UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of Id2 and Id3 in memory $CD8^+$ T cells differentiation and natural killer T cell homeostasis

A dissertation submitted in partial satisfaction of the
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in

Biology

by

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The Dissertation of Cliff Yang Yang is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

To my parents,

Ouyang Jinying and Daichang Yang

Thank you for enduring the hardships of immigration

to give me the opportunity for a better life

To my wife,

Danielle Wang

Thank you for your love and care

and

helping me to grow up (a bit)

EPIGRAPH

Never let your sense of morals keep you from doing what is right.

Isaac Asimov

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ABSTRACT OF THE DISSERTATION

The role of Id2 and Id3 in memory $CD8^+$ T cells differentiation and natural killer T cell homeostasis

by

Cliff Yang Yang

Doctor of Philosophy in Biology

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Professor Ananda Goldrath, Chair
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During infection, naive $CD8^+$ T cells differentiate into effector cells armed to eliminate pathogens and memory cells that provide protection from reinfection. How the transcriptional program regulating terminal differentiation to short-lived effector/memory cells versus long-lived memory cells is initiated and sustained is not clearly defined. Here, we identify long-lived memory precursors prior to the peak of $CD8^+$ T cell expansion through the use of reporter mice that allow us to monitor expression of inhibitor-of-DNA-binding (Id)2 and Id3, key antagonists of E protein transcription factors. $Id3^{hi}$ long-lived memory precursors can be identified prior to expression of cell-surface receptors known to predict memory potential and display a gene-expression profile consistent with this lineage. Deficiency in Id2 or Id3 results in the loss of distinct

populations of $CD8^+$ effector and memory T cells, demonstrating unique roles of these transcriptional inhibitors. Furthermore, we provide a connection between inflammatory cytokines, known to influence memory subset differentiation, with the inverse regulation of Id2 and Id3 expression. These data provide novel insights into how external cues control gene expression and implicate unique roles for Id2 and Id3 in $CD8^+$ T cell biology.

Natural Killer T cells expressing an invariant T cell receptor (*i*NKT) regulate activation of both innate and adaptive immunity in many contexts. *i*NKT cells accumulate in the liver and rapidly produce prodigious amounts of numerous cytokines upon activation, impacting the immune response to viral infection, immunosurveillance for malignant cells and liver regeneration. However, little is known about the factors controlling *i*NKT homeostasis, survival and hepatic localization. Here, we report that the absence of the transcriptional regulator Id2 resulted in a severe, intrinsic defect in the accumulation of hepatic *i*NKT cells. Id2-deficient *i*NKT cells showed increased cell death in the liver, although migration and functional activity were not impaired in comparison to Id2-expressing *i*NKT cells. Id2-deficient *i*NKT cells exhibited diminished expression of CXCR6, a critical determinant of *i*NKT cell accumulation in the liver, and of the anti-apoptotic molecules bcl-2 and bcl-X_L, compared to Id2-sufficient *i*NKT cells. Furthermore, survival and accumulation of *i*NKT cells lacking Id2 expression was rescued by deficiency in bim, a key pro-apoptotic molecule. Thus, Id2 was necessary to establish a hepatic *i*NKT cell population, defining a novel role for Id2 and implicating the Id targets, E protein transcription factors, in the regulation of *i*NKT cell homeostasis.

Chapter 1

Introduction to memory $CD8^+$ T cells

1.1 Memory $CD8^+$ T cells

$CD8^+$ T cells are part of the adaptive immune response necessary for elimination of intracellular pathogens such as bacteria, viruses, and protozoan parasites. Upon encounter with a pathogen, naïve $CD8^+$ T cells proliferate and differentiate into effector $CD8^+$ T cells, which target and kill infected cells[1-3]. During this massive expansion phase, $CD8^+$ T cells up-regulate many molecules involved in clearing the pathogen, including cytokines such as IFN-γ and TNFα, and cytolytic molecules such as perforin and granzyme B. Following this expansion phase, the majority of the effector $CD8^+$ T cells die, leaving a small number of memory $CD8^+$ T cells that persist and are able to respond more rapidly to the same pathogen upon secondary challenge. This memory $CD8^+$ T cell response, combined with humoral immunity, is able to control a secondary infection and quickly eliminate the pathogen, often without clinical symptoms.

However, the molecular pathways contributing to memory $CD8^+$ T cells formation are still unclear. Most of the available vaccines elicit robust antibody responses, but relatively weak $CD8^+$ T cell responses. For example, in people who received smallpox vaccines, only about half the vaccinated group display detectable antigen-specific memory $CD8^+$ T cells a year after vaccination[4]. Understanding this complex process is crucial for rational design of T cell specific vaccines and immunotherapy.

1.2 Models of memory $CD8^+$ T cell differentiation

There are several competing models describing the relationship between effector and memory $CD8^+$ T cell memory and effector lineages. Some argue that the initial priming of the naïve $CD8^+$ T cells by the antigen presenting cells provides the crucial instruction to lineage commitment[1]. The timing of this key decision is still under debate. However, there is some evidence supporting the idea that naïve $CD8^+$ T cells, to some extent, can be programmed during the initial antigen exposure[1, 5]. Others suggest the programming occurs at the first division, as the first two daughter cells from an activated $CD8^+$ T cell inherit different amounts of receptors and other molecules[6].

Another model suggests initial antigen presentation is not the only factor influencing short-lived effector versus long-lived memory cell generation. Rather, it can be argued that lineage commitment is most likely due to combination of the signals received over time by the naïve cell and its progenitor effectors. In this school of thought, early effector $CD8^+$ T cells have the potential to become terminally differentiated effector or long-lived memory $CD8^+$ T cells, depending on when and what signals they receive. It has been proposed that memory $CD8^+$ T cells are derived from the effector T cell pool[1]. Memory $CD8^+$ T cells can be derived from effector $CD8^+$ T cells that were transferred into new hosts[7]. Furthermore, one single naïve cell can differentiate into effectors and memory subsets[8, 9].

The focus of this section is not to judge the validity of each model, but rather to provide a context for the important question: what influences the $CD8^+$ T cell decision to commit to the effectors or memory lineage. Extrinsic factors, such as prolonged inflammatory signals[10], or antigen stimulation[11], can push early effectors cells into a more

terminally differentiated effector subset. This will be discussed in more detail in section 1.4, and the transcriptional factors thought to be involved in this process will be discussed in section 1.5. Extrinsic and intrinsic factors are not mutually exclusive to each model and can be and have been incorporated into multiple different models.

1.3 Cell surface molecules that identify memory precursor subsets

Not all $CD8^+$ T cells are created equal. A useful and convenient way to identify different subsets is by cell surface molecule expression. Many cell surface molecules have been proposed as markers for memory-precursor $CD8^+$ T cells, cells defined as having great potential to differentiate into long-lived memory $CD8^+$ T cells.

The first cell surface markers used to define $CD8^+$ T cell subsets were proposed in 1999. Memory T cells can be divided into central memory and effector memory $CD8^+$ T cell subsets, based on the expression of L-selectin (CD62L) and CC-chemokine receptor 7 (CCR7)[12]. Naive T cells express high levels of CD62L and CCR7; however, after activation, CD62L and CCR7 are rapidly down-regulated[13]. During the immune response, CD62L is re-expressed by some subsets of effector and memory $CD8^+$ T cells. It is thought that $CD62L^{hi}CCR7^+$ (central) memory cells migrate more efficiently to peripheral lymph nodes, whereas $CD62L^{lo}CCR7^-$ (effector) memory cells are more likely to be found in tissues such as liver and lungs[14]. Central memory $CD8^+$ T cells are shown to have a greater proliferative potential than effector memory cells[15]. In addition, following antigen clearance, effector memory $CD8^+$ T cells can convert to central memory $CD8^+$ T cells[15]. Although it is generally agreed that this conversion is due to the degree of antigen stimulation, there is considerable debate over whether this conversion means central and effector memory cells are not distinct subsets[14].

Besides CD62L and CCR7, a series of cell surface markers have been discovered to be indicative of better memory $CD8^+$ T cell formation potential. It was first reported that a subset of effector $CD8^+$ T cells that express high levels of IL-7 receptor α chain (CD127) were precursors of long-lived memory cells[16]. More recently, killer cell lectin-

like receptor G1 (KLRG1) was identified as a marker to discern memory T cell precursors from effectors; $KLRG1^{lo}CD127^{hi}$ $CD8^+$ T cells are thought to be long-lived memory precursors[10]. CD127 and KLRG1, used in combination, have been informative in predicting memory potential, in a variety of different infection models. In IL-7-deficient mice, CD127 expression and kinetics of $CD8^+$ T cell memory formation remained unperturbed[17]. Moreover, forced expression of CD127 in effector $CD8^+$ T cells does not affect memory $CD8^+$ T cell formation[18]. However, it is likely that CD127 is necessary but not sufficient for $CD8^+$ T cells memory formation. Similarly, the loss of KLRG1 does not impact $CD8^+$ T cell memory formation[19], and the exact molecular mechanism of how KLRG1 exerts its function is unknown.

During the early stages of lymphocytic choriomengitis virus (LCMV) infection, expression of CD25 (IL-2 receptor α chain) has also been identified as indicative of memory potential, whereas $CD25^{lo}$ effector cells are thought to preferentially differentiate into memory $CD8^+$ T cells. Although the loss of IL-2 signaling has little effect initially on the number of developing effector and memory cells, the composition of effector subsets is altered and the secondary responses are compromised[20].

Cell surface markers for memory precursors can be indicative of memory potential but may bear little functional relevance. Thus, it is important for us to elucidate the molecular pathways involved in memory formation. The following two sections will explore extrinsic and intrinsic factors found to be required for $CD8^+$ memory T differentiation.

1.4 Extrinsic factors contributing to $CD8^+$ T cell memory formation

Environmental cues have significant effects on the number and composition of the memory population. Cytokines are the key messengers between various cell types during the immune response, and $CD8^+$ T cells require a number of different cytokines for survival, expansion and differentiation.

Common γ chain cytokines (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) bind to multimeric receptors but share the same γ receptor[21]. IL-2 and IL-15 have distinct α receptor but share the same IL-2/15Rβ receptor. Common γ chain cytokines activate mutiple signaling pathways including, Jak-STAT, phosphinositide 3-kinase (PI3K)/Akt, and RAS-mitogen-activated protein kinase (MAPK)[21, 22].

In naïve $CD8^+$ T cell homeostasis, IL-7 plays a dominant role in supporting T cell survival. IL-$7^{-/-}$ and IL-$7R\alpha^{-/-}$ mice have greatly reduced thymocyte and peripheral T cell numbers[23, 24]. IL-7Rα is expressed by resting T cells and blocking IL-7 with an IL-7 neutralizing antibody shortens their lifespan[25]. Conversely, since naïve T cells express little or no IL-2 or IL-15 specific receptors, IL-2 and IL-15 appear to play minimal roles in naïve $CD8^+$ T cell homeostasis[26].

During $CD8^+$ T cell activation, IL-7Rα is down-regulated, while IL-2Rα (CD25) and IL-2/15Rβ (CD122) are up-regulated[27]. However, the absence of IL-2, IL-7 or IL-15 does not have significant impact on early T cell activation[21]. The complex dynamics involving competition of these cytokines is likely to modulate different aspects of T cell activation, in a context-specific manner. In contrast, inflammatory signals, such as IL-12 or type I interferons, are required for naïve $CD8^+$ T cells to undergo optimal expansion, acquire full effector functions, and differentiate into memory $CD8^+$ T cells[28]. On the

other hand, IL-2 signals during priming are required for secondary expansion of memory $CD8^+$ T cells[20].

IL-7 also mediates an obligatory survival signal for memory T cells[21]. The survival and proliferation of memory $CD8^+$ T cells are diminished when transferred to IL-$7^{-/-}$ mice[25]. Memory $CD8^+$ T cells could be generated normally in the absence of IL-15, but the gradual loss of memory cells suggests that IL-15 is also essential for memory T cell homeostasis[29]. Additional evidence suggests IL-15 supports the basal proliferation of memory T cells[30].

IL-2, IL-7 and IL-15 also play a role in memory differentiation. It was reported that sustained IL-2 receptor signaling during expansion drove terminal-effector differentiation[31]. Interestingly, the provision of IL-15 or IL-2, during contraction led to the preferential accumulation of $KLRG1^{hi}$ $CD127^{lo}$ $CD8^+$ T cells, whereas provision of IL-7 instead favored the accumulation of $KLRG1^{lo}$ $CD127^{hi}$ $CD8^+$ T cells[32]. IL-10 and IL-21 have also been implicated in the memory differentiation pathways. In the absence of either IL-10 and IL-21 or STAT3, $CD8^+$ T cells retain terminal effector differentiation states and fail to mature into protective memory T cells that contain self-renewing central memory T cells[33]. The interplay between cytokines such as IL-2, IL-7, IL-15 and IL-21 during memory differentiation could be quite complex, since they share some common receptors and activate multiple STAT proteins.

Besides the common γ chain cytokine family, several other cytokines are also important for generation of memory T cells. Selective attenuation of TGFβ signaling in T cells increased the numbers and multiple functions of antiviral $CD8^+$ T cells and enabled rapid eradication of the persistence-prone virus and memory generation[34]. Pro- or

anti-inflammatory cytokines such as IL-10, IL-12 and type I interferons had also been shown to be influencing memory formation[35-38]. As a general rule, more inflammatory signals and antigen stimulation tend to drive the differentiation of terminal effectors[36]. In summary, multiple cytokines influence the transition from effector to memory $CD8^+$ T cells differentiation and it is unclear how the compensatory and competing signals are balanced downstream at the transcriptional level.

1.5 Transcription factors crucial to $CD8^+$ T cell memory formation

Drastic changes in gene-expression patterns occur when naïve $CD8^+$ T cells differentiate into effector and memory $CD8^+$ T cells[39, 40]. Only a few transcription factors have been found to directly influence the differentiation of memory $CD8^+$ T cells. T-bet, a T-box-binding transcription factor, is essential for the acquisition of $CD8^+$ T cell effector function[41, 42]. T-bet was originally discovered as a key transcriptional factor in Th1 $CD4^+$ T lineage development[43]. Loss of T-bet leads to the accumulation of $KLRG1^{lo}$ $CD127^{hi}$ subset[10]. Inflammatory cytokines such as IL-12 induces T-bet expression and drives the differentiation of $KLRG1^{hi}$ $CD127^{hi}$ subset[10]. Another similar T-box binding transcription factor, Eomes was also found to have some redundant functions as T-bet in $CD8^+$ T effector cells[41]. However, loss of Eomes has little impact in memory $CD8^+$ T cell subset differentiation, though the long-term survival of the memory $CD8^+$ T cells was compromised[44].

A transcriptional repressor, B-lymphocyte induced maturation protein-1 (Blimp-1), has been also found to be required for the proper formation of memory $CD8^+$ T cells[45, 46]. Blimp-1 was first described as a critical regulator of B cell effector and memory differentiation as well as master regulator of follicular helper T cell differentiation[47, 48]. Blimp-1 deficiency promoted the acquisition of memory $CD8^+$ T cells properties by effector cells[45, 46]. Just like T-bet, loss of Blimp-1 leads to the accumulation of $KLRG1^{lo}$ $CD127^{hi}$ subset[45, 46]. Interestingly, Id3 is a known direct target of Blimp-1[49, 50]. Yet the role of Id3 in memory $CD8^+$ T cell differentiation has not been explored previously.

1.6 The role of E and Id proteins in the immune system

The E protein family of transcription factors and their natural antagonists, Id proteins, regulate the commitment to the lymphoid lineages at several developmental stages[51]. E proteins are a subclass of the basic helix-loop-helix (HLH) family of transcriptional factors, which form homodimers or heterodimers and bind specifically to DNA sequences containing E box sites. Id proteins belong to the HLH family which lack the basic DNA-binding domain. They form heterodimers with E proteins and inhibit their DNA-binding activity.

E proteins regulate the lineage fate of early thymocyte progenitors (NK, γδ and αβ), the rearrangement of α and β TCR chains, and the transition from DN to SP and DP to SP. E proteins also exert similar functions in B cell development. In contrast, the inhibition of E proteins by Id proteins, especially by Id2 and Id3, promotes thymocyte proliferation, maturation and survival by regulating E protein activity[51].

Id2-deficient mice have normal numbers of αβ T cells as well as normal splenic structure and $CD4^+$ to $CD8^+$ ratio. However they lack hepatic NKT cells, NK cells, lymph nodes, Langerhans cells, and a subset of intraepithelial lymphocytes (IEL) and CD8α-dendritic cells[52-54]. Id3-deficient mice have defects in αβ T cell selection as well as massive expansion of γδ T cells, follicular-helper-like T cells and innate T cells[49, 55-57]. However, the role of Id2 and Id3 in $CD8^+$ T cell response to infection has been largely unexplained until now.

1.7 The role of Id2 in $CD8^+$ T cell response to infection

It was recently discovered that Id2 is essential for the survival of late effector $CD8^+$ T cells and the proper formation of memory following infection[58]. Id2 expression is up-regulated in effector $CD8^+$ T cells and subsequently maintained at a high level in memory $CD8^+$ T cells. Although Id2-deficient naive $CD8^+$ T cells recognize antigen and proliferate normally early after infection, effector $CD8^+$ T cells do not accumulate because the cells are highly susceptible to apoptosis[58].

Additionally, the small population of Id2-deficient effector T cells that do survive rapidly acquire the central memory phenotype ($CD62L^{hi}$), suggesting that Id2 might regulate the formation of memory $CD8^+$ T cell subsets[58]. However, the expression pattern and functions of Id2 and Id3 in naïve, effector and memory $CD8^+$ T cells are poorly defined. It is of interest to see how the expression of Id2 influence the commitment from effector to memory $CD8^+$ T cells. Furthermore, there is little information regarding how Id2 and Id3 expression is regulated in the context of mature T cell activation.

1.8 Regulation of Id proteins

Several studies have investigated the regulation of Id3 in thymocytes. Id3 expression was increased in response to TCR-mediated signals[59]. In addition, Id3 transcription was downregulated in Ras-dominant negative mice[59]. Also, inhibition of c-Raf with a chemical inhibitor blocked the induction of Id3. Additional evidence suggest that Ras/ERK/MAPK cascade, as well as an immediate transcriptional factor, EGR-1, are involved in the regulation of Id3[59].

Id3 expression is also regulated by the TGF-β family of cytokines[60]. TGF-β, a pleiotropic cytokine, requires expression of Id3 to inhibit the growth and survival of B lymphocyte progenitors. In embryonic stem cells, Id2 and Id3 transcription are activated by bone morphogenetic protein 4 (BMP4)[61]. Lastly, c-myc was also shown to induce Id2 expression in several cell lines[62]. However, the regulation of Id2 and Id3 in mature T cells, especially effector $CD8^+$ T cells, is largely unknown.

1.9 Summary and key questions

During infection, naive $CD8^+$ T cells differentiate into effector cells armed to eliminate pathogens and memory cells that provide protection from reinfection. How the transcriptional program regulating terminal differentiation to short-lived effector/memory cells versus long-lived memory cells is initiated and sustained is not clearly defined.

Historically cell surface markers are used to identify subsets of effector $CD8^+$ T cells which are more likely to differentiate into long-lived memory $CD8^+$ T cells. A number of surface molecules, such as CD127 and KLRG1, were used extensively for that purpose. However, those surfaces markers are not deterministic of memory $CD8^+$ T cell differentiation, thus provide few clues to the transcriptional programming of memory $CD8^+$ T cells.

Studies have begun to examine the transcriptional control of memory $CD8^+$ T cell differentiation. The role of Id2 and Id3 in memory $CD8^+$ T cell differentiation to this point is largely unknown. This thesis will address the differential expression of Id2 and Id3 and how these transcription factors direct effector $CD8^+$ T cells into memory $CD8^+$ T cells. Furthermore, the studies described here will assess the regulation of Id2 and Id3 in $CD8^+$ T cells during the immune response and how environmental cues such as cytokines influence Id2 and Id3 expression.

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Chapter 2

Id2 and Id3 control the formation of memory $CD8^+$ T cell subsets

2.1 Introduction

Infection with intracellular pathogens initiates proliferation of naive $CD8^+$ T cells and their differentiation into cytotoxic, cytokine-secreting effector T cells with a short half-life[1]. After infection is resolved, the majority of effector $CD8^+$ T cells die; however, a small fraction differentiates into memory cells that persist for prolonged periods. Upon pathogen re-exposure, these memory T cells respond with enhanced proliferation, cytotoxicity and cytokine secretion, often providing life-long protection against previously encountered pathogens[2, 3]. Given the importance of $CD8^+$ T cell memory, the identity of factors dictating whether cells will join the memory $CD8^+$ T cell pool has become a key immunological question.

Memory $CD8^+$ T cells are thought to derive from the effector T cell pool[4-6]. During the effector phase, precursors of long-lived memory T cells display substantial differences in gene expression and cell surface phenotype compared to effectors cells[7, 8]. While it is clear that environmental cues such as exposure to pro-inflammatory cytokines[9-13] and duration or intensity of antigen exposure strongly impact the number and composition of the memory T cell population[14, 15], the transcriptional connection between the inflammatory environment and the transition to long-lived memory T cells is an area of intense investigation. Indeed many cytokines, such as IL-2, IL-7, IL-15, IL-12, IFN-γ, IFN-α/β, and TGF-β, influence the transition from effectors to memory cells, in many cases favoring the generation of effector and short-lived memory cells versus long-lived memory cells[9-13].

In recent years, cell surface receptors have proved useful in distinguishing terminally differentiated short-lived effector-memory cells from those more likely to transition into long-lived memory cells. Markers of $CD8^+$ T cells with short-lived effector or longer-lived memory potential include: CD62L (L-selectin), CD127 (IL-7 receptor α chain), KLRG1 (killer cell lectin-like receptor G1), CXCR3, CD43 and CD25 (IL-2 receptor α chain)[7, 16-19]. Many of these surface proteins indicate but are not required for the differentiation of memory $CD8^+$ T cells. For example, IL-7-receptor signaling is permissive but not instructive to the formation of memory $CD8^+$ T cells, and KLRG1 is dispensable for $CD8^+$ T cell differentiation[20, 21]. Moreover, the loss of IL-2 signaling has little effect initially on the number of developing effector and memory cells, though the composition of the effector subsets is altered and the secondary responses are compromised[18, 22-24]. Therefore, it is important to explore memory potential beyond a surface phenotypic 'read-out' and identify the transcriptional programs that control the differentiation of memory $CD8^+$ T cells.

Recent work has begun to elucidate some of the transcriptional regulators required for $CD8^+$ memory formation. Both T-bet and Blimp-1 are required for terminal effector $CD8^+$ T cell differentiation and loss of either of these proteins leads to an accumulation of $KLRG1^{lo}$ $CD127^{hi}$ long-lived memory precursors[7, 25-27]. In contrast, the transcription factors TCF-1 and Eomes, both contribute to the maintenance of the long-lived memory precursor subset[28, 29]. Additionally, we recently demonstrated that the transcriptional inhibitor Id2 is essential for the survival of effector $CD8^+$ T cells and their memory formation following infection[30]. However, the interplay between these different

transcription factors and the signals that drive their expression during $CD8^+$ memory T cell differentiation remains under investigation.

Id proteins negatively regulate the DNA-binding activity of E protein transcription factors[31]. Both E and Id proteins play multiple roles in lymphocyte development and homeostasis. Although there are four Id proteins in mammals, Id2 and Id3 are the family members predominantly expressed in lymphocytes. Deficiency in either leads to numerous defects in the generation of immune cell types. Loss of Id2 impairs development of lymphoid tissue inducer T cells, NK cells, NKT cells, CD8α dendritic cells, and subsets of intraepithelial lymphocytes[32-34]. Id2 plays a role in $CD8^+$ T cell differentiation during infection, suggesting a broader function in differentiation of mature lymphocytes[30]. Id3 deficiency leads to distinct defects in αβ thymocyte selection as well as expansion of γδ, T_{FH}-like and innate T cell subsets[35-39]. However, little is known about the role of Id3 in the $CD8^+$ T cell response to infection. Although the signals that control Id2 expression are not understood, it is known that both T cell receptor (TCR) signaling and TGF-β can influence Id3 expression, and that E protein transcription factors and Blimp-1 are direct transcriptional regulators of the *Id3* gene[40, 41].

To clearly delineate the roles of Id2 and Id3 during the $CD8^+$ T cell immune response, we generated Id2-YFP and Id3-GFP knock-in reporter lines. Using these tools, 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}. This 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. 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. Finally, we showed that both Id2 and Id3 expression were regulated by inflammatory cytokines, providing a link between the cytokine milieu and regulation of the transcriptional activity guiding $CD8^+$ T cell fate during infection.

2.2 Dynamic expression of Id2 and Id3 during the infection

Id2 supports survival of $CD8^+$ effector T cells[30] and Id3 was upregulated in surviving Id2-deficient T cells but it was not clear if this represented compensatory upregulation and/or the presence of a distinct $Id3^{hi}$ population. To understand better the relationship of Id2 and Id3 expression by individual $CD8^+$ T cells during infection, we inserted the coding region of yellow fluorescent protein (YFP) into the first exon of the *Id2* locus, resulting in a mutated locus where YFP expression was under control of the Id2 regulatory elements (**Fig. 2.1a**). We used the Id2-YFP reporter mouse line in combination with an Id3-GFP reporter mouse line generated using an identical strategy[35]. Mice heterozygous for the $Id2^{Y/+}$ and $Id3^{G/+}$ knockin alleles generate equivalent $CD8^+$ T cell responses compared to wild type C57BL/6 littermates (**Fig. 2.1b**), and display comparable Id2-YFP and Id3-GFP reporter expression in numerous cell types, consistent with previous reports and relative mRNA levels determined by qPCR (**Fig. 2.2**)[30].

Naive polyclonal and ovalbumin (OVA) peptide-specific OT-I TCR transgenic $CD8^+$ T cells express readily detectable amounts of both Id2-YFP and Id3-GFP reporters (**Fig. 2.2a** and **Fig. 2.3a**). To study the co-regulation of Id2 and Id3 expression during the immune response, we transferred $Id2^{Y/+}Id3^{G/+}$ $CD45.1^+$ OT-I $CD8^+$ T cells to C57BL/6 hosts or infected $Id2^{Y/+}Id3^{G/+}$ mice with recombinant vesicular stomatitis virus or *Listeria monocytogenes* expressing chicken ovalbumin (OVA) (VSV-OVA and Lm-OVA respectively), both self limiting infections that are rapidly cleared (**Fig. 2.2** and **Fig. 2.3**). We monitored the levels of Id2-YFP and Id3-GFP in antigen-specific $CD8^+$ T cells identified by flow cytometry. $Id2^{Y/+}Id3^{G/+}$ OT-I $CD8^+$ T cell population responding to VSV-OVA infection *in vivo* showed a substantial down-regulation of Id3-GFP (MFI ~5-

fold lower than naive) and a more moderate down-regulation of Id2-YFP compared to their naive counterparts (**Fig. 2.3a** and **b**). Id3-GFP expression was more dynamic, with prominent Id3-GFP^{hi} and Id3-GFP^{lo} subsets emerging just prior to or coincident with the appearance of KLRG1^{hi} and CD127^{hi} populations, the contraction of the effector response, and the emergence of CD8^{+} memory T cells (**Fig. 2.3a**). In the case of Lm-OVA infection, OT-I cells showed a similar regulation of Id2 and Id3 reporters with a delay in the up-regulation of Id3-GFP expression that was consistent with the generation of more KLRG1^{hi} effector cells and later re-expression of CD127 (**Fig. 2.3c**). In our analysis of polyclonal antigen-specific CD8^{+} T cells identified by MHC class I tetramers (H-2K^{b}-OVAp), we found that Id3-GFP re-expression occurred at later time points for both infections. However, Id3-GFP levels increased as the frequency of CD127^{hi} cells increased, as it was the case for OT-I cells (**Fig. 2.2**). We sorted OT-I cells on days 0, 7 and 15 of infection and observed their expression of Id2 and Id3 mRNA by qPCR. Notably, relative expression of the Id2-YFP and Id3-GFP reporters was consistent with relative mRNA expression (**Fig. 2.3d**). We also transferred increasing numbers of OT-I cells into C57BL/6 recipients, thus accelerating CD8^{+} T cell differentiation to a long-lived memory phenotype[42], and found that Id3-GFP was upregulated and Id2-YFP down-regulated more quickly as the number of transferred OT-I cells was increased (**Fig. 2.3e**). Thus, delayed upregulation of Id3-GFP expression correlated with time points where memory T cells emerge from the effector population, suggesting a possible role for Id3 late in CD8^{+} T differentiation to memory cells.

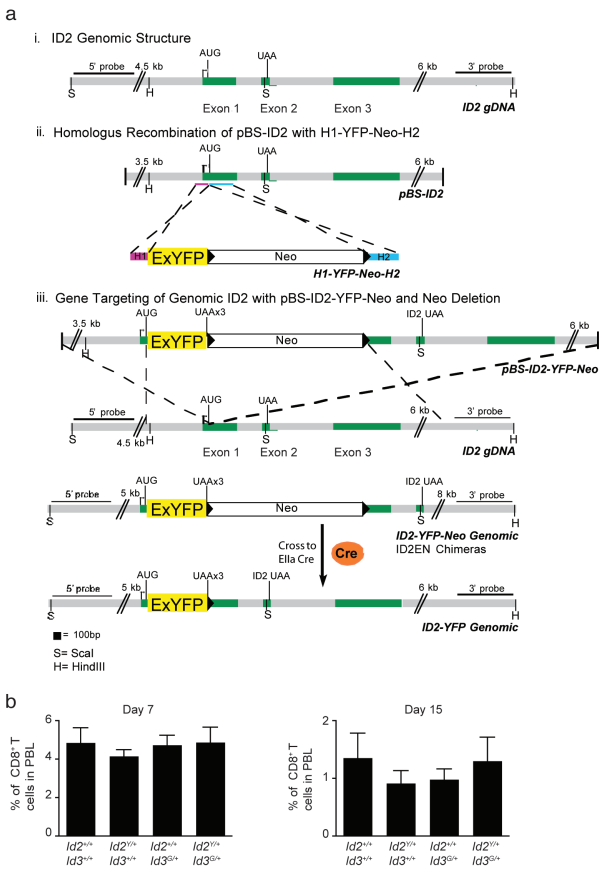

Figure 2.1 Generation of Id2-YFP knockin reporter. (a) i) The genomic structure of *Id2* with exons highlighted in green, the transcriptional start site indicated with an arrow, and the translational start and stop sites indicated by AUG and UAA respectively. Restriction enzyme sites utilized for Southern Hybridization screening are indicated, S = *ScaI*, H = *HindIII*, as well as the location of probes. ii) Homologous recombination of pBS-Id2 with H1-YFP-Neo-H2 in a bacterial system. Recombineering was employed to produce the targeting construct pBS-Id2-YFP-Neo. iii) Gene targeting of genomic Id2 with pBS-Id2-YFP-Neo as occurs in the embryonic stem cells followed by the deletion of the Neo cassette by *EIIa* driven *cre* recombinase in the mouse. **(b)** $Id2^{+/+}Id3^{+/+}$, $Id2^{Y/+}Id3^{+/+}$, and $Id2^{+/+}Id3^{G/+}$, $Id2^{Y/+}Id3^{G/+}$ mice were infected with VSV-OVA and antigen-specific $CD8^+$ T cells from the blood were analyzed at indicated days after infection. Bar graphs indicate percent of antigen specific $CD8^+$ T cells. Data are pooled from 5 independent experiments with 2-3 mice each group. Error bars indicate SEM.

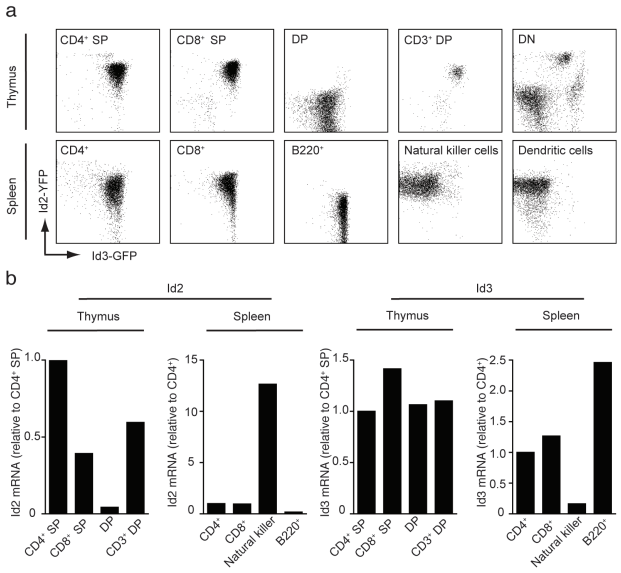

Figure 2.2 Expression of Id2-YFP and Id3-GFP in naive lymphocytes. (a) Thymocytes and splenocytes from naive *Id2*$^{Y/+}$ *Id3*$^{G/+}$ mice were analyzed for Id2-YFP and Id3-GFP expression. Representative FACS plots from 4 independent experiments with 2-3 replicates. **(b)** Indicated populations were sorted from naive wild type C57BL/6 mice and expression of Id2 (left) and Id3 (right) transcripts were analyzed by qPCR. Data are representative of 2 independent experiments.

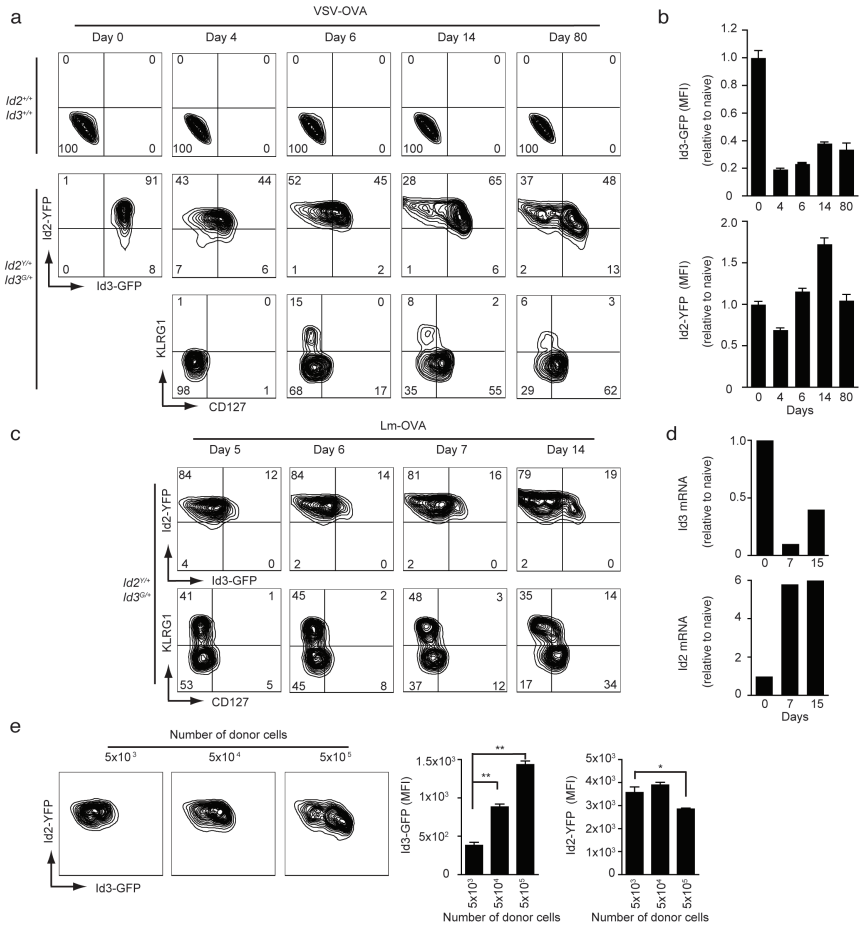

Figure 2.3. Analysis of Id2-YFP and Id3-GFP reporter expression in CD8$^+$ T cells during infection reveals distinct effector populations. (a) Flow cytometry plots of Id2-YFP and Id3-GFP expression by CD8$^+$ OT-I T cells. CD45.2$^+$ C57BL/6 mice received *Id2*$^{Y/+}$*Id3*$^{G/+}$ CD45.1$^+$ OT-I (2.5 x 10^4) cells 1 day before infection with VSV-OVA and CD8$^+$ CD45.1$^+$ splenocytes were analyzed at days indicated after infection. Data are representative of six independent experiments n = 2 - 3 mice per group. **(b)** MFI of Id3-GFP and Id2-YFP of *Id2*$^{Y/+}$*Id3*$^{G/+}$ OT-I cells responding to VSV-OVA infection. **(c)** Flow cytometry plots of Id2-YFP and Id3-GFP by CD8$^+$ OT-I T cells. CD45.2$^+$ C57BL/6 mice received *Id2*$^{Y/+}$*Id3*$^{G/+}$ CD45.1$^+$ OT-I (2.5 x 10^4) cells 1 day before infection with Lm-OVA, and CD8$^+$ CD45.1$^+$ splenocytes were analyzed on indicated days after infection. Data are representative of 3 independent experiments n = 2 - 3 mice per group. **(d)** Time course of Id2 and Id3 mRNA induction during *in vivo* activation of CD45.1$^+$ OT-I T cells. CD45.2$^+$ C57BL/6 mice received *Id2*$^{Y/+}$*Id3*$^{G/+}$ CD45.1$^+$ OT-I (1 x 10^4) cells 1 day before infection with VSV-OVA and OT-I T cells were sorted with >98% purity from the spleens of host mice. **(e)** Flow cytometry plots and MFI of Id2-YFP and Id3-GFP on day 7 of infection with indicated numbers of CD45.1$^+$ OT-I T cells transferred. CD45.2$^+$ C57BL/6 mice received 5 x 10^3, 5 x 10^4, or 5 x 10^5 *Id2*$^{Y/+}$*Id3*$^{G/+}$ CD45.1$^+$ OT-I cells 1 day before infection with VSV-OVA. Data are representative of 3

independent experiments n = 2 - 4 mice per group. Error bars indicate SEM; *, $p<0.05$, **, $p<0.005$, ***, $p<0.001$.

2.3 Id3^{hi} phenotype correlates with long-lived memory

We next examined the relationship between Id2-YFP and Id3-GFP reporter levels and expression of a surface phenotype characteristic for precursors to long-lived memory cells (CD62L, CD127) or short-lived effector-memory cells (KLRG1) (**Fig. 2.4a**). OT-I CD8^{+} T cells with the highest levels of Id3-GFP were largely CD127^{hi}KLRG1^{lo}CD62L^{hi} by day 7 of infection, a phenotype associated with long-lived memory cells and their precursors. Conversely, Id3-GFP^{lo} cells expressed more KLRG1 and lower levels of CD62L, a phenotype associated with short-lived effector cells (**Fig. 2.4a**). A similar relationship among phenotypic markers was also observed for antigen-specific polyclonal cells on day 7 and 14 of infection with Id3^{hi} cells expressing more CD127 and Id3^{lo} cells skewed to expression of KLRG1 (**Fig. 2.5**). We also examined cytokine production by Id3-GFP^{hi} effector cells relative to Id3-GFP^{lo} cells and found they produced more IL-2, consistent with the CD127^{hi} KLRG1^{lo} subset, in addition to increased production of IFN-γ and TNF (**Fig. 2.4b**).

Prolonged IL-2 exposure and higher levels of CD25 are inversely correlated with the differentiation of long-lived memory cells[18]. Thus, we also examined CD25 expression by Id3-GFP^{hi} and Id3-GFP^{lo} cells. We observed higher CD25 expression by OT-I cells during the early stages in VSV-OVA and Lm-OVA infections, but had difficulty finding bimodal expression of CD25 as reported in Lymphocytic choriomeningitis virus (LCMV) infection[18]. Nevertheless, we gated on CD25^{+} and CD25^{-} populations and found little difference in Id2- or Id3-reporter expression (**Fig. 2.6a**). T-bet protein expression was slightly lower in the Id3-GFP^{hi} subset compared to Id3-GFP^{lo} cells (**Fig. 2.6b**). Thus, we observed a strong correlation between high levels of Id3-GFP

expression in $CD8^+$ effector T cells at the peak of infection, and the phenotype and cytokine producing function of memory precursor cells.

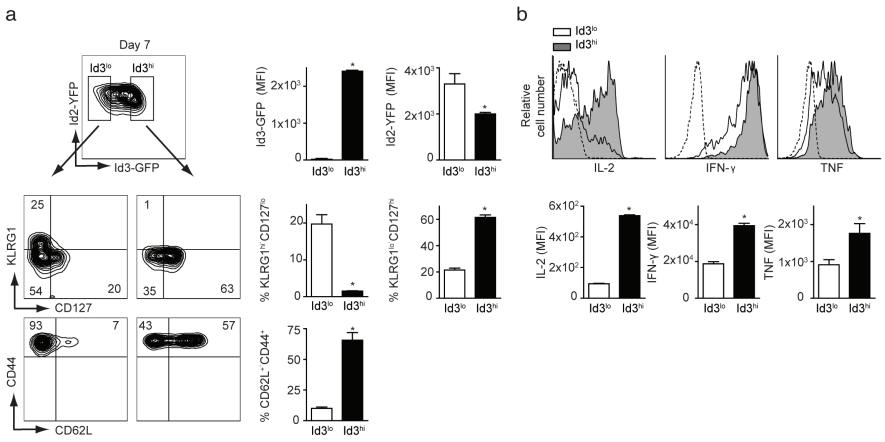

Figure 2.4. Relative expression of Id2-YFP and Id3-GFP during infection correlates with effector and memory precursor subsets. CD45.2^{+} C57BL/6 mice received *Id2*$^{Y/+}$*Id3*$^{G/+}$ CD45.1^{+} OT-I (5 x 10^{4}) cells 1 day before infection with VSV-OVA. **(a)** Flow cytometry plots (left) of the expression of KLRG1, CD127, CD44 and CD62L by Id3-GFP^{hi} and Id3-GFP^{lo} donor cells on day 7 of VSV infection. Numbers indicate percentage of cells in each quadrant. Bar graphs (right) indicate MFI or average percent of KLRG1^{lo}CD127^{hi}, KLRG1^{hi}CD127^{lo} or CD44^{hi} CD62L^{hi} subsets. **(b)** Representative flow cytometry plots (top) and MFI (bottom) of IL-2, IFN-γ and TNF production by splenocytes restimulated for 6 h *in vitro* with OVA peptide on day 7 after VSV infection. Dotted line indicates unstained cells. Data are representative of 4 independent experiments n = 2 - 3 mice per group. Error bars indicate SEM; * p<0.001.

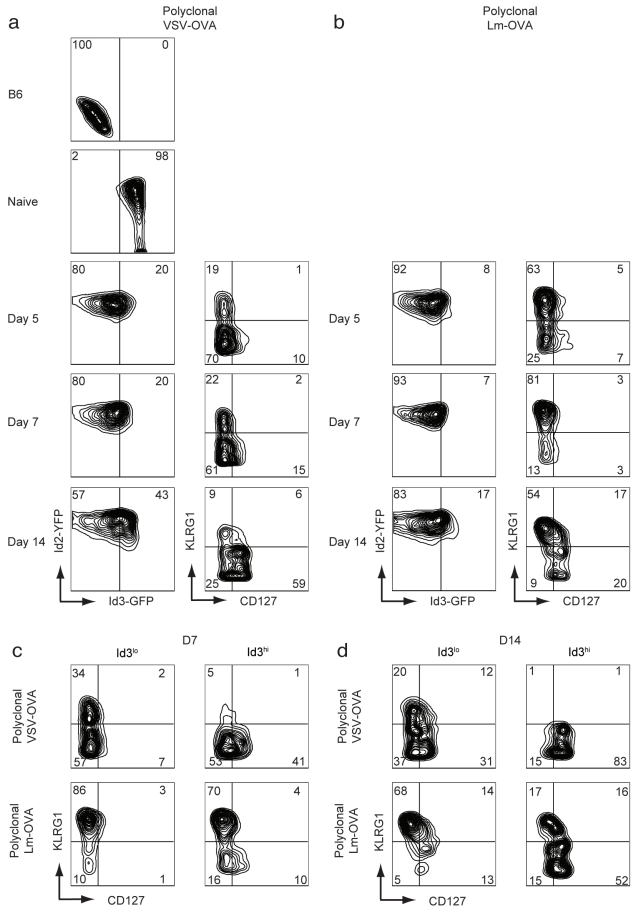

Figure 2.5 Analysis of Id2-YFP and Id3-GFP reporter expression in endogenous CD8$^+$ T cells during infection. FACS plots of Id2-YFP and Id3-GFP expression by polyclonal antigen specific T cells. *Id2*$^{Y/+}$*Id3*$^{G/+}$ mice were infected with VSV-OVA **(a)** or Lm-OVA **(b)** and on days indicated, antigen-specific CD44$^+$ B220$^-$ CD8$^+$ T cells were identified by H-2K^b-OVAp tetramer staining of splenocytes. **(c)** FACS plots (left) of the expression of KLRG1 and CD127 by Id3-GFP^{hi} and Id3-GFP^{lo} donor cells on day 7 or 14 **(d)** of infection. Numbers indicate percentage of cells in each quadrant. Data are representative of 4 independent experiments n = 1-3 mice per group.

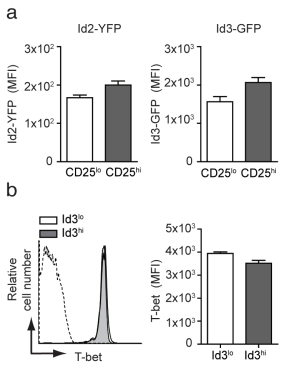

Figure 2.6. Correlation of CD25 and T-bet with Id2-YFP and Id3-GFP. (a) CD45.2$^+$ C57BL/6 mice received *Id2*$^{Y/+}$*Id3*$^{G/+}$ CD45.1$^+$ OT-I (2.5 x 10^4) cells 1 day before infection with Lm-OVA and CD8$^+$ CD45.1$^+$ splenocytes were analyzed at days 4 after infection. Bar graphs represent average MFI of Id2-YFP (left) and Id3-GFP (right) of CD25^{hi} and CD25^{lo} subsets. Data are representative of 4 independent experiments with 2-3 mice. **(b)** CD45.2$^+$ C57BL/6 mice received *Id2*$^{Y/+}$*Id3*$^{G/+}$ CD45.1$^+$ OT-I (5 x 10^4) cells 1 day before infection with VSV-OVA and CD8$^+$ CD45.1$^+$ splenocytes were analyzed at days 7 after infection. Representative FACS plots (left) and MFI (right) of T-bet intracellular staining. Unstained samples were used as negative control. Data are representative of 3 independent experiments with n = 2 - 3 mice. Error bars indicate SEM.

2.4 Id3^{hi} effectors differentiate into long-lived memory cells

To determine the relative memory potential of the effector subsets expressing Id3-GFP, we sorted Id3-GFP^{hi} and Id3-GFP^{lo} OT-I effector cells on day 5 of VSV or Lm infection, and transferred each in equal numbers ($1x10^5$) into infection-matched hosts. We sorted effector cells devoid of surface KLRG1, CD127, CD62L, and CD25 (**Fig. 2.7a**), to ensure we were assessing the memory capacities of cells based on Id3 expression, prior to the upregulation of memory-associated surface receptors. We analyzed the expression of KLRG1 and CD127 by flow cytometry 3 and 6 days after transfer (effectively days 8 and 11 of Lm-OVA infection) in both spleen and liver (**Fig. 2.7b** and **c**). We observed increased differentiation to the CD127^{hi} KLRG1^{lo} subset by Id3-GFP^{hi} effectors and to the CD127^{lo} KLRG1^{hi} subset by Id3-GFP^{lo} effectors, with >6-fold more KLRG1^{hi} cells differentiating from the Id3-GFP^{lo} compared to Id3-GFP^{hi} effector cells at day 3. Conversely, Id3-GFP^{hi} effectors yielded >2-fold higher proportion of CD127^{hi} cells than Id3-GFP^{lo} effectors at day 6.

To determine if the failure of Id3-GFP^{lo} cells to accumulate was due to diminished proliferative capacity, OT-I cells responding to VSV-OVA were transferred to infection-matched host mice that were treated with BrdU on days 2-4 after transfer and proliferation by each subset was assessed (**Fig. 2.7d**). Id3-GFP^{hi} and Id3-GFP^{lo} populations proliferated equally, suggesting that the differences in the absolute number of cells recovered after transfer were due to differential survival.

Finally, we directly tested the potential of Id3-GFP^{hi} OT-I cells responding to VSV-OVA infection to generate memory by re-challenging hosts with Lm-OVA and following the secondary response of both Id3-GFP^{hi} and Id3-GFP^{lo} populations.

Strikingly, the Id3-GFP^{hi} donor cells generated significantly more secondary effectors throughout the infection, with 3-5-fold greater expansion compared to Id3-GFP^{lo} donor cells (**Fig. 2.7e**). These data demonstrate that the Id3-GFP^{hi} effector subset preferentially differentiates into long-lived memory cells.

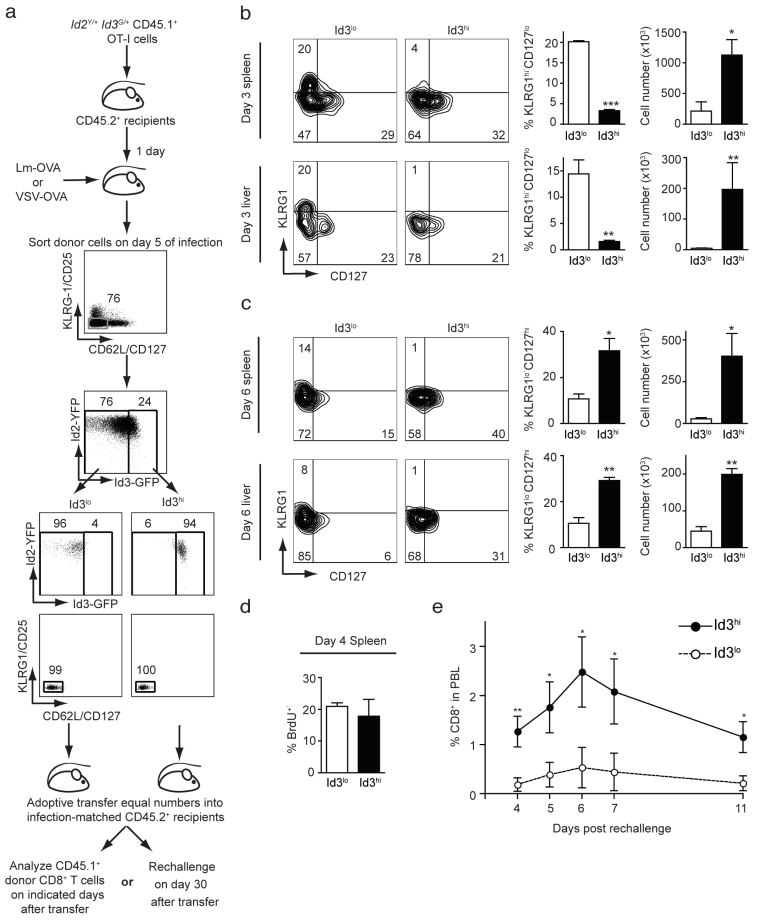

Figure 2.7. The Id3-GFP^{hi} effector CD8^{+} T cell population contains long-lived memory precursor cells prior to expression of KLRG1 or CD127. (a) Experimental design. CD45.2^{+} C57BL/6 mice received *Id2$^{Y/+}$Id3$^{G/+}$* CD45.1^{+} OT-I (2.5 x 10^{4}) cells 1 day before infection with LM-OVA or VSV-OVA. On day 5 after infection, CD25^{-}CD62L^{-}CD127^{-}KLRG1^{-} OT-I cells were sorted based on Id3-GFP expression and equal numbers of Id3-GFP^{hi} or Id3-GFP^{lo} cells were adoptively transferred to infection matched CD45.2^{+} C57BL/6 hosts. **(b)** Flow cytometry plots (left) of KLRG1 and CD127 expression by Id3^{lo} and Id3^{hi} CD45.1^{+} OT-I cells from spleen and liver on day 3 and day 6 **(c)** after transfer of sorted cells from Lm-OVA infection. Numbers in quadrants indicate percentage of cells in each. Bar graphs indicate average percentage of KLRG1^{lo}CD127^{hi} or KLRG1^{hi}CD127^{lo} subsets (middle) or total number of donor cells recovered from spleen and liver (right). Data are representative of 3 independent experiments n = 2 - 3 mice per group. **(d)** BrdU incorporation of sorted OT-I cells responding to VSV-OVA 4 days after transfer. Recipient mice were given BrdU water for 2 days before harvest. **(d)** Kinetics of CD8^{+} T cell expansion of Id3^{lo} and Id3^{hi} CD45.1^{+} OT-I cells in the PBL upon re-challenge with Lm-OVA. Transferred OT-I cells were sorted from VSV-OVA infection. Data are representative of 3 independent experiments n = 3 - 5 mice. Error bars indicate SEM; *, p<0.05, **, p<0.005, ***, p<0.001.

2.5 Memory gene-expression program of Id3^{hi} effector cells

As Id proteins influence transcriptional activity, we next asked if the gene-expression profile of Id3-GFP^{hi} and Id3-GFP^{lo} OT-I effector cells foreshadowed their respective fates as long-lived memory and short-lived effector cells. We transferred and sorted OT-I cells as described (**Figure 2.7**) and analyzed the Id3-GFP^{hi} and Id3-GFP^{lo} effector subsets by microarray on day 5 of infection. We found that even at this early stage of infection, Id3-GFP^{hi} effector cells had a strikingly different gene-expression pattern than the Id3-GFP^{lo} subset, with nearly 400 transcripts differentially expressed by >1.5 fold; 261 genes up-regulated (red) and 137 genes down-regulated (blue) by Id3-GFP^{hi} compared to Id3-GFP^{lo} day 5 effector cells (**Fig. 2.8**). Despite being sorted as negative for expression of characteristic surface memory T cell surface markers, the Id3-GFP^{hi} subset exhibited a strong correlation of gene expression with the CD127^{hi} day 8 effector subset, which has been shown to include memory precursors (previously published microarray data[7]) (**Fig. 2.8**). We also cross-referenced our microarray data with ChIP-Seq data in which E2A occupancy at promoter and enhancer regions in T cells was identified[43]. Here we observed overlap between genes differentially expressed in Id3-GFP^{hi} and Id3-GFP^{lo} cells and genes with known E2A occupancy in T cells (**Fig. 2.8c**, green), indicating that Id3 may directly regulate the transcription of target genes by blocking the activity of E proteins at the promoter and/or enhancer regions. Strikingly, when we compared gene-expression profiles of OT-I effector cells (day 6) to OT-I memory cells (day 100), we found that 77% of the genes preferentially expressed by Id3-GFP^{hi} cells correlated with genes expressed at higher levels by memory compared to effector cells (**Fig. 2.8d**, red). Inversely, 80% of the genes preferentially expressed by

Id3-GFP^{lo} cells correlated with genes enriched in effector compared to memory cells (**Fig. 2.8d**, blue). When we focused on expression of genes with particular interest in CD8^{+} effector and memory subsets, numerous transcripts associated with memory cells were already expressed by day 5 Id3-GFP^{hi} effector cells including: *Il7r, Sell, Il2, Bcl2,* and *Ccr7*, while transcripts associated with terminal differentiation and short-lived effector-memory cells were already down-regulated including: *Gzmb, Klrg1,* and *Prdm1* were already down-regulated (**Fig. 2.8e**). Furthermore, most of these selected genes demonstrated E2A binding across putative enhancer elements (highlighted in green)[43]. Differential expression for a subset of genes was also confirmed by qPCR (**Fig. 2.8f**). Thus, as early as day 5 of infection, differential expression of *Id3* mRNA predicts memory potential and cells that have 'turned on' the appropriate genetic profile to become long-lived memory cells.

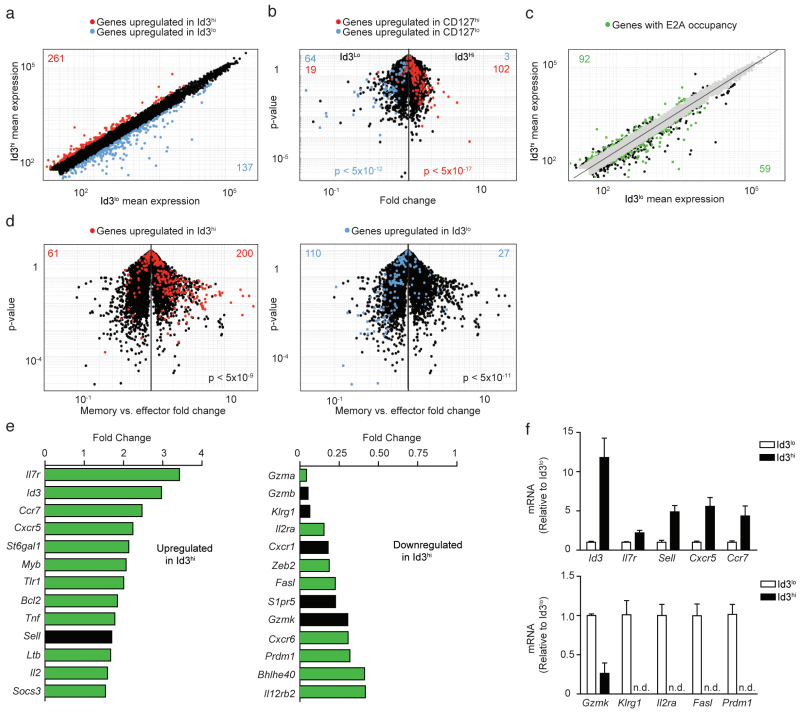

Figure 2.8 Early Id3 expression correlates with memory potential. Gene-expression analysis of CD8$^+$ effector cells. CD45.2$^+$ mice received *Id2*$^{Y/+}$*Id3*$^{G/+}$ CD45.1$^+$ OT-I (2.5 x 10^4) cells 1 day before infection with VSV-OVA. On day 5 after infection, CD25$^-$CD62L$^-$CD127$^-$KLRG1$^-$OT-I cells were sorted based on Id3-GFP expression. **(a)** Normalized expression values for Id3-GFP^{hi} versus Id3-GFP^{lo} CD8$^+$ effector cells. Numbers in corners indicate genes expressed ≥1.5-fold higher in Id3-GFP^{hi} cells (red) or 1.5-fold higher in Id3-GFP^{lo} cells (blue). Data are the average of 2 independent data sets. **(b)** 'Volcano' plot representation of data from **(a)** red indicates genes >2-fold up-regulated in IL-7R^{hi} cells and blue indicates genes that are >2-fold up-regulated in IL-7R^{lo} cells. Numbers represent genes that correlate with either Id3-GFP^{hi} (right) versus Id3-GFP^{lo} (left) expression. p values shown indicate Chi-square results. **(c)** Normalized expression of Id3-GFP^{hi} versus Id3-GFP^{lo} genes where genes with E2A occupancy identified by ChIP-Seq are highlighted in green. **(d)** Gene expression by effector (day 6) and memory (day 100) OT-I cells responding to VSV-OVA infection. Genes up-regulated (red) or down-regulated (blue) ≥1.5-fold in Id3-GFP^{hi} compared to Id3-GFP^{lo} cells are highlighted and numbers represent those genes up- or down-regulated in memory cells. p values shown indicate Chi-square results. **(e)** Fold change of normalized expression values for Id3-GFP^{hi} versus Id3-GFP^{lo} cells for selected genes. Genes with E2A occupancy are highlighted in green. **(f)** Relative mRNA levels measured by qPCR for selected genes, Id3-GFP^{hi} filled bars, Id3-GFP^{lo} open bars. n.d. = not detected. Data are the average of triplicate measurements, error bars indicate SEM, and are representative of 2 experiments.

2.6 Defective long-lived memory formation with loss of Id3

To investigate whether Id3 was required for the normal generation of memory cells, we generated OT-I homozygous knockin (*Id3*$^{G/G}$) mice, resulting in Id3-deficient TCR transgenic donor cells. Id3-deficient mice have a complex phenotype, including excessive IL-4 production by innate cell types[37, 38]. We sought to minimize the impact of T cell extrinsic effects by generating mixed bone marrow chimeras in which wild type and *Id3*$^{G/G}$ OT-I bone marrow were mixed equally and transferred to lethally irradiated recipients. As a result, naive *Id3*$^{G/G}$ OT-I cells from the reconstituted chimeras were CD44^{lo} and comparable to wild type cells (**Fig. 2.9**). We then mixed the *Id3*$^{G/G}$ (CD45.1^{+}) and *Id3*$^{+/+}$ (CD45.1.2^{+}) OT-I cells at a ratio of 1:1, transferred into C57BL/6 CD45.2^{+} hosts, and followed the response to VSV-OVA infection. We monitored the expansion of OT-I cells in peripheral blood lymphocytes (PBL) over time by flow cytometry (**Fig. 2.10a**). In the early expansion phase and at the peak of infection, the *Id3*$^{G/G}$ OT-I cell population expanded and were phenotypically comparable to their *Id3*$^{+/+}$ OT-I counterparts (**Fig. 2.10a**). However, at later stages, *Id3*$^{+/+}$ cells showed a clear advantage over the Id3-deficient CD8^{+} T cells. When we examined the ratio of donor cells in PBL, Id3$^{+/+}$ cells were 3-4-fold greater than Id3-deficient cells 60 days after infection. Notably, the two populations were similar in surface phenotype with respect to KLRG1 and CD127 expression on day 60 (**Fig. 2.10b**). However, there were significantly fewer *Id3*$^{G/G}$ donor cells compared to *Id3*$^{+/+}$ donor cells recovered from the spleen by day 60, and the number of cells bearing the phenotype of long-lived memory cells (CD127^{hi} KLRG1^{lo}) generated by *Id3*$^{+/+}$ donor cells was ~3-4-fold greater than by *Id3*$^{G/G}$ donor cells (**Fig. 2.10b**). Similar results were obtained when *Id3*$^{G/G}$ and *Id3*$^{+/+}$ OT-

I cells were compared after transfer into separate hosts (data not shown). When we examined the *Id3*$^{G/G}$ memory cells that were present at day 80 in more detail, we found that they underwent normal basal homeostatic proliferation, as measured by BrdU incorporation, and displayed normal amounts of the intracellular survival factor Bcl-2 (**Fig. 2.10c**). The memory cells lacking Id3 expression produced more IFN-γ and TNF upon *in vitro* restimulation (**Fig. 2.10d**), which is consistent with the observation that Id3 helps restrain differentiation to effector phenotypes in naive T cells[35]. Thus, Id3 is required for normal development and/or survival of long-lived memory CD8$^+$ T cells.

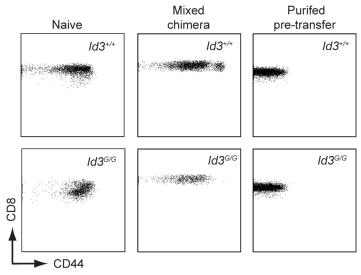

Figure 2.9 Generation of *Id3$^{G/G}$* OT-I mixed chimeras. CD45.1^{+} Id3$^{+/+}$ or Id3$^{G/G}$ OT-I bone marrow were mixed equally with CD45.2^{+} C57BL/6 bone marrow and adoptively transferred into lethally irradiated C57BL/6 CD45.1.2^{+} hosts. FACS plots from OT-I cells from spleens of naive OT-I mice (left), chimeras after 12 weeks of reconstitution (middle), and purified for CD44^{lo} populations before adoptive transfer (right).

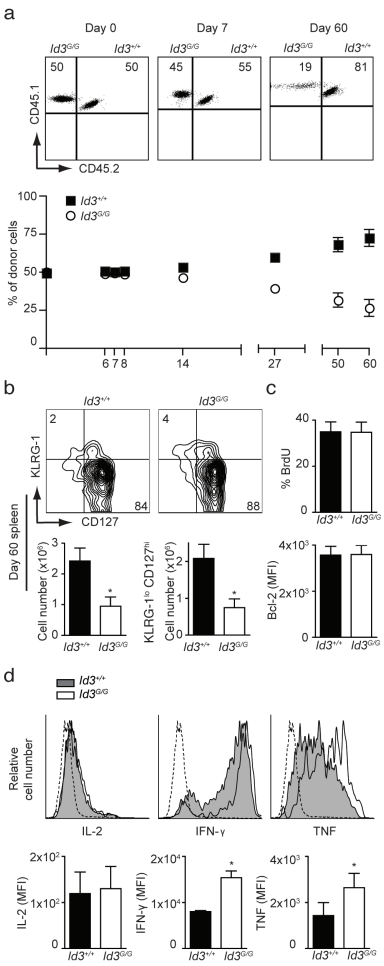

Figure 2.10 Id3-deficiency results in defective formation of long-lived CD8$^+$ memory T cells. CD45.2$^+$ mice received equal numbers of CD44^{lo} *Id3*$^{+/+}$ CD45.1.2$^+$ and *Id3*$^{G/G}$ CD45.1$^+$ OT-I cells 1 day before infection with VSV-OVA. **(a)** Representative flow cytometry plots (top) and time course (bottom) of CD8$^+$ T cell population expansion of *Id3*$^{+/+}$ CD45.1$^+$ and *Id3*$^{G/G}$ CD45.1.2$^+$ OT-I cells over time in the PBL. **(b)** Phenotype (top) and total number (bottom) of *Id3*$^{+/+}$ CD45.1$^+$ and *Id3*$^{G/G}$ CD45.1.2$^+$ OT-I cells recovered from the spleen on day 60 post infection. Data are representative of 2 independent experiments n = 5 - 6 mice per group. **(c)** BrdU incorporation (top) and Bcl-2 expression (bottom) of *Id3*$^{+/+}$ CD45.1$^+$ and *Id3*$^{G/G}$ CD45.1.2$^+$ OT-I cells recovered from the spleen on day 80 post infection. Recipient mice were given BrdU water for 7 days before harvest. **(d)** Representative flow cytometry plots (top) and MFI (bottom) of IL-2, IFN-γ and TNF production by splenocytes restimulated for 8 hours *in vitro* with OVA peptide on day 80 after infection. Dotted line indicates unstained cells. Data are representative of one experiment n = 3 mice per group. Error bars indicate SEM; *, p<0.005, **, p<0.001.

2.7 Higher Id3 expression and loss of KLRG-1^{hi} cells without Id2

Previous studies showed that Id2 was essential for the accumulation and survival of effector CD8^{+} T cells, however we were interested in how loss of Id2 might affect Id3 expression. We crossed *Id2*$^{Y/Y}$ and *Id3*$^{G/G}$ knockin lines to generate *Id2*$^{Y/+}$*Id3*$^{G/+}$, *Id2*$^{Y/Y}$ *Id3*$^{G/+}$, and *Id2*$^{Y/+}$*Id3*$^{G/G}$ mice. Due to early lethality of Id2-deficiency, we transferred fetal liver cells (CD45.2^{+}) into lethally irradiated CD45.1.2^{+} recipients. After 10 weeks of reconstitution, we infected the fetal liver chimeras with Lm-OVA and monitored the expression of Id3-GFP and Id2-YFP by antigen-specific CD8^{+} T cells identified by MHC class I tetramer H-2K^{b}-OVA peptide in the spleen (**Fig. 2.11**). In the absence of Id2 expression (*Id2*$^{Y/Y}$ *Id3*$^{G/+}$), Id3-GFP is upregulated at least 100-fold compared to *Id2*$^{Y/+}$*Id3*$^{G/+}$ effector cells (**Fig. 2.11a**). In contrast, Id2-YFP is relatively unchanged when Id3 is not expressed. We also examined the phenotype of *Id2*$^{Y/Y}$ antigen-specific CD8^{+} T cells and found that the few Id2-deficient cells that did survive did not express KLRG1 and were CD127^{hi} (**Fig. 2.11b**). Thus, upon loss of Id2, Id3-GFP is over-expressed by a small population of cells that bear the phenotype of long-lived memory cells. Together, our data suggest that the balance of Id2 and Id3 expression controls the differentiation of KLRG1^{hi} effector and CD127^{hi} memory subsets and that Id2 either directly or indirectly regulates Id3 expression.

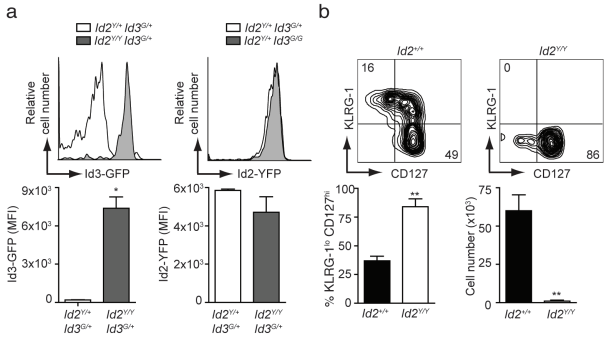

Figure 2.11 Id2-deficient CD8$^+$ T cells upregulate Id3 and fail to generate short-lived effector-memory cells. *Id2$^{Y/+}$Id3$^{G/+}$*, *Id2$^{Y/Y}$Id3$^{G/+}$*, and *Id2$^{Y/+}$Id3$^{G/G}$* fetal liver chimeras were infected with Lm-OVA. **(a)** Histograms indicate reporter expression in splenic antigen-specific CD8$^+$ T cells on day 15 after infection. Data are representative of 4 independent experiments n = 2 - 4 mice per group. **(b)** FACS plots of KLRG1 and CD127 expression by *Id2$^{Y/Y}$* or *Id2$^{+/+}$* splenic antigen-specific CD8$^+$ T cells on day 15 after infection. Data are representative of 3 independent experiments n = 2 - 5 mice per group. Error bars indicate SEM; *, p<0.005, **, p<0.001.

2.8 Inverse regulation of Id2 and Id3 by cytokines

Numerous cytokines are known to influence the generation and survival of effector and memory $CD8^+$ T subsets over the course of infection. To understand how extracellular signals may influence expression of Id proteins, we examined the impact of cytokines on Id2-YFP and Id3-GFP reporter expression. We activated $Id2^{Y/+}Id3^{G/+}$ OT-I splenocytes *in vitro* with irradiated OVA peptide-pulsed splenocytes with or without indicated cytokines and monitored reporter expression by flow cytometry on day 4 of culture. IL-2, IL-12, and IL-21 increased mean fluorescent intensity (MFI) of Id2-YFP by at least 2-5-fold but decreased the MFI of Id3-GFP by at least 3-fold compared to media alone (**Fig. 2.12a**). Next, we examined the effects of IL-2 and IL-12 on Id2-YFP and Id3-GFP expression *in vivo*. We sorted naive $Id2^{Y/+}Id3^{G/+}$ OT-I $CD8^+$ cells, transferred them to IL-12-sufficient or -deficient hosts, and followed reporter expression during the response to infection with Lm-OVA (**Fig. 2.12b**). In agreement with our *in vitro* data indicating that IL-12 lowers Id3 expression, Id3-GFP was upregulated by antigen-specific $CD8^+$ T cells in IL-12-deficient hosts, but no significant differences in Id2-YFP were detected. This upregulation of Id3-GFP was consistently observed throughout the time course (data not shown). To test the role of IL-2 signaling on Id2-YFP expression, we generated $Id2^{Y/+}$ bone marrow chimeras that were either CD25-sufficient or -deficient. $Id2^{Y/+}Il2ra^{+/+}$ or $Id2^{Y/+}Il2ra^{-/-}$ bone marrow was mixed equally with congenically distinct wild-type bone marrow and transferred to wild-type hosts. Following reconstitution, mixed chimeras were infected with VSV-OVA and Id2-YFP levels monitored (**Fig. 2.12c**). On days 7 and 12 of infection, Id2-YFP expression was 2-3-fold higher when IL-2 signaling by antigen-specific $CD8^+$ T cells was intact (**Fig. 2.12c**

and data not shown). Finally, we wanted to determine if CD127-mediated signals control the upregulation of Id3 by $CD8^+$ memory precursors. Id3-GFP expression was unaltered even when CD127 was blocked throughout the entire infection (**Fig. 2.13**), further suggesting that Id3 is expressed prior to and independent of IL-7 receptor expression.

To investigate whether *Id2* expression is directly regulated by cytokines through STAT-mediated signals, we examined its promoter for STAT binding sites. We found 2 potential STAT binding sites in the *Id2* promoter using VISTA genome browser (pipeline.lbl.gov). We examined STAT binding activity at the *Id2* promoter by immunoprecipitation chromatin isolated from OT-I $CD8^+$ T cells on day 7 of infection with STAT4 and STAT5-specific antibodies (**Fig. 2.12d**). The STAT sites in the *Id2* promoter were enriched >3-fold when immunoprecipitated with anti-STAT4 and STAT5 antibodies relative to IgG control. Thus, Id2 expression may be directly regulated by cytokines such as IL-2, IL-12 and IL-21. Together these data provide evidence of complex co-dependent roles for Id2 and Id3 proteins in the regulation of effector and memory T cell formation during the immune response.

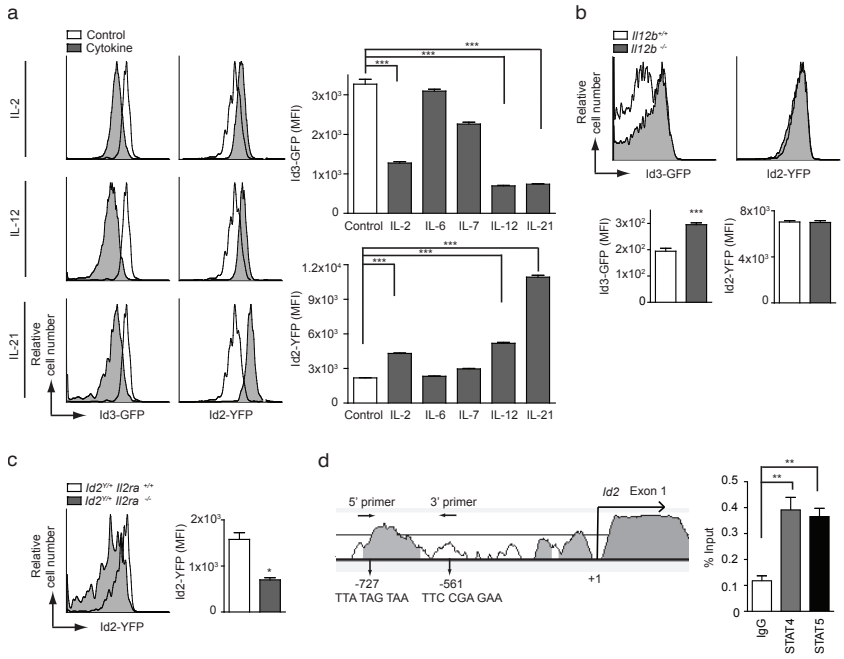

Figure 2.12 Id3-GFP and Id2-YFP expression are inversely co-regulated by cytokines. (a) Histograms (left) and MFI (right) indicating Id2-YFP and Id3-GFP expression by $Id2^{Y/+}Id3^{G/+}$ CD45.1$^+$ OT-I cells cultured with OVA-peptide pulsed APCs for 3-4 days with indicated cytokines. Gray-filled histograms indicate cells cultured with cytokines. Data are representative of 4 independent experiments with 3 replicates. **(b)** Id3-GFP and Id2-YFP expression by $Id2^{Y/+}Id3^{G/+}$ CD45.1$^+$ OT-I cells adoptively transferred into CD45.2 C57BL/6 or IL-12-deficient mice and analyzed on day 15 after infection. Data are representative of 2 independent experiments n = 4 - 5 mice per group. **(c)** $Id2^{Y/+}Il2ra^{+/+}$ or $Id2^{Y/+}Il2ra^{-/-}$ bone marrow was mixed equally with wild-type C57BL/6 bone marrow and adoptively transferred into lethally irradiated hosts. 8-12 weeks after reconstitution, chimeras were infected with VSV-OVA and splenic antigen-specific CD8$^+$ T cells from the donor were analyzed on day 12 of infection. Histogram (left) and MFI (right) indicate Id2-YFP expression in $Il2ra^{+/+}$ and $Il2r^{-/-}$ cells. Data are representative of 2 independent experiments n = 3 - 5 chimeras. **(d)** Human-mouse sequence alignment of *Id2* from VISTA genome browser (left) and chromatin immunoprecipitation (right) from wild type OT-I cells on day 7 of infection with antibody to STAT4, STAT5 or immunoglobulin G (IgG), followed by quantitative PCR analysis of input or precipitated DNA containing indicated binding sites. Data are pooled from three independent experiments n = 5 mice. Error bars indicate SEM; *, $p<0.05$, **, $p<0.005$, ***, $p<0.001$.

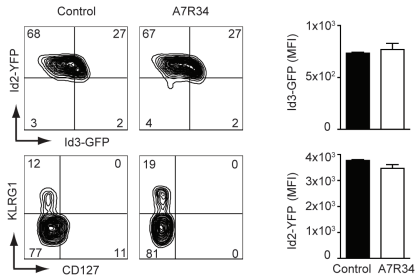

Figure 2.13 Expression of Id3-GFP and Id2-YFP by CD8$^+$ T cells after blocking with anti-IL-7Rα antibodies. FACS plots (left) and MFI (right) of Id2-YFP and Id3-GFP expression by CD8$^+$ OT-I T cells. CD45.2$^+$ C57BL/6 mice received *Id2*$^{Y/+}$*Id3*$^{G/+}$ CD45.1$^+$ OT-I (2.5 x 10^4) cells 1 day before infection with VSV-OVA and CD8$^+$ CD45.1$^+$ splenocytes were analyzed at day 5 after infection. Recipient mice were given 0.5 mg mAb A7R34 (anti-IL-7Rα) or control rat IgG intraperitoneally 1 day before infection and every other day after. Data are representative of 2 independent experiments with n = 3 mice per group. Error bars indicate SEM.

2.9 Discussion

Memory cells originate from effector cells, requiring induction and careful regulation of a distinct transcriptional program; however, the factors influencing this transition are yet to be fully understood. Progress in this area will reveal basic principles governing developmental lineage commitment but is also likely to identify molecular pathways with relevance for clinical endeavors such as vaccine design. To those ends, the work we describe here demonstrates that the relative expression levels of two transcriptional regulators, Id2 and Id3, determine the fate of differentiating $CD8^+$ T cells after activation. High expression of Id3 preferentially guides the transition to memory T cells, while low expression of Id3 leads to differentiation of effector T cells. We show that upregulation of Id3 precedes the traditional markers of memory precursor T cells, indicating that the relative expression of Id3 and Id2 may be the most relevant phenotypic and functional determinant of memory T cells.

Historically, the confirmation of a phenotypic marker for identifying a population containing memory precursor cells has been enrichment of memory cell formation when sorted cells are adoptively transferred into a secondary host. Indeed, we demonstrate that early after infection sorted Id3-GFP^{hi} effector cells preferentially give rise to long-lived memory cells while Id3-GFP^{lo} cells differentiate into short-lived effector cells. Id3 was downregulated by $CD8^+$ T cells early during the effector phase of the immune response and then slowly upregulated and maintained in memory cells. Importantly, the change in Id3 expression occurred prior to the upregulation of KLRG1 and CD127 on the cell surface; that is, the sorted Id3-GFP^{lo} and Id3-GFP^{hi} populations in our studies were negative for both KLRG1 and CD127. Notably, others have shown that expression of

high levels of CD25 or KLRG1 negatively correlates with memory potential whereas those effector cells expressing CD127 or CD62L contain more memory precursors[8, 17, 18, 23, 24, 44]. The timing and degree of expression of these markers occurs variably during the first week of the immune response depending on the infection and precursor frequency of responding cells. Here we find that using Id3-GFP levels affords the ability to identify memory precursors prior to the differential expression of surface markers in the context of VSV and *L. monocytogenes* infection. Effector cells with an Id3-GFP^{hi} phenotype were primarily KLRG1^{lo} CD127^{hi} CD62L^{hi} and produced more IL-2 than Id3-GFP^{lo} Id2-YFP^{hi} cells (a hallmark property of memory cells). Ultimately, we found that the Id2- and Id3-expressing population accumulated, preferentially converted to the KLRG1^{lo}CD127^{hi} memory phenotype, and responded better to secondary challenge than the Id3^{lo} population. In agreement with the observation that Id2 expression was more prominent in short-lived effector-memory CD8^{+} T cells, while Id3 expression was more prominent in long-lived memory precursors, we found that Id2 deficiency resulted in a loss of KLRG1^{hi} cells and Id3 deficiency led to a loss of CD127^{hi} memory cells. Thus, Id2 and Id3 differentially regulate effector and memory cell survival and generation and their expression can be used to predict which precursors will seed the memory compartment.

How do Id proteins regulate CD8^{+} T cell differentiation? The mechanism may be directly related to their inhibition of E protein target genes. Our transcriptional profiling analyses found that the gene-expression profile of Id3-GFP^{hi} cells and Id3-GFP^{lo} cells were strikingly similar to those of CD127^{hi} memory precursor cells and CD127^{lo} effector cells, respectively. Remarkably, many of the ‘signature’ genes expressed in late stage

memory cells (day 100) were already preferentially expressed in the Id3^{hi} memory precursor cells. Thus, upregulation of Id3 correlates with the establishment of a gene-expression profile associated with long-term memory cells. Many of the genes differentially expressed between Id3-GFP^{hi} and Id3-GFP^{lo} cells were identified through an independent study as possessing promoters and enhancers bound to E2A. For example, *Il7r, Socs3, Cxcr5, Ccr7, Il2ra, Cxcr6* and *Id3* itself are known E2A targets[35, 43, 45]. Based on these observations, we suggest that suppression of E protein DNA binding activity is essential to promote developmental progression towards the long-term memory cell fate.

It is known that different E proteins have diverse functions in thymocyte development, and our data support the idea that Id2 and Id3 play distinct roles in CD8^{+} T cell differentiation[46]. Indeed, the fact that each knockout has a defect in memory cell differentiation indicates that Id2 and Id3 play non-redundant roles in memory cell differentiation. Id3-deficient cells were outcompeted by wild type cells and produced diminished numbers of memory cells when transferred into a secondary host. Id2-deficiency also resulted in a reduced memory pool, but the few remaining cells rapidly acquired a KLRG1^{lo}CD127^{hi} phenotype. This dichotomy may be explained by the striking upregulation of Id3 in Id2-knockout CD8^{+} T cells. Consistent with this model are recent observations that E2A binding sites are located in putative regulatory regions across the Id3 locus[35, 43]. Nevertheless, Id3 was unable to compensate completely for the deficiency in Id2-deficient cells, perhaps because Id3 and Id2 bind with different affinities to E protein family members. In such a scenario, cells expressing both Id3 and Id2 may have distinct patterns of gene expression as compared to cells only expressing

Id3 or Id2. Notably, Id2 levels remained unchanged in Id3-knockout $CD8^+$ T cells, suggesting Id3 is regulated by Id2 but not *vice versa*.

Our identification of functionally relevant, cell-intrinsic regulators of memory T cell differentiation will now enable exploration of the molecular mechanisms by which extrinsic signals influence memory cell differentiation. As an initial step, we examined this issue by monitoring how cytokines known to influence $CD8^+$ T cell differentiation affect the expression of Id2 and Id3. IL-2, IL-12 and IL-21 upregulated Id2 expression and down-regulated Id3 expression. These results are consistent with published data suggesting IL-2 and IL-12 signaling drive $CD8^+$ T cells towards a more terminally differentiated state[7, 18]. Our data indicate that STAT4 and STAT5 bind to putative regulatory elements across the *Id2* locus, indicating that cytokine-mediated STAT activation may directly modulate Id2 expression. In contrast, these cytokines may indirectly regulate Id3 expression through modulation of Id2 expression. The regulation of Id3 expression is likely to be complex. In addition to possible feedback regulation by E protein activity, Id3 mRNA abundance is also modulated by the ERK-MAP kinase as well as TGF-β pathways[40, 47]. Previous studies have shown that Blimp-1 acts to antagonize Id3 expression in lymphocytes[48]. This regulation is consistent with the notion that low levels of Id3 promote an effector-like state whereas high abundance of Id3 and low levels of Blimp-1 activate a long-term memory program of gene expression[25, 26]. Using our reporter mice, we observed perturbations in Id2 and Id3 in $CD8^+$ T cells lacking T-bet, suggesting possible regulation of Id proteins by T-bet (unpublished). There is also evidence of *Id2* and *Tbx5* (another T-box family member) acting in the same molecular pathway during cardiac conduction system development[49]. Ultimately, it will

be important to determine how cytokines and transcriptional factors act in concert with E proteins to modulate the expression of Id2 and Id3 in order to properly orchestrate the developmental progression from the naive to effector and memory cell fates.

In conclusion, our study identifies a new phenotypic and functional marker associated with commitment of $CD8^+$ T cells to differentiate into long-lived memory cells. These results, as well as the tools we employed to obtain them, lay the foundation for future studies aimed at identifying other factors regulating Id and E proteins, as well as their downstream targets during both acute and chronic infections. Such information will be useful in developing effective T cell-specific vaccines and immunotherapy.

Chapters 2 contains portions of the material as it appears in Nature Immunology, Yang CY, Best JA, Knell J, Yang E, Sheridan AD, Jesionek AK, Li HS, Rivera RR, Lind KC, D'Cruz LM, Watowich SS, Murre C, Goldrath AW. The transcriptional regulators Id2 and Id3 control the formation of distinct memory $CD8^+$ T cell subsets. 12:1221-1229. (2011). The dissertation author is the first author of this paper.

2.10 Material and methods

Mice. Mice 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. Id3-GFP mice were a gift from C. Murre and were generated as described[35]. Id2-YFP mice were generated as described in Supplementary Figure 1 and were backcrossed at least ten times to the C57BL/6 background. Id3-GFP and Id2-YFP mice were crossed with OT-I TCR-transgenic mice, which recognizes OVA_p (amino acids 257–264) presented by H-$2K^b$. Wild-type male C57BL/6 mice and B6.SJL mice, congenic for the CD45.1 allele, were obtained from The Jackson Laboratory and in some cases crossed to generate F1 mice expressing both CD45.1 and CD45.2. *Il-2ra*$^{-/-}$ *and Il-12b*$^{-/-}$ mice were obtained from The Jackson Laboratory.

Adoptive transfer and infection. Mixed bone marrow chimeras were generated by the transfer of 5 x 10^6 wild-type and 5 x10^6 Id3-knockout $B220^-CD4^-CD8^-$ bone marrow cells into lethally irradiated (1,000 rad) recipient mice. Wild-type donor cells were $CD45.1^+$, Id3-knockout OT-I donor cells were $CD45.2^+$ and recipient mice were $CD45.1.2^+$. Id3-knockout and Id3-wild-type OT-I splenocytes from reconstituted bone marrow chimeras were mixed at a ratio of 1:1 (1 x 10^4 $V\alpha2^+CD8^+$ T cells each) and were transferred together into the appropriate recipients (as determined by expression of congenic markers). For the generation of fetal liver chimeras, 5~10 x 10^6 E14-E15.5 fetal liver cells were injected i.v. into lethally irradiated (1,000 rad) recipient mice. All chimeras were used at least 10 weeks after bone marrow transfer. For analysis of the primary immune response, mice were infected with 5 x10^3 colony-forming units of recombinant Lm-OVA or 1 x 10^5 plaque forming units of recombinant VSV-OVA. Mice

that received adoptive transfer of T cells were infected one day after transfer. For secondary challenge each mice received 5 x 10^3 colony forming units of recombinant Lm-OVA 35 days after primary infection.

Flow cytometry. Single-cell suspensions were prepared from spleen, liver or blood. The following antibodies were used (all antibodies from eBioscience unless otherwise specified): phycoerythrin-indotricarbocyanine–conjugated anti-CD45.2 (104), anti-CD127 (A7R34) iT_{Ag} MHC Tetramer (T03000; Beckman Coulter), anti-Bcl-2 (556537, BD Biosciences) ; peridinin chlorophyll protein–conjugated anti-CD45.1 (A20); peridinin chlorophyll protein–cyanine 5.5–conjugated anti-CD8 (53-6.7) and anti-B220 (RA3-6B2); allophycocyanin-conjugated anti- KLRG1(2F1), anti-CD25 (3C7), anti-IFNγ (XMG1.2), anti-TNFα (MP6-XT22), anti-IL2 (6080103022 Miltenyl Biotec), anti-CD44 (IM7), anti-CD4 (L3T4; BD Pharmingen), anti-BrdU (51-9000019AK, BD Biosciences) and anti-CD8 (53-6.7; Biolegend); phycoerythrin/Cy7 conjugated anti-CD127 (A7R34), anti-CD62L (MEL-14); Alexa Fluor 750–conjugated anti-CD8 (104), anti-CD44 (IM7), anti-CD62L (MEL-14); and Pacific Blue-conjugated anti-CD45.1 (A20). Samples were collected on a FACS LSRII, FACS Fortessa or FACS Aria (BD Biosciences) fitted with custom mirrors from Omega Filters (510/21 with 502LP or 505LP for GFP, 530/30 with 525LP for YFP) and were analyzed with FlowJo software (TreeStar). For intracellular staining, splenocytes were incubated for a total of 6 h at 37 °C at a density of 4 x10^6 cells per well in RPMI 1640 media (Mediatech) containing 10% (volume/volume) bovine growth serum (HyClone) with 10 nM OVAp. After 3 h, 1 ul/ml of brefeldin A (GolgiPlug; BD Biosciences) was added and cultures were incubated for an additional 3 h. Cells were collected and stained with surface antibodies, fixed and permeabilized in

Cytofix-Cytoperm buffer (BD Biosciences) and stained intracellularly as indicated. For BrdU detection, mice were fed with 0.8mg/ml of BrdU in water for indicated days and spleenocytes were stained as indicated by BrdU Flow kit (BD Biosciences). For *in vitro* cytokine assays, 1×10^4 $Id2^{y/+}Id3^{G/+}$ OT-I splenocytes were incubated with 5×10^5 OVA peptide pulsed, irradiated (1200 rads) splenocytes in 48-well plates in 500 μl RPMI media with 10% (vol/vol) FBS, 50 μM β-mercaptoethanol and L-glutamine–penicillin–streptomycin. All cytokines were added at 100 ng/ml except IL-12 (10 ng/ml) at the start of culture. Cells were collected between day 3 and 4.

Quantitative PCR and chromatin immunoprecipitation. Donor cells were sorted as indicated. RNA was extracted with TRIzol reagent (Invitrogen), treated with DNAse (Ambion), and cDNA was generated using SuperScript III kit (Invitrogen). The abundance of mRNA was assessed by quantitative PCR with nonspecific product detection (SYBR Green; Stratagene) using primers that amplify in a linear relationship with primers for 'housekeeping' genes. Results were normalized to expression of GAPDH transcripts. Chromatin-immunoprecipitation assays were done as described with 10 ng DNA for each immunoprecipitation[50]. A polyclonal antibody specific for STAT4 (C-20) or STAT5 (C-17) (Santa Cruz Biotechnology) was used to precipitate STAT4-DNA or STAT5-DNA complexes, and rabbit immunoglobulin G antibody (2729; Cell Signaling Technology) was used as a negative control. Immunocomplexes were bound to protein G–Sepharose beads (Cell Signaling Technology) and were washed 4 times. DNA was eluted and purified and was analyzed by quantitative PCR to detect putative STAT binding sites in the *Id2* gene. *Id2* ChIP primer sequence: 5’ TGT GCA AAC CCC ACT

AAT GA. 3’ CGC TTT TGG GAA GTC ACA TT. qPCR primer sequences are listed in Supplemental Table 1.

Microarray analysis. Donor cells were sorted as described in Fig. 3. RNA was extracted with TRIzol and was amplified twice with the MessageAmp RNA Amplification kit (Ambion). RNA was labeled with biotin using the BioArray HighYield RNA Transcript Labeling kit (Enzo Diagnostics) and was purified with an RNeasy Mini kit (Qiagen). The resulting cRNA was hybridized to GeneChip Mouse Gene 1.0 ST arrays and raw CEL files were obtained. Data were normalized and analyzed with the GenePattern software suite.

Statistical analysis. Statistical significance was determined with two-tailed unpaired Student’s t-test. P values for microarray were determined using chi squared test.

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Chapter 3

Introduction to natural killer T cells

3.1 Introduction to natural killer T cells

Natural killer T (NKT) cells, a distinct T lymphocyte lineage, produce copious amounts of multiple cytokines such as IL-4 and IFNγ upon activation[1, 2]. Those cytokines can influence a variety of immune cells in both adaptive and innate immunity. NKT cells are also able to directly exert cytotoxic function as effector $CD8^+$ T cells. Thus, a wide range of immune responses are influenced by NKT cells and the absence of NKT cells exacerbates bacterial, viral and parasitic pathologies[3].

NKT cells are traditionally categorized into three main subsets. Type I express the Vα14 (mouse) or Vα24 (human) invariant TCR, which recognizes glycolipid antigens such as alpha-galactosylceramide (αGalCer) presented by MHC class I-like molecule CD1d[4]. Type I NKT cells are also called *i*NKT cells. Type II NKT cells are CD1d-reactive NKT cells with non-Vα14 TCR and Type III are CD1d-independent NKT with diverse TCRs. A majority of NKT cells are Type I and can be identified using CD1d tetramers loaded with αGalCer. In the C57BL/6 background, most *i*NKT cells can also be identified by expression of NK1.1 and TCRβ, but not all functionally mature NKT cells.

3.2 Development of natural killer T cells

NKT cell develop in the thymus from double positive (DP) $CD4^+$ $CD8^+$ thymocytes. Rearrangement of the canonical Vα14-Jα18 TCR by developing DP thymocytes allows the positive selection of *i*NKT cells, mediated by the recognition of CD1d on neighbor thymocytes[2-4]. When becoming detectable by the CD1d tetramer, developing *i*NKT cells are defined in four stages based on several surface molecule markers. The earliest detectable subset of *i*NKT cells is called stage 0, with notable CD24 and CD69 expression. Then *i*NKT cells proceed to downregulate CD24 (stage 1), and then upregulate CD44 (stage 2), and lastly NK1.1 (stage 3). The last stage often occurs in the peripheral after stage 2 NKT cells have migrated to the peripheral from the thymus. During maturation, *i*NKT cells upregulate expression of many markers of T cell activation (especially similar to $CD8^+$ T cells), such as CD44, CD69, CD122, NK1.1 and KLRG1[2-4].

3.3 Transcriptional factors regulating NKT cell development and homeostasis

The rearrangement of distal TCRα gene segments Vα14 to Jα18 is crucial for the origin of *i*NKT cells[6]. In conventional T cell development, DP thymocytes must successfully rearrange TCRα chain for a functional TCR. The rearrangement first occurs between proximal Vα and Jα segments, however, if the proximal rearrangement fails, distal rearrangement occurs. The canonical Vα14-Jα18 *i*NKT TCR is only possible from the result of distal rearrangement[7].

An E protein transcription factor, HEB, is essential for *i*NKT cell development[8]. *i*NKT cells failed to develop in the absence of HEB. Deficiency in HEB blocked *i*NKT cell development at their earliest developmental stage (stage 0). All HEB-deficient thymocytes lacked distal Jα gene rearrangements, including the canonical rearrangement required for *i*NKT cell development[8].

The orphan nuclear receptor transcriptional factor, RORγt, is also needed for *i*NKT cell development[9]. RORγt induces expression of the anti-apoptotic molecule Bcl-xL which allows DP thymocytes to survive long enough for distal TCRα rearrangements to occur. RORγt-deficient thymocytes show decreased Bcl-xL expression, diminished survival, and an absence of distal TCRα rearrangements, including the canonical Vα14-Jα18 chain necessary for *i*NKT cell development. Interestingly, loss of HEB expression also resulted in diminished levels of RORγt and Bcl-xL mRNA[8].

Promyelocytic leukemia zinc-finger transcription factor (PLZF), as a key factor in establishing the *i*NKT cell lineage and effector functions[10, 11]. It is expressed in developing *i*NKT cells as early as stage 0. Deficiency in PLZF lead to a block in *i*NKT stage 1 and 2 and results in a drastic reduction of *i*NKT cells in the thymus, spleen and

liver. Very few *i*NKT cells that did accumulate in the peripheral are immature and produced significantly less IL-4 and IFNγ upon activation[10, 11].

The calcinurin-NFAT-Egr2 pathway, activated by TCR-mediated signals, are also shown to be important for *i*NKT cell development[12]. Lack of Egr2, an early growth response transcriptional factor, resulted in impaired development and accumulation of *i*NKT cells. The block in development appears to occur after stage 0. The nuclear factor thymocyte selection-associated HMG box protein (TOX), which is induced by calcineurin-mediated TCR signaling in DP thymocytes, was also shown to be required for *i*NKT cell development[13].

The bHLHZip transcription factor myelocytomatosis oncogene (c-Myc) has also been shown to be required for *i*NKT cell development[14, 15]. C-Myc deficient *i*NKT cells are blocked between stage 0 to 2. Runt related transcription factor 1 (Runx1) is also essential for *i*NKT cell development from DP thymocytes[7]. In its absence, *i*NKT cell development is blocked at the stage 0. Several members of the NF-κB family are also implicated in different stages of *i*NKT cell development[16].

The T box transcription factor, T-bet, is essential for *i*NKT cell development during the last stage of thymic maturation (stage 2 to 3)[7]. In the absence of T-bet, *i*NKT cells accumulate at stage 2 and fail to acquire effector functions, such as the ability to produce IFN-γ and kill targets. Interestingly, T-bet is also needed for the acquiring of effector functions in effector $CD8^+$ T cells[17].

Mice deficient in the zinc finger transcription factor GATA-3 were shown to have a loss of *i*NKT cells in the spleen and liver but not in the thymus[18]. The development of

conventional $CD4^+$ T cells and the $CD4^+$ subset of *i*NKT cells were severely blocked in all tissues including the thymus[18].

3.4 Summary and key questions

Many transcriptional factors are found to be involved in the development of NKT cells. HEB, a member of the E protein family, was crucial for the NKT cell lineage commitment. However, the role of Id proteins in NKT cell development remains unexplored. Furthermore, most of the recent discoveries had been focused on thymic development of NKT cells, very few transcriptional factors specific for the homeostasis of the NKT cells in peripheral organs and the activation of NKT cells had been identified. The next chapter will explore the role of Id2 in NKT homeostasis.

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Chapter 4

Id2 controls survival of hepatic natural killer T cells

4.1 Introduction

NKT cells are a distinct T lymphocyte lineage that are rapidly activated to produce cytokines that influence many cell types either upon recognition of antigen or in inflammatory settings, allowing them to functionally link the innate and adaptive immune responses [1-3]. NKT cells develop in the thymus from $CD4^+CD8^+$ progenitors and pass through three developmental stages defined by expression of CD44 and NK1.1[1, 2]. The last maturation step for many NKT cells involves upregulation of NK1.1 and usually occurs in the periphery after $CD44^{hi}$ $NK1.1^-$ NKT cells have exited the thymus[1,2]. During maturation NKT cells upregulate expression of many markers of T cell activation (including CD44, CD69, CD122) and receptors normally expressed by NK cells (such as KLRG1 and NK1.1)[3]. The NKT cell population can be divided into three main subsets: Type I are the well-studied Vα14 invariant population (*i*NKT); Type II include CD1d-reactive NKT cells with diverse non-Vα14 T cell receptors; Type III are CD1d-independent NKT cells[1, 2, 4]. The majority of *i*NKT cells are activated through interaction of their TCR with glycolipid antigens, such as alpha-galactosylceramide (αGalCer), presented by the MHC class I-like molecule CD1d on antigen presenting cells and can be detected with αGalCer-loaded CD1d tetramers[1, 3, 5]. Several of these glycolipids are derived from microorganisms and mediate *i*NKT cell activation during infection[6]. Upon activation, *i*NKT cells rapidly secrete a diverse set of cytokines representative of multiple $CD4^+$ helper T cell subsets, notably including IFN-γ and IL-4, and therefore influence a wide range of immune responses and disease states[3]. Many signaling molecules, transcription factors, cytokines, and chemokines are involved in *i*NKT cell development, survival, and trafficking[1]. Notably, mutations in the transcription factor T-bet and IL-15

both disrupt *i*NKT cells late in their maturation coinciding with their acquisition of effector functions[7, 8]. Mature *i*NKT cells show a pattern of localization distinct from other T cells and are found preferentially in the liver as well as spleen, bone marrow and thymus but less so in the lymph nodes[5]. CXCR3 and its ligand, CXCL9, are particularly important for *i*NKT cell trafficking to the periphery from the thymus[9]. The chemokine receptor, CXCR6, and its transmembrane ligand, CXCL16, have been shown to be important for *i*NKT cell accumulation in the liver, either by affecting maturation or provision of an essential survival signal[10-13]. LFA-1, a member of the β2 integrin family of adhesion molecules, has also been shown to influence accumulation of *i*NKT cells in liver[14, 15]. However, which specific signals preferentially recruit *i*NKT cells to the liver or support their survival and maturation are not well elucidated.

We noted that: 1) *i*NKT cells share phenotypic and functional properties with $CD8^+$ memory T cells and NK cells, 2) T-bet and IL-15 are required for NK, $CD8^+$ T cell and *i*NKT cell maturation as each cell type acquires effector function, and 3) Deficiency in the transcriptional regulator Inhibitor of DNA-binding-2 (Id2) results in a development block in the transition from NK precursor to a mature NK cells and in $CD8^+$ T cell effector/memory formation at a similar point in maturation[16-18]. Thus, we hypothesized that Id2 would also regulate *i*NKT cell homeostasis/maturation.

The E/Id protein family of transcriptional regulators has been implicated in many aspects of lymphocyte development[19-21]. E proteins are basic helix-loop-helix transcriptional activators/repressors that regulate lymphocyte development by binding to DNA at E-box sites, regulating expression of genes crucial to developmental progression and enforcing key developmental checkpoints[19-21]. E protein DNA-binding activity can

be negatively regulated by Id proteins, which heterodimerize with E proteins and prevent binding to target sequences[19]. One of the Id proteins, Id2, has been shown to be crucial for development of multiple immune cell types[16-18]. Id2-deficient mice lack Peyer's patches, peripheral lymph nodes, mature NK cells, and show diminished numbers of $CD8\alpha^+$ dendritic cells, TCR αβ IELs, and Langerhans cells[16-18]. Recently, we reported that Id2 plays key role in regulating the $CD8^+$ T cell response to infection, where Id2-deficiency resulted in increased apoptosis of effector cells and reduced formation of $CD8^+$ memory T cells[17]. Id2 mRNA was found to be expressed at a ~5-fold higher level in mature *i*NKT cells compared to $CD4^+$ T cells, suggesting that it may function in this cell type[22]. Despite the many known effects of Id2 on lymphoid cells, its function in *i*NKT cells not known.

4.2 $Id2^{KO}$ NKT cells fail to accumulate in liver and bone marrow

We compared the levels of Id2 mRNA expression by quantitative PCR (qPCR) among purified populations of naive and effector $CD8^+$ T cells, NK cells, and total $NK1.1^+TCR\beta^+$ NKT cells sorted from spleen. Id2 mRNA expression was elevated in NK, NKT, and effector cells compared to naïve $CD8^+$ T cells (Fig. 4.1A). As Id2 is a key factor in terminal maturation of NK cells and survival of effector $CD8^+$ T cells[16-18, 23], the high mRNA expression in NKT cells suggested that it could also influence NKT cell development or survival. Interestingly when we compared Id2 mRNA levels between hepatic and splenic NKT cells, expression was similar between the two populations, while Id3 mRNA was dramatically lower in hepatic NKT populations (Fig. 4.1A). Thus, mRNA expression suggested that Id2 would be the relevant Id family member for hepatic NKT cells.

To determine the impact of Id2 deficiency on the NKT cell compartment, we analyzed fetal liver or bone marrow chimeras reconstituted with either Id2-deficient ($Id2^{KO}$) or Id2-sufficent ($Id2^+$) hematopoietic cells. We generated chimeras to study the hematopoietic system as Id2-deficiency leads to severe runting and neonatal death, perhaps due to defects in development of adipose tissue[17, 24]. Examination of $Id2^{+/-}$ and $Id2^{+/+}$ reconstituted chimeras showed no differences in their NKT populations (Fig.4.2), thus experiments included both compared to $Id2^{KO}$ chimeras. NKT cell populations were identified by expression of the donor congenic marker (CD45.2), NK1.1 and TCRβ (referred to as NKT) or by staining with CD1d tetramers[3], which identify the NKT cell subset using the canonical Vα14 TCR (referred to as *i*NKT). It should be noted that the $NK1.1^+TCR\beta^+$ NKT population identifies the mature cells within all three NKT subsets

while CD1d tetramer identifies NK1.1$^{+/-}$ type I *i*NKT cells and a portion of the less abundant type II subset. Chimeras reconstituted with Id2^{KO} donor cells showed a striking reduction in the percentage the *i*NKT subset in the liver and bone marrow compared to controls receiving Id2^{+} donor cells, while a decreased percentage for total NKT cells (NK1.1^{+}TCRβ^{+}) was observed in all tissues (Fig. 4.1B, C). We observed the most severe defect in the liver, where the percentage of total NKT cells recovered from mice reconstituted with Id2^{KO} cells was ~10-fold lower than in chimeras that received Id2^{+} cells (Fig. 4.1C) and where the percentage of the *i*NKT cell population was ~5-fold reduced (Fig. 4.1C). Similarly, analysis of absolute NKT cell numbers revealed a ~20-fold reduction of NK1.1^{+}TCRβ^{+} NKT cells and ~6-fold reduction in CD1d tetramer^{+} *i*NKT cells recovered from livers of chimeras that received Id2^{KO} compared to Id2^{+} donor cells (Fig. 4.1D). In bone marrow, we found a significantly lower frequency and total number of CD1d tetramer^{+} *i*NKT cells in Id2^{KO} compared to Id2^{WT} reconstituted recipients; a similar but not statistically significant trend was observed for NK1.1^{+}TCRβ^{+} NKT cells (Fig. 4.1C, D). While significant differences in frequency of NK1.1^{+}TCRβ^{+} NKT cells were observed in the thymus and spleen, this was not maintained the analysis of absolute cell numbers (Fig. 4.1C, D). As previously reported[16-18, 23], the percentage of TCRβ^{+} NK1.1^{-} T cells (Fig. 4.1B) or CD4^{+} and CD8^{+} T cells was not altered by Id2-deficiency in any of the tissues examined, but the NK cell population (NK1.1^{+}TCRβ^{-}) was absent (Fig. 4.1B). Given the consistent and dramatic effect resulting from the loss of Id2 expression on hepatic NKT cells, we focused primarily on this population.

The final maturation step for many NKT cells is indicated by upregulation of NK1.1 expression and can be completed after cells leave the thymus[1-3]. To investigate the

possibility of an Id2-mediated defect in NKT cell development, the percentages of mature and immature NKT cells, assessed by CD44 and NK1.1 expression, among hepatic and thymic *i*NKT cells from $Id2^{KO}$ and $Id2^{+}$ chimeras were analyzed. There were no significant differences in the percentages or absolute numbers of thymic *i*NKT cells at any stage of development between $Id2^{KO}$ and $Id2^{WT}$ cells, indicating that early development of *i*NKT cells in the thymus was not affected by loss of Id2 (Fig. 4.1E). In contrast, examination of the *i*NKT cell subsets in the liver revealed a lower percentage of mature $CD44^{hi}$ $NK1.1^{+}$ *i*NKT cells and a corresponding 2-fold increase in the percentage of immature $CD44^{hi}$ $NK1.1^{-}$ *i*NKT cells in the $Id2^{KO}$ chimera (Fig. 4.1E). However, a dramatic loss in the absolute number of cells was apparent for both $CD44^{hi}$ $NK1.1^{-}$ and $CD44^{hi}$ $NK1.1^{+}$ *i*NKT populations, indicating that a failure to accumulate *i*NKT cells was not likely due simply to impaired upregulation/expression of NK1.1 as the $NK1.1^{-}$ precursors were also diminished (Fig. 4.1E).

Importantly, we also found that the defective accumulation of $Id2^{KO}$ *i*NKT cells was not rescued in mixed chimeras where $Id2^{+}$ donor cells were also present (Fig. 4.3A). Here, even when progenitors were mixed and allowed to reconstitute congenically distinct recipients, the $Id2^{KO}$ donor cells gave rise to ~10-12-fold fewer *i*NKT cells compared to the $Id2^{+}$ cells within the same recipient. Thus, the presence of $Id2^{+}$ cells did not restore the NKT cell population, highlighting an intrinsic role for Id2 in supporting the formation of an *i*NKT cell population. Furthermore, we did not observe any differences in the expression of CD1d, a molecule important for NKT cell maturation, between the $Id2^{KO}$ and $Id2^{+}$ donor cells in thymus, bone marrow, spleen or liver (Fig. 4.3B).

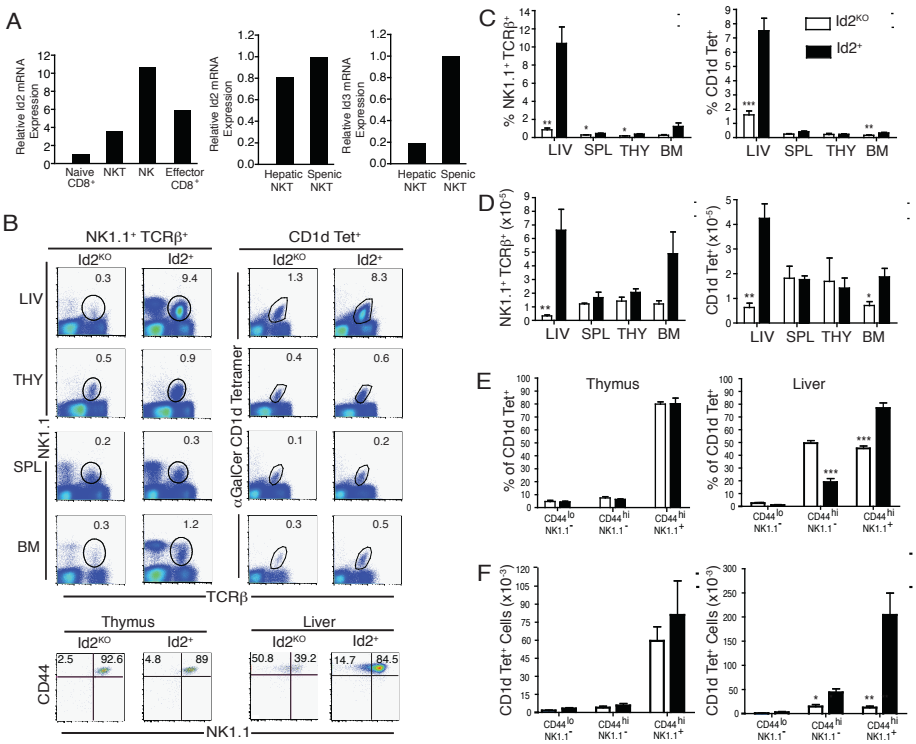

Figure 4.1 Id2^{KO} chimeras have fewer NKT cells in the liver and bone marrow. (A) qPCR was performed on sorted populations of naïve CD8$^+$ T cells, NK cells, NK1.1$^+$ TCRβ$^+$ NKT cells, and effector CD8$^+$ T cells (K^b-OVAp$^+$) on day 7 of infection with Lm-OVA. Data are representative of two independent experiments. (B) Representative flow cytometry plots identifying NKT cells as CD45.2$^+$ NK1.1$^+$TCRβ$^+$ or CD1d tetramer$^+$TCRβ$^+$ lymphocytes from indicated tissues of Id2^{KO} or Id2$^+$ chimeras (upper). Representative flow cytometry plots showing CD44 and NK1.1 expression by CD1d tetramer$^+$ Id2^{KO} or Id2$^+$ NKT cells from thymus and liver (lower). Data are representative of all Id2^{KO} and Id2$^+$ chimeras analyzed in ≥4 experiments. (C) Average (± SEM) percentage of NK1.1$^+$ TCRβ$^+$ or CD1d tetramer$^+$ TCRβ$^+$ NKT cells among donor-gated lymphocytes recovered from indicated tissues harvested from 6-14 Id2^{KO} and Id2$^+$ chimeras. (D) Absolute numbers of NK1.1$^+$ TCRβ$^+$ or CD1d tetramer$^+$ NKT cells recovered from indicated tissues of Id2^{KO} and Id2$^+$ chimeras. Data are the average (± SEM) of ≥4 or more Id2^{KO} and Id2$^+$ pairs of chimeras. The P value for the NK1.1$^+$ TCRβ$^+$ percentages and absolute cell numbers was p = 0.06 for bone marrow. (E) Percent and (F) total number of cells for indicated developmental stages of NKT cells determined by assessing expression of NK1.1 and CD44 on TCRβ$^+$CD1d tetramer$^+$ lymphocytes recovered from chimeras. The average (± SEM) of ≥5 Id2^{KO} and Id2$^+$ chimeras analyzed in two experiments are graphed. Statistical significance was determined using unpaired two-tailed t test where *, P < 0.05, **, P < 0.005, ***, P < 0.0005.

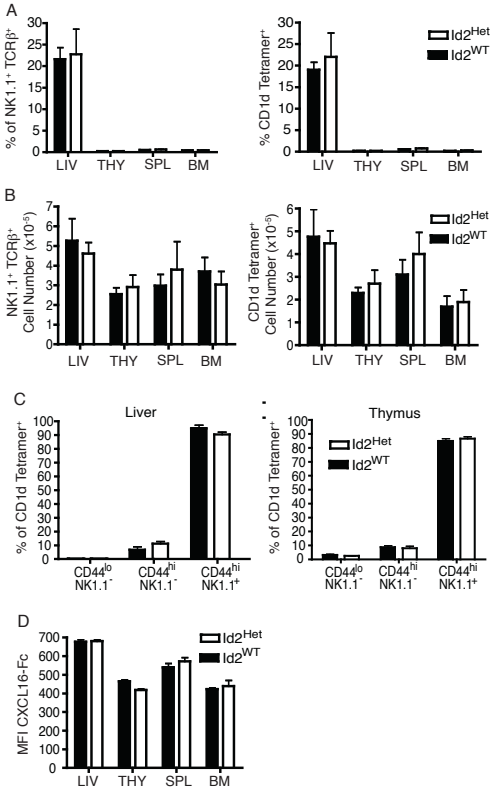

Figure 4.2 Id2$^{+/+}$ and Id2$^{+/-}$ mice generate equivalent NKT cell populations. (A) Percentage of NK1.1^{+}TCRβ^{+} and CD1d tetramer^{+} NKT cells in the liver, thymus, spleen, and bone marrow of 6-week old Id2$^{+/+}$ and Id2$^{+/-}$ mice. (B) Absolute cell numbers of NK1.1^{+} TCRβ^{+} or CD1d tetramer^{+} NKT cells for indicated tissues. (C) Percentage of αGalCer-loaded CD1d tetramer^{+} NKT cells for indicated developmental subsets. Hepatic and thymic NKT cells were gated on expression of TCRβ and αGalCer-loaded CD1d tetramer, then examined for expression of NK1.1 and CD44. (D) Mean fluorescence of CXCL16-Fc binding to NK1.1^{+} TCRβ^{+} NKT cells in the liver, thymus, spleen, and bone marrow. All data shown are the average (± SEM), n = 3 Id2^{WT} and 3 Id2^{+}, *, P < 0.05, **, P < 0.005, ***, P < 0.0005. Statistical significance determined using unpaired two-tailed t test.

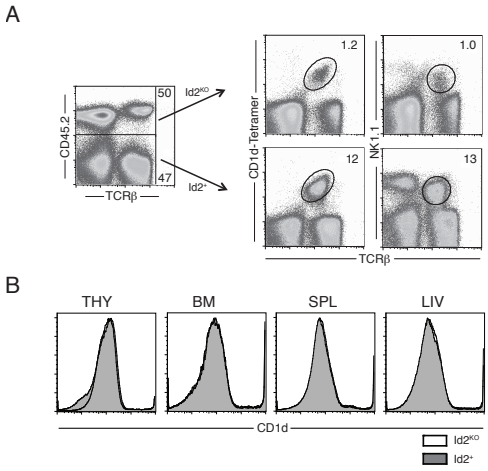

Figure 4.3 Id2^{KO} NKT cell defect is intrinsic. Analysis of Id2^{KO}/Id2^{WT} mixed chimeras. **(**A) Id2^{KO} (CD45.2) and Id2^{WT} (CD45.1) NKT cells were isolated from livers of mixed chimeras and identified by expression of congenic marker, NK1.1, TCRβ, or CD1d tetramer. (B) CD1d surface staining of lymphocytes from thymus, bone marrow, spleen or liver from Id2^{KO} or Id2^{WT} mixed chimeras; Id2^{KO} unfilled and Id2^{WT} shaded. Data are representative of at least two independent experiments with n = 3-4 Id2^{KO} or Id2^{WT}pairs.

4.3 $Id2^{KO}$ NKT cells display an activated phenotype

In order to determine if the few $Id2^{KO}$ NKT cells that were generated could function upon activation, we injected $Id2^{KO}$ or $Id2^{+}$ reconstituted chimeras with 2 μg of αGalCer. Mice were sacrificed 1.5 hours after injection, at which point IFNγ and IL-4 production by hepatic and splenic *i*NKT cells was assessed using intracellular cytokine staining and FACS analysis. $Id2^{KO}$ *i*NKT cells produced similar levels of cytokines after activation compared to their $Id2^{+}$ counterparts (Fig. 4.4A). *i*NKT cells also express many cell-surface markers characteristic of activated/memory T cells ($CD69^{+}$, $CD44^{hi}$, $CD122^{+}$)[1-3]. To characterize Id2-deficient *i*NKT cells further, expression of indicated phenotypic markers by mature *i*NKT cells was assessed. $Id2^{KO}$ *i*NKT cells and their wildtype counterparts expressed similar levels of CD122 (IL-2Rβ), CD44, and CXCR3 (Fig. 4.4B). However, we observed lower levels of CD69 and CD43, which may indicate partially impaired activation and/or maturation (Fig. 4.4B). Thus, the small numbers of mature $Id2^{KO}$ *i*NKT cells that can be identified in the liver have some of the phenotypic markers of maturation and can respond to stimulation with αGalCer *in vivo*.

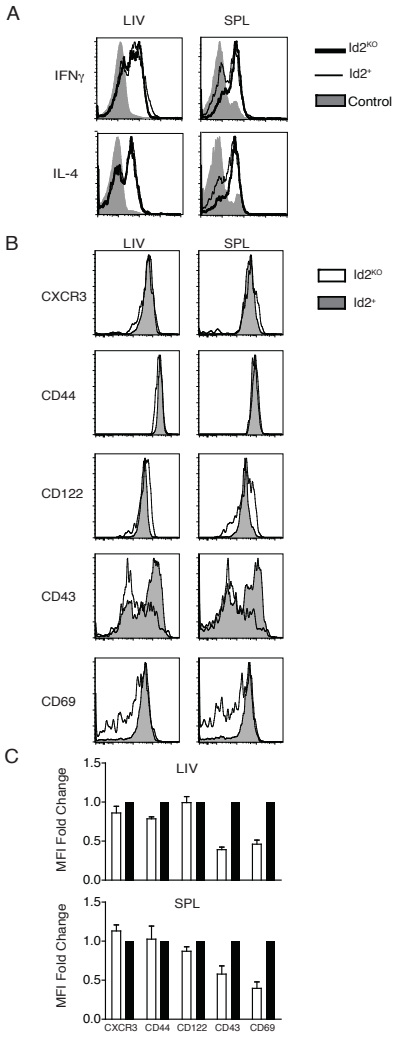

Figure 4.4 Phenotype and function of Id2-deficient NKT cells. (A) Intracellular cytokine staining was used to detect IFN-γ and IL-4 expression by NKT cells harvested from $Id2^{KO}$ or $Id2^{+}$ chimeras that were given 2 μg αGalCer i.p. 1.5 hours prior to sacrifice. NKT cells from $Id2^{KO}$ chimeras (thick black line), $Id2^{+}$ chimeras (thin black line), or unstimulated control B6 mice (gray shaded) were identified by $CD45.2^{+}$ $TCR\beta^{+}$ $NK1.1^{+}$ expression. Data are representative of 3 independent experiments with n = 2 $Id2^{KO}$ and $Id2^{+}$ pairs. (B) Representative flow cytometry plots displaying expression of indicated cell surface molecules by lymphocytes recovered from spleen and liver of $Id2^{KO}$ (unfilled) or $Id2^{+}$ (shaded) donor cells obtained from chimeras. Mature NKT cells were identified by CD45.2, CD1d tetramer and NK1.1 expression. (C) Average (± SEM) fold change of geometric mean fluorescence intensity between $Id2^{KO}$ and $Id2^{WT}$ *i*NKT cells for indicated activation marker. Data are representative of 4 pairs of $Id2^{KO}$ and $Id2^{+}$ chimeras analyzed in 2 independent experiments.

4.4 $Id2^{KO}$ NKT cells undergo increased apoptosis in liver but show normal migration

To assess whether $Id2^{KO}$ *i*NKT cells failed to accumulate in the liver due to impaired migration to this tissue, we transferred $CD45.2^+$ $B220^-CD8^-$ splenocytes from $Id2^{KO}$ or $Id2^+$ chimeras into $CD45.1^+$ host mice and measured the percentage of donor NKT cells recovered in the spleen and liver after 24 hours. Whether identifying the NKT population by expression of NK1.1 and TCRβ or by CD1d tetramer, comparable to increased percentages $Id2^{KO}$ and $Id2^+$ NKT cells were recovered from spleens and livers of host mice, indicating that trafficking to the peripheral organs by the $Id2^{KO}$ *i*NKT cells was not impaired and was perhaps enhanced (Fig. 4.5A).

The level of apoptosis among NKT cells was next evaluated; lymphocytes from liver and spleen were harvested from $Id2^{KO}$ or $Id2^+$ chimeras and stained for Annexin V. More $Id2^{KO}$ than $Id2^+$ hepatic $NK1.1^+TCR\beta^+$ cells stained Annexin V^+ (~60% vs. ~15%), while no differences were observed between the splenic populations or between conventional T cells in liver or spleen, indicating that many of the hepatic $Id2^{KO}$ NKT cells were undergoing apoptosis (Fig. 4.5B). Normal cytokine production by $Id2^{KO}$ NKT cells (Fig. 4.4) in the context of dramatically higher cell death is perhaps surprising; however, activation with αGalCer may rescue or accelerate cell death, leaving only functional cells to assay. It was not possible to measure Annexin V staining for *i*NKT cells as CD1d tetramer staining was lost with the conditions required for Annexin V binding. These results indicated that the lower percentage of *i*NKT cells observed in the livers of $Id2^{KO}$ chimeras was due to impaired survival.

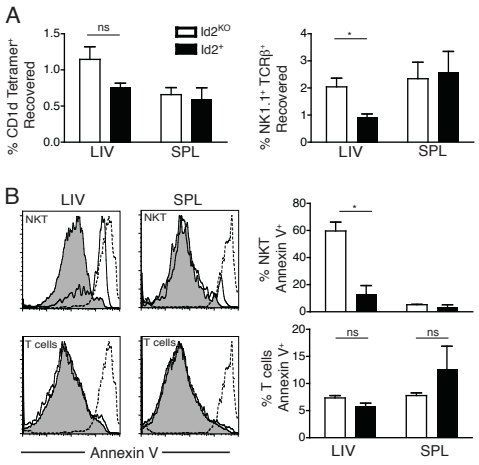

Figure 4.5 Absence of Id2 does not affect NKT cells migration, but results in increased apoptosis. (A) $Id2^{KO}$ and $Id2^{+}$ NKT cells were isolated from livers and spleens of host mice and identified by expression of congenic marker ($CD45.2^{+}$), NK1.1, and TCRβ, or CD1d tetramer. Data shown is average (± SEM), (n = 6-7 for $NK1.1^{+}$ $TCR\beta^{+}$ migration assay, n = 3 for CD1d $tetramer^{+}$ migration assay). (B) Lymphocytes were isolated from $Id2^{KO}$ and $Id2^{+}$ chimeras and were stained with Annexin V and analyzed by flow cytometry. (Left) Representative plots for donor gated $Id2^{KO}$ (unfilled) or $Id2^{+}$ (shaded) $NK1.1^{+}TCR\beta^{+}$ NKT cells or $NK1.1^{-}TCR\beta^{+}$ T cells or dead cells (dotted line). (Right) Average (± SEM) of % Annexin V positive cells (n = 3). Data are representative of 3 independent experiments each with n = 2-3 $Id2^{KO}$ and $Id2^{+}$ pairs. Statistical significance was determined using unpaired two-tailed t test where *, $P < 0.05$.

4.5 $Id2^{KO}$ NKT cells have diminished expression of CXCR6, bcl-2 and bcl-X_L

CXCR6, a chemokine receptor, and its ligand, CXCL16, are essential for *i*NKT cell accumulation in the liver[13, 14]. CXCR6 is expressed at high levels by resting *i*NKT cells and at intermediate levels by effector and memory T cells[22]. To determine if Id2 deficiency affected CXCR6 expression by NKT cells, we first analyzed CXCR6 mRNA by qPCR, comparing expression by NKT cells from $Id2^{KO}$ or $Id2^{+}$ reconstituted chimeras. Both hepatic and splenic $Id2^{KO}$ *i*NKT cells showed a ~8-10-fold reduction in CXCR6 mRNA expression compared to $Id2^{+}$ *i*NKT cells (Fig. 4.6A left), suggesting that Id2 influences CXCR6 expression in this cell type. We next examined surface expression of the CXCR6 protein using a Fc fusion protein of its ligand, CXCL16-Fc, as described previously[12]. NKT cells from $Id2^{KO}$ chimeras showed dramatically reduced levels of CXCL16-Fc binding in both spleen and liver (Fig. 4.6A right), while the total conventional T cell population expressed low levels of CXCR6 that were slightly lower among $Id2^{KO}$ cells (Fig. 4.7A). As CXCR6 has been suggested to promote survival of hepatic *i*NKT cells, $Id2^{KO}$ NKT cells may fail to receive an essential survival signal due to lower CXCR6 expression[10, 11].

However, reduced CXCR6 expression does not provide a complete explanation for the $Id2^{KO}$ *i*NKT cell defect as CXCR6-and CXCL16-deficient mice show a less severe loss of NKT cells[10, 11] (Fig. 4.7). In our study of $Id2^{KO}$ $CD8^{+}$ effector cells, we also observed a defect in survival that correlated with diminished bcl-2 expression and enhanced bim expression[17]. Further, bim has been implicated in negatively regulating survival of activated *i*NKT cells[25]. To determine if Id2 deficiency affected expression of bcl-2 family members in *i*NKT cells, we used qPCR to compare the relative mRNA

levels of a panel of pro- and anti-survival molecules[17]. We found that bcl-2 and bcl-X_L mRNA levels within sorted splenic and hepatic $Id2^{KO}$ and $Id2^{+}$ NKT cells were consistently ~2-3-fold decreased by hepatic $Id2^{KO}$ but not splenic $Id2^{KO}$ NKT cells when compared to $Id2^{WT}$ cells (Fig. 4.8B and C, left). We further confirmed, by intracellular staining, that down-regulation of bcl-2 and bcl-X_L protein expression by $Id2^{KO}$ cells was liver-specific (Fig. 4.8B and C, right) and NKT cell specific, as conventional $Id2^{KO}$ T cells showed similar expression to $Id2^{WT}$ T cells (Fig. 4.7). Interestingly, CXCR6-deficient hepatic NKT cells expressed lower levels of intracellular bcl-2 protein, but displayed normal levels of bcl-X_L (Fig. 4.8), further suggesting that Id2 regulates factors in addition to CXCR6 to support NKT cell survival. The preferential down-regulation of these two anti-apoptotic molecules in liver provide a mechanistic explanation for the liver-specific defect found for $Id2^{KO}$ NKT cells.

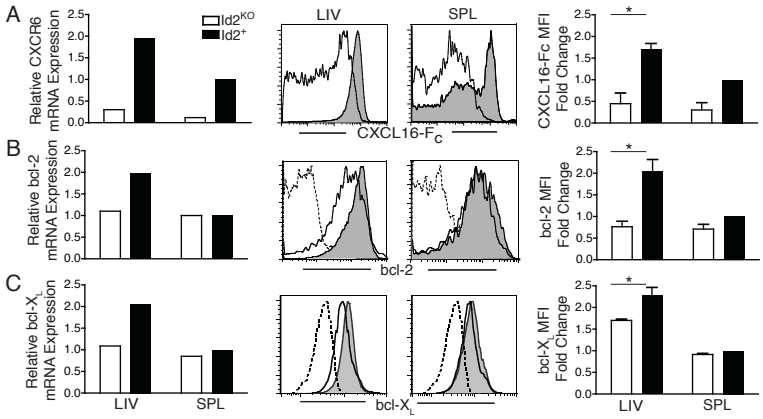

Figure 4.6 Hepatic NKT cells in Id2^{KO} chimeras have diminished expression of CXCR6, bcl-2 and bcl-X$_L$. Relative mRNA and protein expression of (A) CXCR6 (B) bcl-2 (C) bcl-X$_L$. qPCR was performed on sorted NKT cells from Id2^{KO} or Id2^{+} chimeras based on expression of CD45.2, NK1.1 and TCRβ. CXCR6, bcl-2, and bcl-X$_L$ mRNA expression for each sample was normalized to splenic Id2^{+} NKT cells. (Left) qPCR data are representative of two independent sorts with n = 3-4 chimeras. (Center) For flow cytometry NKT cells were identified by expression of congenic marker, NK1.1, and TCRβ. Isotype controls dashed lines; Id2^{KO} unfilled; Id2^{+} shaded. Flow cytometry data are representative of ≥4 pairs of Id2^{KO} and Id2^{+} chimeras. (Right) Average (± SEM) fold change of geometric mean fluorescence intensity between Id2^{KO} and Id2^{WT} NKT cells. Statistical significance was determined using unpaired two-tailed t test where *, $P < 0.05$.

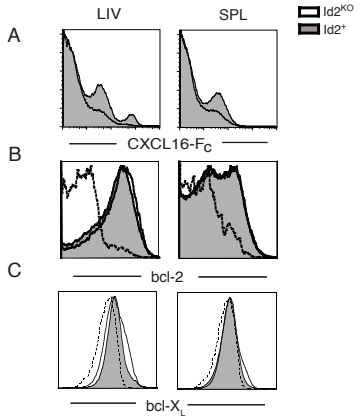

Figure 4.7 Expression of CXCR6, bcl-2 and bcl-XL by conventional Id2^{KO} and Id2^{WT} T cells. Expression of (A) CXCR6, (B) bcl2 and (C) bcl-X_L determined by flow cytometry. Conventional T cells from samples shown in Fig. 5 were identified by expression of congenic marker and were NK1.1^{-}TCRβ^{+}. Isotype controls dashed lines; Id2^{KO} unfilled; Id2^{+} shaded. Flow cytometry data are representative of ≥4 pairs of Id2^{KO} and Id2^{+} chimeras.

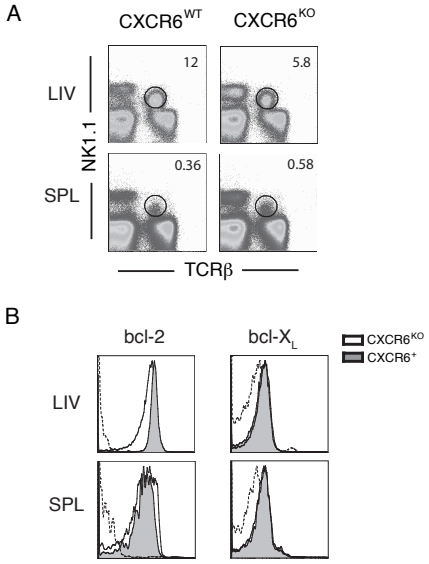

Figure 4.8 Less dramatic defect by CXCR6-deficient NKT cells. (A) Representative flow cytometry plots identifying NKT cells by NK1.1 and TCRb expression in liver of $CXCR6^{WT}$ or $CXCR6^{KO}$ mice. (B) Representative histograms of intracellular staining for bcl-2 or bcl-X_L by $NK1.1^{+}TCRb^{+}$ NKT cells as gated in (A). Isotype controls dashed lines; $Id2^{KO}$ unfilled; $Id2^{+}$ shaded. Flow cytometry data are representative of 3 $CXCR6^{WT}$ or $CXCR6^{KO}$ pairs.

4.6 Bim deficiency rescues $Id2^{KO}$ NKT cell defect

To determine if the lower levels of anti-apoptotic molecules was the cause of the of $Id2^{KO}$ *i*NKT cell defect, we tested if inhibition of the mitochondrial pathway of apoptosis through loss of bim could rescue the phenotype. We generated chimeras reconstituted with $Id2^{WT}bim^{+/-}$, $Id2^{KO}bim^{+/+}$, $Id2^{+}bim^{-/-}$ or $Id2^{KO}bim^{-/-}$ fetal liver cells. Chimeras reconstituted with $Id2^{KO}bim^{-/-}$ donor cells showed a dramatic recovery of the *i*NKT cell population in the liver compared to chimeras receiving $Id2^{KO}bim^{+/+}$ donor cells (Fig. 4.9). The percentage of CD1d tetramer^{+} *i*NKT and NK1.1^{+}TCRβ^{+} NKT cells recovered from mice reconstituted with $Id2^{KO}bim^{-/-}$ cells was ~4-5 fold higher than in chimeras that received $Id2^{KO}bim^{+/+}$ cells (Fig. 4.9A and B). Similarly, analysis of absolute NKT cell numbers revealed a ~8-fold increase in both NK1.1^{+}TCRβ^{+} NKT cells and CD1d tetramer^{+} *i*NKT cells recovered from livers of chimeras that received $Id2^{KO}bim^{-/-}$ donor cells compared to $Id2^{KO}bim^{+/+}$ donor cells (Fig. 4.9C). However, the number of donor *i*NKT cells in the thymus and spleen of recipients that received bim-deficient donor cells was not significantly altered compared recipients that received $Id2^{WT}$ donor cells, suggesting the rescue is not due to increased thymic output in the absence of bim (4.10). The minor difference in percentage and number between $Id2^{KO}bim^{-/-}$ and $Id2^{+}bim^{-/-}$ hepatic NKT cell populations was not significant. Thus, the removal of bim-mediated cell death overcomes the survival defect of $Id2^{KO}$ NKT cells.

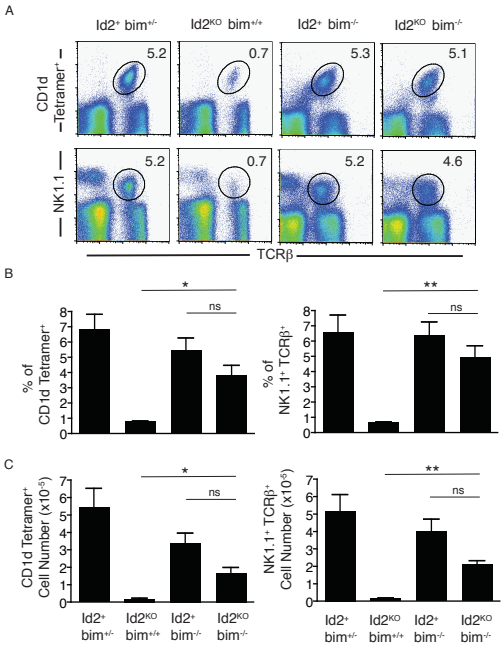

Figure 4.9 Bim deficiency rescues hepatic Id2^{KO} NKT cells. (A) Representative flow cytometry plots identifying NKT cells by congenic marker, NK1.1 and TCRβ or CD1d tetramer staining of hepatic lymphocytes recovered from Id2^{+}bim$^{+/-}$, Id2^{KO}bim$^{+/+}$, Id2^{+}bim$^{-/-}$, or Id2^{KO} bim$^{-/-}$ fetal liver chimeras. (B) Average percentage (± SEM) of indicated populations from (A). (C) Absolute cell number for indicated populations from (A). Data are representative of three independent experiments with n = 3-5 per group. Statistical significance determined using unpaired two-tailed t test where P < 0.01, *, P < 0.005, ***, ns > 0.05.

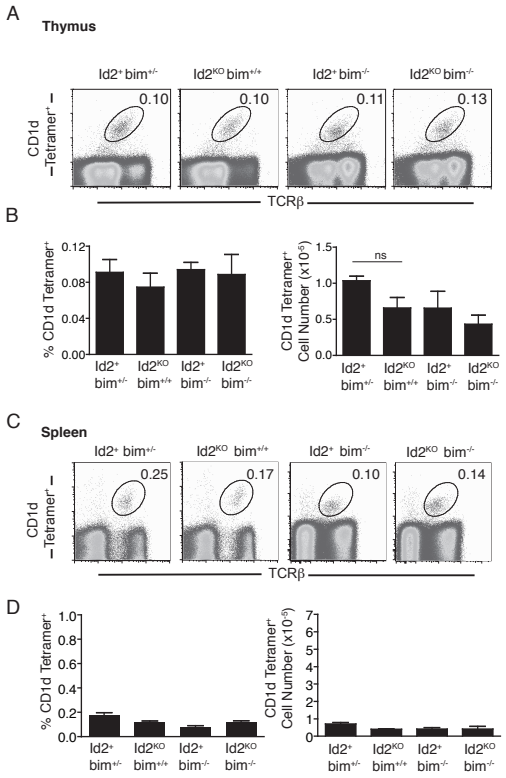

Figure 4.10 Analysis of NKT cell populations in thymus and spleen of chimeras reconstituted with Id2^{WT} or Id2^{KO} bim-deficient donor cells. Analysis of lymphocytes recovered from Id2^{+}bim$^{+/-}$, Id2^{KO}bim$^{+/+}$, Id2^{+}bim$^{-/-}$, or Id2^{KO} bim$^{-/-}$ fetal liver chimeras. (A) Representative flow cytometry plots identifying NKT cells by TCRβ and CD1d tetramer staining of thymus. (B) Average percentage (± SEM) of indicated populations from (A). (C) Representative flow cytometry plots identifying NKT cells by TCRβ and CD1d tetramer staining of spleen. (D) Average percentage (± SEM) of indicated populations from (C). Data are representative of three independent experiments with n = 3-5 per group. Statistical significance determined using unpaired two-tailed t test where ns > 0.05.

4.7 Discussion

We have shown that Id2-deficiency results in perturbations of NKT cell homeostasis including a dramatic reduction in accumulation of both Vα14*i* and total NKT cells in the liver (Fig. 4.1). Id2-deficient NKT cells displayed increased apoptosis compared to their Id2-sufficient counterparts in the liver, suggesting that Id2 expression promotes their survival (Fig. 4.5). These results highlight a novel role for Id2 in NKT cell homeostasis and are reminiscent of defects observed in NK and $CD8^+$ T cells where a requirement for Id2 occurs at late stages of maturation upon acquisition of effector function, coincident with down-regulation of Id3[16, 17, 22, 26].

Much like the defect we observe in the absence of Id2, $CXCR6^{KO}$ and $CXCL16^{KO}$ mice[10, 11, 13], have a significant loss of hepatic but relatively normal *i*NKT cell populations in other tissues. Interestingly, we found that $Id2^{KO}$ NKT cells showed substantially reduced expression of CXCR6 in all tissues tested (Fig. 4.7). The liver-specific *i*NKT cell defect in $CXCR6^{KO}$ mice has been proposed to result from increased apoptosis[10] and impaired maturation[11]. We observed increased apoptosis (Fig. 4.5) and diminished numbers of both $NK1.1^-$ and $NK1.1^+$ $Id2^{KO}$ NKT cells (Fig. 4.1) compared to their $Id2^+$ counterparts in the liver. This observation suggests lower CXCR6 expression deprives Id2-deficient hepatic *i*NKT cells of an essential survival signal, preventing accumulation of mature cells (Fig. 4.6). Thus, splenic and thymic *i*NKT cells may depend on different survival signals, which do not require CXCR6 expression. Finally, we did not observe any defects in cytokine production by the Id2-deficient *i*NKT cells, while $CXCR6^{KO}$ and $CXCL16^{KO}$ NKT cells were found to have decreased cytokine

production, indicating that the residual levels of CXCR6 expressed by $Id2^{KO}$ NKT cells were sufficient to mediate more normal maturation and activation.

Interestingly, we observed a more severe hepatic phenotype than the $CXCR6^{KO}$ mice and find fewer *i*NKT cells in the bone marrow unlike $CXCR6^{KO}$ mice, suggesting that reduced CXCR6 expression may not fully explain the severity and extent of the *i*NKT cell defect in Id2-deficient mice. We found that bcl-2 and bcl-X_L were both expressed at lower levels in the $Id2^{KO}$ *i*NKT cells that localized to the liver, supporting a role for Id2 in promoting cell survival (Fig. 4.6). Loss of expression of pro-survival molecules could not be accounted for solely by down-regulation of CXCR6 expression as $CXCR6^{KO}$ *i*NKT cells had less bcl-2 but normal bcl-X_L expression (Fig. 4.7). Of note, the $Id2^{WT}$ hepatic *i*NKT cells expressed higher levels of these anti-apoptotic molecules compared to their splenic counterparts, perhaps indicating that *i*NKT cells require greater protection from apoptosis when localized to the liver. Importantly, the survival and accumulation defect of $Id2^{KO}$ *i*NKT cells was largely rescued by eliminating bim, a key molecule promoting apoptosis of lymphocytes, including *i*NKT cells, emphasizing the role of Id2 in regulating survival by influencing components of the intrinsic apoptotic pathway (Fig. 4.9).

Studies addressing Id2 function in NK cell development demonstrate that Id proteins are required to control E protein activity, which is accomplished cooperatively by Id2 and Id3 during development but by Id2 alone at later stages as Id3 is downregulated[16, 26]. Interestingly, Id2 mRNA levels were equivalent between NKT cells recovered from spleen and liver, while Id3 levels were ~5-fold higher in splenic compared to hepatic NKT cells in wild type mice, perhaps explaining the normal NKT

population in the $Id2^{KO}$ spleen and why Id2 is crucial for hepatic the NKT population (Fig. 4.1B). Future experiments will be required to establish if the sole function of Id2 in NKT cell homeostasis is to diminish E protein activity and whether activity of E protein transcription factors may influence NKT cell development or function. It is well established that high levels of E protein expression can lead to programmed cell death and that developmental progression and survival require modulation of E protein activity by Id proteins[20, 27, 28]. Furthermore, bim is known to be target directly induced by E proteins[29]. In agreement, the apoptotic death of $Id2^{KO}$ $CD8^{+}$ T cells resulting from a failure to attenuate E protein activity has been correlated with upregulation of pro-apoptotic bim expression and diminished anti-apoptotic bcl-2 expression[17, 29]. Thus, we hypothesize that the regulation of E proteins, which is mediated, at least in part, by Id2 expression controls NKT cell survival by balancing expression of bcl-2 family members.

Chapters 4 contains portions of the material as it appears in Proceedings of National Academy of Science USA, Monticelli LA, Yang Y, Knell J, D'Cruz LM, Cannarile MA, Engel I, Kronenberg M, Goldrath AW. Transcriptional regulator Id2 controls survival of hepatic NKT cells. 106(46): 19461–19466. The dissertation author is the co-first author of this paper.

4.8 Material and Methods

Mice. Mice 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. $Id2^{KO}$ mice were generated as previously described[12] and maintained on the C57/BL6 background. For the generation of fetal liver chimeras, 5-10 x 10^6 $Id2^{KO}$, $Id2^{+/-}$, or $Id2^{+/+}$ E14-E15.5 fetal liver cells and 5-10 x 10^5 RAG^{KO} cells were injected i.v. into lethally irradiated (900 RAD) recipient mice of a distinct CD45 congenic marker. Bone marrow chimeras were generated by transferring 5-10 x 10^6 $B220^-CD4^-CD8^-$ bone marrow cells obtained from $Id2^{KO}$ or $Id2_+$ fetal liver chimeras into lethally irradiated recipient mice of a distinct congenic marker. For the generation of mixed fetal liver chimeras, 5x 10^6 $Id2^{KO}$ or $Id2^{WT}$ E14.5 $CD45.2^+$ fetal liver cells and 5 x 10^6 $Id2^{WT}$ $CD45.1^+$ $B220^-CD4^-CD8^-$ bone marrow cells were injected i.v. into lethally irradiated (900 RAD) recipient $CD45.1^+$ mice. All chimeras were rested for at least eight weeks to allow reconstitution of the host. Mixed bone marrow chimeras were generated by transferring 6 x 10^6 $B220^-CD4^-CD8^-$ bone marrow cells obtained from $CD45.2^+$ $Id2^{KO}$ or $Id2^{WT}$ fetal liver chimeras and 4 x 10^6 $Id2^{WT}$ $CD45.1^+$ $B220^-CD4^-CD8^-$ bone marrow cells into lethally irradiated recipient $CD45.1^+$ mice. All mixed chimeras were rested for at least ten weeks to allow reconstitution of the host. Lymphocytes from CXCR6-deficient ($CXCR6^{KO}$) mice[10] were kindly provided by Dr. Matloubian.

Flow Cytometry: Single cell suspensions were prepared from liver, thymus, spleen, and bone marrow of chimeras. Hepatic NKT cells were isolated as previously described[15]. Fc receptors were blocked with anti-FcγRII/III (24G2) and then stained with the indicated monoclonal antibodies described in Supplemental Methods. The following

fluorochrome-conjugated monoclonal antibodies were used: TCRβ FITC (clone H57-597), CD45.1 FITC (A20), CD45.2 FITC (104), CD43 FITC (1B11) (Biolegend), CD69 PE (H1.2F3), ICOS PE (7E.17G9), CD1d Tetramer PE (NIH Tetramer Core Facility (mCD1d/PBS57)) or generated as previously described (30), TCRβ PE (H57-597), CD122 PE (TM-b1), IFNγ PE (XMG1.2), IL-4 PE (11B11), CXCR3 PE (1C6/CXCR3) (BD Biosciences), goat anti-human IgG Fc_γ PE (Jackson Immunoresearch), CD45.1 PeCy7 (A20), NK1.1 PerCP Cy5.5 (PK136), CD45.2 PerCP Cy5.5 (104), NK1.1 APC (PK136), TCRβ APC (H57-597), CD45.2 APC (104), CD44 APC, Klrg1 APC (2F1), Annexin V APC (Invitrogen), CD45.2 Alexa 750 (104). CXCL16-Fc fusion protein was generously provided by Dr. M. Matloubian and used as previously described (6). Intracellular staining of bcl-2 and bcl-X_L were performed with BD Cytofix/Cytoperm Plus Kit. PE hamster Anti-mouse bcl-2 (3F11) monoclonal antibodies and isotope control PE hamster IgG (A19-3) were purchased from BD Pharmigen; bcl-X_L (54H6) Rabbit mAb (Alexa Fluor 488 Conjugate) and isotype control Rabbit IgG (DA1E) mAb (Alexa Fluor 488 Conjugate) were purchased from Cell Signaling Technology. Samples were collected on FACSCalibur or FACSAria (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Quantitative PCR: Relative levels of mRNA among indicated populations of sorted $CD45.2^+NK1.1^+TCR\beta^+$ NKT cells were compared by qPCR. Id2, Id3, Bcl-2 and Bcl-X_L mRNA levels were assessed using non-specific product detection (SYBR Green, Stratagene). CXCR6 mRNA expression was detected using a TaqMan probe and primer sequences specific for CXCR6 (TaqMan Gene Expression Assay, Applied Biosystems). Id3 Forward: GACTCTGGGACCCTCTCTC, Id3 Reverse:

ACCCAAGTTCAGTCCTTCTC, Id2 Forward: ACCAGAGACCTGGACAGAAC, Id2 Reverse: AAGCTCAGAAGGGAATTCAG. Primers for bcl-2 and bcl-XL have been previously described (17). Samples were normalized to HPRT or GAPDH expression.

Migration Assay: Spleens were harvested from $Id2^{KO}$ or $Id2^{+}$ chimeras and $B220^{+}$ $CD8^{+}$ splenocytes were depleted using MACS columns (Miltenyi Biotec). 13-18 x 10^6 $Id2^{KO}$ or $Id2^{+}$ $B220^{-}$ $CD8^{-}$ splenocytes were injected i.v. into CD45.1 hosts. Recipient mice were sacrificed 24 h after adoptive transfer and lymphocytes recovered from liver and spleen were analyzed by flow cytometry to determine the percentage of donor Id2 NKT cells (CD45.2) recovered.

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Chapter 5

General discussion

5.1 Survival pathways and differentiation programs controlled by Id2 and Id3

Id2 is not required for thymic T cell or natural killer T cell development. However, Id2 had been shown to mediate the survival of both $CD8^+$ effector/memory T cells and peripheral natural killers T cells. In $CD8^+$ T cells, loss of Id2 results in a severe defect in the effector $CD8^+$ T cell population and subsequently, diminished memory $CD8^+$ T cell population[1]. The apoptotic death of $Id2^{KO}$ $CD8^+$ T cells resulting from a failure to attenuate E protein activity has been correlated with upregulation of pro-apoptotic bim expression and diminished anti-apoptotic bcl-2 expression[1].

In natural killer T cells, bcl-2 and bcl-X_L were both expressed at lower levels in the $Id2^{KO}$ *i*NKT cells that localized to the liver, supporting a role for Id2 in promoting cell survival (Fig. 4.6). The survival and accumulation defect of $Id2^{KO}$ *i*NKT cells was largely rescued by eliminating bim, a key molecule promoting apoptosis of lymphocytes, including *i*NKT cells, emphasizing the role of Id2 in regulating survival by influencing components of the intrinsic apoptotic pathway (Fig. 4.9).

In thymic NKT cell development, HEB was shown to regulate bcl-X_L. Bcl-X_L was downregulated in HEB-deficient natural killer T cells. mRNA for RORγt and Bcl-x_L were less abundant in HEB-deficient double positive (DP) cells than in wild-type DP cells. Furthemore, provision of Bcl-x_L transgene restored the development of *i*NKT cells.

It is well established that high levels of E protein expression can lead to programmed cell death and that developmental progression and survival require modulation of E protein activity by Id proteins[2]. Furthermore, bim is known to be target directly induced by E proteins[3]. Thus, we hypothesize that the regulation of E proteins,

which is mediated, at least in part, by Id2 expression, controls cell survival by balancing expression of bcl-2 family members.

Id3 however, appear to play an entirely different role than Id2. Id3 deficiency leads to distinct defects in αβ thymocyte selection as well as expansion of γδ, T_{FH}-like and innate T cell subsets[4-8]. Id3 is also required for memory $CD8^+$ T cells differentiation. The mechanism may be directly related to their inhibition of E protein target genes. Transcriptional profiling analyses found that the gene-expression profile of Id3-GFP^{hi} cells and Id3-GFP^{lo} cells were strikingly similar to those of $CD127^{hi}$ memory precursor cells and $CD127^{lo}$ effector cells, respectively. Thus, upregulation of Id3 correlates with the establishment of a gene-expression profile associated with long-term memory cells. Many of the genes differentially expressed between Id3-GFP^{hi} and Id3-GFP^{lo} cells were identified through an independent study as possessing promoters and enhancers bound to E2A. For example, *Il7r, Socs3, Cxcr5, Ccr7, Il2ra, Cxcr6* and *Id3* itself are known E2A targets[3, 4, 9]. Based on these observations, we suggest that suppression of E protein DNA binding activity is essential to promote developmental progression towards the long-term memory cell fate. Id2 may play an essential role in cell survival through regulation of the bcl-2 family, while the presence of Id3 may regulate various other genes for differentiation.

5.2 Id2 and Id3: Same or different?

Id2 and Id3 belong to the same helix-loop-helix family of transcriptional factors. Although the structures of Id2 and Id3 has not being solved, they are share over 90% similarity based on amino acid sequence comparison. It is known that different E proteins have diverse functions in thymocyte development, and our data support the idea that Id2 and Id3 play distinct roles in $CD8^+$ T cell differentiation[10]. Indeed, the fact that each knockout has a distinct defect in memory cell differentiation indicates that Id2 and Id3 play non-redundant roles in memory cell differentiation. Id3-deficient cells were outcompeted by wild type cells and produced diminished numbers of memory cells when transferred into a secondary host. Id2-deficiency also resulted in a reduced memory pool, but the few remaining cells rapidly acquired a $KLRG1^{lo}CD127^{hi}$ phenotype. This dichotomy may be explained by the striking upregulation of Id3 in Id2-knockout $CD8^+$ T cells. Consistent with this model, recent observations that E2A binding sites are located in putative regulatory regions across the Id3 locus[4, 9]. Nevertheless, Id3 was unable to compensate completely for the deficiency in Id2-deficient cells, perhaps because Id3 and Id2 bind with different affinities to E protein family members. In such a scenario, cells expressing both Id3 and Id2 may have distinct patterns of gene expression as compared to cells only expressing Id3 or Id2.

5.3 Regulation of Id proteins

In $CD8^+$ T cells, Id2 and Id3 expression are regulated by various cytokines. IL-2, IL-12 and IL-21 upregulated Id2 expression and down-regulated Id3 expression. These results are consistent with published data suggesting IL-2 and IL-12 signaling drive $CD8^+$ T cells towards a more terminally differentiated state[11, 12]. STAT4 and STAT5 bind to putative regulatory elements across the *Id2* locus, indicating that cytokine-mediated STAT activation may directly modulate Id2 expression. In contrast, these cytokines may indirectly regulate Id3 expression through modulation of Id2 expression.

The regulation of Id3 expression is likely to be complex. In addition to possible feedback regulation by E protein activity, Id3 mRNA abundance is also modulated by the ERK-MAP kinase as well as TGF-β pathways[13, 14]. Previous studies have shown that Blimp-1 acts to antagonize Id3 expression in lymphocytes[15]. This regulation is consistent with the notion that low levels of Id3 promote an effector-like state whereas abundance of Id3 and low levels of Blimp-1 activate a long-term memory program of gene expression[16, 17].

Id2 and Id3 expression are perturbed in $CD8^+$ T cells lacking T-bet, suggesting possible regulation of Id proteins by T-bet (unpublished). There is also evidence of *Id2* and *Tbx5* (another T-box family member) acting in the same molecular pathway during cardiac conduction system development[18]. Ultimately, it will be important to determine how cytokines and transcriptional factors act in concert with E proteins to modulate the expression of Id2 and Id3. Such information will be useful in developing effective T cell-specific vaccines and immunotherapy.

5.4 References

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