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The role of Ly108 isoforms in lymphocyte activation

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TABLE OF CONTENTS

11. Immunopreciptiation and Western blotting
12. Stimulation of cell lines and measuring cell death
13. RT-PCR
14. Bioinformatics
15. Statistical methods

RESUMEN

El Ly108 es una molécula homófila que se expresa en la membrana plasmática, pertenece a las moléculas de la familia del SLAM, e igual que muchas de éstas se asocia a SAP (proteína asociada a SLAM), una proteína adaptadora intracelular que modula las respuestas inmunitarias humorales. El Ly108 se viene describiendo como crucial para el desarrollo de células invariantes NKT y la citotoxicidad de los linfocitos T citotóxicos. Se ha prestado especial atención a la expresión y función de Ly108 desde que se identificaron múltiples isoformas, algunas de las cuales se expresan de forma diferencial en distintas cepas de ratón. En esta tesis estudiamos la expresión y la función de Ly108 usando anticuerpos monoclonales y una proteína de fusión de Ly108, ambos generados por nosotros. Además, con éstos muestro un estudio destallado de los perfiles de expresión en la mayoría de los principales subconjuntos de células inmunitarias. También encuentro efectos coestimuladores o coinhibidores sobre la función de las células T a través de Ly108 dependiendo del reactivo utilizado y del modo de administración. A continuación, se compara la función de las isoformas individuales. Anteriormente habíamos demostrado que la isoforma Ly108-H1 que descubrí podía proteger contra la enfermedad en un modelo de ratón congénito de Lupus. En el trabajo que presento aquí, utilizo líneas celulares para definir aún más la función de Ly108-H1 en comparación con otras isoformas. Demuestro que Ly108-H1 inhibe la producción de IL-2, mientras que tiene poco efecto sobre la muerte celular. Con un método refinado, muestro que se detecta la fosforilación de Ly108-H1 y que éste se mantiene unido a SAP. Finalmente, propongo que Ly108-H1 puede regular la señalización a dos niveles al retener la capacidad de unirse a sus ligandos extracelulares (otras moléculas Ly108) así como a ligandos intracelulares como SAP, e inhibiendo posiblemente las vías descendentes. Además, muestro la detección de Ly108-3 en células primarias que esta isoforma también se expresa de forma diferencial entre cepas de ratón. La presencia de motivos de unión adicionales y de un SNP no sinónimo en Ly108-3 amplía aún más la diversidad entre cepas murinas. En conjunto este trabajo subraya la importancia de conocer las isoformas, ya que la homología inherente puede suponer un reto a la hora de interpretar los datos de expresión de ARNm y proteínas, especialmente porque la expresión alternativa de ARNm o *splicing* afecta potencialmente a la función de la proteína.

ABSTRACT

Ly108 is a homophilic cell surface molecule that binds SLAM-associated protein (SAP), an intracellular adapter protein that modulates humoral immune responses. Furthermore, Ly108 is crucial for the development of invariant natural killer T (iNKT) cells and CTL cytotoxicity. Significant attention has been paid towards expression and function of Ly108 since multiple isoforms were identified, some of which are differentially expressed in mouse strains. We generated monoclonal antibodies and a Ly108 fusion protein to study expression and function of Ly108. I show detailed expression profiles on most major immune cell subsets. I also found co-stimulatory or co-inhibitory effects on T cell function that is dependent on the reagent used and the mode of delivery. I went on to compare the function of individual isoforms. We have previously shown that the Ly108-H1 that I previously discovered could protect against disease in a congenic mouse model of Lupus. Here, I use cell lines to further define Ly108-H1 function in comparison with other isoforms. I show that Ly108-H1 inhibits IL-2 production while having little effect upon cell death. With a refined method, I could detect phosphorylation of Ly108-H1 and show that SAP binding is retained. I propose that Ly108-H1 may regulate signaling at two levels by retaining the capability to bind its extracellular ligands (other Ly108 molecules) as well as intracellular ligands such as SAP, thereby possibly inhibiting downstream pathways. In addition, I detected Ly108-3 in primary cells and show that this isoform is also differentially expressed between mouse strains. The presence of additional binding motifs and a non-synonymous SNP in *Ly108-3* further extends the diversity between murine strains. This work highlights the importance of isoform awareness, as inherent homology can present a challenge when interpreting mRNA and protein expression data, especially since alternatively splicing potentially affects protein function.

ABREVIATIONS

AA - Amino Acid
AICD - Activation Induced Cell Death
APC - Antigen Presenting Cells
BLAME - B-lymphocyte Activator Macrophage expressed
CD - Cluster of Differentiation
CFA - Complete Freund's Adjuvant
CMV - Cytomegalovirus
CRACC - CD2-like Receptor Activating Cytotoxic Cells
CT - Cycle Threshold
CTL - Cytotoxic T Lymphocyte
DC - Dendritic Cell
EAT-2 - Ewing's sarcoma-associated transcript-2
ELISA - Enzyme-Linked Immunosorbent Assay
ERT - EAT-2-related transducer
FACS - Fluorescence-Activated Cell Sorting
FBS - Fetal Bovine Serum
FITC - Fluorescein Isothiocyanate
GPI - Glycosylphosphatidylinositol
GWAS - Genome wide association studies
HRP - Horseradish Peroxidase
Ig - Immunoglobulin
IP - Immunoprecipitation
IL-2 - Interleukin 2
ITSM - Immunoreceptor Tyrosine-based Switch Motif
kDa - Kilodalton
KLH - Keyhole Limpet Hemocyanin
MHC - Major Histocompatibility Complex
iNKT - Invariant Natural Killer T
PBS - Phosphate-Buffered Saline
PCR - Polymerase Chain Reaction
PMA - Phorbol 12-Myristate 12-Acetate)
RT-PCR - Reverse Transcription Polymerase Chain Reaction
SAP - SLAM-associated protein
SHIP - SH2-containing inositol phosphatase
SHP-1 - SH2 domain-containing protein tyrosine phosphatase
SLAM - Signaling Lymphocytic Activation Molecule
SLAMF - SLAM family
SLE - Systemic Lupus Erythematosus
SNP - Single Nucleotide Polymorphism
TCR - T Cell Receptor
Th - T Helper
TLR - Toll-like receptors
WB - Western Blot
XLP - X-liked lymphoproliferative disease

INTRODUCTION

1. Ly108 and other SLAM-family members

Ly108, (SLAMF6 or NTB-A in humans), is a glycoprotein cell surface receptor expressed by a range of immune cell types, including T cells, B cells, NK cells, iNKT cells and dendritic cells [1–5]. It has emerged as a key player in immune modulation, being implicated in facilitating NK cell cytotoxicity, influencing T cell activation and cytokine responses, as well as playing a major role in iNKT cell development and the formation of long term humoral immunity [1,2,4,6–8]. It is a member of the group of cell surface transmembrane protein receptors commonly referred to as the Signaling Lymphocytic Activation Molecule (SLAM)-family (Figure 1). The SLAM-family of cell surface receptors consists of nine proteins: SLAM (CD150, SLAMF1, IPO-3), CD48 (SLAMF2, BLAST-1), Ly-9 (CD229, SLAMF3, Lgp1), 2B4 (CD244, SLAMF4), CD84 (SLAMF5, MAX.3), Ly108 (CD352, SLAMF6, SF2000, NTB-A in humans), SLAMF7 (CD319, CRACC), SLAMF8 (BLAME, CD353), and SLAMF9 (CD2F-10, SF2001, CD84-H1). These receptors have broad, but slightly different expression profiles and present varying and important functions in the immune system [9–15].

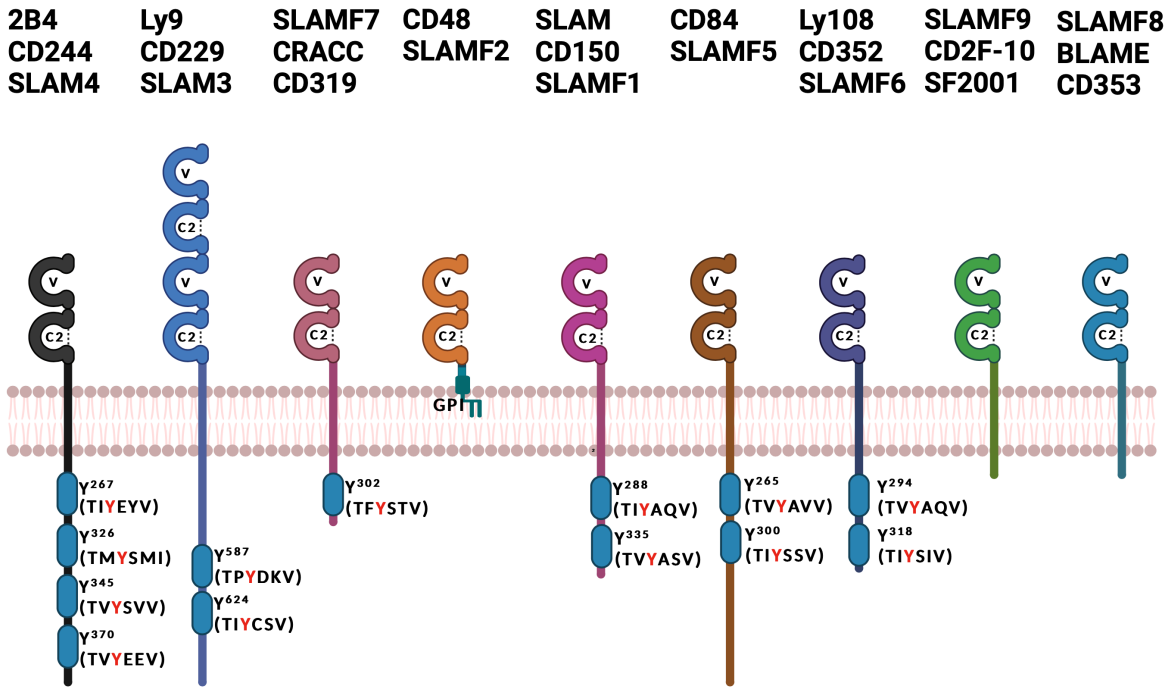

Figure 1. Structural organization of the SLAM family members in mouse. Members of the SLAM family are shown as cartoons in order of their position in the mouse genome. They have a variable-type membrane-distal immunoglobulin domain (V) and a constant-type membrane-proximal immunoglobulin domain (C2) with disulfide bonds (dotted area in C2 domains). All members of the SLAM family have a transmembrane anchoring zone, except SLAMF2 which binds to the membrane through GPI. Those members with a large cytoplasmic region carry one or more copies of an ITSM motif (blue). Next to each ITSM motif, the position of the amino acid tyrosine (Y) susceptible to phosphorylation is indicated, in addition to the location of Y within the ITSM sequence (in red). SLAMF8 and SLAMF9 have a shorter cytoplasmic region, and lack ITSM motifs. The figure shows one representative isoforms of each mouse SLAM family members. Adapted with kind permission from Dr S. Romero Pinedo

The immune system evolved primarily to fight infection but also helps protect from cancer and promotes healing. It consists of organs, tissues, cells and signaling molecules that have developed to sense and kill microbes and cancerous cells, as well as dispose of damaged tissue. Immune cells arise primarily in bone marrow and can travel throughout the body through blood and lymph vessels, sometimes residing for long periods in specialized organs. Cells can signal or 'communicate' by excreting and sensing signaling molecules such as cytokines and chemokines. Immune cells can also signal through the interaction of their cell surface with other cells. It is at this interface between cells of the immune system that Ly108 operates.

Immune cells are generally divided into belonging to the innate or the adaptive immune system. This division is not absolute, with NK and iNKT cell types good examples of cells that bridge innate and adaptive immunity [16,17].

The innate immune system is typically the first line of defense against pathogens, because its response is quicker and less specific, or restricted. Cells of the innate immune system act by ingesting and/or killing pathogens that been recognized due to components of microbes (i.e., bacteria, viruses, fungi) that are often shared. These microbial components are called pathogen-associated molecular patterns (PAMPs) because they can activate the immune system through receptors, such as those from the TLR or NOD/CARD families ([18–20]).

The adaptive immune system can be considered more refined in as much as it has cells that have been selected, programmed, or educated to respond to microbes in a more specific way. This can be by using antibodies, or direct cytotoxicity, process that usually involve a high-affinity antigen receptor such as the B cell receptor or T cell receptor. The developing cells of the adaptive immune system need to be selected in order to prevent harm to the body itself. Firstly, there needs to be a check early in the development of B-and T lymphocytes, as this is when cells are tested whether they are autoreactive or 'self-recognizing'. Autoreactive cells can be harmful as they can cause autoimmunity by attacking the body directly or producing antibodies. A second situation where some control is needed, is during the resolution of an immune response; both innate and adaptive. When microbes or malignancies are killed by immune cells, there is always some damage done to the cells involved, or surrounding tissue. Once the pathogens have been killed the immune response needs to resolve, or calm down, in order to prevent further unnecessary damage while the clearance of debris takes place.

The control mechanisms to prevent overactivity or autoimmunity are extensive and are in place at multiple levels. Following are some examples.

At the genomic level, there is regulation due to imperfect duplication of genes resulting in differences that can counteract the response by serving as decoys or providing opposite signals. This may be the case with Ly108 as one of the SLAM-family members.

At the transcriptional level, regulation may also be achieved by differential splicing, with the Ly108-H1 isoform being a good example of. There are also proteins like IL-1 and HMGB1 that, although generally pro-inflammatory, can also bind receptors that dampen the immune response [20].
At the cellular level, prevention of autoreactive B and T cells is obtained by their deletion or 'killing' by overstimulation at an early checkpoint. This happens when their respective B or T cell receptors happen to recognize a self-antigen during selection. One exception to this rule are iNKT-cells which survive, even though they recognize a self-antigen presented to them in the thymus during development, but this will be dealt with later in the thesis.
Other regulatory mechanism at the cellular level come in the form of regulatory cells such us T-regs which are programmed to counter the immune response through cell surface interaction or the production of anti-inflammatory cytokines. While these are just a few examples, we will expand on those that involve Ly108 later in the thesis.

In sum, because of their broad expression on the collective network of immune cells, SLAM family members are able to form an integral part of the immune system's molecular machinery, influencing immune responses and serving as potential targets for therapeutic interventions.

1.1. General characteristics

Investigating the roles and interactions within the SLAM family is crucial for gaining insights into the intricate network of signaling pathways and regulatory mechanisms that collectively contribute to immune cell modulation. In turn, this knowledge is essential for unraveling Ly108 functions within the broader context of immune regulation. Although the role of all of the SLAM family members in the immune system is too extensive to be discussed in detail in this thesis, I will here focus on the most common themes regard providing stimulation to lymphocytes, T cell help to B cells, NK-mediated cell targeting. For broader and extensive overviews I refer to the publications from Ma et al, Calpe et al and Cannons [10–12].

To effectively discuss and understand the research into Ly108's function, three important characteristics shared by SLAM-family members must be taken into consideration: 1) genomic organization, 2) homophilic interaction, and 3) common signaling pathways.
Firstly, the genes encoding the SLAM-family are located on the long arm of Chromosome 1 and are probably a result of duplication. Seven members are contiguous within a ~400 MB region, with *Slamf8* and *Slamf9* separated from this region by 0.5 Mb (see Figure 2). Individual contributions in mouse models are therefore not easily delineated using interbreeding or classical knockout strategies. This is especially challenging considering this locus is associated with susceptibility to autoimmunity [21].

Improved strategies were later implemented, such as using embryonic stem cells from the B6 background or targeted mutation using CRISP-Cas9 technology[22,23].

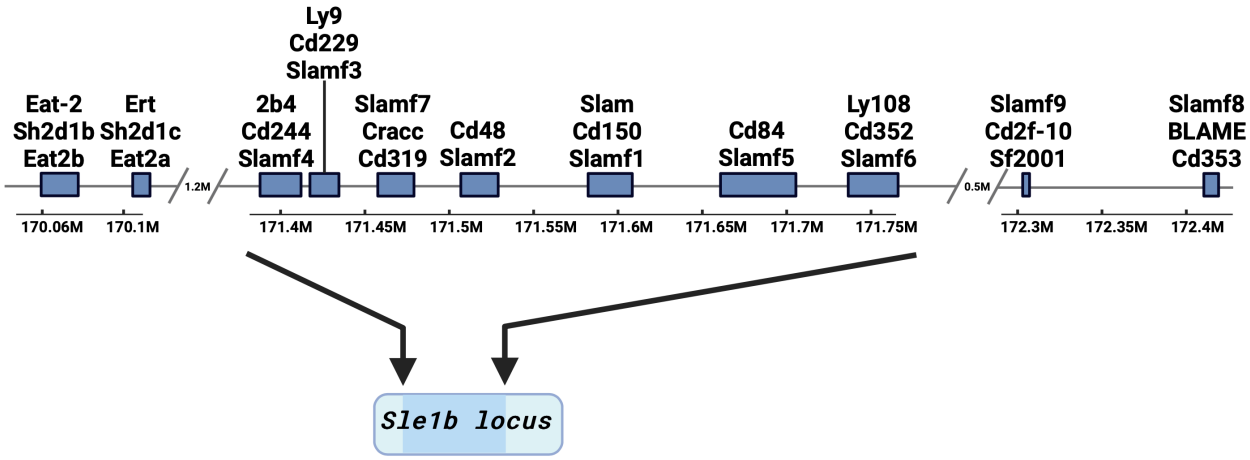

Figure 2. Genomic organization of the SLAM-family on mouse chromosome 1. The upper portion shows a region of mouse chromosome 1 and the position of the SLAM-family receptors and the EAT-2 loci. Filled boxes represent blocks of sequence containing a complete set of gene exons. The lower portion shown by arrows indicate the region that composes the Sle1b locus which is an important murine systemic lupus erythematosus (SLE) susceptibility locus explained further on in introduction. Genomic markers and their positions in chromosome 1 are noted in the map according to the GRCm39 assembly.

Secondly, six of the nine SLAM-family members are self-ligands (including Ly108/NTBA) meaning that their extracellular domains bind in a homophilic fashion. This makes it challenging to study signaling using traditional methods such as fusion proteins, antibody mediated stimulation, or cell culture as these could result in activation or suppression of bi-directional signaling.

Thirdly, most members of the SLAM-family have immunoreceptor tyrosine-based switch motifs (ITSM) in their cytoplasmic tails enabling them to bind SLAM-associated protein (SAP) and EAT-2 (Figure 2), as well as SHP-1, SHP-2, SHIP-1 and Csk [24–27]. Because of these common signaling pathways, studies involving SAP-deficient humans and mice have given us incredible insight into the function of some SLAM-family members. The role of individual SLAM-family members can however be obscured in experiments as other members could compensate their function due to shared signaling pathways. This phenomenon is often referred to as redundancy.

1.2 Expression, structure, and surface ligands

Most SLAM family receptors are broadly expressed on various immune cells, including T cells, B cells, natural killer cells, dendritic cells, monocytes, and macrophages suggests a crucial role in coordinating immune responses across multiple cell types, potentially influencing both innate and adaptive immunity

In contrast, SLAMF8 and SLAMF9 exhibit more restricted expression patterns within immune cells, suggesting potential specialized functions within specific immune responses.

SLAM-family members are structurally similar and have varying, but often overlapping patterns of expression on immune cells. The extracellular domain of the SLAM-family proteins consists of a set of two immunoglobulin-like domains; one C-like, and one V-like, except in the case of SLAMF3 which has two sets of immunoglobulin-like domains in series (see Figure 1). These structures are common in a much wider number of immune-related proteins belonging to the Ig-superfamily. Most SLAM-family members are self-ligands with the exceptions to this homophilic binding being 2B4 and CD48 which are ligands of each other, and SLAMF9 whose ligand remains unidentified.

In the cases studied, binding to their ligand takes place at the outermost V-like domain.

1.3 Intracellular adaptors and signalling

Except for CD48, which is attached to the cell surface membrane through GPI, all SLAM-family members have a transmembrane domain and an intracellular tail. Most SLAM-family members have one or more motifs that are known as ITSMs which consist of a tyrosine embedded in a consensus motif consisting of TxYxxV/I/L. The SLAM-family members that have ITSMs, and are therefore considered 'classical', include SLAM, 2B4, CD84, Ly108 (NTB-A in humans) and SLAMF7. The less typical members, that do not have ITSMs, are CD48, SLAMF8 and SLAMF9. These ITSMs enable the binding of intracellular proteins most closely linked to SLAM-family function called SLAM associated protein (SAP, also known as sh2d1a), EAT-2 (also called EAT-2A or sh2d1b1). In the mouse there is a second EAT-related protein called EAT-2-related transducer (ERT) or EAT-2B [25,28]. These all act as molecular adaptors bridging the SLAM receptors with downstream signaling molecules. These adaptors lack enzymatic activity, but their importance lies in their ability to bind to the cytoplasmic tail of SLAM-family members via their SH2 domain. In most cases, after engagement, the tyrosines in the ITSMs of the SLAM family members become phosphorylated which facilitates the binding to the SH2 domains of SAP and EAT-2/ERT [29–32] (Figure 3). SAP can bind and recruit the Src kinases Fyn, establishing a connection with downstream signal transduction networks and contributing to the regulation of immune cell activation, differentiation, and effector functions [9,33]. This interaction is also unusual, as it does not involve canonical SH3 or SH2 interactions. Instead, an arginine at position 78 (R78) in the SH2 domain of SAP binds with Fyn [30,34]. The SAP SH2 domain surface which interacts with Fyn, does not overlap with the SLAM-binding groove of the SH2 domain of SAP. Not only does SAP binding provide an activating signal, it prevents a inhibiting signal provided by phosphatases that would otherwise bind to the SLAM-receptor [14].

Despite the structural similarities, the SH2 domain of EAT-2 does not contain an R78, and therefore does not bind or link Fyn to the SLAM-receptor in the same way as SAP. Instead, EAT-2 has tyrosines that mediate downstream signals after binding to a SLAM-receptor, and involves Phospholipase C-gamma (PLCγ), a different adaptor to that of SAP [28,35].

In the most direct comparison of expression of these molecules RT-PCR was performed on mRNA from immune cells [28]. SAP was detectable in thymocytes, splenic T cells and NK cells, while EAT-2 mRNA was detected in NK cells, macrophages and dendritic cells. The closely related protein ERT was only detected in NK cells.

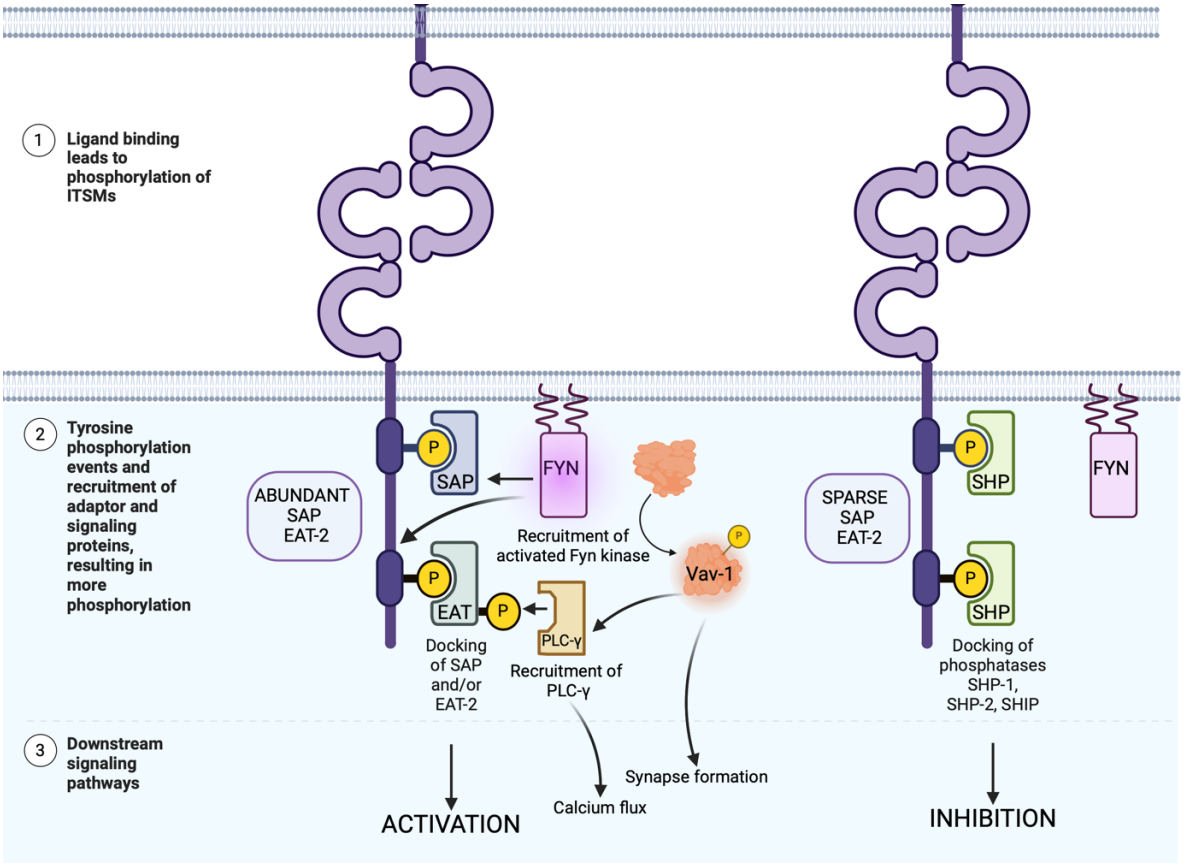

Figure 3. A graphical representation of the dual functions of SLAM-family receptors and SAP adaptors. The order of signaling events is indicated on the left (1-3). The pathway shown on the left represents the activating functions in the presence of SAP and/or EAT-2. Here we see SAP adaptors binding to the ITSMs of SLAM receptors, followed by recruitment of the Src family kinase Fyn. Fyn-mediated phosphorylation of downstream signaling molecules like Vav-1, leads to immune cell activation. On the right we show inhibitory functions with little or no SAP or EAT-2. Here we see the association of SLAM receptors with phosphatases (SHP-1, SHP-2, SHIP) in the absence of SAP, leading to inhibitory signalling.

Other proteins than can bind to ITSMs are the phosphatases SHP-1, SHP-2 and SHIP and the kinase Csk, and these are more often linked to inhibitory receptors or signals. SHP-1, for example, is a protein tyrosine phosphatase that is activated after lymphocyte activation and is able to bind and dephosphorylate other signaling proteins, as well as activating enzymes in NK cells [36]. In a different manner, Csk suppresses T cell activation by phosphorylating the inhibitory tyrosine residues of the Lck and Fyn [37].

The fact that some proteins bind better than others to ITSMs must be taken into consideration, as the mere lack of their expression can have significant impact on function by allowing the binding of another protein [14].

1.4 Immune processes influenced by SLAM-family members

Due to the large body of data it is not possible to describe all SLAM-family members in detail. Broadly speaking, the interaction of SLAM-family proteins with their ligands on other immune cells results in signals that are important for selection, differentiation, and activation.

1.4.1 Function of the SLAM-family as a whole

Over the years antibodies and knockout mice for all SLAM-family and SAP-family members have been generated to study function, but the most exciting findings have been shown by targeting different combinations of these proteins simultaneously.

To study the role of SLAM-family members while also addressing issues regarding redundancy, Cre-lox and CRISP-Cas technology allowed for the targeted deletion of multiple SLAM-family members.

Using CRISPR-Cas gene editing technology the Dong laboratory simultaneously targeted the individual genes of 2B4, Ly9, SLAMF7, CD48, SLAM, CD84 and Ly108 [38]. This resulted in deficiency of these 'classical' SLAM-family members (all excluding SLAMF8 and SLAMF9). Studies in these mice showed an defective NK and iNKT cell development, but normal humoral response when checking T follicular helper cells (TFh) development [38,39]. In addition, the lack of SLAM-family members unexpectedly resulted in increased phagocytosis of hematopoietic cells by macrophages due to the lack of a so-called 'don't eat me signal' [40].

In a separate effort in the Veillette laboratory, a large portion of the major SLAM locus was removed by introducing loxP sites before the 2B4 gene and after Ly108 gene, followed by Cre-mediated deletion

of the almost 400 kilobases of genome between [41]. This resulted in the removal of the same SLAM-family members done with the CRISPR-Cas method. Studies in these mice showed an important role for the SLAM-family in NK cells, iNKT cell development, as well as the humoral response [41–43]. The apparent discrepancy in the results regarding humoral response are probably due to studying long-term end-points including memory B cells and antibody responses [44].

Another use for these animals was for a *'delete and replace'* strategy whereby single SLAM-family genes could be studied by reintroduction of their expression in the otherwise SLAM-family deficient background. In this way it was shown, again, how important Ly108 is for iNKT cell development [43]. The transfection of Ly9 and CD48 into SLAM-family receptor deficient target cells also showed a new function for these genes: their importance in avoiding phagocytosis [40].

In another strategy, three genes were targeted in two separate efforts. In the first, a triple knockout was generated by deleting the genomic region containing SLAM, CD84 and Ly108. These animals showed a significant, but not absolute, defect in iNKT development and an increase in antigen specific IgG titers and plasma cell numbers [22,45]. In a separate approach the triple knockout was generated using CRISP-Cas technology which simultaneously targeted SLAM, CD84 and Ly108. These animals also had the near-total defect in iNKT cell development, but showed no enhanced antibody response [46]. Either way, neither mutated strain had the defective humoral response seen in SLAM-family receptor deficient mice.

Another approach that indirectly examines SLAM-family function utilized SAP and EAT-2 knockouts, as targeting these signaling pathways could also disrupt multiple SLAM-family members simultaneously.

Initial analyses of the SAP knockout mice showed increased short-term immunological activity after lymphotropic choriomeningitis virus (LCMV) infection [47,48]. In depth analyses that followed showed that while SAP expression is not required for early B-cell help or isotype switching, it is required for the development of memory B cells and long-lived plasma cells [49,50]. SAP knockout mice also have impaired both NK and cytotoxic CD8 cell mediated killing of hematopoietic target cells [51,52]. Surprisingly, SAP deficient NK cells display enhanced killing of non-hematopoietic cells [6]. Another striking finding was the total block of iNKT cell development also seen in SAP knockouts, data that pre-dated that of the SLAM-family knockouts [53–55].

EAT-2 signalling is not completely understood, but is also generally considered a positive regulator, particularly in NK cells. It enhances cytotoxic responses and cytokine production upon activation of SLAM family receptors such as NTB-A, 2B4, CD84 [35,56–58]. EAT-2 seems to exert its effects by promoting polarization of cytotoxic granules towards the cells surface [35]. However, other studies in

the knockout mice showed that EAT-2 is a negative regulation of natural killer cell function [28]. These differences are different to align, but there is added complexity due to the presence of ERT that, together with concurrent SAP expression, probably also results in redundancy [25,28].

SAP/EAT-2/ERT triple knockouts helped to address this and showed that these proteins work together in the effective surveillance of hematopoietic tumor cells by NK cells together with the SLAM-family members 2B4, Ly108, SLAMF7 and Ly-9 [59]. This collaboration supports NK cell cytotoxicity through SAP by promoting the formation of immunological synapses and to enhance the killing of target cells [59,60] . It facilitates the activation of cytotoxic T CD8+ lymphocytes (CTLs) as well [52].

1.4.2 Function of individual SLAM-family members

SLAM (CD150, SLAMF1, IPO-3)

SLAM has two extracellular Ig-like domains, an transmembrane domain and an intracellular tail with three tyrosines, two of which are embedded in ITSM sites [6111]. It is expressed on T cells, B cells, dendritic cells, macrophages, and platelets [61–65]. It is a self-ligand, but is also the receptor of the measles virus [66]. It has been shown to be important in a number of processes that revolve around T and B cell activation, selection of iNKT cells, and is also a bacterial sensor that regulates the killing of Gram-negative bacteria [1,61,6768]. There is also evidence that it can provide a negative signal in dendritic cells [69]. SLAM was originally shown to be a stimulating molecule on T cells using monoclonal antibodies, but there is some doubt as to whether this may have instead be caused by the blocking of a negative signal provided by the homophilic SLAM-SLAM interactions [10,61].

CD48 (SLAMF2, BLAST-1)

CD48 is expressed on almost all immune cells, and is involved in an equally wide variety of immune functions [70]. CD48 has two surface ligands; a high affinity ligand 2B4 (CD244) and CD2. It can bind ligands in a *trans* manner in the interface between APCs and T cells, providing stability to the immune synapse[71]. It can also augment T cell receptor signaling by binding to CD2 in a *cis* manner i.e. on the same cell [72]. Structurally, CD48 is GPI linked to the cell surface and therefore does not have an intracellular tail. Nevertheless, signaling can be mediated due to the fact that CD48 is located in lipid rafts, and may associate with other signaling proteins such as Lck, resulting in T cell receptor signaling and IL-2 production [73,74]. CD48 plays a role in antimicrobial immunity and was the first SLAM family member shown to engage microbes, binding the bacterial protein FimH, although this actually results in intracellular *E.coli* survival [75–77] . CD48 is also involved in the antiviral response, particularly through

its binding with 2B4, although the interaction can result in activating or suppressing signals [78]. The type of response is, in-part, linked to the level of SAP expression [26]. In addition, cells infected by HIV express low levels of CD48 thereby avoiding being targeted by NK cells [5]. These interactions and mechanisms also lie at the basis for the exciting results from studies of anti-tumor immunity (see 2B4 section). It is implicated in autoimmunity where in a genome wide analysis of patients with multiple sclerosis, CD48 was identified as a candidate for further studies [79]. There is also some evidence that CD48 plays a role in allergic disorders with there being increased CD48 expression on eosinophils from skin, blood or nasal polyps from atopic patients [80–82].

Ly9 (CD229, SLAMF3, Lgp100)

Ly9 is expressed on thymocytes and B and T lymphocytes in mouse and human, as well as monocytes in mouse [83–88]. Ly9 knockouts (generated in 129 stem cells) showed slight T cell defects as well as increased iNKT cell numbers (when bred to the Balb/c background) [87,89,90]. There was also an expansion of 'innate like' T- and B cells. This could mean that Ly9 is a negative regulator of 'innate like' lymphocytes. Of interest is that Ly9 can not only bind SAP or EAT-2, it also binds the intracellular proteins Grb2 and μ2 that control its internalization after stimulation [91–94]. Also of note is that Ly9 has two alleles which differentiate strains of mice along the line of the SLAM-locus haplotypes important in the studies of Lupus in mice [21,95,96]. The different alleles are not directly implicated in the development of lupus, as Ly9 knockout mice on different genetic backgrounds still developed auto-antibodies [97]. Recently, Ly9 was shown to provide a 'don't eat me' signal to macrophages [40].

2B4 (CD244, SLAMF4)

2B4 is expressed on various cell subsets including NK cells, CD8 T cell subsets, γδ-T cells, monocytes, basophils and eosinophils [98]. The most prominent function of 2B4 is its role in antiviral and antitumor immunity, both of which are driven by cytotoxicity mediated by NK and CD8 cells [78]. Different studies have shown that 2B4 can provide positive or negative signals, depending on the organism, situation or cell subsets examined [78,98–100]. The most feasible explanation for this is that in the presence of high levels of CD48 and SAP, 2B4 engagement results in an activating signal, while in the absence of SAP a negative signal is provided [14]. This may also be why CD48-2B4 signaling provides a 'don't eat me' signal to macrophages [40]. An added complexity is that 2B4 has isoforms. There are two isoform of 2B4 in the mouse which differ in their intracellular domains and provide opposing signals, probably due to

the longer isoform having additional ITSMs [101,102]. There are also two isoforms of 2B4 in human (2B4-A and 2B4-B) but these differ in their extracellular domains [103]. A difference in signal was attributed to a variance in binding affinity for CD48 [104].

CD84 (SLAMF5, MAX.3)

CD84 is structurally similar to SLAM and Ly108 as it has two IgG domains, a transmembrane region and an intracellular tail with two ITSMs [105]. It is expressed on most, if not all, leucocytes including B cells, T cells, platelets, monocytes, dendritic cells [65,88,105–107]. CD84 is required for effective T-B cell adhesion, and disruption of this results in an impaired germinal center formation and reduced T follicular helper cells [107–109]. While monoclonal antibodies against CD84 increased IFN-γ secretion of human lymphocytes in a co-stimulatory manner, T cells from the CD84 knockout mouse had no defect in proliferation or cytokine production [107,108] , indicating complex regulatory mechanisms and warranting further studies.

SLAMF7 (CRACC, CD319, CS1, 19A)

SLAMF7 is expressed on NK cells, iNKT cells, B cells, DCs, macrophages and activated T cells. It is also expressed on a small fraction of double negative thymocytes [58]. It is an activating receptor on NK cells, and this function is dependent on EAT-2, but not SAP [58]. It was also shown to have an inhibitory effect on T cells. SLAMF7 is a self-ligand and binding is required for the phagocytosis of hematopoetic tumor cells by macrophages in a SAP/EAT-2 independent manner [42]. SLAMF7 is highly expressed in multiple myeloma cells and a humanized antibody, elotuzumab, is capable of promoting the destruction of these tumor cells [110]. Elotuzumab enhances the killing of tumor cells in two ways, 1) by stimulating NK cells by engaging their surface SLAMF7, or 2) antibody mediated cytotoxicity when recognizing SLAMF7 on tumor cells, and binding of the Fc fraction to the CD16 activation receptor of NK cells [14]. In recent publication it was shown that due to a *cis* binding with CD47, SLAMF7 on tumor cells could be hidden from macrophages expressing SLAMF7, and phagocytosis avoided [111].

SLAMF8 (BLAME, CD353)

What we know about SLAMF8 so far is that is also a homophilic receptor, has a relatively short intracellular tail that lacks known binding motifs, and that its expression and function is focused around phagocytic cells such as neutrophils, macrophages and dendritic cells [112–114]. SLAMF8

expression is induced on macrophages by bacteria or IFN-γ and was shown to negatively regulate Nox-2 activity in bacterial phagosomes [113]. This negative regulation is PI3K dependent, and in the absence of SLAMF8 there is increased microbial killing [114]. There is also increased *in vivo* migration of dendritic cells, macrophages and neutrophils in SLAMF8$^{-/-}$ mice [115]. Possibly contradicting this is that SLAMF8 knockout significantly suppressed the recruitment of immune cells in a murine arthritis model and that SLAMF8 inhibition also alleviated ischemia/reperfusion-induced myocardial injury and oxidative stress [116,117].

SLAMF9 (CD2F-10, SF2001, CD84-H1)

The SLAMF9 gene is found close to that of SLAMF8 and the protein is similar to SLAMF8 in that it had an extracellular and transmembrane region with a short intracellular tail without SAP binding motifs [118–120]. Although SLAMF9 mRNA was originally found in monocytes, dendritic cells, T cells and B cells, more detailed analysis has shown predominant expression in dendritic cells and certain macrophages [118,119,121–123]. Notably, after using monoclonal antibodies, expression seemed to be particularly apparent on plasmacytoid dendritic cells and in SLAMF9-knockout mice there was disruption of plasmacytoid dendritic cells (pDC) homeostasis and function [121,123].

2. The role of Ly108 and SAP in the immune response

Ly108, also commonly known as SLAMF6 or NTB-A in humans, plays a crucial role in immune regulation. As mentioned before, its expression is most apparent on lymphocytes but it is also expressed on dendritic cells [1–5]. During cell surface interactions between some of these cells, homophilic binding of Ly108 molecules support processes such as NK cell mediated killing, iNKT cell development, and germinal center formation [1,8,43].

Just like other SLAMs, its signaling is mediated through SAP through the ITSMs in Ly108s intracellular tail [10,49]. The understanding of the exact function and expression of Ly018 is challenging due to the presence of isoforms that arise due to alternative splicing. We now know of four Ly108 has isoforms with identical extracellular domains that act as self-ligands in the immune synapse [2,3,124]. Therefore, before delving deeper into complexities, it is imperative to elaborate on isoforms as they are variably expressed and have different intracellular tails.

Later in the section, we will focus on the role of Ly108 in immunity and disease as pay special attention to the emerging role of isoforms as these could have implications for studying SLAM-family members as therapeutic targets in immunology and onco-hematology.

2.1 Ly108 isoforms and tools used to study them

The discovery and initial characterization of Ly108 took place during a search for p53-regulated genes, and described the isolation of two isoforms, Ly108-l and Ly108-s, that are a result of alternative splicing [124]. Alternative splicing is a mechanism allowing a single gene to produce multiple mRNA transcripts and subsequent protein isoforms. This process allows for inclusion, exclusion, or partial utilization of exons during RNA processing resulting in variations of the final mRNA sequences. This flexibility allows cells to produce a more diverse array of proteins, some with unique functions, adding a layer of adaptability and complexity. Alternative splicing contributes to immune cell development and function, and has been described in other SLAM-family members, including NTB-A, the human version of Ly108 [101,103,119,125–130].

The Ly108-l mRNA transcript is longer than Ly108-s and they were therefore presumably designated because of this (long and short). These isoforms were later referred to as Ly108-1 and Ly108-2, respectively, which is perhaps more appropriate as the Ly108-1 protein is actually shorter than that of Ly108-2 (see figure 4 C). The reason for the discrepancy between relative length of cDNA transcript

and protein is that *Ly108-1* (Ly108-1) has a large untranslated region. These two original isoforms share identical extracellular and transmembrane sequences, as well as a large part of their cytoplasmic tails (Figure 4C). The common tail sequences include one ITSM, which are likely the most important motifs for function. Alternative splicing after exon 7 results in mutually exclusive exons and Ly108-1 and Ly108-2 having different distal ends of their cytoplasmic tails (see figure 4A).

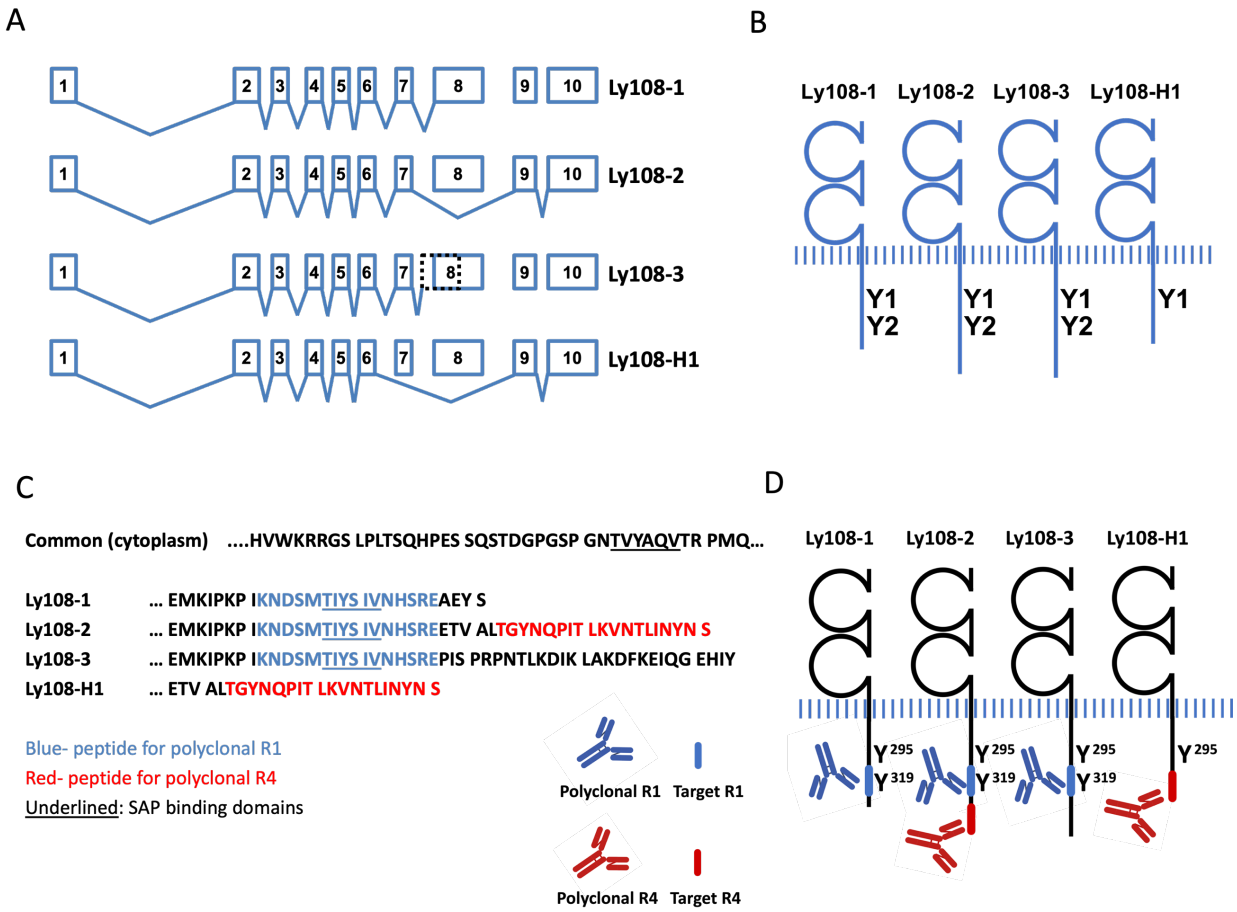

Figure 4. Schematic comparison of Ly108 isoforms. (A) Exon-intron organization of murine ly108 with alignment of Ly108 isoforms. The Ly108-2 isoform is generated by skipping exon 8 with transcription of exon 9 and 10. Ly108-3 is the result of an alternative splice acceptor site in intron 7 resulting in a frame shift. Ly108-H1 is the result of skipping exon 7 and 8. (B) Illustration of Ly108 isoforms with the position of the tyrosines corresponding to ITSMs (SAP/EAT-2 binding sites) indicated. (C) Alignment of amino acid sequences from the cytoplasmic domains of Ly108 showing SAP binding sites (underlined) and the sequences used to generate polyclonal antibodies R1 (blue) and R4 (red). (D) Illustration of Ly108 isoforms with the position of the tyrosines and target sites of the polyclonal antibodies R1 (blue) and R4 (red).

A third isoform; Ly108-3, of which still little is known, was briefly described in the supplemental data of a publication from the Veillette group [2]. We know from the sequence that it is formed by an alternative 3' splice site for exon 8 and contains two ITSMs like Ly108-1 and Ly108-2. Also shown is that it is phosphorylated after cross-linking to a level in-between that of Ly108-1 and Ly108-2. Due to the importance of isoforms put forward in two high-impact publications [96,131], we set out to examine Ly108 and its isoforms at the protein level.

As there were no antibodies available for the detection of Ly108 at that time, we set out to generate these. We immunized a Ly108 deficient mouse ($Ly108^{\Delta E2+3}$) with wild type murine splenocytes before fusing with myeloma cells. For screening purposes, we produced a fusion protein that combined the extracellular domain of Ly108 fused with a human Fc segment (Ly108Fc). Two hybridoma clones, 13G3-19D and 330-AJ, were later used for antibody production. Because both these clones were selected by their reactivity to the extracellular domain of Ly108, they recognize all isoforms. These monoclonal antibodies have been used to study Ly108 surface expression as well as function by us and other groups [1,22,32,39,40,52,132–135].

To examine the first two isoforms at the protein level, I generated polyclonal antibodies directed at segments of the intracellular tail by immunizing rabbits with corresponding peptides. I used one peptide with a sequence that was common to Ly108-1 and Ly108-2 which resulted in polyclonal R1. We also used a second peptide that, at that time, was thought to be specific for Ly108-2 to generate polyclonal R4. The targets of these antibodies are shown in the sequence alignment of Figure 4C and illustrations of Figure 4D.
In order to determine protein levels of individual isoforms we immunoprecipitated and deglycosylated Ly108 and, with polyclonal R4, surprisingly found a new isoform now known as Ly108-H1[3]. Based on our understating of the protein sequences and where polyclonal R1 and polyclonal R4 mapped to, we predicted that Ly108-H1 was an isoform that resulted from skipping exon 7. This was also possible because skipping exon 7 would nevertheless keep the tail sequence in frame, something that was needed for it to remain a target for polyclonal R4. These deduced characteristics enabled us to quickly confirm and clone Ly108-H1 with a full-length RT-PCR with cDNA from a B6 mouse. It is noteworthy that at the time I submitted the sequence we were calling the isoform Ly108-3 (Rietdijk, Wang, Terhorst. Genbank EU591721). We changed the name to Ly108-H1 after the name Ly108-3 was allocated to another isoform[2]. Alignment of the all Ly108 isoform amino acid sequences, together with the targets of our previously generated polyclonal antibodies lead us to believe that Ly108-3 should also be detectable with antibody R1, as it contains the target sequence. Ly108-3, however, is very similar in molecular weight to Ly108-2 (39.1 vs 38.6 kDa, respectively) and this may have been why we did not

previously see it. We had not identified this isoform during our studies, and questioned whether this was the case.

There are four human isoforms of NTB-A found in mRNA databases, three of which were recently studied [130,136]. One isoform is the canonical form, with extra and intracellular domains similar to those of mouse Ly108, while the other two isoforms have partially truncated extracellular domains. One of these isoforms provided a stimulatory signal, which could oppose the regulatory signal of the canonical NTB-A. In addition, the alternative splicing of NTB-A could be steered by the use of antisense oligonucleotides, thereby increasing anti-tumor effect of tumor infiltrating lymphocytes [130].

2.2 Ly108 expression and that of its isoforms

NTB-A (named as it was a NK, T, and-B-antigen) expression was originally described in humans on NK, T-and B cells, as well as thymocytes [137]. It is also present on eosinophils but no expression was found on monocytes or granulocytes in the original publications [4,82] . In the mouse, Ly108 expression has been more extensively studied and the expression profile is similar to that of human. One matter that has yet to be resolved is the expression on macrophages. Expression of NTBA-A was shown on monocytes, but results from the mouse are not clear. Ly108 expression was not detected by Zhong *et al* on peritoneal macrophages, while later it was found by Zarama *et al* [2,138]. Perhaps this discrepancy is due to the fact that expression can be up- or downregulated by activation, and seems to be particularly high on polarized (M2) macrophages [135,138] .

Ly108 is expressed by almost all cells in the thymus, with the highest levels on early thymocytes i.e. double negative and double positive thymocytes [1]. One notable exception we found was that iNKT cells, which express Ly108 in immature phases, effectively lose Ly108 expression at the final stage of their thymic development only to regain it once in the periphery [1]. In the periphery, all CD4 T cells, CD8 T cells, and B cells express Ly108, with slightly higher levels on B cell [2]. Expression on these cells was increased after stimulation with PMA and ionomycin (and anti-CD3/CD28 in T cells or anti-IgM or LPS in B cells). Staining of freshly isolated NK cells showed a small subset of Ly108 positive cells, with a percentage that increase from 11% to 17% upon stimulation with poli I:C. In contrast to the NTB-A data from humans, these cells lost Ly108 expression when propagated [2]. In another study, Ly108 expression was also found on NK cells with decreasing levels when examining sequential development stages [6]. Dendritic cells also express Ly108, while macrophages do not [1,2]. Before the availability of antibodies, we had detected Ly108 by RT-PCR in neutrophils, but this has yet to be shown at the protein level [139].

We also found differences in surface expression of Ly108 when comparing different mouse strains. Strains with SLAM-haplotype 1 (B6, MOLF/EiJ) expressed more Ly108 than strains with SLAM-

haplotype 2 (Balb/c, 129, Sle1b) [3]. Similar results were found when measuring mRNA by quantitative PCR (TaqMan) or immunoprecipitated protein by western blot [3]. One explanation for this could be the presence of an additional isoforms: Ly108-H1, found exclusively in SLAM-haplotype 1 strains. Notably, this also explains why Ly108-H1 was not discovered together with the original two isoforms, as mRNA was isolated from mice with a Balb/c background [124].

Strain specific differences in isoform expression had previously been found with quantitative PCR examining Ly108-1 and Ly108-2 in Lupus prone strains (see SLE section below) [96]. We, however, showed Ly108-1 mRNA levels to be comparable between B6, 129, and Sle1b mice when we performed RT-PCR [3]. This was not in agreement with quantitative PCR data from other studies showing higher levels of Ly108-1 in 129 and Sle1b mice [32,96,131]. A possible explanation for this discrepancy lies in sequences shared between Ly108-1 and the yet-to be discovered Ly108-3 isoform. Using oligonucleotides with targets common to both isoforms would have resulted in amplification of both during quantification. As noted by others, this was the case for Ly108-2 where mRNA levels initially measured would have been overestimated because of the then-unknown sequence homology with Ly108-H1 [32], and may have also been the case with protein levels [3].

There is no published data on expression of the Ly108-3 isoform in primary cells.

2.3 Ly108 signaling

Engagement of Ly108 in the presence of SAP results in Ly108 being phosphorylated. This also results in better binding of SAP, in a process that is supported by Fyn, presumably by to binding of Fyn, as is the case with SLAM [2,24,32]. Stimulation through Ly108 also results in phosphorylation of Vav-1 and c-Cbl, which is similar to what is seen with 2B4, but not SLAM [2]. When comparing Ly108 isoforms Ly108-1 to Ly108-2, Zhong and colleagues saw more phosphorylation of Ly108-1, Vav-1 and c-CBL in stably transfected BI-141 cell lines. These levels of phosphorylation again correlated with the level of SAP binding. When comparing three Ly108 isoforms (Ly108-1/2/3), they saw that Ly108-3 had a high level of phosphorylation, which was between Ly108-1 and Ly108-2. No further data has been published on the Ly108-3. Ly108-H1 was also compared to Ly108-1 and Ly108-2 and shown to have undetectable levels of phosphorylation [32]. We, however, expected the SAP binding to the one ITSM present in Ly108-H1 to be preserved based on analysis using mutation of tyrosines in NTB-A, and others in SLAM [29,56].

Ly108 and NTB-A have been shown to bind to EAT-2 in a yeast two-hybrid system, but only in conjunction with Fyn [120]. NTB-A also binds SAP and EAT-2 in process that is phosphorylation dependent, with both molecules binding at the same time to different sites [56]. NTB-A was also shown to bind SHP-1 and SHP-2 in NK cells, a finding that was not seen in T cells [1374].

Taking a step back though, signaling is initiated when Ly108 binds to other Ly108 molecules with their Ig-V-like extracellular domains expressed on the cell surface. This interaction help stabilize the immunological synapse and enhances signaling and is crucial for at least three of its most important functions: 1) killing of infected or malignant cells through contact with by NK and cytotoxic T cells; 2) thymic development of iNKT cells through contact with CD1d expressing thymocytes; and 3) engagement of Tfh cells and B cells to enable the formation of germinal centers.

2.4 Ly108 and cytotoxicity

Ly108 promotes the killing of malignant cells in two ways. Firstly, via direct killing of malignant hematopoietic by NK and cytotoxic CD8 T cells, and secondly via indirect killing of non-hematopoetic malignant cells after the education of NK cells. NTB-A was originally identified in an attempt to isolate and characterize new activating surface proteins on human NK cells [137]. In this and other studies it was shown that homotypic interaction between Ly108 molecules on NK and target cells resulted in increased cytotoxicity, cytokine production and proliferation. Ly108 appears to play a role in forming a stable immune synapse between the cytotoxic CD8 cell and the target B cell, a process that is dependent on the interactions between its tyrosine residues and SAP [52]. In the absence of SAP and EAT-2, SHP-1 is instead recruited to Ly108 and 2B4 leading to a negative signaling and impaired synapse organization [52]. In apparent contrast, because it only happens in the absence of SAP, Ly108 promotes the education of NK cells allowing for the killing of non-hematopoietic cells, something that is not dependent on stabilization of the immune synapse [6]. There is also a role for EAT-2 in NTB-A mediated activation, which is probably independent of SAP [56]. NTB-A is now a therapeutic target for the treatment of chronic lymphocytic leukemia as it is expressed on both human and mouse CCL cells which responded to antibody treatment [140]. There is also some evidence however that, at least in humans, NTB-A is an inhibitory checkpoint molecule in CD8 cell mediated anti-tumor activity [141,142].

2.5 Ly108 and iNKT cell development

The lack of SAP results in an absence of invariant iNKT cells in humans and mice [53–55]. The selection of iNKT cells in the thymus is a clear example of how SLAM-family members can determine cell fate. iNKT cells are a sub-population of T cells that play an important role in the immune response by rapidly producing cytokines. The T cell receptor of these cells does not react to peptides, but to lipids presented during selection in the thymus, as well as during activation in the periphery. iNKT cells are positively selected if they recognize lipids presented by the MHC-like molecule CD1d, presented on the surface of cortical thymocytes.

Because of defects in iNKT cells from SAP and Fyn knockout mice, we studied the available SLAM-

family knockout mice [1]. Ly108 and SLAM knockout mice had reduced numbers of iNKT cells, and using a bone marrow chimera model, our colleagues in the Bendelac lab were able to study the role of Ly108 and SLAM simultaneously in iNKT cell development. We showed how homotypic interactions in the synapse between CD1d expressing thymocytes and iNKT precursors were needed for effective iNKT development [1]. Our hypothesis at that time was that, because NKT cells are positively selected, Ly108 and SLAM provided a positive signal through SAP. In an unexpected demonstration of Ly108's importance in iNKT cell development, crossing the Ly108-knockout with in SAP-knockout mice, restored the iNKT cell defect [8]. This was explained as an interaction that can provide a signaling switch. Later, the Veillette group showed that the expression of Ly108 could correct the iNKT cell developmental defect present in SLAM-family receptor deficient mice, but that this was due to its providing a negative signal [43]. While this agrees with the current understanding that Ly108 is a negative regulator of the immune response, it is challenging to align all the above findings. While the expression pattern of Ly108 in developing iNKT cells is quite striking, we do not know of any data on the differential expression of Ly108 isoforms.

2.6 Ly108 and the humoral response

The generation of an effective humoral response with potent and specific antibodies is a fundamental requirement for long lasting immunity after exposure to an infection or vaccine. The defining steps of antibody formation take place in the germinal centers that form in stages involving intricate interactions between dendritic cells, T cells and B cells. A significant finding was that the lack of an effective humoral response, the immune defect most apparent in X-liked lymphoproliferative disease (XLP), seems dependent on the interaction between SAP and Ly108 [8]. It has become clear that this is due to a CD4 T helper cell defect associated with SAP deficiency and is characterized by a lack of antigen specific germinal center follicular helper CD4 T cells [49,143]. While there is no defect in the humoral response in Ly108 deficient mice [8,139], the publication of Kageyama and colleagues was able to provide the most significant, yet surprising evidence of the importance of Ly108 and SAP interactions when they showed that the deletion of Ly108 in SAP-deficient mice, restored humoral immunity [8].

There is little new data on isoforms in Ly108 function. The most direct comparison was only of phosphorylation levels showing Ly108-3 having a level of phosphorylation between that of Ly108-1 and Ly108-2 [2]. While mutagenesis of Ly108 has been used to study the role of individual tyrosines in NK function and the humoral response, none of the constructs emulate the Ly108-H1 isoform [2,8]. While only an indirect comparison, NTB-A mutant constructs lacking the second ITSM showed that the second tyrosine was essential for NK cell function and promoted adhesion [56,144].

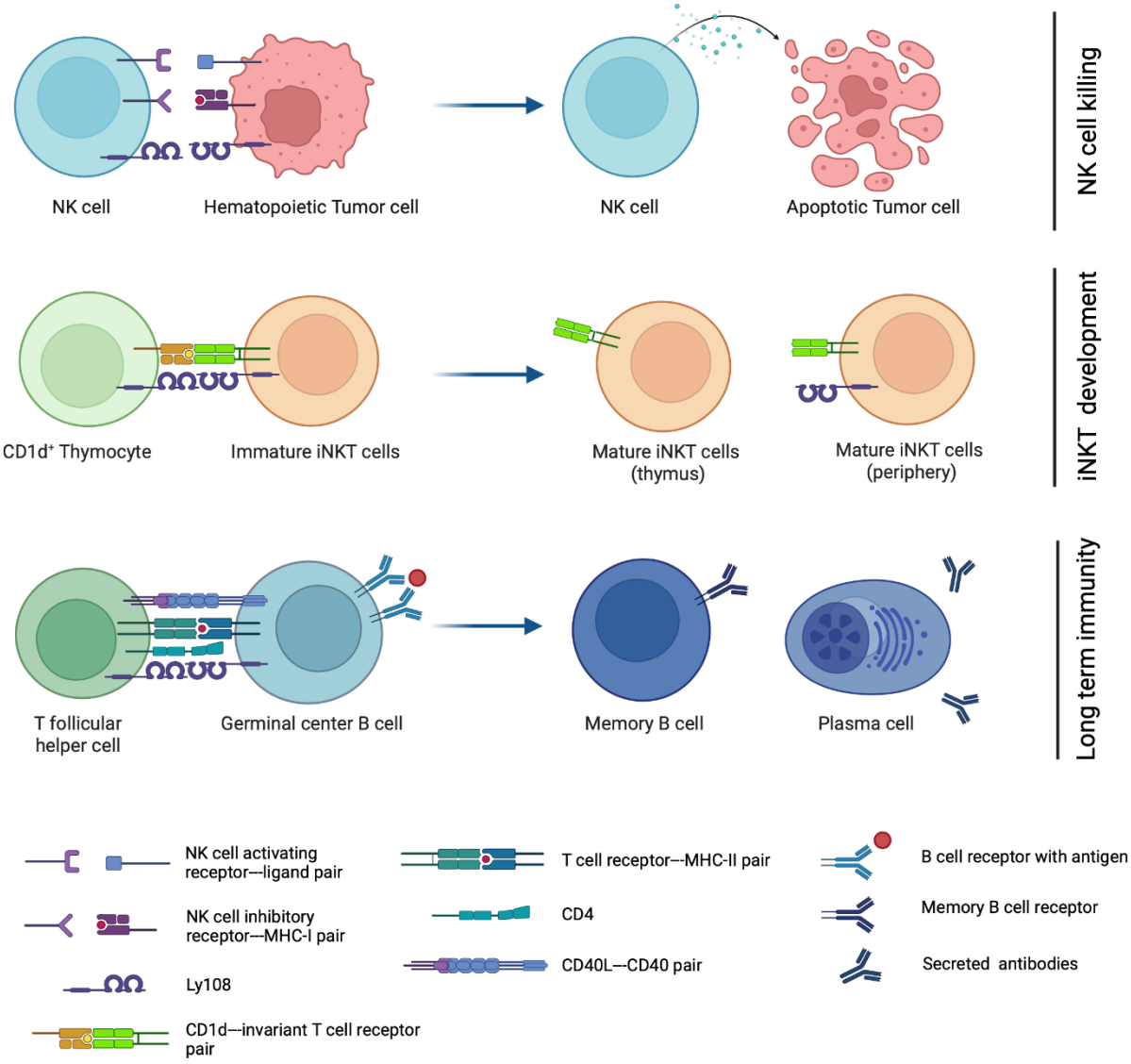

Figure 5. Immunological processes involving Ly108. Homophilic interaction between Ly108 molecules present in the immune synapse promotes NK cell killing (top). During early iNKT cell development, Ly108 on immature iNKT cells interacts with Ly108 on thymocytes that present lipid antigen with CD1d (middle). Ly108 interactions provide signals that, together with SAP result in the development of long-term B cell memory and antibody response (bottom).

3. The role of Ly108 and SAP in disease

3.1 X-linked lymphoproliferative disease as a result of SAP deficiency

The most significant demonstration of the importance of SLAM family members in the immune response can be found in the clinical course of X-linked lymphoproliferative disease (XLP) in humans, which is caused due to deletion or mutations of sh2d1a, the gene encoding SAP [29,145–147]. XLP is characterized by immune dysfunction which can present in a number of ways, the most common being dysgammaglobulinaemia with recurrent infections, malignant lymphoma, and hemophagocytic

lymphohistiocytosis that is usually due to fulminant infectious mononucleosis (FIM) following an Epstein-Barr Virus (EBV) infection [148,149].

The acute, and most severe presentation of XLP is hemophagocytic lymphohistiocytosis that is characterized by extreme systemic immune activation and dysregulation, and is associated with significant mortality, often within weeks after onset. In most cases, the acute severe presentation of XLP arises due to an inappropriate response to EBV, with excessive immune cell activation resulting in a cascade of proinflammatory cytokines and immune cells that, in turn, cause severe damage to liver, spleen and bone marrow[10,149–151]. Due to improved protocols involving immunosuppressive treatments and subsequent hematopoietic stem cell transplant (HSCT), the overall mortality rate has decreased from 75% to 28.6% [149].
Because of the significant risks involved in HSCT, it remains uncertain whether it should be performed in patients who have mild manifestations of disease, even though they are still at risk for severe complications of treatment. Another potential treatment using reconstitution of SAP-deficient stem cells by gene transfer with lentiviral vectors seems feasible as this was shown to correct immune defects in mice[152]. Indeed, a similar approach recently yielded reassuring results in children with otherwise fatal conditions due to deficiency of adenosine deaminase or a-L-iduronidase [153,154].

Some of the specific immunological defects shown in humans with XLP have been put forward as contributing factors in the clinical course. The one most likely to be involved in the acute stage of FIM is impaired cytotoxicity by NK and T cells, resulting in impaired killing of EBV infected cells. This could also lie at the basis of the propensity to develop lymphoma [155,156]
Another factor that could contribute to an inadequate early response to viral infection in humans lacking SAP is the total lack of invariant iNKT cells [53–55]. These cells are known to rapidly produce cytokines as an innate immune response but also contribute to the humoral response [157–159].
A striking finding in humans and mice deficient of SAP is the lack of an effective humoral response[9,10,151]. While patients have reduced numbers of memory B cells and hypogammaglobulinemia, more important is the significant defect in antibody class switching. This is evident in the ineffective generation of antibodies after immunization in humans and mice, as well as response to viral infections.

It is tempting to think that what has been shown in SAP knockout mice could also be applied to patients with XLP; this being that by targeting NTB-A in hematopoietic stem cells, iNKT cell and humoral defects could be reversed, possibly by RNA-interference (knockdown) or CRISPR-Cas.

3.2 Genome wide screening studies in human disease

Systemic Lupus Erythematosus (SLE) and rheumatoid arthritis (RA) are autoimmune diseases characterized by the loss of tolerance towards antigens which can result in the development of autoantibodies, autoreactive immune cells and overreactive immune cells [160,161].

A key element in the pathogenesis of SLE is the development of autoreactive antibodies, particularly against nuclear antigens [162]. Because these and other antigens are broadly expressed all organs in the body can be affected. The resulting symptoms and signs vary greatly among patients, as does the severity of disease, but the parts of the body most often involved include skin, joints, and kidneys as well as hematologic disturbances such as thrombosis. While the etiology is complex and multifactorial, heritability has most recently been estimated to be around 43%[163,164]. Genetic analysis has shown significant linkage to the HLA genes in the MHC region, in addition to at least 138 non-HLA risk alleles contributing towards the development of SLE [165,166]. While there are many susceptibility loci identified by genome wide association studies (GWAS), these can explain less than 30% of cases with the individual contribution of most risks alleles being slight[167,168].
In a different approach examining the genetic contribution, an interbred mouse model of SLE was used to identify genomic regions associated with the development of anti-DNA antibodies [21,169,170]. One of these regions is on the long arm of chromosome 1 containing the SLAM-family of proteins [171]. The significance of this locus was later confirmed in a human study focusing on this region in sibling pairs with SLE[172].

Rheumatoid arthritis is an autoimmune disease primarily involving the synovial lining of the joints [173]. Inflammation can result in bone destruction as well as lead to systemic inflammation with secondary extra-articular organ involvement. GWAS have identified multiple susceptibility loci for rheumatoid arthritis, with the MHC Class-II HLA-DRB1 allele estimated to contribute to susceptibility in more that 30% of cases [174,175]. Recently, NTB-A was also identified as a susceptibility locus in a Korean population[176]. This was added to the growing list of now more than 100 loci identified by GWAS, some of which including those found using extensive re-analysis of existing data[177]

3.3 A role for murine Ly108 isoforms in SLE

As mentioned above, an interbred mouse model of SLE was used to detect genomic regions contributing to the development of autoantibodies, one of which was on chromosome 1. Through extensive backcrossing and interbreeding, and because of naturally occurring homologous recombination within this region, detailed mapping could be performed by screening for disease. It was due to this that the Sle1 locus on chromosome 1 could later be separated into three distinct risk loci that were termed Sle1a, Sle1b and Sle1c[171]. When comparing the impact of each locus on disease progression, it was shown that Sle1b contributed the most to increased immunoglobulin levels, lymphocyte activation markers and the development of anti-chromatin antibodies[171]. The genes in the Sle1b locus, including SLAM-family genes, were examined in more detail by sequence analysis and mRNA expression measurement[96]. When comparing B6 wild-type mice to that from B6 mice with the Sle1b locus (B6.Sle1b) there were significant differences found in protein sequences, as well as mRNA expression levels.

Ly108 was identified as the strongest candidate because of the greatest difference in mRNA levels between B6 wild-type and B6.Sle1b [3]. A more detailed mRNA analysis performed on the two Ly108 isoforms known at that time, seemed to show that the increased levels in B6 mice were due to higher levels of Ly108-2 (also known as Ly108-s)[96,124]. In retrospect, we now know, that because of significant mRNA sequence homology, Ly108-2 levels would have been overestimated due the presence of Ly108-H1, an isoform later found exclusively in B6 mice[3,32]. Nevertheless, additional functional analysis of the two known isoforms provided valuable insight into differences potentially contributing to the development of SLE[96,131]. The basis of functional differences between Ly108-1 and Ly108-2 were explored due to the demonstration of reduced B-cell tolerance in B6-Sle1b mice that were bred to express a B-cell receptor transgene[131]. This was shown to be due to impaired B cell deletion, anergy and receptor revision. To mimic some of these findings, *in vivo* transfection experiments were designed to study the two isoforms, with Ly108-2 expressing cells showing stronger calcium flux and B cell receptor mediated cell death. It seemed at the time therefore, that higher levels of Ly108-2 could explain why B6 mice were protected against the development of SLE.

While attempting to compare Ly108-1 and Ly108-2 at the protein level, I later identified Ly108-H1 and showed it was absent in Sle1b mice[3]. Because of the association between Lupus, the Sle1b locus, and the absence of Ly108-H1 we introduced a single copy into these mice. We were able to ameliorate the development of Lupus with one of the notable findings that there was reduced T cell response in these mice after stimulation.

AIMS

1. Determine the expression of Ly108 on immune cell subsets.

2. Examine the impact of individual Ly108 isoforms on T cell activation.

3. Characterize the protein-protein interactions mediated by the different Ly108 isoforms, particularly in the context of the SAP-dependent pathways.

4. Determine whether the Ly108-3 isoform is expressed as a protein in primary cells, and explore the differences in Ly108 isoform expression between wild type and the Sle1b lupus-prone mouse strains.

5. Develop and utilize genetically modified mouse models expressing individual Ly108 isoforms to dissect their specific contributions to immune cell biology and autoimmunity.

6. Search for novel strain-specific sequence variations.

MATERIALS AND METHODS

1. Generation of Ly108Fc fusion protein

The extracellular region of Ly108 was amplified from thymus cDNA using primers that introduced the restriction sites KpnI and BamHI. After digestion with these restriction enzymes the PCR product was ligated into the pCDNA vector, adjacent to a previously inserted sequence of the human Fc fragment. Transient expression was screened for in COS-7 cells and stable expression (for production) in 293F cells by WB of cell lysates and supernatant using Goat anti-human-IgG-HRP. Ly108Fc was purified from supernatant with protein G agarose before elution and dialysis.

2. Generation of monoclonal anti-Ly108

Monoclonal antibodies against Ly108 were generated by immunizing a Ly108-deficient mouse with wild type (*wt)* thymocytes. Splenocytes were fused with NS1 myeloma cells and were selected based on supernatant's reactivity with plate bound Ly108-Fc, as judged by ELISA. After three rounds of subcloning and limiting dilution, two clones (13G3-19D and 330-AJ, both IgG2a) were selected based on reactivity with Ly108-Fc, as judged by ELISA (see Figure 6). We refer to these antibodies as 13G3 and 330 in this work. Antibodies were produced in bioreactor flasks (CELLine, Sigma Aldrich) and purified from supernatant with protein G agarose before elution and dialysis.

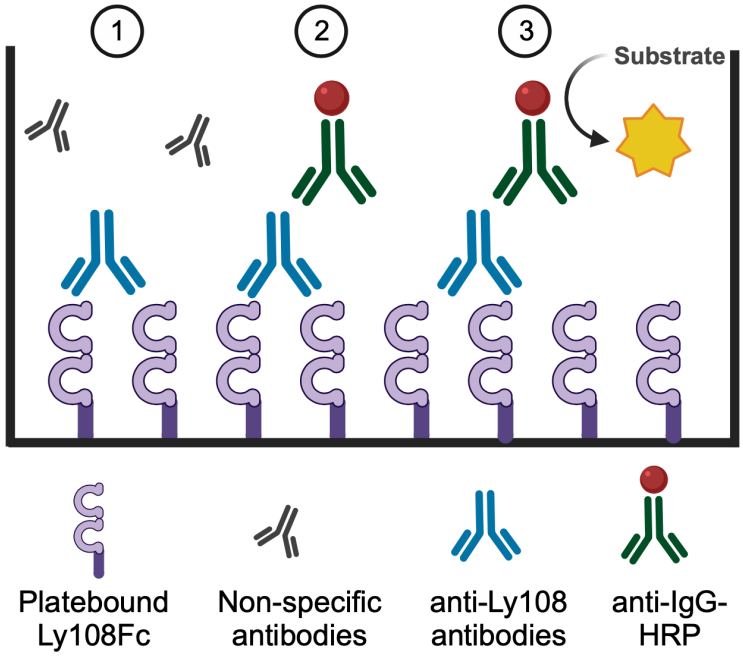

Figure 6. Illustration of the sandwich ELISA developed to screen hybridoma supernatants. From left to right there is (1) binding of anti-Ly108 antibodies to the capture peptide Ly108Fc (human Fc). (2) Detection of bound antibodies by secondary antibody against mouse IgG-HRP (3) addition of substrate for the HRP to provide visible signal. Created with BioRender.com.

3. Mice

C57BL/6 (B6) mice were purchased from Jackson and 129SvEvTac (129) mice from Taconic. B6.Sle1b (Sle1b) mice were kindly provided by Dr L. Morel. All animals were housed in the animal facility of the Beth Israel Deaconess Medical Center under approved protocols.

Transgenic mice expressing Ly108 isoforms were generated in a manner previously described [178]. Briefly, individual Ly108 isoforms (Ly108-1 and Ly108-H1) were amplified from a thymus of a C57BL/6 mice using primers that introduce unique XmaI restriction sites. Amplified products were cloned into pCR2.1 TOPO (Invitrogen) before subcloning into the human CD2 cassette. Removal of vector backbone (prokaryotic DNA) was performed by digestion prior to purification by agarose gel electrophoresis and dialysis. Purified DNA was injected into fertilized C57BL/6 oocytes. Screening of founders was performed by PCR and these were crossed with Ly108 knockout mice to have animals expressing one isoform of Ly108.

We had also generated Ly108-2 transgenics, but this line was lost to breeding difficulties.

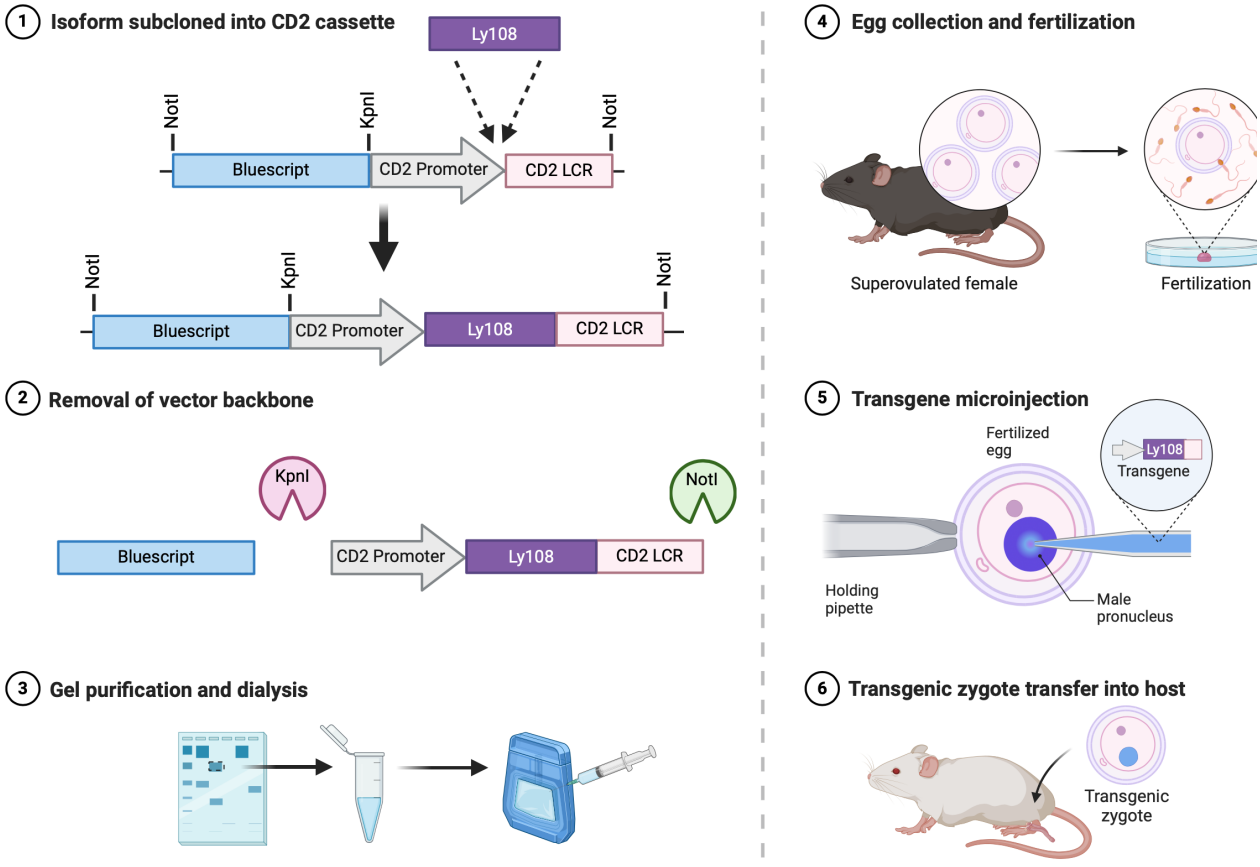

Figure 7. Graphical summary of the steps used to generate Ly108 isoform transgenic mice. In numerical order we see the major steps in creating, purifying, and genetic modification of mice to express a single isoform. Created with BioRender.com.

4. Flow cytometry

Single cell suspensions of thymocytes were made by mashing tissue between frosted slides before filtering. Mashed spleens were treated with red cell lysis buffer (Sigma-Aldrich) before filtering. Neutrophils were isolated from bone marrow or peritoneum (4 h after injection with 2 ml of 5% Brewer's thioglycolate medium). Bone marrow or thioglycolate peritoneal lavage was washed three times in HBSS/5% FCS. Neutrophils were then isolated by discontinuous Percoll gradient centrifugation. Cells were labeled with biotinylated or Cy5 conjugated anti-Ly108 (13G3) or IgG2a isotype control (Beckman Coulter) together with different combinations of the following directly conjugated antibodies after blocking Fc receptors with anti-CD16/32 and 20% rabbit-serum: CD8-Pacific Blue, CD4-APC-Alexa750, B220-APC-Alexa750, IgM-APC, AA4.1-PECy7, CD21-Pacific Blue (eBioscience), CD3-FITC, IgD-FITC, CD23-PE, CD45RB-PE, CD25-FITC, CXCR5-PE, GL7-FITC, Fas-PE, CD11c-FITC, NK1.1-PerCP-Cy5.5 (BD Pharmingen). NKT cells were detected by PE-conjugated PBS57-loaded CD1d tetramer provided by the NIH Tetramer Facility. Data was acquired with FACScan, LSR II or FACSCanto cytometers with HTS option (BD Pharmingen) and analysed using Cellquest (BD Pharmingen) and FlowJo software (Treestar). Dead cells were excluded upon DAPI uptake (except in cell death studies) and doublets were discriminated using forward and side scatter height and area (except in the case of the conjugation experiment)

5. Cell-cell conjugates and FACS

iNKT cell conjugate studies were performed with single cell suspension of thymocytes. To identify double positive thymocytes that could potentially be (lipid) antigen presenting cells, one set of cells were stained with CD4 PE-Cy7 and CD8-Pacific Blue. To identify iNKT cells, as well as analyse Ly108 and NK1.1, a separate portion of cells were stained with CD1d tetramer-PE, NK1.1-PerCP-Cy5.5 and Ly108-Cy5. These cells were gently mixed and centrifuged and incubated for 30 minutes before fixing with 2% paraformaldehyde. Samples were FACSed on a LSR II cytometer. A fixed and then conjugated sample served as one control, showing very few conjugated cells. Cell conjugates were identified by particles that were FACS positive for CD4, CD8 and the CD1d tetramer (see Figure 14).

6. *In vitro* stimulation assays for primary cells

Co-stimulation of purified T cells with anti-Ly108 was performed with negatively selected splenic T cells (total T cells or CD4 T cells) or positive selection of naïve CD4 cells using CD62L microbeads (Miltenyi Biotech). Cells were stimulated with the indicated antibodies. For plate bound antibody stimulation 96 well plates were incubated with PBS and the indicated antibody before rinsing. For cytokine production cells were cultured at $1x10^6$/ml and supernatants were collected after 48 hours. 3H-labeled thymidine (New England Nuclear, North Billerica, MA) was added at 1 μCi/well (37 KBq) of a 96-well plate for the final 16-hour incubation, after which cells were harvested on glass filter paper,

and radioactivity was measured in a Wallac 1450 Microbeta liquid scintillation counter (Wallac, Gaithersburg, MD). Concentrations of cytokines were measured by capture enzyme-linked immunosorbent assay (ELISA) using OptEIA ELISA sets (BD Pharmingen) according to the protocol provided by the manufacturer. The measured optical density units were converted at an absorbance of 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

7. CD45RB^{hi} transfer model of murine colitis

$CD4^{+}CD45RB^{high}$ T cells were isolated from the spleens of naive C57BL/6 wt mice by cell sorting. After confirming that the isolated cells were >95% pure, 5 x 10^{5} sorted cells were suspended in 200 µl of sterile PBS and injected intravenously into Rag-$1^{-/-}$ recipient mice of same age and sex as the donor mice (see Figure 8) Mice were injected intraperitoneally with 500 µg of Ly108Fc, anti-Ly108 antibodies or control mouse IgG (Jackson Immunoresearch Laboratories) twice a week starting from the day of adoptive transfer. The recipient mice were weighed twice weekly. All mice were sacrificed when signs of diarrhea, hunching, and wasting disease appeared in the control. Disease activity index (DAI) was compiled as sum of four parameters: hunching and wasting were scored 0 or 1, stool consistency 0 –3, and colon thickening 0 –3 with higher scores representing more severe colitis. Histological specimens were obtained from the proximal, middle, and distal colon. The histology scores were assigned in a blinded manner by our participating pathologist, Dr. A. K. Bhan. The sections were scored for the presence of crypt abscesses (0–1), the degree of mucosal thickness (0–3), and the degree of inflammatory infiltrate (0–3). The maximum score for DAI was 8 and for histological index 7.

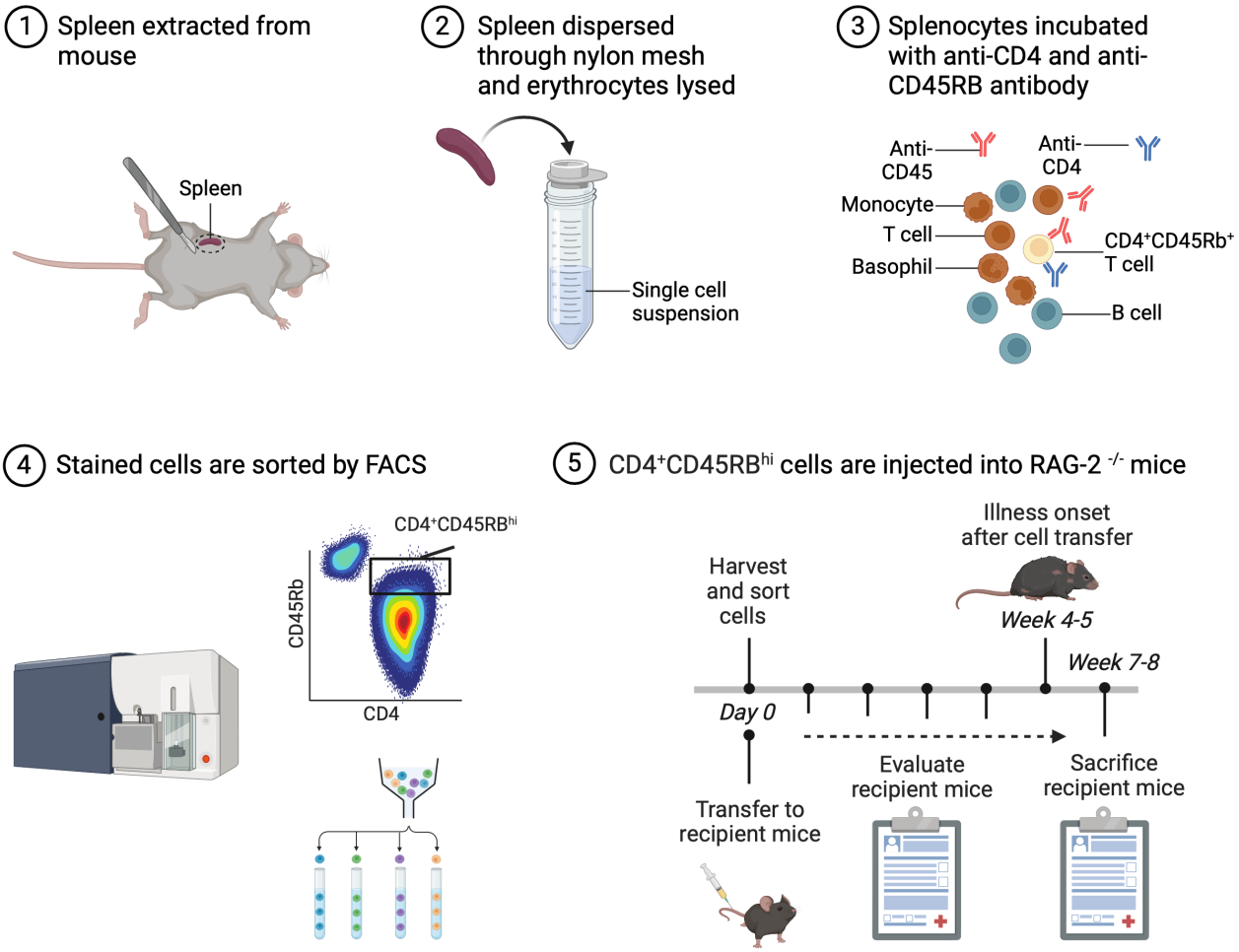

Figure 8. Schematic of the CD4^{+}CD45RB^{high} T cell transfer model of murine colitis. Here we show the major steps in the model, from isolating and transferring cells to evaluating disease. Created with BioRender.com.

8. Generation of polyclonal antibodies

Ly108 antisera were generated by immunizing four female New Zealand White rabbits with peptides conjugated via a N-terminal cysteine to KLH (Pierce, Illinois). Antiserum from rabbits 1 and 2 (R1 and R2) which recognizes Ly108-1, Ly108-2 and Ly108-3 was generated against the peptide Cys-KNDSMTIYSIVNHSRE. Antiserum from rabbits 3 and 4 (R3 and R4) recognizing Ly108-2 and Ly108-H1 was directed against the peptide Cys-ALTGYNQPITLKVNTLINYNS. 2ml Complete Freunds adjuvant (CFA; Sigma) to 2 ml of 0.5 mg/ml purified protein antigen in PBS at 4°C and mixed until an emulsion developed. Animals received an intradermal injection with 1ml of emulsion. A single booster injection was given after 4 weeks and rabbits bled two weeks later. The blood sample was allowed to stand 4 hr at room temperature for clot formation after which the serum was decanted and stored at −20°C. Using an ELISA we determined that antisera R1 and R4 had the best detection of Ly108.

9. Transient transfections

Ly108 isoforms were amplified by PCR with primers introducing XhoI and XbaI restriction sites and cloned into pCR2.1-TOPO (Invitrogen) before subcloning into the mammalian expression vector PCI-neo (Promega). Ly108-1 and Ly108-2 cDNA was kindly provided by Dr. E. Ruley. Ly108-H1 was amplified from C57BL/6 thymus cDNA.

Thymocytes from 129Sv mice were transfected by electroporation as previously described [179]. Briefly, 20-30x10^6 cells were resuspended in 250ml RPMI, 10% FBS and mixed with 50 ml RPMI containing 100 mg DNA and 10mM HEPES. Electroporation was performed in 4mm cuvettes with a single 360V pulse for the duration of 10ms. Cells were used for experiments one day after transfection,

WEHI-231 cells (ATCC, Rockville, MD) were transfected by AMAXA methodology according to the manufacturers protocol. Cells were rested for 24 hours before experiments.

10. Generation of cell lines stably expressing Ly108 isoforms

BI-141 cells were maintained in complete media (RPMI 10% Fetal Calf Serum) supplemented with glutamine and penicilline/streptomycine. Stable SAP transfectants were obtained by transfection of a SAP IRES GFP retroviral vector followed by cell sorting. For transfection of Ly108 isoforms, 1-2x10^7 BI-141 cells were transfected by electroporation (250V, 960µF) with 10µg of plasmid DNA in 400µL OptiMEM (Gibco) using a cuvette with a 4mm electrode gap (Biorad). To isolate stable transfectants, cells with surface expression were sorted by FACS (BD-Aria) after 48-hours and maintained in media

containing the selection antibiotic G418 (0.6mg/ml) for one week before a second round of cell sorting. Individual clones were isolated by limiting dilution in selection media. There were variations of expression levels (see Figure 9). DO11.10 cells (kindly provided by Dr J. Buhlman) were transfected by AMAXA technology using the manufacturers protocol for EL-4 cells.

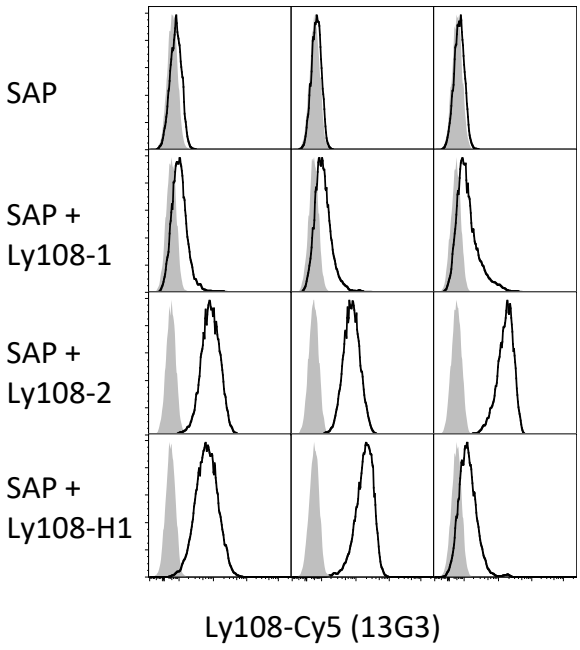

Figure 9. Ly108 expression in BI-141 clones stably expressing Ly108 isoforms. Clones shown are SAP (Mock): A1, A2, A3. Ly108-1: A1, A5, D4. Ly108-2: C1, C4, C6. Ly108-H1: A7, A17, A18.

11. Immunoprecipitation and Western blotting.

To phosphorylate Ly108 and perform co-immunoprecipitations assays, cells were treated with pervanadate for 10 minutes before lysis. Lysed cells were solubilized with the detergent Brij 98. Ly108 was precipitated from lysates with anti-Ly108 (13G3-19D) and protein-G agarose (Invitrogen) followed by denaturing in Glycoprotein Denaturing buffer. Proteins were separated on a 4-12% gradient SDS-PAGE gel with MOPS running buffer (Invitrogen). After transfer to PVDF membrane, Western blotting was performed with the indicated antibody. Phosphotyrosine was detected by Western blot using the monoclonal antibody 4G10 (Upstate). These were followed by species specific secondary HRP-conjugated antibodies (Jackson Immunoresearch). Reactivity was detected by chemiluminescence with Supersignal (Pierce).

For the separation of isoforms (as shown in Figure 10) Ly108 was precipitated from Brij 98-lysates with anti-Ly108 (13G3) and protein-G agarose (Invitrogen) [3]. Upon incubation in the Glycoprotein Denaturing buffer G7 (New England Biolabs) the immuno purified proteins were resolubilized in NP-40 prior to deglycosylation at 37°C for 2 hours with immobilized Carbohydrate Binding Domain-PNGase-F fusion protein (CBM-PNGaseF), generously donated by Dr Anthony Warren (UBC, Canada). We used this immobilized PNGaseF because there was cross-reactivity between our polyclonals and pure PNGaseF, which has a similar molecular weight to Ly108. Isoforms were separated on a 12% continuous SDS-PAGE gel with MOPS running buffer (Invitrogen). After transfer to PVDF membrane, western blotting was performed with indicated rabbit primary and anti-rabbit, light chain specific secondary HRP-conjugated antibody (Jackson Immunoresearch). Reactivity was detected by chemiluminescence with Supersignal (Pierce). Quantification was performed with ImageJ (Fiji) [180].

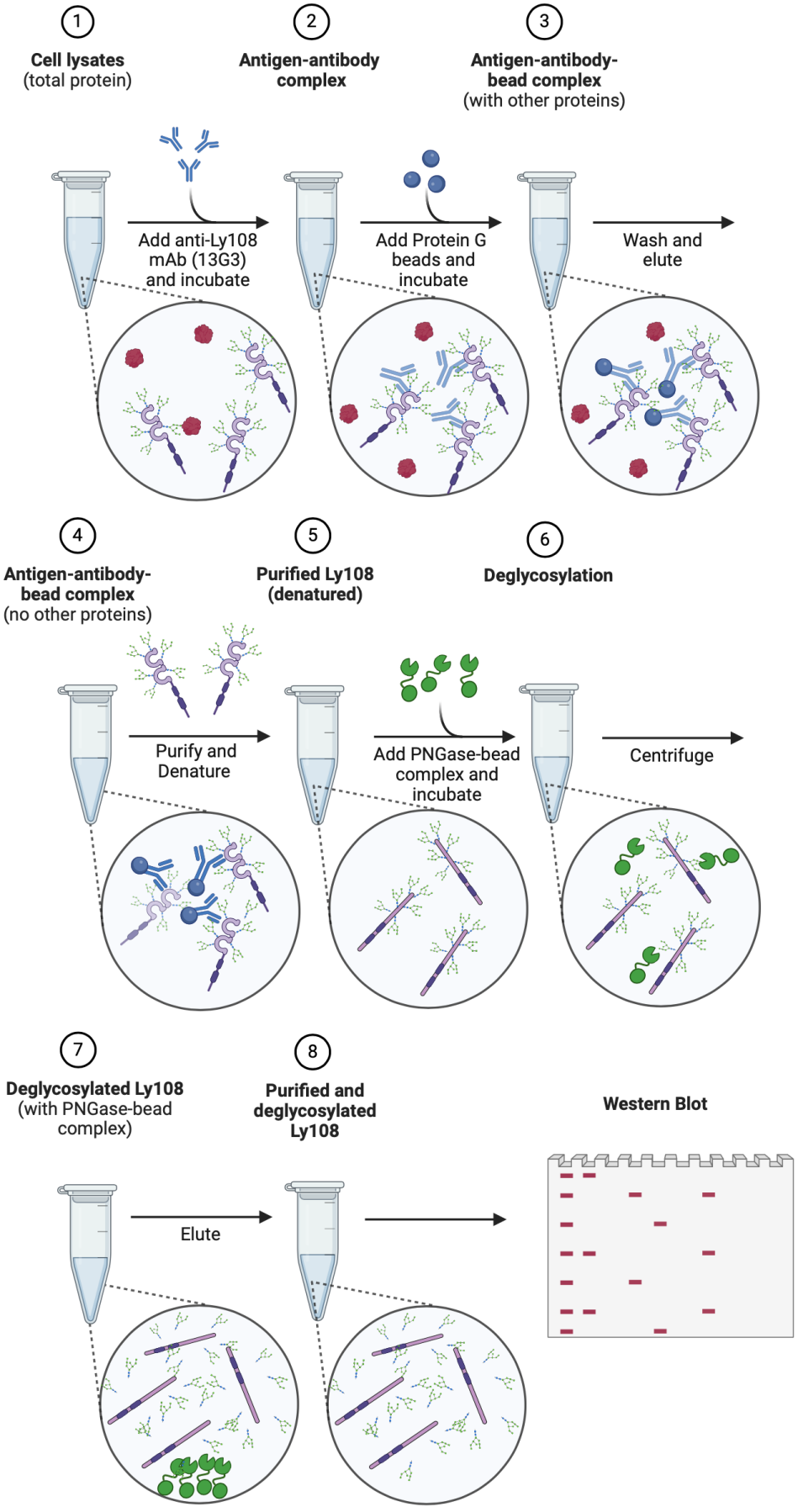

Figure 10. Graphical representation of the experimental procedure used for the immunoprecipitation, and deglycosylation of Ly108 isoforms used for Western blotting. Created with BioRender.com.

12. Stimulation of cell lines and measuring cell death

For cytokine production and anti-CD3 induced cell death BI-141 and D011.10 cells were stimulated with the indicated amounts of plate bound anti-CD3 (2C11) for 20 hours in complete media. Cell death was determined by DAPI uptake and flow cytometry. Cytokines were analyzed by ELISA (BD Pharmingen) as above.

WEHI-231 were transfected with individual isoforms. After 24 hours cells were stimulated with anti-IgM for 24 hours. Apoptosis was determined by FACS analysis by using AnnexinV binding and DAPI exclusion. Values shown are relative to unstimulated cells mock transfected with empty vector.

For apoptosis induction in thymocytes, 2 x10^6 thymocytes were stimulated with 2.5mg/ml plate bound anti-CD3 and 5mg/mL soluble anti-CD28 (manufacturer) or aLy108 (13G3) for 3 hours before FACS analysis with Annexin V and DAPI.

Apoptosis was determined by FACS analysis by using AnnexinV binding and DAPI exclusion. Apoptotic index was calculated by comparing the experimental groups to unstimulated cells mock transfected with an empty vector.

13. RT-PCR.

RNA was extracted from cells using the RNeasy kit (QIAGEN) or TRIZOL (Invitrogen). A shorter RT-PCR of Ly108-3 and Ly108-1 was performed with forward primer: 5'-TCATTCCAGAGAGCCCATTT-3' and reverse primer 5'-GAAGGATCCAGGCTGAAGTG-3'. A full-length RT-PCR of Ly108-1 and Ly108-3 was performed on cDNA templates with the following primers with introduction of restriction sites:

Ly108-start: 5′-GGCTCGAGATGGCTGTCTCAAGGGCT-3′;

Ly108-1-end: 5′-GGTCTAGATTAAGAGTATTCG-GCCTCTCTGG-3′

before subcloning into vectors for sequencing. Sequences were determined at the Beth Israel Deaconess Medical Center Sequence facility and nucleotide sequences were assembled and aligned using Vector NTI Advance (Invitrogen). Each RT was performed with the Protoscript cDNA kit (New England Biolabs, Inc.).

Quantitative PCR was performed directly on RNA samples on the PRISM 7700 Sequence Deterctor System with Taqman One-Step Master Mix (Applied Biosystems). The differences (Δ) between the CT values of the gene of interest and housekeeping gene (18S) were used to determine Relative Expression using the formula $2^{-(\Delta CT)}$. Primer and probe sequences are as follows:

5': TGGTCTGGCTCTTTCCACTTG

3': GGGTCTGAGCTGCTCTGTGAA

TaqMan® probe: CTTCTGCCTCGGCTCAGGGAGTGAA

14. Bioinformatics

Alignment of Ly108 sequences was performed with the help of BLAST from the National Center for Biotechnology Information (NCBI)[181] and SnapGene software (www.snapgene.com).

The PhosphoSitePlus database was used to determine potential phosphorylation sites at www.phosphosite.org [182].

The Eukaryotic Linear Motif (ELM) resource was used to predict binding with other proteins with searches using the tail sequence of Ly108 isoforms before filtering hits flanking the non-synonymous SNP (www. http://elm.eu.org) [183].

To predict structural differences that may occur due to the non-synonymous SNP in Ly108-3 we used PEP-FOLD3 with the last 50 amino acids of the tail (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/) [184,185].

15. Statistical methods

Differences in disease in the CD45RB^{hi} model were analyzed using the Mann-Whitney test.

For the BI-141 stimulation experiments, multiple comparisons were analyzed using the non-parametric Kruskall-Wallis test with the Dunn's post-hoc test. Single comparisons (DO11.10 experiments) were performed using the Mann-Whitney test. Statistical analysis was performed using GraphPad Prism version 9.4.1 for macOS (GraphPad Software, San Diego, California USA. www.graphpad.com).

RESULTS

1. Expression of murine Ly108

Using the monoclonal antibodies we had generated (330-AJ and 13G3-19D), I set out to extend our studies to determine expression on additional immune cells, examining different sources, subsets as well as stimulatory conditions.

1.1 Expression on neutrophils and dendritic cells

One of the first cell types I examined were neutrophils, as research from our lab had shown that neutrophils from Ly108 deficient mice were deficient in microbial killing, suggesting that Ly108 could be expressed in these cell types[139]. I had previously shown Ly108 expression in bone marrow derived neutrophils at the transcriptional level by RT-PCR on mRNA. Neutrophils had been isolated by discontinuous Percoll gradient centrifugation and were usually 95% pure. To determine, whether Ly108 would also be expressed at the protein level in neutrophils, I set out to detect Ly108 expression by FACS, using Gr-1 as a neutrophil surface marker and our antibodies. Different sources of neutrophils were used. First, I investigated Ly108 expression on the membrane of neutrophils from bone marrow or blood (Figure 11A and 11B). Neutrophils are produced in the bone marrow through a process called granulopoiesis. Initially, they are released into the bloodstream as mature but relatively quiescent cells. These neutrophils are often referred to as "circulating" or "resting" neutrophils. Interestingly, I found that Ly108 expression was not expressed in the membrane of these Gr1+ cells. Next, I examined Ly108 expression in thioglycolate-elicited peritoneal neutrophils. Thioglycolate is a commonly used agent in experimental models to induce inflammation and recruit immune cells, including neutrophils, into the peritoneal cavity in animal studies. When thioglycolate is injected intraperitoneally, it triggers a localized inflammatory response, leading to the recruitment of neutrophils from the bloodstream into the peritoneal cavity. The neutrophils recruited to the peritoneal cavity in response to thioglycolate are activated neutrophils, who have undergone changes in their morphology, surface receptor expression, and functional responses to become more efficient at combating pathogens and resolving inflammation. FACs analyses of this population, showed that Ly108 is expressed on a small subset of thioglycate elicited peritoneal neutrophils (Figure 11C). Taken together, this shows that whereas there is no surface expression on resting neutrophils from bone marrow or blood, there is some low expression of Ly108 on a small subset of thioglycolate elicited neutrophils. This suggests that Ly108 might only be present in certain subsets of activated neutrophils.

Current data on Ly108 expression on macrophages is somewhat conflicting (see references [2,135,138] and introduction). We had performed some early experiments using antibody 13G3-19D to test whether we could detect Ly108 but, because of inconclusive and preliminary results, I cannot present reliable data.

When examining another phagocytic cell type, dendritic cells, I found high expression of Ly108 as was also shown by Zhong and Veillette [2]. This was also found when examining other dendritic cell subsets such as those with myeloid, lymphoid, or plasmacytoid surface markers (X. Romero, L Wang, unpublished data).

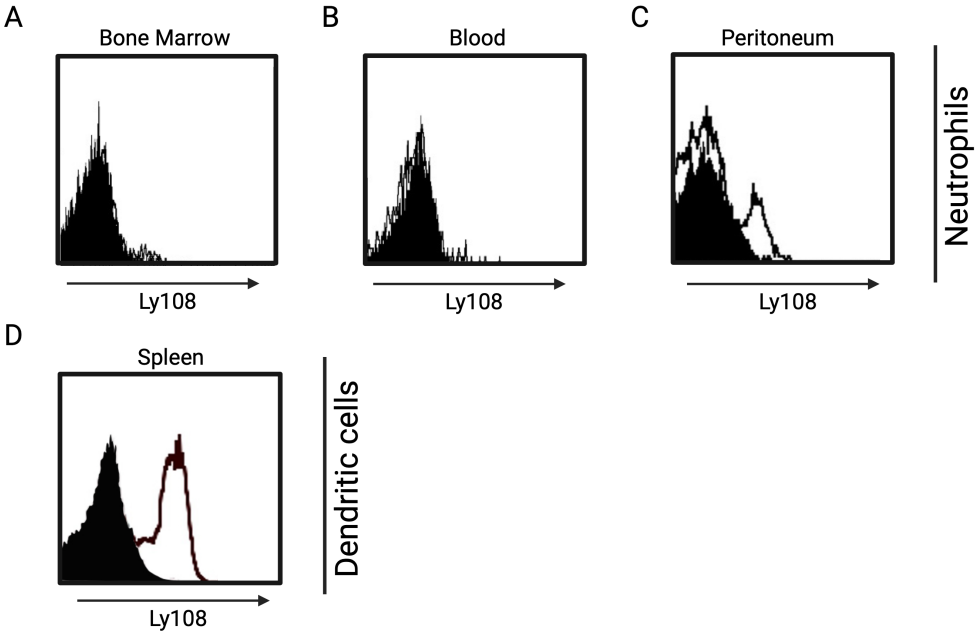

Figure 11. Surface expression of Ly108 on neutrophils and dendritic cells. Neutrophils from bone marrow (A), peripheral blood (B) and peritoneum (C) were gated on using Gr-1 expression. (D) Dendritic cells were gated on with CD11c and Ly108 expression is shown (F). Expression of Ly108 (empty histograms) is shown using antibody 13G3-19D compared to an isotype control (filled histograms).

1.2 Expression in lymphocytes

To further examine and compare the Ly108 expression on lymphocytes I extended our analysis from what had already been shown on thymocytes and developing NKT cells [1]. To better perform these experiments, I directly conjugated the antibodies to the Cy5 fluorophore and used an unspecific IgG2a isotype, as well as cells from the Ly108 knockout mouse, as controls.

In Figure 12A, I show high expression on early (double negative and double positive) thymocytes. The levels drop slightly during maturation (single positive CD8 or CD4 cells) to a level corresponding to that of peripheral T cells as shown in Figure 12B and in previous publications [1,32].

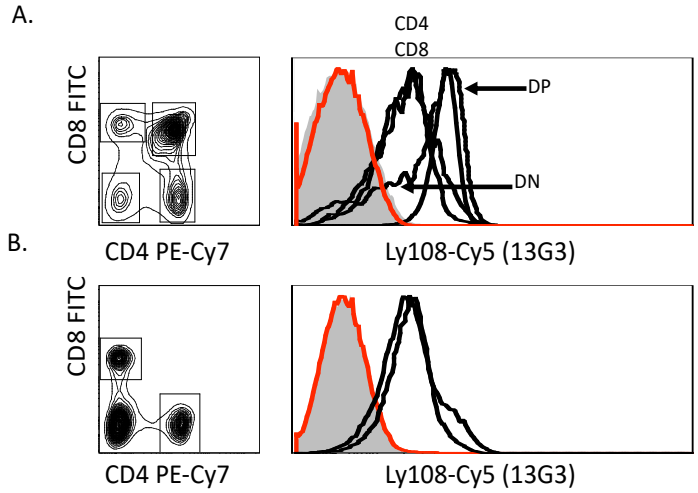

Figure 12. Surface expression of Ly108 on subsets of thymocytes and T cells. Thymocyte and splenic suspensions from B6 mice were stained with directly conjugated antibodies against cell surface markers and analysed by flow cytometry. Thymocyte (A), and splenocyte (B) subsets were gated as indicated and expression of Ly108 determined on the indicated populations.

We extended the FACS analysis to include additional immune cell subsets, as shown in Figure 13. Here, we saw relatively high and comparable Ly108 expression in all T and B cell subsets. There was also low expression on NK cells (Figure 13C) as previously shown by the Veillette group [2].

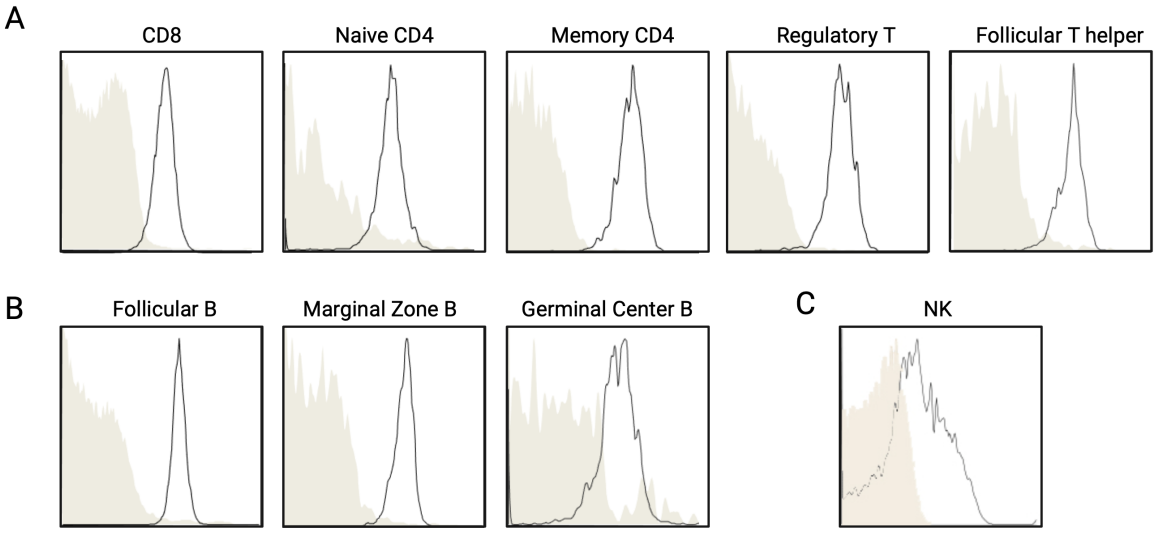

Figure 13. Ly108 surface expression on specialized lymphocyte subsets. Ly108 expression was determined by FACS analysis on populations gated by the surface markers in brackets. (A) T cells: Naïve CD4 (CD3+CD4+CD45RB^{hi}CD25-), Memory CD4 (CD3+CD4+CD45RB^{lo}CD25-), Regulatory T cell (CD3+CD4+CD25+), Follicular T helper (CD4^{+}CXCR5^{+}). (B) B cells: Follicular B cell (IgD^{hi}IgM^{lo}CD21^{int}), Marginal Zone (IgD^{lo}IgM^{hi}CD21^{hi}), Germinal Center B cell (GL-7^{hi}Fas^{hi}). (D) NK cell (NK1.1+CD1d-Tetramer-).

An interesting finding during our previous studies of the role of SLAM and Ly108 in iNKT cell selection was that Ly108 expression seemed to fall during this process, and low expression was seen at the final stage of iNKT cell development in the thymus [1]. In Figure 14A we show this decreasing Ly108 expression in three stages of development by FACS. Next, I sorted these three different populations and isolated mRNA to determine whether the decrease at the surface was attributable to a decrease in Ly108 transcriptional levels. Using primer-and-probe sets that amplify all four isoforms of Ly108, as well as a set that maps to the UTR of Ly108-1 and Ly108-3, we provide additional evidence that there was a fall in transcription levels during iNKT cell selection stages.

We hypothesized that immature iNKT cells would tend to bind to CD1d+CD4+CD8+ thymocytes, which present lipid antigen to iNKT cells during selection, while mature iNKT cells would lose their binding with CD1d+CD4+CD8+ thymocytes. By using cell conjugation to discriminate between immature and mature NKT cells I saw that there was Ly108 expression on conjugated iNKT cells, while there was little on the unconjugated controls (Figure 14E). While these results support my hypothesis, a potential caveat is that Ly108 positive cells are more likely to form conjugates in general, and that this experiment is not necessarily an accurate reflection of what happens in the thymus. As an additional control I examined NK1.1 expression on conjugated versus unconjugated iNKT cells but did not see a clear difference (Figure 14F). I do not have an explanation for this, but spectral interference may be a contributing factor.

Importantly, the expression of Ly108 alone was able to restore the iNKT cell defect seen in the SLAM family Receptor knockout mouse (SFR-KO), while the absence of Ly108 alone was able to restore the iNKT cell defect seen in the SAP knockout mouse [438]. Therefore, the dynamic expression of Ly108 and SAP may be of extreme importance for iNKT development.

In summary, our generation of monoclonal antibodies allowed us to determine the expression profile of Ly108. We show high expression on all lymphocytes and dendritic cells, and low levels on stimulated neutrophils. We were also able to confirm the downregulation of Ly108 in NKT cells at the final stages of thymic development.

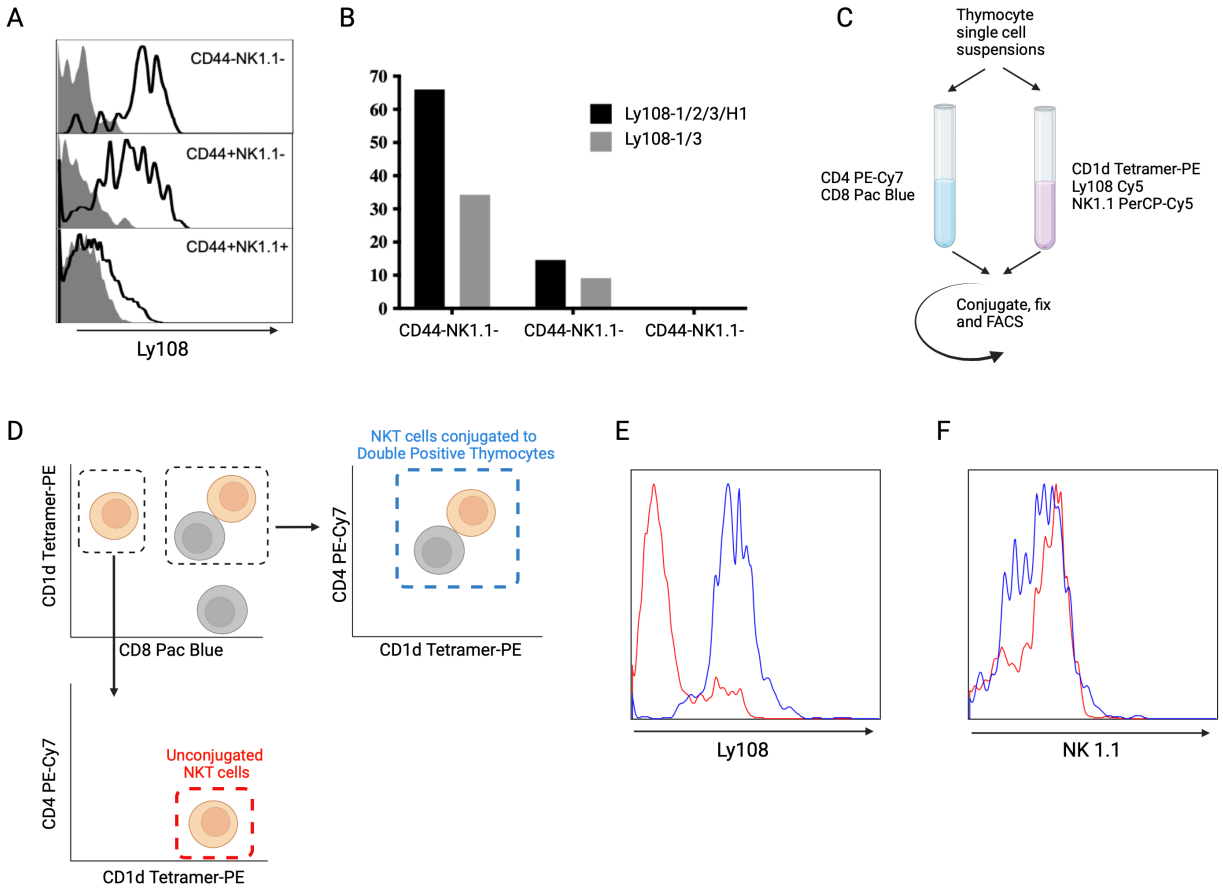

Figure 14. Expression of Ly108 on thymic iNKT-cells. (A) Surface expression of Ly108 was measured on developing iNKT cells by gating on PBS57-CD1d-tetramer positive cells with maturation stages based on CD44 and NK1.1 expression. The top panel indicates immature iNKT cells, the middle panel transitional iNKT cells, and the bottom panel mature iNKT cells. (B) mRNA expression of Ly108 in developing thymic iNKT cells was determined by qPCR (TaqMan) in cell populations that had been sorted by FACS, using the same markers as in A. Black bars represent the amount of total Ly108 i.e. all isoforms while grey bars represent only Ly108-1 and Ly108-3. Experimental setup (C) and gating strategy (D) used to determine surface expression of Ly108 on developing iNKT cells (orange), based on their conjugation to DP thymocytes (grey). Expression of Ly108 (E) and NK1.1 (F) on conjugated iNKT cells (blue histogram) or unconjugated iNKT cells (red histogram).

2. The role of Ly108 on T cells

An early report on the human homologue of Ly108 reported a co-stimulatory effect of a cross reactive anti-NTB-A antibody, as increased proliferation and IFN-γ was seen in human and mouse T cells [4]. While we have not seen any additional experiments with this antibody, additional support for a role of Ly108 in T cells stimulation was shown as there was significantly less IL-4 production from Ly108 deficient mice after CD3/CD28 stimulation [139].

2.1 The effect of anti-Ly108 antibodies on *in vitro* co-stimulation

To confirm these findings and test our antibodies, we stimulated T cells with anti-CD3, anti-CD3 in combination with anti-CD28, in the presence and absence of anti-Ly108 (clones 330 or 13G3). In the absence of anti-CD3, stimulation of T cells with anti-Ly108 antibodies alone did not result in increased T cell proliferation or IL-2 production (data not shown).

In contrast, we saw an increase of proliferation when anti-Ly108 was added to T cells stimulated with anti-CD3 and anti-CD3/anti-CD28, as shown in Figure 15A and 15B. The effect on IL-2 production seemed to depend on the mode of delivery, where plate-bound anti-Ly108 resulted in less IL-2 production (Figure 15C) in contrast to soluble antibody that had increased IL-2 (Figure 15D).

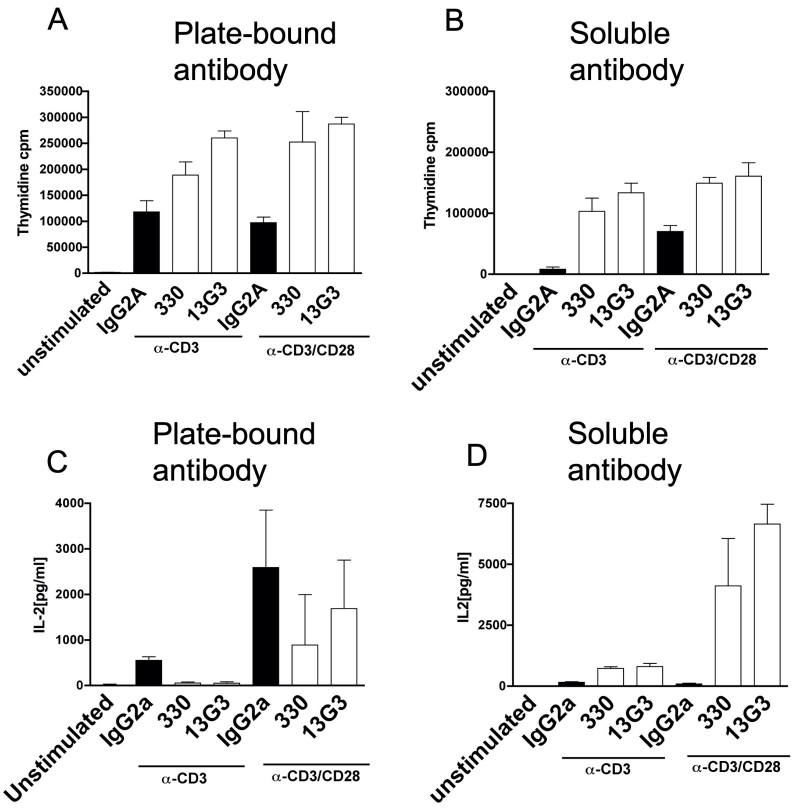

Figure 15. Co-stimulaton of purified T cells by anti-Ly108. Negatively selected T cells were stimulated with the indicated antibodies and assayed for proliferation by thymidine incorporation (A and B) or ELISA for IL-2 production (C and D). anti-Ly108 antibodies (330, 13G3) or isotype control were either plate-bound (A and C) or soluble (B and D).

We performed additional experiments with CD4 cells and saw similar results. While anti-Ly108 had a co-stimulatory effect on proliferation whether plate-bound or soluble (Figure 16A and 16B), IL-2 production was reduced with plate-bound antibody (Figure 16C)

While we do not have more data that can further explain this, it may be related to the cross-linking possibly provided by plate-bound antibody or interference between intercellular homophilic Ly108 interactions provided by soluble antibody.

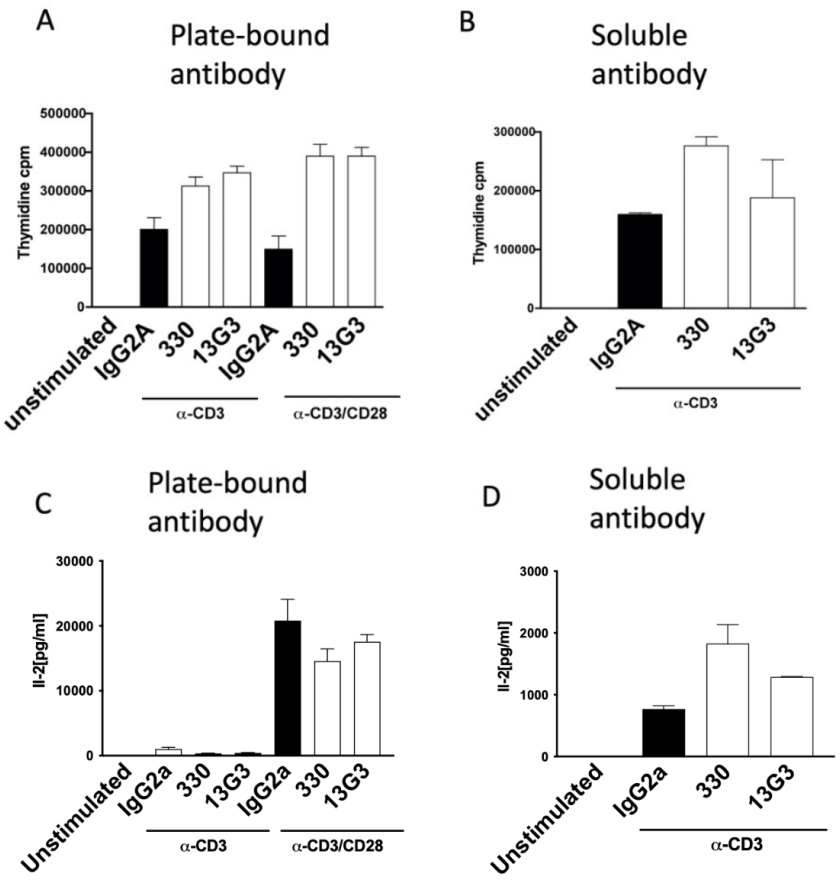

Figure 16. Co-stimulaton of purified CD4 T cells by anti-Ly108. Negatively selected CD 4 T cells were stimulated with the indicated antibodies and assayed for proliferation by thymidine incorporation (A and B) or ELISA for IL-2 production (C and D). anti-Ly108 antibodies (330, 13G3) or isotype control were either plate-bound (A and C) or soluble (B and D).

2.2 The effect of anti-Ly108 antibodies on *in vivo* co-stimulation

The interaction between Ly108 molecules expressed on the surface of T cells and those on antigen presenting cells has been shown to help form the immune synapse, although there are conflicting results as to whether the co-stimulation provided is a positive or negative signal [8,144,186]. Blocking of more well-known co-stimulatory pathways such as the B7-CD28 pathway can ameliorate autoimmune conditions

through preventing a second signal after MHC-TCR interactions [187]. We previously used the CD45RB-high T cell transfer model of murine colitis to study co-stimulatory molecules and proinflammatory cytokines with soluble receptors or monoclonal antibodies [188,189]. A graphical representation of the model is shown in Figure 17A and detailed in the methods section. Because the lack of $CD4^+CD25^+$ regulatory T cells is the main cause of disease in this model, we compared Ly108 expression on both these, as well as the $CD4^+CD45RB^{hi}$ effector cells and saw high, but comparable levels on both type of cells (Figure 17B and 17C). In addition, we determined that Ly108 mRNA was increased in the colons of mice with colitis compared to healthy mice (Figure 17D)

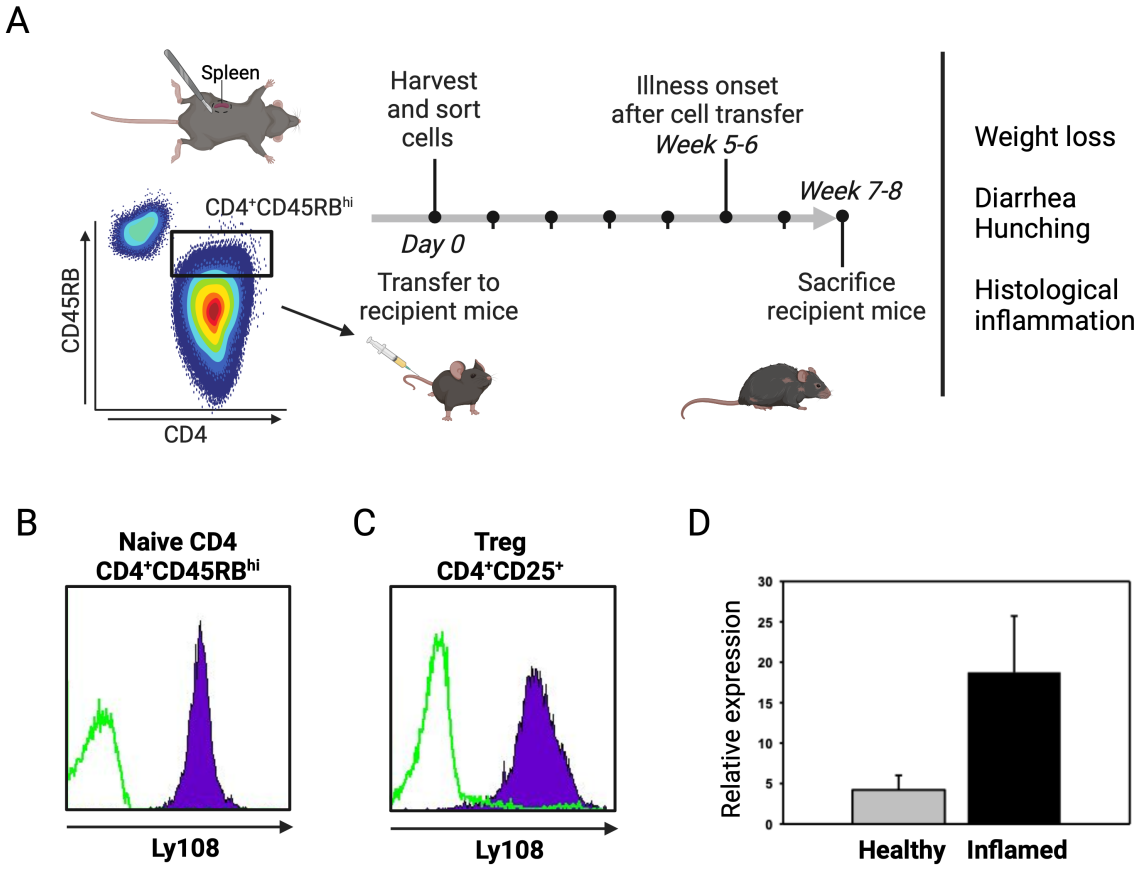

Figure 17. Ly108 expression in the $CD45RB^{hi}$ transfer model of murine colitis. (A) Graphical representation of the model. (B) Surface expression of Ly108 on naïve CD4 T cells ($CD4^+CD45RB^{hi}$) and (C) $CD4^+CD25^+$ regulatory T cells (Treg) as determined by FACS. (D) Expression of Ly108 in whole colon from healthy mice (grey columns) and inflamed colons from the $CD45RB^{hi}$ model (black columns). Created with BioRender.com.

Considering the role of NTB-A and Ly108 on T cells activation as well as increased expression in inflamed colon, we examined whether administration of a soluble Ly108 would have an effect on the model. We used the Ly108Fc described earlier, which is the extracellular domain of Ly108 fused with a human Fc segment. This can bind Ly108 on the surface of cells, presumably interfering with homophilic interactions.

As shown in Figure 18, while there was a trend showing reduction of disease activity in the Ly108Fc group, only one parameter (disease activity index) showed a statistically significant difference (Figure 18C)

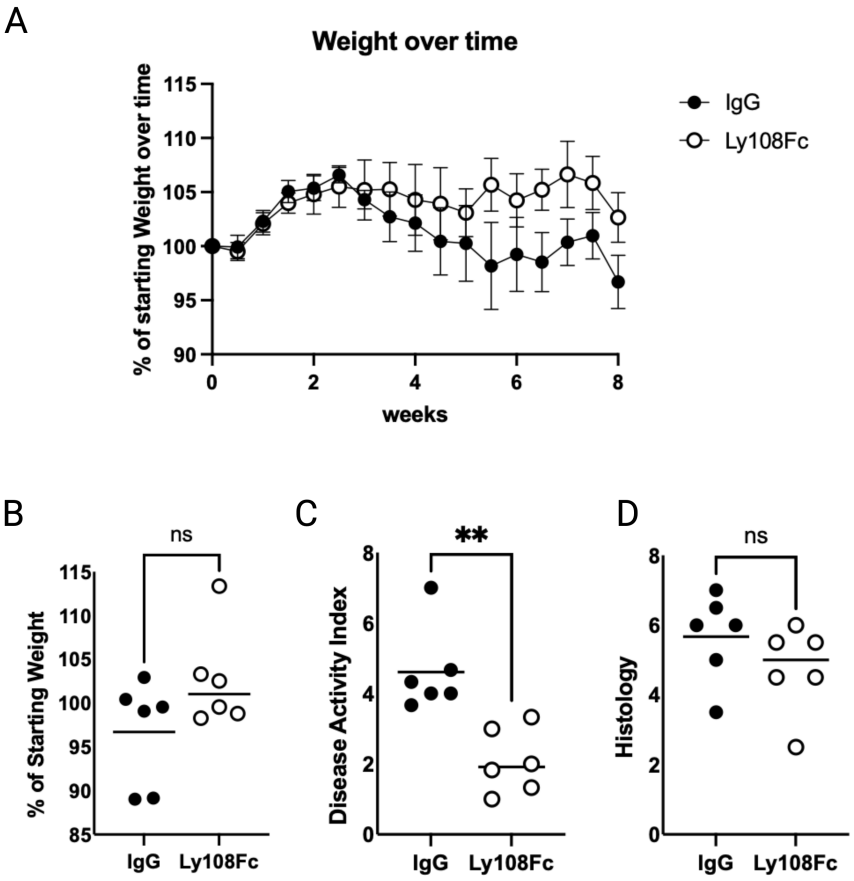

Figure 18. Administration of Ly108Fc in the CD45RB^{hi} model of murine colitis. (A,B) Weights of individual animals were measured twice weekly in both the Ly108Fc treated group (open circles) as the IgG control group (filled circles). Upon sacrifice, Disease Activity Index (C) and Histology (D) were scored. Results are from a single experiment.

We then proceeded to test anti-Ly108 antibodies 13G3-19D and 330-AJ in the model. Although there was significant variation of disease severity, administration of the anti-Ly108 antibodies in the CD45RB^{hi} model did not appear to reduce disease as judged by weight loss and disease activity. When examining histology, we surprisingly saw granuloma formation in almost all of the animals that had been treated with anti-Ly108 antibodies (Figure 19A). Granulomas are tightly clustered collections of macrophages associated with several diseases but could be especially significant in this case as they are

associated with Crohn's disease. This finding was something that, to our knowledge, has not been described in this model.

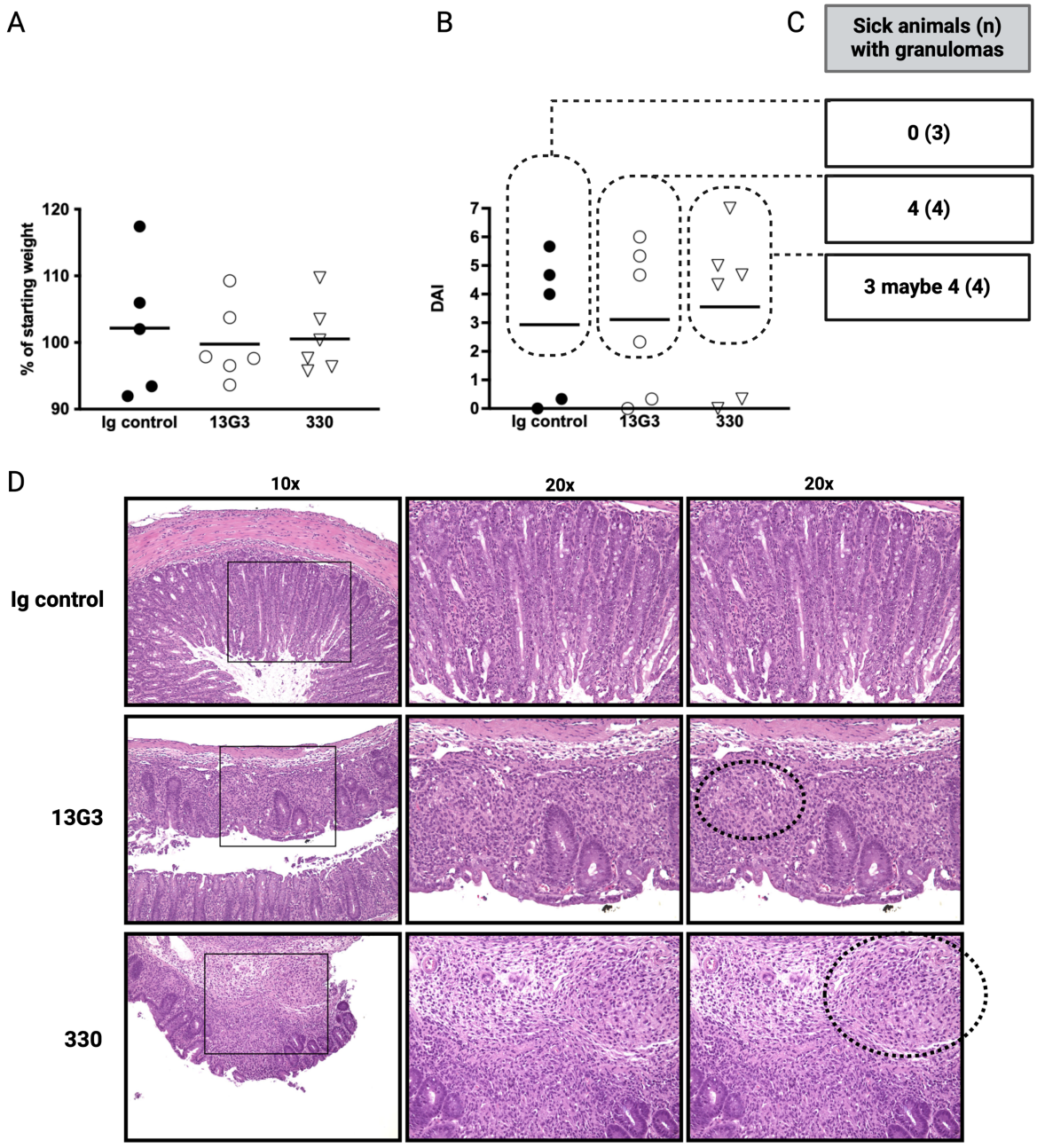

Figure 19. Administration of anti-Ly108 in the CD45RBhigh model of murine colitis. (A, B) Weights and Disease Activity Index upon sacrifice. (C) Presence of granulomas in colon histology. (D) Representative histology of sick mice with granulomas indicated by dashed circles in the 20x images to the right

In summary, we found a curious difference of *in vitro* IL-2 production by T cells after anti-Ly108 exposure that was based on the mode of delivery *i.e*. soluble or plate-bound. We also found a slight, but insignificant, indication that Ly108Fc could protect against T cell mediated disease, while the anti-Ly108 antibodies, if anything, made the disease worse.

While these were intriguing preliminary results, we directed our resources to focus on Ly108 isoforms because of their emerging significance.

3. Signaling differences between Ly108 isoforms

The first detailed studies on Ly108 signaling pathways compared the two original isoforms (Ly108-1 and Ly108-2). This work examined the effect of crosslinking on downstream phosphorylation where Ly108-2 provided a weaker signal than Ly108-1 as showed in multiple experiments [2]. In this same work, Ly108-3 was shown to have a signaling strength intermediate to that of Ly108-1 and Ly108-2 (see supplemental figure 4 of Zhong et al [2]). This is, to our knowledge, the first and only description of Ly108-3. Our discovery of Ly108-H1 followed, and Dutta and colleagues went on to show that Ly108-H1 was not phosphorylated in the thymus, while Ly108-1 and Ly108-2 were [32]. We set out to study these processes with regard to Ly108 isoform phosphorylation and SAP binding. SAP binding to Ly108 is important for its signaling and has been shown to be dependent on phosphorylation [2,32]. We expected phosphorylation of Ly108-H1 and phosphorylation-dependent SAP-binding based on previous studies that utilized targeted mutations of ITSMs to characterize SLAMF1, NTB-A and Ly108 [30,56,120].

3.1 Alignment of Ly108 isoforms highlight potential signaling differences

We performed sequence alignment of the four Ly108 isoforms to determine if there were any significant differences that could explain the functional and biochemical differences previously reported between isoforms [2,3,32,131]. In Figure 20A we show the alternative splicing and exon use of the ly108 gene (for convenience duplicated from introduction). When mapping the SAP binding sites (underlined in Figure 20B) Ly108-H1 uniquely has only one ITSM, while the other isoforms have two. Also evident is that, while the mRNA sequences between Ly108-1 and Ly108-3 are very similar, Ly108-3 has a different and unique peptide sequence in its tail due to a frame shift [2].

Searching the PhosphoSitePlus database with the tails of Ly108 isoforms showed, in addition to the described ITSMs, various tyrosines assigned as putative phosphorylation sites [182]. When comparing these predictions, it remains apparent that the most striking difference with regards to phosphorylation is the absence of a second ITSM in Ly108-H1 (Figure 20C and 20E). Because phosphorylation is linked to SAP binding, as well as a general measure for signaling, we proceeded to compare the levels of phosphorylation in Ly108-H1 to Ly108-1 and Ly108-2.

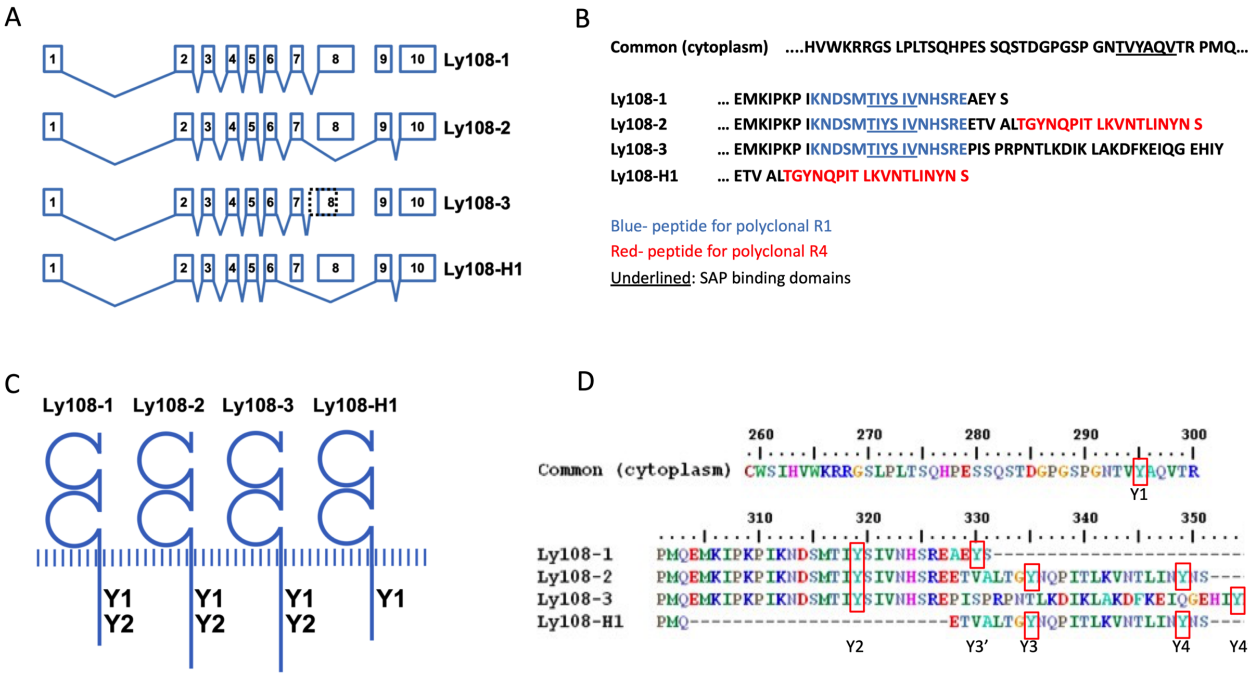

Figure 20. Schematic comparison of Ly108 isoforms. (A) Exon-intron organization of murine ly108 (slamf6) with alignment of Ly108 isoforms. (B) Alignment of amino acid sequences from the cytoplasmic domains of Ly108 showing SAP binding sites (underlined) and the sequences used to generate polyclonal antibodies R1 (C) Illustration of Ly108 isoforms with the position of ITSMs. (D) ITSMs and plausible phosphorylation sites in the different Ly108 variants. Tyrosine residue annotation using Ly108-2 as reference appear in position 295, 319, 335 and 349 (Y1-Y4). Other tyrosines shown are Y3' in Ly108-1 and Y4' in Ly108-3.

3.2. Differential phosphorylation and SAP binding of Ly108 isoforms.

For the next experiments, I used the BI-141 T-cell hybridoma, an antigen-specific mouse T-cell hybridoma cell line that produces IL-2 and undergoes apoptosis in response to TCR stimulation. It is widely used in immunological experiments, in elucidating the mechanisms of TCR but also other cell surface molecules, in their role modulating T-cell activation and function. Using these BI-141 cells transfected with SAP and individual Ly108 isoforms, we confirmed that Ly108-2 is less phosphorylated than Ly108-1, as shown in Figure 21A and 21B [2,32]. Similarly to Dutta *et al* we saw almost no detectable phosphorylation of Ly108-H1, but saw a slight signal using quantitation software (last column Figure 2B) [32]. In contrast to their findings, we show in Figure 21A and 21C that Ly108-H1 can effectively bind SAP, although this was after treatment with the phosphatase inhibitor pervanadate. This prompted us to question whether phosphorylated Ly108-H1 had escaped detection because of dilution in the naturally occurring smeared band of the glycosylated form of the protein. To address this, we performed separate experiments to concentrate the immunoprecipitated Ly108 isoforms by deglycosylation with PNGaseF.

This resulted in clear bands, and detectable phosphorylation of Ly108-H1, as seen in Figure 21D (top panel). Quantification of relative phosphorylation proved difficult as the pan anti-Ly108 monoclonal antibody 13G3-19D does not detect deglycosylated Ly108 well, and neither polyclonal detects all four isoforms. To estimate total Ly108 we used the pervanadate treated Ly108-2 to normalize the R1 and R4 results. With this method, we see Ly108-2 to be slightly more phosphorylated than Ly108-1 and Ly108-H1. These results, however, may be further confounded as R1 unexpectedly showed a higher signal in pervanadate treated lanes. SAP binding to the three isoforms was confirmed and the levels shown in Figure 21D (bottom panel) and Fig. 21F are consistent with those seen in the previous experiment. SAP binding to Ly108-H1 could also be seen in thymocytes from transgenic mice (Fig. 21G).

These results show, therefore, that Ly108-H1 can be phosphorylated when using pervanadate, after which it effectively binds SAP, even though detection of Ly108 phosphorylation was challenging.

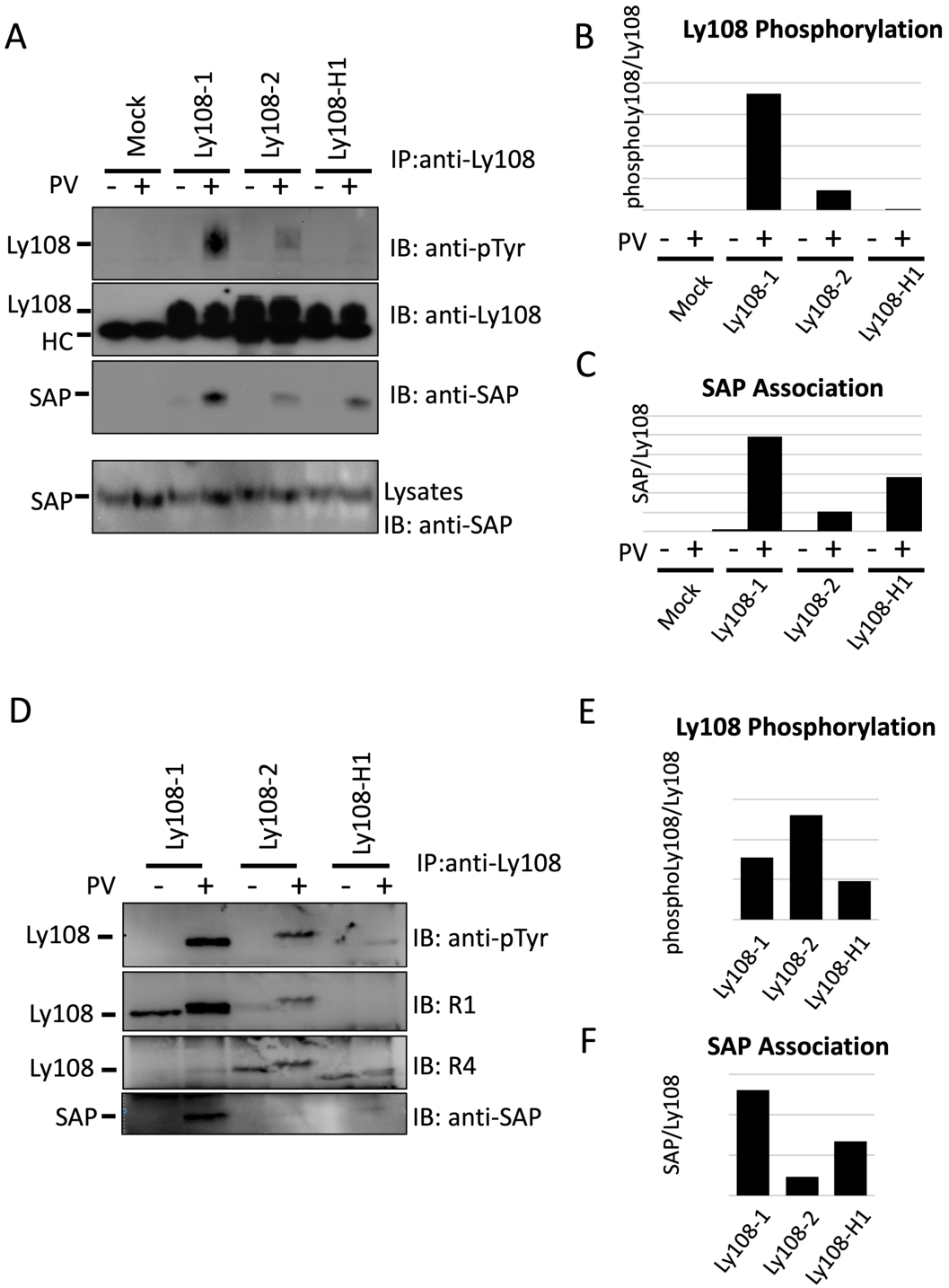

Figure 21. Differential phosphorylation and SAP binding by Ly108 isoforms. (A) Induction of Ly108 phosphorylation and SAP binding with pervanadate (PV) treatment of BI-141 cells expressing individual isoforms. Ly108 isoforms were immunoprecipitated and probed with anti-pTyr (top panel). The amount of total Ly108 was determined by reprobing with mouse anti-Ly108 mAb 13G3 (upper middle panel). The Ly108-SAP association was determined by probing the Ly108 immunoprecipitates with anti-SAP (lower middle panel). Levels of SAP in whole lysates were determined by probing with ant-SAP (bottom panel). Result are representative of three experiments (B) Phosphorylated of Ly108 was quantified and shown relative to total Ly108. Results of quantification are expressed as arbitrary units. (C) SAP association quantified relative to total Ly108. (D) Improved detection of phosphorylated Ly108-H1. Pervanadate treatment, immunoprecipitation and immunoblotting were performed as above but precipitates were subjected to deglycosylation with PNGase-F in an intermediary step. Note that the gel shift due to phosphorylation is now more evident. A single experiment was performed. (E) Quantification of phosphorylation of Ly108 is shown relative to total Ly108. Total Ly108 was estimated with R1 and R4 by using Ly108-2 to normalize obtained values from pervanadate treated lanes. (F) SAP association was quantified relative to total Ly108 in the pervanadate treated lanes. Results of quantification are expressed as arbitrary units.

4. Functional differences between Ly108 isoforms

The first comparison of function between Ly108-1 and Ly108-2 was performed by the Mohan group with calcium flux experiments that suggested that Ly108-2 provided a stronger signal than Ly108-1. This corresponded with an increased Activation-Induced Cell Death (AICD) they found in the WEHI-231 cell line transfected with Ly108-2 compared to Ly108-1 [131]. These experiments suggested that Ly108-2 could protect against autoimmunity by providing a signal that would reduce the threshold needed for deletion of autoreactive B cells. We, however, went on to show that the novel isofom Ly108-H1 could suppress the activation of autoimmune T cells in two autoimmune models, thereby possibly abrogating disease [3,22]. We set out to directly compare these three isoforms in T cells.

4.1 Suppression of cytokine production by Ly108-H1

To define the role of Ly108-H1 in T cell function we measured cytokine production in clones of the BI-141 cell line that were transfected to stably express SAP and the Ly108 individual isoforms, in a manner previously described [30,74]. In line with phosphorylation experiments we compared clones expressing Ly108-H1 to those with Ly108-1 and Ly108-2, as well as clones lacking Ly108 (Mock).

The stimulation with anti-CD3 of BI-141 clones expressing SAP and Ly108 resulted in less IL-2 production than those expressing SAP alone. We consistently saw that Ly108-H1 expression resulted in the greatest reduction of IL-2 production (Figure 22A). A less pronounced reduction in IL-2 was also observed with Ly108-1 expressing clones when compared to control and Ly108-2 expressing clones, although we cannot exclude that this result may be influenced by lower levels of Ly108 on the transfected cells (Figure 9). There was no effect on IFN-γ production (data not shown). To confirm the reproducibility of our findings, we also tested the effect of Ly108-H1 on IL-2 secretion, using another Ly108 negative T-cell line, the DO11.10 T-T hybridoma cells line. This is another widely used murine (mouse) T cell hybridoma cell line that is specific for the ovalbumin 323-339 peptide presented by the MHC class II molecule I-A. To that end, we stably transfected Ly108-H1 or empty vector control) and proceeded to activate the cells with ant-CD3. Although not significant, we saw some inhibition of IL-2 production in the Ly108-H1 cells when compared to the empty vector control (Figure 22B).

Thus, collectively, these experiments suggest that Ly108-H1 expression on T cells significantly reduces IL-2 production upon anti-CD3 stimulation compared to other Ly108 isoforms and control clones.

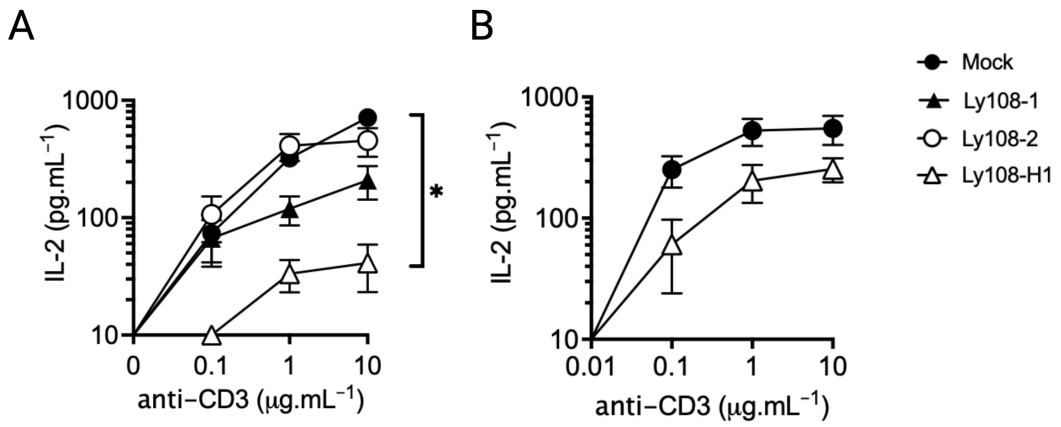

Figure 22. Suppression of IL-2 production by Ly108-H1 in T cell lines. (A+B) Individual clones of BI-141 cells stably expressing SAP and individual isoforms were stimulated with plate-bound anti-CD3. After 20 h IL-2 (A) or (B) IFN-gamma was determined by ELISA. Data points indicate mean +/- SEM of three separate clones. Each clone was assayed in triplicate. Similar results were obtained in three separate experiments. We performed a Kruskal-Wallis test resulting in $p<0.0001$. Shown is the Dunn's post-test significance comparing individual columns: * $p<0.05$. Note the logarithmic scale. (B) IL-2 production in DO11.10 cells stably expressing Ly108-H1 or the empty vector pCI-neo were performed as above. Data points indicate mean +/- SEM of four separate clones. Each clone was assayed in triplicate. Similar results were obtained in four separate experiments.

4.2 Differential Activation-Induced Cell Death due to Ly108 isoforms

Next, we investigated whether the IL-2 reduction in Ly108-H1 cells could be due to early cell death or apoptosis. Stimulation with anti-CD3 of BI-141 cells (stably transfected with Ly108-H1 and SAP) did not result in increase of AICD. Interestingly, we did observe increased AICD with Ly108-2 as seen in Figure 23A. We reproduced this experiment in the DO11.10 T/T hybridoma cells stably transfected with Ly108-H1, and as in the BI-141 cells, Ly108-H1 did not enhance AICD in DO11.10 cells (Figure 23B). As mentioned above, there is a theory that Ly108-2 could lower the threshold for AICD and apoptosis in immature B cells. In order to test the role of Ly108-H1 in immature T cells and B cells we performed some limited experiments to analyze the effect of individual isoforms on apoptosis using thymocytes and the WEHI-231 cell line as previously described for the two original Ly108 isoforms [131]. The WEHI-231 is a murine B cell lymphoma cell line that has been widely used as a model system to study B cell biology, immunoglobulin (Ig) gene expression, and B cell differentiation.

Here too we observed that overexpression of Ly108-2 resulted in more apoptosis than Ly108-1, while Ly108-H1 did not show a clear pattern of enhancement (Fig. 23C and 23D).

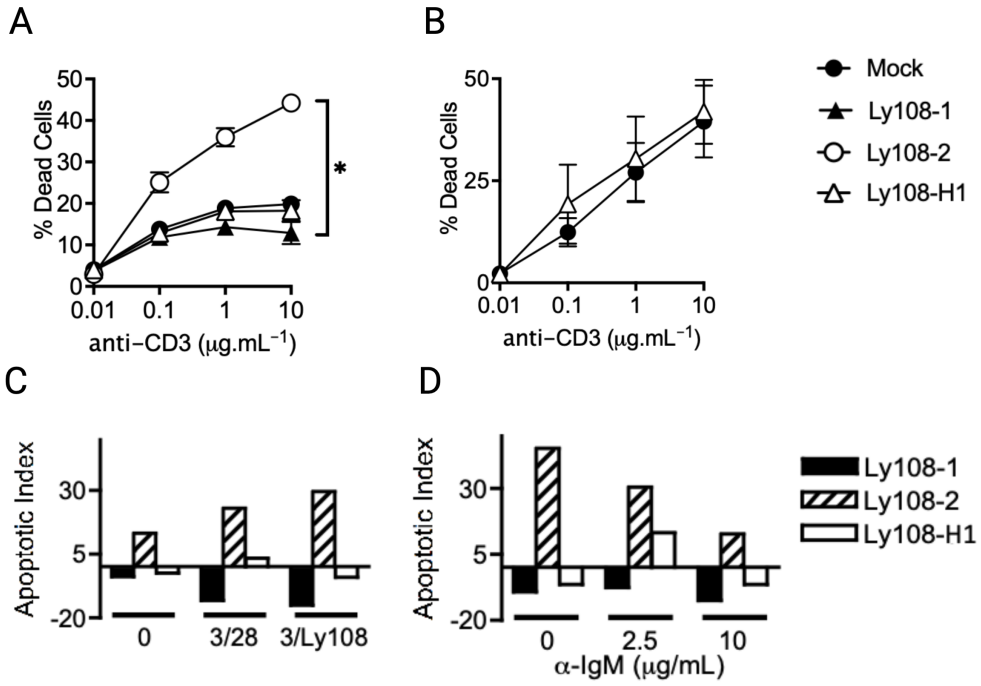

Figure 23. Lack of effect on AICD and apoptosis by Ly108-H1 (A) AICD was determined by FACS in BI-141 cells stimulated with anti-CD3 using DAPI as a marker for dead cells. Each clone was assayed singularly with data from 10,000 cells collected. Data points indicate mean +/- SEM of three separate clones. Similar results were obtained in two separate experiments. (B) AICD in DO11.10 cells stably expressing Ly108-H1 or the empty vector pCI-neo was performed as above. Each clone was assayed singularly with data from 10,000 cells collected. Data points indicate mean +/- SEM of 5 separate clones. Similar results were obtained in two separate experiments. (C) Thymocytes from 129Sv mice were transfected with individual isoforms by electroporation. The following day cells were stimulated with 25ug/mL plate bound anti-CD3 and 5ug/mL soluble anti-CD28 or anti-Ly108 (13G3) for 3 hours. (D) WEHI-231 were transfected with individual isoforms. After 24 hours cells were stimulated with anti-IgM for 24 hours. Apoptosis was determined by FACS analysis by using AnnexinV binding and DAPI exclusion. Values shown are relative to unstimulated cells mock transfected with empty vector.

In an attempt to reproduce the suppressive qualities of Ly108-H1 in primary cells, we examined T cells from Ly108-H1 and compared them to Ly108-1 transgenic mice. We had generated these mice using a CD2 cassette which drives expression in lymphocytes [178]. We had also generated Ly108-2 transgenics, but this line was lost to breeding difficulties. By crossing transgenic lines with Ly108 knockout mice we aimed to compare individual isoforms. Although very few animals were available for analysis, we were able to obtain some insightful data regarding the suppressive effects of this isoform in primary T cells.

We saw reduced proliferation of naïve CD4 cells from CD2-Ly108-H1 transgenics mice when stimulated with anti-CD3 and anti-CD28, in comparison to wt mice or transgenic mice with other isoforms (Figure 24A).

We were also able to use these mice to confirm SAP binding to Ly108-1 and Ly108-H1 after pervanadate treatment and show again that there is less phosphorylation of Ly108-H1 (Figure 24B)

Because of the breeding challenges and concerns about the effect of varying transgene copy numbers in the individual lines, we suspended this project. We instead proceeded to use Bacterial Artificial Chromosomes (BAC) transgenics as they only possess one copy of the candidate transgene. This provided other Ly108-H1 T cell data mentioned above [3,22].

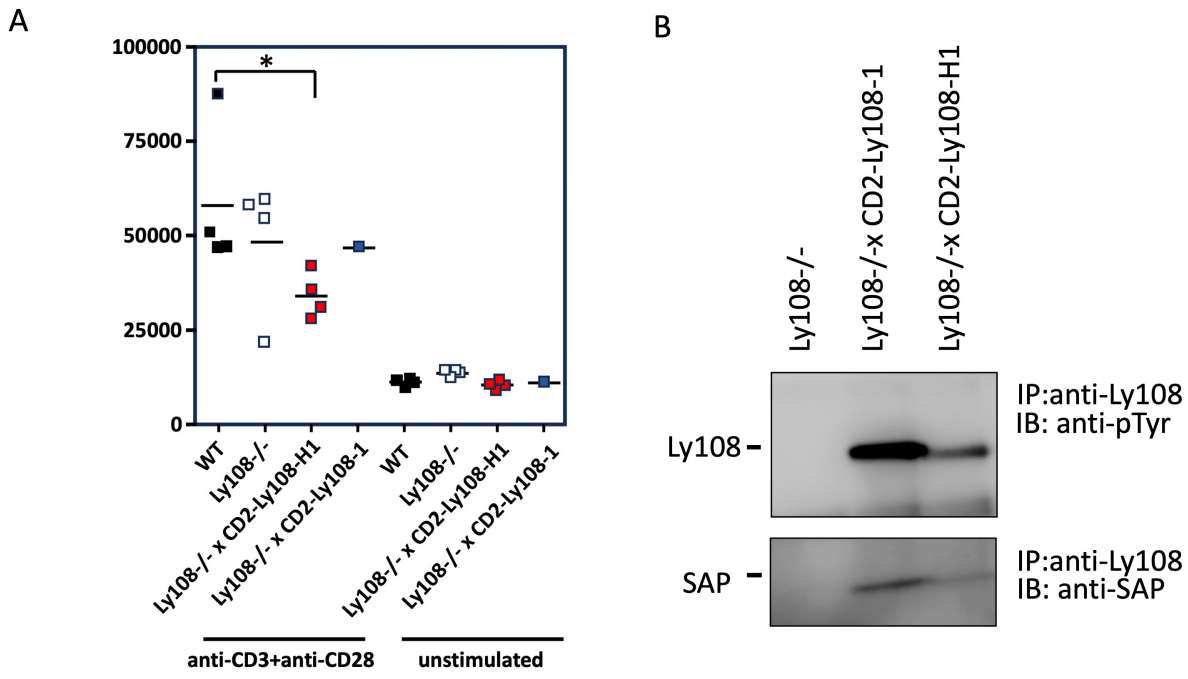

Figure 24. Data from CD2-Ly108-isoform transgenics. (A) In vitro proliferation of naïve (CD62L+) CD4+ lymphocytes was determined by H^3 -thymidine incorporation after stimulation with antiCD3 (0.3µg/ml) and anti-CD28 (10µg/ml). Ly108-H1 expressing T cells (B) Ly108-H1 expressed as a transgene in Ly108-/- mice binds SAP. Thymocytes were treated with pervanadate and deglycosylation, immunoprecipitation and western blotting was performed as in Figure 21D

In summary, our findings support those of Kumar and colleagues for Ly108-2 regarding AICD, while demonstrating that there does not seem to be a role for Ly108-H1 therein [131]. Expression of Ly108-H1 resulted in profoundly suppressed IL-2 production in the BI-141 T cell line and reduced proliferation of T cells from transgenic mice.

5. Detection of phosphorylated isoforms, including Ly108-3, in primary cells

Phosphorylation of Ly108 seems to be needed for SAP binding and differences between the phosphorylation of some of the individual isoforms have previously been shown [2,32]. One significant finding in the work from Schwartzberg and colleagues was that Ly108 is phosphorylated in the intact thymus, but that phosphorylation is lost when single cell suspensions are examined [32]. In primary cells detailed comparison of levels of phosphorylation has not been performed and, in fact, Ly108-3 expression has not been shown.

5.1 Differential phosphorylation of Ly108 isoforms in primary cells

To confirm that SAP does not bind unphosphorylated Ly108 in primary cells we established co-immunoprecipitation conditions with cell suspensions of murine thymocytes. As shown in Figure 25A, using our monoclonal antibody (13G3-19D) that binds all four isoforms, we were unable to co-immunoprecipitate SAP unless the cells were treated with pervanadate. Due to the dependance of SAP binding on Ly108 phosphorylation we set out to compare phosphorylation of Ly108 isoforms in primary cells. We took two approaches to compare phosphorylation as well as detect Ly108-3. First, based on previous findings and predictions, we knew that Ly108-3 could be phosphorylated and therefore detected using an anti-phosphotyrosine antibody [2]. Second, together with the targets of our previously generated polyclonal antibodies shown in Figure 4, lead us to believe that Ly108-3 should also be detectable with antibody R1 (Fig. 1B, target shown in blue). R1 had initially been generated to detect Ly108-1 and Ly108-2, but Ly108-3 contains an identical target sequence and is very similar in molecular weight to Ly108-2 (39.1 vs 38.6 kDa, respectively). Both strategies required pervanadate treatment, as well as deglycosylation and additional separation of isoforms by SDS-PAGE. In the top panel of Figure 25B, three bands of phosphorylated Ly108 are visible in the lane from the Lupus prone, SLAM haplotype-2 Sle1b mice. Three corresponding bands are also visible in the middle panel of membranes re-probed with R1 and correspond with Ly108-1, Ly108-2 and Ly108-3 as indicated. As expected, re-probing with R4 in the lower panel resulted in only one band (Ly108-2) in Sle1b mice and two bands (Ly108-H1 and Ly108-2) in B6 mice. Consistent with previous studies, Ly108-1 is the most heavily phosphorylated, followed by Ly108-3 and then Ly108-2 [2,32]. Ly108-H1 phosphorylation in this experiment is not clearly seen (Figure 25B, top panel), and barely visible on the original films, which is also consistent with previous findings [32].

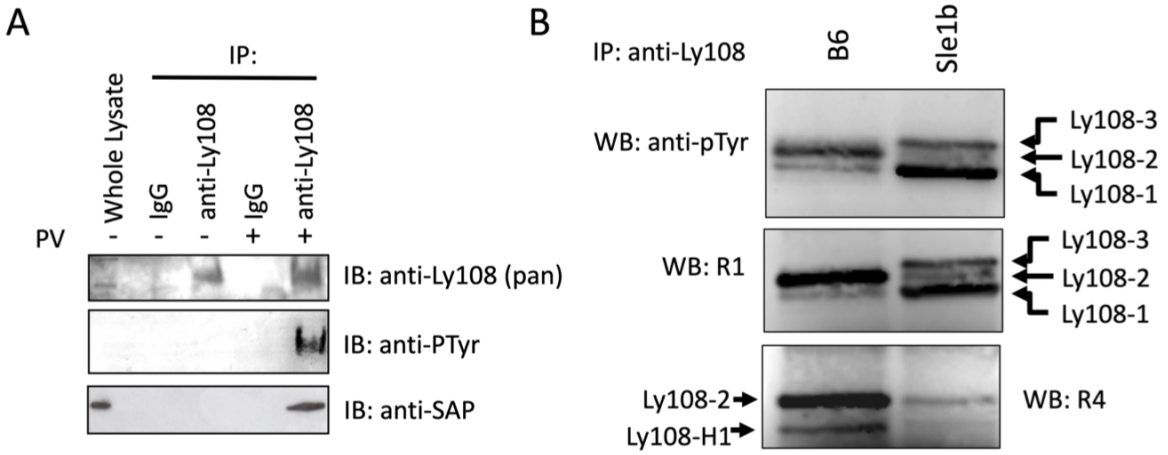

Figure 25. Ly108 isoforms, including Ly108-3, are differentially expressed and phosphorylated in primary cells. (A) Suspended thymocytes from B6 mice were treated where indicated for 20 minutes with pervanadate (PV+) before lysis, after which immunoprecipitation was performed with the indicated antibodies or isotype control (IgG). After SDS-PAGE and transfer, membranes were blotted for Ly108 and SAP. Phosphorylated Ly108 was detected on stripped membranes that were re-probed with anti-phosphotyrosine. (B) Thymocytes from the indicated strains were treated with pervanadate before lysis. Ly108 was immunoprecipitated (IP) and deglycosylated. After separation by SDS-PAGE and transfer to membrane, phosphorylated isoforms were detected by anti-phosphotyrosine antibody by western blotting (WB). Further reprobing was performed with polyclonals R1 and R4 antibody to demonstrate specificity. Individual isoforms are indicated with the help of arrows.

5.2 Differential expression of Ly108-3 between mouse strains

While it seems that Ly108-3 is preferentially expressed in Sle1b mice at the protein level, the experimental conditions are not suitably for accurate comparison.

To test sensitivity of the R1 antibody for Ly108-3 from B6 mice and address potential conformational changes due to this SNP, we used immunoprecipitated B cell lysates that had not been treated with pervanadate, as this has been reported to reduce Ly108 expression [32]. We also compared B6 to 129 wild-type mice to see if Ly108-3 expression was associated with SLAM haplotype-2. Here too we could see a discrete band of a protein slightly larger than Ly108-2 (Fig. 26A elbow arrow) in 129 mice, that is absent when re-probed with R4 (Fig. 26A lower panel). This detection pattern is in agreement with that expected of Ly108-3 and confirms that R1 also recognizes unphosphorylated Ly108-3. The increased expression of Ly108-3 protein seems therefore to be common in haplotype-2 mice.

Because the differential Ly108-3 expression had only been partly addressed, we performed a semi-quantitative RT-PCR on cDNA from B6 and Sle1b mice using primers that amplify Ly108-1 and Ly108-3 with amplicons of slightly different lengths. As seen in Figure 36D we do see Ly108-3 mRNA present in B6 mice, although it is more prominent in Sle1b.

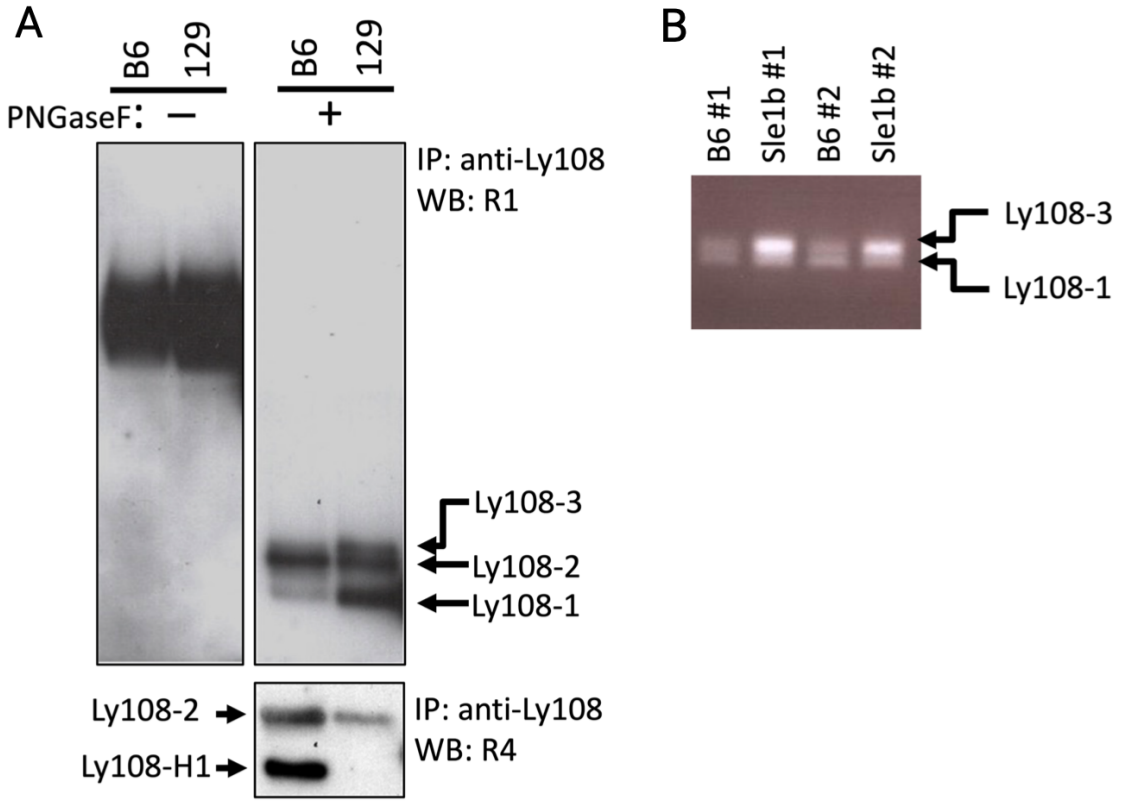

Figure 26. Detection of Ly08-3 in primary cells (A) Immunoprecipitation of Ly108 from unstimulated B cells from the indicated mouse strains. Some samples were deglycosylated (in the right panels) and western blotting was performed with the polyclonal antibodies R1 (top panels) or R4 (bottom). Elbow arrow directed at a band corresponding to Ly108-3. (B) RT-PCR products using oligonucleotides corresponding to exon 7-8 and template cDNA from thymus show bands of 227bp and 241bp corresponding to Ly108-1 and Ly108-3, respectively. Two mice from each strain indicated were used.

6. Characterization of a non-synonymous SNP in Ly108-3

In our search for an explanation for differences in expression we became aware of a non-synonymous single nucleotide polymorphism (SNP) in Ly108-3: rs31534295. Two characteristics of this SNP caught our attention. First, it was situated just downstream of the second ITSM and target of polyclonal R1. Second, the polymorphism results in a proline to leucine substitution, which likely results in significant conformational changes. For these reasons we examined it more closely.

6.1 Haplotype specific SNP distribution

Shown in Figure 27A is the site of rs31534295 and the resulting alignment with B6 after our sequencing of Ly108-3 from B6 and Sle1b mice. I searched the Mouse Genome Database and found that the SNP variant present in Sle1b mice is shared with other SLAM haplotype-2 mouse strains, as shown in Figure 27B [190,191]. This is along the line of distribution shared by Ly108-H1 expression that I found in some strains [3]. While these results are perhaps not surprising, it was important to ascertain that the distribution is haplotype specific as this may partially explain expression data from the past.

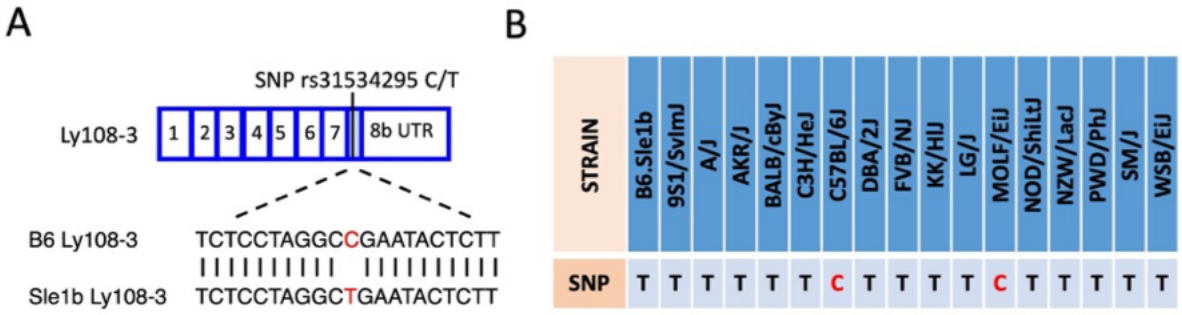

Figure 27. Characterization of the non-synonymous SNP rs31534295 in Ly108-3. (A) Exon organization of *Ly108-3* showing the location of the non-synonymous SNP rs31534295 (in red) and alignment of a short portion of Sle1b derived *Ly108-3* with B6. Shown is the SNP and 10bp flanking sequence. (B) SNP distribution across various mouse strains.

6.2 Predictive analysis of SNP significance

One aspect pointing to the potential significance of SNP rs31534295 is its proximity to a SAP binding site (Figure 28A) Because I expected the proline to leucine substitution to alter the secondary structure of Ly108/3, I modelled the intracellular tail of Ly108-3 using the PEP-FOLD4 software (Figure 28B) [184,185,192,193]. When comparing the secondary structure predictions of Ly108-3 from Haplotype 1 to that of Haplotype 2 I saw a slightly higher probability for an alpha helix in the Haplotype 2 derived tail

(Figure 28B). The difference in predicted change is not large and not clearly visible in the predicted 3D structure (Fig 28C).

As a structural change could have implications for interaction between the tail of Ly108-3 and intracellular adapter proteins, I set out to determine if this region contained any sequences that correspond to protein binding sites. In another computational approach I used the Eukaryotic Linear Motif (ELM) resource to predict protein interaction sites[183]. I used the tail sequence of Ly108-3 as input and limited the result to those directly surrounding the SNP. Using this approach, I identified six protein-binding sites in this region (Fig 28D) These motifs were conserved between Haplotype 1 and Haplotype 2 *i.e.* there was no change in binding site due to the non-synonymous SNP. This does not, however, rule out that binding may be altered due to conformational changes in this region of the protein.

In summary, I describe another strain specific difference in the ly108 gene which, as a non-synonymous SNP in proximity to important binding motifs, could have a significant effect on signaling, and perhaps immune function.

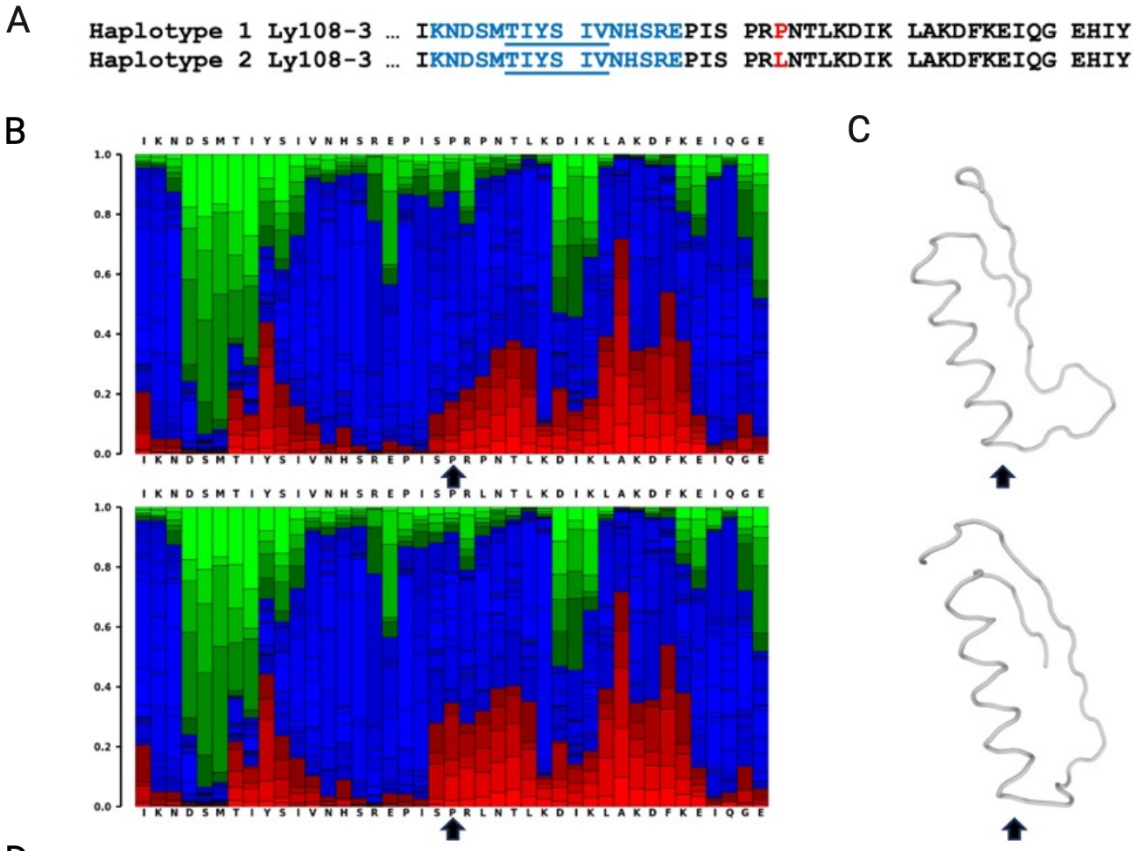

Figure 28. Prediction of potential significance of SNP rs31534295. (A) The resulting amino acid substitution (in red) in the tail of Ly108-3 is shown in relation to the second SAP binding site (underlined) and target of polyclonal antibody R1 (blue). (B) Secondary structural prediction of tail region shown by a local structure prediction profile with the probability of types of secondary structure represented using the following color code: red: alpha helical, green: extended, blue: coil. Above is haplotype 1 sequence, below haplotype 2 (C) Examples of predicted 3D structures. Above is haplotype 1 sequence, below haplotype 2 ((D) ELM predictions from searches using the indicated tail sequence of Ly108-3 and filtered for hits flanking the non-synonymous SNP.

DISCUSSION

This thesis describes work I performed to study Ly108, a SLAM-family member that is expressed on immune cells, has SAP binding sites, and is implicated in autoimmunity. Importantly, Ly108 has a valuable role in the generation of high-affinity antibodies and long-term B cell memory, a process that is dependent on the interaction with SAP [8]. At the commencement of my work not much was known about Ly108, the murine homologue of NTB-A. Using diverse tools, I generated anti-Ly108 antibodies and fusion proteins to extend the knowledge on its expression data, as well as examine their effect of on T cell function. Because of accumulating data emphasizing the significance of Ly108 isoforms I also wanted to compare their expression, function, levels of phosphorylation, as well as their ability to bind SAP. My discovery of the novel isoform Ly108-H1 resulted in data that suggested that it could prevent autoimmunity by suppressing T cell functions [3]. I had hypothesized that it would somehow suppress T cell activation and, in this work, I set out to compare it to other isoforms. I also hypothesized that Ly108-H1 would be able to bind SAP, even though others had not seen this. I then went on to study Ly108-3, an isoform of which little is known, and predicted that it could be detected in primary cells.

1. Expression of Ly108

Although we had generated expression data using the monoclonal antibody 13G3-19D early after it was made, only little was deemed relevant for publication [1,3]. A cell panel with expression of Ly108 was published by Zhong *et al* with one potentially relevant subset absent, this being neutrophils. My work contributes to our knowledge of Ly108 expression in two ways. First, I show that Ly108 is only present on a small subset of activated neutrophils. Second, the drop of Ly108 expression in mature thymic NKT cells seems to coincide with their uncoupling from the double positive $CD1d^{+}$ thymocytes during selection.

This second finding was especially interesting, because of fascinating results from the Crotty and Veillette laboratories. We had known from earlier work that SAP was crucial for NKT cell development [53–55]. We had also shown that Ly108 expression dropped upon maturation in the thymus, and was partially responsible for NKT cell development [1]. What the other groups later showed was intriguing. Firstly, Kageyama and colleagues showed that crossing of the Ly108 knockout with the SAP knockout restored the NKT defect [8]. The authors propose that Ly108 and SAP form a switch that provides a survival signal in NKT cell development. Further evidence for an important role of Ly108 was shown when it was reintroduced in mice deficient in NKT cells because of deletion of multiple SLAM-family members [43]. In elegant experiments, Lu and colleagues show that SLAM-family members, and Ly108 in particular, provide an inhibitory signal to NKT cells just after positive selection in the thymus thereby supporting their survival. While a unifying explanation is still lacking, it is tempting to hypothesize that the downregulation of Ly108 late in thymic NKT cell development (Figure 14) could be due to a

mechanism needed to ensure NKT cell survival. Ly108 may, therefore, play an important role in NKT cell selection by preventing negative signaling through inhibitory pathways rather than promoting positive selection via activating signals as was previously thought.

2. Effect of Ly108 antibodies and Ly108 fusion protein *in vitro* and *in vivo*

In previously published T cell co-stimulation experiments, the presence of monoclonal antibodies against NTB-A, together with anti-CD3, lead to increased T cell proliferation and cytokine secretion (IL-2 and IFN-γ) [4,144]. However, it was not clear in all of the experiments whether the antibodies used were plate bound or soluble.

In the mouse, Dutta *et al* showed that stimulation of double positive thymocytes with plate bound anti-Ly108 (13G3) augmented T cell receptor signals in thymocytes [194]. Thus, I also tested our antibodies (13G3 and 330) in co-stimulatory experiments. However, in our study I used T cells and both plate bound as well as soluble anti-Ly108 antibodies. This approach aimed to determine if differences due to the mode of delivery could influence the results, which is crucial for interpreting findings from our subsequent *in vivo* experiments. I hypothesized that plate-bound antibodies could cross-link Ly108 while soluble antibodies could perhaps interfere with Ly108 homophilic interactions. Interestingly I saw increased T cell proliferation in both groups but saw slight inhibition of IL-2 in the plate-bound samples. While my experiments do not provide a conclusive explanation for these differences, they may be important to consider when designing future experiments or contemplating therapeutic applications of antibodies.

Valdez *et al* used a Ly108-fusion protein to delay the onset of antigen-induced experimental allergic encephalomyelitis (EAE) in a mouse model of multiple sclerosis [4]. Our Ly108Fc also showed some suppression of disease in the CD45RB^{hi} model of murine colitis (Figure 18). I also performed *in vivo* experiments were also performed with 13G3 and 330 in this CD45RB^{hi} model of colitis. I did not see any suppression of disease and, if anything, saw signs of more severe disease. This was not apparent in the standardized disease parameters, but instead in histology that showed the presence of granulomas. Granulomas are associated with a few disorders with the most relevant in this case being Crohn's Disease (CD) and Chronic Granulomateus Disease (CGD). CD is a multifactorial inflammatory bowel disease, but certain mechanisms have been linked to impaired control of microbes [195]. CGD is a condition characterized by increased susceptibility to infections that results from defective killing of bacteria and fungi due to ineffective generation of reactive oxygen species [9]. This is interesting to consider because SLAM and Ly108 have been implicated in the generation of reactive oxygen species [68,139]. Thus, the formation of granulomas in the CD45RB^{hi} model due to administration of Ly108

antibodies could potentially be due to impaired microbial control or increased inflammation. However, the exact cause is not yet known and warrants further investigation.

Our results, for the most part, align with previous research with NTB-A and Ly108 demonstrating that our anti-Ly108 antibodies T cell proliferation and IL-2 production, but suppress IL-2 when the antibodies are plate bound. Reconciling different data on cell stimulation with antibodies poses significant challenges due to variations in experimental conditions, antibody specificities, and biological contexts. These aspects are further compounded by the complexity of Ly108 signaling involving the homophilic binding and the downstream effects influenced by the presence of SAP. Thus, interpreting the collective findings requires careful consideration of these variables to draw meaningful conclusions about Ly108's role in immune regulation.

3. Ly108-H1 suppresses T cell activation, but does not enhance cell death

Previous work supported a protective role for Ly108-2 in the protection against Lupus-like autoimmunity in mice. This protection might stem from augmented B cell receptor signaling, which could lower the threshold for self-reactive immature B cells to undergo negative selection [131]. We later demonstrated that introduction of a *Ly108-H1* transgene, or transferring Ly108 expressing T cells into Lupus-susceptible congenic mice could ameliorate disease and dampen T and B cell activation [3]. In my thesis I show that Ly108-H1 had a pronounced inhibitory effect on IL-2; thus providing support for a T cell mediated regulation of inflammation.

The mechanism proposed by Kumar *et al* by which Ly108-2 is thought to protect against autoimmunity involves the promotion of activation induced apoptosis or death in immature B cells [131]. This work was based on erroneous findings that there was more *Ly108-2* mRNA in B6 mice as compared to Sle1b mice, since this was studied before the discovery of Ly108-H1 [3,96]. I examined the role of Ly108-H1 in AICD in T and B cell lines as well as immature thymocytes. My findings support those of Kumar and colleagues for Ly108-2, specifically showing increased AICD. In contrast, my results suggest that Ly108-H1 does not play a role in this mechanism. While it is possible that Ly108-2 and Ly108-H1 protect against autoimmunity in two different, and seemingly opposing ways, I propose that Ly108-H1 may be more important than the other isoforms in the context of murine SLE for two reasons. First, the strain-specific differences of *Ly108-2* do not seem as robust as that of Ly108-H1. This is because *Ly108-2* mRNA levels initially measured would have been overestimated because of the then-unknown sequence homology with *Ly108-H1* [32]. Second, Ly108-H1 has been the only isoform shown to protect against murine SLE *in vivo*, although there was still some residual disease [3].

4. Ly108 isoforms have different levels of phosphorylation and bind SAP

The ability to bind the adapter proteins SAP is of utmost importance to the function of those SLAM-family members with ITSMs [11,197]. A comparison of binding motifs in Ly108 isoforms shows that Ly108-H1 is unique in that it has one ITSM, while other Ly108 isoforms have two. Except in the case of SLAM, the tyrosines embedded in the ITSMs need to be phosphorylated in order to bind SAP. Previous analysis of Ly108-H1 showed no tyrosine phosphorylation and little SAP binding [32]. However, we were able to detect phosphorylated Ly108-H1 after treatment with pervanadate, as well as SAP binding. I hypothesize that the observed phosphorylation of Ly108-H1 seen represents the tyrosine contained within the first ITSM, as SAP binding was phosphorylation dependent. I also propose that the second ITSM is crucial for further signaling as was shown with SLAM, where SAP binding resulted in Fyn-T recruitment, and phosphorylation of distally located tyrosines [30,34]. In addition, it was shown that the second ITSM of SLAM was crucial for signaling through phosphorylation of the downstream mediators Dok-1, Dok-2, and SHIP-1 [30]. The second ITSM is also essential for the function of NTB-A in NK cells [56]. I propose, therefore, that differences between isoforms will be more pronounced if we were to extend the analysis of downstream signaling comparing isoforms because Ly108-H1 lacks a second ITSM but does bind SAP.

5. Detection of Ly108-3 and demonstration of additional strain specific differences

After alignment of the amino acid sequences for all isoforms we correctly predicted that Ly108-3 could be isolated and detected by our antibodies in primary cells, and now knew that all isoforms could be phosphorylated. In addition to deglycosylation, we were able to use the individual differences in phosphorylation of isoforms to better separate them. This allowed for detection by our polyclonal antibody R1 as well as the anti-phosphotyrosine antibody 4G10. Interestingly, we could not clearly detect Ly108-3 protein in B6 lysates but could see it in Sle1b and 129 mice. We analyzed mRNA from B6 and Sle1b mice, and although we did find Ly108-3 expressed in B6 mice it was relatively low. It is therefore possible that low levels of mRNA resulted in undetectable Ly108-3 in B6 mice. We do not know the reason for the differences in mRNA. One factor possibly contributing to reduced Ly108-3 protein in B6 mice could be the extra proline near to two others. Proline can slow down translation because it reacts slower as a donor or receptor to other amino acids. Its structure also makes it more difficult to position itself into the ribosome during incorporation of amino acids into the growing chain [198]. Structural changes due to the extra proline could also influence binding of adaptor proteins, something that would be of great interest to attain.

6. The impact of isoforms

By using gene prediction algorithms, analysis of the raw genome sequence served as a starting point in the discovery of new putative proteins. In the recent years, various additional algorithms that predict transcription and splice variants have been developed [199]. More importantly, much new mRNA sequence data has been generated with next generation sequencing technologies such as hybridization based microarrays and RNA-seq [200,201]. Analysis of the mouse transcriptome first predicted that 39% of murine genes have multiple transcripts [202]. This has subsequently been extended in humans using high-throughput genome-wide analyses showing that about 90% of genes generate multiple protein isoforms through alternative splicing [203204]. Of note, a number of processes involving the immune system are influenced by alternative splicing and a recent publication has even described how isoforms in immune cells are implicated in disease [125,205].

It is therefore not surprising that most of the SLAM-family receptors show alternative splicing, with five SLAM family members having isoforms that vary in their cytoplasmic tails [11]. This can result in a forms lacking some or all ITSMs leading to reduced ability to bind SAP or EAT-2 [10,128206]. While not all alternative splicing results in missing ITSMs, other important signalling motifs such as the distal tyrosine in Ly108-1 may be lost. These differences are not only important for our understanding of biology, they can also be put to use. For example, the observation that one isoforms of NTB-A had an opposite signal to others was used as a strategy to increase anti-tumor activity of lymphocytes. This was done by steering the expression towards this stimulatory isoform using antisense nucleotides [130].

Another extremely exciting line of research regards the interaction of SLAM-family members with microbes [13]. SLAM, CD48 and Ly108 have been shown to be the receptors for microbial ligands [66,6813375]. Of particular interest, particularly when viewed in the light of isoform research, is the discovery that certain cytomegalovirus (CMV) strains express homologues of CD48, Ly9 and Ly108 [207,208209]. These homologues were captured from the host during evolution and can assist in viral entry, as well as inhibit the antiviral response [210]. For example, one of the CD48 homologues is soluble and released from infected host cells after which it inhibits NK cell mediated killing through disruption of CD48-2B4 interaction [211].

7. Final remarks

The research presented in this this thesis provides a comprehensive analysis of the Ly108 molecule and its various isoforms. I was able to provide detailed expression of Ly108 on immune cells, examine the co-stimulatory, or rather co-inhibitory, role of Ly108, and profile the differences between individual

isoforms.

The findings presented in this thesis underscore the complexity of Ly108's role in immune regulation. Studies on Ly108 and NTB-A have resulted in interesting but seemingly conflicting results. Reconciling different data on Ly108 and NTB-A function poses significant challenges due to variations in experimental conditions, specificity of reagents, and biological contexts. Further compounding the complexity are the downstream signaling pathways, where the effects of Ly108 stimulation would vary depending on the presence of signaling molecules such as SAP and EAT-2. Thus, further research, as well as careful interpretation of the collective findings, is needed to draw meaningful conclusions about Ly108's role in immune regulation.

Finally, the differential expression and function of Ly108 and NTBA-A isoforms add an additional layer of complexity, making it a challenging but promising target for therapeutic interventions in immunology, onco-hematology and infectious diseases. Future research should focus on further elucidating the specific roles of isoforms and their potential as therapeutic targets.

CONCLUSIONS

1. Ly108 is widely expressed on the surface of immune cells in the mouse. Two exceptions are a subset of developing NKT cells in the thymus, where Ly108 is temporarily low at the mature thymic stage and neutrophils which only express Ly108 in a subset of cells after stimulation.

2. Monoclonal antibodies against Ly108 cause increased T cell proliferation and IL-2 production when in solution, while IL-2 production is inhibited when Ly108 is crosslinked with plate bound antibodies. These antibodies appear to cause increased immune activation in a T cell transfer model of disease, while Ly108Fc appears to inhibit disease. It is not clear whether these antibodies activate a co-stimulatory mechanism of Ly108 or block an inhibitory mechanism.

3. When expressed in a T cell hybridoma, Ly108-H1 significantly decreases IL-2 cytokine production than Ly108-1 and Ly108-2.

4. Ly108-H1 can be phosphorylated and does bind SAP.

5. Ly108-3 is expressed in primary cells and can be phosphorylated

6. Haplotype-2 mice, including the Lupus prone Sle1b mice have higher levels of Ly108-3 mRNA and protein. A haplotype-specific non-synonymous SNP of Ly108-3 results in an extra proline which may affect expression, structure and function.

CONCLUSIONES

1. Ly108 se expresa ampliamente en la superficie de las células inmunitarias de ratón con dos excepciones notables: un subconjunto de células NKT en el timo, donde Ly108 muestra una expresión temporalmente baja en la etapa tímica más madura; y en un subconjunto de neutrófilos que expresan Ly108 después de la estimulación.

2. El entrecruzamiento de Ly108 con anticuerpos unidos a la placa inhibe la producción de IL-2, mientras que el tratamiento con anticuerpos disueltos en el medio causa un aumento en la proliferación de células T y la producción de IL-2 Estos anticuerpos parecen causar un aumento en la activación inmunitaria en un modelo de enfermedad por transferencia de células T, mientras que Ly108Fc parece inhibir la enfermedad. No está claro si estos anticuerpos activan un mecanismo coestimulador de Ly108 o bloquean un mecanismo inhibidor.

3. La sobreexpresión de Ly108-H1 en un híbridoma de células T reduce significativamente la producción de la citoquina IL-2 en comparación con la sobreexpresión de Ly108-1 y Ly108-2.

4. Ly108-H1 puede fosforilarse y se une a SAP

5. Ly108-3 se expresa en células primarias y puede ser fosforilado.

6. Los ratones del haplotipo-2, incluidos los ratones propensos al lupus Sle1b, tienen niveles más altos de ARNm y proteína de Ly108-3. Un haplotipo especifico con un SNP no sinónimo de Ly108-3 resulta en una prolina adicional que puede afectar la expresión, estructura y función.

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