LINEAGE SPECIFIC ROLE FOR ADHESION AND DEGRANULATION PROMOTING ADAPTOR PROTEIN (ADAP) IN iNKT CELL DEVELOPMENT AND FUNCTIONS

By

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ABSTRACT

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Invariant natural killer T (iNKT) cells comprise a sub-lineage of T lymphocytes that express an "invariant" T cell receptor (TCR) with specificity for glycolipid antigens when presented in complex with the MHC class I molecule CD1d. Following TCR engagement, iNKT cells rapidly secrete cytokines and transactivate the anti-tumor functions of dendritic cells (DC), natural killer (NK), T and B cells as well as directly kill tumor cells. Although TCR-CD1d interactions are required for iNKT cell functions, the signaling mechanisms that co-operate with the TCR to promote maximal iNKT cell responses remain poorly understood. Collective studies from our laboratory and others have established that the adaptor protein SAP and its signaling partner, Fyn is critical for iNKT cell development and functions. We investigated the role of ADAP (adhesion and degranulation promoting adaptor protein), a Fyn-binding protein in iNKT cell development and functions. Our studies demonstrate that ADAP is critical for homeostatic maintenance of these cells in peripheral organs. Furthermore, we observe that iNKT cytokine production, and bystander immune cell activation are remarkably reduced in *Adap*$^{-/-}$ mice. These findings are not restricted to the murine system, as *ADAP*-silenced human iNKT cells also have significantly impaired cytokine release. Importantly, we demonstrate that ADAP is essential for TCR-induced iNKT cell cytotoxicity against leukemia targets *in vitro* and *in vivo*. Collectively, our data suggest that ADAP plays a unique linage-specific role in iNKT cells. These studies will have an impact by establishing new paradigms for iNKT cell signaling and offering insights into how iNKT cells can be best activated to enhance host immunity and treat cancer.

I dedicate this thesis to my Mother, Father, Sister & Grandparents;
and of course, my dog, Milo.

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I write here to express my deepest gratitude to those who have helped me along the way, for the successes I have had are largely due to them. I first must thank my mentor, Dr. Rupali Das, as she has taken me under her wing and gone beyond her call of duty to teach me how to question, conduct, and present among the realm of research. Her willingness to help me in all areas, inside and outside of research, has helped improve my scientific literacy, self-discipline, and communication. Her support more than anything else has exponentially excelled any improvements thus far. Most of all, she has instilled some of her passion for science into me, a gift which will last a lifetime.

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PREFACE

The primary motivator, and basis of my research stems from clinical stories and experiences I have witnessed, and brought me to the ongoing search for a "cure" for cancer. Dr. Rupali Das has enlightened me on the emerging potential of immunotherapy, and its successes and deficits. I performed these studies with hopes of one day contributing to advancement and discovery of more powerful, and directed medicine.

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KEY TO ABBREVIATIONS

^{51}Cr	Chromium-51
ADAP	Adhesion and degranulation-promoting adaptor protein
Ag	Antigen
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cell
CD	Cluster of differentiation
CPM	Counts per minute
CRACC	CD2-like receptor-activating cytotoxic cells
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DC	Dendritic cell
DN	Double negative
DP	Double positive
E:T	Effector to target ratio
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked immunosorbent assay
EVH1	Drosophila enabled/vasodilator-stimulated phosphoprotein homology 1
FACS	Fluorescence activated cell sorting
FSC	Forward scatter
FYB	Fyn-binding protein

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H&E	Hematoxylin and eosin
IACUC	Institutional Animal Care and Use Committee
IFN-γ	Interferon gamma
IGB3	Isoglobotriosylceramide
IL	Interleukin
iNKT	Invariant natural killer T-cell
LAT	Linker for activation of T-cells
LFA	Lymphocyte function-associated antigen
Liv	Liver
Luc	Luciferase
Mac-1	Macrophage-1 antigen
MACS	Magnetic-activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MTOC	Microtubule-organizing center
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NLS	Nuclear localization sequence
NSG	Non-obese diabetic (NOD) scid gamma
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PH	Pleckstrin homology domain

PLZF	Promyelocytic leukemia zinc finger protein
PRAM-1	Promyelocytic leukemia retinoic acid receptor-alpha regulated adaptor molecule-1
RORγt	RAR-related orphan receptor gamma
SAP	SLAM-associated protein
SD	Standard deviation
SEM	Standard error of the mean
siRNA	Short-interfering ribonucleic acid
SKAP55	Src kinase-associated phosphoprotein
SKAP55R	Src kinase-associated phosphoprotein-related protein
SLAM	Signaling lymphocytoic activation molecule
SLAP-130	SH2 domain-containing Slp-76-associated phosphoprotein of 130 kDa
SLP-76	SH2 domain containing leukocyte protein of 76kDa
SMAC	Supramolecular activation complex
Spl	Spleen
SSC	Side Scatter
TAM	Tumor-associated macrophage
TBI	Total body imaging
Tcon	Conventional T-cell
TCR	T-cell receptor
TEC	Thymic epithelial cells
Tet	Tetramer
ThPOK	Th inducing POZ-Kruppel factor
Thy	Thymus

TME	Tumor microenvironment
TRAIL	Tumor necrosis factor (TNF)-related apoptosis-inducing ligand
ZAP-70	Zeta-chain-associated protein kinase 70
αGC	Alpha-galactosylceramide

OVERALL INTRODUCTION

Natural Killer T (NKT) cells comprise a unique subset of T lymphocytes that differ in both their development and functional requirements, as compared to conventional T-cells (Tcons) [1]. As both NKT and T-cells develop in the thymus, they are by definition T-cells, however they differ in their selection process. It is well studied that Tcons undergo a selection process by peptide antigen presentation by major histocompatibility complex (MHC), of class I, or II on the surface of epithelial cells in the thymus. In contrast, NKT cells develop by selection of an endogenous glycolipid antigen presented by $CD4^{+}CD8^{+}$ thymocytes, termed double-positive (DP) thymocytes [1, 2], as shown in Figure 1. Presentation of the glycolipid is by a monomorphic MHC class I-like molecule, termed CD1d [1]. This engagement is a positive selection process for NKT cells, as only T-cell progenitors expressing a highly invariant T-cell receptor (TCR), will engage with the CD1d and commit to becoming an invariant NKT (iNKT) cell [1]. In addition, iNKT cells express NK-cell surface markers, such as NK1.1, and possess an innate-like memory [3]. Much like NK cells, iNKT cells contain lytic molecules for direct cytolysis [4]. Unique to iNKT cells, they contain preformed vesicles for Th1 and Th2 cytokines, which upon activation, are secreted rapidly and in a large amounts [5, 6].

iNKT classifications

Mature iNKT cells can be further divided into sub-types, based on their co-receptor expression, and cytokine release profile. As a result of their T-cell lineage, mature iNKT cells can either be $CD4^{+}$ or $CD4^{-}CD8^{-}$ (double negative, DN) [5, 6], that can vary in their functional phenotypes. Another classification of NKT cells, is dependent on their cytokine release profile, Th1, Th2, or Th17 [7]. At large, release of Th1 cytokines, including IFN-γ and IL-12, are commonly considered inflammatory inducing cytokines, as they recruit and assist in activation of other

effector immune cells. Th2 cytokines, on the other hand are commonly referred to as anti-inflammatory, and include cytokines such as IL-4, IL-5, and IL-13 that usually help to suppress an immune response. However these cytokines can sometimes be detrimental to the host in certain conditions such as in asthma and allergic diseases [8]. Lastly, the pro-inflammatory Th17 group of cytokines, such as IL-17, IL-21, and IL-22 [9, 10], are currently under investigation to ascertain their roles in modulating iNKT cell functions [8]. Thus, iNKT cells are designated as NKT-1, NKT-2, or NKT-17, corresponding to their Th1, Th2, or Th17 cytokine release profiles, respectively. While iNKT cells can release various amounts of cytokines in different settings, the type of release can be skewed which is largely dependent on the glycolipid antigen presented and the co-stimulatory molecules at play. Invariant NKT cells, as described, contain a highly restricted TCR repertoire, consisting of Vα14 that usually pairs with Vβ8.2, 2, or 7 in mice and Vα24 and Vβ11 in humans [11, 12]. This invariant TCR is the phenotype of Type-I or invariant NKT (iNKT) cells. These iNKT cells are dependent on the transcription factor promyelocytic zinc finger (PLZF) in order to develop into their fully functional, TCR-restricted phenotype [13, 14]. The TCR of iNKT cells are exclusively reactive to glycolipid antigens, which is commonly modeled by use of α-galactosylceramide (αGC) when presented by CD1d on an antigen-presenting cell (APC) [15]. The use of this reagent has excelled the study of iNKT cells, as CD1d tetramers loaded with αGC has allowed for this population to be more accurately analyzed by flow cytometry [15]. These iNKT cells are commonly found in highest incidence in the liver lymphocytes of both mice and humans, at about 30% and 1% respectively, however much less studied in humans [16, 17]. They are also found in about 1% of thymic and splenic lymphocytes in mice, and about .01% in humans [18, 19]. Additionally, iNKT cells can be found in circulating peripheral blood mononuclear cells (PBMCs), at around 1% in both mice and

humans, however this number can vary greatly on different factors[19]. Another subtype of NKT cells classified as Type-II NKT cells express more variable TCR αβ chain combinations [20]. While they are still CD1d restricted, they are activated by sulfatide form of a glycolipid [20]. Additionally, they can lack expression of NK1.1, however are still classified as a NKT cell due to their CD1d dependence for activation. As iNKT cells release both Th1 and Th2 cytokines, they can act as a protector against certain infectious diseases, while promoting others such as asthmatic allergy. Type-II NKT cells appear to play dual roles as well, however seem to play beneficial roles in regulation of autoimmune diseases, but have suppressive roles in in host immunity against cancer, which is usually opposing to the protective role mediated by type I or iNKT cells.

Invariant NKT cells in disease states

Invariant NKT cells can either confer protection or mediate promotion of different disease states. Certain studies have shown iNKT cells to play a role in the clearance of some infectious diseases, whether it be bacterial, parasitic, or viral, although these studies are not fully confirmed [21-23]. However, studies in different murine models, that lack of iNKT cells demonstrate greater incidence and proliferation of the infection, indicating that iNKT cells play some key role in the protection from infection, most likely by indirect mechanisms. Continuing, iNKT cells have been shown to play a protective role against autoimmune induced diseases, such as type-1 diabetes, multiple sclerosis and rheumatoid arthritis [24-27]. In each case, primarily via cytokine release, certain subtypes of iNKT cells are present in greater incidence at the autoimmune target sites resulted in slower progression of the disease. However, due to the dual cytokine profile of iNKT cells, studies have also shown that release of Th1 cytokines can recruit

effector cells that amplify the disease progression. This leaves more room for iNKT cells to be studied and manipulated to prevent progression of pathological diseases. In certain asthmatic conditions modeled in mice, secretion of Th2 cytokines by iNKT exacerbated the airway hyper-reactivity, suggesting a detrimental role for these cells in the amplification of asthmatic conditions [28]. Studies have further shown that manipulation of iNKT cells to skew their cytokine profile to a Th1 release, have deterred the incidence of asthma [29]. More so, previous studies have shown iNKT cells have direct cytotoxic effects against tumor targets, as well as indirect activation of other cytolytic effectors. Activation of iNKT cells by αGC presentation has been shown to be sufficient in recruiting cytolytic effectors such as NK cells and $CD8^+$ T-cells, however this response was short-lived as iNKT cells soon became unresponsive or anergic [30, 31]. Anergic iNKT cells are in a state such that they are functionally inactivated, but still present [31]. To prevent this state, incorporation of DCs pulsed with αGC resulted in a more sustained attack on the tumor, most likely due to the co-stimulation from the DC, such as CD40-CD40L interactions [30]. At large, shown in both murine and human cancers, increased iNKT cell incidence results in a slower progression of various tumor types [32, 33]. In contrast, iNKT cell-deficient mice ($Cd1d^{-/-}$ or $J\alpha18^{-/-}$) are highly susceptible to spontaneous, carcinogen-induced or adoptively transferred tumors and reconstitution of the iNKT cell compartment in these animals results in prevention of slower tumor formation. These studies coupled with data from retrospective clinical studies highlight the importance of iNKT cells in tumor control [34, 35]. While this is true, iNKT cells can play a paradoxical role in cancer, as it can both promote and suppress the immune responses for recruitment or lack there of cytotoxic effectors [36]. The suppression of tumor cells occurs through a two-pronged attack, direct and indirect cytotoxicity. Tumors expressing CD1d can present a glycolipid and be directly lysed by iNKT cells through

release of perforin and Granzyme-B, or by Fas Ligand or TRAIL interactions [37]. Additionally, the recruitment of $CD8^+$ T and NK cells by Th1 cytokine release amplifies the cytotoxic response [38]. More so, iNKT cells also possess the ability to kill tumor associated macrophages (TAMs), which function to keep the tumor microenvironment in an immunosuppressive state, hiding it from immuneosurveillance [39]. On the contrary, release of Th2 cytokines by Type-II NKT cells, or other subtypes of NKT cells, can also result in immunosuppression and thus deterred tumor clearance [40]. Taken together, iNKT cells play a prominent role in the protection, detection, and clearance of cancerous cells. Further characterization of the mechanism(s) of how iNKT cells function, or can be altered to function, in different tumor types can be critical in understanding how to eliminate cancers using novel immunotherapeutic approaches.

Signaling mechanisms of iNKT cells

Although it is well recognized that iNKT cells play critical role in host immunity against several pathogens or malignant cells, little is known regarding the signals that promote iNKT functions. Previous studies from our laboratory demonstrated for the first time that the adaptor molecule, SAP (Signaling Lymphocytic Activation Molecule [SLAM]-associated protein) is essential for not only iNKT cell linage development but also for TCR-induced iNKT cell lysis of multiple cellular targets *in vitro* and *in vivo*, where mice reconstituted with SAP-null iNKT cells exhibit extremely poor control of T-cell lymphoma growth. TCR-induced iNKT cell lysis was dependent upon Fyn, which often functions in conjunction with SAP to promote cell activation. Importantly, siRNA-mediated silencing of SAP or FYN in human iNKT cells significantly impaired TCR-induced target cell lysis. Collectively, these findings highlight the central role for

SAP-associated signals in iNKT cell cytotoxicity. ***Thus the goal of the current study was to identify and characterize the downstream signaling molecules that regulate iNKT cell development and functions. To that end, we investigated the role of the adaptor protein ADAP (Adhesion and Degranulation-Promoting Adapter Protein) in iNKT cell development and functions.*** The results obtained from these studies are significant as they increase our understanding of iNKT cell biology and provide insights into how iNKT cell functional responses can be further enhanced to improve host immunity against various diseases, specifically for the treatment of hematopoietic cancers such as leukemia and lymphoma.

ADAP regulates lymphocyte development and functions

In this study, we specifically chose to investigate the role of ADAP in iNKT cell development and function, as it is well established that upon TCR stimulation, ADAP binds to Fyn to modulate T cell functional responses. It was originally cloned as a phosphoprotein that inducibly binds SLP-76 and Fyn upon TCR stimulation [41, 42]. Accordingly, ADAP was formerly known as Fyb (Fyn binding protein) or SLAP 130 (Slp76-associated adaptor protein of 130kD) [43]. ADAP contains a large central proline-rich domain, an enabled vasodilator-stimulated phosphoprotein homology 1-binding domain [42], three putatively phosphorylated tyrosine residues, and a C-terminal SH3-like domain [42]). Two isoforms, 120 and 130 kDa, differing by a single, differentially spliced exon of 138 bp, have thus far been identified [42, 44], as shown in Figure 2. Early studies overexpressing ADAP in the Jurkat human T cell line implicated ADAP in TCR-induced IL-2 production [42]. ADAP has also been shown to be phosphorylated downstream of β_1 integrin ligation in Jurkat T cells [45, 46]. Studies in ADAP-deficient mice demonstrate that ADAP is required for optimal passage through multiple thymic developmental

checkpoints and that ADAP may modulate TCR-dependent adhesive functions that are critical for thymocyte development and selection. Upon TCR activation, ADAP-deficient peripheral T cells have intact proximal signaling but inefficiently up-regulate early activation markers CD25 and CD69, fail to produce IL-2, and proliferate poorly in response to TCR stimulation [47]. ADAP-deficient T cells also demonstrate impaired inside-out activation of integrins, with decreased clustering of LFA-1 and impaired adhesion to ICAM-1 upon TCR ligation [48]. Employing ADAP-deficient mice (*Adap*$^{-/-}$) and gene-silencing approaches in human iNKT cells, we specifically examined the requirement of ADAP in iNKT cell development and maturation as well as it role in direct and indirect immune-surveillance against cancer cells.

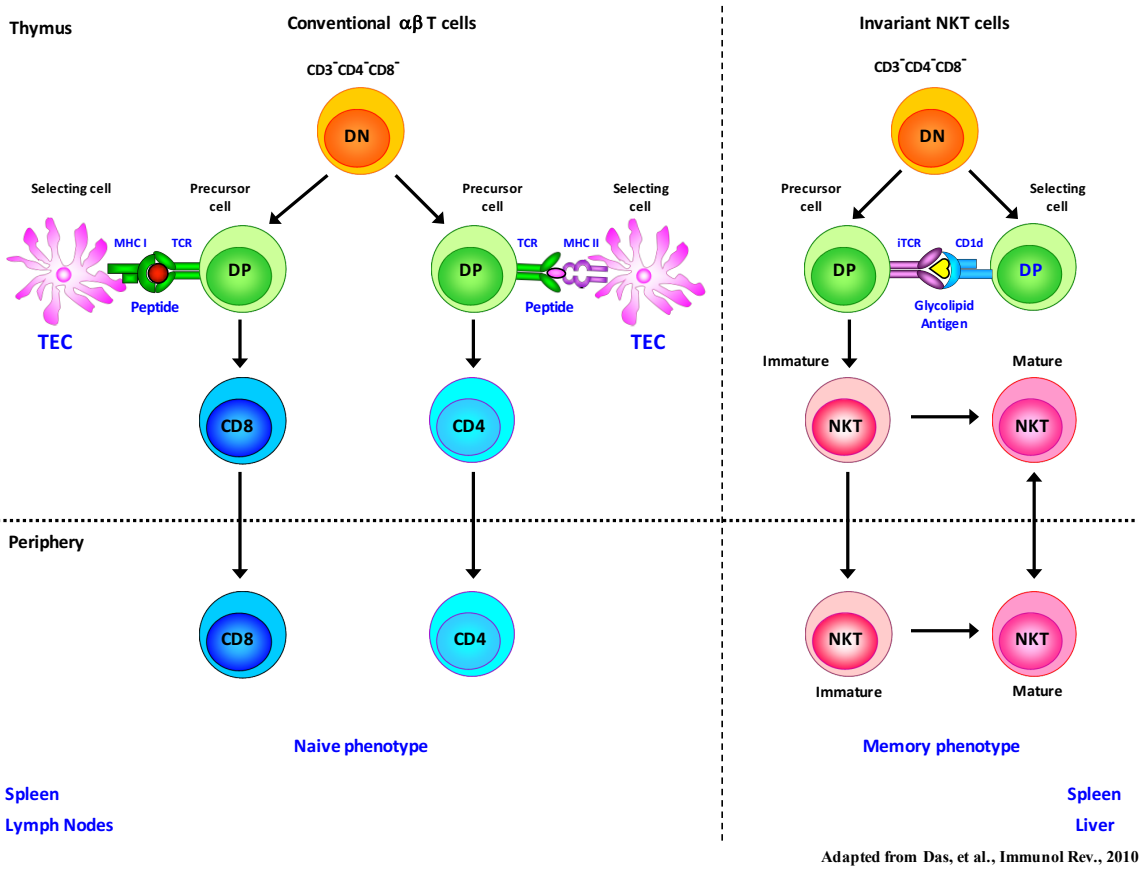
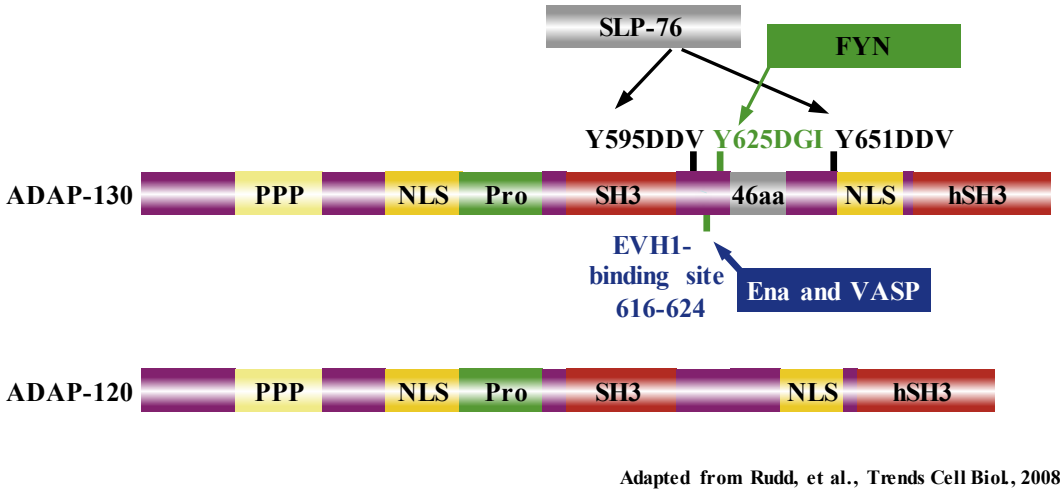


Figure 1. Schematic view of iNKT cell versus conventional αβ T cell development.

NKT cell precursors diverge from conventional αβ T cells at the $CD4^+CD8^+$ double-positive (DP) stage. The invariant TCR α-chain (iTCR) on iNKT cell precursors recognizes glycolipid antigens presented by the CD1d molecule expressed at the surface of "selecting" DP thymocytes. In contrast, $CD4^+$ and $CD8^+$ T cells express diverse TCRs that recognize peptide antigens presented by MHC class II or class I molecules, respectively, expressed by thymic cortical epithelial cells (TEC). Mature thymic emigrants of the conventional αβ T cell lineage have a naïve phenotype, as opposed to iNKT cells, which acquire a memory/effector phenotype during thymic development. Immature NKT cells can acquire the NK1.1 marker after emigration to

Figure 1. (cont'd)

peripheral tissues or within the thymus. While a subset of $NK1.1^+$ iNKT cells remain in the thymus as "long term-residents", some also migrate to the periphery.



Adapted from Rudd, et al., Trends Cell Biol., 2008

Figure 2. Molecular domains of ADAP and their binding partners.

ADAP and its major molecular domains as shown, within its two major isoforms, 130 kD (top) and 120 kD (bottom). ADAP interaction sites with SLP-76 and Fyn at tyrosine sites 595 & 651 and 625, respectively. EVH1 shown to bind to 616-624 sites. Abbreviations: PPP, proline-rich domain; NLS, nuclear localization sites; EVH1, Ena (enabled)-VASP (vasodilator-stimulated phosphor-protein) homology-1; PH, Pleckstrin homology domain; SH3, Src homology 3 domain.

CHAPTER 1

ADAP REGULATES HOMEOSTATIC MAINTENANCE OF iNKT CELLS BUT NOT ITS THYMIC DEVELOPMENT OR MATURATION

Introduction

Invariant natural killer T (iNKT) cells comprise a rare lymphocyte sub-lineage with phenotypic and functional properties similar to T and NK cells. Much like T-cells which develop in the thymus, development of iNKT cells also begin from a lymphoid progenitor [49]. During ontogenesis, iNKT cells diverge from the conventional T cells at the stage when T-cells express both CD4 and CD8 co-receptors, termed as double positive (DP) cells [49]. The T-cell receptor (TCR), in general, can express highly variable alpha (α) and beta (β) chain combinations following V(D)J recombination [49]. However, in the case of iNKT cells, the TCR consists of highly invariant Vα14-Jα18 chains that often pair with Vβ 8.2, 7, or 2 in mice and Vα24-Jα18 and Vβ11 in humans [49]. While conventional T cells are selected by peptide antigens in complex with major histocompatibility complex (MHC) class I or II molecules present on the surface of thymic epithelial cells, iNKT cells develop following selection by self-glycolipid antigens in complex with the MHC class I–like molecule CD1d, when presented by DP thymocytes [15]. Although still unclear, it is thought the endogenous glycolipid is or likely to be similar to iGb3 (isoglobotrisylceramide) [49].

Developmental stages of iNKT cells in the thymus

After positive selection, when a cell is committed into becoming an iNKT cell, it enters a naïve stage, also termed stage 0, when phenotypically it is $CD24^{hi}$, $CD44^{lo}$, and $NK1.1^{neg}$ [1, 50] as shown in Figure 1. As this cell continues to develop into stages 1 through 3, it undergoes further changes in the surface expression of CD24, CD44 and NK1.1 as: expressing $CD24^{lo}$, $CD44^{lo}$, and $NK1.1^{neg}$ (stage 1), $CD24^{lo}$, $CD44^{hi}$, and $NK1.1^{neg}$ (stage 2) and $CD24^{lo}$, $CD44^{hi}$, and $NK1.1^{pos}$ (stage 3) [50]. In addition, CD4 is expressed exclusively from stages 0-3, where at the

end of stage 3, CD4 will remain expressed, or be down regulated, resulting in mature, $CD4^+$ or $CD4^-CD8^-$ (double negative; DN) iNKT cells [1]. At this stage, iNKT cells can remain in the thymus, or migrate to secondary lymphoid organs such as the spleen, and lymph nodes [51] or the liver and lungs. Migration to these organs is determined by a balance of chemokine receptor CXCR6 expression on iNKT cells, and CXCL16 (ligand for CXCR6) on resident organs, and expression is determined by the niche of the immune milieu in each of these organs [51, 52].

Transcriptional regulation of iNKT cells

While several transcription factors that regulate iNKT cell development and maturation are shared with T cells, there are some unique transcription factors that exclusively regulate the developmental pathway of iNKT cells. In particular, mice deficient in *Rag-1* or *Rag-2* genes lack iNKT cells, as theses genes are required for V(D)J recombination into the invariant TCR [53]. In addition, any genetic deletion of the proper TCRα/β chains of iNKT cells or any of the TCR signaling molecules, such as Lck, ZAP-70, LAT, and SLP-76, results in diminished iNKT cells [54-57]. Along the stages of development for iNKT cells, the first major genetic requirement is expression of transcription factors RORγt and AP-1, required for expression of CD4 and CD8 before stage 0 [58, 59]. The transition from stage 0 to stage 1 requires a key regulator of iNKT cells called promyelocytic zinc finger protein (PLZF), unique to iNKT cells in the thymus [14, 60]. Mice deficient in this transcription factor result in decreased iNKT cell incidence in the thymus in mice [14]. Moreover, iNKT cells deficient in PLZF result in decreased functional capacity, including failure to secrete Thi and Th2 cytokines upon activation, emphasizing its importance to iNKT cells [14]. The transition from stage 1 to 2 and beyond to full maturation is required by multiple transcription factors, including Egr2, NF-κB, c-Myc,

GATA-3, and ThPOK being required for NK1.1 expression as well as iNKT functionality and migration [61-65]. One of the final maturation factors required is the T-Box transcription factor (T-bet) whose expression increases from stage 1-3, resulting in functionally mature, iNKT cells [66, 67].

SLAM-SAP-Fyn axis in iNKT cell ontogeny

While transcriptional regulation is quite well defined, the signaling mechanisms they induce and regulate in iNKT cell development are yet to be fully understood. In particular, an additional required signaling event must occur during the DP selection process while TCR is engaged with CD1d. A family of receptors, known as SLAM (signaling lymphocytic activation molecule) receptors mostly act as self-ligands for each other, inducing activation of many required transcription factors downstream [68]. The family consists of varied expression of SLAM, Ly108, Ly-9, CD84, and CRACC of which its ligand is the same homotypic molecule, and 2B4, which uniquely binds to CD48, a heterotypic molecule [68]. While it is well characterized that SLAM-150 and Ly108 have known to be important for iNKT development, expression of other SLAM family receptors on iNKT cells, their intracellular signaling pathways are yet to be fully understood. However, a molecule downstream of all of these SLAM receptors, termed SAP (SLAM associated protein) is required in expression and functions of its downstream components [68]. This adaptor protein is required for an appropriate interaction with target molecules, and immunological synapse formation [68]. More so in the thymus, SAP is an indispensable molecule for iNKT development, as mice deficient in SAP lack any iNKT cells whatsoever [68]. After a SLAM receptor is bound, SAP recruits another protein, termed Fyn, which is a tyrosine kinase that is required for the downstream signaling events to occur [68].

Thus, in addition to TCR ligation in the thymus, the SLAM-SAP-Fyn axis is required as well for proper iNKT development [68].

Earlier studies claimed that the adapter protein ADAP (a well-known Fyn-binding protein) is dispensable for conventional T cell development [43], however, data from more recent studies demonstrate that ADAP is required for optimal development of TCRαβ cells in the thymus, although it is dispensable for splenic B cell [69] and NK cell development [70]. Interestingly, it is now known that ADAP not only regulates intraepithelial innate T cells, CD8αα homeostasis in the small intestinal epithelium, it might also play a role in the development of non-T hematopoietic lineages [69]. In this study, we used a flow cytometry-based approach to determine the role of ADAP in iNKT cell development, maturation, homeostatic maintenance as well as subset distribution. We observed that while ADAP is dispensable for the development of mature iNKT cells in the thymus, it is required for the migration and homeostatic maintenance of these cells in the spleen as well as the liver. Our studies indicate that the decreased number of iNKT cells in the periphery could be partly due to the reduced expression of CXCR6 in thymic iNKT cells. We also demonstrate that the decrease in iNKT cells in the spleen and livers of $Adap^{-/-}$ mice is independent of CD11a (α chain of LFA-1) and CD11b ((α chain of Mac-1) expression.

Materials and Methods

Mice

C57BL/6 (B6), and Vα14-Jα18 transgenic mice Tg(Cd4-TcraDN32D3)1Aben (Vα14), were purchased from Jackson Laboratories (Bar Harbor, ME). *Adap*$^{-/-}$ mice on B6 background were kindly provided by Dr. Gary Koretzky (Weill Cornell University, New York, NY). All animal studies were approved by the Campus Animal Resources facilities of Michigan State University (MSU) and used under IACUC standards.

Antibodies and Flow Cytometry

The antibodies used for immunofluorescence staining included CD4, CD8, NK1.1, CD44, CD3, and CD56, CD11a, CD11b (BD Biosciences, San Jose, CA); TCRβ, CD24, CXCR6, and fluorochrome attached isotype matched IgG1 and IgG2b (BioLegend, San Diego, CA); Vα14 and Vβ11 (Beckman Coulter, Brea, CA). Fluorochrome conjugated CD1d-tetramer (CD1d-Tet) loaded with glycolipid antigen (PBS57), or unloaded controls were provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). All flow data were collected on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Isolation of purified populations of invariant NKT (iNKT) cells

Single-cell suspensions were prepared from the liver, spleen and thymus. Hepatic mononuclear cells were first isolated using density centrifugation with Percoll (GE, Piscataway, NJ) and then stained with NK1.1 and TCRβ antibodies and sorted for NK1.1^{+}TCRβ^{+} cells using BD Influx (BD Biosciences) to obtain pure populations of iNKT cells. For spleens and thymi, iNKT cells

were enriched by depletion of $B220^+$ B and $CD8^+$ T cells using anti–B220 and anti-CD8 magnetic beads, respectively, (Miltenyi Biotec; Auburn, CA) per manufacturer's instructions. Subsequently, iNKT cells were flow-sorted from these enriched lymphocyte fractions as described above. Cells obtained using this method were routinely >97% $NK1.1^+$ and $TCR\beta^+$ and were also largely iNKT cells (>92%), when stained using PBS57-loaded CD1d tetramers.

Human iNKT cell expansion

Human blood samples (de-identified) were obtained from Gulf Coast Regional Blood Center (Houston, TX). Human peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque (GE Healthcare) and subsequently cultured in complete AIM-V media (Gibco, Waltham, MA) with 10% fetal bovine serum (FBS), alpha-galactosylceramide, (αGC) (Enzo Lifesciences, Farmingdale, NY) at 500 ng/mL and recombinant human (rh) IL-2 (Peprotech, Rocky Hill, NJ) at 50U/mL. On the fourth day, rhIL-15 (Peprotech) and rhIL-2 were added at 10ng/mL and 10U/mL respectively to the culture. After four more days, human iNKT cells were purified by staining with FITC-conjugated anti-Vα24 antibody, followed by anti-FITC magnetic beads, per manufacturer's instructions (Miltenyi Biotech). Post-sort FACS analysis revealed >95% PBS57-CD1d tetramer reactive cells.

Western Blotting

Cells were lysed in RIPA lysis buffer (Thermo Scientific; Rockland, IL) with complete protease inhibitors (Roche; Basel, Switzerland) as per manufacturer's instructions. Protein concentration was determined by Bradford assay (Biorad; Hercules, CA); 30μg of total protein was resolved by 8% SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare). Blots were probed

with the following antibodies: anti-ADAP and anti-GAPDH-HRP (Cell Signaling Technologies, Danvers, MA). Bound antibodies were detected using the appropriate anti-IgG coupled to HRP (Biorad, Hercules, CA) and ECL reagents (GE Healthcare).

Quantitative real-time PCR

Total RNA was extracted from purified iNKT cells from thymi and livers of *Adap*$^{-/-}$ and B6 mice using TRIzol solution (Invitrogen). RNA (1 μg) was reverse transcribed into cDNA using SuperScript III in a 20 μl reaction volume according to manufacturer's instructions (Applied Biosystems; Foster City, CA). Real-time quantitative PCR was performed using Quant Studio™ 3 system (Applied Biosystems), validated Taqman ADAP and GAPDH primers and Fast Advanced Master Mix according to manufacturers' instructions. Relative gene expression data (fold change) between samples was accomplished using the ΔΔCt method and GAPDH was used as the internal reference control [68] .

Statistics

Student's *t*-test and One-way ANOVA with Tukey's post-hoc test were used as indicated in the figure legends. Significance is shown as *($p<0.05$), **($p<0.01$), or ns for non-significant values. All statistical data was determined using GraphPad Prism (GraphPad, San Diego, CA).

Results

ADAP is dispensable for the thymic development of iNKT cells but is essential for their maintenance in the spleen and liver

Given that SAP and Fyn are both required for proper development of iNKT cells [71], and that ADAP is a known binding partner of Fyn [72], we sought to determine the role of ADAP in iNKT cell development, using *Adap*$^{-/-}$ mice and C57BL/6 (B6) as wild type controls. The burst of iNKT cell development occurs during the first few weeks of post-natal life [73], so we allowed mice to mature until 8 weeks of age. We then investigated the distribution and cell numbers of iNKT cells in the thymus, spleen, and livers of age and sex-matched animals from each genotype by flow cytometry analysis. The percentage iNKT cell populations were identified as TCRβ^+CD1d-tetramer$^+$ (Figure 3A). While the incidence of iNKT cells in the thymus of B6 was comparable to those from *Adap*$^{-/-}$ mice (Table 1), these frequencies were significantly reduced in the peripheral organs (Fig. 3A and B) of *Adap*$^{-/-}$ mice. Consistent with previous studies and as depicted in Figure 3B, the incidence of iNKT cells in the spleen and liver of B6 mice were (0.72%±0.12%) and (16.6%±2.0%) respectively. In contrast, this was two- and over three-fold reduced in spleen (0.35%±0.06%) and liver (5.1%±0.4%), respectively of *Adap*$^{-/-}$ mice. Similarly, the total number of thymic iNKT cells in B6 mice was indistinguishable from those of *Adap*$^{-/-}$ mice (Table 2). However, iNKT cells in spleen and liver of *Adap*$^{-/-}$ mice were consistently and significantly (for livers) reduced as compared to their wild-type counterparts (Figure 3C and Table 2). These results show that ADAP is not required for iNKT development within the thymus, but becomes required as the iNKT cells migrate and maintain themselves in the peripheral organs.

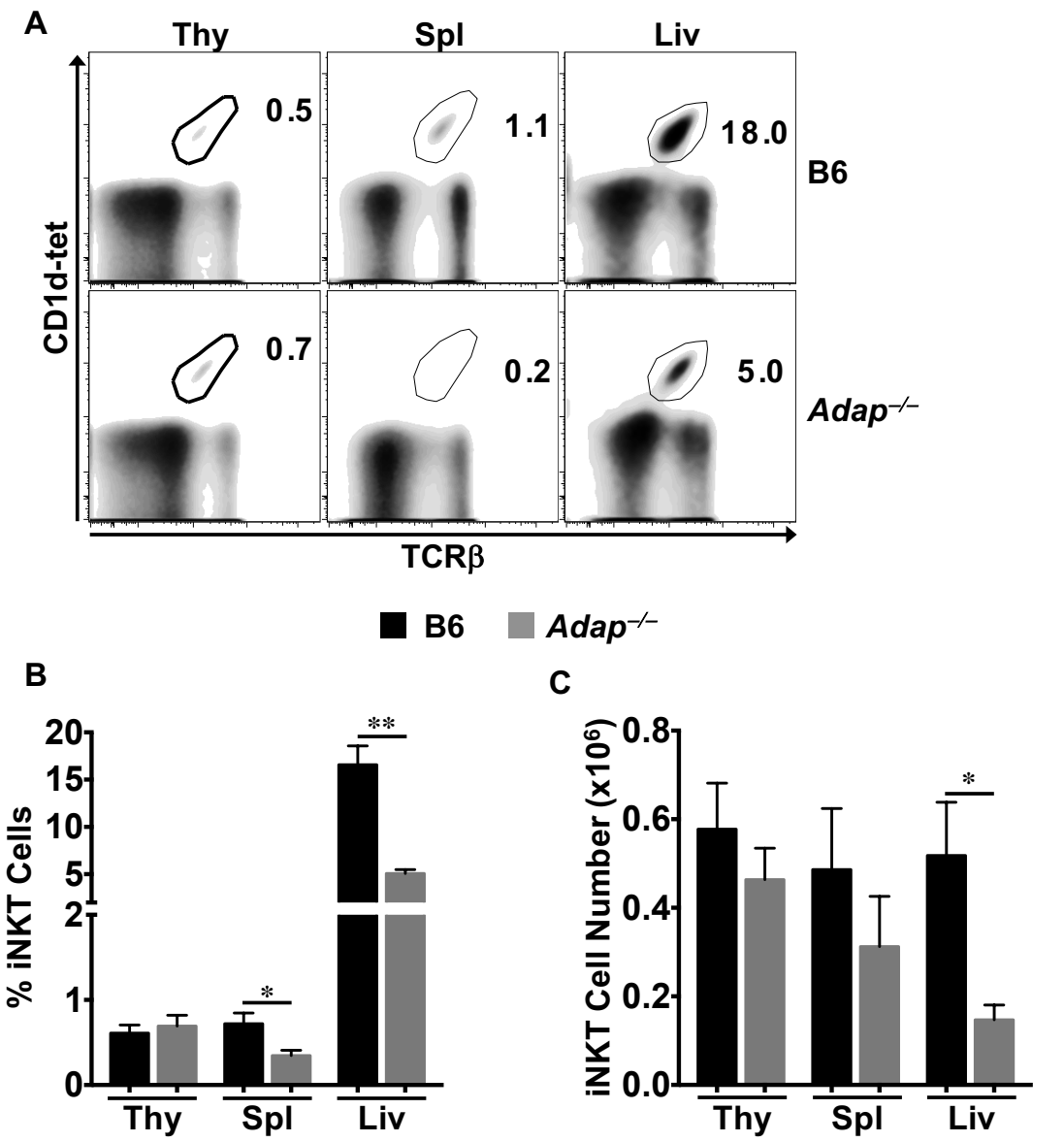


Figure 3. iNKT cell incidence and absolute numbers are reduced in the spleen and liver of *Adap*$^{-/-}$ mice. (A) Populations of iNKT cells were identified by FACS analysis of PBS57-CD1d tetramer^{+} TCRβ^{+} cells in the thymus (Thy), spleen (Spl), and liver (Liv) of B6 and *Adap*$^{-/-}$ mice. Representative density plots are from 5 separate experiments. (B) Average frequency (percent of total live cells) and (C) absolute number of PBS57-CD1d tetramer^{+} TCRβ^{+} iNKT cells from the organs of B6 and *Adap*$^{-/-}$ mice were pooled. Data is presented as the mean of each group ±

Figure 3. (cont'd)

standard error of the mean (SEM) of at least 10 mice per organ, per genotype. Significance was determined by unpaired ***t***-test between B6 and *Adap*$^{-/-}$ mice for each organ. (*p<0.05, **p<0.01).

Table 1. Frequency of iNKT cell subsets in various organs

Frequency	Thy		Spl		Liv	
	B6	***Adap*$^{-/-}$**	**B6**	***Adap*$^{-/-}$**	**B6**	***Adap*$^{-/-}$**
iNKTs	0.61±0.09	0.69±0.13	0.72±0.13	**0.35±0.06***	16.6±2.0	**5.1±0.4***
CD44^{hi}NK1.1^{hi}	79.3±8.28	76.28±6.7	83.9±6.3	68.98±4.12	95.7±1.2	84.4±2.72
CD4^{+}	43.72±5.55	42.45±6.0	75.12±4.05	67.23±4.41	75.1±1.87	72.7±1.41
DN	38.13±2.4	37.0±4.05	20.0±2.71	27.5±2.54	23.0±1.91	25.3±1.42

*p<0.05

Table 2. Absolute cell number of iNKT cell subsets in various organs

Absolute Cell Number (x10^{6})	Thy		Spl		Liv	
	B6	***Adap*$^{-/-}$**	**B6**	***Adap*$^{-/-}$**	**B6**	***Adap*$^{-/-}$**
iNKTs	0.58±0.1	0.46±0.07	0.49±0.14	0.31±0.11	0.5±0.1	**0.15±0.03***
CD44^{hi}NK1.1^{hi}	0.55±0.08	0.42±0.08	0.4±0.16	0.25±0.11	0.59±0.16	**0.18±0.02***
CD4^{+}	0.27±0.04	0.2±0.04	0.32±0.13	0.22±0.09	0.47±0.12	**0.15±0.01***
DN	0.25±0.04	0.18±0.03	0.12±0.06	0.12±0.06	0.13±0.03	0.05±0.00

*p<0.05

ADAP is not required for iNKT cell maturation or subset distribution

To precisely and quantitatively determine the stages of iNKT cell development affected by the absence of ADAP, we analyzed various maturation markers expressed during defined stages of development and migration from the thymus [50] by flow cytometry. Accordingly, $TCR\beta^{+}CD1d\text{-}tetramer^{+}$ iNKT cells from thymus, spleen, and liver of B6 and *Adap*$^{-/-}$ mice were analyzed for their surface expression of CD24, CD44, and NK1.1 (Figure 4). No significant differences in either frequency (Figure 4A-C) or absolute number (Figure 4D) of iNKT cells in stage 0 ($CD24^{hi}CD44^{-}$), stage 1 ($CD24^{lo}CD44^{-}$), stage 2 ($CD24^{-}CD44^{hi}$) or stage 3 ($CD44^{hi}NK1.1^{+}$) were observed in *Adap*$^{-/-}$ mice compared to wild-type controls, in any of the organs. Based on the CD4 expression, two iNKT cell subsets can be distinguished: $CD4^{+}$ and $CD4^{-}CD8^{-}$ (double negative [DN]). We analysed whether lack of ADAP could affect the development and/or tissue distribution of one particular iNKT cell subset. Comparison of the frequency and absolute number of iNKT cells in B6 and *Adap*$^{-/-}$ mice revealed a significant reduction in both $CD4^{+}$ and DN iNKT cells in the livers of *Adap*$^{-/-}$ mice (Figure 5C, E). However, there was no skewing toward one particular subset in the thymus or peripheral organs (Figure 5A-E, Table 2). Taken together, our data suggests that ADAP is not required for iNKT cell maturation or subset development. Importantly, although iNKT cell numbers are diminished in the spleen and livers of *Adap*$^{-/-}$ mice, they are fully mature.

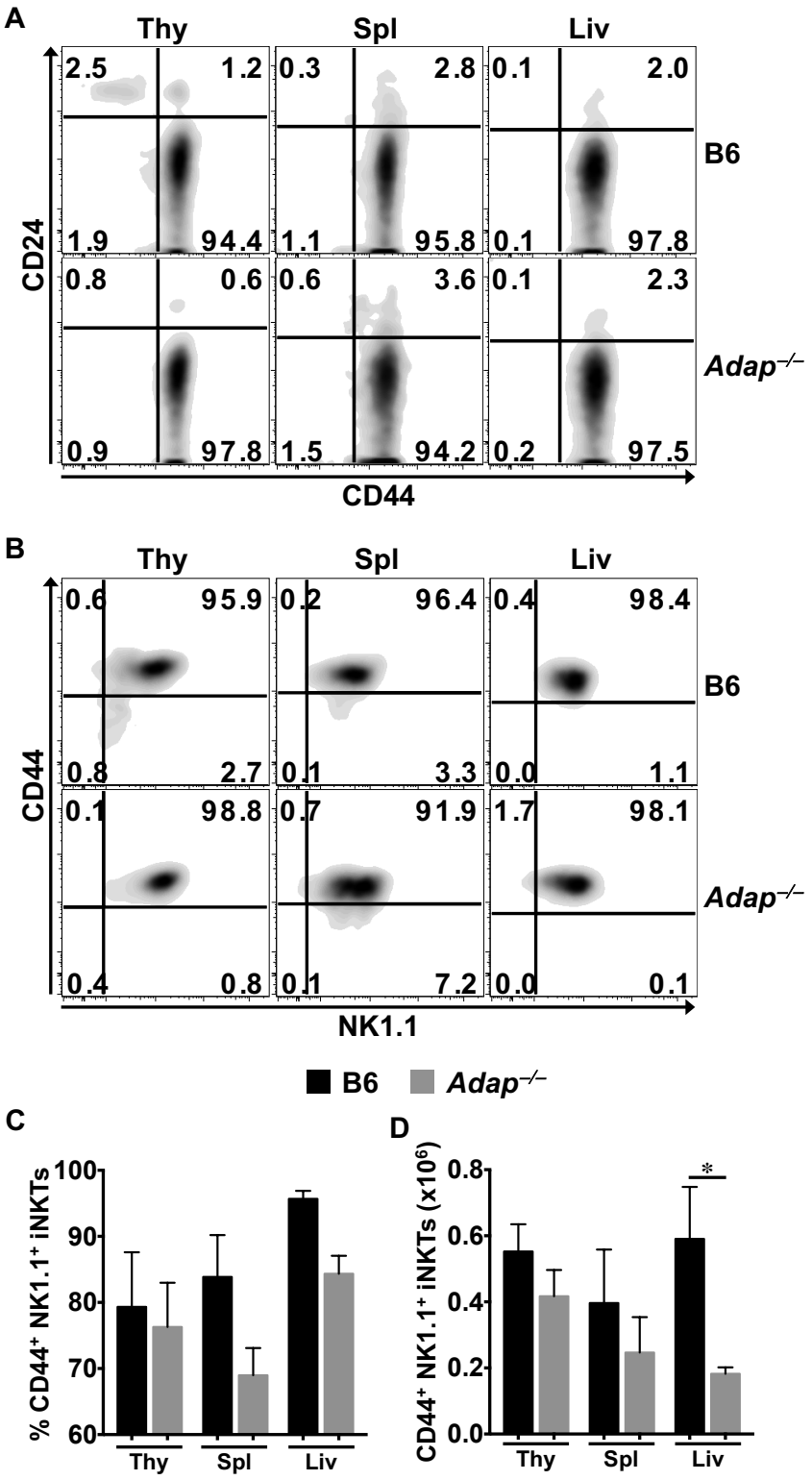


Figure 4. ADAP is neither required for the early nor late stages of iNKT cell maturation.

(A, B) Representative density plots for CD24 and CD44 (A); CD44 and NK1.1 (B) expression in

Figure 4. (cont'd)

iNKT cells within the thymus (Thy), spleen (Spl), and liver (Liv) of B6 and *Adap*$^{-/-}$ mice. (C-D) Pooled data from 3 experiments showing percent (C) and absolute number (D) of CD44^{hi} NK1.1^{+} iNKT cells (n=6 for each condition) in the various organs. Data is presented as mean ± SEM. Statistical analysis was performed by unpaired ***t***-test between B6 and *Adap*$^{-/-}$ mice for each organ (*$p<0.05$).

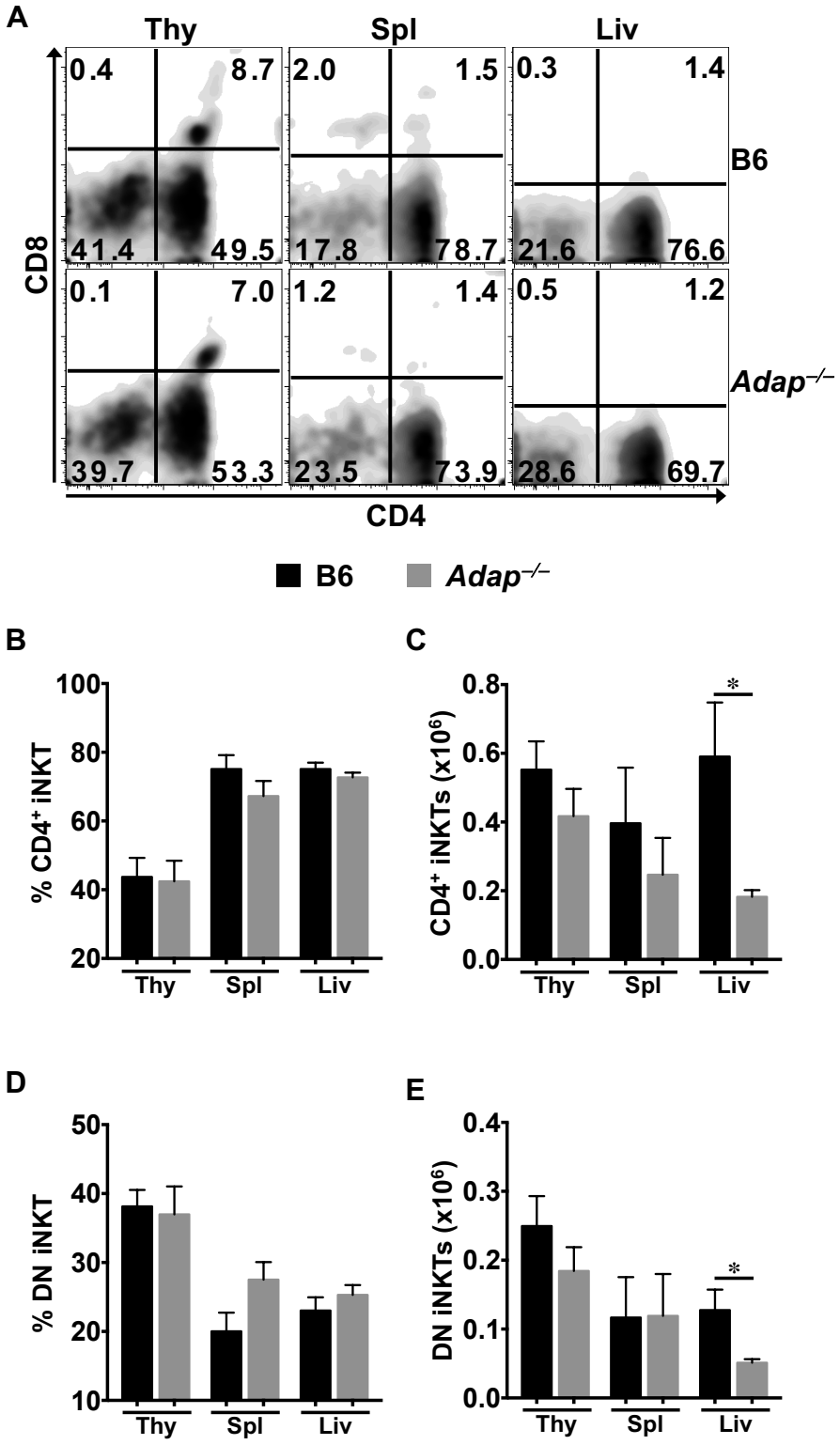


Figure 5. ADAP is not required for subset distribution of iNKT cells. (A) Representative FACS plots of CD4 and CD8 (gated on iNKT cells) in B6 and *Adap*$^{-/-}$ mice within the thymus

Figure 5. (cont'd)

(Thy), spleen (Spl), and liver (Liv). (B-E) Pooled data from 3 experiments show percent (B, D) and absolute cell numbers (C, E) of $CD4^+$ (B, C) and DN (D, E) iNKT cells. Data is shown as mean ± SEM, with n=6 for each condition. Statistical significance was determined by unpaired ***t***-test between B6 and $Adap^{-/-}$ mice for each organ. (*$p<0.05$).

Altered CXCR6 expression on thymic and splenic iNKT cells of *Adap*$^{-/-}$ mice

CXCR6, a chemokine receptor, which is expressed on iNKT cells, controls the selective accumulation of these cells in the liver, in part by transmitting survival signals [51]. To investigate a potential role for CXCR6 in the reduced frequency of iNKT cells in the spleen and liver of *Adap*$^{-/-}$ mice, we examined the expression of this chemokine receptor on these cells in B6 and *Adap*$^{-/-}$ mice. As shown in Figure 6A, the percentage of CXCR6 expressing iNKT cells were significantly decreased in thymus and spleen of *Adap*$^{-/-}$ mice when compared to B6 animals (Table 3). Interestingly, while there was no difference in the incidence of CXCR6^{+} hepatic iNKT cells (Figure 6B), the absolute numbers of CXCR6^{+}iNKT cells were significantly reduced in the livers of *Adap*$^{-/-}$ mice (Figure 6C). Taken together, our data indicate that one of the possibilities for the reduced numbers of iNKT cells in the livers of *Adap*$^{-/-}$ mice could be due to the reduced expression of this chemokine receptor on thymic iNKT cells in these mice. However, the *Adap*$^{-/-}$ iNKT cells that do home to the liver, express CXCR6.

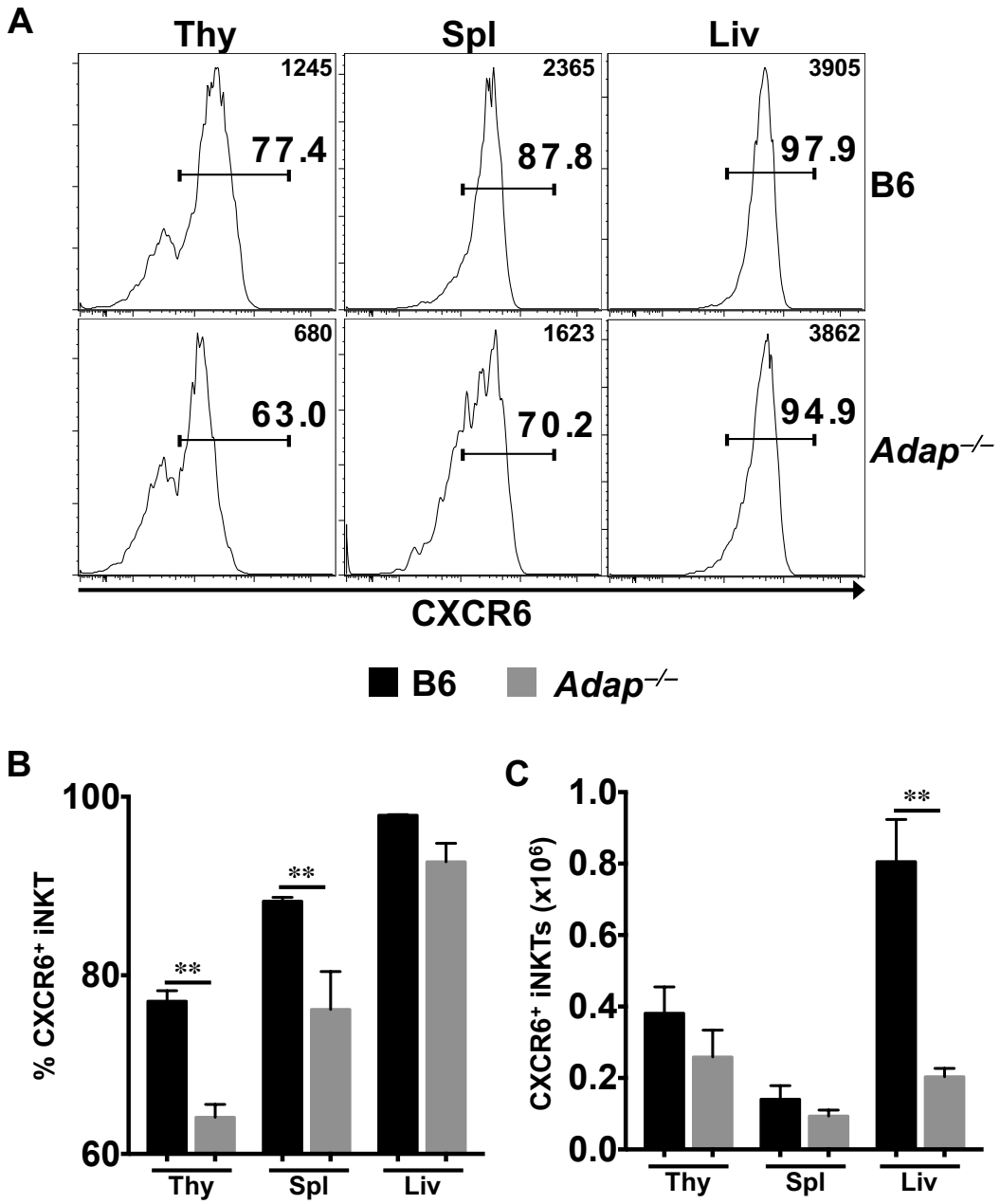


Figure 6. Reduced expression of CXCR6 in iNKT cells of *Adap*$^{-/-}$ mice. (A) Representative histograms depicting CXCR6 expression in iNKT cells from the thymus (Thy), spleen (Spl), and liver (Liv) of B6 and *Adap*$^{-/-}$ mice. Numbers on gate shows percentage greater than isotype controls, per organ. MFI of representative histograms are shown in the upper right corner of each plot. Percentages (B) and absolute numbers (C) of CXCR6^{+} iNKT cells from each organ

Figure 6. (cont'd)

and genotype were pooled from 2 experiments (n= 4-9 mice per genotype). Data is presented by mean ± SEM. Significance was determined by unpaired ***t***-test between B6 and *Adap*$^{-/-}$ mice for each organ (**p<0.01).

Table 3. Frequency and Absolute cell number of CXCR6^{+} iNKT cells

	Thy		Spl		Liv	
	B6	***Adap*$^{-/-}$**	**B6**	***Adap*$^{-/-}$**	**B6**	***Adap*$^{-/-}$**
Frequency	77.1±1.17	**64.1±1.4***	88.3±0.43	**76.2±4.3***	97.9±0.06	92.72±2.06
Absolute Cell Number (x10^{6})	0.38±0.07	0.26±0.08	0.14±0.04	0.09±0.02	0.81±0.12	**0.2±0.02***

*$p<0.05$

Expression of integrin CD11a and CD11b is normal in iNKT cells of *Adap*$^{-/-}$ mice

Previous studies have demonstrated clearly that iNKT cells are retained locally within the liver or spleen through constitutive interactions of integrins such as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) with their natural ligand–intercellular adhesion molecule 1 (ICAM-1) [74, 75]. However, as shown in Figure 7A-C, we observed no difference in either the frequency or the absolute number of CD11a^{+} or CD11b^{+} iNKT cells in the *Adap*$^{-/-}$mice when compared to those in B6 mice, suggesting that decreased iNKT cells in the peripheral organs of *Adap*$^{-/-}$ mice is not likely due to integrin expression.

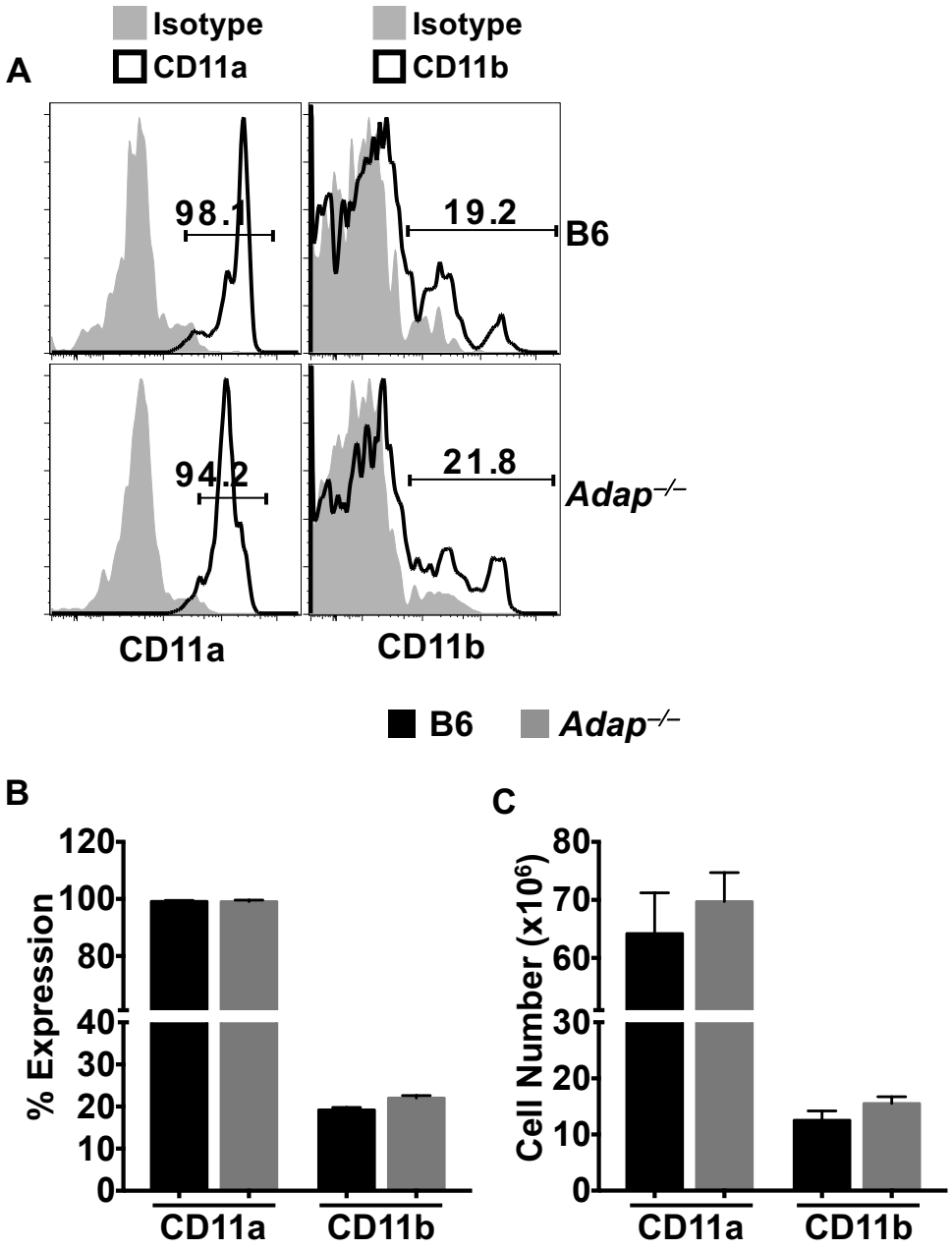


Figure 7. Expression of CD11a and CD11b is not altered in *Adap*$^{-/-}$ iNKT cells. (A) Representative histograms of CD11a (left) and CD11b (right) expression in iNKT cells from the spleens of B6 and *Adap*$^{-/-}$ mice. Numbers within histograms depict percent positive expression for CD11a or CD11b. Positive gate was determined by incidence greater than isotype controls, per organ. Percentage (B) and total numbers (C) of iNKT cells positive for CD11a and CD11b

Figure 7. (cont'd)

surface expression is shown. Data is presented as mean ± SEM from 1 of 2 experiments with n= 3-9 mice tested per genotype.

Discussion

Adapter molecules mediate protein-protein interactions in signal transduction cascades. These signaling cascades translate information from cell surface receptors into cellular responses. It is known that the adaptor ADAP plays a role in integrin-mediated signaling pathways that regulate thymocyte development as well as adhesion and functional responses in T lymphocytes. However, little is known about the role of ADAP in iNKT cell development or functions. In the current study, we examined the role of ADAP in the development of iNKT cells using a genetically engineered mouse lacking this molecule. Here, we provide evidence that although the development or maturation of iNKT cells is not affected by the lack of ADAP, their numbers in the liver and spleens are significantly reduced in *Adap*$^{-/-}$ mice. Thus our findings reveal a unique linage-specific role of ADAP in iNKT cell homeostatic maintenance.

Similar to conventional T cells, development of iNKT cells occurs in the thymus. These cells are absent in nude mice [1, 76], fail to develop in thymectomized mice [77, 78], and are first detected in the thymus in the perinatal period [49, 50, 79, 80]. Both conventional αβ T cells and iNKT cells proceed through the same early triple negative ($CD3^-CD4^-CD8^-$) stage and have almost identical signaling requirements for the development of their respective precursors [81]. Rather, it is during the double positive ($CD4^+CD8^+$) stage that Vα14 to Jα18 rearrangement and invariant TCR expression occurs, which dictate iNKT lineage commitment [2, 58, 82, 83]. iNKT cell development at the DP stage is highly dependent on signals generated by engagement of the SLAM family (SLAMf) of surface receptors, which are expressed on developing iNKT cell precursors as well as cortical DP thymocytes. The SLAM receptors include SLAM, 2B4, CD84, Ly9, Ly108, and CRACC (CD2-like receptor activating cytotoxic cells). With the exception of

2B4, which interacts with CD48 as its ligand, these receptors all function in a homotypic manner and bind to like receptors with high affinity [83]. SLAM receptor signaling is mediated SAP, which binds via its phosphotyrosine-binding pocket to one or more conserved tyrosine-based motifs present within the SLAM receptor cytoplasmic tails [84]. Using a distinct region of its SH2 domain centered at arginine 78, SAP also binds to the Src family tyrosine kinase Fyn, leading to an increase in kinase activity and generation of a SLAM receptor-SAP-Fyn-mediated tyrosine phosphorylation signal [85, 86]. Evidence for the crucial role of SLAM-SAP signaling in iNKT cell development came from studies wherein patients with the immunodeficiency X-linked lymphoproliferative disease (XLP), who harbor inactivating mutations in the gene encoding SAP, and $Sap^{-/-}$ mice, lack iNKT cells [71, 87, 88]. Interestingly, similar results are seen in mice lacking Fyn or expressing a mutant version of SAP that cannot bind to Fyn (Sap^{R78A}) [55, 89]. It is currently unclear as to how SLAMf receptor-SAP signals coordinate with those emanating from the TCR to foster proper iNKT cell development.

ADAP binds to both SH2-containing leukocyte phosphoprotein of 76 kDa (SLP-76) and the Src-family kinase Fyn [42, 90]. As both these molecules control iNKT cell development, we postulated that ADAP is a likely downstream candidate of the SAP-Fyn axis and/or is required for TCR –induced signals during development. ADAP is clearly required for efficient maturation of thymocytes undergoing TCR-dependent positive and negative selection [90]. ADAP protein and mRNA are upregulated during the DP-to-SP transition, a phase of thymocyte maturation driven by signals emanating from the TCR [91]. In addition, ADAP is specifically increased in $TCR\beta^{high}$ and $CD69^{high}$ DP subsets, suggesting that the TCR signals known to enhance expression of these surface proteins may also be driving augmentation of ADAP protein levels.

These observations are intriguing because they suggest that protein levels of ADAP and of its binding partner SLP-76 are regulated largely in parallel. SLP-76 is a hematopoietic adaptor protein that is absolutely required for pre-TCR and TCR-dependent signaling leading to thymocyte maturation [92]. The lack of a requirement for ADAP in the development of iNKT cells despite the fact that they clearly express ADAP protein suggests the presence of an ADAP-independent signaling cascade. These observations are similar to those seen in other non-conventional T lymphocytes, such as the CD8αα T cells, the most abundant among intestinal intraepithelial lymphocytes (IEL). The marked reduction in contribution by ADAP-deficient cells to the IEL CD8αα T cell compartment in mixed chimeras suggests a defect either in homeostasis or in migration/homing of these cells. Indeed, our results indicate that the reduced iNKT cell numbers in the peripheral organs of *Adap*$^{-/-}$ mice may be in part due defective migration/homing of these cells to the liver and spleen.

Following positive selection, NKT cells progress down a pathway of maturation that is marked by the sequential acquisition of specific patterns of surface marker expression: CD24, CD44 and NK1.1. The most immature iNKT cells are defined as CD24^{hi}CD44^{lo}NK1.1^{-} (Stage 0), followed by CD24^{lo}CD44^{lo}NK1.1^{-} (Stage 1), CD24^{lo}CD44^{hi}NK1.1^{-} (Stage 2), and finally CD24^{lo}CD44^{hi}NK1.1^{+} (Stage 3) [93]. In the absence of ADAP, we neither observed a defect in transition of the immature iNKT cells to the mature stages nor did we observe preferential skewing to a particular subset development. The most significant changes observed in our study were the alterations in peripheral iNKT compartment in *Adap*$^{-/-}$ mice. ADAP deficiency specifically impacted cells within the liver and spleen but not the thymus. *Adap*$^{-/-}$ iNKT cells exhibit a peripheral homeostatic defect most likely due to altered tissue-specific migration and/or

retention. The migration and retention of iNKT cells within peripheral tissues is mediated by a complex interplay between integrins (LFA-1 [94] and Mac-1 [95] and chemokines (CXCL16 [96]). We observed no reduction in the levels of CD11a or CD11b integrin expression on *Adap*$^{-/-}$ *i*NKT cells derived from all tissues. However, we did observe significant reduction in the frequency of CXCR6^{+} cells in the thymus of *Adap*$^{-/-}$ mice. Although CXCR6 is not required for thymic iNKT cell development, it plays a vital role in the accumulation of iNKT cells in the liver via interaction with its ligand, CXCL16. The redistribution of iNKT cells to bone marrow of *Cxcr6*$^{-/-}$ mice suggests a role for CXCR6 in iNKT cell homing or retention in the liver and lungs. Thus the reduced frequency of CXCR6 on thymic iNKT cells of *Adap*$^{-/-}$ mice explains in part, the reduced number of iNKT cells in the spleens and livers. Furthermore, the reduced frequency of CXCR6 on splenic iNKT cells of *Adap*$^{-/-}$ mice suggests that although thymic iNKT cells migrate to the spleen, reduced CXCR6 expression fail to retain them in the spleen.

Interestingly, we observed that the incidence of CXCR6 on liver iNKT cells were indistinguishable between B6 and *Adap*$^{-/-}$ mice. Of note, the reduced numbers of iNKT cells in the liver and/or spleen did not coincide with increased accumulation of these cells in the thymus or peripheral lymph nodes. Taken together, we believe that thymic iNKT cells that express CXCR6 home to the spleen and liver. However either due to lack of CXCL16 expression in the respective tissues or due to their inability to maintain surface expression of CXCR6 in the absence of ADAP, these cells fail to either proliferate or survive rather than migrating to the lymph nodes. Our studies thus favor a role for ADAP in proliferation and/or survival of iNKT cells, in part via CXCR6 expression. Consistent with this notion, Geissmann et al. [51] reported that *Cxcr6*$^{-/-}$ iNKT cells exhibited enhanced apoptosis in culture. However, other studies [96]

exhibited no differences in the apoptosis (annexin V staining) of cultured *Cxcr6*$^{+/+}$ and *Cxcr6*$^{-/-}$ iNKT cells [96]. In addition, anti-CXCL16 treatment does decrease survival of resident liver NKT cells, as cell numbers remained the same as in isotype-treated mice [96]. In addition to chemokines and integrins, peripheral iNKT homeostasis is dependent on a steady provision of co-stimulatory signals. While thymic iNKT cell development depends on CD28-B7 interactions [97], peripheral survival relies on co-stimulatory signals delivered through ICOS/ICOS-L interaction [98]. Whether ADAP is involved in CD28-mediated signaling or regulates ICOS signaling has not yet been established.

Although T cells and NK cells share a common lymphoid progenitor in the bone marrow, their molecular requirements for development are distinct. While T cell maturation requires pre-TCR- and TCR-mediated signal transduction in addition to signals mediated by select cytokines [e.g. IL-7 [99]], NK cells primarily rely on cytokine or growth factor signaling components for their development [100]. Normal NK development and distribution in *Adap*$^{-/-}$ mice suggest that ADAP does not regulate signaling by cytokines that utilize the common gamma chain [IL-2 and IL-15 [101]]. Given that ADAP signals downstream of ITAM-associated molecules [e.g. TCR [102] and the FcεRI [103]] rather than of cytokine receptors, it is not surprising that ADAP is indispensable for NK cell development.

Taken together, our findings define a pivotal role for ADAP in peripheral *i*NKT homeostasis: ADAP is important for *i*NKT migration to the peripheral tissues and is involved in promoting cellular retention within the tissues, probably in part by regulating chemokine receptor CXCR6 expression. While ADAP is required for the homeostasis of iNKT cells, the mechanism(s)

involved are yet to be defined. Studies are currently underway to identify whether the decreased peripheral iNKT cell numbers in *Adap*$^{-/-}$ mice are likely due a marked reduction in homeostatic proliferation or higher susceptible to apoptosis or both.

CHAPTER 2

ADAP REGULATES INVARIANT NATURAL KILLER T CELL ACTIVATION AND IMMUNOMODULATORY FUNCTIONS

Introduction

Invariant natural killer T (iNKT) cells are innate-like lymphocytes as they exhibit characteristics of both innate and adaptive immune cells. However, unlike conventional T cells, iNKT cells bear a semi-invariant TCR that recognizes different lipid antigens presented by monomorphic CD1d molecules. Soon after the TCR-antigen-CD1d engagement, iNKT cells rapidly secrete copious amounts of cytokines, mainly IFN-γ and IL-4, a characteristic reminiscent of innate immune responses [104-107]. Besides the CD1d-dependent mode, iNKT cells are also activated by cytokines, which is common when the lipid antigen is weakly immunogenic [108]. In a physiological context, iNKT cells become activated by microbial or self-lipid antigens bound to CD1d molecules. For example, isoglobotriosylceramide (iGB3), a neutral glycosphingolipid, has been identified as a weak self-lipid antigen for human and murine iNKT cells [21, 109, 110], although its role as the only positive-selecting self-lipid in the thymus remains controversial [21, 109, 110]. Lysophospholipids and glycosphingolipids have been shown to be self-lipid antigens in different contexts [21, 111, 112]. Self-lipid antigens are weakly immunogenic and iNKT cell activation in this case is often largely driven by IL-12 and IL-18. Although CD1d-activated iNKT cells can undergo further activation *via* cytokines secreted from matured DCs, cytokines IL-12 and IL-18 are alone sufficient to activate iNKT cells [113, 114].

Unlike peptide-MHC restricted conventional T cells, which emerge from the thymus "naïve" iNKT cells leave the thymus fully matured and able to perform their effector functions without priming [115-118]. Depending on how the iNKT cells are activated, this can include both regulatory cytokines (e.g., IL-4, IL-10) [115-118] and/or pro-inflammatory cytokines (e.g., IL-2, IL-17, TNF-α, and/or IFN-γ) [115-118]. Since iNKT cells respond rapidly and without the need

for priming, they are some of the first cells within an immune response to be activated and therefore act as a "bridge" between the innate and adaptive immune systems. As shown in Figure 8, iNKT cell activation via TCR engagement or IL-12 or both causes iNKT cells to upregulate IL-12 receptor and CD40L, while also inducing maturation and production of IL-12 in dendritic cells (DCs). This IL-12 release then in turn greatly increases IFN-γ production by iNKT cells, leading to a positive feedback loop for Th1 immunity [115-118]. Additionally, this maturation of DCs leads to trans-activation of NK cells and increased MHC class I and II antigen presentation to T and B cells, allowing for both innate and adaptive immune responses to be established [115-118].

Since iNKT cells are uniquely placed at the interface between innate and adaptive immunity, they have a tremendous influence in shaping immune responses. Although the frequency of iNKT cells in humans ranges from 0.01 to 0.1% in peripheral blood (lower than in mice), this frequency is still orders of magnitude higher than that of naïve peptide-specific T cells [119, 120]. Cytokine stimulation and cognate interaction between iNKT cells and dendritic cells (DCs), B cells, neutrophils, and macrophages often polarizes these cells toward a pro-inflammatory phenotype [121-123]. In general, $CD4^+$ iNKT cells are able to express more Th2-related cytokines like IL-4, although they can express as much Th1 cytokines at the same time [115-119]. Human DN iNKT cells are biased toward a Th1-related phenotype, and preferentially make IFN-γ. However, $CD4^+$ iNKT cells can make IFN-γ and DN iNKT cells can make IL-4, at least partially depending on the stimuli given [115-119]. Mouse iNKT cells are less clearly biased. Relatively Th1-like iNKT cells tend to be enriched within the spleen and liver, while Th2-like iNKT cells are associated with the lungs and intestine [115-119]. There are also Th17-

like iNKT cells that express cytokines like IL-17 and are enriched within the lungs, intestine, lymph nodes, and skin [124]. A recently described subset, adipose iNKT cells tend to make anti-inflammatory cytokines like IL-10 and unlike other mature iNKT cells, lack PLZF [125, 126]. Because iNKT cells can rapidly produce IFN-γ, IL-4, or both, they have been found to play a role in various diseases by establishing a Th1- or Th2-based immune response. However, the mechanisms that control iNKT cell functions are poorly understood.

In light of the shared functional properties of T, NK and iNKT cells, and the requirement for ADAP in efficient but specific functions of T [43, 127] and NK cells [70, 128], we hypothesized that ADAP would also play a role in iNKT cell activation and immunomodulatory functions. ADAP is a positive regulator of T cell activation and is required for both T cell proliferation and IL-2 production. The role of ADAP in NK cell cytokine production seems controversial. While earlier studies showed that ADAP is dispensable for NK cell cytokine secretion [70], more recent studies demonstrate that signaling by Fyn-ADAP via the Carma1-Bcl10-MAP3K7 signalosome exclusively regulates inflammatory cytokine production in NK cells [128]. In this study, using B6 and *Adap*$^{-/-}$ mice as well as purified iNKT cells from these animals, we demonstrate that ADAP is required for both iNKT cell cytokine production as well as its release. We also demonstrate that in the absence of ADAP, murine iNKT cells fail to modulate activation of T, B and NK cells as to promote maturation of dendritic cells (DC). Importantly, this observation is not limited to murine iNKT cells, as gene silencing of *ADAP* in human iNKT cells also results in significant reduction in cytokine levels in culture supernatants.

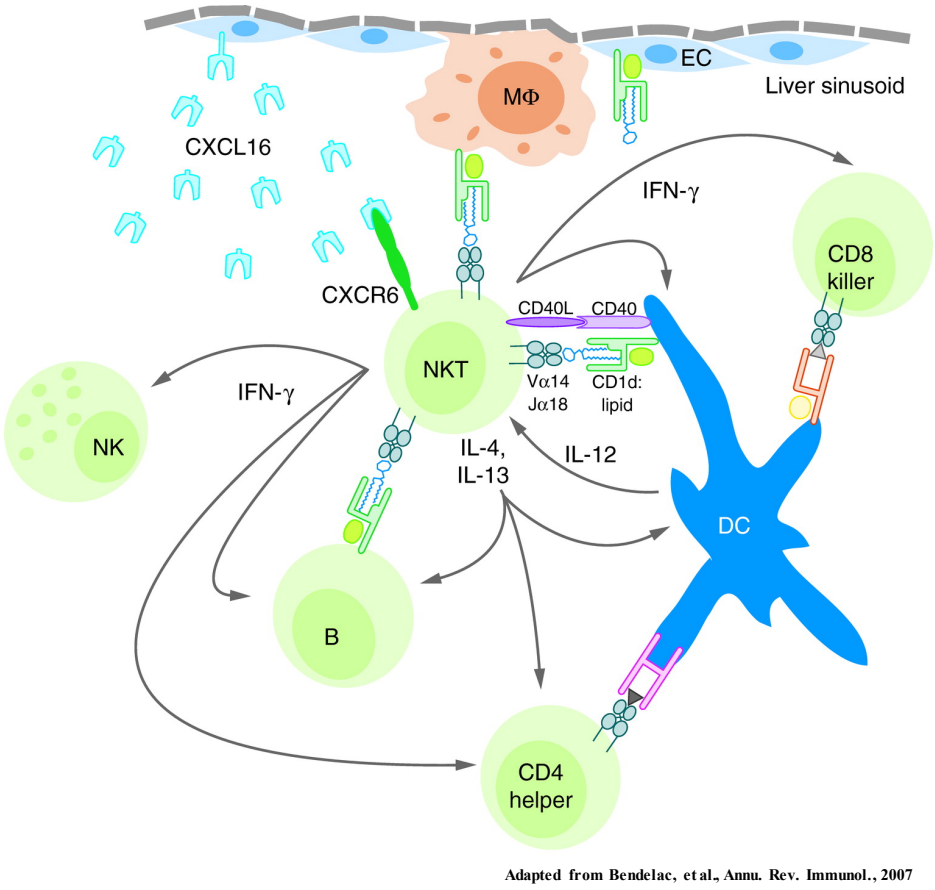


Figure 8. Cellular interactions between iNKT cells and other immune cells

Invariant NKT cells are activated by TCR engagement with a glycolipid presented by CD1d on dendritic cells (DC), B cells, or macrophages (MΦ). Additional co-stimulation between CD40L on iNKT cells and DCs or DC release of IL-12 amplifies the activation of iNKT cells. iNKT cell release of IFN-γ recruits and activates NK cells and CD8 killer T-cells. Release of IL-4 and IL-13 helps recruit CD4 helper T-cells. Migration of iNKT cells to the liver is by interaction between CXCR6 on iNKT cells and CXCL16 on resident liver sinusoids.

Materials and Methods

Mice

Mice were used as described in Chapter 1.

Reagents

EL4 T lymphoblastic cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Recombinant human (rh) IL-2 was from Peprotech (Rocky Hill, NJ) and rhIL-15 from Sigma Aldrich (St. Louis, MO). KRN7000 (αGC) and its analog, OCH were purchased from Enzo Life Sciences (Farmingdale, NY). Antibody list includes anti: NK1.1, CD69, CD3, CD56, IL-4, IFN-γ, CD11b, CD11c, and B220 (BD Biosciences, San Jose, CA); TCRβ, (BioLegend, San Diego, CA); Vα14 and Vβ11 (Beckman Coulter, Brea, CA). CD1d-tetramer loaded with glycolipid antigen PBS57, was provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Murine and human iNKT cells were obtained as in Chapter 1.

Flow cytometry and Western Blotting

Immunofluorescence staining, cell sorting and flow cytometry as well as SDS-PAGE and western blotting were performed as described in the "Materials and Methods" of Chapter 1.

Transient gene silencing in human iNKT cells

Human iNKT cells were subjected to a transient gene knockdown as per protocol provided in the Amaxa Human T-cell Nucleofector Kit (Lonza, Basel, Switzerland). Briefly, $2x10^6$ human iNKT cells were resuspended in 100μL nucleofector solution, and transfected with 300 pmol of control (Invitrogen) or *ADAP*-specific (SMARTpool: ON-TARGETplus FYB siRNA; Dharmacon,

Lafayette, CO) siRNA oligonucleotides by using Amaxa nucleofection protocol O-17. Cells were immediately transferred to complete RPMI media supplemented with 10% FBS in the presence of 50U/mL rhIL-2, for 48 hours at 37ºC before use. Knockdown efficiency was verified by western blotting using human specific antibodies against ADAP and GAPDH (loading control).

***In vivo* cytokine production**

For *in vivo* stimulation, mice were injected i.p. with 4 μg of PBS57 in 300 μl of sterile PBS. After 2-4 h, serum was collected from mice by retro-orbital bleeds and analyzed for cytokines using specific sandwich enzyme-linked immunosorbent assay (ELISA; BD OptEIA™). All samples were run in duplicate. Splenocytes and liver lymphocytes were isolated and stained to detect cell surface markers or intracellular cytokines, as indicated in the respective section.

***In vitro* cytokine assay**

Purified iNKT cells ($80x10^3$ cells in 100 μL) were co-cultured with EL4 cells ($80x10^3$ cells in 100 μL) that were either left untreated or loaded with PBS44 (100ng/mL), in a total volume of 200 μL. After 24 hours, culture supernatant was harvested and analyzed for cytokine release by ELISA (BD OptEIA™). Optical density was measured at 450 nm on a FlexStation3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA) and converted into sample concentration through SoftMax Pro Software (Molecular Devices).

Statistics

Statistical analysis was performed as described in the "Materials and Methods" section of Chapter 1.

Results

ADAP is required for TCR-induced iNKT cell cytokine production

A primary role of iNKT cells is to modulate the activation and recruitment of other immune cells, which is accomplished by release of both Th1 and Th2 cytokines [129]. Injection of αGC into B6 mice leads to iNKT cell activation [130], resulting in the rapid production of both Th1- and Th2-type cytokines. To investigate whether deletion of *Adap* influences iNKT cell cytokine responses, B6 and *Adap*$^{-/-}$ mice were injected i.p. with PBS44, an analogue of αGC. After 2 hours, serum was collected and analyzed for the presence of cytokines. Both IFN-γ and IL-4 were markedly reduced in the serum from *Adap*$^{-/-}$ mice, whereas robust levels of both the cytokines were found in B6 mice (Figure 9A-C). To investigate whether the reduced serum cytokine levels were due to the lower number of cytokine producing iNKT cells in *Adap*$^{-/-}$ mice, defect(s) in their ability to produce cytokine, or a combination of both factors, splenocytes and liver lymphocytes were harvested and evaluated for iNKT cell cytokine production by intracellular staining. Using this approach, we observed that both B6 and *Adap*$^{-/-}$ mice produced detectable levels of IFN-γ and IL-4 directly *ex vivo* (Figure 9D-G). However, the frequency of IFN-γ or IL-4-producing iNKT cells in the spleen and liver of *Adap*$^{-/-}$ mice were significantly reduced as compared to those from B6 controls (Figure 9H, I). These data indicate that in the absence of ADAP, the reduced cytokine levels in the serum could be either due to lower number of IFN-γ or IL-4-producing iNKT cells in *Adap*$^{-/-}$ mice or their impaired ability to release cytokines following challenge with glycolipid antigen, or both.

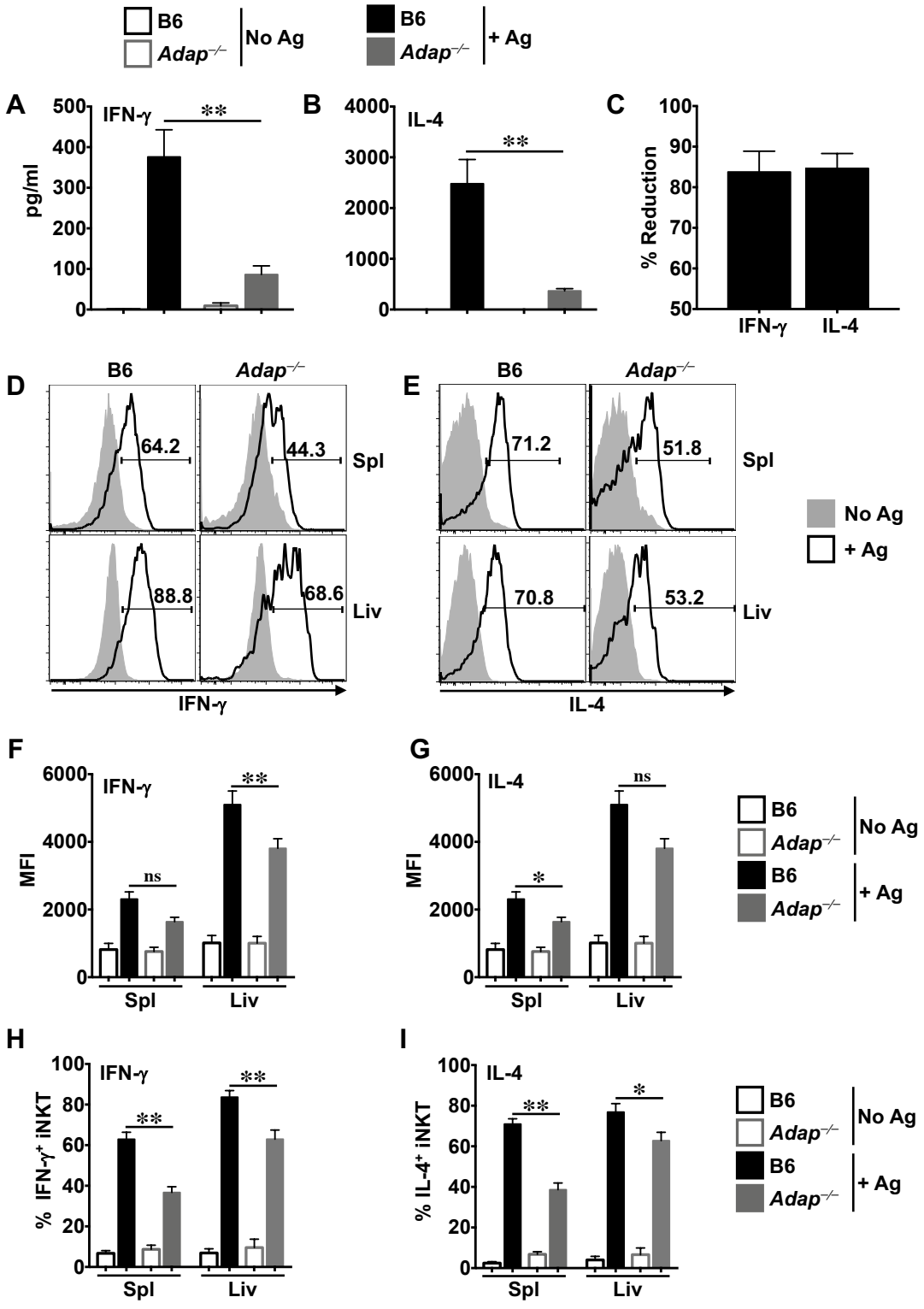


Figure 9. ADAP is required for adequate cytokine production and release in iNKT cells.

Age and sex matched B6 and *Adap*$^{-/-}$ mice were injected intraperitoneally (i.p) with 4μg of

Figure 9. (cont'd)

PBS44 or left untreated. After 2 hours, serum was collected and IFN-γ (A) and IL-4 (B) levels were measured by ELISA. Data represent the mean ± SEM from 9 independent experiments. (C) Mean percent reduction ± SEM in the serum levels of IFN-γ and IL-4 in PBS44–injected *Adap*$^{-/-}$ mice as compared to similarly treated B6 mice. Data are averaged from 9 independent experiments. (D-G) After 2 hours, the percentages of splenic and hepatic iNKT cells producing IFN-γ (D) and IL-4 (E) directly *ex vivo* were analyzed by intracellular cytokine staining and flow cytometry. Representative histograms are shown in D, E as indicated. Bar graphs show MFI (F, G) and % positive iNKT cells (H, I) for IFN-γ (F, H) and IL-4 (G, I) in the spleen and liver. Data in F-I represent the mean ± SEM and are pooled from 4 independent experiments. Significance was determined by unpaired *t*-test. *$p<0.05$,**$p<0.01$. MFI: mean fluorescence intensity

ADAP regulates iNKT cell cytokine release

In order to determine if the cytokine release defect in *Adap*$^{-/-}$ mice was due to an intrinsic iNKT cell defect, or because of a decreased numbers of these cells in the peripheral organs, equal numbers of purified B6 and *Adap*$^{-/-}$ iNKT cells were cultured with antigen-pulsed or unpulsed EL4 cells. After 18 hours, iNKT cell cytokine production was assessed. B6 iNKT cells secreted detectable levels of IFN-γ and IL-4 when cultured with lipid-antigen loaded but not unloaded EL4 cells. Compared to B6, *Adap*$^{-/-}$ iNKT cells produced significantly less cytokines when cultured with antigen-loaded cells establishing that ADAP regulates both Th1 and Th2 cytokine release by iNKT cells (Figure 10). These findings also help explain the reduced *in vivo* cytokine responses of PBS44-injected *Adap*$^{-/-}$ mice.

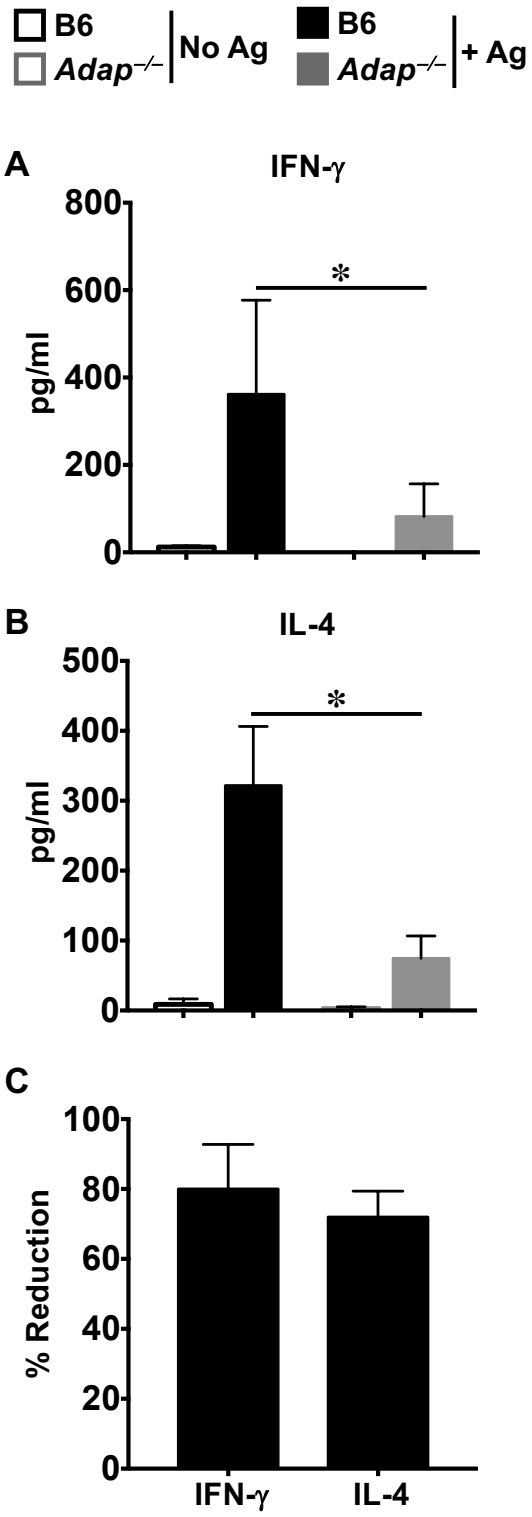


Figure 10. ADAP-deficient iNKT cells produce less cytokines in response to antigen-pulsed EL4 cells *in vitro*. iNKT cells from B6 or *Adap*$^{-/-}$ mice were co-cultured with either PBS44-

Figure 10. (cont'd)

loaded or unloaded EL4 cells. iNKT and target cells were mixed at a 1:1 ratio in a total volume of 200μL and incubated overnight at 37°C. After 24 hours, culture supernatants were harvested and analyzed for IFN-γ (A) and IL-4 (B) levels by ELISA. (C) Percent reduction in cytokine (IFN-γ and IL-4) release by *Adap*$^{-/-}$ iNKT cells as compared to those from B6 mice. Data in A-C represent the mean ± SEM from 3-6 experiments. Statistical significance was determined by unpaired two-tail *t*-test. * $p < 0.05$.

ADAP is required for human iNKT cell cytokine release

To examine whether ADAP is also required for human iNKT cell cytokine production, we expanded and purified iNKT cells from the blood of normal donors [68] and examined their ability to produce Th1 and Th2 cytokines. Gene expression of *ADAP* in human iNKT cells was significantly lower than in conventional T cells; however both the lymphocyte lineages expressed comparable protein levels of ADAP (Figure 11A, B). Interestingly, both primary T and iNKT cells express varying levels of both the isoform ADAP (Figure 11B). Human iNKT cells exhibit robust cytokine responses against PBS44-loaded but not unloaded EL4 cells [68]. Expanded iNKT cells from 1 donor was independently transfected with *ADAP*-specific siRNA or left untransfected (WT) and used for *in vitro* cytokine assay. *ADAP*-silenced cells expressed almost no ADAP protein (Figure 11C) and similar to murine iNKT cells, exhibited a significant reduction in cytokine release (Figure 11D, E). Together, these studies indicate that ADAP is a critical mediator of human iNKT cell cytokine response.

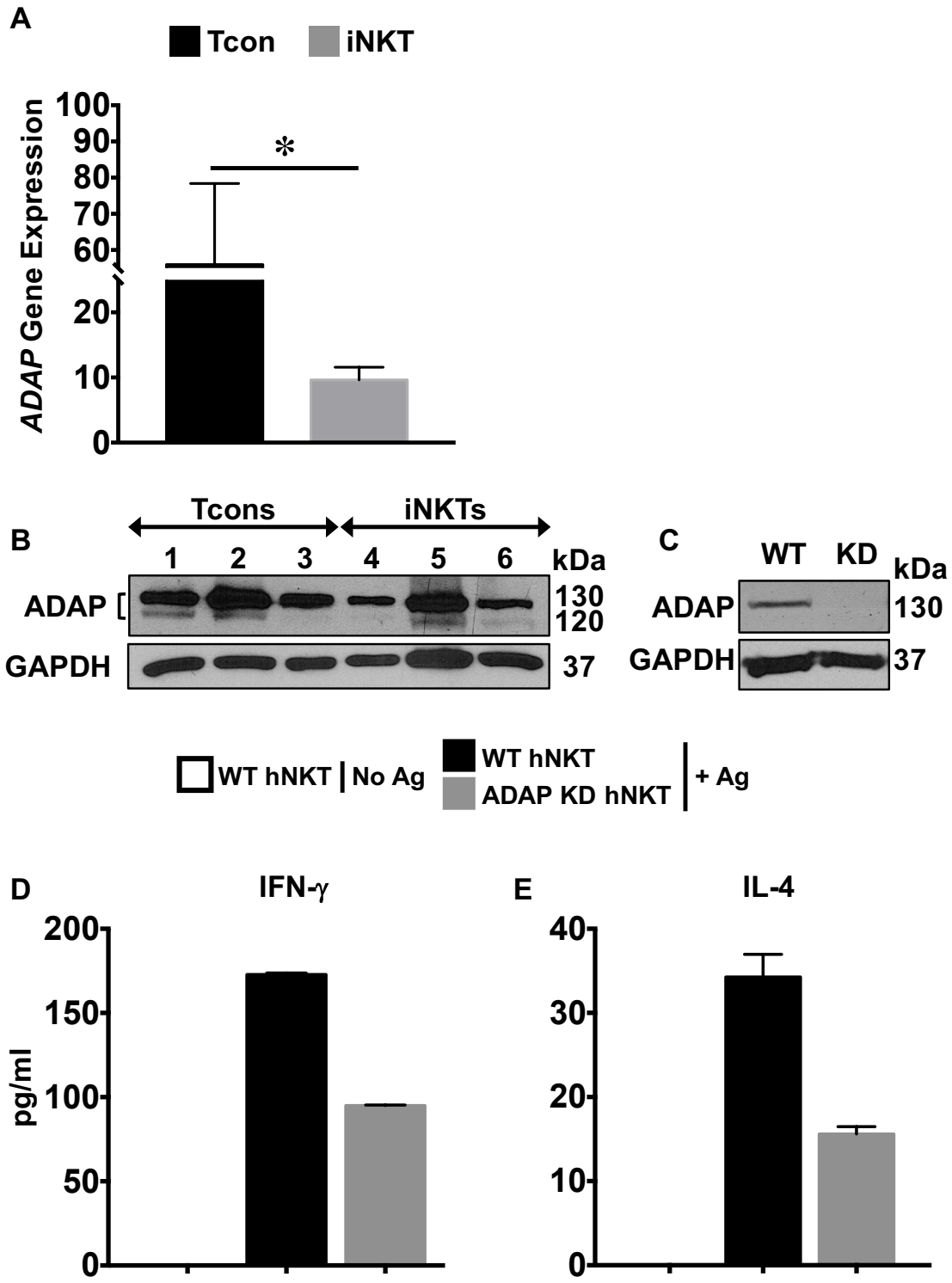


Figure 11. Silencing *ADAP* gene expression in human iNKT cells results in reduced cytokine production. (A) Gene expression of *ADAP* in human conventional T-cells (Tcons) and

Figure 11. (cont'd)

human iNKT cells expanded from PBMCs from human donors. (B) Representative western blotting of ADAP (top) and GAPDH (bottom) in human T (lanes 1-3) and iNKT (lanes 4-6) cells from three separate human PBMC samples. (C) Human iNKT cells were transfected with *ADAP*-specific siRNA (KD) or left untransfected (WT). After 48 hours, knockdown of ADAP expression was assessed by immunoblotting. (D, E) Untransfected or *ADAP*-silenced human iNKT cells were co-cultured with either PBS44-loaded or unloaded EL4 target cells. iNKT and target cells were mixed in a 1:1 ratio in a total volume of 200μL and incubated overnight at 37°C. After 24 hours, culture supernatants were harvested and analyzed for IFN-γ (D) and IL-4 (E) levels by ELISA. Data is from 1 donor and is represented as mean ± SD.

ADAP is required for TCR-induced iNKT cell-mediated bystander cell activation.

The secretion of cytokines by iNKT cells contributes to the *in vivo* activation of other immune cell lineages [131]. Therefore, we next investigated whether *Adap*$^{-/-}$ mice showed abnormalities in iNKT cell cytokine–dependent events. Animals were injected with PBS44 and 4 hours later, lymphocytes harvested and examined for up-regulation of the CD69 activation marker on splenic dendritic cells (DC), T, B, and NK cells and production of IFN-γ by splenic and liver NK cells. In B6 mice, PBS44 induced robust up-regulation of CD69 on T, B, NK and DCs (Figure 12A), stimulated the production of IFN-γ by ~20-30% of splenic and liver NK cells (Figure 12C-E), as well as exhibited robust serum cytokine responses. However, most of these antigen-induced responses were markedly reduced in *Adap*$^{-/-}$ mice, indicating that ADAP is required iNKT cell-mediated immunomodulatory functions.

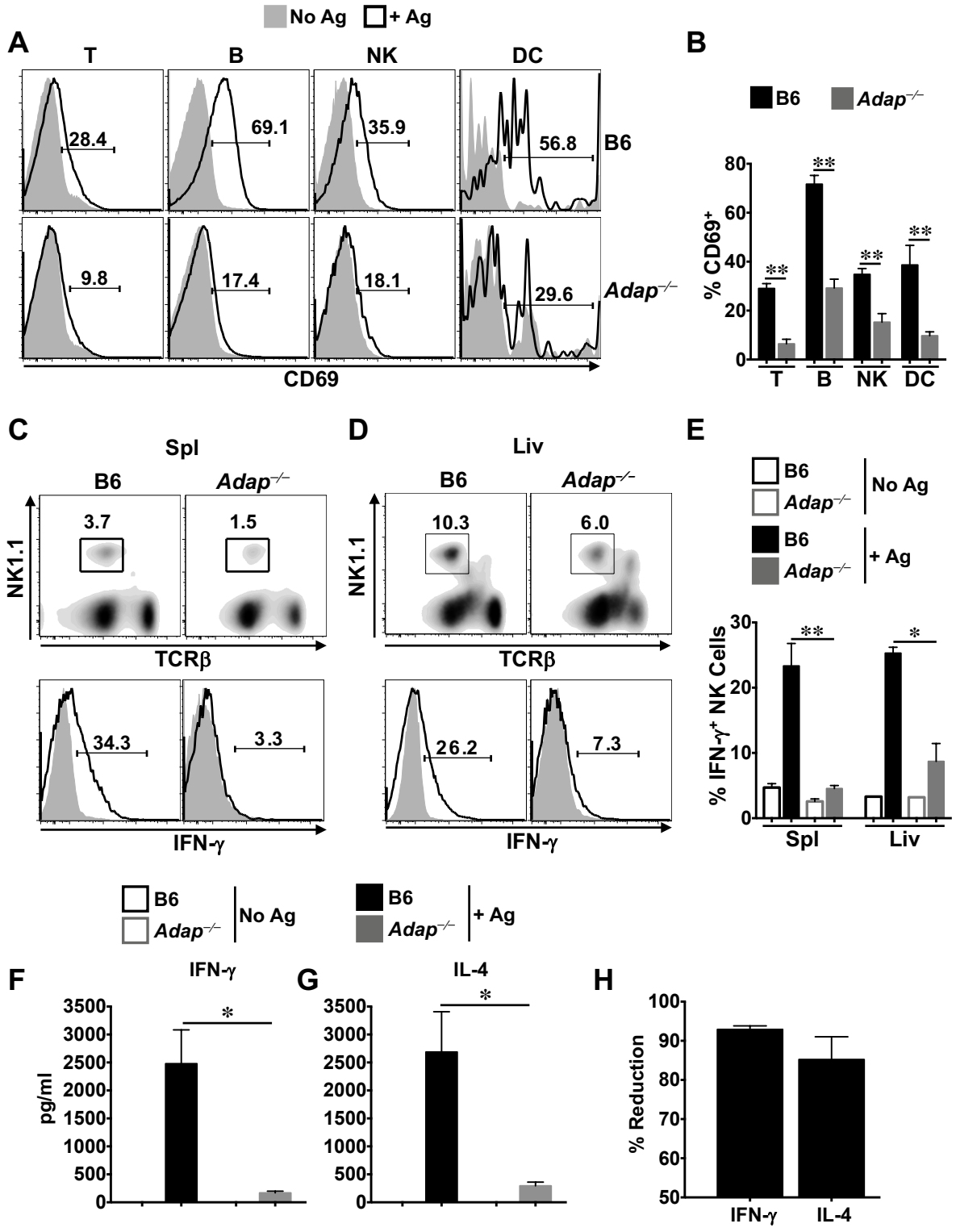


Figure 12. ADAP is required for iNKT-cell mediated transactivation of immune cells. Age and sex-matched B6 and *Adap*$^{-/-}$ mice were injected i.p with 4μg of PBS44 (Ag) or left untreated

Figure 12. (cont'd)

(No Ag). After 4 hours, splenocytes were analyzed for the percentage of $TCR\beta^+$ (T), $B220^+$ (B), $NK1.1^+$ (NK), and $CD11c^+$ (DC) cells expressing CD69. Representative histograms are shown in (A). (B) Bar graph showing % positive T, B, and NK cells and DC expressing CD69 following activation, is pooled from 2 independent experiments. Splenocytes (C) and intrahepatic lymphocytes (D) were stained with NK1.1 and TCRβ antibodies and the percentage of $NK1.1^+TCR\beta^-$ cells (as gated in the *upper density plots*) producing IFN-γ directly *ex vivo* was determined by intracellular staining and flow cytometry. Data are representative of 2 experiments with 5-9 mice of each genotype analyzed per condition. (E) Bar graph showing % IFN-γ^+ NK cells in the spleen and liver are pooled from 2 independent experiments. Serum was collected and IFN-γ (F) and IL-4 (H) levels were measured by ELISA. Data represent the mean ± SEM from 2 independent experiments. (H) Mean percent reduction ± SEM in the serum levels of IFN-γ and IL-4 in PBS44-injected $Adap^{-/-}$ mice as compared to similarly treated B6 mice. Data are averaged from 2 independent experiments. Significance was determined by unpaired *t*-test. *$p<0.05$, **$p<0.01$.

Discussion

As one of the hallmark functions of iNKT cells is the large magnitude of cytokines released upon activation, understanding the intercellular signaling requirements becomes crucial. iNKT cells possess mechanisms of both conventional T-cells and NK cells, and in regards to ADAP, it is essential for each. Previous studies have shown ADAP to be required for normal levels of IL-2 release from $CD4^+$ T-cells[43], and IFN-γ from NK cells [128]. Consistent with the role of ADAP in T & NK cells, our studies have shown that iNKT cells are critically dependent on ADAP to both produce and release cytokines upon activation as shown in Figure 9. However, our studies reveal that there was a great defect in cytokine release than cytokine production by $Adap^{-/-}$ iNKT cells, in both major Th1 and Th2 cytokines, IFN-γ and IL-4, respectively. This leads us to believe that there is a greater requirement for ADAP in the functional cytokine release from iNKT cells than there is for production of cytokines. We have previously shown a decreased number of iNKT cells in the spleen and liver of $Adap^{-/-}$ mice (Chapter 1, Figure 3), thus leading to the possibility that this may be the cause of the decreased serum cytokine levels we observed. To test this possibility, we performed an *in vitro* cytokine release assay, as to control for the number of iNKT cells. As our findings showed significant reductions of IFN-γ and IL-4 on a per cell basis, this aided to our mode of thought that ADAP is a critical requirement for iNKT cell cytokine release. Our findings in mice are recapitulated in human iNKT cells. Using equal numbers of wild type (untransfected) and *ADAP* gene-silenced human iNKT cells, we observed a similar dependence on ADAP for cytokine (IFN-γ and IL-4) release following activation.

Previous studies have characterized the requirements of vesicular release of cytokines from both NK cells and T-cells. In particular, NK cells and T-cells alike are dependent on several Rab family proteins such as Rab11, GTPase required for proper vesicular trafficking and exocytosis of cytokine loaded vesicles [132]. Additionally, several classes of syntaxins, such as syntaxin 11, may be required for the docking and release of such cytokine vesicles of T-cells and NK cells for vesicular release as well [133]. Additional data from our lab shows the gene expression as determined by real-time PCR, of Rab11 GTPase is relatively decreased in *Adap*$^{-/-}$ iNKT cells as compared to B6, where as the syntaxin levels are unchanged. While gene expression may or may not be changed, the functionality of these proteins may be dependent on ADAP. As is important with what is termed "inside-out" signaling, the requirement of ADAP for integrin LFA-1 reorganization and proper immune synapse formation may be required for integrin dependent signaling for vesicle trafficking and release [48]. As it is yet to be studied the importance of these vesicular release proteins in iNKT cells, it is possible ADAP may be influencing these; future studies are warranted in these areas for expression of these proteins.

Invariant NKT cells are known to have pre-formed mRNA for IFN-γ and IL-4 to assist in the rapid translation and production upon activation. This mechanism is separate of the preformed cytokines, ready for immediate release. Thus, due to the fact we observe some release, albeit much less, of IFNγ and IL4 post in vivo iNKT cell activation, it is possible ADAP is playing influence into the gene expression of these pre-formed mRNAs. These processes are thought to be regulated by a combination of several transcription factors, such as PLZF and NFκB, and ThPOK [14, 62, 64]. While also yet to be fully understood, it remains possible ADAP is regulating the amount of mRNA encoding for IFN-γ and IL-4 by influencing these transcription

factors, thus dampening the release of each. Of note, it has been shown ThPOK expression in iNKT cells is required for development $CD4^+$ iNKT cells as well secretion of both IFN-γ and IL-4 [64, 134]. Due to our observations of decreased CD4 iNKT cells in *Adap*$^{-/-}$ mice (Chapter 1 Figure 4), and decreased cytokine secretion, it is consistent with our data that ADAP is possibly influencing expression of ThPOK. More so, an upstream transcriptional regulator of ThPOK, called GATA-3, is found to act downstream of TCR signaling. Additionally, it has been shown that a lack of this transcription factor resulted in apoptosis in iNKT cells, as seem by a decreased survival in the liver [135]. As ADAP acts downstream of TCR as well, it is possible the decreased homeostasis or proliferation seen in iNKT cells in *Adap*$^{-/-}$ mice, is because of influence on GATA-3, however future studies in these areas are warranted.

The premier role of cytokine release by iNKT cells is to recruit and assist in the activation of other bystander immune cells. Consistent with our hypothesis, decreased cytokine release from iNKT cells resulted in the decreased activation of other immune cells, as shown by CD69 expression. As ADAP is expressed in T-cells [136], NK-cells [70], and dendritic cells (DCs) [137], is possible the complete lack of ADAP in these mice is influencing the activation of these cells by cytokine dependent mechanisms. However, as we observed a significant decrease in B-cell activation, this can more accurately explain the inherent defect in iNKT transactivation ability, as B-cells do not express ADAP [138]. More so, as iNKT cells can signal for differentiation of DCs into a mature state through cytokine dependent mechanisms, iNKT cell cytokine release is further required for immune regulation [139]. Additionally, NK cells depend on ADAP for only the release of Th1 cytokines, we can confer the decreased serum release of IFN-γ in *Adap*$^{-/-}$ mice was dependent on both NK cells and iNKT cells. More so, the defect in

secretion of IL-4 in the serum must be from iNKT cells alone, as no other immune cells will produce Th2 cytokines within 4 hours of αGC administration.

Taken together, we find ADAP to be essential in the release and to a lesser magnitude production of both Th1 and Th2 cytokines by iNKT cells. As this is the primary method of bridging the adaptive and innate immune systems, a lack of this function emphasizes importance of ADAP in iNKT cell immune functions. We have shown the requirement of ADAP in the transactivation of bystander immune cells, by iNKT cell activation alone, highlighting a critical role for this molecule in regulation of host immunity in several disease states.

CHAPTER 3

ADAP IS INDISPENSABLE FOR INVARIANT NATURAL KILLER T CELL ANTI-TUMOR RESPONSES

Introduction

Previous studies have shown that iNKT cells aid in protection from tumors, through multiple mechanisms. Heterozygous mutations in the well-studied tumor suppressor p53, results in increased tumor incidence, the susceptibility to which increases in the absence of iNKT cells [140]. Similarly, carcinogen-induced tumor incidence was much greater in mice lacking iNKT cells (*Cd1d*$^{-/-}$) as compared to mice with normal iNKT cells [141]. On the contrary, reincorporation of wild-type iNKT cells into iNKT deficient mice halted growth of the carcinogen-induced tumors [141]. In addition, activation of endogenous, wild-type iNKT cells by injection of αGC alone decreased the growth of spontaneously-induced [142] or carcinogen-induced tumors [143]dependent on release of IFN-γ. In human models, cytotoxic iNKT cells were able to enhance the cytotoxic T-cell lysis of Epstein-Barr Virus-positive (EBV^{+}) human tumors by means of IFN-γ release [144]. Taken together, iNKT cells appear to play a vital role in the control of tumor burden in both mice and humans.

iNKT cells are an enticing cell type for means of tumor control, as they possess multiple ways of inducing anti-tumor effects. In particular, iNKTs main mode of killing involves the recruitment and activation of other cytolytic immune cells, such as natural killer cells (NK cells) and CD8^{+} T-cells (cytotoxic T-cells), by means of release of IFNγ and expression of the co-stimulatory molecule CD40 [145, 146]. These mechanisms result in dendritic cell (DC) activation, a professional antigen presenting cell for iNKT cells, which assist in the responses of both cytotoxic and helper T-cells [141], by IL-12 release [141]. These methods of killing are termed indirect iNKT tumor control (Figure 13A). However, iNKT cells also possess the mechanisms of direct tumor lysis (Figure 13B). In particular, upon iNKT cell activation by TCR interaction

with a glycolipid presented by CD1d, iNKTs will upregulate and release cytolytic molecules such as perforin and Granzyme B [147, 148]. In addition, they upregulate and localize the surface death receptors Fas ligand and TRAIL, of which induce apoptosis in the target cell when bound [149]. Although it is known that the iNKT cytolytic functional responses are highly dependent on CD1d-glycolipid antigen presentation interaction with a functional TCR, the signaling mechanisms that regulate these cytotoxic responses are not completely understood. However, studies have shown both the protein SAP and Fyn, which interact with each other downstream of SLAM family receptors upon iNKT cell activation, are essential in the cytotoxic functional response of iNKT cell [68]. In this study, we examined the role of ADAP (adhesion and degranulating-promoting adaptor protein) in iNKT cell cytotoxicity, as it is a known binding partner of Fyn. These studies are important as better characterization of the intracellular signaling for optimal activation of iNKT cells will lend greater understanding of the use of iNKT cells in tumor control and prevention.

To understand the role of ADAP in iNKT cytotoxic functions, we sort-purified iNKT cells from B6 and *Adap*$^{-/-}$ mice and tested their ability to target EL4 T lymphoma cells both *in vitro* and *in vivo*. Our results demonstrate that ADAP is required for murine iNKT cell cytotoxicity *in vitro* as well as iNKT cell-mediated control of tumor growth *in vivo*.

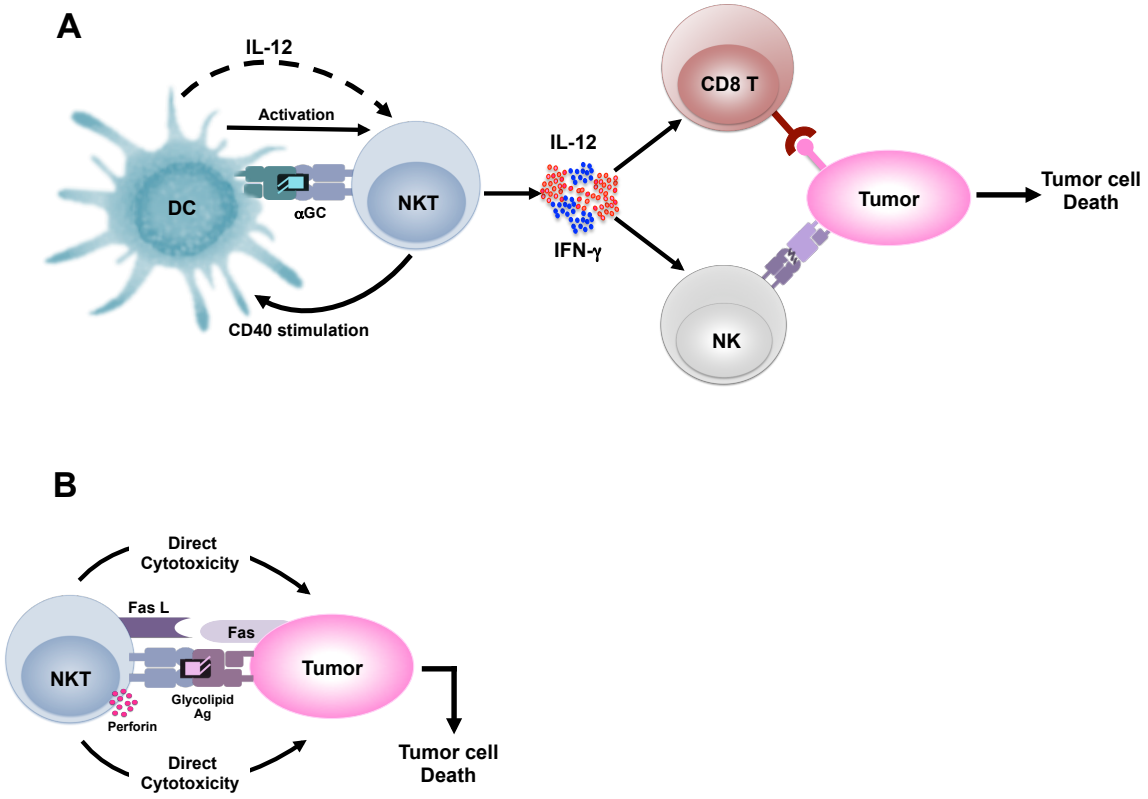


Figure 13. Anti-tumor mechanisms of iNKT cells. (A) Indirect mechanism of iNKT cell cytotoxicity. The cross talk between iNKT cells and antigen presenting cells (such as DCs), presenting a tumor-derived glycolipid, leads to the activation of iNKT cells, IFN-γ release and CD40 stimulation. This iNKT cell-derived IFN-γ induces DC production of IL-12, which further augments IFN-γ production by iNKT and NK cells, and serves to stimulate $CD8^+$ T cell- and NK cell-dependent killing of tumor cells. (B) iNKT cells recognize glycolipid antigens presented by CD1d on tumor cells and mount direct cytotoxicity via perforin/granzyme exocytosis or Fas-Fas ligand (Fas L) interactions.

Materials and Methods

Mice

Mice were used as described in Chapter 1, with the addition of NOD.*CgPrkdc*^{scid} *Il2rg*^{tm1Wjl}*/SzJ* (NSG) mice, which were purchased from Jackson Laboratories (Bar Harbor, ME), and bred at MSU animal facility. All Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC), MSU.

Reagents and Antibodies

Reagents and antibodies used are as described in Chapters 1 & 2, with the addition of Granzyme-B and Thy1.2 (BioLegend, San Diego, CA); Perforin, Fas L, and TRAIL (BD Bioscience, San Diego, CA). Luciferase-expressing EL4 (EL4-Luc) was purchased from Caliper Life Sciences (Hopkinton, MA). EL4-Luc cells were thoroughly tested and certified to be pathogen-free prior to injection into NSG mice.

In vitro Cytotoxicity assay

Invariant NKT cell cytotoxicity was evaluated by ^{51}Cr-release assay. EL4 cells were used as target cells (T, 1x 10^6) were labeled with 100μCi ^{51}Cr (Na_2CrO_4; Perkin Elmer; Waltham, MA) for 1-2 h at 37°C and then washed extensively. ^{51}Cr-labeled targets were either loaded with PBS44 (100ng/mL) or left untreated; then co-cultured in triplicate with effector cells (E) at varying E:T ratios. After 16 h, supernatants were sampled (experimental counts per minute [CPM]) and applied to LumaPlates (Perkin Elmer). Cultures of target cells alone were assayed in a similar fashion to determine spontaneous release (Spont CPM). Total CPM was assessed by

complete lysis of target cells by using 0.1% IGEPAL (Sigma Aldrich) in water. Percent specific lysis was calculated using the following formula:

% Specific lysis = 100 x [Experimental CPM – Spont CPM]/[Total CPM – Spont CPM]

Human iNKT cell expansion, purification and gene silencing

As described in Chapter 1 and 2.

***In Vivo* tumor model**

Age-matched NSG mice received purified iNKT ($4x10^5$) cells from either B6 or *Adap−/−* animals via tail vein injection. Animals in control group received no iNKT cells. Three days later (Day 0), mice in all the groups were challenged with PBS44-loaded EL4-Luc cells ($1x10^5$). Tumor dissemination was visualized by bioluminescence imaging (BLI) at different time points post tumor cell injection. Two weeks later, mice were sacrificed; livers and spleens were removed and weighed. Tumor clearance was evaluated using *ex vivo* BLI, flow cytometry and histology. Mice were euthanized during the study if they developed hind-limb paralysis.

***In vivo* and *ex vivo* BLI**

For *in vivo* BLI, mice were given an i.p injection of D-luciferin (Caliper Life Sciences; 150 mg/kg body weight) and then anesthetized with isoflurane gas using a Xenogen XGI Gas Anesthesia System. Ten minutes after injection of the substrate, mice were imaged in groups using the IVIS Imaging system (Xenogen; Baltimore, MD). To image specific tissues for tumor involvement, mice were injected with an additional dose of luciferin (50 mg/kg body weight). Five minutes later, animals were killed; spleens and liver were harvested and imaged. Tissue

processing and imaging were conducted within a period of 3 minutes to minimize signal decay. For both *in vivo* and *ex vivo* BLI, data were collected until either the mid-range of the linear scale or the maximal exposure settings were achieved (f stop 1, large binning and 120 s). Imaging data were analyzed and quantified with Living Image v3.2 Software (Xenogen).

Histologic analysis

Representative samples of liver were obtained from all the animals in each cohort, washed in PBS and fixed in 10% formalin. Samples were then embedded in paraffin, cut into 5-μm-thick sections and stained with hematoxylin and eosin (H&E). Digital images of sections were obtained using a Nikon Eclipse 50i (Nikon, Minato, Tokyo, Japan) and INFINITY-3, using INFINITY ANALYZE 6.5.4 software (Lumen*era* Corporation, Ottawa, Ontario, Canada).

Statistics

Student's *t*-test and one-way or two-way ANOVA with Tukey's post-hoc test were used as indicated. Significance shown as *($p<0.05$), **($p<0.01$), or ns for non-significant values. All statistical data was determined using GraphPad Prism (GraphPad, San Diego, CA).

Results

ADAP is required for optimal TCR-induced iNKT cell cytotoxicity *in vitro*.

The SAP and Fyn axis is required for robust murine and human iNKT cell cytotoxicity and this requirement is conversed among other cytotoxic effectors, including T as well as NK cells. However, ADAP-deficient NK cells exhibit no cytotoxic defects, and the role of ADAP in $CD8^+$ T cells remains undefined. To address whether ADAP regulates iNKT cell cytotoxicity, we evaluated the expression of death promoting proteins in B6 and $Adap^{-/-}$ iNKTs and observed that they expressed comparable levels of perforin, granzyme B, FasL and TRAIL-R (Figure 14). Next, we assessed the killing ability of iNKT cells sort-purified from the livers of B6 and $Adap^{-/-}$ mice. iNKT cells were placed in culture with ^{51}Cr-labeled EL4 T-lymphoma cells that had been loaded with OCH (weak antigen, Figure 15A) or PBS44 (strong agonist, Figure 15B) or left unloaded. Regardless of the antigen used, B6 iNKT cells exhibited potent killing of OCH or PBS44 loaded but not unloaded EL4 cells (Figure 15A, B). In contrast, $Adap^{-/-}$ iNKT cells exhibited diminished killing, that was 46 ± 9% (OCH) and 52 ± 10% (PBS44) less than the activity of B6 cells (E:T ratio 20:1) (Figure 15C). Together, these studies reveal that ADAP is required for optimal *in vitro* TCR-induced iNKT cell cytotoxic responses.

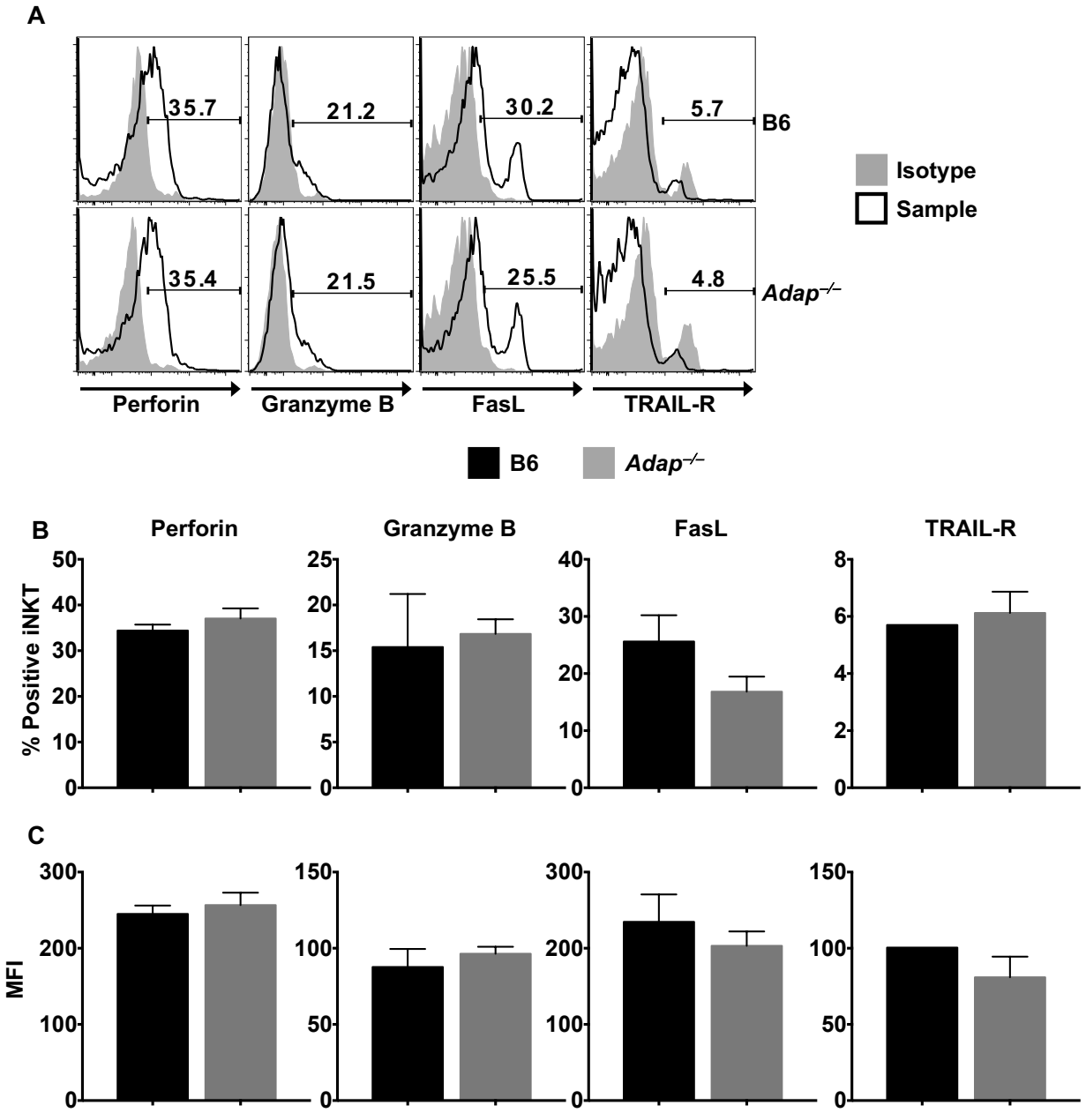


Figure 14. Basal expression of death receptors and lytic molecules are not altered in *Adap*$^{-/-}$ iNKT cells. (A) Splenocytes from B6 and *Adap*$^{-/-}$ mice (gated on CD1d-tetramer^{+}TCRβ^{+}iNKT cells) were analyzed for surface expression of FasL (CD95L) and TRAIL receptor-R2. Cells were also fixed, permeabilized and stained for intracellular levels of perforin and granzyme B. Representative flow cytometric data from 1 of 2 independent experiments is shown. (B) Percent

Figure 14. (cont'd)

% iNKT cells and (C) MFI for the lytic molecules and death receptors are shown as indicated. Data is presented as mean ± SD from 1 of 2 independent experiments (n=5-7 per genotype).

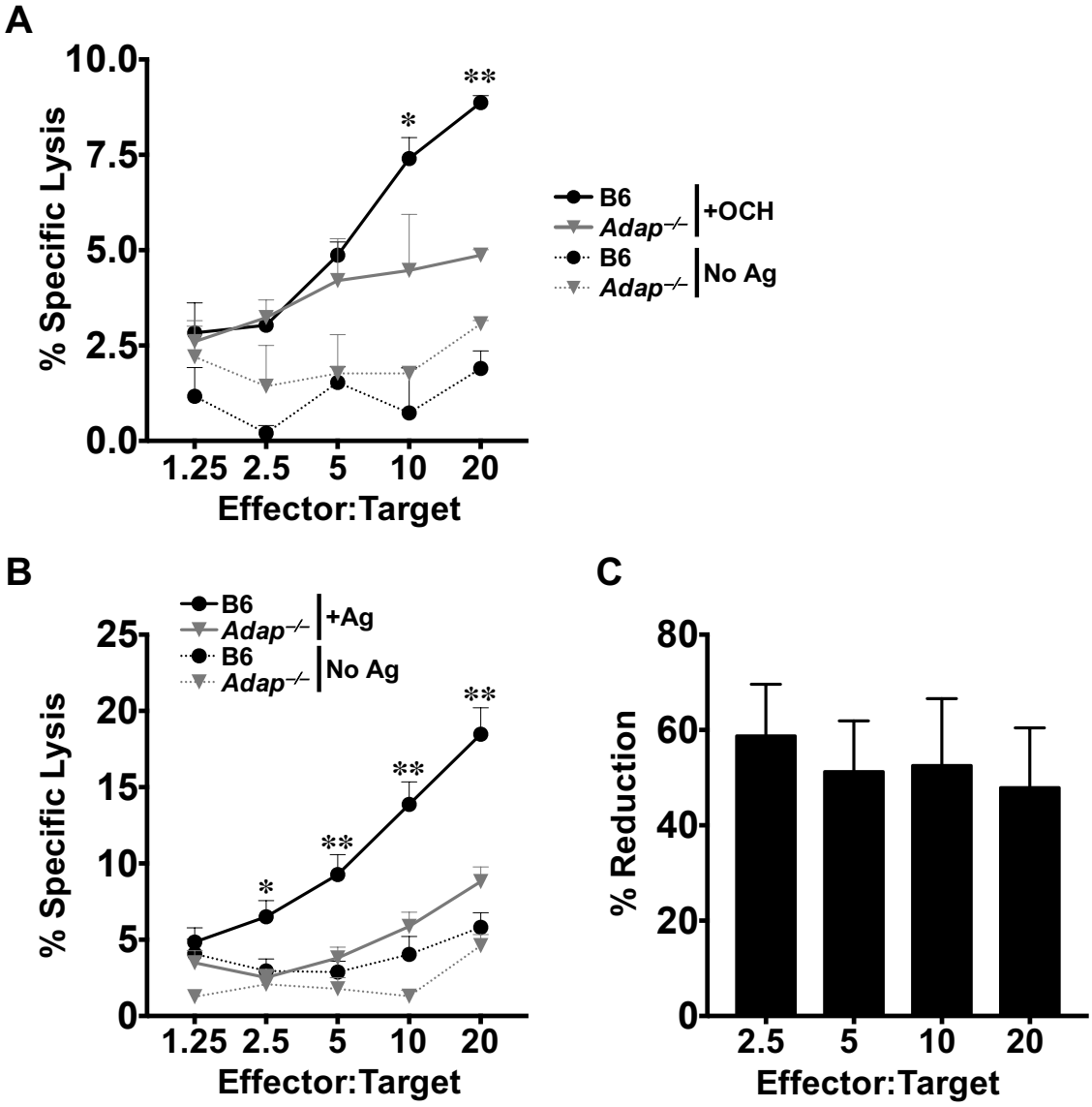


Figure 15. *Adap*$^{-/-}$ iNKT cells exhibit defective killing of antigen-pulsed target cells *in vitro*. EL4 target cells were pulsed with 100 ng/ml of antigen: OCH (A) or PBS44 (B) or left untreated. Cytolysis of OCH-loaded (A) or PBS44 (B) or –unloaded EL4 cells by B6 or *Adap*$^{-/-}$ iNKT cells. (C) Mean percent reduction ± SEM in cytolysis of PBS44-loaded EL4 cells by *Adap*$^{-/-}$ iNKT cells compared to B6 iNKT cells. Data in A is from 1 representative experiment and data in B, C are averaged from 5 independent experiments. Statistical significance was determined by two-way ANOVA test. * $p < 0.05$, ** $p < 0.01$

ADAP is required for iNKT cell-mediated control of tumor growth *in vivo*.

Having observed that ADAP was required for optimal *in vitro* anti-tumor cytotoxic responses, we next sought to address whether it was also required for iNKT cell-mediated tumor clearance *in vivo*. For these studies, immunodeficient NOD-SCID IL-2Rγ$^{-/-}$(NSG) mice were reconstituted or not with purified B6 or *Adap*$^{-/-}$ iNKT cells and 3 days later challenged with PBS44-loaded luciferase-expressing EL4 cells (EL4-LUC). Tumor dissemination was monitored by bioluminescence imaging (BLI) and tumor cell burden and iNKT reconstitution by flow cytometric and histological analyses 3 weeks after tumor cell injection. Compared to mice that received no iNKT cells, animals receiving B6 iNKT cells exhibited a significantly lower tumor burden as determined by lower live animal radiance (Figure 16A, B), and reduced radiance (Figure 16C, D) and weights (Figure 16E) of tumor-bearing organs. Similarly, flow cytometric analyses of B6 iNKT cell-reconstituted mice demonstrated a lower number of tumor cells in the liver and spleen (Figure 16F-I) and histological analyses showed very few if any tumor cell aggregates (Figure 16L). Consistent with their *in vitro* cytolytic defects, *Adap*$^{-/-}$ iNKT cells exhibited poorer control of EL4 cell growth *in vivo* compared to B6 iNKT cells (Figure 16A-I, L). Of note, there was no significant difference in the number of B6 or *Adap*$^{-/-}$ iNKT cells recovered from the organs of tumor-bearing mice (Figure 16J, K). Thus, the suboptimal *in vivo* clearance of tumor is not due to the inability of *Adap*$^{-/-}$ iNKTs to traffic to the tumor site, but instead due to their impaired cytotoxic capacity.

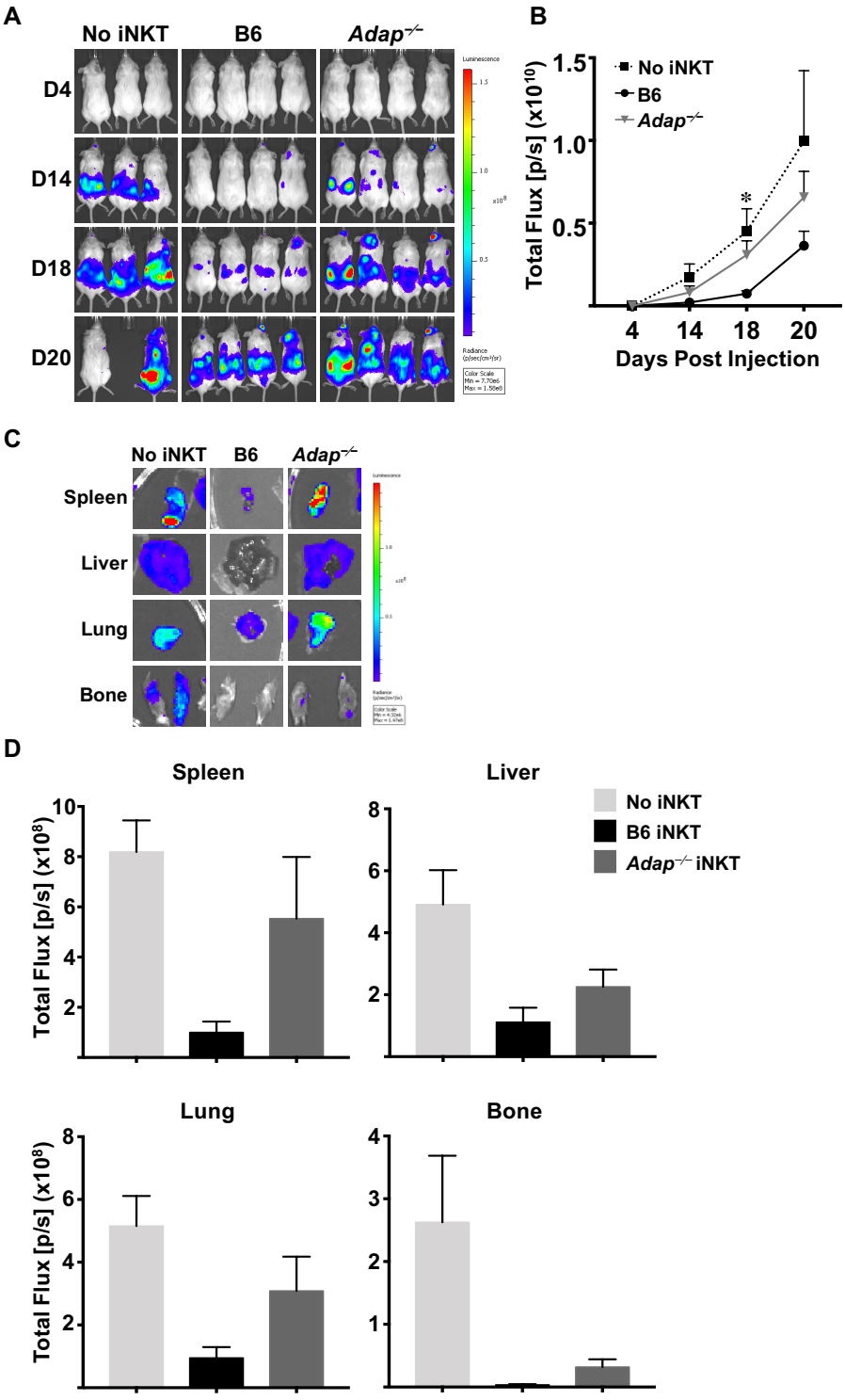
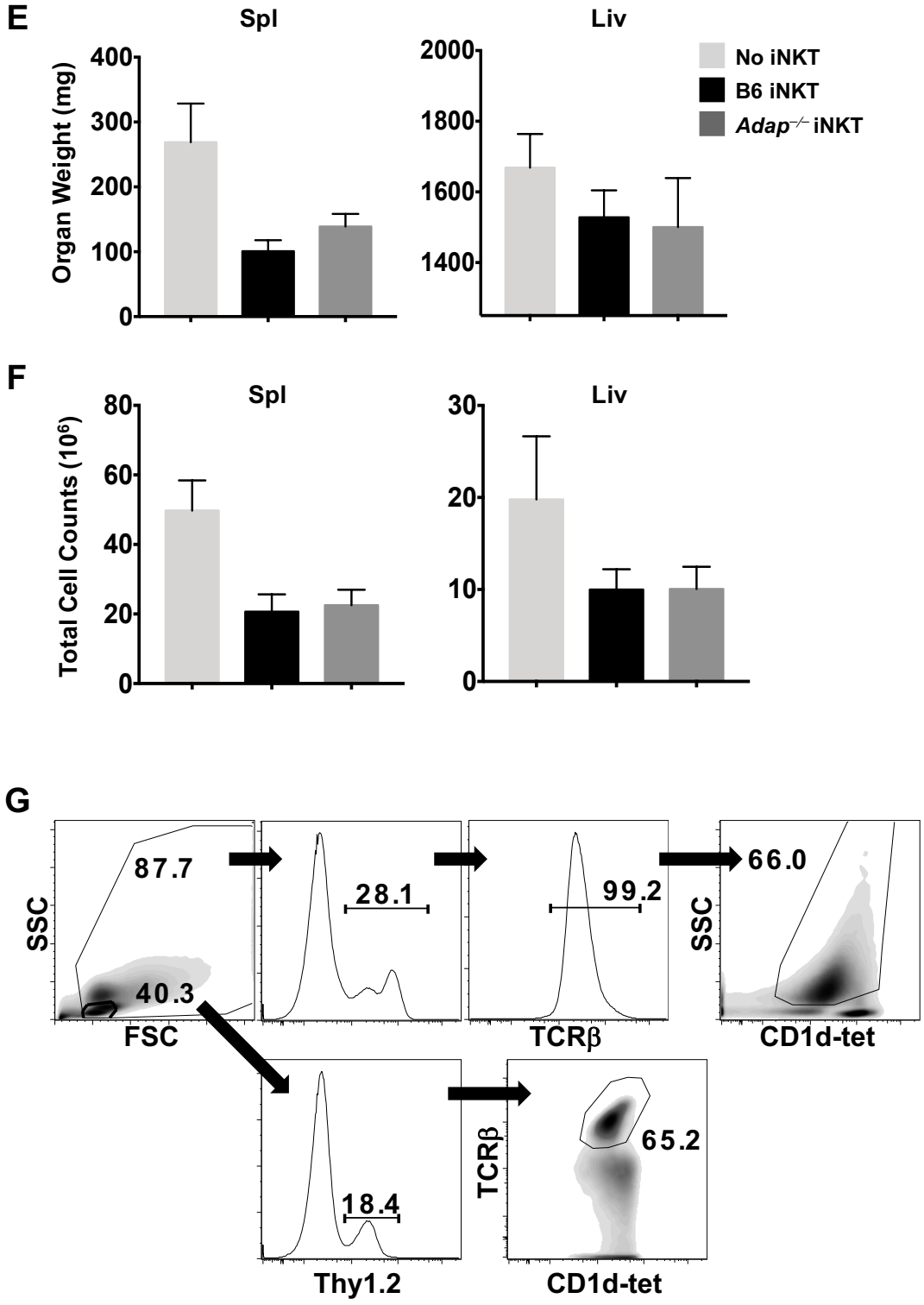


Figure 16. ***Adap*-deficient iNKT cells exhibit defective *in vivo* control of antigen-pulsed EL4 cells.** NSG mice were reconstituted or not (No iNKT cells) with $4x10^5$ iNKT cells (B6 or $Adap^{-/-}$). Three days later, mice were challenged with $1x10^5$ EL4-Luc cells. (A) Whole body

Figure 16. (cont'd)

distribution of EL4-Luc cells over the first 3 weeks using *in vivo* BLI. (B) Quantification of BLI images at serial time points. Data points are average radiance emitted from 4 mice per group. (C) Total radiance of spleen, liver, lung and bones from a representative mouse from each group is shown. Organ (D) *ex vivo* BLI, (E) weights and (F) total cell counts are shown (spleens and livers). (G) Gating strategy for EL4 and iNKT cell analysis from the liver is shown. (H-K) Percent (H, J) and absolute number (I, K) of EL4 (H, I) and iNKT cells (J, K) was determined in the spleens and livers of mice by flow cytometry. (L) Tumor burden in the liver was assessed histologically. Black arrows indicate tumor aggregates. Data are pooled from 2 experiments with 3-4 mice in each cohort and error bars represent SEM. Statistical significance was determined by two-way ANOVA. * $p < 0.05$, ** $p < 0.01$

Figure 16. (cont'd)



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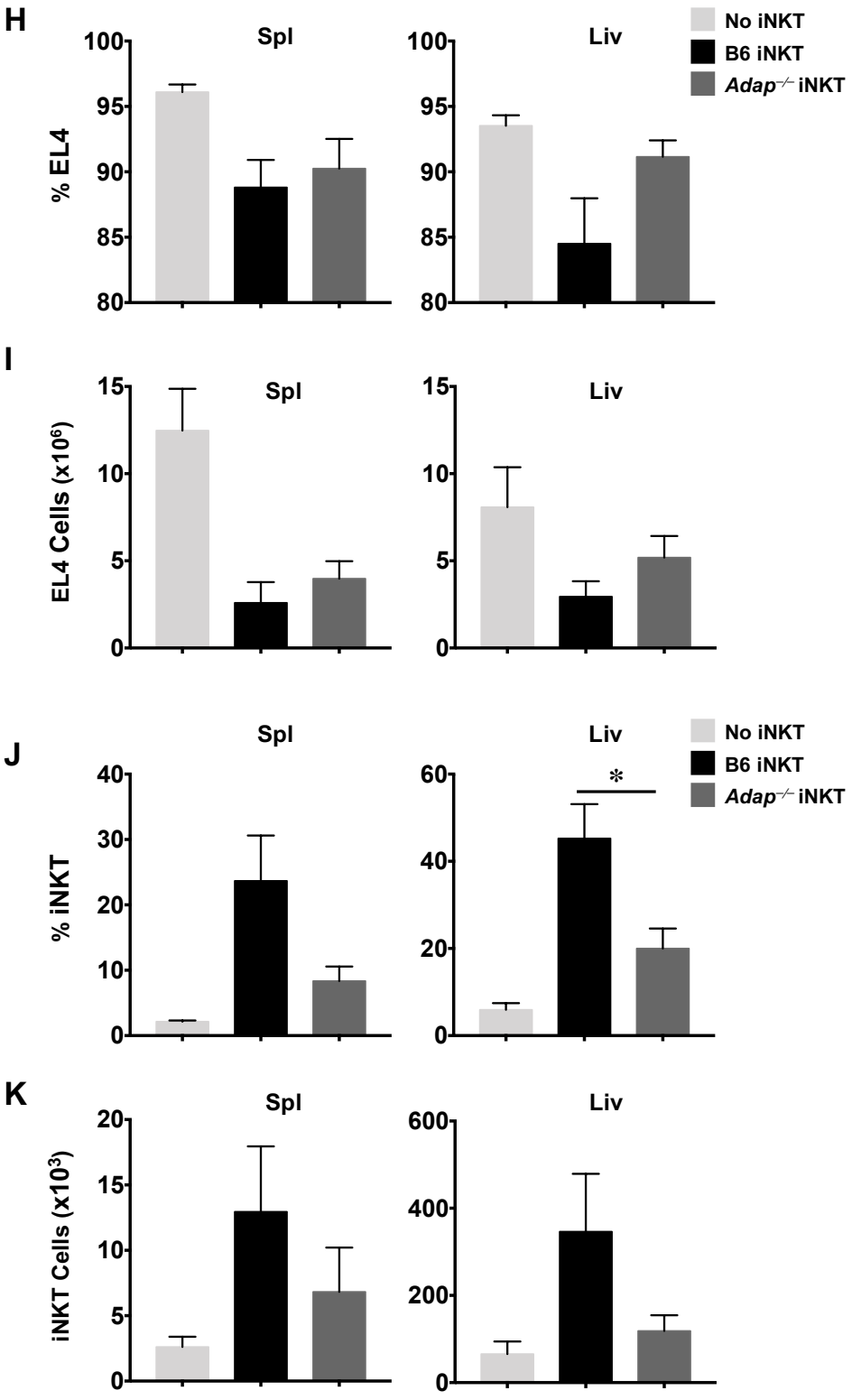

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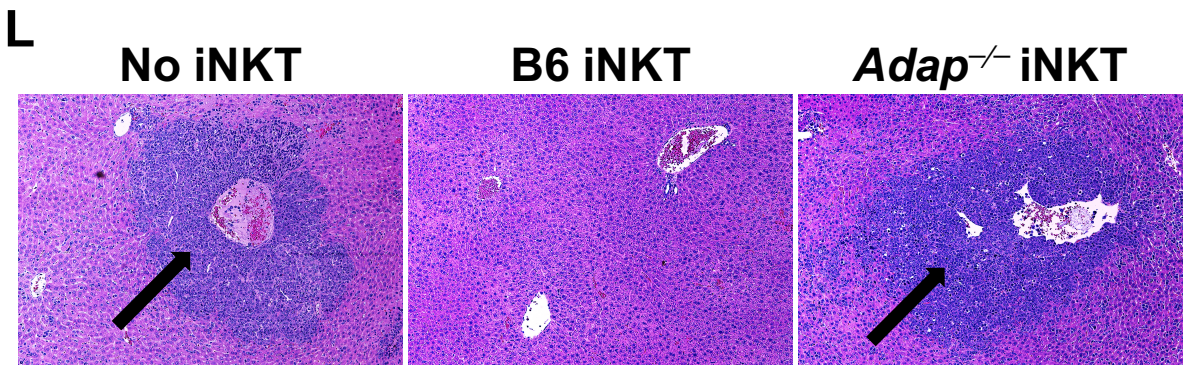

Table 4. Total flux from *ex vivo* organ BLI

Total Flux [p/s](x10^8)	No iNKT	B6 iNKT	*Adap*$^{-/-}$ iNKT
Spleen	8.19±1.26	1.0±0.43	5.53±2.46
Liver	4.91±1.11	1.11±0.47	2.26±0.56
Lung	5.15±0.96	0.95±0.35	3.08±1.09
Bone	2.62±1.06	0.03±0.01	0.32±0.12

Table 5. Frequency of EL4 and iNKT cells in various organs *ex vivo*

	No iNKT		B6 iNKT		*Adap*$^{-/-}$ iNKT	
Frequency	**Spl**	**Liv**	**Spl**	**Liv**	**Spl**	**Liv**
EL4	96.1±0.58	93.5±0.1	88.81±2.1	84.5±3.5	90.3±2.3	91.15±1.3
iNKT	2.14±0.18	5.94±1.51	23.7±6.9	45.3±7.86	8.35±2.2	**19.0±4.58***

* $p < 0.05$

Table 6. Absolute cell number of EL4 and iNKT cells in various organs *ex vivo*

Absolute Cell Number	No iNKT		B6 iNKT		*Adap*$^{-/-}$ iNKT	
	Spl	**Liv**	**Spl**	**Liv**	**Spl**	**Liv**
EL4 (x10^6)	12.8 ±2.4	8.01±2.3	2.6±1.19	2.95±0.88	4.0±1.0	5.1±1.23
iNKT (x10^3)	2.6±0.78	65.4±28.9	12.95±5.0	346.3±132.6	6.8±3.34	118.4±36.1

Discussion

Lymphocyte signaling cascades responsible for anti-tumor cytotoxicity and inflammatory cytokine production must be tightly regulated in order to control an immune response. Disruption of these cascades can cause immune suppression as seen in a tumor microenvironment, and loss of signaling integrity can lead to autoimmunity and other forms of host-tissue damage. Therefore, understanding the distinct signaling events that exclusively control specific effector functions of "killer" lymphocytes (T and NK cells) is critical for understanding disease progression and formulating successful immunotherapy. Elucidation of divergent signaling pathways involved in receptor-mediated activation has provided insights into the independent regulation of cytotoxicity and cytokine production in lymphocytes. Specifically, the Fyn signaling axis represents a branch point for killer cell effector functions and provides a model for how cytotoxicity and cytokine production are differentially regulated. While the Fyn–PI(3)K pathway controls multiple functions, including cytotoxicity, cell development, and cytokine production, the Fyn–ADAP pathway preferentially regulates cytokine production in NK and T cells. In this study, we provide evidence that unlike in T or NK cells, ADAP regulates both cytokine as well as cytotoxic responses of iNKT cells. The primed nature of iNKT cells in addition to their multiple mechanisms of cytolysis make them prime candidates for the immune surveillance of human tumors. While this is true, the intracellular signaling requirements of these cells are less well known.

In regards to ADAP, we found it to be required for direct cytolysis by murine iNKT cells. This is consistent with our previous studies regarding SAP and Fyn, as a lack of either protein significantly reduces target cell lysis of iNKT cells [68]. As ADAP binds to Fyn, this extends

our previous understanding of the signaling pathways for iNKT cell cytolysis. Although SAP and ADAP differentially regulate iNKT cell development, their requirement for iNKT cell cytolytic activity might be similar or overlapping. Both SAP and ADAP are required for stable conjugate formation between T cell and the antigen-presenting cell [150]. SAP has been shown to be required in the formation of a functional lytic synapse, which is requires actin polymerization and movement of the microtubule organization center at the site of contact [68]. Interestingly, ADAP links TCR signaling to reorganization of the actin cytoskeleton. As seen in T-cells upon activation, ADAP engages with Evl of the Ena/VASP family of proteins, WASP (Wiskott-Aldrich syndrome protein), and the Arp2/3 complex, inducing actin rearrangement, which may play a role in TCR clustering and signal strength [151, 152]. In our studies, we observed an inherent defect in cytotoxicity by *Adap*$^{-/-}$ iNKT cells, which was independent of TCR signal strength. Consistent with this notion, *Adap*$^{-/-}$ iNKT cells failed to mount a robust cytotoxic response both in the presence of a weak or strong agonist like OCH (truncated version of αGC) and αGC respectively. ADAP is unique in that it has binding partners of two separate pathways; ADAP binds to Fyn downstream of SLAM family receptors [68, 86] and to phosphorylated Slp76 downstream of the TCR [153]. As Fyn is required for iNKT cell cytotoxicity upon TCR engagement, and Slp76 is in NK cells [154], it leads us to believe ADAP expression may be a converging point of each pathway.

Previous studies reveal that *in vitro* and *in vivo* lysis of EL4 T cell lymphoma relies on iNKT cell expression of perforin where as Fas/FasL pathway is required for iNKT cell-mediated control of CD1d-transfected A20 B-lymphoma cells. However, A20-CD1d cells express significantly higher levels of CD1d and Fas compared to EL4s [155, 156], and thus they may induce more

FasL upregulation on iNKT cells and/or be more susceptible to FasL-mediated apoptosis. Loss of perforin expression does not fully abolish EL4 lysis *in vitro*, while loss of both perforin and FasL does. Therefore perforin and FasL both contribute to iNKT cell cytotoxicity. Taken together, results from these studies imply that the mechanism of iNKT cell-mediated tumor control may be modulated by several variables, including the levels of cytotoxic proteins and ligands expressed by the iNKT cells, the apoptosis-inducing receptors borne by the targets, and other external factors that influence iNKT:target cell interactions. We observed comparable levels of both cytotoxic molecules (perforin and granzyme) and death-promoting receptors (FasL and TRAIL) on B6 and *Adap*$^{-/-}$ iNKT cells, suggesting that the cytolytic defect exhibited by and *Adap*$^{-/-}$ is independent of the levels of cytotoxic proteins.

Our most clinically relevant finding was that ADAP is required for the tumor protective properties of iNKT cells *in vivo*. We demonstrate that ADAP expression in iNKT cells is required for direct EL4 tumor control. This was apparent by increased total body as well as organ specific BLI in animals reconstituted with *Adap*$^{-/-}$ iNKT cells. While monitoring expansion of the tumor by live animal imaging, allowed us to monitor growth over time at a macroscale, FACS analysis allowed us to quantitate EL4 growth in the spleen and liver. Histological assessment of liver also showed a greater visualized infiltration (black arrow) of EL4 cells (dark purple) near the blood vessels of the organ. As the EL4 cell line is a T-cell lymphoma, it does not commonly form solid tumors, but will greatly accumulate around highly vascularized areas, especially in a T-cell deficient niche as in the NSG liver. Consistent with our hypothesis, we observed a decrease in the number of reconstituted *Adap*$^{-/-}$iNKT cells in both the spleen and liver as compared to B6. This leads us to further believe that ADAP may be required for the

peripheral maintenance or proliferation of iNKT cells, as described in Chapter 1. Taken all together, we conclude ADAP is required in iNKT cells for direct cytolysis and tumor immuneosurveillance *in vivo.*

Understanding iNKT cell signaling becomes of vital importance when trying to modulate or adapt them for tumor regulation. Comparative analysis of certain tumor types, in particular breast cancer, have found correlatively lower metastasis rates with CD1d expressing tumors [157], indicating an tumor control role of iNKT cells. More so than just direct tumor lysis, iNKT cells can inhibit tumor growth by other mechanisms. Particularly, iNKT cells can kill both myeloid-derived suppressor cells (MDSCs) [158] and tumor associated macrophages (TAMs) [39], both of which can skew the tumor microenvironment to an immunosuppressive state, and promote tumor growth. Future studies are warranted as to the role ADAP plays in these functions, however our findings lead us to believe it to be important in each. More so, as it has been studied in cytotoxic $CD8^+$ T-cells, deficiencies in ADAP resulted in a decrease in PD-1 expression, and thus enhanced the anti-tumor response by the T-cells [159]. Future studies understanding how ADAP can be influencing iNKT cell tumor protection will become vital in clinical immunotherapies. In conclusion, we found ADAP in iNKT to be required in the direct cytotoxicity to tumor targets, as seen in our *in vitro* models. Given that ADAP is dispensable for NK cell cytotoxicity and has no known role in T cell anti-tumor response, our studies highlight a lineage –specific role for ADAP in iNKT cell cytotoxicity.

OVERALL DISCUSSION

The application of iNKT cells have been implicated in clinical settings for protection for various cancer types. It has been observed that greater incidences of iNKT cells have correlated with decreased rates of tumor growth and metastasis in various solid tumors such as colon [32], lung [33], head and neck[160], and neuroblastomas [161]. Additionally, as thought be a protective mechanism of the tumor by deterring immuneosurveillance, iNKT cells are shown to be greatly reduced in circulation independent of tumor type or load [162]. It is common for tumors to influence the tumor microenvironment (TME) to an immunosuppressive state, the ability of iNKT cells to secrete Th1 cytokines assists in the recruitment and activation of other immune cells for attack against a tumor. Additionally, iNKT cells can directly kill CD1d positive tumors or tumor-associated macrophages (TAM), which assist in the immunosuppressive TME [39]. Due to the nature of Th1 and Th2 cytokine release by iNKT cells, understanding how to influence these responses by regulating the signaling proteins downstream of TCR activation proves crucial in future cancer immunotherapies.

In particular to our study, we found ADAP to required for both the release of cytokines, and cytotoxic functions of iNKT cells, thus highlighting its importance in the intracellular signaling requirements of iNKT cells. While we observe these functional defects, it is yet to be fully determined the mechanisms of which ADAP is regulating. However, it is likely that ADAP is regulating the functional immune synapse in iNKT cells, as was previously shown in T-cells. T-cell conjugation with an antigen presenting cell was shown to be dependent on ADAP co-localization with integrin LFA-1 and supramolecular activation cluster (SMAC) [163]. While these ADAP dependent mechanisms could be regulating iNKT cell functions, it is likely multi-faceted, as ADAP functions downstream of multiple signaling pathways. In particular, ADAP

binds to Slp-76 downstream of TCR [151], and to Fyn downstream of SLAM-R[68], and possibly cytokine receptor signaling. This highlights ADAP as a possible convergence point, of multiple pathways, thus better characterizing its role in iNKT cell function. ADAP serves as a binding protein, and other binding interactions downstream may be the cause of its functional dependencies. In particular, a protein termed SKAP55 (src kinase-associated phosphoprotein of 55-kD), known to constitutively bind to ADAP in T-cells, is specifically required for LFA-1 clustering [164, 165]. More so, ADAP is required to prevent degradation of SKAP55 [166]. Preliminary studies in our lab have found similar findings in iNKT cells; diminished SKAP55 expression in $Adap^{-/-}$ mice. While not yet fully understood, this data leads us to believe that SKAP55 may be a functional regulatory protein in iNKT cells a well. A protein with nearly identical structure to SKAP55, called SKAP55R, is thought to play similar roles as SKAP55 [165]. Preliminary studies from our lab reveal that SKAP55R have greater basal expression than SKAP55 in iNKT cells, and more so SKAP55R expression increases upon polyclonal TCR activation, while SKAP55 levels are unchanged. Whether SKAP55 and/or SKAP55R preferentially bind to ADAP following stimulation to regulate iNKT cell functions remain to be determined.

A structural homologue of ADAP that is thought to play a role in integrin-mediated signaling cascade for integrin-dependent is termed PRAM (PML-retinoic acid receptor alpha regulated adaptor molecule 1) [167, 168]. PRAM shares multiple similar binding partners with ADAP, including Slp-76, Lyn (structurally similar to Fyn) and SKAP55R (structurally similar to SKAP55)[167, 168]. While similar in many ways to ADAP, PRAM differs in its expression within cell types, as PRAM is most commonly expressed in myeloid immune cell lineages such

as neutrophils, whereas ADAP is more commonly expressed in lymphoid cells. However, is interesting to note that while iNKT cells express PRAM, basal expression of ADAP is 4-5 fold higher in *Pram*$^{-/-}$ mice. Furthermore, our preliminary analysis demonstrates that *Pram*$^{-/-}$ iNKT cells to have greater antigen-induced functional response, as seen by increased direct specific lysis and cytokine release *in vitro*. Given that both ADAP and PRAM can bind to Slp-76 as well as SKAP55 and/or SKAP55R, it is likely that ADAP and PRAM compete for binding with either of these molecules and thereby regulate the functions of each other. Alternatively, it is also possible that ADAP and PRAM function co-operatively to promote iNKT cells functions. Studies are currently underway to gain mechanistic insights into how ADAP regulates iNKT cell homeostatic maintenance, cytokine production and release as well as has the ability to mount robust target cell lysis both *in vitro* and *in vivo*.

Cancer immunotherapy is aimed at harnessing the power and specificity of the immune system in the treatment of specific malignancies. Based on their combined ability to kill tumor cells and promote the anti-tumor functions of NK and $CD8^+$ T cells, iNKT cells represent an excellent candidate for incorporation into novel immunotherapeutic trials for cancer. Indeed, several clinical trials demonstrate that iNKT cell therapies are well tolerated, transiently activate the human immune system, and promote objective yet partial tumor responses. Although encouraging, there is need for improvement. At present, the challenge lies in better understanding how iNKT cells recognize and respond to tumors and developing more effective methods to promote their anti-tumor functions. By further defining the role of ADAP in murine and human iNKT cell functions, these studies will provide new and important insights into the signals controlling iNKT interaction with, and responses to, normal, malignant and/or infected cells.

These studies will have an impact by establishing new paradigms for iNKT cell signaling and offering insights into how iNKTs can be best activated to enhance host immunity and treat cancer.

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