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2008

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

A role for transcriptional regulator Id2 in natural killer T cells

A thesis submitted in partial satisfaction of the requirements for the degree of Master
of Science

in

Biology

by

Laurel Anne Monticelli

Committee in charge:

Professor Ananda Goldrath, Chair
Professor Cornelius Murre
Professor David Traver

2008

The thesis of Laurel Anne Monticelli is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2008

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ACKNOWLEDGEMENTS

First of all, I want to thank Ananda for her encouragement and mentorship over the past couple years. Under her guidance, I've developed a passion for research that has motivated me to pursue a doctorate in immunology. I must also give a huge thank you to former postdoc Michael Cannarile for spending the time and effort to train me when I joined the lab as an inexperienced undergraduate. His expertise on the Id2 project was crucial to my progress in the lab.

Thanks to all the members of the Murre and Hedrick labs for their support and willingness to share reagents, a special thanks to Kees Murre for providing the Id2-knockout mouse line. I'd also like to thank Mitch Kronenberg and Isaac Engel at LIAI for their generosity in providing the CD1d tetramer and advice on the NKT cell project. Thanks to Mehrdad Matloubian at UCSF for giving us the CXCL16-Fc fusion protein.

I couldn't have done any of this research without the friendship and support of my fellow Goldrath Gophers. Thanks to Kitty Cheung, my late night lab buddy, for her help with injections. Thanks to Jamie Knell for Chipotle runs, puppy cuddling, and willingness to drive me home from lab no matter how late at night. Thanks also to Adam Best for rides home and always offering help in lab whenever I asked. Thanks to Cliff Yang for kindly sharing some of his data for this thesis. I was lucky I could count on postdocs Mark Rubinstein, Louise D' Cruz, and Miriam Werneck for their expertise whenever I had questions about my experiments. Thanks especially to Miriam for critical reading of this thesis. I must also thank all the past members of the lab, including Nick Lind, Kristin Camfield, and Alison Sheridan, for their friendship

and help during my first year. The past two years have been an amazing experience and simply a lot of fun, and I couldn't have asked for better labmates to spend it with.

Thanks of course to Mom, Dad, Alisa, and Papa for their support and unwavering confidence in me. Thanks to my friends, especially Andie and Mona, for keeping me sane during the past year and putting up with my crazy work schedule.

ABSTRACT OF THE THESIS

A role for transcriptional regulator Id2 in natural killer T cells

by

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Master of Science in Biology

University of California, San Diego, 2008

Professor Ananda Goldrath, Chair

Natural Killer T (NKT) cells are a critical component of the body's immune system and function to activate both innate and adaptive immune cells. NKT cells are T lymphocyte lineage cells that develop in the thymus and express a variety of both NK and T cell surface markers. Mice deficient in the inhibitor of DNA binding protein-2 (Id2) fail to develop natural killer cells, CD8 α^+ dendritic cells, $\alpha\beta$ IELs, Langerhans cells, and lack lymph nodes. Absence of Id2 also affects the CD8 $^+$ T cell immune response to infection, resulting in reduced survival of effector CD8 $^+$ T cells and impaired memory formation. The role of Id2 in NKT cell physiology has not been addressed.

Here, I show that bone marrow chimeras reconstituted with Id2-deficient hematopoietic stem cells have fewer NKT cells in the liver, spleen, thymus, and bone marrow compared to chimeras generated with Id2-sufficient HSCs, with the most severe defect (10-fold reduction) occurring in the liver. Despite their diminished numbers, Id2-deficient NKT cells produce cytokines after activation with α -galactosylceramide, suggesting normal functional activity, and have similar expression levels of multiple maturation markers compared to Id2-sufficient chimeras. However, Id2-deficient NKT cells have lower mRNA and cell surface expression of the chemokine receptor CXCR6, which is thought to have a critical role in NKT cell accumulation in the liver. The NKT cell defect in Id2-deficient chimeras is not due to an inability of cells to migrate to the peripheral tissues, but could be the result of increased cell death. Early NKT cell development is not affected by Id2 deficiency, possibly due to compensation by Id3, but maturation in the periphery is impaired. These experiments point to a role for Id2 in regulating a CXCR6-mediated survival/maturation signal essential for accumulation of NKT cells in the liver.

Introduction

NKT cells play critical roles in the body's immune system by preventing autoimmunity, clearing microbial infections, and aiding in tumor surveillance^{1,2}. NKT cells are T lymphocyte lineage cells that have properties of both natural killer cells and conventional T cells, allowing them to function as a link between the innate and adaptive immune systems¹⁻³. Upon infection, they rapidly secrete a variety of cytokines that induce the activation of NK cells, macrophages, dendritic cells, B cells, and memory T cells¹⁻⁴. NKT cells were originally defined by their cell-surface expression of both an $\alpha\beta$ T cell receptor and NK cell surface markers, such as NK1.1 and Ly49 receptors in the C57BL/6 background¹⁻⁶. However, NKT cells also express other cell-surface markers characteristic of activated/memory T cells (CD69⁺, CD44^{hi}, CD62L^{lo}, CD122⁺)³⁻⁵. NKT cells are activated through interaction of their TCR with glycolipid antigens presented by the MHC-like molecule CD1d on antigen presenting cells¹⁻⁶. These cells can be activated by the marine sponge-derived glycolipid, α -galactosylceramide (α GalCer), or by a weaker endogenous glycosphingolipid called isoglobotrihexosylceramide (iGb3)^{1,3,4,6}. Upon activation, NKT cells produce an incredibly diverse set of cytokines representative of all the CD4⁺ helper T cell subsets, including Th1 cytokines (IFN γ , TNF α), Th2 cytokines (IL-4, IL-10), and IL-17²⁻⁶.

NKT cells can be divided into three main subsets, Type I are the well-studied V α 14i invariant population, Type II includes CD1d-reactive NKT cells with diverse non-V α 14 T cell receptors, and Type III are CD1d-independent NKT cells¹⁻⁶. Most NK1.1⁺ NKT cells express a semi-invariant TCR with a canonical V α 14-J α 18 rearrangement associated with V β 8, V β 7, or V β 2¹⁻⁶. In the C57BL/6 background,

most NKT cells can be identified by expression of NK1.1 and TCR β , but not all NKT cells express NK1.1¹⁻⁶. All invariant (V α 14i) NKT cells can be identified using CD1d tetramers loaded with α GalCer.

NKT cells develop in the thymus from CD4⁺CD8⁺ double-positive progenitors and can become either CD4⁺ NKT cells or CD8⁻CD4⁻ double-negative NKT cells¹⁻⁵. There are three stages of NKT development defined by expression of CD44 and NK1.1. Stage 1 cells are CD44^{lo} NK1.1⁻, stage 2 cells increase CD44 expression to become CD44^{hi} NK1.1⁻, and stage 3 mature cells are CD44^{hi} NK1.1⁺^{1-3,5}. Massive proliferation occurs between stages 1 and 2, followed by positive selection by CD1d-expressing cortical thymocytes^{1-3,5}. The last maturation step involving upregulation of NK1.1 usually occurs in the periphery after CD44^{hi} NK1.1⁻ NKT cells have left the thymus. However, a small population of NKT cells complete maturation in the thymus and remain as long-term thymic resident mature NKT cells^{1-3,5}. NKT cells are found in highest percentage in the liver (up to 20% of lymphocytes in some mouse strains), followed by the spleen and bone marrow (up to 2.5%), thymus (~0.5%), and peripheral blood (>0.5%)⁴.

Many transcription factors, cytokines, and chemokines are involved in NKT cell development, survival, and trafficking. The number of NKT cells is dramatically reduced in mice deficient in IL-2R β , IL-15, IL-15R α , IRF-1, Fyn, Ets-1, lymphotoxin (LT), and T-bet¹⁻⁵. The absence of the T-box transcription factor T-bet causes a severe reduction in the number of V α 14i NKT cells due to a block between stage 2 and 3 of development^{1,5,7,8}. T-bet^{-/-} NKT cells do not express a high level of CD122 (IL-2R β), indicating that the defect could be attributed to a failure to proliferate in response to

the cytokine IL-15^{5,7,8}. IL-15 itself plays a critical role in the maturation and survival of NKT cells, deficiencies in any component of the IL-15 signaling pathway leads to a substantial loss of mature NKT cells^{1,2,5,7,8,9}. The lack of mature NKT cells in IL-15^{-/-} mice is more likely due to impaired survival and homeostasis than a complete block in NKT cell development⁹. In addition to transcription factors and cytokines, chemokines also play a significant role in trafficking and survival of NKT cells in the periphery^{10,12}. NKT cell emigration from the thymus and localization patterns in the periphery are dependant on chemokines; CXCR3 and its ligand CXCL9 are particularly important for NKT trafficking to the periphery from the thymus¹⁰. Additionally, the chemokine receptor CXCR6 and its transmembrane ligand CXCL16 have been shown to be critical for NKT cell accumulation in the liver, either by affecting migration or through providing an essential survival signal^{11,12}.

Due to their relationship to both NK cells and conventional T cells, NKT cells can be regulated by a number of genes important for either cell lineage. We were interested in determining if NKT cells could be regulated by the helix-loop-helix family of transcriptional activators and inhibitors, which have been implicated in many aspects of lymphocyte development¹⁴⁻¹⁸. E proteins are basic helix-loop-helix transcriptional activators/repressors that regulate lymphocyte development by binding to DNA at E-box sites (CANNTG) as homodimers or heterodimers to drive transcription¹⁴⁻¹⁶. The four E proteins, HEB, E2-2, and E12/E47 splice variants of E2A, can be negatively regulated by another class of HLH proteins called Inhibitor of DNA-binding proteins (Id)¹⁴⁻¹⁶. Id proteins lack the DNA binding domain and therefore cannot bind to DNA directly, but they can inhibit transcription by

heterodimerizing with E proteins to prevent binding to DNA. Id proteins are widely expressed throughout the lymphoid tissues and have been shown to regulate lineage commitment of early thymocyte progenitors, $\alpha\beta$ TCR chain rearrangement, and B cell development¹⁴⁻¹⁸.

One of the Id proteins, Id2, has been shown to be crucial for development of several immune cell types¹⁴⁻¹⁹. Id2-deficient mice lack Peyer's patches and cannot form peripheral lymph nodes due to the absence of lymphoid tissue-inducing (LTi) cells¹⁴⁻¹⁹. Additionally, Id2 is essential for development of mature NK cells and Id2-deficient mice have few CD8 α^+ dendritic cells, $\alpha\beta$ IELs, or Langerhans cells¹⁶. Id2-deficient mice have typical CD4 $^+$ /CD8 $^+$ T cell ratios and no obvious defects in splenic architecture¹⁶. Recently, Id2 has been shown to have a role in regulating the CD8 $^+$ T cell response to infection²⁰. The absence of Id2 results in increased apoptosis of effector CD8 $^+$ T cells and reduced memory formation²⁰. Id2 is expressed at a ~5-fold higher level in mature NKT cells compared to CD4 $^+$ T cells, suggesting that Id2 may be an important regulator of this cell type²¹. Despite the many known effects of Id2 on lymphoid cells, its possible role in NKT cells has not been investigated.

Here, I examined the possible roles for Id2 in development, migration, and survival of NKT cells. I found that the absence of Id2 in the hematopoietic lineage results in a defect in accumulation of NKT cells in the liver, spleen, thymus, and bone marrow. NKT cells from Id2-deficient bone marrow chimeras are functionally normal, producing cytokines upon activation with α GalCer, and can successfully migrate to the peripheral tissues. However, Id2-deficient NKT cells have lower expression of the chemokine receptor CXCR6 and show increased apoptosis of NKT cells in the liver.

Early NKT cell development in the thymus does not appear to be affected by the lack of Id2, possibly due to compensation by Id3, but maturation of NKT cells in the liver may be affected. These results point to a role for Id2 in regulating a CXCR6-mediated survival signal for NKT cells in the liver and possibly affecting NKT cell maturation in the periphery.

Results

Id2 is highly expressed in natural killer and natural killer T cells

Id2 is expressed in both T lineage and NK lineage immune cells¹⁴⁻²⁰. To examine the relative expression of Id2 in NKT, NK, and CD8⁺ T cells, quantitative PCR was performed on cDNA from sorted pure populations of CD8⁺ T cells, NK1.1⁺ TCR β ⁻ NK cells, and CD1d tetramer⁺ NK1.1⁺ TCR β ⁺ mature NKT cells from a wildtype C57BL/6 mouse. Id2 was expressed at a higher level in NK and NKT cells compared to CD8⁺ T cells (Figure 1). Id2 expression in NK cells was ~ 8-fold higher than in CD8⁺ T cells, while Id2 expression in NKT cells was 2-3-fold higher than CD8⁺ T cells. The high level of Id2 expression in NKT cells and the documented NK cell defect in Id2-deficient mice prompted us to investigate the NKT cell compartment in mice lacking Id2.

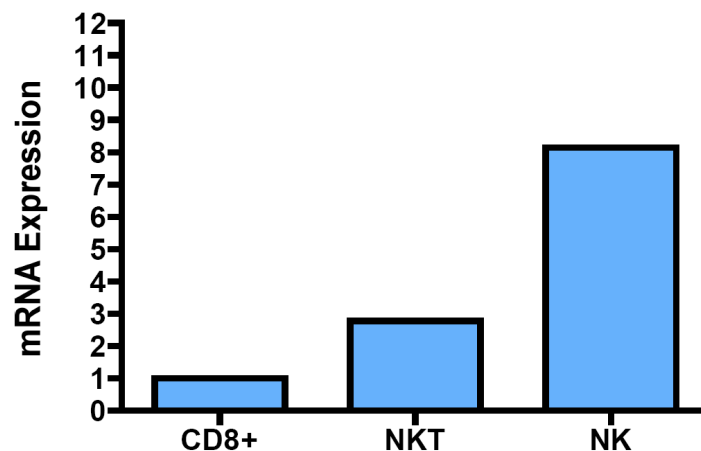


Figure 1: Id2 is highly expressed in NK and NKT cells. Quantitative PCR was performed using cDNA from sorted cell populations of CD8⁺ T cells, CD1d tetramer⁺ NK1.1⁺ TCR β ⁺ NKT cells, and NK1.1⁺ NK cells from a C57BL/6 mouse.

* Data generously provided by Cliff Yang, unpublished

Generation of Id2-deficient fetal liver and bone marrow chimeras

In order to examine the possible role of Id2 in natural killer T cells, we studied the NKT population in the Id2-deficient mouse bred on the C57BL/6 background (generous gift of Dr. Cornelius Murre, UCSD). However, Id2-deficient mice on the C57BL/6 background typically die within one week of birth due to unknown reasons²⁰. Since NKT cells do not reach significant percentages in the peripheral tissues until three weeks of age³, I generated fetal liver and bone marrow chimeras to study the NKT cell populations in the absence of Id2. Timed matings were set up between Id2-heterozygous mice and pregnant females were sacrificed two weeks later to obtain E14 embryos. Embryos were genotyped by PCR and Id2^{KO} or Id2-heterozygous fetal liver cells combined with bone marrow cells from a RAG-knockout mouse (to provide non-lymphoid cell populations normally absent as a result of Id2-deficiency) were injected i.v. into lethally irradiated host mice of a different congenic marker to distinguish the donor cells. Subsequently, Id2^{KO} or Id2^{Het} bone marrow chimeras were generated by transfer of bone marrow cells from Id2 fetal liver chimeras into lethally irradiated host mice of a different congenic marker. Mice were rested for eight weeks before use in experiments to allow full reconstitution of the host animals' immune systems with donor cells.

Although all experiments presented here were performed using Id2-heterozygote chimeras, limited data from experiments using Id2-wildtype chimeras indicated no substantial difference in the NKT cell compartment between Id2-heterozygote and Id2-wildtype chimeras.

Id2^{KO} chimeras have fewer NKT cells in the liver, spleen, thymus, and bone marrow

To determine if the absence of Id2 causes any defect in the NKT cell compartment, Id2^{KO} and Id2^{Het} reconstituted bone marrow chimeras were sacrificed and percentages of NKT cells were analyzed in the liver, spleen, thymus, and bone marrow by flow cytometry. NKT cells were identified by expression of congenic marker (CD45.2⁺), NK1.1 and TCR β or by expression of a CD1d tetramer loaded with α -GalCer to identify the invariant V α 14i NKT cell subset. Id2^{KO} chimeras have a reduction in the percentage of NKT cells in the liver, bone marrow, spleen, and thymus compared to Id2^{Het} chimeras (Figure 2a,b). The most severe defect was in the liver, where 10-fold fewer NK1.1⁺ TCR β ⁺ NKT cells ($p = 0.0019$) were observed in mice reconstituted with Id2^{KO} cells compared to those that received Id2^{Het} cells.

There was a ~ 4-fold reduction in NK1.1⁺ TCR β ⁺ NKT cells in the bone marrow, a slight decrease in splenic NKT cells, and ~ 2-fold fewer NK1.1⁺ TCR β ⁺ NKT cells in the thymus of Id2^{KO} chimeras (Figure 2c). The NKT cell defect in the V α 14i NKT cell population was similar but less severe than the reduction observed for NK1.1⁺ TCR β ⁺ NKT cells. Id2^{KO} chimeras have approximately 7-fold fewer V α 14i NKT cells than Id2^{Het} chimeras in the liver ($p < 0.0001$), 2-fold less in the bone marrow, comparable percentages in the spleen, and a minor reduction in the thymus (Figure 2d). The difference in the defect between the NK1.1⁺ TCR β ⁺ NKT subset and the CD1d tetramer⁺ TCR β ⁺ NKT population could be the result of Id2-deficiency

affecting the diverse, non-V α 14 T cell receptor NKT population or the CD1d-independent subset more severely than invariant V α 14i NKT cells. As previously reported, the percentage of T cells was not affected by the absence of Id2 in any of the tissues examined, but the NK cell population was dramatically reduced (Figure 2a,b).

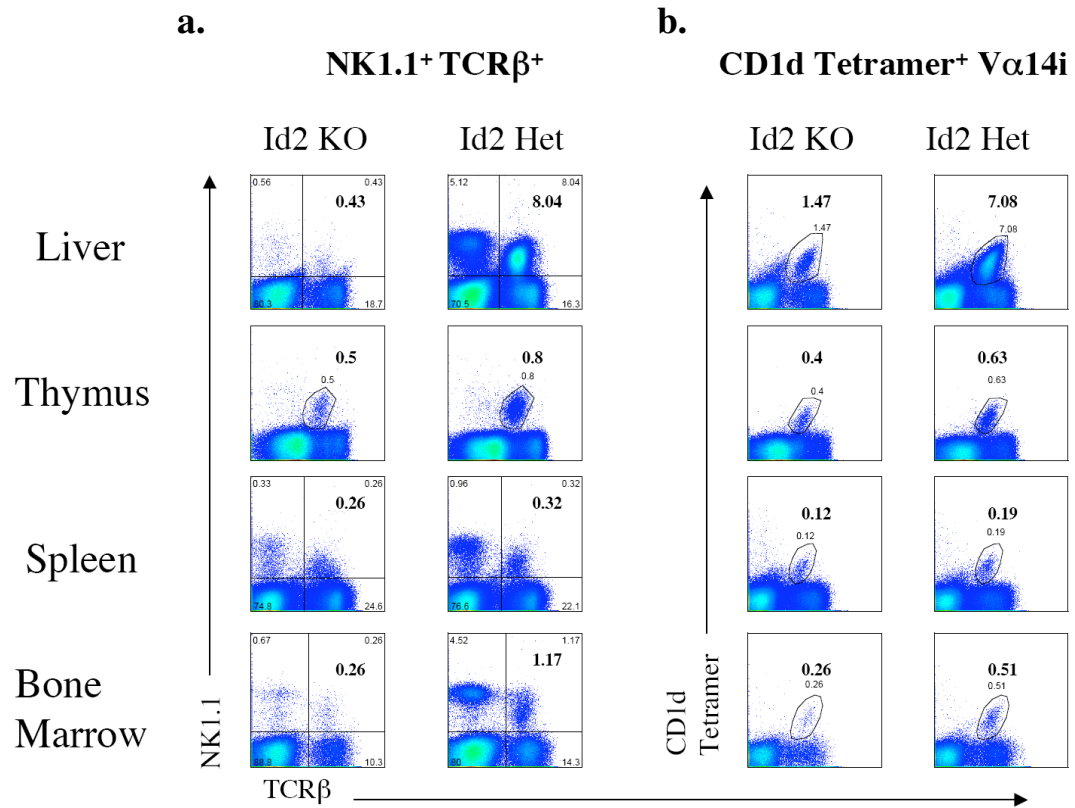


Figure 2: Id2^{KO} chimeras have fewer NKT cells in liver, thymus, spleen, and bone marrow. Cells were obtained from Id2^{KO} or Id2^{Het} bone marrow chimeras and gated on expression of congenic marker (CD45.2⁺). **Figure 2a,b:** Representative flow cytometry plots of NK1.1⁺ TCR β ⁺ (Figure 2a) or CD1d tetramer⁺ TCR β ⁺ (Figure 2b) NKT cells from Id2^{KO} or Id2^{Het} chimeras. Data is representative of all Id2^{KO} and Id2^{Het} chimeras analyzed in more than four experiments. **Figure 2c,d:** Percentage of NK1.1⁺ TCR β ⁺ (Figure 2c) or CD1d tetramer⁺ TCR β ⁺ (Figure 2d) NKT cells from Id2^{KO} or Id2^{Het} chimeras. Results are average ($\pm$ s.d.) of 4-6 Id2^{KO} and Id2^{Het} pairs, statistical significance determined using unpaired two-tailed t test.

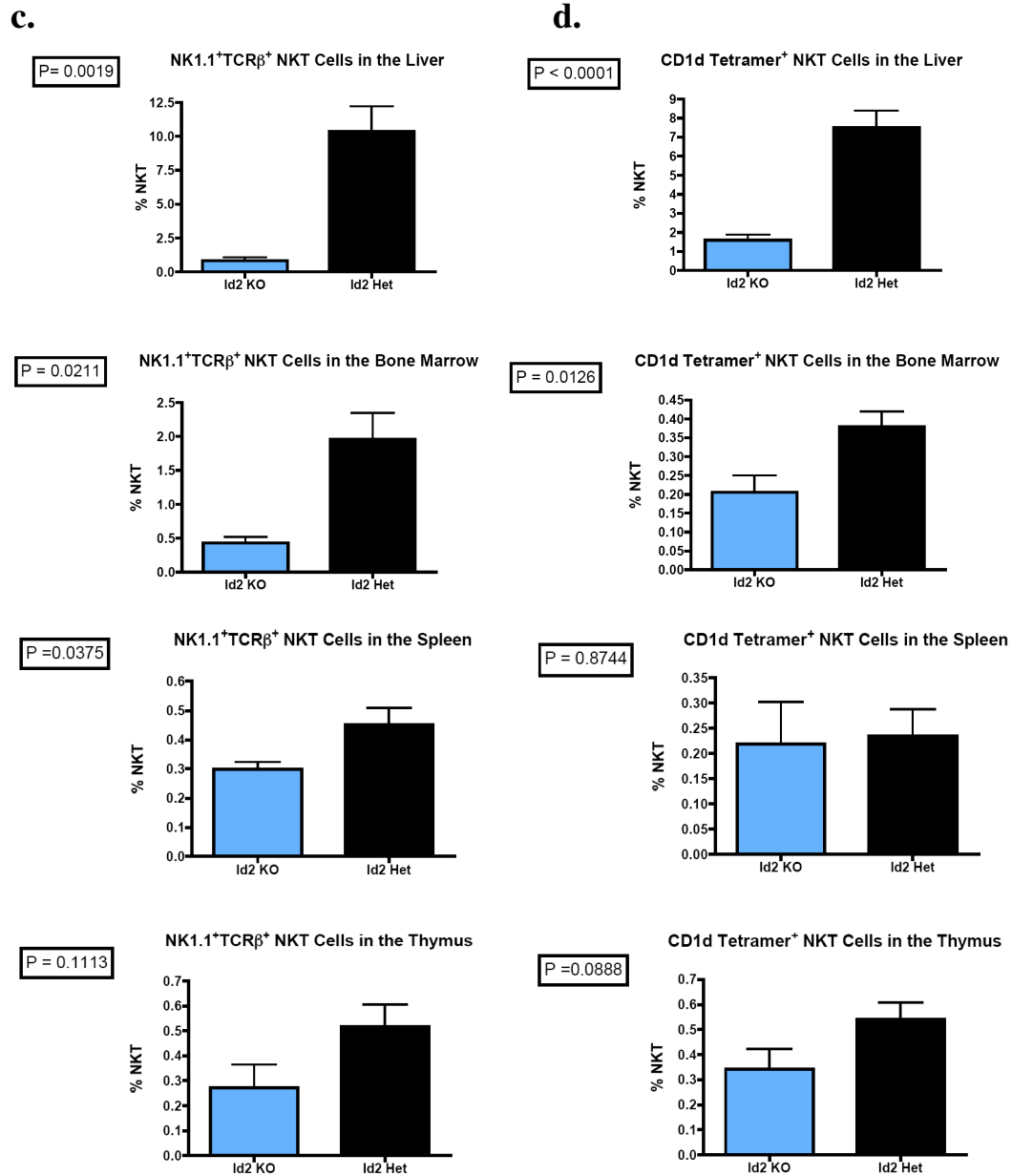


Figure 2: continued: Id2^{KO} chimeras have fewer NKT cells in liver, thymus, spleen, and bone marrow. Cells were obtained from Id2^{KO} or Id2^{Het} bone marrow chimeras and gated on expression of congenic marker (CD45.2⁺). **Figure 2a,b:** Representative flow cytometry plots of NK1.1⁺ TCRβ⁺ (Figure 2a) or CD1d tetramer⁺ TCRβ⁺ (Figure 2b) NKT cells from Id2^{KO} or Id2^{Het} chimeras. Data is representative of all Id2^{KO} and Id2^{Het} chimeras analyzed in more than four experiments. **Figure 2c,d:** Percentage of NK1.1⁺ TCRβ⁺ (Figure 2c) or CD1d tetramer⁺ TCRβ⁺ (Figure 2d) NKT cells from Id2^{KO} or Id2^{Het} chimeras. Results are average (± s.d.) of 4-6 Id2^{KO} and Id2^{Het} pairs, statistical significance determined using unpaired two-tailed t test.

Id2^{KO} NKT cells produce cytokines

NKT cells are characterized by rapid production of cytokines, including IL-4 and IFN γ , after activation *in vivo* by injection of α -GalCer²⁻⁶. In order to determine if the Id2^{KO} NKT cells could function normally upon activation, I injected Id2^{KO} or Id2^{Het} bone marrow chimeras with 2 μ g of α -GalCer i.p. Mice were sacrificed 1.5 hours after injection, at which point production of IFN γ , IL-4, and TNF α by hepatic and splenic NKT cells was assessed through intracellular cytokine staining and FACS analysis.

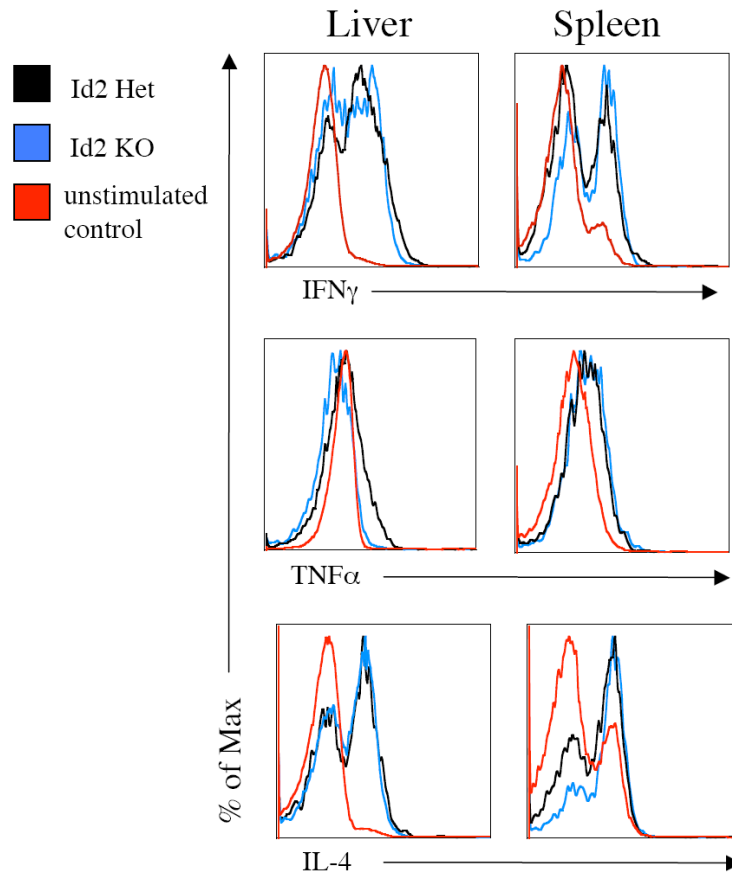


Figure 3: Id2^{KO} NKT cells are functionally normal and can produce cytokines upon activation with α -galactosylceramide. Id2^{KO} or Id2^{Het} chimeras were given 2 μ g α -galactosylceramide i.p. and sacrificed 1.5 hours later for removal of spleen and liver. Cells are gated on CD45.2⁺ TCR β ⁺ NK1.1⁺ and stained intracellularly for production of IFN γ , IL-4, and TNF α . Data is representative of 3 Id2^{KO} and Id2^{Het} pairs from two experiments.

Id2^{KO} NKT cells showed no defect in their ability to produce cytokines after activation (Figure 3). Both Id2^{KO} and Id2^{Het} mice made a substantial amount of IFN γ and IL-4 as well as a small amount of TNF α , indicating that although the percentage of NKT cells in the liver is severely reduced in Id2^{KO} chimeras, the small numbers of NKT cells that accumulate appear to have no defect in responding to stimulation by α -GalCer *in vivo*.

Id2^{KO} and Id2^{Het} NKT cells share similar expression of phenotypic markers

To further examine the NKT cell defect in Id2^{KO} chimeras, splenic NKT cells were stained for cell surface expression of various phenotypic markers to determine any differential expression pattern between Id2^{KO} and Id2^{Het} NKT cells. There were no significant differences in surface expression of any of the markers examined (Figure 4). Id2^{KO} NKT cells expressed equally high levels of CD122 (IL-2R β) as Id2^{Het} cells, indicating that Id2^{KO} NKT cells are potentially competent in their ability to respond to the pro-survival and maturation signals provided by IL-15. Both Id2^{KO} and Id2^{Het} NKT cells have elevated expression of the activation markers CD69 and CD44 in agreement with published data showing the “activated” state of mature NKT cells¹⁻⁶. Both Id2^{KO} and Id2^{Het} NKT cells also showed high expression of CD43, another marker expressed on mature NKT cells⁷.

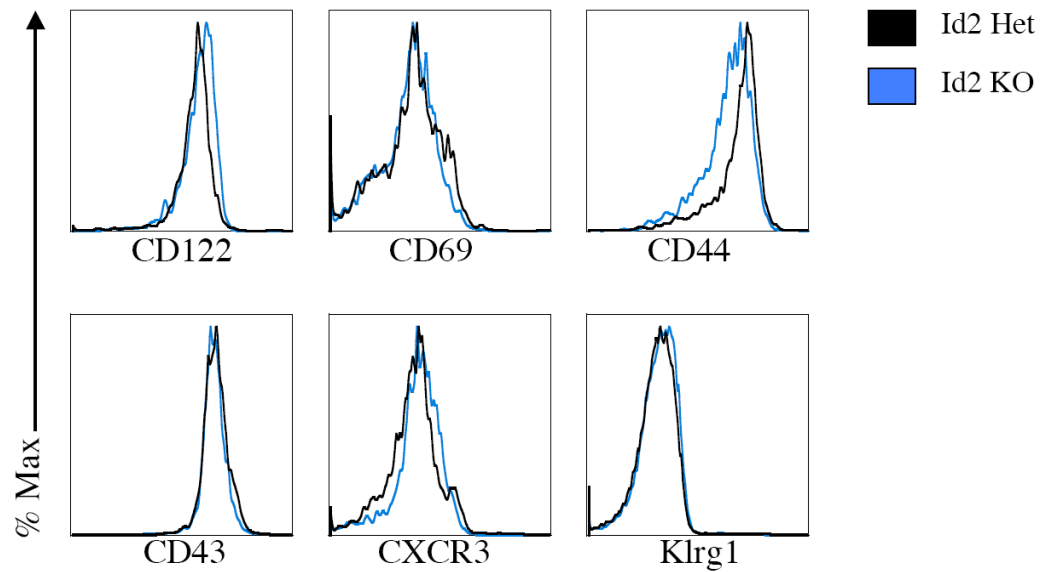


Figure 4: Id2^{KO} and Id2^{Het} NKT cells have similar expression of activation and maturation phenotypic markers. Id2^{KO} or Id2^{Het} splenocytes were obtained from bone marrow chimeras. Cells gated on congenic marker (CD45.2⁺), TCR β , and NK1.1. Data is representative of 2 Id2^{KO} and Id2^{Het} chimeras.

Id2^{KO} NKT cells have high expression of the chemokine receptor CXCR3, a receptor shown to be essential for NKT cell trafficking from the thymus to the periphery¹⁰. Klrp1 (Killer cell lectin-like receptor) is expressed on activated NK cells and on effector CD8⁺ T cells²². Id2^{KO} CD8⁺ T cells are Klrp1^{lo} and lack the subset of Klrp1^{hi} cells found in the Id2^{WT} mouse (unpublished data), but there was no difference in Klrp1 expression between the Id2^{KO} and Id2^{Het} NKT cells. These data suggest that loss of Id2 does not lead to a severe developmental defect.

Lower expression of chemokine receptor CXCR6 mRNA by Id2^{KO} NKT cells

While no remarkable differences in expression of the homing chemokine receptor CXCR3 between Id2^{KO} and Id2^{Het} NKT cells were observed, I next examined expression of CXCR6, another chemokine receptor reported to play an important role in NKT cell localization and survival in the liver. Although migration in NKT cells is thought to be largely dependant on CXCR3/CXCL9, recent studies have identified CXCR6 as a critical determinant for NKT cell accumulation in the liver^{12,13}. Mice deficient in CXCR6 have severely reduced numbers of hepatic NKT cells although NKT cell percentages in the spleen, thymus, bone marrow, and peripheral blood are normal and NKT cell migration to the liver is not affected¹². The failure to accumulate NKT cells in the liver of CXCR6-deficient mice may be the result of increased cell death¹².

To determine if Id2 deficiency affects CXCR6 expression on NKT cells, I analyzed CXCR6 mRNA expression by quantitative PCR. NKT cells from Id2^{KO} or Id2^{Het} bone marrow chimeras were sorted by expression of congenic marker (CD45.2⁺), TCR β , and NK1.1. Quantitative PCR was performed on cDNA from Id2^{KO} and Id2^{Het} liver and spleen NKT cells to determine the mRNA expression level of CXCR6. NKT cells deficient in Id2 had substantially lower CXCR6 expression than Id2^{Het} NKT cells (Figure 5). Hepatic Id2^{KO} NKT cells had ~ 10-fold lower mRNA expression and spleen NKT cells showed a ~ 8-fold reduction compared to Id2^{Het} NKT cells, suggesting that Id2 may be regulating CXCR6 expression in this cell type.

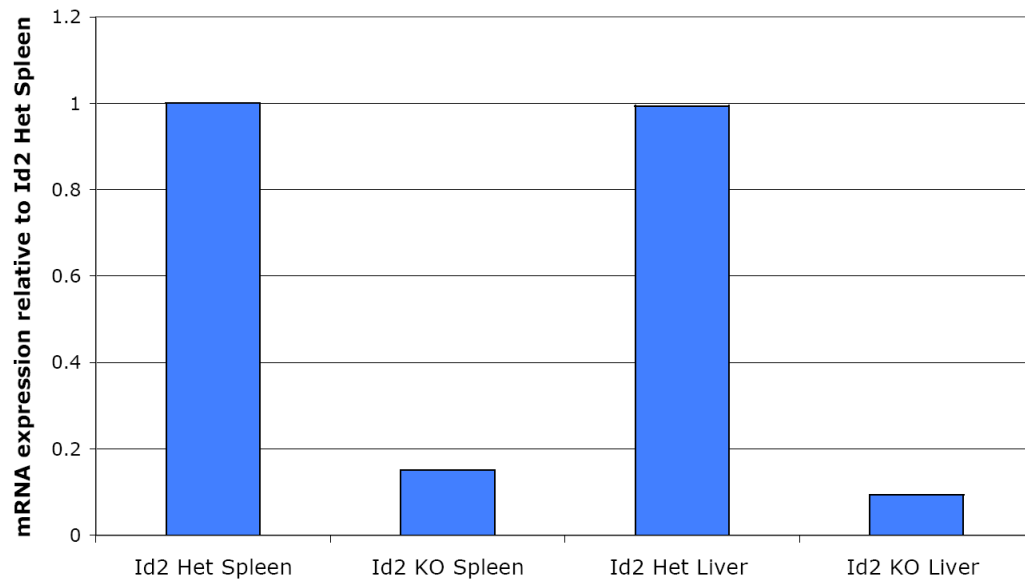


Figure 5: Id2^{KO} NKT cells have lower mRNA expression of the chemokine receptor CXCR6. CD45.2⁺ TCR β ⁺ NK1.1⁺ NKT cells were sorted from Id2^{KO} or Id2^{Het} bone marrow chimeras and cDNA was made by RT-PCR. Quantitative PCR was performed on cDNA using TaqMan probe and primers specific for CXCR6. CXCR6 mRNA expression for each sample was normalized to Id2^{Het} splenic NKT expression level. Data shown is average of n = 4 Id2^{KO} and Id2^{Het} pairs analyzed in two experiments.

CXCR6 mRNA expression is decreased in Id2^{KO} CD8⁺ T cells

The dramatically decreased expression of CXCR6 mRNA in Id2^{KO} NKT cells prompted us to ask if this expression pattern also applied to other T lineage cell types. Previously, Id2 has been described to have an important role in regulating the CD8⁺ T cell immune response to infection²⁰. CXCR6 has been shown to be expressed in both effector and memory CD4⁺ and CD8⁺ T cells, although expression is 18-fold lower than in NKT cells²¹. I used quantitative PCR to analyze the expression of CXCR6 in day 6 effector CD8⁺T cells sorted from the spleens of mice adoptively transferred with Id2^{KO} or Id2^{WT} OT-I transgenic CD8⁺ T cells and infected with *Listeria*-OVA. Similar to NKT cells, CXCR6 mRNA expression was lower in the Id2^{KO} CD8⁺ T cells

compared to wildtype cells (~ 8-fold difference) (Figure 6). These results are in agreement with data obtained from microarray analysis of Id2^{KO} vs Id2^{WT} effector CD8⁺ T cells (data not shown). This data suggests that Id2 may play a role in regulating CXCR6 expression in cell types other than NKT cells as well.

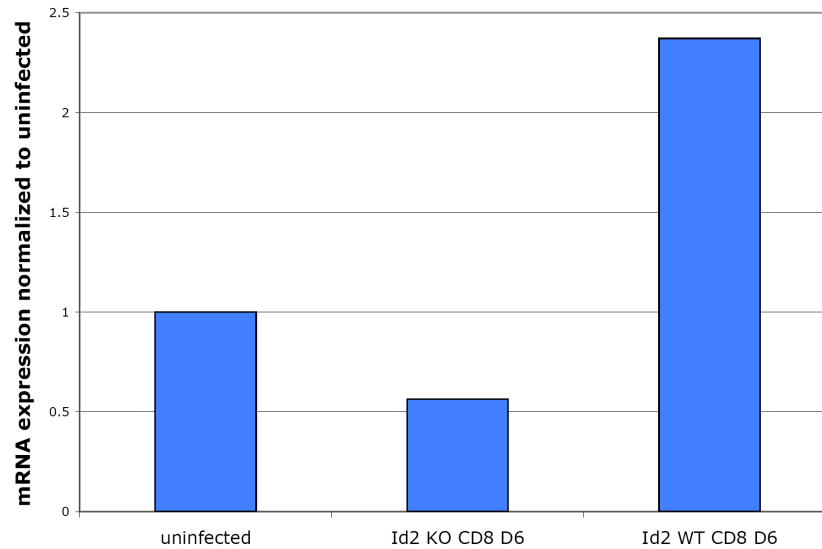
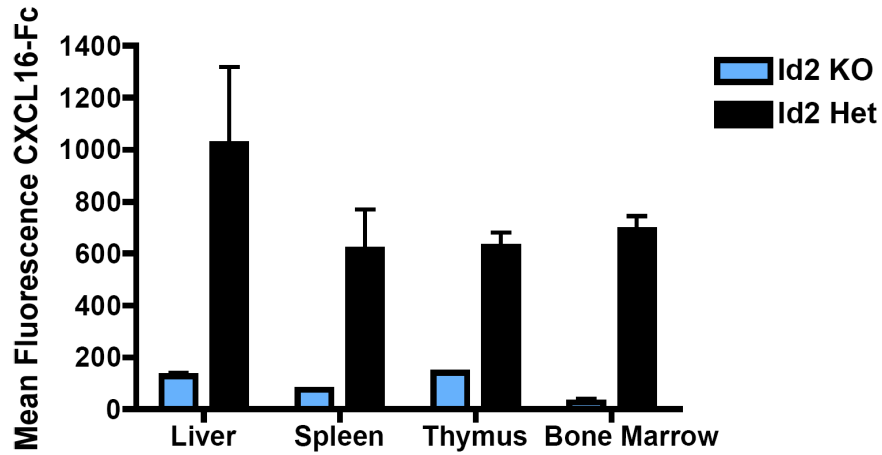


Figure 6: CXCR6 mRNA expression is lower in Id2^{KO} CD8⁺ T cells compared to Id2^{WT} CD8⁺ T cells. 1×10^4 Id2^{KO} or Id2^{WT} OT-I cells were adoptively transferred into host mice that were infected with 5×10^3 cfu Listeria-OVA. Quantitative PCR was performed on cDNA from sorted populations of Id2^{KO} or Id2^{WT} OT-I cells on day 6 of infection.

Reduced CXCL16-Fc binding by Id2^{KO} NKT cells

Although the differential mRNA expression of CXCR6 between Id2^{KO} and Id2^{Het} NKT cells suggested a significant reduction of CXCR6 on the cell surface, I wanted to analyze protein expression directly. Due to the lack of a mouse anti-CXCR6 antibody available for flow cytometry, I examined CXCR6 expression on the surface of NKT cells by measuring binding to its ligand CXCL16 using a CXCL16-Fc fusion protein kindly provided by Dr. Mehrdad Matloubian (UCSF) ¹¹.

a.



b.

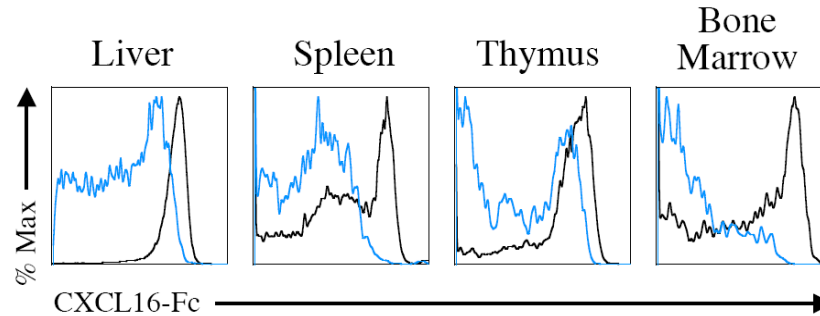


Figure 7: Reduced binding to CXCL16-Fc by Id2^{KO} NKT cells. Id2^{KO} and Id2^{Het} NKT cells were isolated from the liver, spleen, thymus, and bone marrow of bone marrow chimeras and identified by expression of congenic marker (CD45.2⁺), NK1.1, and TCR β . Cells were stained with CXCL16-Fc fusion protein (provided by M. Matloubian) and analyzed by flow cytometry. **a.** Mean fluorescence (average $\pm$ s.d.) of CXCL16-Fc in all four tissues from Id2^{Het} or Id2^{KO} mice. n = 2 pairs of Id2^{KO} and Id2^{Het} mice **b.** Representative flow cytometry plots of CXCL16-Fc expression on Id2^{KO} or Id2^{Het} NKT cells.

TCR β ⁺ NK1.1⁺ NKT cells from Id2^{KO} chimeras showed dramatically reduced levels of CXCL16-Fc binding in all tissues examined (Figure 7). Mean fluorescence CXCL16-Fc staining for Id2^{KO} NKT cells compared to Id2^{Het} cells was 8-fold lower in the liver and spleen, ~ 4-fold lower in the thymus, and nearly 25-fold lower in the bone marrow (Figure 7a). Taken together with the quantitative PCR results, these data suggest that Id2^{KO} NKT cells have substantially reduced expression of CXCR6.

Migration to the peripheral organs is not affected by loss of Id2

CXCR6 has been suggested to play a critical role in the accumulation of NKT cells in the liver, either through providing an essential survival signal or by regulating NKT trafficking¹². To assess whether Id2^{KO} NKT cells fail to accumulate in the liver due to impaired migration, I transferred $13-18 \times 10^6$ CD45.2⁺ B220⁻CD8⁻ splenocytes from Id2^{KO} or Id2^{Het} bone marrow chimeras into CD45.1⁺ host mice to measure the percentage of donor NKT cells recovered in the spleen and liver after 24 hours. Id2^{KO} NKT cells were recovered from spleens and livers of host mice in comparable percentages to Id2^{Het} NKT cells, indicating no defect in the ability of Id2^{KO} NKT cells to traffic to the peripheral organs after transfer (Figure 8). These results are in agreement with previous data showing that NKT cells deficient in CXCR6 appear to have no significant defect in migration to the liver¹².

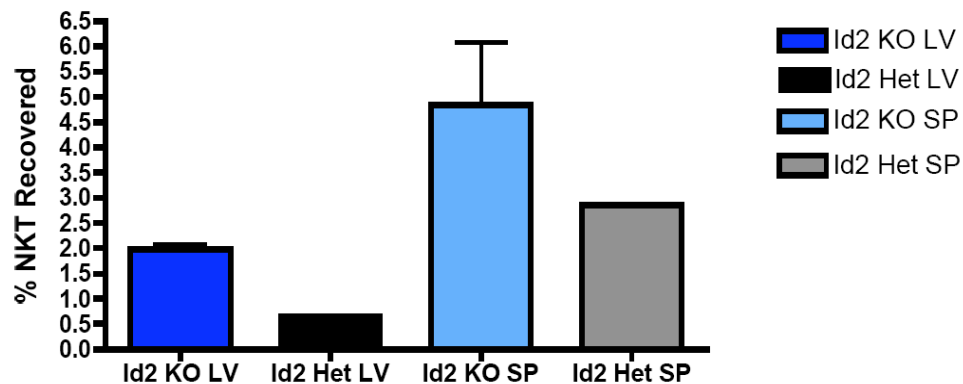


Figure 8: Id2^{KO} NKT cells show no impairment in migration to the liver or spleen after adoptive transfer. Id2^{KO} and Id2^{Het} NKT cells were isolated from livers and spleens of host mice and identified by expression of congenic marker (CD45.2⁺), NK1.1, and TCR β .

Increased apoptosis of Id2^{KO} NKT cells in the liver

Since Id2^{KO} NKT cells do not appear to have a defect in migration to the liver or spleen, we next examined whether the reduced NKT numbers in the liver could be due to increased cell death. Livers were harvested from Id2^{KO} or Id2^{Het} bone marrow chimeras and CD1d tetramer⁺ V α 14i NKT cells were stained for Annexin V to determine the percentage of apoptotic cells. More Id2^{KO} liver NKT cells stained Annexin V⁺ than Id2^{Het} NKT cells, indicating that many of the NKT cells in the liver were undergoing apoptosis (Figure 9). Similar results were obtained for NK1.1⁺ TCR β ⁺ NKT cells (data not shown). These results suggest that the lower percentage of NKT cells observed in the livers of Id2^{KO} chimeras is due, at least in part, to a defect in survival.

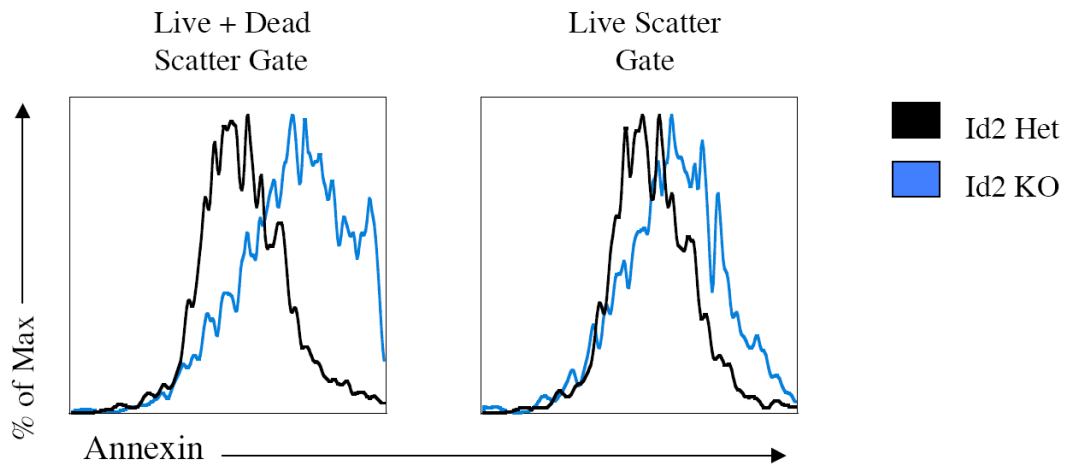


Figure 9: Increased apoptosis of Id2^{KO} NKT cells in the liver. Liver NKT cells were isolated from Id2^{KO} and Id2^{Het} bone marrow chimeras and identified based on expression of congenic marker (CD45.2⁺). TCR β , and CD1d tetramer. Cells were stained with Annexin V and analyzed by flow cytometry. Data is representative of 2 Id2^{KO} and Id2^{Het} pairs.

Early NKT development is not affected by Id2-deficiency

Another possibility for the reduced numbers of NKT cells in Id2^{KO} chimeras is a defect in one of the three stages of NKT cell development. NKT cell development occurs in the thymus and is characterized by three distinct stages based on the expression of NK1.1 and CD44; stage 1: NK1.1⁻ CD44^{lo}, stage 2: NK1.1⁻ CD44^{hi}, stage 3: NK1.1⁺ CD44^{hi 1-6}. Id2^{KO} chimeras would have fewer mature NK1.1⁺ CD44^{hi} NKT cells and correspondingly more immature NK1.1⁻ CD44^{hi} NKT cells if loss of Id2 blocked the last maturation step.

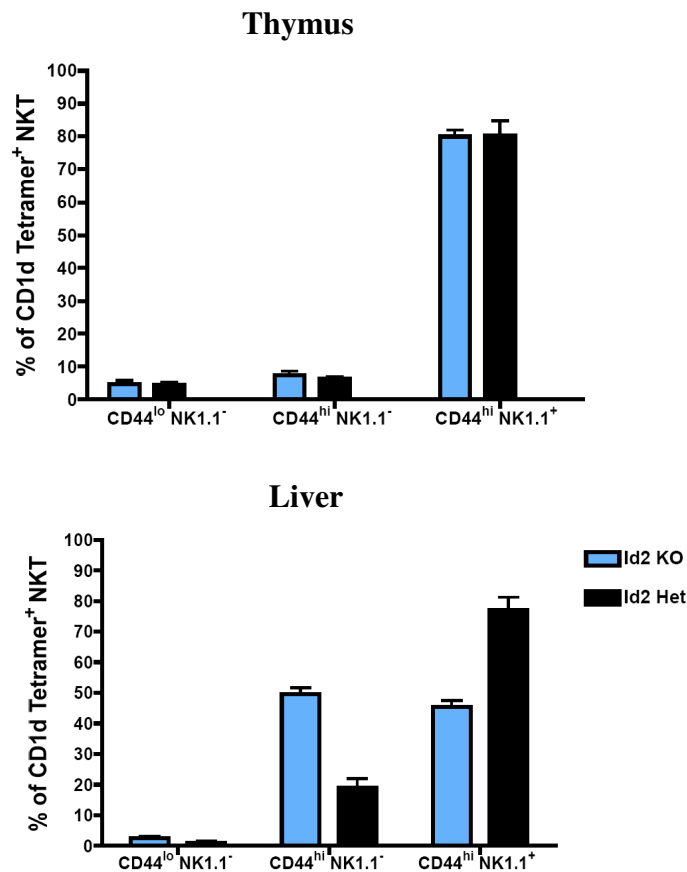


Figure 10: Absence of Id2 does not affect early NKT development in the thymus. Thymic and hepatic NKT cells were obtained from Id2^{KO} or Id2^{Het} bone marrow chimeras. Cells were gated on expression of congenic marker (CD45.2⁺), TCR β , and CD1d tetramer, then examined for expression of NK1.1 and CD44. Data is representative of 5 or 6 Id2^{KO} and Id2^{Het} chimeras analyzed in two experiments.

To investigate the possibility of an Id2-mediated NKT developmental defect, hepatic and thymic NKT cells from Id2^{KO} and Id2^{Het} bone marrow chimeras were analyzed for the percentages of mature and immature NKT subsets. There were no differences in the percentages of thymic NKT cells at any stage of development in Id2^{KO} chimeras compared to Id2^{Het} chimeras, indicating that Id2^{KO} NKT development in the thymus is normal (Figure 10). However, examination of the NKT cells in the liver revealed fewer mature CD44^{hi} NK1.1⁺ NKT cells and an increase in the percentage of immature stage 2 CD44^{hi} NK1.1⁻ NKT cells in the Id2^{KO} chimera. More than 50% of Id2^{KO} NKT cells were immature, compared to ~ 20% in the Id2^{Het} chimera. Together, these data suggest a possible role for Id2 in regulating the maturation step (upregulation of NK1.1) in the liver. Recent studies have shown that CXCR6-deficient mice also have reduced accumulation of mature NKT cells in the liver, indicating that CXCR6 may be involved in final maturation in the periphery²⁴. However, the increase in the percentage of immature NK1.1⁻ cells in Id2^{KO} chimeras could be due entirely to the increased apoptosis of mature hepatic NKT cells or a combination of reduced survival and a defect in maturation.

Id3 is upregulated in Id2^{KO} NKT cells

Another inhibitor of DNA binding protein, Id3, has been proposed to be able to compensate for low levels of Id2 during early stages of NK cell development¹⁷⁻¹⁸. Id3 is upregulated during the early stages of NKT cell development, showing highest expression in the immature NK1.1⁻CD44^{lo} and NK1.1⁻CD44^{hi} NKT developmental

subsets⁸. Id2 expression during NKT development is inversely correlated with Id3, showing upregulation in the mature NK1.1⁺ CD44^{hi} population^{8,21}. To examine the Id3 expression level in the absence of Id2, I performed quantitative PCR on cDNA from sorted populations of NKT cells isolated from the liver or spleen of Id2^{KO} and Id2^{Het} bone marrow chimeras. Id3 mRNA levels were elevated in Id2^{KO} NKT cells from both the liver (~ 14-fold) and the spleen (~ 4-fold) compared to Id2^{Het} NKT cells, indicating that Id3 may be compensating for the lack of Id2 during NKT cell development (Figure 11). Id3 may be a direct target of E proteins and increased E2A activity as a result of Id2-deficiency could lead to higher mRNA expression levels of Id3 (personal communication, C. Murre).

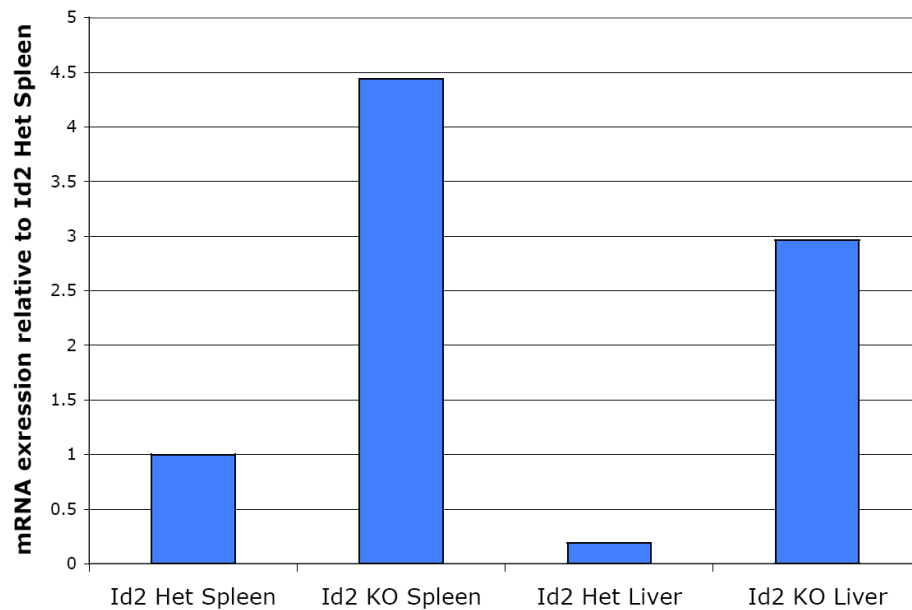


Figure 11: Higher mRNA expression of Id3 in Id2^{KO} spleen and liver NKT cells. NKT cells were sorted from spleens and livers of Id2^{KO} or Id2^{Het} bone marrow chimeras based on expression of congenic marker (CD45.2⁺), NK1.1, and TCR β . Id3 mRNA expression for each sample was normalized to Id2^{Het} splenic NKT expression level. Data shown is average of n = 4 Id2^{KO} and Id2^{Het} pairs analyzed in two experiments.

Discussion

The absence of transcriptional regulator Id2 results in fewer NKT cells in the liver, spleen, thymus, and bone marrow, with the most severe defect occurring in the liver. However, those Id2^{KO} NKT cells that are present can produce cytokines upon activation *in vivo* with α -GalCer and are phenotypically similar to Id2^{Het} NKT cells in expression of CD122, CD44, CD69, Klrg1, CD43, and CXCR3. High expression of CD44, CD69, and CD43 is characteristic of the “activated” memory-like phenotype possessed by mature NKT cells³⁻⁵. Id2-deficient spleen NKT cells express these markers at the same levels as Id2^{Het} cells, suggesting that NKT development in the thymus is not significantly perturbed in the absence of Id2.

Examination of NKT developmental stages in the thymus showed no substantial loss of mature CD44^{hi} NK1.1⁺ Id2^{KO} NKT cells, indicating that deficiency in Id2 does not prevent thymic NKT cells from undergoing final maturation defined by upregulation of NK1.1. However, the ~2-fold increase in the percentage of immature NK1.1⁻ NKT cells in the liver of Id2^{KO} chimeras compared to Id2^{Het} animals suggests that Id2 may play a role in NKT maturation in this peripheral organ. Immature NK1.1⁻ NKT cells in the peripheral organs are usually recent thymic emigrants that have not yet completed the final maturation step from developmental stage 2 to stage 3^{1-3,5}. The signals that regulate this step are largely unknown. If Id2 is necessary for the maturation of NKT cells in the liver, the absence of Id2 would prevent the immature NKT cells from upregulating NK1.1. Interestingly, CXCR6-deficient mice also have a dramatic reduction in mature hepatic NKT cells and increased immature NKT cell population, very similar to the defect seen in the Id2^{KO} chimeras²⁴. Alternatively, the

increase in the percentage of immature NKT cells in the liver could simply be the result of the loss of mature NKT cells due to apoptosis after failing to receive a survival signal through CXCR6. Since these mechanisms are not mutually exclusive, it is possible that the reduced accumulation of hepatic NKT cells in the Id2^{KO} chimera is the result of a defect in both maturation and survival. Another explanation for the reduction of NKT cells in Id2^{KO} chimeras is a failure of the cells to proliferate, but the slow turnover of NKT cells in the periphery makes it unlikely that a defect in proliferation could be responsible for the significant absence of Id2^{KO} NKT cells in the liver¹.

CXCR6, also known as Bonzo or STRL33, is expressed at high levels by resting NKT cells and at lower levels by effector and memory T cells²¹. Its ligand, CXCL16, is a transmembrane chemokine expressed in spleen, thymus, liver, and lung, as well as on dendritic cells and macrophages¹¹⁻¹³. CXCL16 is an unusual chemokine in its expression on the cell membrane, allowing it to function as an adhesion molecule for cells expressing CXCR6¹³. While it is suspected that CXCR6 may have a role in NKT migration within the liver, NKT cells show strong chemotaxis to chemokines other than CXCL16, most notably CXCL9 and SDF-1^{10,13}. Despite low CXCR6 expression, Id2^{KO} splenic NKT cells showed no impairment in their ability to migrate to the spleen or liver, indicating that other chemokines may be responsible or can compensate for NKT trafficking to the peripheral organs. CXCR6-deficient NKT cells are still able to crawl along the liver sinusoids and show no impairment in their ability to migrate to the liver¹². Id2^{KO} NKT cells do express high levels of CXCR3 on their cell surface, a chemokine receptor known to induce NKT chemotaxis. Mice deficient

in CXCR6 have a dramatic decrease in hepatic NKT cells, but NKT cell populations in other tissues are normal¹². The liver NKT cell defect in CXCR6-deficient mice is thought to be due to increased apoptosis and impaired maturation^{12,24}, similar to the defect reported here for Id2-deficient chimeras. The increase of apoptotic NKT cells and decrease in mature cells in the livers of Id2^{KO} chimeras supports the hypothesis that CXCR6 mediates a survival and/or maturation signal necessary for NKT accumulation in the liver. CXCR6 expression is also reduced in Id2^{KO} CD8⁺ T cells, suggesting that Id2 regulates CXCR6 expression in multiple lymphoid cell lineages. Interestingly, the defect in effector CD8⁺ T cell response to infection in the absence of Id2 is due to increased cell death similar to the NKT cell defect²⁰. However, CXCR6 is expressed at 18-fold lower levels in T cells compared to NKT cells and is not thought to be an essential chemokine receptor for CD8⁺ T cell trafficking²¹.

Interestingly, we found that Id2^{KO} NKT cells have dramatically higher Id3 expression than Id2^{Het} cells, implying that Id3 may compensate for the lack of Id2. Id3 is downregulated as Id2 is upregulated during the later stages of NKT cell development, leaving Id2 as the main regulator of E protein activity in mature NKT cells⁸. The high expression of Id3 in early NKT developmental stages could be compensating for the lack of Id2, thereby preventing a block in early development. Id3 has also been proposed to compensate for Id2 in NK cell development¹⁷⁻¹⁸. Further investigation using Id2-Id3 compound deficient mice could help address the role of Id proteins in NKT cell development and establish whether attenuation of E protein activity is necessary for NKT cell development and/or required for NKT cell survival in the periphery.

A plausible hypothesis for how Id2 regulates expression of CXCR6 is that Id2 normally prevents E proteins from binding to the regulatory elements of CXCR6, thereby allowing CXCR6 to be expressed on NKT cells to receive a survival and/or maturation signal critical for NKT cell accumulation in the liver. In the absence of Id2, E protein activity is unchecked, leading to the downregulation of CXCR6 mRNA expression in NKT cells. The lack of CXCR6 cell surface expression deprives hepatic NKT cells of an essential survival signal, resulting in apoptosis of NKT cells in the liver. However, Id proteins may have other targets in addition to E proteins, making it possible that Id2 regulation of CXCR6 expression is independent of E protein activity²³. Future studies should determine if E proteins bind to E-box sites within the CXCR6 promoter or intronic sequences. Additionally, E proteins have many other targets that could be contributing to the NKT cell defect besides CXCR6¹⁴⁻¹⁶. Mice deficient in E2A or E2-2 do not show any defect in NKT cell populations from all tissues examined (data not shown), indicating that E proteins may not be essential for NKT development, maturation, or survival. However, it is also possible that multiple E proteins are functioning together to regulate NKT cell maturation and the loss of one E protein could be compensated by the activity of another E protein. Examination of NKT cell development and function in Id2-Id3 and E2A-HEB compound deficient mice should help answer many of these questions to further elucidate the roles of Id and E proteins in NKT cell physiology.

Materials and Methods

Mice. Mice were bred and housed in specific pathogen-free conditions at the University of California, San Diego in accordance with animal care policies. Id2^{KO} mice were generated as previously described²⁰ and maintained through breeding heterozygous mice. For the generation of fetal liver chimeras, timed matings were performed between Id2^{Het} mice, embryos were removed and genotyped at E14-15. 5-10 x 10⁶ Id2^{KO} or Id2^{Het} fetal liver cells and 5-10 x 10⁵ RAG-/- cells were injected i.v. into lethally irradiated (900 RAD) recipient mice of a different congenic marker. Bone marrow chimeras were created by transferring 5 x 10⁶ B220⁺CD4⁺CD8⁻ bone marrow cells obtained from Id2^{KO} or Id2^{Het} fetal liver chimeras into lethally irradiated recipient mice of a separate congenic marker. All fetal liver chimeras and bone marrow chimeras were given antibiotics in the drinking water for three weeks following irradiation and were rested for eight weeks before use in experiments.

Isolation of NKT cells from livers: Livers were perfused with PBS through the aorta to remove NKT cells circulating in the blood. Livers were mashed through a 70µm cell strainer and washed twice with PBS supplemented with 5% bovine growth serum. The pellet was resuspended in 34% Percoll (Sigma) and spun at 1750 rpm for 25 min at RT to remove hepatocytes. The pellet was washed with 5% BGS in PBS and any contaminating red blood cells were removed using ACK red blood cell lysis buffer.

Flow Cytometry: Single cell suspensions were prepared from liver, thymus, spleen, and bone marrow of Id2^{KO} or Id2^{Het} chimeras. Cellular Fc receptors were blocked for

five minutes using unconjugated mouse antibody to Fc γ II/III (BD Biosciences Pharmingen). The following fluorochrome-conjugated monoclonal antibodies were used: TCR β FITC, CD45.1 FITC, CD45.2 FITC, CD43 FITC, CD69 FITC, CD1d Tetramer PE (NIH Tetramer Core Facility), TCR β PE, CD122 PE, CXCR3 PE (BD Biosciences), goat anti-human IgG Fc γ PE (Jackson ImmunoResearch), NK1.1 PerCP Cy5.5, CD45.2 PerCP Cy5.5, TCR β APC, CD45.2 APC, CD44 APC, Klrg1 APC, Annexin V APC (Invitrogen), CD45.2 Alexa 750. All antibodies were purchased from eBioscience unless specified. Cells were collected on FACS Calibur or FACS Aria (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Annexin staining: Liver NKT cells were isolated as described above and incubated with Fc-block for 5 minutes. Cells were stained with TCR β FITC, CD1d Tetramer PE, and CD45.2 PerCP Cy5.5 on ice in the dark for 30 minutes. Cells were washed once with Annexin V binding buffer, resuspended in Annexin V buffer with Annexin V APC, and incubated in the dark at RT for 15 minutes. Samples were collected within 1 hour of staining.

CXCL16-Fc staining: Single cell suspensions were made from liver, spleen, thymus, and bone marrow of Id2^{KO} or Id2^{Het} chimeras and incubated with Fc-block for 15 minutes on ice. 1:200 dilution of CXCL16-Fc fusion protein (gift of M. Matloubian, UCSF) was added to samples and incubated for 30 minutes on ice. Cells were washed twice with FACS buffer (2% BGS and 0.01% azide in PBS) and stained with a secondary antibody (PE goat anti-human IgG Fc γ blocked with 2% normal mouse and rat serum) for 20 minutes on ice in the dark. Samples were washed twice with FACS

buffer and stained with CD45.1 FITC, NK1.1 PerCP Cy5.5, and TCR β APC on ice in the dark for 15 minutes.

Activation of NKT cells and Intracellular Cytokine Staining: Id2^{KO} or Id2^{Het} bone marrow chimeras were injected i.p. with 2 μ g α -galactosylceramide and sacrificed 1.5 hours later. Mice were perfused with PBS, livers and spleens were collected for flow cytometry analysis. Cells were stained for production of intracellular cytokines IFN γ PE, IL-4 PE, TNF α APC directly *ex vivo* (no additional stimulation) using the Cytofix/Cytoperm Plus kit protocol (BD Biosciences).

Quantitative PCR: Livers and spleens were harvested from Id2^{KO} or Id2^{Het} bone marrow chimeras and CD45.2⁺ NK1.1⁺ TCR β ⁺ NKT cells were sorted on the FACS Aria. RNA was extracted from sorted cells using TRIzol solution (Invitrogen) and treated with DNA-free kit (Ambion) to remove contaminating DNA. cDNA was generated using RT-PCR with SuperScript III kit (Invitrogen) following manufacturer's instructions. Quantitative real time PCR for Id3 was done by non-specific product detection (SYBR Green, Stratagene) using the following primer sequences: Id3 Forward: GACTCTGGGACCCTCTCTC, Id3 Reverse: ACCCAAGTTCAGTCCTTCTC. Samples were normalized to the housekeeping gene HPRT. CXCR6 mRNA expression was detected using a TaqMan probe and primer sequences specific for CXCR6 (TaqMan Gene Expression Assay, Applied Biosystems).

Migration Assay: Spleens were harvested from Id2^{KO} or Id2^{Het} bone marrow chimeras and splenocytes were depleted using MACS columns (Miltenyi) with B220 biotin and CD8 biotin to remove mature B cells and CD8⁺ T cells. 13-18 x 10⁶ Id2^{KO} or Id2^{Het} B220⁻ CD8⁻ splenocytes were injected i.v. into CD45.1 hosts. Recipient mice were sacrificed 24 hours after adoptive transfer, livers and spleens were removed for flow cytometry analysis. Cells were stained with CD45.1 FITC, TCR β PE, NK1.1 PerCP Cy5.5, and CD45.2 APC to measure the percentage of donor Id2 NKT cells (CD45.2⁺) recovered.

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