THE ROLE OF NATURAL KILLER T CELLS IN X-LINKED LYMPHOPROLIFERATIVE DISEASE

by

BRIAN KAI-HO CHUNG

B.Sc., University of British Columbia, 2002

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

February 2012

ABSTRACT

X-linked lymphoproliferative disease (XLP) is a fatal immunodeficiency syndrome characterized by fulminant infectious mononucleosis (IM), uncontrolled lymphoproliferation and B cell malignancies following infection with the Epstein-Barr virus (EBV). In the majority of affected individuals, XLP is caused by mutations to the gene Src homology 2-domain 1A (*SH2D1A)*, which encodes for signaling lymphocyte activation molecule-associated protein (SAP). Studies in SAP-deficient ($SAP^{-/-}$) mice and in humans with XLP indicate that SAP is necessary for the normal function of several immune cell populations including natural killer (NK) cells, $CD4^+$ helper T (T_H) cells and $CD8^+$ cytotoxic T lymphocytes (CTL). Surprisingly despite these immune cell defects, individuals with XLP appear immuno-competent against most pathogens and only develop disease upon exposure to EBV. Why EBV infection specifically triggers XLP in humans with defective SAP expression is not fully understood, however, we observed that $SAP^{-/-}$ mice and XLP subjects completely lack the immune subset of natural killer T (NKT) cells. Given that NKT cells are known to play an important role in controlling immune responses and that XLP subjects generate dysregulated immune responses following EBV infection, we hypothesized that NKT cells may directly control the proliferation of EBV-infected B cells. To test this hypothesis, we established an *in vitro* experimental system using human NKT cell lines and EBV-transformed tonsillar B cells. EBV transformation of naïve B cells into lymphoblastoid cell lines (LCL) resulted in the downregulation of the NKT cell antigen-presenting molecule CD1d and correlated with the inability of LCL to activate NKT cells, even in the presence of the superagonist α-galactosylceramide (αGalCer). By contrast, LCL with induced CD1d expression strongly

activate NKT cell effector functions even in the absence of αGalCer. Collectively, these results demonstrate that NKT cells may be critical for controlling early EBV replication in B cells prior to CD1d downregulation and suggest that the absence of NKT cells may underlie the immune dysregulation in XLP subjects following EBV infection.

PREFACE

A version of Chapter 2 has been published. Chung, BK., Aoukaty, A., Dutz, J., Terhorst, C., Tan, R. (2005). Signaling lymphocyte activation molecule-associated protein controls natural killer T cell functions. J. Immunol. 174(6): 3153-7. BKC performed experiments; BKC, AA, JD and RT designed the research and analyzed results; CT provided $SAP^{-/-}$ mice; BKC generated the figures; BKC and RT wrote the manuscript.

A version of Chapter 3 has been submitted for publication review. Chung, BK., Allan, LL., Zheng, D, Nie, JC., Biggs, C., Kozak, FK., Priatel, JJ., van den Elzen, P., Tan, R. (2011). Natural killer T cells control EBV-infected B cells. PLOS Pathogens. BKC, DZ, JCN, CB performed experiments; BKC, LLA, JJP, PvdE and RT designed the research and analyzed results; FKK provided tonsil samples; BKC generated the figures; BKC, JJP and RT wrote the manuscript.

All animal experimentation was carried out with adherence to the guidelines presented by the University of British Columbia Animal Care Committee. Canadian Council on Animal Care Approval was granted under the certificate number: #A03-0001, #A05-1507 and #A05-1143.

The collection of all human tonsil samples was carried out with adherence to the guidelines presented by the University of British Columbia Children's and Women's Clinical Research Ethics Board (UBC CREB). UBC CREB approval was granted under the certificate number: #H06-03256.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

7-AAD	7-amino-actinomycin
αα	alpha-alpha
αGalCer	α-galactosylceramide
β	beta
γδ	gamma-delta
APC	antigen presenting cells
ATRA	all-trans retinoic acid
BCR	B cell receptors
$CD1d^{-/-}$	CD1d-deficient
CFA	complete Freund's adjuvant
CLP	common lymphoid progenitors
CMP	common myeloid progenitors
CMV	cytomegalovirus
CR2	complement component receptor 2
CRACC	CD2-like receptor activating cytotoxic T cells
CTL	$CD8^+$ cytotoxic T lymphocyte
DC	dendritic cells
EAE	experimental allergic encephalomyelitis
EAT2	Ewing's sarcoma-associated transcript-2
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
ER	endoplasmic reticulum
ERT	EAT2-releated transducer
Ets	E26 transformation-specific
FasL	Fas ligand

$Fyn^{-/-}$	Fyn-deficient
Fyn	Fyn tyrosine kinase
GD3	disialoganglioside
GC	germinal centers
GI	gastrointestinal tract
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSC	hematopoietic stem cells
HSV	herpes simplex virus
ICOS	inducible T cell costimulatory
IFN-γ	interferon-gamma
Ig	immunoglobulins
iGb3	isoglobotrihexosylceramide
IL	interleukin
IM	infectious mononucleosis
iNKT	invariant natural killer T
ITK	IL-2-inducible T cell kinase
ITSM	immunoreceptor tyrosine-based switching motif
KSHV	Kaposi-associated herpesvirus
LCL	lymphoblastoid cell lines
LCMV	lymphocytic choriomeningitis
LEF	lymphoid enhancer-binding factor
LMP	latent membrane protein
MHC	major histocompatibility complex
MHV-68	murine herpesvirus 68
MIR	modulator of immune recognition
NK	natural killer

NKT	natural killer T
NOD	non-obese diabetic
PAMP	pattern-recognition molecular patterns
PIX	PAK-interacting exchange factor
PKC-θ	protein kinase C-θ
PML	polymorphonuclear leukocyte
PPAR	peroxisome proliferator-activated receptor
PRR	patter-recognition receptors
OVA	ovalbumin
RAG	recombination-activating gene
RAR	retinoic acid receptor
RasGAP	RAS-GTPase-activation
RSV	respiratory syncytial virus
SAP	signaling lymphocyte activation molecule (SLAM)-associated protein
$SAP^{-/-}$	SAP-deficient
SCID	severe combined immune deficiency
SH2D1A	Src homology 2-domain 1A
SHIP	SH2-domain-containing inositol-5-phosphate
SLAM	signaling lymphocyte activation molecule
SLE	systemic lupus erythematosus
TCF	T cell factors
TCR	T cell receptors
TNF-α	tumor necrosis factor-alpha
T_H	$CD4^+$ helper T lymphocyte
T_H17	IL-17-producing $CD4^+$ helper T cells
T_{regs}	regulatory T cells
VDJ	variable diverse joining

VSV	vesicular stomatitis virus
VV	vaccina virus
VZV	varicella-zoster virus
XLP	X-linked lymphoproliferative disease

ACKNOWLEDGMENTS

It is my great pleasure to offer my enduring gratitude to the faculty, staff and my fellow students at the University of British Columbia who have inspired me to continue my work in the field of immunology and to everyone who has contributed to this thesis through their generous advice, support and encouragement. I've been very fortunate to form lasting relationships with an incredible number of talented and intelligent people who have offered invaluable advice and inspiration that have made my graduate studies a memorable experience.

I owe particular thanks to my supervisor Rusung Tan for providing excellent scientific guidance throughout my time as a graduate student. I deeply appreciate your generously encouragement, knowledge of immunology and patience. Your mentorship has greatly influenced my understanding of every aspect of research.

I would like to thank all the members of my supervisory committee: Cheryl Wellington, Jan Dutz and Alice Mui, for supporting me over all of these years. Your constructive criticism, encouraging support and patience has been deeply appreciated.

I have been very fortunate to collaborate with a number of fantastic groups during my graduate studies that have provided amazing opportunities for exploring novel avenues of research. I would like to particularly thank: Cox Terhorst at Harvard, Shawn Li at Western Ontario University, Marc Horwitx and Peter van den Elzen at the University of British Columbia.

Research could not be possible without the cooperation, dedication and hard work of past and present members of the laboratory and research institute: Ala Aoukaty, Erica Lee, Qin Ouyang, Jacqueline Trudeau, Tracy Chandler, Justin Lee, Sebastian Ko, Catherine Biggs, Johnny Nie, Hiromi Shimizu, John Priatel, Lynn Huang, Xiaoxia Wang, Rashmi Gupta, Jason Hung, Huilian Qin, Ash Marwaha, Spencer Staiger, Lenka Allan, Dong-Jun Zheng, Lisa Xu, Rosemary Delnavine and a recent addition to the family, Mia Samurai Schnitzel.

I owe a very special thank you to my parents who have given me every opportunity to achieve success and who have always supported my scientific career aspirations.

CHAPTER 1

INTRODUCTION

1.1 The immune system

The immune system is a complex collection of specialized cells that have evolved to recognize and protect against a diverse variety of parasites, fungi, bacteria and viruses that cause disease. In addition to combating foreign pathogens, the immune system discriminates between healthy cells and those that have undergone mutation, eliminating cells that potentially may develop into cancer.

Given the heterogeneity of pathogens and mutated cells, many types of immune cells with unique abilities have evolved to prevent disease. Immune cells are broadly classified as belonging to either the innate or adaptive immune system based on the immediacy of their cellular response, the specificity of their response and whether the responsive cell develops immunological memory following infection. Cells of the innate ('natural') immune system respond quickly after infection and recognize non-specific danger signals also known as pathogen-associated molecular patterns (PAMP) using pattern-recognition receptors (PRR) (1). However, cells of the innate immune system fail to show improved responsiveness when re-challenged with the same pathogen and therefore exhibit no immunological memory. By contrast, immune cells that respond later during infection, demonstrate strong specificity and progressively improve their functional response with each successive exposure to the same pathogen are associated with the adaptive ('acquired') immune system. Although cells of the innate and adaptive immune system differ by the aforementioned criteria, cells of each immunological arm utilize similar mechanisms to initiate and modulate immune responses including direct cell-to-cell contact and the secretion of soluble molecules known as chemokines and cytokines. Studies of immunocompromised individuals and in genetically engineered

animal models demonstrate that cells of both the innate and adaptive immune system are critical for the development of optimal immune responses.

1.1.1 Origin of immune cells

All of the specialized cells that comprise the innate and adaptive immune system originally arise from pluripotent common hematopoietic stem cells (HSC) that reside in the bone marrow (2). HSC are distinguished by their unique capacity to self-renew and differentiate into common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). CLP and CMP are oligopotent precursors that give rise to highly differentiated unipotent cells of the innate and adaptive immune system. NK cells, T lymphocytes (T cells) and B lymphocytes (B cells) differentiate from CLP. Macrophages, neutrophils, eosinophils and basophils arise from CMP. Dendritic cells (DC) can differentiate from both CLP and CMP (3).

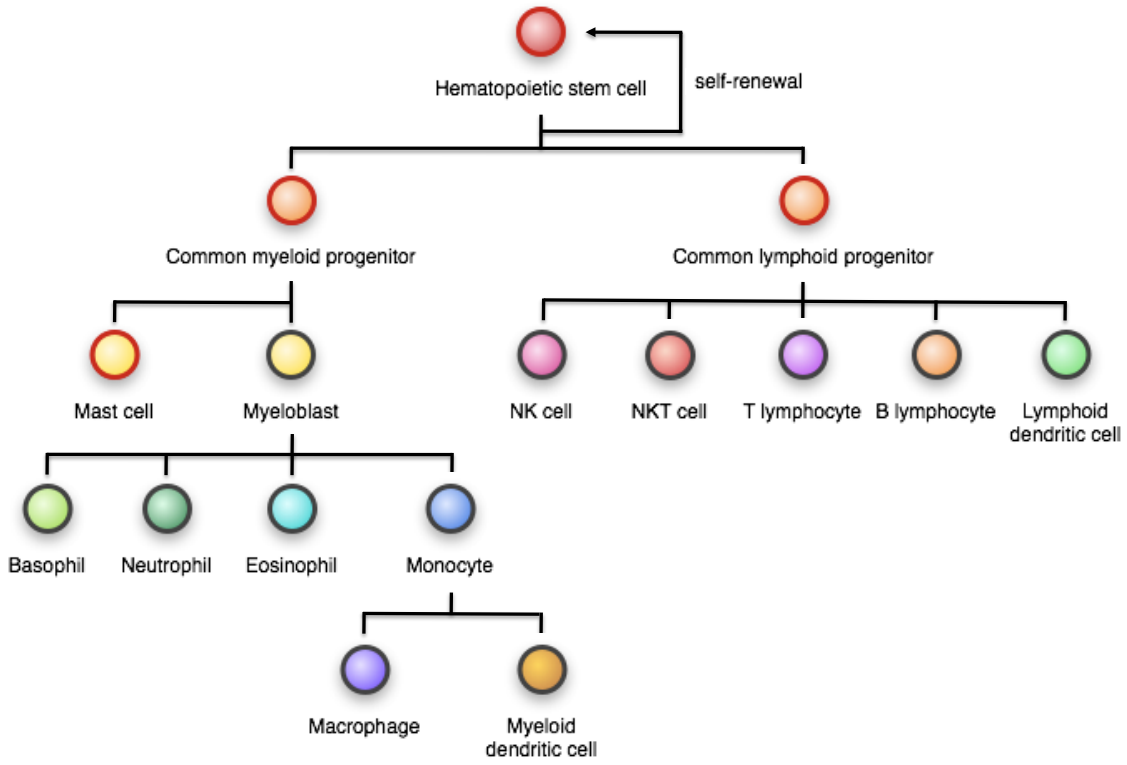


Figure 1.1 Hematopoiesis in humans. Hematopoietic stem cells have the capacity to self-renew and give rise to common myeloid and lymphoid progenitors that differentiate into the specialized cells of the immune system.

1.2 Cells of the innate immune system

NK cells, macrophages, neutrophils, eosinophils, basophils and DC constitute the major cell populations of innate immunity. Innate immune cells are essential for providing the initial defense against pathogens and for triggering adaptive immune responses.

1.2.1 NK cells

NK cells are important for detecting and eradicating virus-infected and transformed cells (4-6). NK cells are constitutively activated *in vivo* enabling them to respond early during the course of infection. Unlike T cells and B cells, NK cells do not express T cell antigen receptors (TCR) or B cell antigen receptors (BCR) on their cell surface. Rather, NK cells express a combination of germline-encoded activating and inhibiting receptors that activate or suppress NK cell effector function. Hence, the activation of NK cells is dependent on the balance of overall signaling between activating and inhibiting receptors.

NK cells are cytotoxic and primed to kill appropriate targets, including virus-infected and transformed cells, via the proximal release of perforin and granzyme proteins. The release of perforin by NK cells was originally thought to induce the formation of pores in the plasma membrane of target cells, thereby allowing granzymes to enter the cytoplasm of target cells and induce apoptosis (7). However, more recent reports suggest that granzymes can induce programmed cell death independently of perforin by directly binding granzyme receptors or forming granzyme complexes that are internalized into the target cell (8). NK cells also have the capacity to secrete large quantities of pro-inflammatory chemokines and cytokines such as interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) that can directly inhibit viral replication, recruit other immune cells to the site of infection and strongly regulate adaptive immunity through the maturation of DC (9).

Following activation, NK cells clonally proliferate and survive for at least several

days. Recent data suggests that some NK cell subsets may have the ability to survive for months and demonstrate immunological memory when re-challenged with the same pathogen (10, 11). These observations suggest that some NK cell subsets may possess abilities that are typically associated with both innate and adaptive immunity but these findings are still preliminary.

1.2.2 Macrophages

Macrophages are long-lived phagocytes and localize to many tissues and organs throughout the body. Macrophages differentiate from circulating monocytes and are recruited by chemokines to the site of infection. Macrophages enter inflamed tissue between the endothelium of blood vessel linings and engulf pathogens as well as dead/dying cells. Macrophages, as well as DC and B cells, are considered professional antigen presenting cells (APC) because they process and present antigens in the context of major histocompatibility complex (MHC, or human leukocyte antigen [HLA]) class II molecules. Antigens presented by MHC class II molecules can promote the expansion of pathogen-specific helper T cell (T_H) subsets that are critical for establishing adaptive immunity (12).

1.2.3 Neutrophils, eosinophils and basophils

Neutrophils, eosinophils and basophils (often referred to as granulocytes) are short-lived phagocytes that migrate within minutes to areas of inflammation. These phagocytes have distinctive multi-lobed nuclei and are collective known as polymorphonuclear

leukocytes (PML). Activation of granulocytes results in the release of their cytoplasmic granules, which contain a milieu of anti-microbial molecules and pro-inflammatory chemokines/cytokines. Cytoplasmic granules of neutrophils, eosinophils and basophiles can be visually distinguished on the basis of histological staining (13).

1.2.4 Dendritic cells

DC are professional APC that process and present peptide antigens on MHC class I and class II molecules at the cell surface. Intracellular proteins including those synthesized by viruses and intracellular-replicating bacteria are processed by DC into short peptides (8-10 amino acids) and presented by MHC class I. These peptide-MHC class I complexes are recognized by $CD8^+$ T cells, a subset of lymphocytes specialized in killing infected cells. Alternatively, extracellular proteins such as those derived from parasites, fungi and extracellular-replicating bacteria are degraded within endosomes into longer peptides (13-25 amino acids) and are presented in the context of MHC class II. T_H cells activated by the appropriate peptide-MHC class II complexes become licensed to promote the differentiation and proliferation of naïve B cells to produce antibodies.

Immature DC are phagocytic and localize in areas exposed to the environment such as the skin, lungs and gastrointestinal (GI) tract. Immature DC express high levels of PRR but relatively low levels of peptide-MHC complexes and the co-stimulatory molecules CD40, CD80 (B7.1) and CD86 (B7.2). Upon PAMP recognition, DC become activated and migrate to lymph nodes, specialized immune organs located throughout the body. Here, mature DC directly interact and stimulate the activation of other immune cells. Due to their high level of MHC and co-stimulatory molecule expression, mature

DC can activate the differentiation and proliferation of naïve $CD4^+$ and $CD8^+$ T cells (14).

1.3 Cells of the adaptive immune system

The adaptive immune system is composed of highly specialized lymphocytes known as B cells and T cells. Immune responses elicited by B cells are referred to as humoral whereas protective immunity elicited by T cells is known as cell-mediated.

1.3.1 B cells

B cells are responsible for generating immunoglobulins (Ig, or antibodies) that bind specific antigens and neutralize the dissemination of foreign pathogens following an infection. Immature B cells differentiate from CLP located in the bone marrow and undergo development in processes referred to as positive and negative selection. Immature B cells that fail to recognize antigen die by neglect whereas those that strongly recognize self-antigen undergo apoptosis or become anergic to prevent the development of self-reactive antibodies. Positive and negative selection ensures that naïve B cells in the periphery are capable of responding to foreign but not to self-antigen thereby mitigating the possibility of autoimmunity.

B cells are defined by the surface expression of unique membrane-bound Ig molecules known as the BCR that capture and signal the recognition of cognate antigen. Every B cell expresses thousands of BCR with identical antigen specificity on their cell surface. By contrast, the antigen specificity of BCR on different individual B cells is

unique meaning that the collective repertoire of B cells has the potential to recognize a wide variety of potential antigens.

The enormous diversity of BCR is a result of the random rearrangement of germline-encoded Ig genes known as antigen receptor gene rearrangement or variable diverse joining (VDJ) recombination (15). VDJ recombination occurs early during B cell development prior to the emigration of B cells from the bone marrow and requires the expression of the lymphocyte-specific recombination-activating gene-1, -2 (RAG1/2) (16, 17). Given that VDJ recombination also occurs in T cells, individuals with impaired RAG1/2 expression have significantly reduced numbers of B cells and T cells and develop severe combined immune deficiency (SCID) syndrome (18).

Activated B cells produce antigen-specific antibodies and can also act as professional APC to stimulate T cell responses. B cells that bind cognate antigen circulate to the draining lymph node where they interact with T cells and differentiate into short-lived antibody-secreting plasma cells. The activation of naïve B cells into plasma cells generally requires two signals, BCR recognition of cognate antigen and co-stimulatory signals from T_H cells. Some cognate antigens can independently activate B cells through the BCR without T_H cell help. However, most BCR-stimulated B cells become anergic without T_H cell co-stimulation and fail to mature into antibody-secreting plasma cells (19).

Five distinct antibody isotypes are produced by plasma cells: IgA, IgD, IgE, IgG and IgM. Each antibody isotype has a unique set of functional properties that are best-suited for neutralizing a specific type of pathogen. The production of a particular antibody isotype is based on the co-stimulation signals received from T_H cells and from

the surrounding cytokine milieu. Plasma cells are short-lived and survive two to three days but a fraction of antibody-secreting cells mature into long-lived antigen specific memory B cells. These cells persist for the lifetime of the individual and respond vigorously when re-exposed to cognate antigen.

1.3.2 T cells

T cells also express antigen-specific receptors called TCR but do not produce antibodies and instead play a central role in cell-mediated immunity. Unlike B cells that undergo selection in the bone marrow, immature pre-T cells (or thymocyte precursors) migrate from the bone marrow to the thymus to undergo positive and negative selection. Analogous to B cell development, the process of thymocyte selection eliminates strongly self-reactive T cells and allows T cells capable of responding to foreign antigen to survive. The development of T cells is defined in stages by the expression of the glycoproteins CD4 and CD8 on the cell surface. Thymocytes that successful complete positive and negative selection in the thymus, migrate into the periphery as either single positive $CD4^+$ or $CD8^+$ T cells.

1.3.2.1 $CD4^+$ helper T cells

$CD4^+$ T cells are referred to as T_H cells and are responsible for establishing and optimizing immune cell responses during infection. Like all naïve T cells, T_H cells require two signals to become activated: first, TCR on the plasma membrane must encounter cognate peptide-MHC class II molecules presented on the surface of APC such

as DC, B cells or macrophage; second, the co-stimulatory molecules CD28 (expressed on T cells) and CD80 and CD86 (expressed on APC) must interact and provide secondary signaling. When naïve $CD4^+$ T cells receive both signals, T_H cells will secrete the growth factor interleukin (IL) -2 and proliferate. A small proportion of activated T_H cells will mature into memory T_H cells that upon secondary challenge elicit full effector functions without the need of additional co-stimulation.

T_H cell responses are phenotyically categorized by how they modulate the immune response and by what cytokines they produce. Activated T_H1 cells promote cell-mediated immunity important for fighting intracellular pathogens by secreting IFN-γ that triggers the cytotoxicity function of NK cells and $CD8^+$ T cells. By contrast, T_H2 cells release IL-4 that promotes the production of antibodies by plasma cells and inhibits the production of IFN-γ. Several unique T_H cell subsets have been recently identified including: regulatory T cells (T_{regs}) that suppress adaptive immune responses (20), IL-17-secreting helper T cells (T_H17) that are associated with promoting inflammation and autoimmunity (21), and follicular helper T cells (T_{FH}) that mediate the expansion of B cells and the formation of germinal centres (22).

1.3.2.2 Cytotoxic $CD8^+$ T cells

$CD8^+$ T cells are also known as cytototoxic T lymphoctyes (CTL) and play a similar role to NK cells because they recognize and eliminate virus-infected and transformed cells. However, the kinetics, mechanism and outcome of $CD8^+$ T cell activation differs in several ways to NK cells: First, naïve $CD8^+$ T cells show poor cytotoxicity and require TCR recognition of cognate peptide-MHC class I complexes and

CD28 co-stimulation to become effective killers. NK cells do not require priming and have the capacity to kill target cells immediately. Second, because CTL express antigen-specific TCR, CTL are able to discriminate between healthy and infected/transformed cells. NK cells do not possess such a capacity and indiscriminately kill healthy or infected/transformed cells that have decreased or lost the expression of MHC class I molecules. Third, activation of $CD8^+$ T cells results in the creation of memory CTL that are long-lived. By contrast, activated NK cells die relatively quickly and the majority of NK cells exhibit no memory upon re-challenge.

Although the activation of CTL and NK cells differs, both immune subsets kill target cells by releasing perforin and granzymes. Cells targeted by perforin and granzymes trigger apoptotic programmed cell death. CTL also can induce apoptosis in target cells through the upregulation of Fas ligand (FasL), a TNF family member that binds the death receptor Fas (CD95) expressed on target cells. Hence, CTL and NK cells together cooperate to eliminate infected and transformed cells regardless of whether the target cell has maintained or downregulated the expression of MHC class I.

1.4 Innate-like lymphocytes - bridging innate and adaptive immunity

Although innate immunity limits the replication and dissemination of pathogens, sterilizing immunity against most microbes requires the activation of the adaptive immune system. As such, several studies have focused on delineating which cell types are responsible for establishing adaptive immunity with the ultimate goal of developing effective immunotherapies. DC, macrophages and NK cells have been targeted as candidates for immunotherapy as these immune populations respond quickly to signs of

infection and also possess the ability to strongly regulate adaptive immunity. However, DC, macrophages and NK cells lack the expression of antigen-specific receptors that may hamper their ability to generate a robust pathogen-specific response. Several studies have recently identified a number of lymphocyte populations that express antigen-specific receptors but quickly activate their effector functions without priming and co-stimulation. These innate-like lymphocytes which include: natural killer NKT T cells, gamma-delta ($\gamma\delta$) T cells, CD8-alpha-alpha ($\alpha\alpha$) T cells, marginal zone B cells and B1 B cells, may represent evolutionary intermediates that serve to bridge innate and adaptive immunity (23).

Unlike innate immune cells, innate-like lymphocytes express antigen receptors and in contrast to conventional lymphocytes, their antigen receptor often recognizes carbohydrates or glycolipids and not peptides. Innate-like lymphocytes also preferentially reside in the gut epithelium or in specialized niches of organs in higher frequencies (24).

1.5 Natural killer T cells

NKT cells, also known as CD1d-restricted T cells, are a subset of innate-like lymphocytes with phenotypic resemblance to both NK cells and conventional $CD4^+$ and $CD8^+$ T cells. NKT cells recognize lipid antigens presented on the monomorphic MHC class I-like molecule CD1d and are restricted to CD1d expression on double-positive thymocytes for development (25, 26). TCR stimulation of NKT cells activates the rapid release of T_H1 and T_H2 cytokines that has been shown to promote DC maturation and the activation of NK cells and conventional $CD4^+$ and $CD8^+$ T cells.

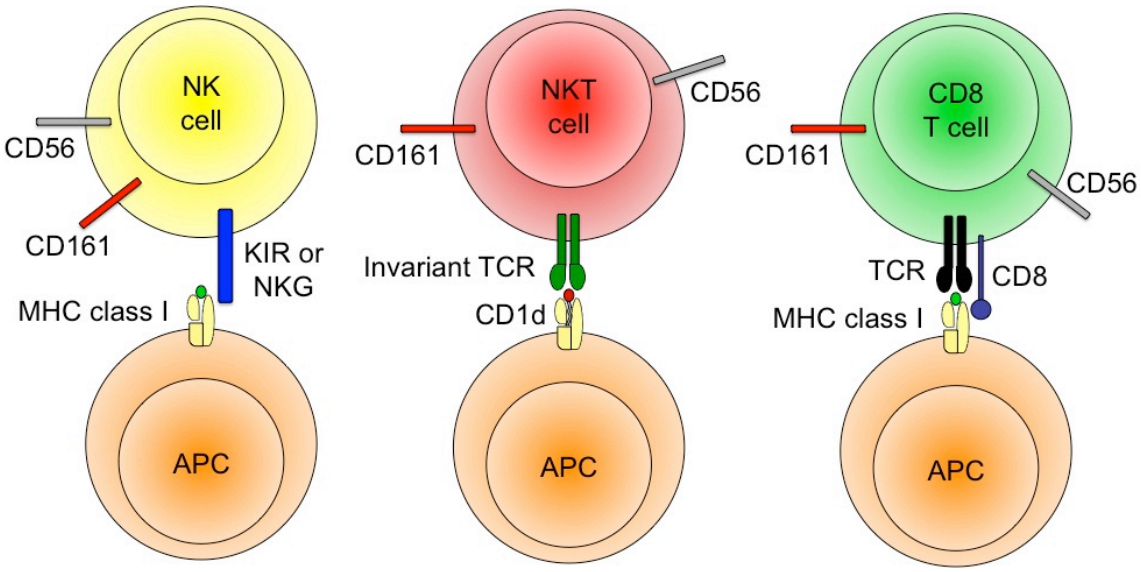


Figure 1.2 NKT cells share phenotypic resemblance with NK cells and CD8$^+$ T cells but differ in their antigen specificity. NKT cells express many of the same surface receptors as NK cells and CD8$^+$ T cells such as CD56 and CD161. In contrast to conventional T cells that recognize peptides presented by MHC molecules, NKT cells express an invariant TCR that recognizes lipid antigens presented by CD1d molecules.

1.5.1 Invariant (type 1) and non-invariant (type 2) NKT cells

Conventional CD4$^+$ and CD8$^+$ T cells express heterodimeric antigen-specific TCR composed of α and beta (β) subunits. Thus, the overall diversity of the TCR repertoire results from a combination of antigen receptor gene rearrangement mediated by RAG-1/-2 and by the stochastic pairing of various αβ chains.

All NKT cells are CD1d-restricted but are delineated as invariant NKT (iNKT, or type 1) cells or non-invariant (type 2) NKT cells based on the usage of TCR αβ subunits

and whether they are capable of recognizing the glycolipid, a potent synthetic NKT cell agonist originally isolated from a marine sea sponge extract (27). Human iNKT cells express an invariant TCR α-chain encoded by Vα24/Jα18 genes paired with the Vβ11 β-chain. Mouse iNKT cells express the invariant TCR α-chain Vα14/Jα18 coupled with Vβ2, Vβ7 and Vβ8 β-chains (28). Both human and mouse iNKT cells universally recognize the αGalCer. Conversely, non-invariant NKT cells utilize a diverse combination of TCR αβ subunits and are not activated by αGalCer. iNKT and non-invariant NKT cells both express NK receptors belonging to the NKR-P or Ly49 gene families, appear activated or memory-like ($CD44^{hi}CD122^{hi}$) (29, 30) and secrete similar cytokines following stimulation *in vitro* (31). iNKT cells preferentially localize to the thymus and liver whereas non-invariant NKT cells are found in greater numbers in the spleen and bone marrow (32).

Table 1.1 Overview of type 1 (invariant) and type 2 (non-invariant) NKT cells

Feature	Type 1 NKT cells (invariant)	Type 2 NKT cells (non-invariant)
TCR αβ-chain usage	Vα24-Jα18 + Vβ11 (humans) Vα14-Jα18 + Vβ2, 7, 8 (mice)	diverse (humans)
Surface receptors	NKR-P or Ly49 NK cell receptors $CD44^{hi}CD69^{hi}CD122^{hi}$	NKR-P or Ly49 NK cell receptors $CD44^{hi}CD69^{hi}CD122^{hi}$
Localization	thymus + liver	spleen + bone marrow
Frequency (% of T cells)	30% in liver, 2-3% in spleen, 0.5% in lymph nodes, thymus, blood	unknown
Lipid activation	αGalCer, microbial glycosphinolipids, GD3, iGb3,	sulfatide
Cytokine profile	IL-4, IFN-γ	IL-4, IFN-γ, IL-13

1.5.2 Development of NKT cells

NKT cells originate from the same pluripotent CLP as conventional $CD4^+$ and $CD8^+$ T cell but differ in their development in many respects. Immature thymocytes that ultimately become conventional $CD4^+$ and $CD8^+$ T cells undergo positive and negative selection against peptide-MHC molecules expressed on thymic epithelial cells. By contrast, positive selection of NKT cells is restricted to the presentation of glycolipids by monomorphic CD1d molecules expressed on $CD4^+CD8^+$ double-positive (DP) thymocytes (33, 34). Mice deficient in CD1d expression ($CD1d^{-/-}$ mice) lack NKT cells (35) but mice that exclusively express CD1d on DP thymocytes have normal NKT cell

frequencies (36). These findings indicate that CD1d expression on DP thymocytes is required for NKT cell development but expendable for homeostasis of NKT cells in the periphery (37).

Negative selection of NKT cells is also CD1d-dependent as the overexpression of CD1d alters TCR β-chain usage and generates less responsive NKT cells (38). Furthermore, CD1d expression on thymic epithelial cells enhances negative selection of NKT cells and cannot promote positive selection (39). The majority of NKT cells that have undergone successful thymic selection migrate to the periphery as $CD4^+$ or $CD4^-$ NKT cells with each subset having distinct functional characteristics (40-42).

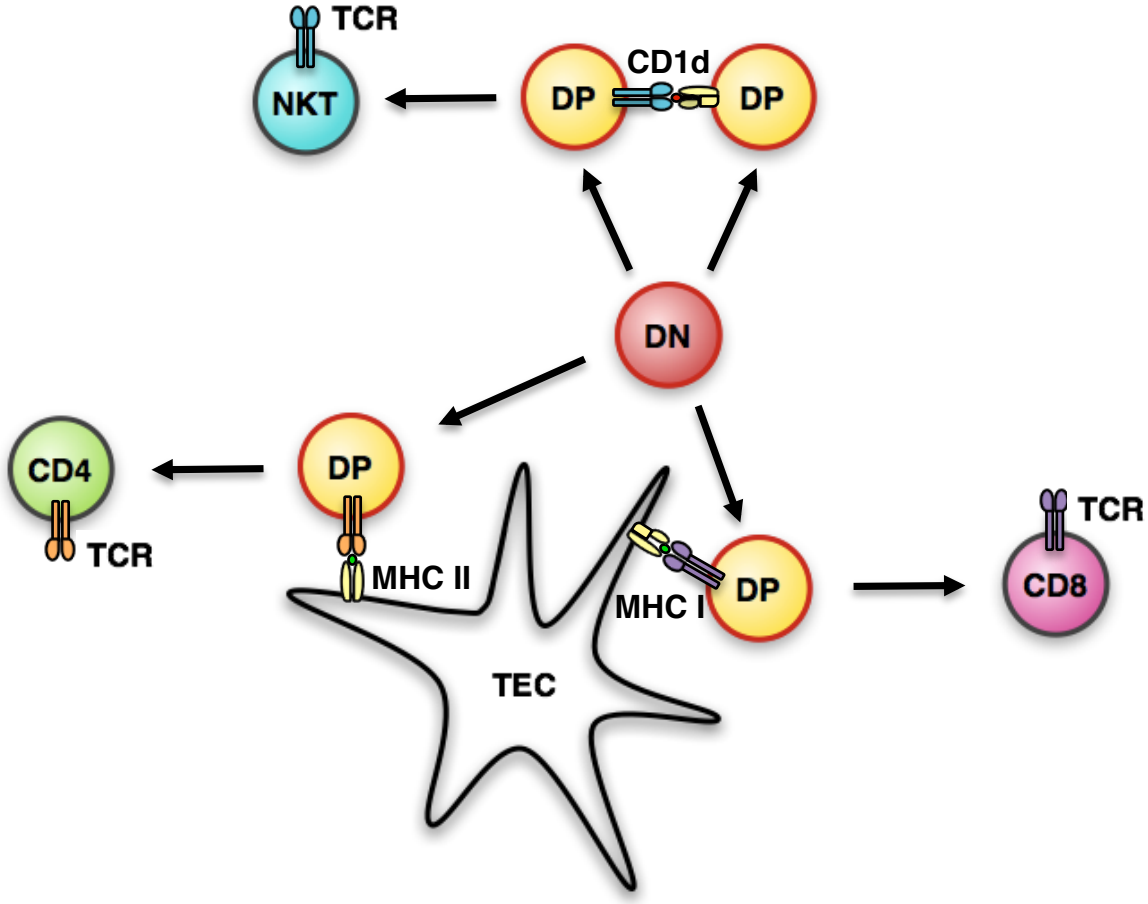


Figure 1.3 Development of NKT cells. Natural killer T (NKT) cells develop in the thymus from $CD4^+CD8^+$ double positive (DP) thymocytes that have undergone random T cell receptor (TCR) gene rearrangement. Conventional $CD4^+$ (CD4) or $CD8^+$ (CD8) T cells undergo positive selection when their TCR binds with the appropriate avidity to peptide–MHC class I or II molecules expressed on thymic epithelial cells (TEC). DP thymocytes that recognize lipid-CD1d molecules expressed on DP thymocytes differentiate into NKT cells.

1.6 CD1d – lipid presentation to NKT cells

CD1d is a member of the CD1 family of antigen presenting molecules whose evolutionary conservation predates the separation between mammals and avian species (43, 44). In humans, the CD1 family contains the five members: CD1a, CD1b, CD1c, CD1d and CD1e, whereas mice express only the CD1d isotype (45). CD1d molecules show structural similarity to MHC class I molecules and are composed of a α-chain associated with a β2-microglobulin molecule. The α-chain of CD1d contains a hydrophobic antigen-binding domain that enables CD1d to present glycoplipids to the canonical TCR of NKT cells (33, 46, 47).

1.6.1 Professional APC express CD1d

Similar to MHC class II, CD1d is preferentially expressed on the surface of professional APC in the periphery (48, 49). The molecular pathways involved in regulating CD1d expression on professional APC are poorly defined although several studies have recently identified key players. The E26 transformation-specific (Ets) family of transcription factors, specifically Elf-1 in B cells and PU.1 in DC and macrophage, have been shown to regulate CD1d expression (50). Furthermore, the activation of the transcription factor peroxisome proliferator-activated receptor (PPAR)-γ has also been demonstrated to increase the level of CD1d expression on DC (51). PPAR-γ regulates several key enzymes involved in the metabolism of retinoids into all-trans retinoic acid (ATRA), a ligand for the family of retinoic acid receptor (RAR) transcription factors. Along with PPAR-γ, direct activation of RAR-α in DC also leads to an increase of CD1d

expression (52). These discoveries are significant given that several PPAR-γ and RAR-α synthetic agonists, including rosiglitazone and ATRA, are administered therapeutically and could be inadvertently modulating the activation of NKT cells.

1.6.2 Lipid antigen processing and presentation on CD1d

Lipid antigens presented by CD1d are derived from both endogenous ('self') and exogenous ('foreign') origins. Following synthesis and assembly in the endoplasmic reticulum (ER), CD1d molecules are loaded with self-lipids during their transport to the cell surface and are recycled constitutively between the plasma membrane and the endosomal pathway (53). Self and foreign lipids are exchanged and actively loaded onto CD1d molecules in the late endosome/lysosomal compartment by saposins (54).

The trafficking of CD1d molecules between the plasma membrane and endosomal compartments is highly dependent on the cytoplasmic tyrosine-based sorting motif of CD1d and the association of CD1d with the MHC class II invariant chain (Ii) (55). Mice with truncated CD1d molecules lacking the tyrosine-based sorting motif have reduced NKT cell numbers and impaired NKT cell effector functions (56). Likewise, DC that lack Ii expression but have full-length CD1d show a decreased capacity to activate NKT cells. These data indicate that both the tyrosine-based sorting motif of CD1d and the expression of Ii are required for the physiological presentation of lipid antigens by CD1d.

1.6.3 Endogenous NKT cell ligands

Identifying the endogenous ligands that activate NKT cells in the periphery and

mediate positive selection in the thymus has proven to be challenging. NKT cells are believed to recognize self-lipids because of experiments that showed tumor-derived lipid extracts loaded onto CD1d molecules activated NKT cells (30). Human disialoganglioside (GD3) was the identified as the first self-lipid ligand after mice immunized with GD3 developed GD3-specific NKT cells which secreted IL-4 and IFN-γ (57). In addition to tumor-derived lipids, NKT cells cultured with IL-12 also appear to recognize lipids of self-origin presented by CD1d on DC (58). More recently, human NKT cells have been shown to recognize cholinated lyso-phospholipids, including lyso-phosphatidylcholin and lyso-sphingomyelin (59). Lyso-phospholipids are present at elevated levels during inflammation and may represent a novel class of antigenic self-lipids that alert NKT cells to infectious pathogens.

Despite extensive efforts, the identity of the self-lipid(s) required for the positive selection of NKT cells remains elusive. Isoglobotrihexosylceramide (iGb3), an endogenous lipid lacking in β-hexosaminidase B-deficient mice, had been previously described as the putative endogenous ligand required for NKT cell development (60). However, the validity of this result was controversial, as several lipid pathways require β-hexosaminidase. Moreover, subsequent studies were unable to detect iGb3 expression in the thymus and iGb3 synthase-deficient mice, which specifically lack iGb3, developed normal frequencies of NKT cells (61, 62). Although iGb3 is unlikely to be the ligand responsible for the thymic selection of NKT cells, the absence of NKT cells in β-hexosaminidase B-deficient mice suggest that endogenous lipids within the lysosomal compartment may be putative candidates (63).

1.6.4 Exogenous NKT cell ligands

The exogenous lipid antigens that activate NKT cells originate from various pathogens but share a common α-anomeric linkage between the sugar moiety and alkyl lipid chain. These α-anomeric linkages are thought to distinguish foreign microbial lipids from mammalian, which are only found in the β-anomeric conformation (64). Several exogenous NKT cell ligands been identified from bacteria, including *Sphingomonas* (α-linked galactosylceramide), *Borrelia burgdorferi* (α-galactosyldiacylglycerols) (65-67) and *Mycobacterium* (phosphatidylinositol mannoside) (68). Lipophosphoglycans derived from the protozoa parasite *Leishmania donovani* can also be presented by CD1d and have been shown to activate NKT cells (69).

The most extensively characterized exogenous iNKT cell ligand, αGalCer, was isolated from the marine sea sponge *Agelas mauritianus* because of its strong anti-tumorgenic properties (27). Crystal structure analysis of αGalCer-CD1d complexes revealed that the alkyl chains of αGalCer are buried deep within the hydrophobic pockets of CD1d and that the polar sugar moiety interacts with the α-chain of the canonical NKT cell TCR (70). These findings indicate that unidentified exogenous lipid antigens may interact with CD1d molecules and be presented to iNKT cells in a similar conformational arrangement.

1.7 Function of NKT cells

The synthetic ligand αGalCer specifically activates iNKT cells and has been used extensively as a tool to investigate the function of NKT cells in various immunological

settings. iNKT cells activated by αGalCer become cytotoxic and simultaneously secrete large quantities of cytokines, including IFN-γ and IL-4. Given that NKT cell activation strongly promotes T_H1 and T_H2 immunity, a role for NKT cells has been studied in autoimmunity, cancer and infectious disease (71).

1.7.1 Autoimmunity and NKT cells

The role of NKT cells in autoimmunity disease has been best characterized in the induced-multiple sclerosis mouse model of experimental allergic encephalomyelitis (EAE) mouse and spontaneous non-obese diabetic (NOD) mouse model of human Type 1 diabetes (T1D). EAE-induced $CD1d^{-/-}$ mice and NOD-$CD1d^{-/-}$ mice show an accelerated onset and greater severity of disease compared to wild-type controls with normal NKT cell numbers (72-74). Furthermore, wild-type mice injected with αGalCer are protected against induced-EAE and disease in NOD mice is delayed with a reduced rate of incidence (72, 75-77).

Although these studies indicate that NKT cells can regulate the incidence and severity of autoimmunity, the direct mechanisms of NKT-mediated protection are unclear. IL-4 released by activated NKT cells have been proposed to skew the immune system from a destructive T_H1 cell-mediated response towards a more protective T_H2 humoral immunity (78, 79). However, recent studies in the systemic lupus erythematosus (SLE) mouse model show that NKT cells suppress auto-antibody production and would therefore be considered protective (80). Despite promoting T_H1 immunity, studies have also shown that IFN-γ from NKT cells, as well as NK cells, can delay the onset and

reduce the incidence of diabetes (81, 82). Cytokines released by NKT cells also mediate the maturation of DC, which could suppress self-reactive lymphocytes and lessen autoimmune disease (83, 84). These findings indicate that the activation of NKT cells likely activates several immunomodulatory pathways that synergistically prevent autoimmunity.

1.7.2 Cancer and NKT cells

NKT cells have been studied comprehensively following the discovery of αGalCer as a potent inhibitor of tumor growth. (27). αGalCer treatments prevent spontaneous cancer development in tumor-susceptible mice and chemically-induced tumor development in various mouse models (85). NKT cells may initiate early anti-tumor responses via the large release of IFN-γ that is necessary for the sequential activation and elimination of tumor cells by NK cells and CTL (86-88). Tumor angiogenesis is also inhibited by IFN-γ released by NKT cells suggesting that NKT cells mediate direct anti-tumorgenic responses (89). Interestingly, the absence of NKT cells also permits the survival of more immunogenic tumor cells suggesting some forms of cancer may evade NKT cell immune surveillance during oncogenesis (90).

Early clinical trials using direct intravenous injections of soluble αGalCer have shown limited anti-tumor responses (91). However, recent clinical studies utilizing the adoptive transfer of αGalCer-pulsed autologous DC has proven more promising in the activation of human NKT cells and delaying tumor progression (92, 93). Hence, therapeutically promoting the activation and expansion of NKT cells in cancer subjects

may augment the normal physiological role of NKT cells in preventing tumor formation.

1.7.3 Helminthes, parasites, bacteria and NKT cells

NKT cells promote protective immunity towards a broad range of microbial pathogens including helminthes, parasites and bacteria (94). In many types of infection, NKT cells mediate their protective immune responses through the rapid and vast production of IFN-γ and/or IL-4. During *Schistosoma mansoni* helminth infections, IFN-γ and IL-4 released by NKT cells mediates protective T_H1 and T_H2 immunity (95). Lung infections with *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* bacteria are more severe in $CD1d^{-/-}$ mice and wild-type mice treated with αGalCer show significantly improved bacterial clearance (96, 97). Bacterial glycolipids synthesized by *Sphingomonas* and *Borrelia burgdorferi* and by the protozoan parasite *Leishmania donovani* presented in the context of CD1d directly activate NKT cells (65-67, 69) suggesting that NKT cells may directly mount antimicrobial responses against foreign pathogens. Collectively, these data indicate that NKT cells are involved during infection and may be necessary for optimal immune responses against numerous pathogens.

1.8 Viruses and NKT cells

More recently, a role for NKT cells has been implicated for establishing immunity during viral infection. Several viruses, including coxsackievirus B3 (Huber S et al., 2003), lymphocytic choriomeningitis virus (LCMV) (98), respiratory syncytial virus (RSV) (99), encephalomyocarditis virus (100) and herpes simplex virus (HSV)-1 (101)

activate NKT cells during infection or exhibit an altered course of infection in $CD1d^{-/-}$ mice. The mechanism(s) by which NKT cells recognize viral infections remains poorly understood. Although some microbial lipids directly activate NKT cells, viruses have not been described to encode or synthesize lipids. Thus, NKT cells may indirectly detect viruses following their activation by cytokines released by DC, such as IL-12, which sensitize NKT cells to weak interactions with self-lipids presented by CD1d (58). Cytokines produced by activated NKT cells stimulate the sequentially activation of NK cells and CTL, immune cells that eliminate virus-infected cells (88, 102). NKT cells may also recognize and kill virus-transformed cells as NKT cells are activated by lipid extracts isolated from tumor cells (103). Although the exact mechanisms by which NKT cells sense viral infection are incompletely understood, overwhelming evidence suggests that NKT cells play an important role during the generation of anti-viral immunity.

1.8.1 Viruses and CD1d

Viruses have evolved various strategies of evading the host immune response. One such strategy employed by many viruses is to alter the peptide presentation by MHC class I and class II at the cell surface, which disrupts the activation of $CD4^+$ and $CD8^+$ T cells (104). Analogously, several studies have reported that viruses also modify the surface expression of CD1d, which strongly suggest that NKT cells have a fundamental role in mediating anti-viral immunity.

The earliest evidence showing that NKT cells play a role during viral infection was observed in mice infected with adenovirus, hepatitis B virus and LCMV (98, 105, 106). Thereafter, vesicular stomatitis virus (VSV) and vaccina virus (VV) infection of

mouse CD1d-transfected fibroblasts was shown to alter the intracellular trafficking of CD1d and inhibit NKT cell activation (107). Additionally, the activation of NKT cells was significantly inhibited due to the modification of CD1d surface expression by the viral proteins Nef and modulator of immune recognition (MIR) synthesized by human immunodeficiency virus (HIV)-1 and Kaposi-associated herpesvirus respectively (108, 109). HSV-1 infection also caused a loss of NKT cell activation because CD1d molecules were sequestering by HSV-1 in the lysosomal compartments and were unable to recycle to the plasma membrane (110). Lastly, NKT cells appear to be critical for regulating the replication of varicella-zoster virus (VZV) as a lack of NKT cells was the only immune defect observed in an individual with a fatal case of disseminated VZV infection (111). Together, these data strongly suggest that viruses intentionally inhibit the expression of CD1d to evade NKT cells and suggest that individuals with defective NKT cell development or impaired NKT cell function may be more susceptible to viral infection.

1.9 XLP

David Purtilo originally described XLP in 1975 following the death of several male siblings and maternally related male relatives from what appeared to be severe IM (112). XLP, originally known as Duncan disease, was later identified as a novel recessive X-chromosomal disorder triggered by acute EBV infection and characterized by fulminant IM, aberrant B and T cell lymphoproliferation, and dysgammaglobulinaemia (113). The clinical complications of XLP are not mutually exclusive in affected males as additional manifestations, notably aplastic anemia and systemic vasculitis have also been reported (114-116). Regardless of the clinical presentation, prognosis for individuals with

XLP remains poor with fewer than 30% of affected males living beyond age ten. Current treatment for XLP is mainly palliative although bone marrow transplantation has proven successful but is associated with serious health risks (117-119).

1.9.1 SAP

Most individuals with XLP have germline mutations in SAP, also known as *SH2D1A*, a 128 amino acid intracellular adaptor molecule expressed in NK cells, NKT cells, $CD4^+$ T cells, $CD8^+$ T cells, and some B cell subsets (120-125). SAP is located on Xq25 of the X-chromosome and shares homology with Ewing's sarcoma-associated transcript-2 (EAT2, also known as *SH2D1B*) in humans and with EAT2-related transducer (ERT, also known as *SH2D1C*) in mice (126, 127). EAT2 and ERT are located on chromosome 1 and genetic analysis of EAT2 and ERT suggests these genes evolved by gene duplication. Given that EAT2 mutations have not been reported in XLP patients, the function of EAT2 appears to be dispensable for regulating the immune response against EBV. Additionally, EAT2-deficient and ERT-deficient mice do not recapitulate the clinical or immunophenotype of XLP patients following viral challenge (128). By contrast, $SAP^{-/-}$ mice develop exaggerated $CD8^+$ T cell responses and hypogammaglobulinaemia, resembling those of XLP patients, when infected with the closest known murine EBV equivalent, murine herpesvirus-68 (MHV-68), or LCMV (25, 129-132).

1.9.2 SAP signaling pathways

SAP comprises four exons that encode a single SH2 domain and short carboxy tail. The SH2 domain of SAP interacts with the immunoreceptor tyrosine-based switching motif (ITSM), a conserved phosphorylation sequence [T/S-x-pY-x-x-V/I] expressed in the cytoplasmic region of signaling lymphocyte activation molecule (SLAM) family immunoreceptors (120, 122, 124, 125). Several nonsense, missense, and splice-site SAP mutations have been reported in XLP patients, all of which affect association of SAP and the ITSM of SLAM family members.

The SLAM receptor family contains six immunomodulatory glycoproteins: *SLAM* (also known as CD150), *Ly-9* (CD229), natural killer, T and B cell antigen (*NTB-A* in humans, *Ly108* in mice), *CD84*, CD2-like receptor activating cytotoxic T cells (*CRACC*), and *2B4* (CD244) (133). SLAM family receptors are presented exclusively on the surface of immune cells and express multiple extracellular immunoglobulin-like domains, a transmembrane region and a cytoplasmic domain containing tyrosine-based motifs. SLAM family members form homotypic receptor-ligand complexes with the exception of 2B4, which binds CD48 (125, 134, 135). Mice that lack the expression of SLAM family members show decreased neutrophil, NK cell, $CD4^+$ and $CD8^+$ T cell function, particularly in regard to cytokine production and cytotoxicity (125). Mice deficient in a single SLAM family member fail to show marked defects in immune cell function suggesting that receptors of the SLAM family may signal using similar pathways (120).

In healthy individuals, homotypic interactions between SLAM family members activate the intracellular phosphorylation of ITSMs and the recruitment of SAP (124). Binding of SAP to SLAM family receptors selectively recruits Fyn, a membrane-bound

tyrosine kinase (136, 137). Fyn phosphorylates the cytoplasmic domain of the SLAM family receptors and activates additional protein complexes essential for downstream signaling (137, 138). Fyn activation in T cells results in activation of Ras-GTPase-activation protein (RasGAP) and nuclear factor κB (NF-κB) whereas Fyn expression in NK cells recruits SH2-domain-containing inositol-5-phosphatase (SHIP) (136, 139-141). SAP also recruits PAK-interacting exchange factor (PIX), a Rac GTP exchange factor important for cell motility (142). Thus, SAP-mediated recruitment of Fyn initiates several signaling pathways involved in the production of cytokines, activation of cytotoxicity and upregulation of cell adhesion molecules (133).

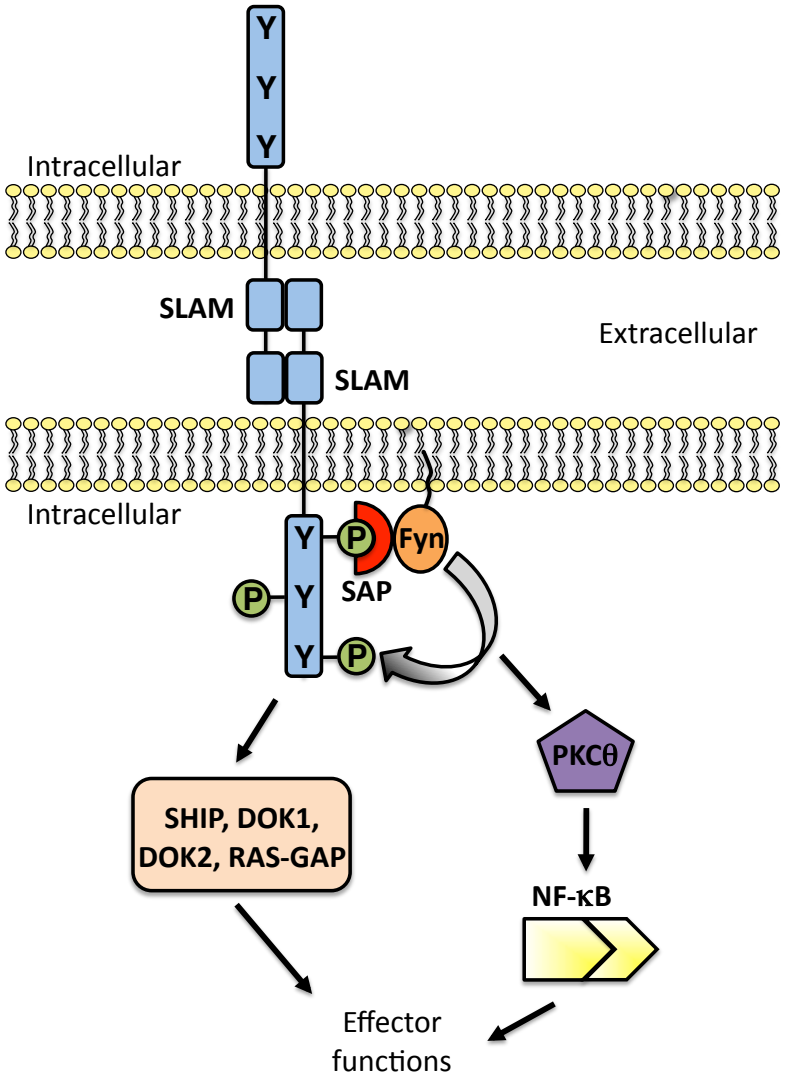


Figure 1.4 SAP signaling pathways. Homotypic interactions between signaling lymphocytic activation molecule (SLAM) receptors stimulates the recruitment of SLAM-associated protein (SAP) and the activation of Fyn tyrosine kinase. SLAM tyrosine residues (Y) phosphorylated by Fyn recruit downstream signaling adaptor proteins, including SH2-domain-containin inositol-5' phosphatase (SHIP), docking protein 1 (DOK1), DOK2 and RAS-GTPase-activating protein (RAS-GAP). SAP also activates the translocation of protein kinase C-θ (PKCθ) to the plasma membrane and nuclear factor-κB (NF-κB) into the nucleus.

1.9.3 Loss of SAP expression in NK cells and CTL

The disruption of the SLAM receptor family-SAP-Fyn signaling cascade in XLP subjects is associated with a number of immune cell defects. NK cells and CTL isolated from XLP subjects show impaired cytotoxicity against autologous B cells infected with EBV *in vitro* suggesting that the clearance of EBV-infected B cells *in vivo* may be reduced (143-145). Given that NK cells and CTL are important for anti-viral immunity, the impaired killing capacity of these subsets likely contributes to the accumulation of virus-infected B cells in XLP subjects. Poor killing of infected B cells may prolong NK cell and CTL activation resulting in overt proliferation and excessive cytokine secretion.

Experiments performed with $SAP^{-/-}$ mice have proven inconclusive in determining whether defects in NK cells and CTL cytotoxic function promote heightened lymphoproliferation following viral challenge *in vivo*. Similar to XLP patients, NK cells and CTL isolated from $SAP^{-/-}$ mice show reduced killing efficacy *in vitro* when compared to cells from wild-type mice (146). Paradoxically, $SAP^{-/-}$ mice infected with MHV-68 or the Armstrong strain of LCMV recover more quickly from viral challenge compared to wild-type controls. The accelerated recovery of $SAP^{-/-}$ mice correlates with a significantly larger $CD8^+$ T cell response and lower viral titres compared to wild-type mice suggesting that $CD8^+$ T cells lacking SAP expression are functionally competent *in vivo* (129, 132). Furthermore, $SAP^{-/-}$ mice challenged with the more virulent LCMV clone 13 strain develop severe immunopathology that is abrogated by depleting $CD8^+$ T cells prior to infection (130). Again, these findings indicate that $SAP^{-/-}$ CTL are functional but suggest that their abnormal proliferation leads to chronic inflammation and a dysregulated immune response.

1.9.4 SAP is required for the differentiation and function of $CD4^+$ T cells

In addition to impaired CTL responses, XLP subjects have significantly reduced numbers of circulating memory $CD4^+$ T cells that express lower levels of inducible T cell co-stimulatory (ICOS, also known as CD278) and produce less IL-10 (147). $SAP^{-/-}$ mice have similar defects in $CD4^+$ T cells including decreased IL-4 and IL-13 production suggesting that SAP is critical for the production of $CD4^+$ T cell cytokines (132, 148). Humoral defects observed in $SAP^{-/-}$ mice, including hypogammaglobinaemia, impaired immunoglobulin isotype switching, loss of peripheral memory B cells and absence of germinal centre formation are restored by the adoptive transfer of wild-type $CD4^+$ T cells into $SAP^{-/-}$ recipients (25, 132, 148-150). Furthermore, targeted deletion of SAP expression in $CD4^+$ T cells or B cells demonstrated that SAP expression in T_{FH} cells is necessary for generating humoral responses whereas SAP expression in B cells is dispensable (131, 151). In the absence of SAP, T_{FH} cells fail to sustain prolong adhesion with B cells, an interaction that is dependent on homotypic interactions between the SLAM-family receptors CD84 and Ly108 (152). Although similar experiments have not been conducted with human T_{FH} cells, XLP subjects also present with equivalent defects in humoral immunity (147, 153). These findings suggest that the effector functions of human $CD4^+$ T cells require SAP expression and that XLP subjects may have impaired function and differentiation of multiple T_H cell subsets.

1.10 EBV

EBV is a member of the human herpesviridae, a large family of DNA viruses that can cause clinical illness in humans during lytic infection. EBV preferentially infects human B cells and epithelial cells, but under some circumstance infects T cells, NK cells and smooth muscle cells (154). EBV specifically targets B cells using glycoprotein (gp) 350, which recognizes CD21 (also known as complement component receptor 2, CR2) expressed on the surface of B cells (155, 156). EBV stabilizes its attachment to the surface of B cells by binding MHC class II via gp42 (157-159). To gain cell entry, EBV activates the core fusion machinery, a complex of three glycoproteins gB, gH and gL that are evolutionarily conserved within herpesvirus family (160). The trimeric gBgHgL complex fuses the viral and endosomal membranes allowing EBV to enter the cell.

EBV-infected B cells express four different profiles of viral gene usage *in vivo*: lytic, latency I, II and III. Virus is continuously produced and released from infected cells undergoing lytic replication (161). Upon infection, resting B cells become proliferating B cell blasts and express viral genes associated with latency III, including EBV nuclear antigen (EBNA) 1-6 and latent membrane protein (LMP) 1 and 2 (162). B cells normally require BCR signaling and co-stimulation from T_H cell subsets. LMP1 and LMP2 allow EBV to mimic both BCR signaling and T_H co-stimulation inducing infected B cells to constitutively proliferate (163, 164). Similar to antigen-activated B cells, EBV-infected B cell blasts migrate to the lymph node follicles and form germinal centers (GC) (165). In these specialized structures, the expression of latent viral genes is reduced (latency II) and infected B cells differentiate into antibody-secreting plasma cells or long-lived memory B cells (166). EBV-infected memory B cells emigrating from the lymph node

turn off viral gene expression (latency I) and persist life-long as circulating resting memory B cells. Some EBV-infected memory B cells re-enter lymph node follicles, turn on LMP1 and LMP2 expression and undergo lytic replication. In this way, EBV continually generates new resting memory B cells and also has the opportunity to infect new individuals.

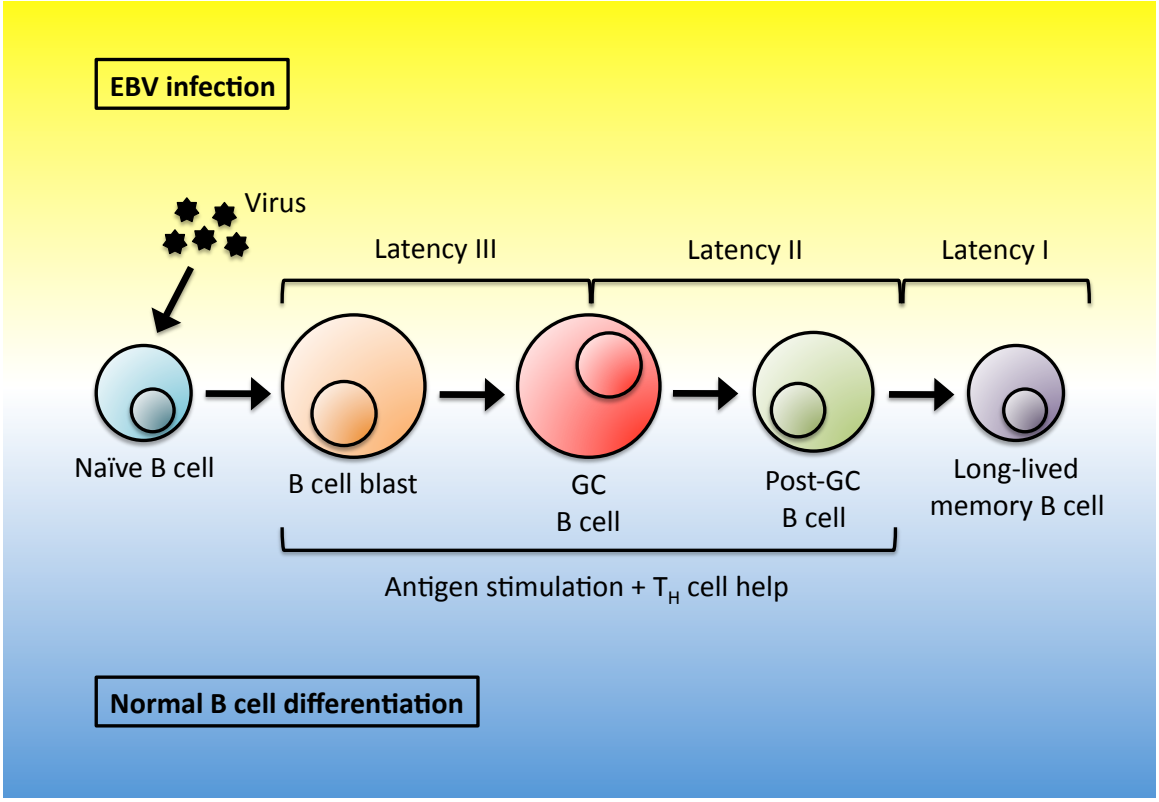


Figure 1.5 EBV infection induces B cell differentiation. Resting B cells infected with EBV become activated B cell blasts (Latency III), migrate to the lymph node follicles and form germinal centers (GC). EBV-infected GC B cells (Latency II) mature into long-lived circulating B cells (Latency I). Normal differentiation of B cells requires appropriate antigen stimulation and co-stimulatory help from T_H cells.

1.11 EBV and XLP

In healthy individuals, EBV infection is generally self-limiting and viral replication is controlled early by NK cells and later by EBV-specific CTL and neutralizing antibodies (167-169). However, individuals with SAP mutations develop XLP, which is characterized by severe IM and EBV-associated lymphomas and carcinomas (112, 170). XLP subjects have impaired NK cell and CTL effector function and these immune subsets are critical for eliminating virus-infected cells. Moreover, individuals with XLP show poor antibody production and Ig isotype class switching but these B cell defects are rescued in $SAP^{-/-}$ mice that receive an adoptive transfer of wild-type naïve $CD4^+$ T cells. These findings indicate that in addition to NK cell and CTL effector function, SAP expression is critical for the differentiation of naïve $CD4^+$ T cells into mature T_H cells *in vivo*. Interestingly, despite multiple defects in NK cell, CTL and T_H cell function, individuals with XLP appear immunological normal prior to EBV exposure. Why XLP subjects demonstrate a unique inability to control EBV infection remains poorly understood.

1.12 Thesis objective and specific aims

A role for SAP in the activation of immune cells was strongly suggested by the initial observations that SAP associates with intracellular domain of the activation immune receptors SLAM (171, 172) and 2B4 (173), and that individuals lacking SAP expression (XLP) develop an uncontrolled proliferation of B cells and T cells following infection by EBV. SLAM and 2B4 are members of the SLAM family of immune receptors and both are highly expressed on the surface of NK cells and $CD8^+$ T cells

(125). NK cells and $CD8^+$ T cells are also important for regulating EBV replication (174); hence, most SAP studies have focused on the effects of SAP expression on NK and T cell signaling and function.

Several groups have demonstrated that SAP mutations lead to NK and T cell dysfunction in patients with XLP (173, 175, 176). NK cell deficiencies include impaired cytotoxicity and IFN-γ secretion that clearly manifest when NK cells are stimulated through 2B4 (177). XLP subjects also have significant T cell defects including poor $CD4^+$ T cell differentiation, reduced IL-10 secretion and defective $CD8^+$ T cell cytokine production and killing (125, 126).

In T cells, SAP specifically recruits Fyn tyrosine kinase to the cytoplasmic domain of SLAM family receptors (121, 123, 124, 136, 140, 178). Fyn induces the phosphorylation of SLAM family receptors, thereby enabling the activation of RasGRP and ultimately the nuclear translocation of NF-κB (137, 138). Signaling by SLAM family receptors provides naïve T cells with the co-stimulation required for the optimal activation of $CD4^+$ and $CD8^+$ T cell effector functions (125). In addition, SAP mutations disrupt SLAM-SAP-Fyn signaling transduction in T cells and may account for many of the immune system abnormalities seen in the clinical course of XLP.

Despite making significant progress towards determining the many distinct molecular roles of SAP, an intriguing question still remains: *why does EBV specifically elicit XLP in boys lacking SAP expression?* EBV primarily infects human B cells but the lack of SAP expression within B cells does not appear to significantly impact B cell function or differentiation intrinsically. In humans, SAP expression is limited to a

fraction of B cells (179)and in mice, SAP is undetectable in most B cells (151). Furthermore, the adoptive transfer of wild-type T cells into $SAP^{-/-}$ mice ameliorates the B cell defects also observed in XLP patients including Ig isotype class switching, GC formation and memory cell generation (25). Together, these findings suggest that SAP expression is not required for the normal function and differentiation of B cells but that SAP is necessary for the optimal activation and differentiation of immune cells dedicated to regulating EBV replication.

As stated previously, $CD8^+$ T cells are likely to be critical for the regulation of EBV replication as massive expansions of EBV-specific CTL are observed after infection (174, 180, 181). Reports showing that EBV-specific CTL from XLP patients have reduced IFN-γ production and lytic activity (145) implied that defective CTL could be responsible for the abnormal EBV susceptibility of XLP patients. Surprisingly, individuals lacking SAP do not develop lymphoproliferative disorders or show heighted vulnerability to other viral infections including cytomegalovirus (CMV) or human immunodeficiency virus (HIV) which also elicit strong CTL responses (182, 183) and can cause life-threatening infections in individuals with other immunodeficiency syndromes (184). These results suggest that SAP may be critical for the function and/or differentiation of additional immune subsets that are critical for controlling EBV replication. Another possible explanation is that boys with SAP mutations encounter EBV first because of the early and ubiquitous nature of the virus, and that they might also develop abnormal lymphoproliferative responses if and when the encountered pathogens such as HIV. Although this latter hypothesis warrants further investigation, we believe that in addition to its described role in NK and T cells, SAP may also be necessary for the

function and/or differentiation of other immune cells that play a critical role in controlling EBV replication.

iNKT cells are innate-like lymphocytes that can regulate the expansions of EBV-specific lymphocytes (185). Whether iNKT cells directly control EBV-infected B cells or whether SAP is required for iNKT cell function is currently unknown. iNKT cells may be critical for the recognition of EBV-infected B cells because an absence of iNKT cells leads to abnormally vulnerabilities to viruses including a fatal case of disseminated VZV infection (111) and EBV-associated lymphoproliferation (186). Furthermore, XLP patients may have reduced numbers or completely lack iNKT cells as SAP directly interacts with Fyn (136) and Fyn-deficient ($Fyn^{-/-}$) mice have severely reduced NKT cell numbers (187).

The overall objective of this project is to determine the role of SAP on iNKT cell development and characterize the role of iNKT cells in regulating EBV immune responses. We hypothesize that SAP is critical for the function and/or development of NKT cells and that the unique susceptibility of XLP subjects to EBV infection reflects defects in NKT cell development and/or impaired NKT cell responses.

To address this hypothesis, we propose the following specific aims:

1. To determine the role of SAP on iNKT cell development by analyzing the frequency and number of iNKT cells in the animal model of XLP ($SAP^{-/-}$ mice) and in peripheral blood samples collected from XLP subjects.

2. To study the expression of the iNKT cell-specific lipid antigen-presentation molecule, CD1d, following infection by EBV.

3. To develop an experimental *in vitro* EBV infection model that can be used to investigate the role of human iNKT cells on the regulation of human B cells transformed by EBV.

CHAPTER 2

SAP IS NECESSARY FOR THE DEVELOPMENT OF MOUSE AND HUMAN INVARIANT NATURAL KILLER T CELLS

2.1 Introduction

XLP is a primary immunodeficiency disorder characterized by fulminant infectious mononucleosis and hemophagocytosis following primary EBV infection and in later stages, by chronic hypogammaglobulinemia, and non-Hodgkin's B cell lymphoma (188). These complications are not mutually exclusive and other expressions of XLP disease have also been reported, notably aplastic anemia and systemic vasculitis (114, 116). Regardless of the clinical presentation, the prognosis for XLP patients remains poor with affected males suffering substantial morbidity and mortality often before adulthood. A major breakthrough in understanding the immunopathogenesis of XLP occurred when mutations of the gene, *SH2D1A* were identified to be responsible for disease (171, 189, 190). *SH2D1A* comprises four exons that encode SLAM-associated protein (SAP), an adaptor molecule of 128 amino acids containing a single SH2 domain and a short carboxy terminus. Many *SH2D1A* mutations, including stop codons, truncations and missense mutations all of which affect the SH2 domain, have been described in patients clinically diagnosed with XLP (121, 123, 124, 191).

A role for SAP in lymphocyte regulation has been suggested by the uncontrolled proliferation of T and B cells seen during the clinical course of XLP disease (188) and by the observation that $SAP^{-/-}$ mice generate abnormally large virus-specific $CD8^{+}$ and $CD4^{+}$ T cell populations following infection with LCMV (192). Other evidence that SAP participates in lymphocyte regulation comes from the discoveries that SAP binds to the intracellular domains of SLAM (171) and 2B4 (173), two immune regulatory molecules primarily expressed on T cells and NK cells respectively. Subsequent studies have also confirmed that interaction of SAP with SLAM mediates critical T cell signaling (193),

that SAP-2B4 interactions underlie abnormal NK cell function in XLP (175, 194, 195), and that $SAP^{-/-}$ mice have an inability to generate memory B cell responses (25).

Despite evidence of abnormal lymphocyte signaling and function in humans with XLP and in $SAP^{-/-}$ mice, a role for SAP in NKT cells, a subset of regulatory lymphocytes has not been described. NKT cells share phenotypic characteristics with NK cells and activated or memory T cells (196) and thus might also be expected to express SAP. In addition, NKT cells are important regulators of immunity and autoimmunity in both mouse and human studies (196, 197). Moreover, Ho et al have reported that CD1d-restricted NKT cells act to regulate EBV-specific lymphocyte expansions providing a possible link between NKT cell function and the large T cell expansions seen in XLP patients following EBV infection (198).

Therefore, we sought to determine a role for NKT cells in the immune dysregulation of $SAP^{-/-}$ mice. To our surprise, we observed that $SAP^{-/-}$ mice have a complete absence of CD1d-restricted invariant NKT (iNKT) cells and that $SAP^{-/-}$ mice injected with the potent NKT cell agonist, αGalCer, fail to develop or activate CD1d-restricted iNKT cells that produce IFN-γ or IL-4. $SAP^{-/-}$ mice were also unable to mount ovalbumin (OVA)-specific cytotoxic T lymphocyte (OVA-CTL) responses when co-injected with OVA and αGalCer. Furthermore, in contrast to healthy individuals, two patients with XLP lacked a population of iNKT cells in their peripheral blood. These findings suggest that SAP is critical for normal iNKT cell development and that the absence of iNKT cells may underlie the immunological abnormalities observed in $SAP^{-/-}$ mice and XLP patients.

2.2 Materials and methods

2.2.1 Mice

C57BL/6 mice 6-8 weeks of age were obtained from Charles River Laboratories (Lasalle, Quebec). $SAP^{-/-}$ mice back-crossed to a C57BL/6 background (192) were maintained in a specific pathogen-free facility in the animal care unit of the British Columbia Research Institute for Women's and Children's Health. SAP gene expression in C57BL/6 (wild-type, WT) and SAP-deficient mice was confirmed by PCR and by immunoprecipitations/western blots (Appendix 1). The Animal Care Committee of the University of British Columbia approved the care and use of these animals.

2.2.2 Patient samples

XLP patient peripheral blood samples were obtained from two boys with a mutation within the second exon of SH2D1A (199). Control blood samples were obtained from healthy donors. The UBC Clinical Research Ethics Board approved the collection of samples and informed consent was obtained from all subjects prior to the collection of blood.

2.2.3 Flow cytometry, antibodies, and tetramers

Sample data were collected using a FACSCalibur flow cytometer and analyzed with CellQuest (Becton Dickinson, San Jose, CA). Anti-CD3-FITC, anti-TCRVβ8.1/8.2-FITC, anti-NK1.1-PE, IgG-PE, anti-B220-PerCP, anti-IFN-γ-APC, and anti-IL4-APC mAbs were purchased from BD Biosciences (San Jose, CA). Anti-CD8-FITC was

purchased from Cedarlane (Hornby, ON). PE-conjugated Kb-$OVA_{254-267}$ tetramer was synthesized according to standard protocols. PE-conjugated CD1d tetramer unloaded and loaded with αGalCer were a gift from Steven Porcelli. For immunostaining, single cell suspensions of lymph nodes, spleen, liver, and thymus were resuspended in PBS containing 0.3% BSA and 0.2% Na_3N. CD1d $tetramer^+$ iNKT cells were stained with loaded or unloaded CD1d tetramer for 60 minutes on ice followed by anti-TCRVβ8.1/8.2-FITC and anti-B220-PerCP for 30 minutes. iNKT cells were stained with anti-NK1.1-PE, or IgG-PE and anti-CD3-FITC and anti-B220-PerCP for 30 minutes on ice. OVA-CTL were stained with a PE-conjugated control tetramer or PE-conjugated Kb-$OVA_{254-267}$ tetramer for 60 minutes on ice followed by anti-CD8-FITC and anti-B220-PerCP for 30 minutes.

2.2.4 Intracellular IFN-γ and IL-4 staining

Mouse liver cells were isolated 2 hours following a single intraperitoneal injection of 4 μg of αGalCer (Kirin Brewery, Gunma, Japan). Liver cells were stained with CD1d tetramer as above and processed for intracellular cytokine staining according to the manufacture's protocol (Fixation/Permeablization Kit with Golgistop, BD Biosciences, San Jose, CA). Cells were stained with anti-IFN-γ-APC or anti-IL-4-APC for 30 minutes on ice.

2.2.5 OVA immunization

Mice were injected subcutaneously on days 1 and 8 with 2 μg of αGalCer and 500 μg of chicken OVA grade VII (Sigma-Aldrich, St.Louis, MO). Peripheral blood lymphocytes were co-stained with anti-CD8 and Kb-$OVA_{254-267}$ tetramers on day 14.

2.3 Results and discussion

2.3.1 $SAP^{-/-}$ mice lack iNKT cells

To determine a role for SAP in iNKT cell function, lymphocytes from $SAP^{-/-}$ mice and C57BL/6 littermates were stained with antibodies to CD3 and NK1.1, surface molecules that mark the iNKT cell population in this mouse strain (196). We found that the frequencies of iNKT cells in the lymph node, spleen, liver and thymus of $SAP^{-/-}$ mice were significantly lower than those of C57BL/6 littermates suggesting that $SAP^{-/-}$ mice lack iNKT cells (Figure 1A). However, although the co-expression of NK1.1 and CD3 has been used frequently to identify iNKT cells, these surface molecules fail to exclusively identify CD1d-restricted iNKT cells (200). Therefore, lymph node, spleen, liver and thymus cells were co-stained with CD1d tetramer and antibody to TCRVβ8 (Figure 1B and 1C). These data confirmed that $SAP^{-/-}$ mice have a complete absence of CD1d-restricted iNKT cells.

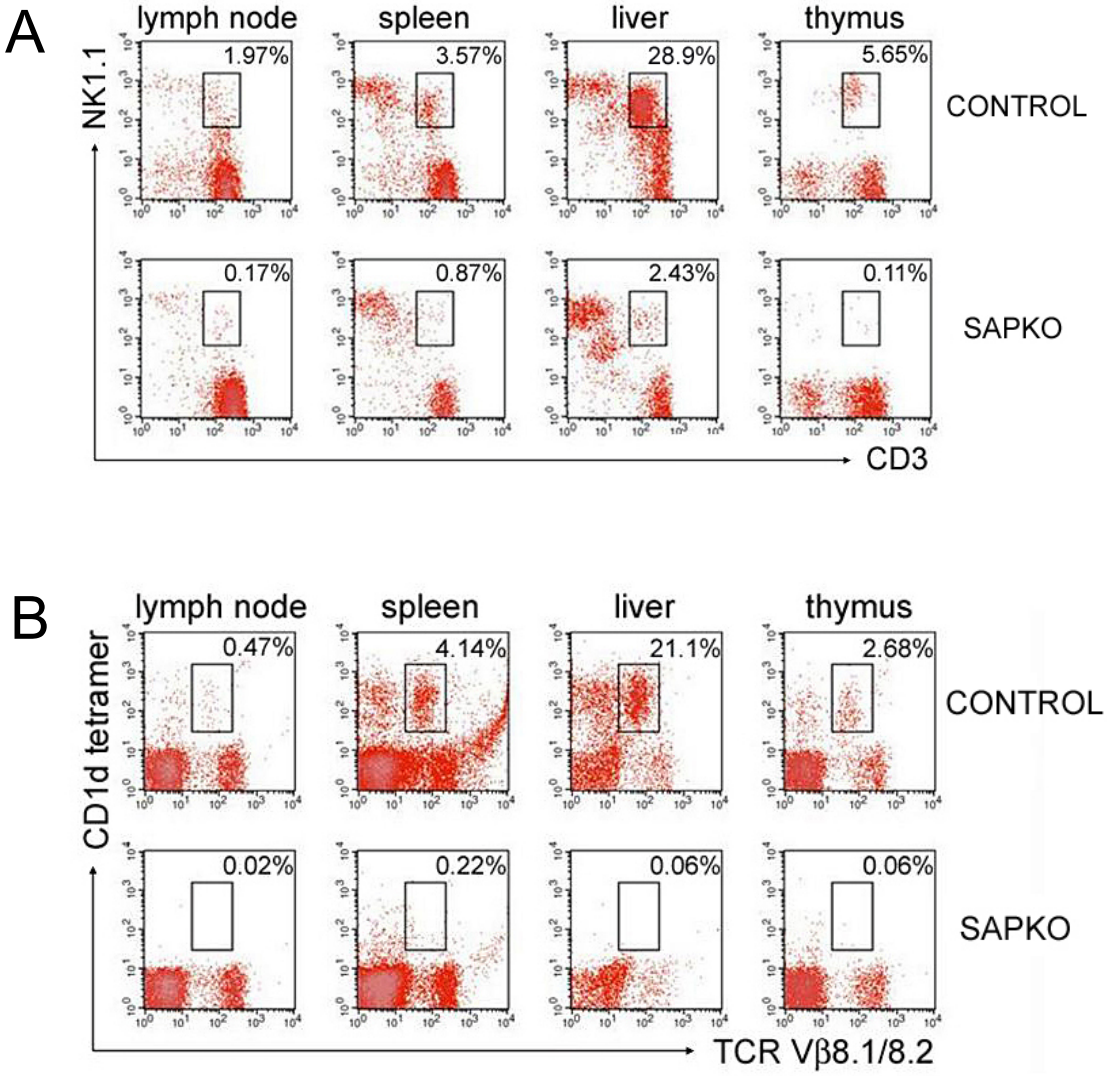

A
lymph node
spleen
liver
thymus
NK1.1
1.97%
3.57%
28.9%
5.65%
CONTROL
0.17%
0.87%
2.43%
0.11%
SAPKO
CD3
B
lymph node
spleen
liver
thymus
CD1d tetramer
0.47%
4.14%
21.1%
2.68%
CONTROL
0.02%
0.22%
0.06%
0.06%
SAPKO
TCR Vβ8.1/8.2
C
LYMPH NODE
LIVER
SPLEEN
THYMUS
Frequency of NKT cells
CONTROL
SAPKO
CD3+/NK1.1+ NKT cells
CD1d-restricted NKT cells

Figure 2.1 SAP$^{-/-}$ mice lack iNKT cells. CD3^{+}/NK1.1^{+} and iNKT cells (CD1d tetramer^{+}/TCRVβ8.1/8.2^{+}) are absent from the lymph node, spleen, liver and thymus of SAP$^{-/-}$ mice (SAPKO) compared to C57BL/6 littermates (CONTROL). Representative FACS plots indicate the frequency of CD3^{+}/NK1.1^{+} [**A**] and iNKT cells [**B**] in the indicated organs. [**C**] Mean percentages of organ-specific CD3^{+}/NK1.1^{+} and iNKT cells (CD1d-restricted NKT cells) in SAP$^{-/-}$ (n=6) and CONTROL mice (n=6) ±SEM are shown, *p<0.05.

2.3.2 SAP$^{-/-}$ mice fail to generate iNKT cell cytokines in response to αGalCer

iNKT cells are known to produce IFN-γ and IL-4 rapidly upon activation and these responses are thought to be important for bridging innate and adaptive immunity (201-204). Given the absence of iNKT cells, we anticipated that these cytokine responses might be absent in SAP$^{-/-}$ mice. To test this hypothesis, SAP$^{-/-}$ mice were given intraperitoneal injections of αGalCer and the liver population of iNKT cells was assayed for production of IFN-γ and IL-4. These cytokines are normally produced rapidly upon αGalCer administration (202, 203). Two hours after a single injection of αGalCer, we observed no production of IFN-γ or IL-4 from the hepatic iNKT cells of SAP$^{-/-}$ mice (Figs. 2A and 2B). In fact, no iNKT cells were seen in SAP$^{-/-}$ mice. In contrast, iNKT liver cells from C57BL/6 mice produced significant amounts of IFN-γ (mean 79.4 ± 3.8%) and IL-4 (mean 26.7 ± 5.3%) following αGalCer administration. These data indicate that SAP$^{-/-}$ mice are unable to mount a rapid iNKT cell cytokine response characterized by production of IFN-γ and IL-4.

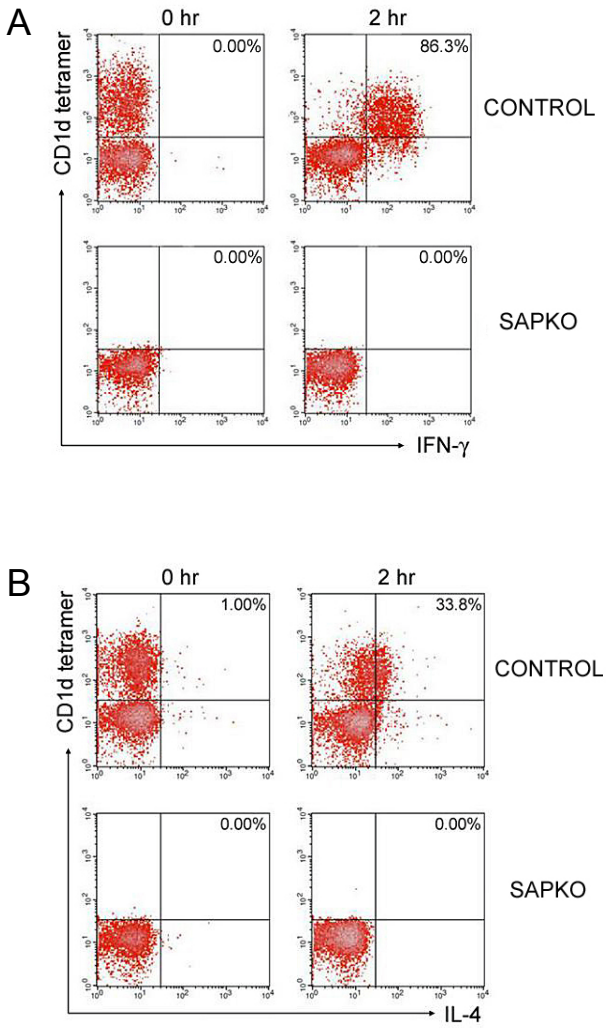


Figure 2.2 SAP$^{-/-}$ mice fail to generate iNKT cell cytokines in response to αGalCer. Hepatic C57BL/6 (CONTROL) and SAP$^{-/-}$ (SAPKO) CD1d-restricted NKT cells (CD1d tetramer^{+}/TCRVβ8^{+}) were isolated 2 hours following intraperitoneal injection of αGalCer and the proportion of IFN-γ [**A**] and IL-4 [**B**] secreting cells was determined.

2.3.3 iNKT-dependent antigen-specific CTL proliferation is defective in $SAP^{-/-}$ *mice*

iNKT cells are functionally important for the generation and regulation of antigen-specific T cell responses. For example, iNKT cells are known to regulate LCMV-induced cytokine production as well as the magnitude of the cell-mediated immune response to an acute viral infection (205). As well, activation of iNKT cells by αGalCer at the time of OVA immunization results in substantial OVA-CTL expansion (206). To determine whether the absence of iNKT cells in $SAP^{-/-}$ mice would affect activation or proliferation of antigen-specific CTL, both $SAP^{-/-}$ and C57BL/6 mice were co-injected with OVA and αGalCer and the OVA-CTL expansion was measured at day 14 using Kb-$OVA_{254-267}$ tetramers. Immunization with OVA and αGalCer produced minimal OVA-specific CTL in $SAP^{-/-}$ mice whereas wild type mice demonstrated significant expansions (Fig. 3). C57BL/6 mice immunized with a control for αGalCer (vehicle alone) and $SAP^{-/-}$ mice injected with OVA but not αGalCer also failed to produce significant OVA-CTL expansions. $SAP^{-/-}$ and C57BL/6 mice immunized with OVA and complete Freund's adjuvant (CFA) produced similar CTL expansions demonstrating that the defective responses seen in αGalCer injected $SAP^{-/-}$ mice were not due to an intrinsic defect in production of antigen-specific CTL. These data indicate that activated iNKT cells promote the generation of antigen-specific CTL and that $SAP^{-/-}$ mice lack the ability to generate CTL in response to immunized antigen and iNKT cell agonist.

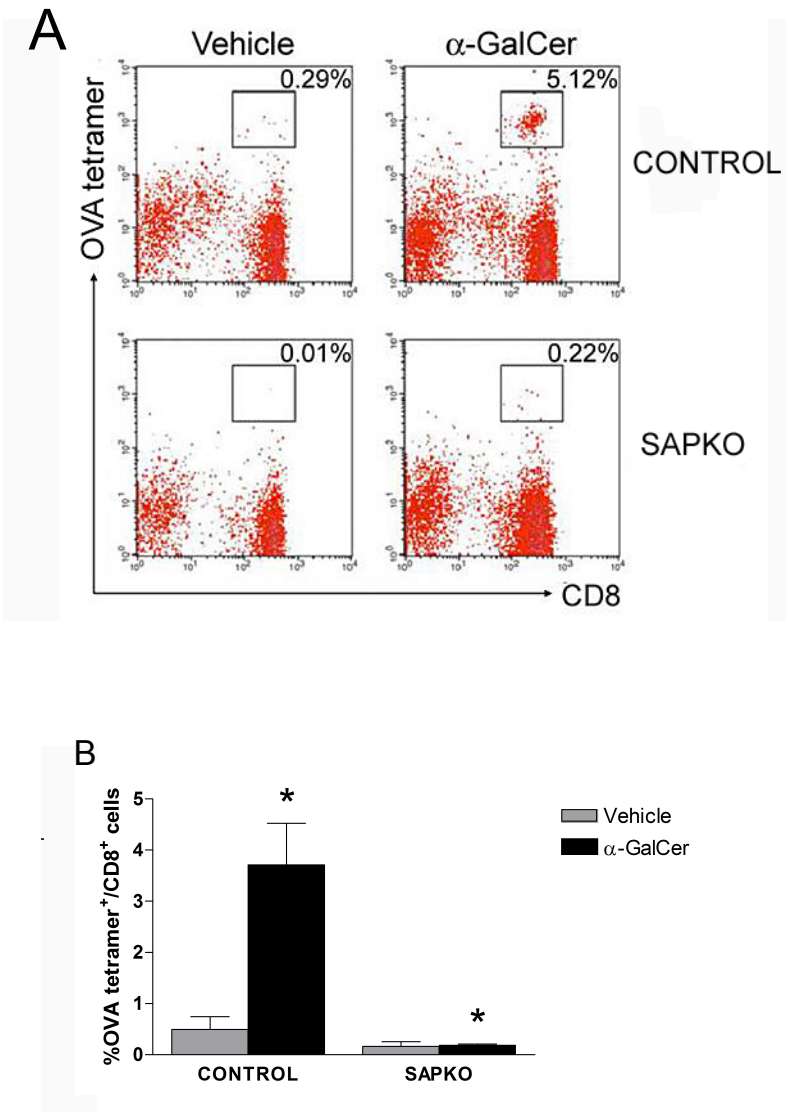


Figure 2.3 iNKT cell-dependent antigen-specific CTL proliferation is impaired in SAP$^{-/-}$ mice. [**A**] Representative FACS plots indicate the frequency of OVA-CTL (Kb-OVA$_{254-267}$ tetramer^{+}/CD8^{+}) in the peripheral blood of C57BL/6 (CONTROL) and SAP$^{-/-}$ (SAPKO) littermates immunized with OVA and vehicle (control for α-GalCer), CFA, or αGalCer. [**B**] Mean frequencies of Kb-OVA$_{254-267}$ tetramer^{+}/CD8^{+} peripheral blood monocytes from immunized CONTROL (n=3) and SAP$^{-/-}$ mice (n=3) ±SEM are shown, *p<0.05.

2.3.4 iNKT cells are absent in XLP patients

To determine whether iNKT cells are absent in XLP patients, two individuals with mutations in the second exon of SAP that are known to produce the clinical phenotype of XLP (199) were studied for the presence of iNKT cells. iNKT cells are normally present at a low but consistent frequency in the peripheral blood of healthy individuals (40, 207). Peripheral blood mononuclear cells from the XLP patients and from eight healthy controls were stained with CD1d tetramer and anti-CD3 to determine the frequency of iNKT cells (Fig 4). A small but reproducible population of CD1d tetramer$^{+/}$CD3^{+} cells was present in the blood of all controls (mean 0.024 +/- 0.005% SEM). These frequencies of iNKT cells are similar to those previously reported (40, 207). In contrast, neither XLP individual had any evidence of iNKT cells in the peripheral blood.

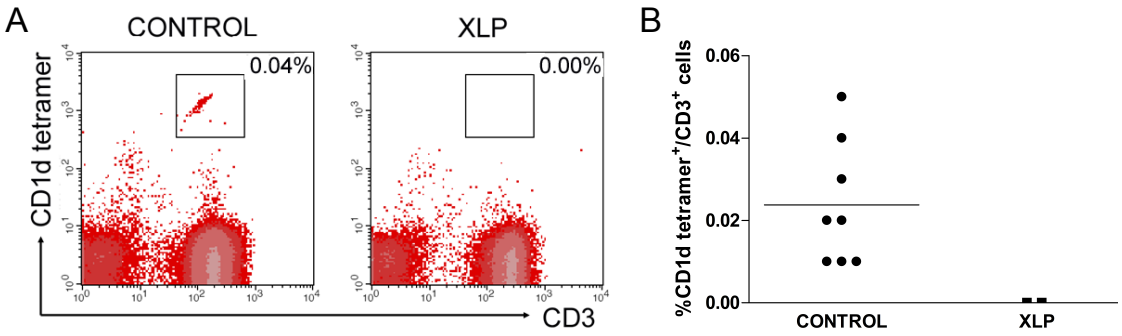


Figure 2.4 iNKT cells are absent in XLP patients. Representative FACS plots compare the frequency of iNKT cells (CD1d tetramer^{+}/CD3^{+}) in the peripheral blood of an XLP patient and healthy control [A]. Healthy controls (n=8, ●) in contrast to XLP patients (n=2, ■) have an infrequent but significant population of iNKT cells in the peripheral blood [B]. The mean frequencies are represented by a horizontal bar, *$p < 0.03$.

Our reported findings indicate that SAP$^{-/-}$ mice and individuals with XLP lack iNKT cells. The broad importance of iNKT cells for immune regulation suggests that the complete absence of this lymphocyte subset underlies some if not many of the pathological immune responses seen in XLP and in SAP$^{-/-}$ mice. For example, iNKT cells bridge innate and adaptive responses by rapidly activating NK cells (204) and by fostering dendritic cell maturation (208-210) and the ability to generate optimal OVA-CTL responses with concomitant αGalCer administration has been directly attributed to the effect of NKT cells on dendritic cell maturation (206). Furthermore, SAP$^{-/-}$ mice infected with LCMV are unable to generate virus-specific memory humoral responses

(25) and the failure to generate long lived plasma cells may reflect a dependence of B cells on iNKT cell factors (79). The requirement for SAP in the development of iNKT cells may be related to the Src family kinase, Fyn as $Fyn^{-/-}$ mice do not develop CD1d-dependent NKT cells (187) and SAP has been shown to be critical for mediating recruitment of Fyn to the SLAM receptor in T cells (136, 140). In addition, $SAP^{-/-}$ mice have been shown to harbor defects in the development of Th2 responses (192) and mice engineered to lack a Fyn kinase binding site on SAP exhibit deficiencies in both IL-4 and IL-13 production (211). These latter defects were attributed to an inability of mutated SAP to bridge Fyn-SLAM association. Therefore, the inability of SAP-deficient mice to generate normal Th2 responses may be related to the inability of Fyn kinase to associate with SLAM family receptors thus leading to abnormal iNKT development.

With regard to the clinical presentation of XLP, we hypothesize that in the absence of the iNKT cell help that is necessary for a rapid NK cell response and for dendritic cell maturation, a sub-optimal anti-viral cellular response occurs. Viral replication would therefore proceed through its early stages unchecked, allowing for a chronic viral state to develop. This model is supported by our clinical observations that boys with XLP have detectable EBV viremia throughout the course of their disease. Viral infection that is not swiftly constrained may lead to the chronic stimulation of virus-specific $CD8^+$ T cells that are unable to efficiently clear virus-infected cells, as in the case of perforin (212) or IFN-γ deficient mice (213).

In conclusion, we show that $SAP^{-/-}$ mice and XLP patients have a complete lack of iNKT cells. The absence of iNKT cells may underlie the virus-induced immune

pathology seen in SAP$^{-/-}$ mice and humans with XLP and SAP must be required for the development of iNKT cells.

CHAPTER 3

INVARIANT NATURAL KILLER T CELLS RECOGNIZE AND KILL B CELLS TRANSFORMED BY EBV

3.1 Introduction

XLP caused by mutations in SAP, is a combined immunodeficiency syndrome characterized by fulminant IM, uncontrolled B/T lymphoproliferation and malignant B cell lymphomas following infection with EBV (214). The observation that both XLP subjects and SAP-deficient mice lack iNKT cells suggests that this subset of innate immune cells might be critical for the regulation of EBV driven lymphoproliferation and EBV transformation of B cells (215-217). Several studies that have documented the importance of iNKT cells in controlling other *herpesvirus* infections support this hypothesis. First, a fatal case of disseminated chicken pox infection due to an attenuated vaccine strain of varicella-zoster virus, also a member of the *herpesvirus* family, has been attributed to a selective loss of iNKT cells (111). Second, mutations of ITK leading to an absence of iNKT cells was linked with EBV-associated lymphoproliferation (186). Third, KSHV and HSV-1 are known to downregulate CD1d, implying that viruses have evolved mechanisms to suppress surface CD1d expression in an attempt to evade recognition and elimination by iNKT cells (109, 110). Although the pathways regulating physiological expression of CD1d are incompletely understood, recent studies with human DC have shown that CD1d transcription is rapidly induced by retinoids (218), a class of vitamin A derivatives regulated by the nuclear retinoic acid receptor-α (RARα). However, the use of RARα-agonists to elevate CD1d expression on virally infected cells has not been previously reported.

Here, we show that EBV transformation of naïve human B cells to LCL results in the downregulation of CD1d expression and loss of iNKT cell recognition. RARα-agonist AM580 induces transcriptional and surface expression of CD1d on LCL, eliciting

iNKT cell effector functions, even in the absence of α-galactosylceramide (αGalCer). These findings suggest that iNKT cells may be critical for controlling EBV replication immediately following B cell infection at a time point when sufficient levels of CD1d are still present. Collectively, our results suggest that iNKT cells could regulate EBV-infected cells through secretion of pro-inflammatory cytokines and by direct cytotoxicity and suggest that RARα-agonists may be used therapeutically to augment NKT cell immune surveillance.

3.2 Materials and methods

3.2.1 Tonsil samples

De-identified tonsil samples were collected from subjects undergoing elective surgery at British Columbia Children's Hospital. Ethics approval was obtained from the University of British Columbia and Children's and Women's Health Centre of British Columbia Review Board (H06-03256). All clinical investigation was conducted in accordance with the principles expressed in the Declaration of Helsinki. Written and oral consents were not required for this study because tonsil specimens were de-indentified with no plan to subsequently link the specimen to an individual subject. Tonsil samples were mechanically homogenized and cell suspensions were isolated by Ficoll-Paque (GE Healthcare) density centrifugation before cryopreservation.

3.2.2 Lymphoblastoid cell lines (LCL) and iNKT cells

LCL were generated by re-suspending tonsillar lymphocytes in EBV marmoset B95.8 supernatant. CD1d-restricted invariant human NKT cell lines BM2a.3, J3N.5 and M0 were maintained in culture as described previously (219).

3.2.3 Antibodies and flow cytometry

The following antibodies were used: anti-human CD1a (HI149), CD1b (M-T101), CD1d (CD1d42), CD3 (HIT39), CD4 (RPA-T4), CD8 (G42-8), CD19 (HIB19), HLA-DR, DP, DQ (TU39) and CD80 (BB1) (all from BD Biosciences). Data was acquired using a FACSCalibur flow cytometer (BD Biosciences) and analyzed by FlowJo 8.7 (Treestar) software.

3.2.4 IFN-γ ELISA and chromium release assays (CRA)

Tonsillar B cells were purified by magnetic bead negative selection (StemCell Technologies) and LCL were treated with 100 nM AM580 (Enzo Life Sciences) for 24 h. Untreated and AM580-treated naïve B cells or LCL were co-cultured at a 1:1 ratio with NKT cells (50,000 cells per well) in 96-well U-bottom plates for 48 h. AM580 (100 nM), αGalCer (10 nM, Kirin Breweries) and blocking CD1d antibody (25 μg/ml, clone 12.1.1.1) (220) were added to NKT cells simultaneously. At 48 h, IFN-γ production was measured using capture ELISA anti-IFN-γ antibody (clone 2G1, Thermo Electron) and biotinylated anti-IFN-γ detection antibody (clone B133.5, Thermo Electron). Untreated LCL and LCL treated with AM580 (100 nM) for 72 h were pulsed with 10 ng/ml

αGalCer overnight. LCL were re-suspended in 125 μCi ^{51}Cr (PerkinElmer) for 1.5 h and ^{51}Cr-labelled LCL (10,000 cells/well) were plated in triplicate to 96-well U-bottom plates. After 4 h, supernatants were measured using a gamma counter (PerkinElmer Wizard). NKT cell specific killing was calculated using the following equation: % specific killing = 100 x (experimental release – spontaneous release)/(maximum release – spontaneous release).

3.2.5 RT-PCR

RNA from C1R cells, C1R-CD1d cells, untreated LCL and LCL incubated for 72 h with AM580 (100 nM) were purified using RNeasy Micro Kits (Qiagen) and cDNA was synthesized using SuperScript First-Strand (Invitrogen). The CD1d and cyclophosphorin A primer sequences have been previously described (221).

3.2.6 Statistical analysis

Statistical significance was calculated using paired Student's *t*-tests (GraphPad Software).

3.3 Results

3.3.1 iNKT cells fail to recognize LCL due to the loss of CD1d

iNKT cells express a restricted subset of T cell receptors (TCR) that recognize hydrophobic lipid and glycolipid antigens presented by the non-polymorphic MHC-like

molecule, CD1d, and are defined by the ability to rapidly execute their effector functions (222). Given the severe IM syndrome that arises in XLP subjects, all of whom lack iNKT cells, we hypothesized that this innate immune subset may play a critical role in the anti-EBV immune responses. To test this hypothesis, we assessed whether naïve B cells or LCL could elicit the activation of three human iNKT cell lines BM2a.3, J3N.5 and M0 (219, 223) in the presence or absence of the iNKT cell antigen, αGalCer (Figure 1A). iNKT cell lines co-cultured with naïve B cells secreted substantial amounts of IFN-γ but only when αGalCer was present. This data indicates that iNKT cells are capable of B cell recognition in the presence of appropriate lipid antigen. By contrast, all three iNKT cell lines failed to secrete IFN-γ when cultured with LCL even in the presence of αGalCer, indicating that immortalization of B cells by EBV impairs their capacity to function as efficient APC for iNKT cells.

Next, we investigated whether the ability of human iNKT cells to respond to naïve B cells, but not LCL, was related to membrane expression of CD1d. Using flow cytometry, CD1d surface levels on LCL were greatly reduced compared to tonsillar B cells that were stained directly *ex vivo* (> 50-fold decrease; MFI: 29.3 ± 1.6 *vs.* 0.55 ± 0.13; Figure 1B). Moreover, CD1d expression on LCL was almost 40-fold lower than naïve B cells that had been cultured for five days (MFI: 21.0 ± 0.76 *vs*. 0.55 ± 0.13). By contrast, the expression of other antigen presentation molecules, MHC class I (HLA-A, -B & -C) and MHC class II (HLA-DR, -DQ & -DP) on LCL, were comparable to levels seen on naïve B cells (Figure 1C) demonstrating that EBV infection and transformation of B cells is associated with selective loss of CD1d expression and abrogation of iNKT cell recognition.

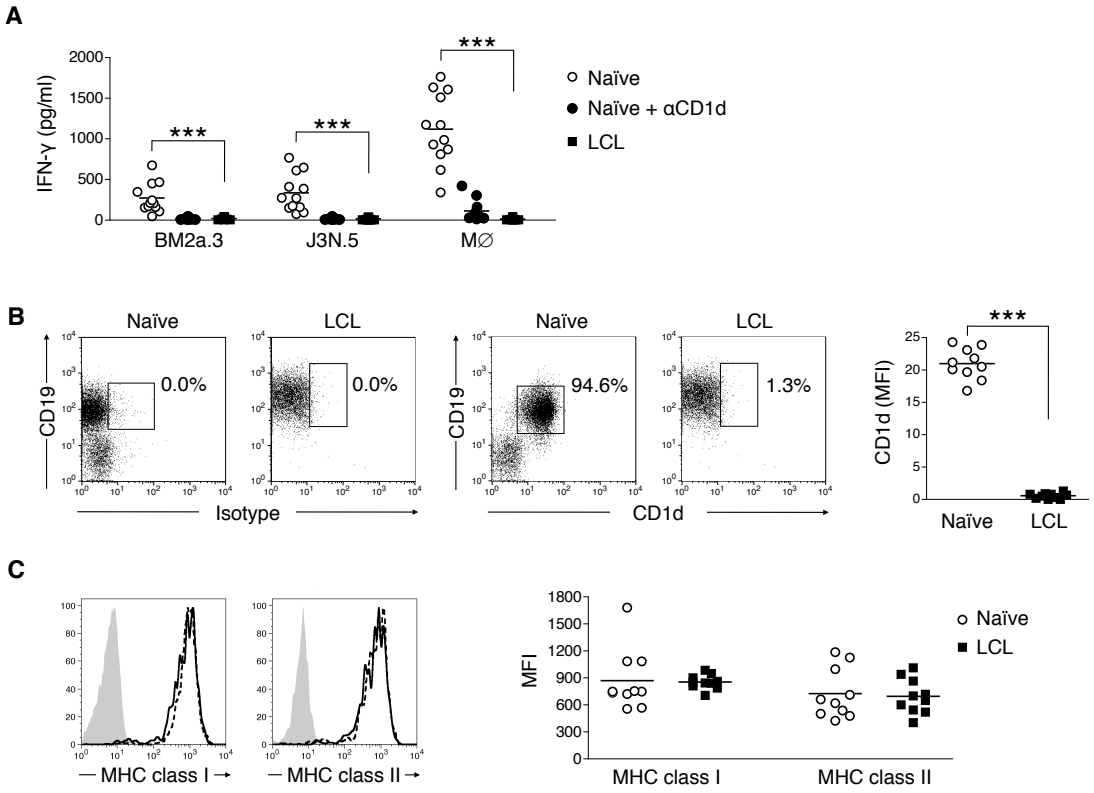


Figure 3.1 LCL downregulate CD1d expression and fail to activate iNKT cells. [**A**] Naïve B cells (○), naïve B cells treated with blocking αCD1d (●) and LCL (■) were loaded with αGalCer and cultured with human iNKT cell lines (BM2α.3, J3N.5, M0) for 48h. IFN-γ produced by iNKT cells was measured by ELISA performed in triplicate. [**B**] Tonsillar mononuclear cells were incubated with or without B95.8 supernatant for 5 days and stained for CD1d expression. Representative CD1d and isotype control staining of naïve B cells and EBV transformed B cells (LCL). MFI values indicate positive CD1d staining above the isotype controls of naïve B cells (○) and LCL (■) generated from unrelated individual tonsil samples, ($n = 10$) ***$p < 0.001$. [**C**] MHC class I (HLA-A, -B, -C) and MHC class II (HLA-DR, -DP, -DQ) expression on naïve B cells (dotted lines) and LCL (solid lines). Isotype control staining is shown as shaded regions. MHC class I

and MHC class II MFI values indicate net staining above the isotype controls for naïve B cells (○) and LCL (■), (n = 10).

3.3.2 RARα-agonist AM580 induces CD1d expression on LCL

The finding that B cells downregulate CD1d following EBV infection and transformation *in vitro* led us to hypothesize that iNKT cells may be important for recognizing newly infected B cells during the brief temporal window prior to loss of CD1d expression *in vivo*. To address this hypothesis, we determined whether iNKT cells could recognize LCL if surface CD1d expression were artificially induced using AM580, an RARα-agonist that has been previously shown to upregulate CD1d transcription and expression on human DC (218). First, CD1d transcription was analyzed in LCL with or without AM580 treatment (Figure 2A). Parental C1R cells (HLA-A and HLA-B negative LCL) and those transfected with CD1d (C1R-CD1d) were used as controls (224). Notably, AM580 induced robust CD1d transcription in LCL, and as expected, C1R-CD1d expressed high levels of CD1d mRNA while message was undetectable in both LCL and parental C1R cells.

Next, we assessed the effect of AM580 on surface CD1d expression of LCL at different time points following exposure. Elevations in CD1d expression were detectable at 24 h and increased steadily until reaching a maximum level at 72 h post-AM580 treatment (Figure 2B). At 72 h incubation with AM580, CD1d levels had increased by 135-fold relative to untreated cells (MFI: 0.31 ± 0.13 *vs.* 42.0 ± 12.8). Hence, RARα activation in LCL induces surface CD1d expression to levels comparable to those seen on naïve tonsillar B cells.

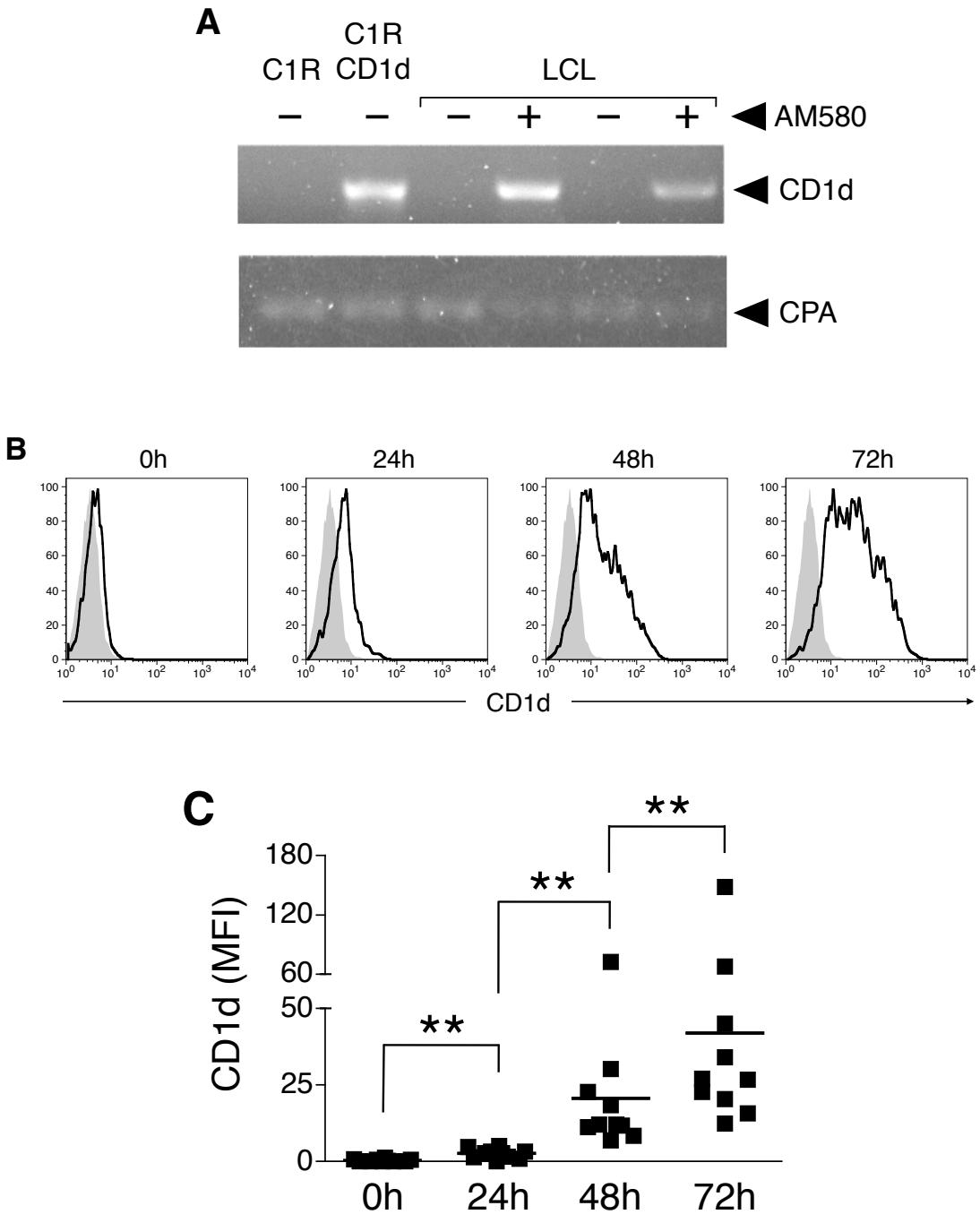


Figure 3.2 RARα-agonist AM580 induces CD1d expression on LCL. [**A**] CD1d transcriptional levels in LCL treated with (+) or without (–) AM580 for 72h was measured by RT-PCR. CD1d-transfected C1R (C1R-CD1d) and C1R cells are shown as positive and negative controls for CD1d expression. Cyclophosphorin A (CSA) was used

as a RT-PCR loading control. [**B**] Representative surface CD1d (solid lines) and isotype control staining of LCL treated with AM580 for 0, 24, 48 and 72h. [**C**] CD1d MFI values (■) indicate positive CD1d staining above the isotype control of LCL generated from unrelated individual tonsil samples, (n = 10) $^{**}p < 0.01$.

3.3.3 Ectopic CD1d expression on LCL activates iNKT cell effector functions

To determine whether CD1d expression induced on the surface of LCL was capable of activating iNKT cells, LCL were treated with AM580 and co-cultured with human iNKT cell lines and αGalCer (Figure 3A). In contrast to untreated LCL, AM580-treated LCL loaded with αGalCer stimulated robust IFN-γ secretion by all three iNKT cell lines tested. Furthermore, the addition of a blocking anti-CD1d antibody suppressed IFN-γ secretion, demonstrating that the iNKT cell activation is dependent on CD1d. Remarkably, AM580-treated LCL induced strong CD1d-dependent production of IFN-γ by iNKT cells even in the *absence* of αGalCer (Figure 3B). These findings indicate that the lack of surface CD1 expression by LCL limits their recognition by iNKT cells.

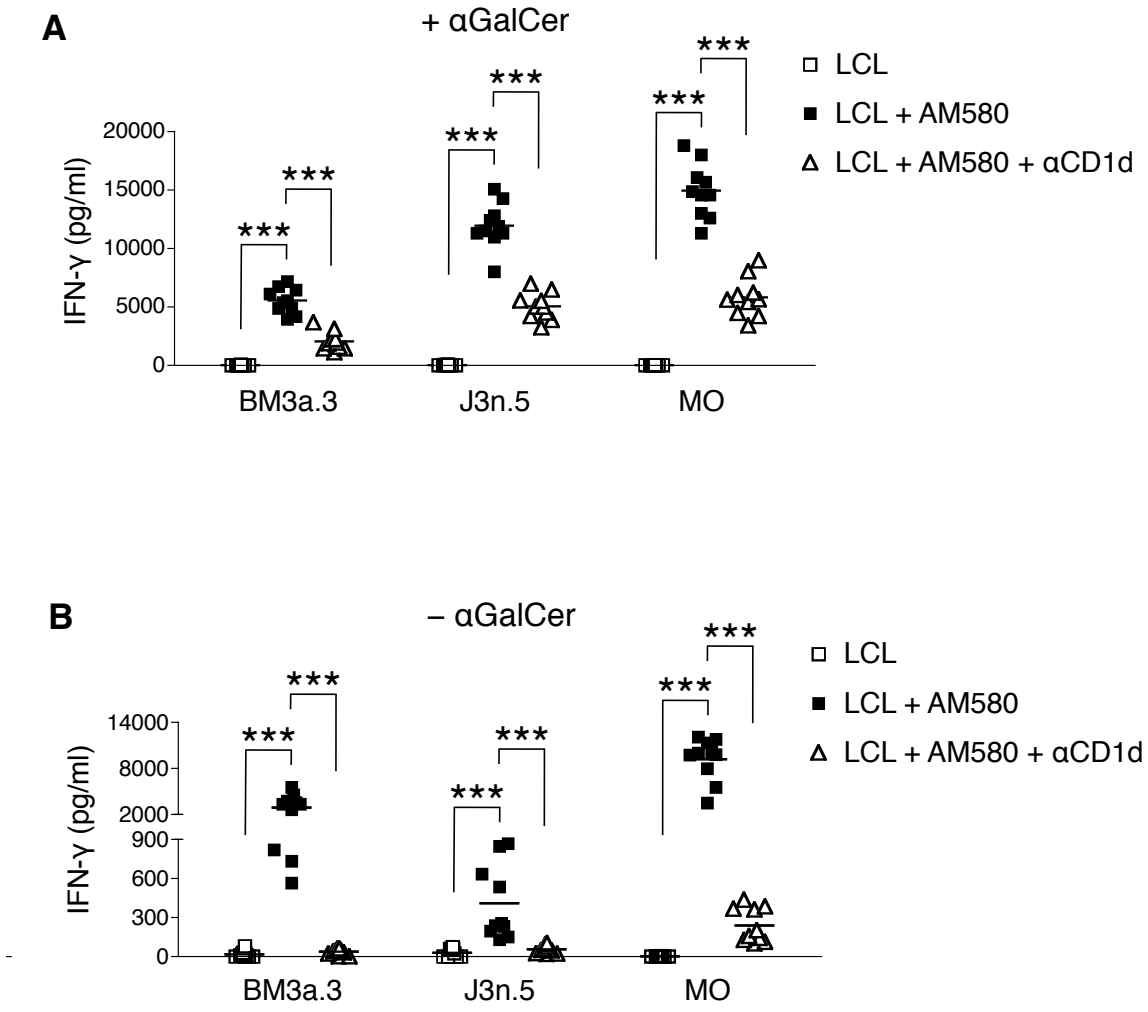


Figure 3.3 Ectopic CD1d expression by LCL activates IFN-γ secretion by iNKT cells. [**A**] IFN-γ production by iNKT cells (BM2α.3, J3N.5, M0) cultured for 48h with αGalCer-loaded LCL (+ αGalCer) pretreated for 24h with (■) or without (□) AM580 and blocking αCD1d antibody (△). [**B**] IFN-γ production by iNKT cells (BM2α.3, J3N.5, M0) cultured for 48h with unloaded LCL (– αGalCer) pretreated for 24h with (■) or without (□) AM580 and blocking αCD1d antibody (△). IFN-γ was measured by ELISA performed in triplicate with LCL generated from unrelated individual tonsil samples ($n = 10$) ***$p < 0.001$.

Finally, we evaluated whether AM580-treated LCL could act as targets of iNKT cell cytotoxicity using standard ^{51}Cr-release assays. All three iNKT cell lines exhibited potent specific killing of AM580-treated LCL that had been pulsed with αGalCer (Figure 4A). By contrast, untreated LCL targets failed to trigger iNKT cell cytotoxicity regardless of whether they had been loaded with αGalCer. Interestingly, AM580-treated LCL incubated with iNKT cells in the *absence* of αGalCer induced low to moderate cytotoxicity by two of the three iNKT cell lines tested (Figure 4B). It is not immediately clear why the different iNKT cell lines exhibited variable killing of AM580-treated targets, but the phenomenon may be attributable to the fine specificity of the TCR and its relative affinity for the glycolipid antigens presented by LCL. Collectively, these experiments indicate that LCL with ectopic CD1d expression are fully capable of activating iNKT cell effector functions and that an endogenous lipid antigen with iNKT cell TCR specificity is induced by EBV infection or transformation.

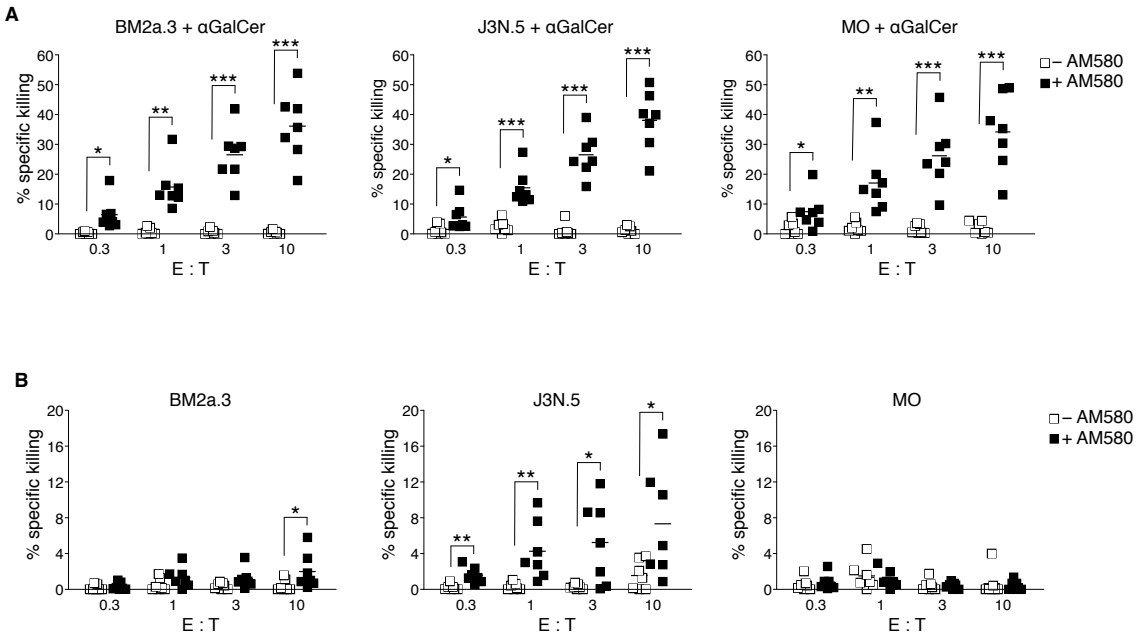


Figure 3.4 iNKT cells kill LCL with induced CD1d expression. [**A**] iNKT cell cytotoxicity against LCL pulsed with αGalCer (+ αGalCer) treated for 72h with (■) or without (□) AM580 and incubated with BM2α.3, J3N.5, M0 for 6h at the indicated effector : target (E:T) ratios. [**B**] iNKT cell cytotoxicity against unloaded LCL treated for 72h with (■) or without (□) AM580 and incubated with BM2α.3, J3N.5, M0 for 6h at the indicated effector : target (E:T) ratios. ^{51}Cr release experiments were performed in triplicate with LCL generated from individual tonsil samples (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001.

3.4 Discussion

The control of EBV replication *in vivo* has been ascribed to natural killer (NK) cells and CTL (154). NK cells, activated by DC, appear to limit the early dissemination of virus through the rapid release of IFN-γ, preventing EBV-transformation of tonsillar B cells (225). In addition to NK cell responses, EBV-specific CTL, whose fundamental importance is underscored by their massive expansions during IM (180) and also by the utility that infusions of virus-specific effector T cells have on EBV-associated malignancies (226), contribute to lifelong control of infection. Several reports have shown that NK cells and CTL in XLP subjects are functionally impaired (143, 144, 227, 228) with Rickinson and colleagues most recently demonstrating that the memory CTL response to EBV is defective in XLP patients who survived initial infection (227).

Our data indicate that a third subset of cytotoxic lymphocytes, iNKT cells, is also likely to play a critical role in regulating the pathogenesis of EBV infection by constraining viral replication during early stages post infection. Several lines of evidence support the proposal that iNKT cells are among the first lymphocyte responders. One, iNKT cells are pre-primed, expressing the activation markers CD69 and CD44, and mobilize rapidly into tissues following antigen exposure (229, 230). Two, iNKT cells provide critical early crosstalk, in the form of IFN-γ release, to NK cells (204) resulting in DC maturation (208). Lastly, we show that iNKT cells must respond to EBV-infected B cells prior to downregulation of CD1d. Thus, we speculate that the early containment of newly infected B cells by iNKT cells may account for the difference between a manageable and an uncontrolled infection, and consequently, humans with rare mutations

leading to a deficiency of iNKT cells have poor outcomes from herpesvirus infections (186).

Retinoids, like AM580, are employed clinically to treat a diverse spectrum of diseases, including the use of ATRA for promyelocytic leukemia(231) and 9-cis retinoic acid for Kaposi's sarcoma (231, 232). The exact mechanisms used by these drugs are not completely known and, in addition to their described roles, may involve the activation of iNKT cells. Our experiments, demonstrating that B cells treated with a RARα agonist were rendered susceptible to iNKT cell-mediated recognition and lysis (Figure 3 and Figure 4), indicate that bolstering iNKT cell responses through enforced expression of CD1d may improve control of EBV. Moreover, these data suggest that the augmentation of NKT cell responses through administration of RARα agonists, either alone or in combination with αGalCer, may prove valuable as adjuvants for stimulating optimal immune responses to EBV and EBV-associated malignancies.

CHAPTER 4

CONCLUSION

4.1 Summary

The recognition and regulation of EBV replication is a dynamic process orchestrated between the cells of the innate ('natural') and adaptive ('acquired') immune system. During viral replication in B cells, PAMP molecules are released and bind to PRR expressed by cells of the innate immune system (233, 234). PRR signaling triggers the effector functions of innate responders including the secretion of immunomodulatory chemokines/cytokines, the production of anti-viral molecules and the phagocytosis of infected cells (4). In addition to mounting the first line of defense against EBV replication, the activation of innate immunity initiates the activation of adaptive immune responses that are largely responsible for the immediate and lifelong regulation of EBV replication (154, 162).

Strong activation of adaptive immunity requires that APC have undergone maturation, a process whereby immature APC upregulate the expression of antigens and co-stimulatory molecules on their cell surface (14). During EBV infection, mature APC that have captured, processed and presented viral antigen migrate to the local draining lymph nodes where they induce the expansion and differentiation of EBV-specific B cells and T cells. Given that cells of the innate immune system recognize early signs of infection and promote the maturation of APC, a rapid and robust innate response is likely critical in the process of limiting early EBV replication and for establishing a timely virus-specific adaptive immune response.

Humans with XLP exhibit a severe and relatively specific vulnerability to EBV infection that is frequently characterized by the development of a life-threatening fulminant IM, uncontrolled B/T cell lymphoproliferation and malignant B cell

lymphomas (235). Individuals with XLP harbor SAP mutations (121, 123, 124) that are responsible for NK, B and T cell defects including impaired cytokine production, lytic activity, Ig isotype switching, memory/long-lived plasma cell generation, and T cell differentiation (122, 125, 126). Although XLP patients present with defects in both innate and adaptive immunity, adaptive immune responses have been extensively studied after EBV infection (236). However, the role of innate immunity remains poorly defined because EBV does not infect nor replicate efficiently in conventional mouse models. In humans, studying early EBV responses is hampered because clinical symptoms associated with EBV infection typically manifest 4-7 weeks after primary exposure, long after innate immune responses have likely occurred (236, 237).

Therefore, to better understand how defects of innate immune cells impact the severity of EBV infection in XLP patients, we established a breeding colony of $SAP^{-/-}$ mice obtained from Cox Terhorst (Harvard University, Mid-Cambridge). Although mice are not permissive to EBV infection, $SAP^{-/-}$ mice infected with LCMV recapitulate many aspects of XLP including the exacerbated proliferation of virus-specific T cells and hypogammaglobulinemia (192), poor memory B cell generation (25), and impaired $CD4^+$ T cell differentiation (132). NK cells from XLP patients show reduced NK cell IFN-γ production and killing (177) but the function of NK cells in $SAP^{-/-}$ mice had not been shown. We broadly hypothesized that NK cells may be critical in the regulation of EBV immunity as NK cells regulate the magnitude of EBV-specific CTL responses (238) and using $SAP^{-/-}$ mice, we investigated the role of NK cells on the generation of antigen-specific CTL responses. However, during the course of our preliminary studies, we observed that a distinct population of $CD3^+NK1.1^+$ cells was absent (Figure 2.1),

particularly in the liver where the expression of $CD3^+NK1.1^+$ lymphocytes predominately corresponds to the immune subset of iNKT cells (28).

To verify these findings, we stained lymphocytes from $SAP^{-/-}$ mice and age-matched wild type controls with the CD1d tetramer that exclusively identifies iNKT cells (229). Similar to our previous results, we found that CD1d $tetramer^+CD3^+$ cells were absent in the lymph node, thymus, spleen and liver of $SAP^{-/-}$ mice (Figure 2.1). Together, these results suggested that SAP is required for the development of iNKT cells at least in mice.

Although our original intention was to investigate the role of NK cells, the serendipitous discovery that $SAP^{-/-}$ mice lacked iNKT cells led us to believe that iNKT cells would also be absent in XLP patients and that iNKT cells may ultimately affect the early regulation of human herpesvirus EBV replication in humans. Our hypothesis was supported by several findings: one, a fatal case of disseminated VZV human herpesvirus infection was linked with a specific loss of iNKT cells (111), two, a pair of consanguineous girls who died from EBV-associated lymphoproliferation lacked iNKT cells (186), and three, surface expression of CD1d was lost/altered upon infection by KSHV and HSV-1 human herpesvirus infections (109, 110).

Because XLP is a rare disease and human experiments are limited in their scope, we sought first to better characterize the absence of iNKT cells in $SAP^{-/-}$ mice. We injected $SAP^{-/-}$ mice with the NKT cell agonist αGalCer and failed to detect IFN-γ or IL-4 production whereas wild-type controls produced significant levels of both cytokines (Figure 2.2). To examine whether the loss of NKT cells could affect the activation of

adaptive immune responses, we co-injected $SAP^{-/-}$ mice and wild-type controls with αGalCer and OVA (Figure 2.3). Unlike wild-type controls, $SAP^{-/-}$ mice failed to mount antigen-specific CTL responses when immunized using an NKT cell-specific adjuvant. However, SAP is not intrinsically required for the proliferation of antigen-specific CTL as $SAP^{-/-}$ and wild-type mice co-injected with CFA and OVA generated similar expansions of antigen-specific CTL. Taken together, these data suggest that the early activation of NKT cells may promote the expansion of antigen-specific immunity.

To determine whether SAP is also required for iNKT cell development in humans, we collected peripheral blood samples from XLP subjects and healthy individuals and assessed the presence of iNKT cells (Figure 2.4). We identified a small but distinguishable population of iNKT cells in healthy controls but were unable to detect any such population in XLP individuals. Although the statistical power of our analysis was limited by the availability of XLP samples, our conclusions were strongly corroborated by two concurrent studies that also observed that iNKT cells were near-absent in a combined total of 25 XLP patients (216, 217). Furthermore, X-chromosome inactivation analysis of a female XLP carrier determined that the mutated SAP allele was inactivated in all of her iNKT cells (216). Together, these data confirmed that SAP is essential for the development of iNKT cells in humans and mice and thus, we hypothesized that the complete absence of iNKT cells in XLP subjects or a partial dysfunction of iNKT cells in healthy individuals may lead to a delayed activation of adaptive immunity following infection by EBV.

To determine the role of iNKT cells during EBV infection, we co-cultured lymphocytes with a laboratory strain of EBV and measured the surface expression of

CD1d. NK cells, $CD4^+$ T cells and $CD8^+$ T cells did not express CD1d before and after incubation with EBV (Appendix 2). By contrast, we observed relatively high levels of CD1d on naive B cells but greatly reduced expression on LCL (Figure 3.1). To verify the expression of CD1d on LCL, naïve B cells and LCL were loaded with αGalCer and co-cultured with iNKT cell lines (Figure 3.1). Unlike naïve B cells, LCL failed to stimulate IFN-γ production from iNKT cell lines despite expressing significant higher levels of the co-stimulatory molecules CD80 and CD86 (Appendix 3). To determine whether EBV transformation specifically downregulates CD1d, we measured the expression level of MHC class I and class II molecules on naïve B cells and LCL (Figure 3.1). Compared to naïve B cells, EBV transformation increased the expression of MHC class I and class II expression on LCL suggesting that EBV may strategically transform B cells to evade iNKT cell surveillance *in vivo*.

To ascertain whether artificially inducing the expression of CD1d sensitizes EBV-transformed B cells to iNKT cell recognition, CD1d expression was upregulated on LCL using the RARα-agonist AM580 (Figure 3.2). Following AM580 treatment, RNA and protein expression of CD1d on the cell surface was detectable by RT-PCR and flow cytometry analysis respectively. To assess the function of CD1d on AM580-treated LCL, IFN-γ production and cytotoxic ability was measured from iNKT cell lines co-cultured with AM580-treated LCL (Figure 3.3 and Figure 3.4). In contrast to untreated LCL, AM580-treated LCL loaded overnight with αGalCer stimulated strong IFN-γ production and were susceptible to killing from each of the three tested iNKT cell lines. Remarkably, NKT cell lines co-cultured with AM580-treated LCL in the absence of αGalCer also activated robust IFN-γ production from all three of the iNKT cell lines. In the absence of

αGalCer, AM580-treated LCL induced moderate iNKT cell cytotoxicity from two of the three iNKT cell lines tested. CD1d expression on AM580-treated LCL is crucial for eliciting NKT cell effector functions as the use of blocking CD1d antibody greatly diminished the IFN-γ production. These data suggest that EBV transformation of B cells triggers the presentation of lipid antigen(s) on CD1d that are capable of activating the effector functions of iNKT cells. Hence, the survival of EBV may be greatly enhanced by the loss of CD1d following the infection and transformation of B cells by EBV.

Based on our data, we speculate that EBV infection of human B cells induces the rapid expression of antigenic self-lipids that are recognized by iNKT cells (Figure 4.1). Upon activation, iNKT cells vigorously produce IFN-γ and likely, a myriad of other cytokines that activate NK cells (204), induce maturation of DC (208-210) and the sequential priming of EBV-specific $CD4^+$ and $CD8^+$ T cells (239). Thereafter, EBV-specific T cells travel to sites of inflammation to control EBV replication before chronic infection is established. EBV-activated $CD4^+$ T cells promote the differentiation and expansion of EBV-specific $CD8^+$ T cells which are critical for the control of EBV replication (180, 226). iNKT cells also produce IL-4 that may directly influence antibody synthesis by B cells (45).

When SAP expression is reduced or completely lost, iNKT cell development is impaired (215-217). In the absence of iNKT cells, we hypothesize that early immune responses to EBV are delayed and sub-optimal. Poor control of EBV replication may result in a greater magnitude of viral replication that alters the infection from a self-limiting disease to a chronic infection associated severe immunopathology.

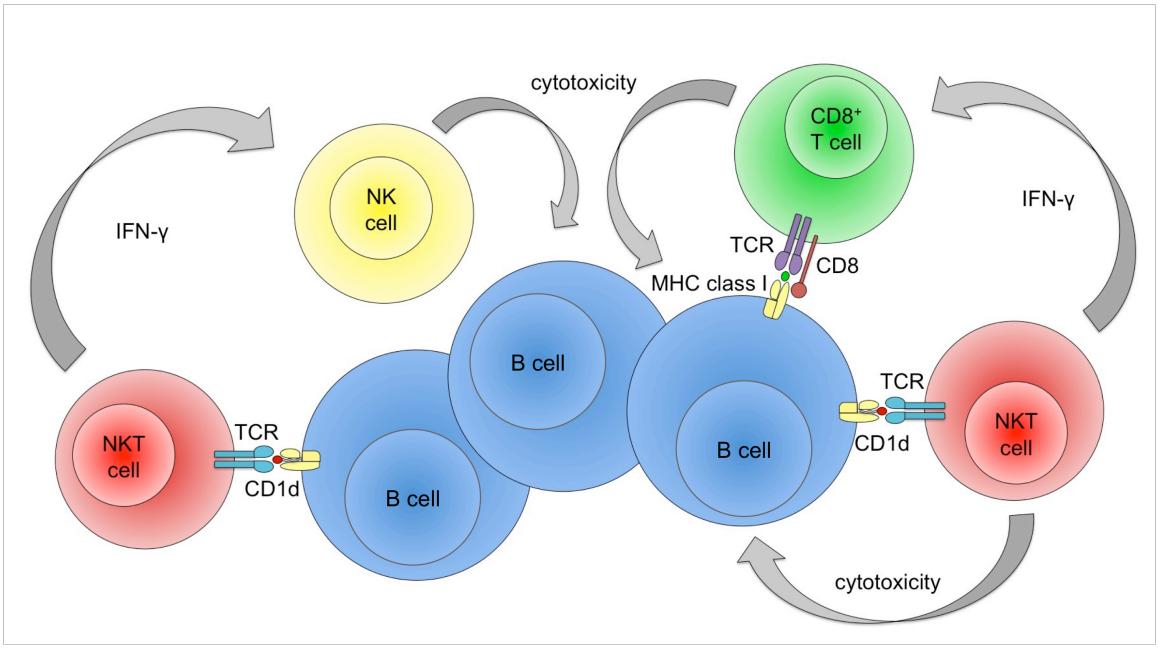


Figure 4.1 Model of iNKT cell activation during EBV infection. EBV infects B cells and induces the surface presentation of lipid antigens that stimulate iNKT cell effector functions including, IFN-γ production and cytotoxicity. The recognition of EBV infection in B cells triggers the early activation of NK cells and the proliferation of EBV-specific $CD8^+$ T cells that are critical for the regulation of EBV replication.

4.2 Significance

EBV is a ubiquitous pathogen and infects more than 90% of adults worldwide (154). In the majority of healthy individuals, EBV infection occurs in early childhood and is often asymptomatic or may present as IM, a self-limiting illness characterized by flu-like symptoms. By contrast, immuno-compromised individuals infected with EBV can develop a number of diseases including, Burkitt's lymphoma, B-cell lymphoproliferative disease, Hodgkin's and non-Hodgkin's lymphomas, nasopharyngeal carcinomas, gastric

carcinoma and oral leukoplakia (236). Individuals with mutations in SAP develop XLP, a rare immunodeficiency syndrome characterized by severe IM, hemophagocytosis, chronic hypogammaglobulinemia and non-Hodgkin's B cell lymphoma resulting in severe morbidity and mortality following EBV infection (240). The serious complications associated with EBV replication in immuno-deficient individuals underscores the critical role that the immune system plays during EBV infection.

Although the adaptive response during EBV infection has been extensively studied, less is known about early innate responses during primary EBV infection. The findings reported herein provide new insights into the role of innate immunity following EBV infection, and specifically, the role of iNKT cells in the control of B cells transformed by EBV. Our data suggests that a lack of iNKT cells may contribute to the poor control of EBV replication in XLP patients and demonstrates that RARα-agonists such as AM580 induce the ectopic expression of CD1d on LCL. The combinatory use of αGalCer and RARα-agonists may have potential implications as novel preventative or therapeutic implications for immuno-compromised individuals who are susceptible or suffer from EBV-associated diseases. Lastly, in addition to viral infection, several studies have reported that iNKT cells are deficient or dysfunctional in individuals who suffer from autoimmune diseases including, diabetes, systemic lupus erthermatosus, athersclerosis and asthma (241). Our results suggest that αGalCer and RARα-agonists may also prove useful as immunotherapies in these auto-inflammatory conditions.

4.3 Future directions

This study highlights the role of SAP in the development of iNKT cells in both humans and mice. Furthermore, this study presences evidence that iNKT cells and CD1d expression may be critical for the recognition and regulation of EBV replication *in vivo*. Our findings provide insights into the possible treatment of EBV-related diseases and other disorders associated with a reduced number or an impaired function of iNKT cells. However, several questions remain to be addressed from this study.

Our findings show that the transformation of naïve B cells by EBV results in the loss of CD1d expression and capacity to activate iNKT cells even when LCL are loaded with αGalCer. However, when CD1d expression was restored by AM580 treatment, we measured iNKT cell activation in the absence of αGalCer suggesting that EBV downregulates CD1d to evade iNKT cell recognition. AM580 treatment alone was not responsible for the increased iNKT cell activation, as naïve B cells treated with AM580 did not elicit IFN-γ production from iNKT cell line (Appendix 4). For these reasons, we speculate that at a certain stage of infection, newly infected B cells express both CD1d and antigenic self-lipids.

Although latent EBV replication is well characterized *in vitro*, lytic EBV replication *in vitro* is poorly defined due to the lack of a fully permissive animal model and because EBV infection of B cells *in vitro* typically induces latent gene expression (239). To address whether CD1d is expressed during EBV lytic replication, we will induce lytic infection in EBV^+ B cells from Martin Rowe (University of Birmingham, UK) transfected with a reporter gene upstream of the promoter region for the early *BMRF1* lytic gene. Unlike LCL that appear to express higher levels of MHC class I and

II compared to naïve B cells, MHC class I and II expression is decreased on cells that express proteins associated with lytic EBV infection (242) suggesting that NK and iNKT cells may play a more important role during this early stage of EBV infection. To the best of our knowledge, CD1d expression on lytically infected B cells has not been determined but we suspect that lytically infected B cells may maintain surface CD1d as protein expression is detected on naïve B cells but not on latently infected LCL. In addition, lytically infected B cells will be co-cultured with various iNKT cell lines to determine whether B cells undergoing lytic EBV replication can activate IFN-γ production and lytic activity.

Although adaptive immunity has been extensively studied following EBV infection, the contribution of iNKT cells *in vivo* remains poorly defined due to the lack of permissive animal models that suitably mimic the course of human disease. Moreover, the direct study of iNKT cells in humans is difficult because the clinical symptoms associated with a poor control of EBV replication typically appear after a lengthy incubation period that precludes the study of early innate responses (236). Thus, to clearly determine the overall contribution of iNKT cells during EBV infection *in vivo*, we propose to generate a humanized mouse model by implanting autologous human fetal thymic and liver tissue into lethally-irradiated immuno-deficient mice that simultaneously receive an adoptive transfer of human $CD34^+$ stem cells isolated from cord blood. Using a similar protocol, immuno-deficient mice are reconstituted with human immune systems which express T cells, B cells, $CD14^+$ monocytes and macrophages, $CD11c^+$ DCs and iNKT cells (243). Following EBV infection, iNKT cells isolated from peripheral blood, spleen and liver will be stained with CD1d tetramer and the expression of various

activation markers such as CD25, CD69 and IFN-γ will be detected by flow cytometry (28).

To determine whether iNKT cells would be useful as a immunotherapeutic target for improving EBV immunity or for vaccine development, we will treat humanized mice with various doses of αGalCer before, at the time of, and after infection with a reporter strain of EBV we received from Richard Longnecker (Northwestern University Medical School, Chicago). The effect of iNKT cell activation on the regulation of EBV will be measured by comparing viral titer using RT-PCR, the magnitude of EBV-specific CTL responses using EBV-specific tetramers and the proportion of EBV^+ B cells by flow cytometry. Furthermore, iNKT cells will be isolated from humanized mice, expanded *in vitro* (219) and adoptively transferred back into humanized mouse recipients before, at the time of, or after EBV infection. Again, the effect on EBV replication could be measured by RT-PCR and flow cytometry.

Lastly, we demonstrated that the RARα-agonist AM580 induces the expression of CD1d on LCL. Previous findings suggest that activation of retinoid pathway leads to the upregulated of CD1d expression on various innate immune cells (51, 52) and our results suggest that similar pathways are conserved in EBV-transformed B cells. How CD1d expression is regulated following EBV transformation of B cells is currently unknown. However, EBV gene products LMP1 and LMP2 are known to activate the nuclear translocation of the transcription factor β-catenin (244, 245) and the co-localization of β-catenin with T cell factors (TCF) and lymphoid enhancer-binding factor (LEF) (246) has also been shown to regulate the expression of CD1d (247). These findings and data showing that the activation of the PPAR-γ pathway induces the proteasomal degradation

of β-catenin (248), we speculate that AM580 upregulates CD1d expression in EBV-transformed B cells by inhibiting the co-localization of β-catenin with TCF/LEF. To test this hypothesis, we will compare the intracellular expression and nuclear co-location of β-catenin and TCF/LEF by image flow technology (249) and traditional immunoprecipitation/Western blot experiments. Data from these experiments may reveal novel signaling molecules and pathways that are targeted by EBV and cause the downregulation of CD1d expression.

REFERENCES

1. Ausubel, F.M. 2005. Are innate immune signaling pathways in plants and animals conserved? *Nat Immunol* 6:973-979.
2. Orkin, S.H., and Zon, L.I. 2008. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* 132:631-644.
3. Kawamoto, H., Wada, H., and Katsura, Y. A revised scheme for developmental pathways of hematopoietic cells: the myeloid-based model. *Int Immunol* 22:65-70.
4. Biron, C.A. 1999. Initial and innate responses to viral infections--pattern setting in immunity or disease. *Curr Opin Microbiol* 2:374-381.
5. Biron, C.A., Nguyen, K.B., Pien, G.C., Cousens, L.P., and Salazar-Mather, T.P. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 17:189-220.
6. Diefenbach, A., and Raulet, D.H. 2002. The innate immune response to tumors and its role in the induction of T-cell immunity. *Immunol Rev* 188:9-21.
7. Marx, J.L. 1986. How killer cells kill their targets. *Science* 231:1367-1369.
8. Bleackley, R.C. 2005. A molecular view of cytotoxic T lymphocyte induced killing. *Biochem Cell Biol* 83:747-751.
9. Brandstadter, J.D., and Yang, Y. Natural killer cell responses to viral infection. *J Innate Immun* 3:274-279.
10. Paust, S., Gill, H.S., Wang, B.Z., Flynn, M.P., Moseman, E.A., Senman, B., Szczepanik, M., Telenti, A., Askenase, P.W., Compans, R.W., et al. Critical role for the chemokine receptor CXCR6 in NK cell-mediated antigen-specific memory of haptens and viruses. *Nat Immunol* 11:1127-1135.
11. Paust, S., Senman, B., and von Andrian, U.H. Adaptive immune responses mediated by natural killer cells. *Immunol Rev* 235:286-296.
12. Mosser, D.M., and Edwards, J.P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958-969.
13. Mantovani, A., Cassatella, M.A., Costantini, C., and Jaillon, S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 11:519-531.
14. Banchereau, J., and Steinman, R.M. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-252.
15. Brack, C., Hirama, M., Lenhard-Schuller, R., and Tonegawa, S. 1978. A complete immunoglobulin gene is created by somatic recombination. *Cell* 15:1-14.
16. Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68:869-877.
17. Shinkai, Y., Rathbun, G., Lam, K.P., Oltz, E.M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A.M., et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68:855-867.
18. Santagata, S., Villa, A., Sobacchi, C., Cortes, P., and Vezzoni, P. 2000. The genetic and biochemical basis of Omenn syndrome. *Immunol Rev* 178:64-74.
19. Minton, K. B cells: Short- and long-term memory. *Nat Rev Immunol* 11:160.

20. Workman, C.J., Szymczak-Workman, A.L., Collison, L.W., Pillai, M.R., and Vignali, D.A. 2009. The development and function of regulatory T cells. *Cell Mol Life Sci* 66:2603-2622.
21. Ouyang, W., Kolls, J.K., and Zheng, Y. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28:454-467.
22. Crotty, S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 29:621-663.
23. Yamagata, T., Benoist, C., and Mathis, D. 2006. A shared gene-expression signature in innate-like lymphocytes. *Immunol Rev* 210:52-66.
24. Bendelac, A., Bonneville, M., and Kearney, J.F. 2001. Autoreactivity by design: innate B and T lymphocytes. *Nat Rev Immunol* 1:177-186.
25. Crotty, S., Kersh, E.N., Cannons, J., Schwartzberg, P.L., and Ahmed, R. 2003. SAP is required for generating long-term humoral immunity. *Nature* 421:282-287.
26. Wu, L., D'Amico, A., Hochrein, H., O'Keeffe, M., Shortman, K., and Lucas, K. 2001. Development of thymic and splenic dendritic cell populations from different hemopoietic precursors. *Blood* 98:3376-3382.
27. Kobayashi, E., Motoki, K., Uchida, T., Fukushima, H., and Koezuka, Y. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* 7:529-534.
28. Van Kaer, L. 2007. NKT cells: T lymphocytes with innate effector functions. *Curr Opin Immunol* 19:354-364.
29. Behar, S.M., and Cardell, S. 2000. Diverse CD1d-restricted T cells: diverse phenotypes, and diverse functions. *Semin Immunol* 12:551-560.
30. Gumperz, J.E., Roy, C., Makowska, A., Lum, D., Sugita, M., Podrebarac, T., Koezuka, Y., Porcelli, S.A., Cardell, S., Brenner, M.B., et al. 2000. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* 12:211-221.
31. Stenstrom, M., Skold, M., Ericsson, A., Beaudoin, L., Sidobre, S., Kronenberg, M., Lehuen, A., and Cardell, S. 2004. Surface receptors identify mouse NK1.1+ T cell subsets distinguished by function and T cell receptor type. *Eur J Immunol* 34:56-65.
32. Apostolou, I., Cumano, A., Gachelin, G., and Kourilsky, P. 2000. Evidence for two subgroups of CD4-CD8- NKT cells with distinct TCR alpha beta repertoires and differential distribution in lymphoid tissues. *J Immunol* 165:2481-2490.
33. Bendelac, A. 1995. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J Exp Med* 182:2091-2096.
34. Coles, M.C., and Raulet, D.H. 2000. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *J Immunol* 164:2412-2418.
35. Mendiratta, S.K., Martin, W.D., Hong, S., Boesteanu, A., Joyce, S., and Van Kaer, L. 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* 6:469-477.
36. Wei, D.G., Lee, H., Park, S.H., Beaudoin, L., Teyton, L., Lehuen, A., and Bendelac, A. 2005. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *J Exp Med* 202:239-248.

37. McNab, F.W., Berzins, S.P., Pellicci, D.G., Kyparissoudis, K., Field, K., Smyth, M.J., and Godfrey, D.I. 2005. The influence of CD1d in postselection NKT cell maturation and homeostasis. *J Immunol* 175:3762-3768.
38. Chun, T., Page, M.J., Gapin, L., Matsuda, J.L., Xu, H., Nguyen, H., Kang, H.S., Stanic, A.K., Joyce, S., Koltun, W.A., et al. 2003. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *J Exp Med* 197:907-918.
39. Schumann, J., Pittoni, P., Tonti, E., Macdonald, H.R., Dellabona, P., and Casorati, G. 2005. Targeted expression of human CD1d in transgenic mice reveals independent roles for thymocytes and thymic APCs in positive and negative selection of Valpha14i NKT cells. *J Immunol* 175:7303-7310.
40. Gumperz, J.E., Miyake, S., Yamamura, T., and Brenner, M.B. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* 195:625-636.
41. Lee, P.T., Putnam, A., Benlagha, K., Teyton, L., Gottlieb, P.A., and Bendelac, A. 2002. Testing the NKT cell hypothesis of human IDDM pathogenesis. *J Clin Invest* 110:793-800.
42. Okai, M., Nieda, M., Tazbirkova, A., Horley, D., Kikuchi, A., Durrant, S., Takahashi, T., Boyd, A., Abraham, R., Yagita, H., et al. 2002. Human peripheral blood Valpha24+ Vbeta11+ NKT cells expand following administration of alpha-galactosylceramide-pulsed dendritic cells. *Vox Sang* 83:250-253.
43. Miller, M.M., Wang, C., Parisini, E., Coletta, R.D., Goto, R.M., Lee, S.Y., Barral, D.C., Townes, M., Roura-Mir, C., Ford, H.L., et al. 2005. Characterization of two avian MHC-like genes reveals an ancient origin of the CD1 family. *Proc Natl Acad Sci U S A* 102:8674-8679.
44. Salomonsen, J., Sorensen, M.R., Marston, D.A., Rogers, S.L., Collen, T., van Hateren, A., Smith, A.L., Beal, R.K., Skjodt, K., and Kaufman, J. 2005. Two CD1 genes map to the chicken MHC, indicating that CD1 genes are ancient and likely to have been present in the primordial MHC. *Proc Natl Acad Sci U S A* 102:8668-8673.
45. Brigl, M., and Brenner, M.B. 2004. CD1: antigen presentation and T cell function. *Annu Rev Immunol* 22:817-890.
46. Bendelac, A. 1995. CD1: presenting unusual antigens to unusual T lymphocytes. *Science* 269:185-186.
47. Bendelac, A., Lantz, O., Quimby, M.E., Yewdell, J.W., Bennink, J.R., and Brutkiewicz, R.R. 1995. CD1 recognition by mouse NK1+ T lymphocytes. *Science* 268:863-865.
48. Park, S.H., Roark, J.H., and Bendelac, A. 1998. Tissue-specific recognition of mouse CD1 molecules. *J Immunol* 160:3128-3134.
49. Roark, J.H., Park, S.H., Jayawardena, J., Kavita, U., Shannon, M., and Bendelac, A. 1998. CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J Immunol* 160:3121-3127.
50. Geng, Y., Laslo, P., Barton, K., and Wang, C.R. 2005. Transcriptional regulation of CD1D1 by Ets family transcription factors. *J Immunol* 175:1022-1029.

51. Szatmari, I., Gogolak, P., Im, J.S., Dezso, B., Rajnavolgyi, E., and Nagy, L. 2004. Activation of PPARgamma specifies a dendritic cell subtype capable of enhanced induction of iNKT cell expansion. *Immunity* 21:95-106.
52. Szatmari, I., Vamosi, G., Brazda, P., Balint, B.L., Benko, S., Szeles, L., Jeney, V., Ozvegy-Laczka, C., Szanto, A., Barta, E., et al. 2006. Peroxisome proliferator-activated receptor gamma-regulated ABCG2 expression confers cytoprotection to human dendritic cells. *J Biol Chem* 281:23812-23823.
53. Hava, D.L., Brigl, M., van den Elzen, P., Zajonc, D.M., Wilson, I.A., and Brenner, M.B. 2005. CD1 assembly and the formation of CD1-antigen complexes. *Curr Opin Immunol* 17:88-94.
54. Zhou, D., Cantu, C., 3rd, Sagiv, Y., Schrantz, N., Kulkarni, A.B., Qi, X., Mahuran, D.J., Morales, C.R., Grabowski, G.A., Benlagha, K., et al. 2004. Editing of CD1d-bound lipid antigens by endosomal lipid transfer proteins. *Science* 303:523-527.
55. Jayawardena-Wolf, J., Benlagha, K., Chiu, Y.H., Mehr, R., and Bendelac, A. 2001. CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. *Immunity* 15:897-908.
56. Chiu, Y.H., Park, S.H., Benlagha, K., Forestier, C., Jayawardena-Wolf, J., Savage, P.B., Teyton, L., and Bendelac, A. 2002. Multiple defects in antigen presentation and T cell development by mice expressing cytoplasmic tail-truncated CD1d. *Nat Immunol* 3:55-60.
57. Wu, D.Y., Segal, N.H., Sidobre, S., Kronenberg, M., and Chapman, P.B. 2003. Cross-presentation of disialoganglioside GD3 to natural killer T cells. *J Exp Med* 198:173-181.
58. Brigl, M., Bry, L., Kent, S.C., Gumperz, J.E., and Brenner, M.B. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol* 4:1230-1237.
59. Fox, L.M., Cox, D.G., Lockridge, J.L., Wang, X., Chen, X., Scharf, L., Trott, D.L., Ndonye, R.M., Veerapen, N., Besra, G.S., et al. 2009. Recognition of lyso-phospholipids by human natural killer T lymphocytes. *PLoS Biol* 7:e1000228.
60. Zhou, D., Mattner, J., Cantu, C., 3rd, Schrantz, N., Yin, N., Gao, Y., Sagiv, Y., Hudspeth, K., Wu, Y.P., Yamashita, T., et al. 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science* 306:1786-1789.
61. Christiansen, D., Milland, J., Mouhtouris, E., Vaughan, H., Pellicci, D.G., McConville, M.J., Godfrey, D.I., and Sandrin, M.S. 2008. Humans lack iGb3 due to the absence of functional iGb3-synthase: implications for NKT cell development and transplantation. *PLoS Biol* 6:e172.
62. Porubsky, S., Speak, A.O., Luckow, B., Cerundolo, V., Platt, F.M., and Grone, H.J. 2007. Normal development and function of invariant natural killer T cells in mice with isoglobotrihexosylceramide (iGb3) deficiency. *Proc Natl Acad Sci U S A* 104:5977-5982.
63. Kjer-Nielsen, L., Borg, N.A., Pellicci, D.G., Beddoe, T., Kostenko, L., Clements, C.S., Williamson, N.A., Smyth, M.J., Besra, G.S., Reid, H.H., et al. 2006. A structural basis for selection and cross-species reactivity of the semi-invariant NKT cell receptor in CD1d/glycolipid recognition. *J Exp Med* 203:661-673.

64. Zajonc, D.M., Maricic, I., Wu, D., Halder, R., Roy, K., Wong, C.H., Kumar, V., and Wilson, I.A. 2005. Structural basis for CD1d presentation of a sulfatide derived from myelin and its implications for autoimmunity. *J Exp Med* 202:1517-1526.
65. Kinjo, T., Nakamatsu, M., Nakasone, C., Yamamoto, N., Kinjo, Y., Miyagi, K., Uezu, K., Nakamura, K., Higa, F., Tateyama, M., et al. 2006. NKT cells play a limited role in the neutrophilic inflammatory responses and host defense to pulmonary infection with Pseudomonas aeruginosa. *Microbes Infect* 8:2679-2685.
66. Kinjo, Y., Wu, D., Kim, G., Xing, G.W., Poles, M.A., Ho, D.D., Tsuji, M., Kawahara, K., Wong, C.H., and Kronenberg, M. 2005. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* 434:520-525.
67. Mattner, J., Debord, K.L., Ismail, N., Goff, R.D., Cantu, C., 3rd, Zhou, D., Saint-Mezard, P., Wang, V., Gao, Y., Yin, N., et al. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 434:525-529.
68. Fischer, K., Scotet, E., Niemeyer, M., Koebernick, H., Zerrahn, J., Maillet, S., Hurwitz, R., Kursar, M., Bonneville, M., Kaufmann, S.H., et al. 2004. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc Natl Acad Sci U S A* 101:10685-10690.
69. Amprey, J.L., Im, J.S., Turco, S.J., Murray, H.W., Illarionov, P.A., Besra, G.S., Porcelli, S.A., and Spath, G.F. 2004. A subset of liver NK T cells is activated during Leishmania donovani infection by CD1d-bound lipophosphoglycan. *J Exp Med* 200:895-904.
70. Borg, N.A., Wun, K.S., Kjer-Nielsen, L., Wilce, M.C., Pellicci, D.G., Koh, R., Besra, G.S., Bharadwaj, M., Godfrey, D.I., McCluskey, J., et al. 2007. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature* 448:44-49.
71. Godfrey, D.I., and Kronenberg, M. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest* 114:1379-1388.
72. Hong, S., Wilson, M.T., Serizawa, I., Wu, L., Singh, N., Naidenko, O.V., Miura, T., Haba, T., Scherer, D.C., Wei, J., et al. 2001. The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat Med* 7:1052-1056.
73. Shi, F.D., Flodstrom, M., Balasa, B., Kim, S.H., Van Gunst, K., Strominger, J.L., Wilson, S.B., and Sarvetnick, N. 2001. Germ line deletion of the CD1 locus exacerbates diabetes in the NOD mouse. *Proc Natl Acad Sci U S A* 98:6777-6782.
74. Wang, B., Geng, Y.B., and Wang, C.R. 2001. CD1-restricted NK T cells protect nonobese diabetic mice from developing diabetes. *J Exp Med* 194:313-320.
75. Miyamoto, K., Miyake, S., and Yamamura, T. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* 413:531-534.
76. Sharif, S., Arreaza, G.A., Zucker, P., Mi, Q.S., Sondhi, J., Naidenko, O.V., Kronenberg, M., Koezuka, Y., Delovitch, T.L., Gombert, J.M., et al. 2001. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. *Nat Med* 7:1057-1062.

77. Singh, A.K., Wilson, M.T., Hong, S., Olivares-Villagomez, D., Du, C., Stanic, A.K., Joyce, S., Sriram, S., Koezuka, Y., and Van Kaer, L. 2001. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* 194:1801-1811.
78. Laloux, V., Beaudoin, L., Jeske, D., Carnaud, C., and Lehuen, A. 2001. NK T cell-induced protection against diabetes in V alpha 14-J alpha 281 transgenic nonobese diabetic mice is associated with a Th2 shift circumscribed regionally to the islets and functionally to islet autoantigen. *J Immunol* 166:3749-3756.
79. Galli, G., Nuti, S., Tavarini, S., Galli-Stampino, L., De Lalla, C., Casorati, G., Dellabona, P., and Abrignani, S. 2003. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. *J Exp Med* 197:1051-1057.
80. Wermeling, F., Lind, S.M., Jordo, E.D., Cardell, S.L., and Karlsson, M.C. Invariant NKT cells limit activation of autoreactive CD1d-positive B cells. *J Exp Med* 207:943-952.
81. Cain, J.A., Smith, J.A., Ondr, J.K., Wang, B., and Katz, J.D. 2006. NKT cells and IFN-gamma establish the regulatory environment for the control of diabetogenic T cells in the nonobese diabetic mouse. *J Immunol* 176:1645-1654.
82. Lee, I.F., Qin, H., Priatel, J.J., and Tan, R. 2008. Critical role for IFN-gamma in natural killer cell-mediated protection from diabetes. *Eur J Immunol* 38:82-89.
83. Chen, Y.G., Choisy-Rossi, C.M., Holl, T.M., Chapman, H.D., Besra, G.S., Porcelli, S.A., Shaffer, D.J., Roopenian, D., Wilson, S.B., and Serreze, D.V. 2005. Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes. *J Immunol* 174:1196-1204.
84. Naumov, Y.N., Bahjat, K.S., Gausling, R., Abraham, R., Exley, M.A., Koezuka, Y., Balk, S.B., Strominger, J.L., Clare-Salzer, M., and Wilson, S.B. 2001. Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets. *Proc Natl Acad Sci U S A* 98:13838-13843.
85. Hayakawa, Y., Rovero, S., Forni, G., and Smyth, M.J. 2003. Alpha-galactosylceramide (KRN7000) suppression of chemical- and oncogene-dependent carcinogenesis. *Proc Natl Acad Sci U S A* 100:9464-9469.
86. Smyth, M.J., Crowe, N.Y., Hayakawa, Y., Takeda, K., Yagita, H., and Godfrey, D.I. 2002. NKT cells - conductors of tumor immunity? *Curr Opin Immunol* 14:165-171.
87. Nakagawa, R., Nagafune, I., Tazunoki, Y., Ehara, H., Tomura, H., Iijima, R., Motoki, K., Kamishohara, M., and Seki, S. 2001. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by alpha-galactosylceramide in mice. *J Immunol* 166:6578-6584.
88. Hayakawa, Y., Takeda, K., Yagita, H., Kakuta, S., Iwakura, Y., Van Kaer, L., Saiki, I., and Okumura, K. 2001. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. *Eur J Immunol* 31:1720-1727.
89. Hayakawa, Y., Takeda, K., Yagita, H., Smyth, M.J., Van Kaer, L., Okumura, K., and Saiki, I. 2002. IFN-gamma-mediated inhibition of tumor angiogenesis by natural killer T-cell ligand, alpha-galactosylceramide. *Blood* 100:1728-1733.

90. Smyth, M.J., Thia, K.Y., Street, S.E., Cretney, E., Trapani, J.A., Taniguchi, M., Kawano, T., Pelikan, S.B., Crowe, N.Y., and Godfrey, D.I. 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* 191:661-668.
91. Giaccone, G., Punt, C.J., Ando, Y., Ruijter, R., Nishi, N., Peters, M., von Blomberg, B.M., Scheper, R.J., van der Vliet, H.J., van den Eertwegh, A.J., et al. 2002. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* 8:3702-3709.
92. Nieda, M., Okai, M., Tazbirkova, A., Lin, H., Yamaura, A., Ide, K., Abraham, R., Juji, T., Macfarlane, D.J., and Nicol, A.J. 2004. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* 103:383-389.
93. Ishikawa, A., Motohashi, S., Ishikawa, E., Fuchida, H., Higashino, K., Otsuji, M., Iizasa, T., Nakayama, T., Taniguchi, M., and Fujisawa, T. 2005. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* 11:1910-1917.
94. Tupin, E., Kinjo, Y., and Kronenberg, M. 2007. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol* 5:405-417.
95. Mallevaey, T., Fontaine, J., Breuilh, L., Paget, C., Castro-Keller, A., Vendeville, C., Capron, M., Leite-de-Moraes, M., Trottein, F., and Faveeuw, C. 2007. Invariant and noninvariant natural killer T cells exert opposite regulatory functions on the immune response during murine schistosomiasis. *Infect Immun* 75:2171-2180.
96. Nieuwenhuis, E.E., Matsumoto, T., Exley, M., Schleipman, R.A., Glickman, J., Bailey, D.T., Corazza, N., Colgan, S.P., Onderdonk, A.B., and Blumberg, R.S. 2002. CD1d-dependent macrophage-mediated clearance of Pseudomonas aeruginosa from lung. *Nat Med* 8:588-593.
97. Kawakami, K., Yamamoto, N., Kinjo, Y., Miyagi, K., Nakasone, C., Uezu, K., Kinjo, T., Nakayama, T., Taniguchi, M., and Saito, A. 2003. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection. *Eur J Immunol* 33:3322-3330.
98. Hobbs, J.A., Cho, S., Roberts, T.J., Sriram, V., Zhang, J., Xu, M., and Brutkiewicz, R.R. 2001. Selective loss of natural killer T cells by apoptosis following infection with lymphocytic choriomeningitis virus. *J Virol* 75:10746-10754.
99. Johnson, T.R., Hong, S., Van Kaer, L., Koezuka, Y., and Graham, B.S. 2002. NK T cells contribute to expansion of CD8(+) T cells and amplification of antiviral immune responses to respiratory syncytial virus. *J Virol* 76:4294-4303.
100. Exley, M.A., Bigley, N.J., Cheng, O., Tahir, S.M., Smiley, S.T., Carter, Q.L., Stills, H.F., Grusby, M.J., Koezuka, Y., Taniguchi, M., et al. 2001. CD1d-reactive T-cell activation leads to amelioration of disease caused by diabetogenic encephalomyocarditis virus. *J Leukoc Biol* 69:713-718.
101. Grubor-Bauk, B., Simmons, A., Mayrhofer, G., and Speck, P.G. 2003. Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant V alpha 14-J alpha 281 TCR. *J Immunol* 170:1430-1434.

102. Raftery, M.J., Winau, F., Giese, T., Kaufmann, S.H., Schaible, U.E., and Schonrich, G. 2008. Viral danger signals control CD1d de novo synthesis and NKT cell activation. *Eur J Immunol* 38:668-679.
103. Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Sato, H., Kondo, E., Harada, M., Koseki, H., Nakayama, T., et al. 1998. Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc Natl Acad Sci U S A* 95:5690-5693.
104. Tortorella, D., Gewurz, B.E., Furman, M.H., Schust, D.J., and Ploegh, H.L. 2000. Viral subversion of the immune system. *Annu Rev Immunol* 18:861-926.
105. Kakimi, K., Guidotti, L.G., Koezuka, Y., and Chisari, F.V. 2000. Natural killer T cell activation inhibits hepatitis B virus replication in vivo. *J Exp Med* 192:921-930.
106. Yang, Y., and Wilson, J.M. 1995. Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo. *J Immunol* 155:2564-2570.
107. Renukaradhya, G.J., Webb, T.J., Khan, M.A., Lin, Y.L., Du, W., Gervay-Hague, J., and Brutkiewicz, R.R. 2005. Virus-induced inhibition of CD1d1-mediated antigen presentation: reciprocal regulation by p38 and ERK. *J Immunol* 175:4301-4308.
108. Cho, S., Knox, K.S., Kohli, L.M., He, J.J., Exley, M.A., Wilson, S.B., and Brutkiewicz, R.R. 2005. Impaired cell surface expression of human CD1d by the formation of an HIV-1 Nef/CD1d complex. *Virology* 337:242-252.
109. Sanchez, D.J., Gumperz, J.E., and Ganem, D. 2005. Regulation of CD1d expression and function by a herpesvirus infection. *J Clin Invest* 115:1369-1378.
110. Yuan, W., Dasgupta, A., and Cresswell, P. 2006. Herpes simplex virus evades natural killer T cell recognition by suppressing CD1d recycling. *Nat Immunol* 7:835-842.
111. Levy, O., Orange, J.S., Hibberd, P., Steinberg, S., LaRussa, P., Weinberg, A., Wilson, S.B., Shaulov, A., Fleisher, G., Geha, R.S., et al. 2003. Disseminated varicella infection due to the vaccine strain of varicella-zoster virus, in a patient with a novel deficiency in natural killer T cells. *J Infect Dis* 188:948-953.
112. Purtilo, D.T., Cassel, C.K., Yang, J.P., and Harper, R. 1975. X-linked recessive progressive combined variable immunodeficiency (Duncan's disease). *Lancet* 1:935-940.
113. Seemayer, T.A., Gross, T.G., Egeler, R.M., Pirruccello, S.J., Davis, J.R., Kelly, C.M., Okano, M., Lanyi, A., and Sumegi, J. 1995. X-linked lymphoproliferative disease: twenty-five years after the discovery. *Pediatr Res* 38:471-478.
114. Dutz, J.P., Benoit, L., Wang, X., Demetrick, D.J., Junker, A., de Sa, D., and Tan, R. 2001. Lymphocytic vasculitis in X-linked lymphoproliferative disease. *Blood* 97:95-100.
115. Kanegane, H., Ito, Y., Ohshima, K., Shichijo, T., Tomimasu, K., Nomura, K., Futatani, T., Sumazaki, R., and Miyawaki, T. 2005. X-linked lymphoproliferative syndrome presenting with systemic lymphocytic vasculitis. *Am J Hematol* 78:130-133.
116. Loeffel, S., Chang, C.H., Heyn, R., Harada, S., Lipscomb, H., Sinangil, F., Volsky, D.J., McClain, K., Ochs, H., and Purtilo, D.T. 1985. Necrotizing

lymphoid vasculitis in X-linked lymphoproliferative syndrome. *Arch Pathol Lab Med* 109:546-550.

117. Filipovich, A.H., Blazar, B.R., Ramsay, N.K., Kersey, J.H., Zelkowitz, L., Harada, S., and Purtilo, D.T. 1986. Allogeneic bone marrow transplantation for X-linked lymphoproliferative syndrome. *Transplantation* 42:222-224.
118. Gross, T.G., Filipovich, A.H., Conley, M.E., Pracher, E., Schmiegelow, K., Verdirame, J.D., Vowels, M., Williams, L.L., and Seemayer, T.A. 1996. Cure of X-linked lymphoproliferative disease (XLP) with allogeneic hematopoietic stem cell transplantation (HSCT): report from the XLP registry. *Bone Marrow Transplant* 17:741-744.
119. Vowels, M.R., Tang, R.L., Berdoukas, V., Ford, D., Thierry, D., Purtilo, D., and Gluckman, E. 1993. Brief report: correction of X-linked lymphoproliferative disease by transplantation of cord-blood stem cells. *N Engl J Med* 329:1623-1625.
120. Calpe, S., Wang, N., Romero, X., Berger, S.B., Lanyi, A., Engel, P., and Terhorst, C. 2008. The SLAM and SAP gene families control innate and adaptive immune responses. *Adv Immunol* 97:177-250.
121. Coffey, A.J., Brooksbank, R.A., Brandau, O., Oohashi, T., Howell, G.R., Bye, J.M., Cahn, A.P., Durham, J., Heath, P., Wray, P., et al. 1998. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene. *Nat Genet* 20:129-135.
122. Ma, C.S., Nichols, K.E., and Tangye, S.G. 2007. Regulation of cellular and humoral immune responses by the SLAM and SAP families of molecules. *Annu Rev Immunol* 25:337-379.
123. Nichols, K.E., Harkin, D.P., Levitz, S., Krainer, M., Kolquist, K.A., Genovese, C., Bernard, A., Ferguson, M., Zuo, L., Snyder, E., et al. 1998. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome. *Proc Natl Acad Sci U S A* 95:13765-13770.
124. Sayos, J., Wu, C., Morra, M., Wang, N., Zhang, X., Allen, D., van Schaik, S., Notarangelo, L., Geha, R., Roncarolo, M.G., et al. 1998. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. *Nature* 395:462-469.
125. Veillette, A. 2006. Immune regulation by SLAM family receptors and SAP-related adaptors. *Nat Rev Immunol* 6:56-66.
126. Engel, P., Eck, M.J., and Terhorst, C. 2003. The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat Rev Immunol* 3:813-821.
127. Latour, S., and Veillette, A. 2004. The SAP family of adaptors in immune regulation. *Semin Immunol* 16:409-419.
128. Roncagalli, R., Taylor, J.E., Zhang, S., Shi, X., Chen, R., Cruz-Munoz, M.E., Yin, L., Latour, S., and Veillette, A. 2005. Negative regulation of natural killer cell function by EAT-2, a SAP-related adaptor. *Nat Immunol* 6:1002-1010.
129. Chen, G., Tai, A.K., Lin, M., Chang, F., Terhorst, C., and Huber, B.T. 2005. Signaling lymphocyte activation molecule-associated protein is a negative regulator of the CD8 T cell response in mice. *J Immunol* 175:2212-2218.
130. Crotty, S., McCausland, M.M., Aubert, R.D., Wherry, E.J., and Ahmed, R. 2006. Hypogammaglobulinemia and exacerbated CD8 T-cell-mediated

immunopathology in SAP-deficient mice with chronic LCMV infection mimics human XLP disease. *Blood* 108:3085-3093.

131. McCausland, M.M., Yusuf, I., Tran, H., Ono, N., Yanagi, Y., and Crotty, S. 2007. SAP regulation of follicular helper CD4 T cell development and humoral immunity is independent of SLAM and Fyn kinase. *J Immunol* 178:817-828.
132. Wu, C., Nguyen, K.B., Pien, G.C., Wang, N., Gullo, C., Howie, D., Sosa, M.R., Edwards, M.J., Borrow, P., Satoskar, A.R., et al. 2001. SAP controls T cell responses to virus and terminal differentiation of TH2 cells. *Nat Immunol* 2:410-414.
133. Schwartzberg, P.L., Mueller, K.L., Qi, H., and Cannons, J.L. 2009. SLAM receptors and SAP influence lymphocyte interactions, development and function. *Nat Rev Immunol* 9:39-46.
134. Brown, M.H., Boles, K., van der Merwe, P.A., Kumar, V., Mathew, P.A., and Barclay, A.N. 1998. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J Exp Med* 188:2083-2090.
135. Latchman, Y., McKay, P.F., and Reiser, H. 1998. Identification of the 2B4 molecule as a counter-receptor for CD48. *J Immunol* 161:5809-5812.
136. Chan, B., Lanyi, A., Song, H.K., Griesbach, J., Simarro-Grande, M., Poy, F., Howie, D., Sumegi, J., Terhorst, C., and Eck, M.J. 2003. SAP couples Fyn to SLAM immune receptors. *Nat Cell Biol* 5:155-160.
137. Chen, R., Latour, S., Shi, X., and Veillette, A. 2006. Association between SAP and FynT: Inducible SH3 domain-mediated interaction controlled by engagement of the SLAM receptor. *Mol Cell Biol* 26:5559-5568.
138. Simarro, M., Lanyi, A., Howie, D., Poy, F., Bruggeman, J., Choi, M., Sumegi, J., Eck, M.J., and Terhorst, C. 2004. SAP increases FynT kinase activity and is required for phosphorylation of SLAM and Ly9. *Int Immunol* 16:727-736.
139. Cannons, J.L., Yu, L.J., Hill, B., Mijares, L.A., Dombroski, D., Nichols, K.E., Antonellis, A., Koretzky, G.A., Gardner, K., and Schwartzberg, P.L. 2004. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kappaB1. *Immunity* 21:693-706.
140. Latour, S., Roncagalli, R., Chen, R., Bakinowski, M., Shi, X., Schwartzberg, P.L., Davidson, D., and Veillette, A. 2003. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. *Nat Cell Biol* 5:149-154.
141. Mikhalap, S.V., Shlapatska, L.M., Berdova, A.G., Law, C.L., Clark, E.A., and Sidorenko, S.P. 1999. CDw150 associates with src-homology 2-containing inositol phosphatase and modulates CD95-mediated apoptosis. *J Immunol* 162:5719-5727.
142. Gu, C., Tangye, S.G., Sun, X., Luo, Y., Lin, Z., and Wu, J. 2006. The X-linked lymphoproliferative disease gene product SAP associates with PAK-interacting exchange factor and participates in T cell activation. *Proc Natl Acad Sci U S A* 103:14447-14452.
143. Dupre, L., Andolfi, G., Tangye, S.G., Clementi, R., Locatelli, F., Arico, M., Aiuti, A., and Roncarolo, M.G. 2005. SAP controls the cytolytic activity of CD8+ T cells against EBV-infected cells. *Blood* 105:4383-4389.

144. Parolini, S., Bottino, C., Falco, M., Augugliaro, R., Giliani, S., Franceschini, R., Ochs, H.D., Wolf, H., Bonnefoy, J.Y., Biassoni, R., et al. 2000. X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. *J Exp Med* 192:337-346.
145. Sharifi, R., Sinclair, J.C., Gilmour, K.C., Arkwright, P.D., Kinnon, C., Thrasher, A.J., and Gaspar, H.B. 2004. SAP mediates specific cytotoxic T-cell functions in X-linked lymphoproliferative disease. *Blood* 103:3821-3827.
146. Bloch-Queyrat, C., Fondaneche, M.C., Chen, R., Yin, L., Relouzat, F., Veillette, A., Fischer, A., and Latour, S. 2005. Regulation of natural cytotoxicity by the adaptor SAP and the Src-related kinase Fyn. *J Exp Med* 202:181-192.
147. Ma, C.S., Hare, N.J., Nichols, K.E., Dupre, L., Andolfi, G., Roncarolo, M.G., Adelstein, S., Hodgkin, P.D., and Tangye, S.G. 2005. Impaired humoral immunity in X-linked lymphoproliferative disease is associated with defective IL-10 production by CD4+ T cells. *J Clin Invest* 115:1049-1059.
148. Czar, M.J., Kersh, E.N., Mijares, L.A., Lanier, G., Lewis, J., Yap, G., Chen, A., Sher, A., Duckett, C.S., Ahmed, R., et al. 2001. Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proc Natl Acad Sci U S A* 98:7449-7454.
149. Hron, J.D., Caplan, L., Gerth, A.J., Schwartzberg, P.L., and Peng, S.L. 2004. SH2D1A regulates T-dependent humoral autoimmunity. *J Exp Med* 200:261-266.
150. Morra, M., Barrington, R.A., Abadia-Molina, A.C., Okamoto, S., Julien, A., Gullo, C., Kalsy, A., Edwards, M.J., Chen, G., Spolski, R., et al. 2005. Defective B cell responses in the absence of SH2D1A. *Proc Natl Acad Sci U S A* 102:4819-4823.
151. Veillette, A., Zhang, S., Shi, X., Dong, Z., Davidson, D., and Zhong, M.C. 2008. SAP expression in T cells, not in B cells, is required for humoral immunity. *Proc Natl Acad Sci U S A* 105:1273-1278.
152. Cannons, J.L., Qi, H., Lu, K.T., Dutta, M., Gomez-Rodriguez, J., Cheng, J., Wakeland, E.K., Germain, R.N., and Schwartzberg, P.L. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* 32:253-265.
153. Malbran, A., Belmonte, L., Ruibal-Ares, B., Bare, P., Massud, I., Parodi, C., Felippo, M., Hodinka, R., Haines, K., Nichols, K.E., et al. 2004. Loss of circulating CD27+ memory B cells and CCR4+ T cells occurring in association with elevated EBV loads in XLP patients surviving primary EBV infection. *Blood* 103:1625-1631.
154. Hislop, A.D., Taylor, G.S., Sauce, D., and Rickinson, A.B. 2007. Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol* 25:587-617.
155. Nemerow, G.R., Mold, C., Schwend, V.K., Tollefson, V., and Cooper, N.R. 1987. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. *J Virol* 61:1416-1420.

156. Tanner, J., Weis, J., Fearon, D., Whang, Y., and Kieff, E. 1987. Epstein-Barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis. *Cell* 50:203-213.
157. Haan, K.M., Kwok, W.W., Longnecker, R., and Speck, P. 2000. Epstein-Barr virus entry utilizing HLA-DP or HLA-DQ as a coreceptor. *J Virol* 74:2451-2454.
158. Li, Q., Spriggs, M.K., Kovats, S., Turk, S.M., Comeau, M.R., Nepom, B., and Hutt-Fletcher, L.M. 1997. Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes. *J Virol* 71:4657-4662.
159. Spriggs, M.K., Armitage, R.J., Comeau, M.R., Strockbine, L., Farrah, T., Macduff, B., Ulrich, D., Alderson, M.R., Mullberg, J., and Cohen, J.I. 1996. The extracellular domain of the Epstein-Barr virus BZLF2 protein binds the HLA-DR beta chain and inhibits antigen presentation. *J Virol* 70:5557-5563.
160. Spear, P.G., and Longnecker, R. 2003. Herpesvirus entry: an update. *J Virol* 77:10179-10185.
161. Gordadze, A.V., Peng, R., Tan, J., Liu, G., Sutton, R., Kempkes, B., Bornkamm, G.W., and Ling, P.D. 2001. Notch1IC partially replaces EBNA2 function in B cells immortalized by Epstein-Barr virus. *J Virol* 75:5899-5912.
162. Thorley-Lawson, D.A. 2001. Epstein-Barr virus: exploiting the immune system. *Nat Rev Immunol* 1:75-82.
163. Caldwell, R.G., Wilson, J.B., Anderson, S.J., and Longnecker, R. 1998. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity* 9:405-411.
164. Gires, O., Zimber-Strobl, U., Gonnella, R., Ueffing, M., Marschall, G., Zeidler, R., Pich, D., and Hammerschmidt, W. 1997. Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule. *EMBO J* 16:6131-6140.
165. Liu, Y.J., Joshua, D.E., Williams, G.T., Smith, C.A., Gordon, J., and MacLennan, I.C. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature* 342:929-931.
166. Babcock, G.J., Hochberg, D., and Thorley-Lawson, A.D. 2000. The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. *Immunity* 13:497-506.
167. Yao, Q.Y., Rickinson, A.B., and Epstein, M.A. 1985. A re-examination of the Epstein-Barr virus carrier state in healthy seropositive individuals. *Int J Cancer* 35:35-42.
168. Tan, L.C., Gudgeon, N., Annels, N.E., Hansasuta, P., O'Callaghan, C.A., Rowland-Jones, S., McMichael, A.J., Rickinson, A.B., and Callan, M.F. 1999. A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers. *J Immunol* 162:1827-1835.
169. Henle, W., Henle, G., and Lennette, E.T. 1979. The Epstein-Barr virus. *Sci Am* 241:48-59.
170. Hamilton, J.K., Paquin, L.A., Sullivan, J.L., Maurer, H.S., Cruzi, F.G., Provisor, A.J., Steuber, C.P., Hawkins, E., Yawn, D., Cornet, J.A., et al. 1980. X-linked lymphoproliferative syndrome registry report. *J Pediatr* 96:669-673.
171. Sayos, J., Wu, C., Morra, M., Wang, N., Zhang, X., Allen, D., van Schaik, S., Notarangelo, L., Geha, R., Roncarolo, M.G., et al. 1998. The X-linked

lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM [see comments]. *Nature* 395:462-469.
172. Cocks, B.G., Chang, C.C., Carballido, J.M., Yssel, H., de Vries, J.E., and Aversa, G. 1995. A novel receptor involved in T-cell activation. *Nature* 376:260-263.
173. Tangye, S.G., Lazetic, S., Woollatt, E., Sutherland, G.R., Lanier, L.L., and Phillips, J.H. 1999. Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J Immunol* 162:6981-6985.
174. Williams, H., McAulay, K., Macsween, K.F., Gallacher, N.J., Higgins, C.D., Harrison, N., Swerdlow, A.J., and Crawford, D.H. 2005. The immune response to primary EBV infection: a role for natural killer cells. *Br J Haematol* 129:266-274.
175. Parolini, S., Bottino, C., Falco, M., Augugliaro, R., Giliani, S., Franceschini, R., Ochs, H.D., Wolf, H., Bonnefoy, J.Y., Biassoni, R., et al. 2000. X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill epstein-barr virus-infected cells [In Process Citation]. *J Exp Med* 192:337-346.
176. Benoit, L., Wang, X., Pabst, H.F., Dutz, J., and Tan, R. 2000. Defective NK cell activation in X-linked lymphoproliferative disease. *J Immunol (Cutting Edge)* 165:3549-3553.
177. Tangye, S.G., Phillips, J.H., Lanier, L.L., and Nichols, K.E. 2000. Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome. *J Immunol* 165:2932-2936.
178. Chen, R., Relouzat, F., Roncagalli, R., Aoukaty, A., Tan, R., Latour, S., and Veillette, A. 2004. Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors. *Mol Cell Biol* 24:5144-5156.
179. Li, C., Chung, B., Tao, J., Iosef, C., Aoukaty, A., Wang, Y., Tan, R., and Li, S.S. 2008. The X-linked lymphoproliferative syndrome gene product SAP regulates B cell function through the FcgammaRIIB receptor. *Cell Signal* 20:1960-1967.
180. Callan, M.F., Steven, N., Krausa, P., Wilson, J.D., Moss, P.A., Gillespie, G.M., Bell, J.I., Rickinson, A.B., and McMichael, A.J. 1996. Large clonal expansions of CD8+ T cells in acute infectious mononucleosis. *Nat Med* 2:906-911.
181. Tomkinson, B.E., Wagner, D.K., Nelson, D.L., and Sullivan, J.L. 1987. Activated lymphocytes during acute Epstein-Barr virus infection. *J Immunol* 139:3802-3807.
182. Harrer, T., Harrer, E., Kalams, S.A., Barbosa, P., Trocha, A., Johnson, R.P., Elbeik, T., Feinberg, M.B., Buchbinder, S.P., and Walker, B.D. 1996. Cytotoxic T lymphocytes in asymptomatic long-term nonprogressing HIV-1 infection. Breadth and specificity of the response and relation to in vivo viral quasispecies in a person with prolonged infection and low viral load. *J Immunol* 156:2616-2623.
183. Jin, X., Demoitie, M.A., Donahoe, S.M., Ogg, G.S., Bonhoeffer, S., Kakimoto, W.M., Gillespie, G., Moss, P.A., Dyer, W., Kurilla, M.G., et al. 2000. High frequency of cytomegalovirus-specific cytotoxic T-effector cells in HLA-A*0201-positive subjects during multiple viral coinfections. *J Infect Dis* 181:165-175.

184. Fischer, A. 2007. Human primary immunodeficiency diseases. *Immunity* 27:835-845.
185. Ho, L.P., Urban, B.C., Jones, L., Ogg, G.S., and McMichael, A.J. 2004. CD4(-)CD8alphaalpha subset of CD1d-restricted NKT cells controls T cell expansion. *J Immunol* 172:7350-7358.
186. Huck, K., Feyen, O., Niehues, T., Ruschendorf, F., Hubner, N., Laws, H.J., Telieps, T., Knapp, S., Wacker, H.H., Meindl, A., et al. 2009. Girls homozygous for an IL-2-inducible T cell kinase mutation that leads to protein deficiency develop fatal EBV-associated lymphoproliferation. *J Clin Invest* 119:1350-1358.
187. Eberl, G., Lowin-Kropf, B., and MacDonald, H.R. 1999. Cutting edge: NKT cell development is selectively impaired in Fyn- deficient mice. *J Immunol* 163:4091-4094.
188. Purtilo, D.T., Grierson, H.L., Davis, J.R., and Okano, M. 1991. The X-linked lymphoproliferative disease: from autopsy toward cloning the gene 1975-1990. *Pediatr Pathol* 11:685-710.
189. Coffey, A.J., Brooksbank, R.A., Brandau, O., Oohashi, T., Howell, G.R., Bye, J.M., Cahn, A.P., Durham, J., Heath, P., Wray, P., et al. 1998. Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene [see comments]. *Nat Genet* 20:129-135.
190. Nichols, K.E., Harkin, D.P., Levitz, S., Krainer, M., Kolquist, K.A., Genovese, C., Bernard, A., Ferguson, M., Zuo, L., Snyder, E., et al. 1998. Inactivating mutations in an SH2 domain-encoding gene in X-linked lymphoproliferative syndrome [In Process Citation]. *Proc Natl Acad Sci U S A* 95:13765-13770.
191. Yin, L., Ferrand, V., Lavoue, M.F., Hayoz, D., Philippe, N., Souillet, G., Seri, M., Giacchino, R., Castagnola, E., Hodgson, S., et al. 1999. SH2D1A mutation analysis for diagnosis of XLP in typical and atypical patients. *Hum Genet* 105:501-505.
192. Wu, C., Nguyen, K.B., Pien, G.C., Wang, N., Gullo, C., Howie, D., Sosa, M.R., Edwards, M.J., Borrow, P., Satoskar, A.R., et al. 2001. SAP controls T cell responses to virus and terminal differentiation of TH2 cells. *Nat Immunol* 2:410-414.
193. Veillette, A. 2003. SAP: a molecular switch regulating the immune response through a unique signaling mechanism. *Eur J Immunol* 33:1141-1144.
194. Tangye, S.G., Phillips, J.H., Lanier, L.L., and Nichols, K.E. 2000. Cutting Edge: Functional Requirement for SAP in 2B4-Mediated Activation of Human Natural Killer Cells as Revealed by the X-Linked Lymphoproliferative Syndrome. *J Immunol* 165:2932-2936.
195. Benoit, L., Wang, X., Pabst, H.F., Dutz, J., and Tan, R. 2000. Defective NK cell activation in X-linked lymphoproliferative disease. *J Immunol* 165:3549-3553.
196. Bendelac, A., Rivera, M.N., Park, S.H., and Roark, J.H. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol* 15:535-562.
197. Kronenberg, M., and Gapin, L. 2002. The unconventional lifestyle of NKT cells. *Nat Rev Immunol* 2:557-568.

198. Ho, L.P., Urban, B.C., Jones, L., Ogg, G.S., and McMichael, A.J. 2004. CD4-CD8alphaalpha subset of CD1d-restricted NKT cells controls T cell expansion. *J Immunol* 172:7350-7358.
199. Dutz, J.P., Benoit, L., Wang, X., Demetrick, D.J., Junker, A., de Sa, D., and Tan, R. 2001. Lymphocytic vasculitis in X-linked lymphoproliferative disease. *Blood* 97:95-100.
200. Hammond, K.J., Pellicci, D.G., Poulton, L.D., Naidenko, O.V., Scalzo, A.A., Baxter, A.G., and Godfrey, D.I. 2001. CD1d-restricted NKT cells: an interstrain comparison. *J Immunol* 167:1164-1173.
201. Yoshimoto, T., and Paul, W.E. 1994. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J Exp Med* 179:1285-1295.
202. Burdin, N., Brossay, L., and Kronenberg, M. 1999. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur J Immunol* 29:2014-2025.
203. Singh, N., Hong, S., Scherer, D.C., Serizawa, I., Burdin, N., Kronenberg, M., Koezuka, Y., and Van Kaer, L. 1999. Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J Immunol* 163:2373-2377.
204. Carnaud, C., Lee, D., Donnars, O., Park, S.H., Beavis, A., Koezuka, Y., and Bendelac, A. 1999. Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J Immunol* 163:4647-4650.
205. Roberts, T.J., Lin, Y., Spence, P.M., Van Kaer, L., and Brutkiewicz, R.R. 2004. CD1d1-dependent control of the magnitude of an acute antiviral immune response. *J Immunol* 172:3454-3461.
206. Hermans, I.F., Silk, J.D., Gileadi, U., Salio, M., Mathew, B., Ritter, G., Schmidt, R., Harris, A.L., Old, L., and Cerundolo, V. 2003. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* 171:5140-5147.
207. van Der Vliet, H.J., Nishi, N., de Gruijl, T.D., von Blomberg, B.M., van den Eertwegh, A.J., Pinedo, H.M., Giaccone, G., and Scheper, R.J. 2000. Human natural killer T cells acquire a memory-activated phenotype before birth. *Blood* 95:2440-2442.
208. Fujii, S., Shimizu, K., Smith, C., Bonifaz, L., and Steinman, R.M. 2003. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* 198:267-279.
209. Fujii, S., Liu, K., Smith, C., Bonito, A.J., and Steinman, R.M. 2004. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* 199:1607-1618.
210. Stober, D., Jomantaite, I., Schirmbeck, R., and Reimann, J. 2003. NKT cells provide help for dendritic cell-dependent priming of MHC class I-restricted CD8+ T cells in vivo. *J Immunol* 170:2540-2548.

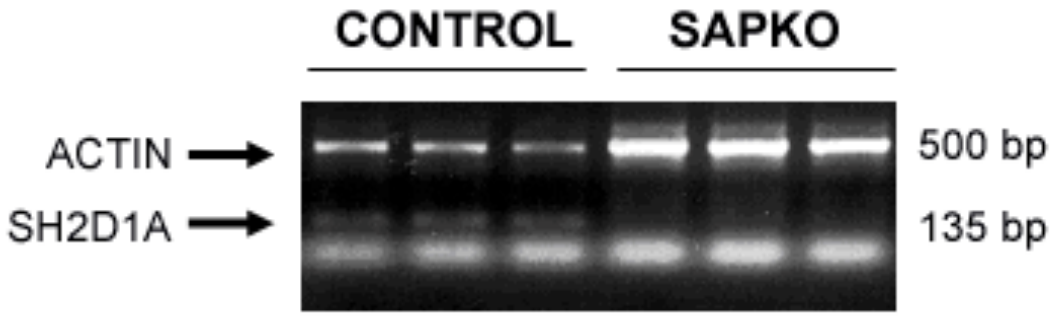
211. Davidson, D., Shi, X., Zhang, S., Wang, H., Nemer, M., Ono, N., Ohno, S., Yanagi, Y., and Veillette, A. 2004. Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine regulation. *Immunity* 21:707-717.
212. Badovinac, V.P., Hamilton, S.E., and Harty, J.T. 2003. Viral infection results in massive CD8+ T cell expansion and mortality in vaccinated perforin-deficient mice. *Immunity* 18:463-474.
213. Weck, K.E., Dal Canto, A.J., Gould, J.D., O'Guin, A.K., Roth, K.A., Saffitz, J.E., Speck, S.H., and Virgin, H.W. 1997. Murine gamma-herpesvirus 68 causes severe large-vessel arteritis in mice lacking interferon-gamma responsiveness: a new model for virus- induced vascular disease [see comments]. *Nat Med* 3:1346-1353.
214. Gaspar, H.B., Sharifi, R., Gilmour, K.C., and Thrasher, A.J. 2002. X-linked lymphoproliferative disease: clinical, diagnostic and molecular perspective. *Br J Haematol* 119:585-595.
215. Chung, B., Aoukaty, A., Dutz, J., Terhorst, C., and Tan, R. 2005. Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. *J Immunol* 174:3153-3157.
216. Nichols, K.E., Hom, J., Gong, S.Y., Ganguly, A., Ma, C.S., Cannons, J.L., Tangye, S.G., Schwartzberg, P.L., Koretzky, G.A., and Stein, P.L. 2005. Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat Med* 11:340-345.
217. Pasquier, B., Yin, L., Fondaneche, M.C., Relouzat, F., Bloch-Queyrat, C., Lambert, N., Fischer, A., de Saint-Basile, G., and Latour, S. 2005. Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J Exp Med* 201:695-701.
218. Szatmari, I., Pap, A., Ruhl, R., Ma, J.X., Illarionov, P.A., Besra, G.S., Rajnavolgyi, E., Dezso, B., and Nagy, L. 2006. PPARgamma controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. *J Exp Med* 203:2351-2362.
219. Allan, L.L., Hoefl, K., Zheng, D.J., Chung, B.K., Kozak, F.K., Tan, R., and van den Elzen, P. 2009. Apolipoprotein-mediated lipid antigen presentation in B cells provides a pathway for innate help by NKT cells. *Blood* 114:2411-2416.
220. Exley, M., Garcia, J., Balk, S.P., and Porcelli, S. 1997. Requirements for CD1d recognition by human invariant Valpha24+ CD4-CD8- T cells. *J Exp Med* 186:109-120.
221. Kenna, T., O'Brien, M., Hogan, A.E., Exley, M.A., Porcelli, S.A., Hegarty, J.E., O'Farrelly, C., and Doherty, D.G. 2007. CD1 expression and CD1-restricted T cell activity in normal and tumour-bearing human liver. *Cancer Immunol Immunother* 56:563-572.
222. Godfrey, D.I., Stankovic, S., and Baxter, A.G. Raising the NKT cell family. *Nat Immunol* 11:197-206.
223. Brigl, M., van den Elzen, P., Chen, X., Meyers, J.H., Wu, D., Wong, C.H., Reddington, F., Illarianov, P.A., Besra, G.S., Brenner, M.B., et al. 2006. Conserved and heterogeneous lipid antigen specificities of CD1d-restricted NKT cell receptors. *J Immunol* 176:3625-3634.

224. Storkus, W.J., Howell, D.N., Salter, R.D., Dawson, J.R., and Cresswell, P. 1987. NK susceptibility varies inversely with target cell class I HLA antigen expression. *J Immunol* 138:1657-1659.
225. Strowig, T., Brilot, F., Arrey, F., Bougras, G., Thomas, D., Muller, W.A., and Munz, C. 2008. Tonsilar NK cells restrict B cell transformation by the Epstein-Barr virus via IFN-gamma. *PLoS Pathog* 4:e27.
226. Heslop, H.E., Slobod, K.S., Pule, M.A., Hale, G.A., Rousseau, A., Smith, C.A., Bollard, C.M., Liu, H., Wu, M.F., Rochester, R.J., et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood* 115:925-935.
227. Hislop, A.D., Palendira, U., Leese, A.M., Arkwright, P.D., Rohrlich, P.S., Tangye, S.G., Gaspar, H.B., Lankester, A.C., Moretta, A., and Rickinson, A.B. Impaired Epstein-Barr virus-specific CD8+ T-cell function in X-linked lymphoproliferative disease is restricted to SLAM family-positive B-cell targets. *Blood* 116:3249-3257.
228. Bottino, C., Falco, M., Parolini, S., Marcenaro, E., Augugliaro, R., Sivori, S., Landi, E., Biassoni, R., Notarangelo, L.D., Moretta, L., et al. 2001. NTB-A [correction of GNTB-A], a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease. *J Exp Med* 194:235-246.
229. Matsuda, J.L., Naidenko, O.V., Gapin, L., Nakayama, T., Taniguchi, M., Wang, C.R., Koezuka, Y., and Kronenberg, M. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* 192:741-754.
230. Vincent, M.S., Leslie, D.S., Gumperz, J.E., Xiong, X., Grant, E.P., and Brenner, M.B. 2002. CD1-dependent dendritic cell instruction. *Nat Immunol* 3:1163-1168.
231. Ohno, R., Asou, N., and Ohnishi, K. 2003. Treatment of acute promyelocytic leukemia: strategy toward further increase of cure rate. *Leukemia* 17:1454-1463.
232. Dezube, B.J. 2000. New therapies for the treatment of AIDS-related Kaposi sarcoma. *Curr Opin Oncol* 12:445-449.
233. Matzinger, P. 2002. The danger model: a renewed sense of self. *Science* 296:301-305.
234. Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature* 449:819-826.
235. Gilmour, K.C., and Gaspar, H.B. 2003. Pathogenesis and diagnosis of X-linked lymphoproliferative disease. *Expert Rev Mol Diagn* 3:549-561.
236. Young, L.S., and Rickinson, A.B. 2004. Epstein-Barr virus: 40 years on. *Nat Rev Cancer* 4:757-768.
237. Vetsika, E.K., and Callan, M. 2004. Infectious mononucleosis and Epstein-Barr virus. *Expert Rev Mol Med* 6:1-16.
238. Su, H.C., Nguyen, K.B., Salazar-Mather, T.P., Ruzek, M.C., Dalod, M.Y., and Biron, C.A. 2001. NK cell functions restrain T cell responses during viral infections. *Eur J Immunol* 31:3048-3055.
239. Ressing, M.E., Horst, D., Griffin, B.D., Tellam, J., Zuo, J., Khanna, R., Rowe, M., and Wiertz, E.J. 2008. Epstein-Barr virus evasion of CD8(+) and CD4(+) T cell

immunity via concerted actions of multiple gene products. *Semin Cancer Biol* 18:397-408.

240. Purtilo, D.T. 1991. X-linked lymphoproliferative disease (XLP) as a model of Epstein-Barr virus-induced immunopathology. *Springer Semin Immunopathol* 13:181-197.
241. van der Vliet, H.J., Molling, J.W., von Blomberg, B.M., Nishi, N., Kolgen, W., van den Eertwegh, A.J., Pinedo, H.M., Giaccone, G., and Scheper, R.J. 2004. The immunoregulatory role of CD1d-restricted natural killer T cells in disease. *Clin Immunol* 112:8-23.
242. Ressing, M.E., Keating, S.E., van Leeuwen, D., Koppers-Lalic, D., Pappworth, I.Y., Wiertz, E.J., and Rowe, M. 2005. Impaired transporter associated with antigen processing-dependent peptide transport during productive EBV infection. *J Immunol* 174:6829-6838.
243. Lockridge, J.L., Chen, X., Zhou, Y., Rajesh, D., Roenneburg, D.A., Hegde, S., Gerdts, S., Cheng, T.Y., Anderson, R.J., Painter, G.F., et al. Analysis of the CD1 antigen presenting system in humanized SCID mice. *PLoS One* 6:e21701.
244. Morrison, J.A., Klingelhutz, A.J., and Raab-Traub, N. 2003. Epstein-Barr virus latent membrane protein 2A activates beta-catenin signaling in epithelial cells. *J Virol* 77:12276-12284.
245. You, S., Zhang, F., Meng, F., Li, H., Liu, Q., Liang, Y., Dong, Y., Yang, W., and Han, A. EBV-encoded LMP1 increases nuclear beta-catenin accumulation and its transcriptional activity in nasopharyngeal carcinoma. *Tumour Biol* 32:623-630.
246. Nelson, W.J., and Nusse, R. 2004. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 303:1483-1487.
247. Chen, Q.Y., Zhang, T., Pincus, S.H., Wu, S., Ricks, D., Liu, D., Sun, Z., Maclaren, N., and Lan, M.S. Human CD1D gene expression is regulated by LEF-1 through distal promoter regulatory elements. *J Immunol* 184:5047-5054.
248. Liu, J., Wang, H., Zuo, Y., and Farmer, S.R. 2006. Functional interaction between peroxisome proliferator-activated receptor gamma and beta-catenin. *Mol Cell Biol* 26:5827-5837.
249. Gandillet, A., Park, S., Lassailly, F., Griessinger, E., Vargaftig, J., Filby, A., Lister, T.A., and Bonnet, D. Heterogeneous sensitivity of human acute myeloid leukemia to beta-catenin down-modulation. *Leukemia* 25:770-780.

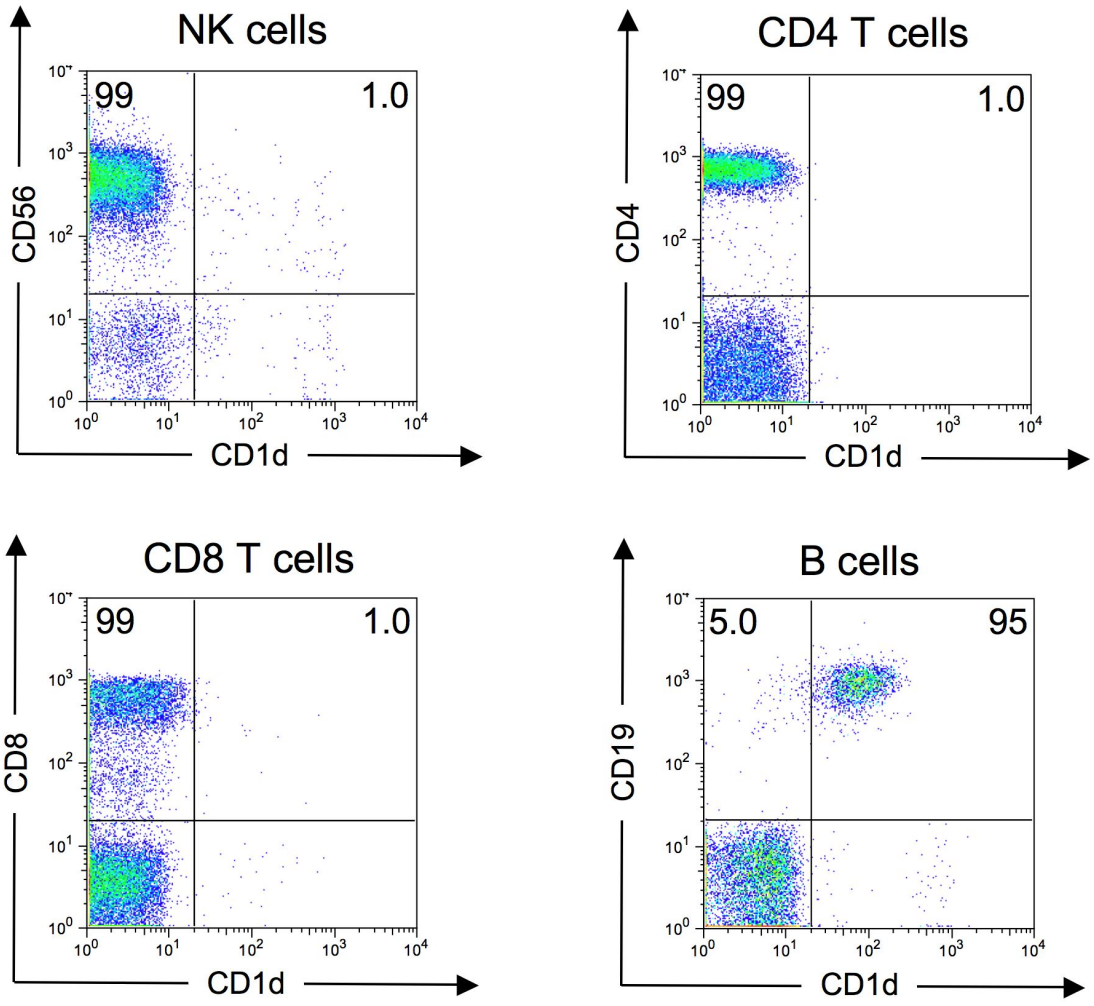
APPENDICES



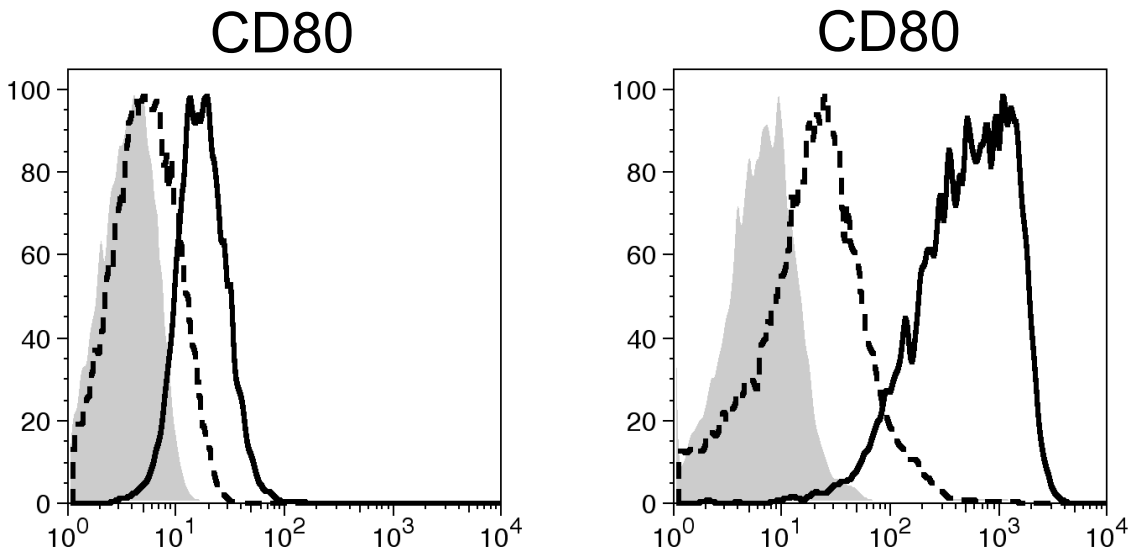
CONTROL: C57BL/6 mice

SAPKO: SAP knockout mice

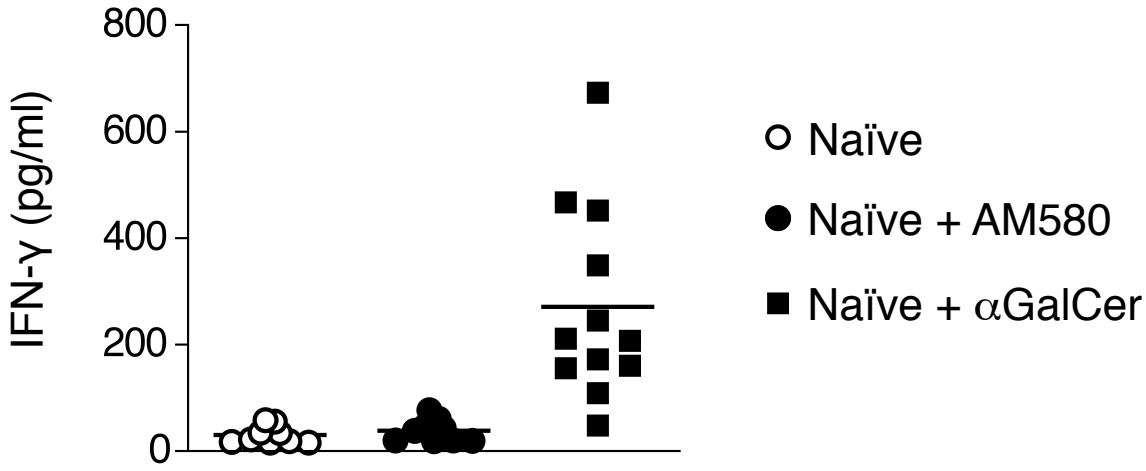
Appendix A. Genotyping of SAP$^{-/-}$ mice. DNA collected from wild-type (control) and SAP$^{-/-}$ mice (SAPKO) ear clippings were digested overnight with Proteinase K and used for PCR genotyping. *SH2D1A* (135bp) is absent from SAPKO mice but present in controls. Actin (500bp) is shown as loading control.



Appendix B. CD1d expression is present on B cells but not NK cells, CD4+ T cells and CD8$^+$ T cells. Tonsillar B cells (CD19$^+$), NK cells (CD3$^-$CD56$^+$), CD4$^+$ T cells (CD3$^+$CD4$^+$) and CD8$^+$ T cells (CD3$^+$CD8$^+$) were stained with anti-CD1d monoclonal antibody and analyzed by flow cytometry. Gated numbers represent the proportion of CD1d-negative vs CD1d-positive cells.



Appendix C. LCL upregulate the expression of the co-stimulatory molecules CD80 and CD86. Naïve B (dotted line) cells and B cells transformed with EBV (solid line) were stained with anti-CD80 and anti-CD86 monoclonal antibodies. Isotype control staining (shaded region) is shown to illustrate background non-specific staining.



Appendix D. Naïve B cells treated with AM580 do not activate NKT cells. Untreated naïve B cells (○, *n* =*9*), naïve B cells treated with AM580 for 48 h (●, *n* = 9) and naïve B cells loaded overnight with αGalCer (■, *n* = 12) were co-cultured with the MO human NKT cell line for 48 h. IFN-γ produced by NKT cells was measured by ELISA performed in triplicate.