Fisher Scientific) and 0.5 mM PMSF (Thermo Fisher Scientific) followed by sonication and isolation of chromatin. Protein G Dynabeads (Life Technologies) were conjugated to antibodies specific to Bcl6 (N-3 and C-19, Santa Cruz). Normal rabbit IgG (cat # sc-2027, Santa Cruz) was used as a control. Chromatin was immunoprecipitated using the conjugated beads, eluted, and reverse crosslinked using 0.3 M NaCl at 65°C overnight. Quantitative PCR was performed on isolated DNA and sample values were given as a percentage of input. qPCR primers: *Id2* forward-ATG AAA GCC TTC AGT CCG GTG, reverse-AGC AGA CTC ATC GGG TCG T; *Gapdh* forward-GGT CCT CAG TGT AGC CCA AG, reverse-AAT GTG TCC GTC GTG GAT CT; *Tcf3* forward-CAT CCA TGT CCT GCG AAG CCA, reverse-TTC TTG TCC TCT TCG GCG T.

Statistical Methods

Statistical tests were performed using Prism 6.0 (GraphPad). Significance was determined by unpaired Student's t-test with a 95% confidence interval.

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