SLAMF Receptors In Health and Disease: Implications for Therapeutic Targeting

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Colophon

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SLAMF Receptors In Health and Disease: Implications for Therapeutic Targeting

SLAMF Recepetoren in gezondheid en ziekte:
Implicaties voor therapeutische benadering en binding

(met een samenvatting in het Nederlands)

Proefschift

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Promotoren: Prof.dr. E.J.H.J. Wiertz
Prof.dr. C. Terhorst

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CHAPTER 1

SLAMF Receptors: Immune Regulators in Health and Disease

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1 Introduction

Effective immune responses develop after a well-orchestrated series of events that include recognition, immune cell interactions and activation/inhibition of signaling pathways. The signaling lymphocyte activation molecule family (SLAMF) of cell surface receptors, which consists of nine transmembrane proteins (SLAMF1-9) expressed at different levels, are involved in viral and bacterial recognition, serve as co-stimulatory molecules at immune synapses, and modulate myeloid and lymphocyte development. SLAMF receptors are homophilic receptors, with the exception of SLAMF2 and SLAMF4, and are only expressed on hematopoietic cells. Their adaptors, SLAM associated protein (SAP) and Ewing's sarcoma-associated transcript 2 (EAT-2), bind to the cytoplasmic tails and control the functions and magnitude of SLAMF receptor signaling. In this review, we summarize the current knowledge on the role of SLAMF receptors in regulating immune functions and recent findings describing how SLAMF receptors can be exploited as drug targets in human malignancies.

2 A Background of the SLAM Family Members and adaptors SAP and EAT-2

2.1 Structure

The SLAM family of immune cell surface receptors is a member of the CD2 subfamily of the immunoglobulin (Ig) superfamily consisting of nine members, SLAMF1-9 [1-4]. SLAMF receptors are type I transmembrane glycoproteins comprised of an extracellular membrane containing an N-terminal V-Ig domain followed by a C2-Ig domain in the extracellular region (this set is duplicated in SLAMF3), a transmembrane region, and an intracellular cytoplasmic tail containing tyrosine based switch motifs (ITSM). Notable exceptions to this structure include SLAMF2, which has a glycosyl-phosphatidyl-inositol (GPI) membrane anchor and like SLAMF8 and SLAMF9 lack ITSM motifs [5-8]. Binding of SLAM associated adaptors; SAP and EAT-2, to cytoplasmic tails of various SLAMFs regulate their function on different immune cells. Expression of SLAMFs and their adaptors is restricted to hematopoietic cells. In addition, the gene loci are located on chromosome 1 in both mice and humans, except SAP, which is located on the X chromosome [9]**(Figure 1)**.

All SLAMFs are homophilic receptors aside from SLAMF2 and SLAMF4, which bind each other [10-12]. The determination of the SLAMF3, SLAMF5 and SLAMF6 crystal structures revealed *in-trans* interactions through their IgV domains (SLAMF3 unpublished data, generously donated by Profs. Steve Almo

and Stanley Nathenson, Albert Einstein College of Medicine **(Figure 2)** [13, 14]. Engagement of SLAMF receptors on immune cells (e.g. APC - T cell) trigger inhibitory or activating signals that modulate cellular responses. Within these homophilic and heterophilic interactions, the binding affinities for each SLAMF varies (SLAMF3 nM, SLAMF5 sub-μM, SLAMF6 ~ 2 μM, SLAMF2/4 ~4 μM, SLAMF1 ~200 μM) which likely contributes to functional differences within the family of receptors [12-15]. In addition to being self-ligands, SLAMF1 also serves as an entry receptor for Measles virus [16, 17] while SLAMF1, SLAMF2 and SLAMF6 have been demonstrated to interact with bacterial components [18-21] (reviewed in detail ref [22]).

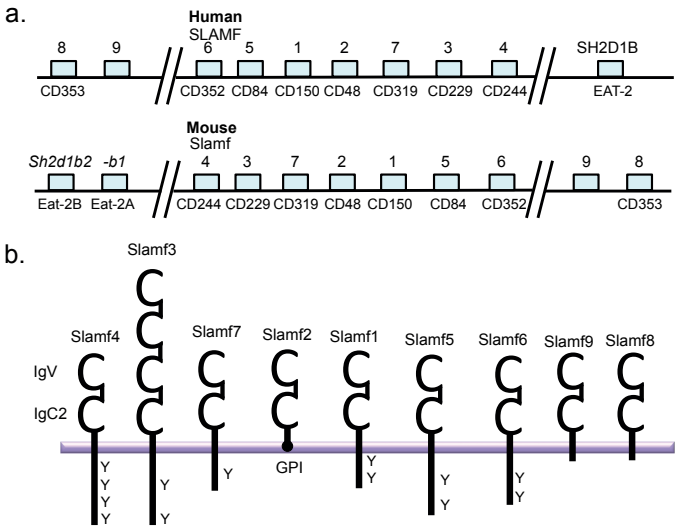

Figure 1. Signaling lymphocyte activation molecule family (SLAMF) of genes and protein. a. Organizational overview of SLAMF gene cluster on chromosome 1 in human and mouse. **b.** SLAMF members consist of an IgV/IgC2 ectodomain, which is duplicated in SLAMF3. While SLAMF2 and SLAMF4 bind each other, other SLAMF receptors are homophilic. Six members of the family contain varying lengths of cytoplasmic tail with ITSM motifs (Y) that can recruit and bind the adaptors SAP and/or EAT-2.

2.2 SAP and X-linked Lymphoproliferative Disease (XLP)

First identified in 1975, X-linked lymphoproliferative disease (XLP) (also known as Duncan's disease) is an extremely rare primary immunodeficiency that mainly manifests in males and is primarily characterized by extreme susceptibility to infection with Epstein-Barr virus (EBV) [23]. However, most XLP patients infected with EBV develop fatal or fulminant infectious mononucleosis due to dysregulated

immune responses, which leads to clonal proliferation and expansion of T and B cells. NK and $CD8^+$ T cell functional defects have been identified in XLP patients likely contributing to the inability to control EBV infections [24-27]. Patients usually exhibit progressive loss of serum IgG and develop B cell lymphomas and dys-gammaglobulinemia [28-31].

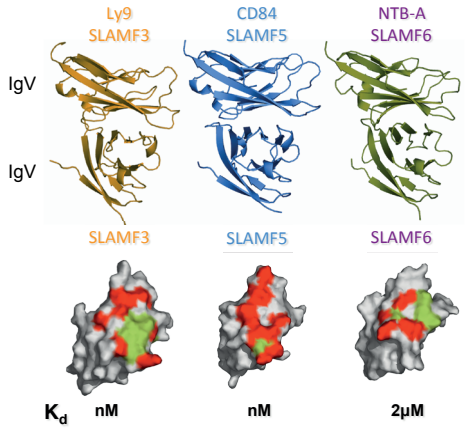

Figure 2. Homophilic engagement of SLAMF3, SLAMF5 and SLAMF6 occurs via interactions of the IgV domains. Specificity of homophilic binding is determined by different surface characteristics. All three SLAMF receptors show different binding affinities. Green = hydrophobic, red= hydrophilic amino acids

Not until twenty years after the description of XLP, the genetic cause was determined by the Terhorst lab: mutations in or deletion of the *SH2D1A* gene, which encodes a 15 kD cytoplasmic protein SAP consisting of a single Src homology 2 (SH2) domain and a 28 amino acid tail [32-35]. In the same publication, SAP was shown to bind to SLAMF1 and subsequent studies showed binding of SAP to ITSM motifs in the cytoplasmic tail of six of the SLAMF receptors **(Figure 3)** [1, 30, 34, 35]. SAP was then identified to be required for recruitment and activation of Src-family kinase FynT upon SLAM ligation [36]. The crystal structure of the SLAM-SAP-Fyn-SH3 ternary complex revealed that SAP binds the FynT SH3 domain through a non-canonical surface interaction and couples Fyn to SLAM receptors **(Figure 4)** [37].

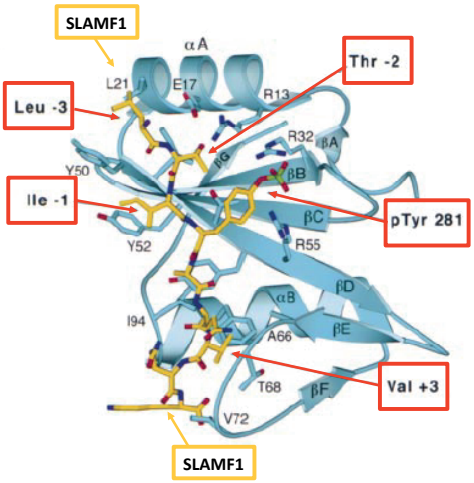

Figure 3. Ribbon diagram showing SAP/SLAMF1 pY281 complex. The bound SLAMF1 phosphopeptide is shown in a stick representation (yellow). Selected SAP residues that form the binding site are shown in blue. SLAMF1 residues N-terminal to pY281 make additional interactions with SAP at pY -3 and pY -1 (positions relative to pTyr281)[34, 35].

SAP is mainly expressed in T cells, NK cells, NKT cells and eosinophils while expression in B cells is found only in some cases [33, 34, 38-40]. Of note, later research also identified mutations in *XIAP*, X-linked inhibitor of apoptosis, which are associated with XLP like disease manifestations in a small number of families [41, 42].

Studies from SAP deficient mice shed light on understanding the basis of XLP. Naïve $CD4^+$ T cells from SAP deficient mice exhibited reduced production of peptide-MHC or T cell receptor (TCR) driven T_H2 cytokines [43-46]. In addition, germinal center formation is significantly impaired and antibody secreting cells (ASCs) and memory B cells are lost, leading to severely reduced levels of serum IgG and IgE [43, 47-49]. Later, these defects were also identified in XLP patients [50, 51].

In SAP deficient mice there appeared to a B cell defect, which was dependent on the genetic background [52] as evidenced by adoptive transfer studies of SAP deficient B cells and WT $CD4^+$ T cells [47]. Although the B cell defect seems secondary to that of $CD4^+$ T cells [48, 49, 51], *in vitro* experiments suggested an intrinsic defect in class switching [40].

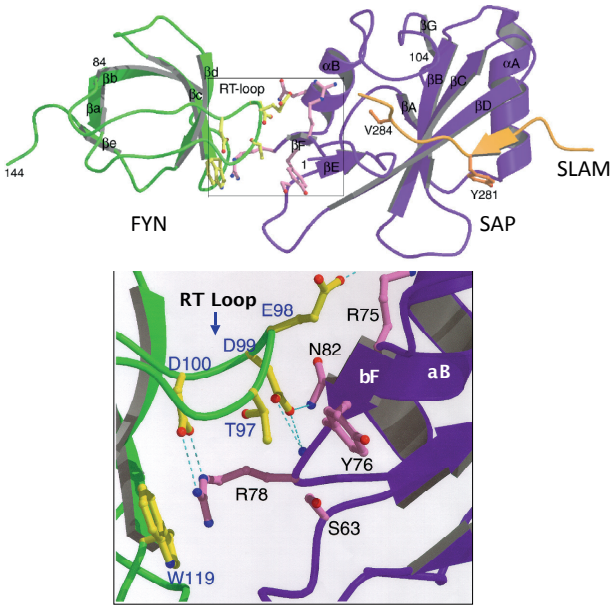

Figure 4. SAP couples Fyn to SLAMF receptors. SAP binds the Fyn SH3 domain through a non-canonical surface interaction (zoomed area). SLAM peptide binds SAP in a 3-pronged mode via the β-strand of N-terminal, Tyr 281 and Val 284.(SLAM = SLAMF1)

$CD8^+$ T cells and NK cell functions are also altered in SAP deficient mice and XLP patients. While human *in vitro* studies suggested SAP is required for induction of cytotoxicity by $CD8^+$ T cells [53], SAP deficient mice presented more virus specific $CD8^+$ T cells in lymphocytic choriomeningitis virus (LCMV) and γ-herpesvirus 68 infections, suggesting SAP inhibits $CD8^+$ T cell responses [54, 55]. Finally, SAP was found to be indispensable for NKT cell development as SAP deficient mice and XLP patients lack NKT cells [56-58].

In summary, SAP signaling is critical for mounting proper immune responses. Moreover, SAP, as well as EAT-2, regulates signaling through SLAMFs by setting thresholds for activation/inhibition and modulating cell-cell interactions and responses. Here, we will highlight the roles of SLAMF receptors in regulating normal immune responses and under various disease conditions.

3 Immune cell Functions of SLAMF Receptors

SLAMF receptors are adhesion molecules that are involved in development of lymphocytes and in orchestrating innate and adaptive immune responses. While contribution of each SLAMF member is unique, they can have compensating or opposing roles in function, which will be discussed in this section.

3.1 SLAMF1 (CD150, SLAM)

Current understanding of the biology of SLAMF1 (SLAM, CD150) demonstrates the importance of this receptor at multiple levels during an immune response. The cytoplasmic tail of SLAMF1 can recruit SAP and EAT-2 upon immune cell activation in both mice and humans [35, 59]. SLAMF1 is widely expressed on B and T cells, macrophages, dendritic cells, platelets and hematopoietic stem cells [60-64]. Expression on B cells starts from pre-B cell stage and is upregulated in plasma cells [65]. In B cell malignancies, SLAMF1 demonstrated heterogeneous expression, with differentially expressed isoforms that may contribute to disease pathogenesis [66].

SLAMF1 ligation during T cell activation is critical for the fate of the T cell. Upon T cell stimulation, SLAMF1 co-localizes with the T cell receptor and the tyrosine residues on the cytoplasmic tail are phosphorylated [67]. However, binding of SAP to the cytoplasmic tail can be in both a phosphotyrosine-independent and dependent manner [67]. Initial studies using monoclonal antibodies against SLAMF1 in conjunction with TCR stimulation induced IFNγ secretion and promoted T_H1 responses and even induced switching of T_H2 cells to T_H0 phenotype [60, 68, 69]. SLAMF1-/- mice exhibited no difference in IFNγ production compared to WT controls, but interestingly IL-4 and IL-13 cytokine levels were reduced indicating compromised T_H2 responses [44, 62]. During germinal center (GC) reactions, T follicular helper cells (T_{FH}) residing within the GCs use SAP signaling, while SLAMF1 is required for IL-4 production by T_{FH} cells to provide optimal help to B cells [70]. This discrepancy *in vitro* and *in vivo* suggests that there may be other SLAMF receptors that can compensate *in vivo* for T_H1 responses in the absence of SLAMF1. The role of SLAMF1 in NKT cells will be discussed in the SLAMF6 chapter.

The findings that upon encountering inflammatory stimuli with lipopolysaccharide (LPS), IL-1β or IFNγ, SLAMF1 is upregulated on antigen presenting cells (APCs), i.e. macrophages and dendritic cells (DCs), led to the notion that SLAMF1 may have a role in regulating APC functions [62, 63, 71]. Interestingly, SLAMF1 was found to interact with the OmpC and OmpF bacterial membrane

proteins of Gram-negative bacteria to facilitate phagocytosis in macrophages [18]. It further regulated the activity of NADPH oxidase (NOX2) complex in phagolysosomal compartments and facilitated the recruitment of autophagy complex containing Beclin-1, Vps34, Vps15 and UVRAG [20]. As autophagy is a universal process by which cells remove misfolded proteins, clear damaged organelles and also eliminate intracellular pathogens [72], expression and signaling through SLAMF1 may play a role in proper regulation of this process. Additionally, a recent study demonstrated that SLAMF1 enhances type I interferon production upon encountering Gram-negative bacteria by modulating MyD88 in a TLR4 independent signaling manner [73]. Taken together, these findings suggest SLAMF1 is a target for controlling inflammatory responses to Gram-negative bacteria through multiple mechanisms.

3.1.1 SLAMF1 is implicated in various Autoimmune Diseases

Autoimmunity is in general defined by the loss of immune tolerance to self-antigens and causes destruction of healthy cells. SLAMF1 has been associated with human inflammatory bowel syndromes including Crohn's disease as well as experimental murine models of colitis [74-76]. Inflamed colon sections from Crohn's disease patients showed enhanced SLAMF1 expression on monocytes and macrophages driven by Toll like receptor (TLR) responses to bacterial ligands as compared to healthy gut tissue [74]. Involvement of SLAMF1 was also demonstrated in an experimental model of colitis where antibodies blocking SLAMF1 homophilic interactions reduced colitis in mice [75].

Pathology of rheumatoid arthritis (RA), characterized by chronic inflammation of the joints, has also been associated with increased expression levels of SLAMF1 on T cells in the synovial fluid and tissue of patients compared to that of healthy individuals [77]. This study suggested SLAMF1 homophilic interactions between T cells and synovial mononuclear cells may contribute to cytokine profile changes in these patients. Additionally, another study found EBV-associated RNA and DNA in the synovial fluid of RA patients and speculated that SAP and SLAM interactions and/or signaling may play an important role due to the link between SAP signaling and EBV infections [78].

Another autoimmune disease where SLAMF1 has been implicated is multiple sclerosis (MS), a condition where immune cells attack myelin sheath [79]. The percentage of T cells expressing SLAMF1 was increased in the blood of MS patients, but the contribution of this to the disease is not yet known [80].

3.2 SLAMF3 (Ly9, CD229)

3.2.1 Structure and Expression of SLAMF3

SLAMF3 is a unique member of the family with its two sets of extracellular IgV/IgC2 domains [81, 82]. While several isoforms of SLAMF3 exist, their contribution to immune responses is not known. Homophilic interaction occurs via the N-terminal IgV domains and induces localization at the immune synapses indicating that SLAMF3 may be important in T-B cell interactions [83]. Indeed, SLAMF3 is expressed on all subsets of B cells and T cells [84, 85]. High expression on NK cells and monocytes are restricted to murine cells [81, 86, 87]. A SLAMF3 homolog, A33, has been identified in genomes of squirrel monkey CMV (SMCMV) and owl monkey CMV (OMCMV), that infect New world monkeys[88]. This was acquired by retrotranscription of virus-host coevolution by the new world monkey CMV.

Similar to other family members, the cytoplasmic tail of SLAMF3 contains ITSM motifs for binding of SAP and EAT-2. Interestingly, SAP has been demonstrated to bind only to human SLAMF3 cytoplasmic tail but not mouse [7, 59, 89]. In addition, Grb2 (growth factor receptor-bound protein 2), a ubiquitous adaptor protein, as well as the μ2 chain of AP-2 adaptor complex, was demonstrated to bind the phosphorylated cytoplasmic tail of SLAMF3 independent of SAP binding site [90, 91]. Regulated by TCR stimulation, recruitment and binding of Grb2 to the SLAMF3 cytoplasmic tail facilitates internalization of the receptor [91]. This interaction appears to be unique among the SLAMF members.

3.2.2 Insights into immune cell functions

Involvement and importance of SLAMF3 in regulating immune cell interactions and functions came from studies using SLAMF3-deficient (Ly9-/-) mice. T cells from Ly9-/- mice presented a mild T_H2 defect *in vitro* along with reduced proliferation upon suboptimal anti-CD3 stimulations [92]. While Ly9-/- mice mounted normal T and B cell responses during *in vivo* viral infection with LCMV [92], the proportion of innate memory $CD8^+$ T cells significantly increased upon post-infection with murine cytomegalovirus (MCMV) [93]. Furthermore, invariant NKT (iNKT) cells are increased in Ly9-/- mice suggesting SLAMF3 is an inhibitory receptor for expansion of innate $CD8^+$ T cells and iNKT cell development [93].

While B cell development in the bone marrow is normal in Ly9-/- mice, splenic transitional 1, marginal zone and B1a B cells are expanded [94]. In accordance

with this increase, T cell independent antibody responses after immunization with 2,4,6-trinitrophenyl-Ficoll were increased. Injecting monoclonal antibodies against SLAMF3 (αSLAMF3) in WT mice resulted in loss of marginal zone B cells and innate like B cells along with down-regulation of CD19/CD21/CD81 complex in an Fc independent manner. Furthermore, aged Ly9-/- mice presented with spontaneous autoantibody production, indicating loss of self tolerance, regardless of background [95]. These findings demonstrate SLAMF3 is a negative regulator of immune responses and may be relevant in targeting B cell related diseases.

3.3 SLAMF6 (CD352, Ly108, NTB-A)

3.3.1 Structure, expression and ligands

SLAMF6 (human: NTB-A, mouse: Ly108) structural details are reviewed elsewhere [3]. In mice, different alternatively spliced forms exist: Ly108.1 and Ly108.2 were first identified, containing one and two additional unique tyrosine motifs, respectively [96, 97]. Later a novel isoform Ly108-H1 was discovered [98]. Tyrosine phosphorylation of the cytoplasmic tail of SLAMF6 leads to recruitment of SAP with high affinity binding, but in the absence of phosphorylation, SAP cannot be recruited [3, 99, 100]. Binding of SAP activates downstream signaling by recruiting the Src family kinase Fyn [37, 101, 102]. This interaction prevents the tyrosine phosphatases SHP1 and/or SHP2 from binding to the cytoplasmic tail and their subsequent negative regulation [34, 35].

SLAMF6 is expressed on a wide variety of immune cells including T cells (also T_{FH}), B cells, NK cells (expressed in human only), double positive thymocytes, eosinophils and neutrophils (mouse only) [6, 38, 96, 103-106]. Furthermore, high expression of SLAMF6 has been determined in various B cell lymphomas, *i.e.* mantle cell and follicular lymphomas [107]. Expression on normal as well as malignant cells suggests that SLAMF6 may be involved in distinct cell-cell interactions in different microenvironments and may be a useful therapeutic target.

Similar to SLAMF3, a SLAMF6 homolog, S1, with a 97% amino acid sequence identity in its ligand binding N-terminal Ig domain have been identified in SMCMV and OMCMV [88]. This was acquired by retrotranscription of virus-host coevolution by the new world monkey CMV. This suggests an immune evasion mechanism of viruses by acquiring the host SLAMF receptors that retain the ligand binding capacity. This allows the interference with host SLAMF functions and induces immunomodulatory actions.

In addition to being a self-ligand, SLAMF6 detects viral and bacterial components as well. For instance, human and mouse SLAMF6 bind the outer membrane proteins OmpC and OmpF, of *E.coli, S. typhimurium*, and in part to *Citrobacter rodentium* [18, 21]. Human SLAMF6 can recognize the influenza haemagglutinin (HA) and Vpu protein of HIV-1, both of which affect NK cell cytotoxicity mediated by SLAMF6 [108, 109].

3.3.2 SLAMF6 signaling in T and B cells

Co-stimulatory molecules play an important role in activation and initiation of proper T cell responses upon TCR engagement. CD28 is the best-known secondary signal necessary for T cell activation. However, studies from CD28 deficient mice suggested additional co-stimulatory molecules play a role as antigen dependent T cell responses were still intact in these mice [110, 111]. In the absence of CD28, stimulation and crosslinking of SLAMF6 with monoclonal antibodies *in vitro* leads to recruitment of SAP to the phosphorylated cytoplasmic tail, and subsequent events lead to T cell proliferation and cytokine production [112]. When SLAMF6 was blocked *in vivo* using a soluble SLAMF6-Fc fusion protein, B cell isotype switching to IgG2a and IgG3 was blocked [112]. In fact, injections of anti-SLAMF6 to WT mice immunized with NP-OVA or in a chronic graft versus host disease (cGVHD) model inhibited T and B cell responses in these distinct *in vivo* models [113, 114].

Involvement of SLAMF6 in regulating immune responses was demonstrated using mice deficient in SLAMF6 [115]. Expression of the SLAMF6 extracellular domain was disturbed by removal of exons 2 and 3 (Ly108 **Δ**E2+3) [115]. *In vivo* infection of Ly108 **Δ**E2+3 mice with *L. mexicana* led to delayed formation of lesions as well as significantly smaller lesions compared to WT controls, indicating a role for Ly108 in innate and adaptive immune responses.

Under normal circumstances, T cell expansion upon infections is constrained by a mechanism called restimulation-induced cell death (RICD), which induces apoptosis of effector T cells during the peak of an immune response [116]. Upon restimulation of these T cells, pro-apoptotic molecules are upregulated, such as FAS ligand and BIM, to induce apoptosis. This mechanism was found to be defective in patients with XLP, mainly due to absence of SAP and defective signaling through SLAMF6 [117]. In RICD, SAP/SLAMF6 signaling was shown to augment TCR signaling to achieve the threshold necessary for RICD. This was facilitated by SAP's recruitment of Lck, but not Fyn, to the cytoplasmic tail [118].

SLAMF6 is also implicated in trogocytosis, the transfer of membrane patches from target to effector T cells [119, 120]. The trogocytosis capacity of $CD8^+$ T cell clones from melanoma patients correlated with their cytotoxic capacity. Furthermore, higher cytotoxic capacity was correlated with increased phosphorylation of SLAMF6 [119]. Enhanced cytotoxicity of these $CD8^+$ T cells could be blocked by an antagonist αSLAMF6, indicating that SLAMF6 co-stimulation plays an active role in T cell functional diversity. As $CD8^+$ T cells from XLP patients are defective in cytolytic activity against EBV-infected B cell targets, SLAMF6 as well as SAP may be involved [53, 121-123]. Mice deficient in SAP presented altered T cell responses including short-lived T-B cell interactions, defective germinal center formation, and humoral immunity [2, 100, 124]. As $CD8^+$ T cells are critical in controlling infections, the role of SAP as well as contributions of SLAMFs were investigated. Studies using SAP deficient mouse T cells showed inefficient killing of B cell target cells, but not others, suggesting SAP is especially critical in T-B cell interactions [125]. These defects were at the level of immune synapse organization and inefficient actin clearance. Conjugation of OVA-pulsed B cells with WT or SAP-/- cytotoxic $CD8^+$ T lymphocytes (CTL) showed significant SHP-1 localization at the immune synapse with SAP-/- CTL compared to WT CTL as quantified from immunofluorescence microscopy images [125]. This suggested that in the absence of SAP, SHP1 is recruited to the cytoplasmic tail of SLAMF6 [126]. When B cell targets were SLAMF6 deficient, killing ability of SAP-/- T cells was rescued demonstrating that SLAMF6 is an important regulator of SAP dependent responses from CTLs. Our current understanding of how SLAMF6 may play a role in T-B cell interactions is summarized in **Figure 5.**

3.3.3 SLAMF6 controls neutrophil functions

Disruption of Ly108 expression in mice (Ly108 **Δ**E2+3) also revealed a role for Ly108 in neutrophil functions [115]. In response to infections with *Salmonella typhimurium*, neutrophils exhibited significantly reduced reactive oxygen species (ROS) production and bacterial killing with increased IL-6, IL-12 and TNF-α production compared to WT controls. Mechanisms of Ly108 signaling involved in ROS production is still not known. While neutrophils are not implicated in XLP and human neutrophils do not appear to express SLAMF6, it may be of interest to investigate whether the expression can be induced upon activation.

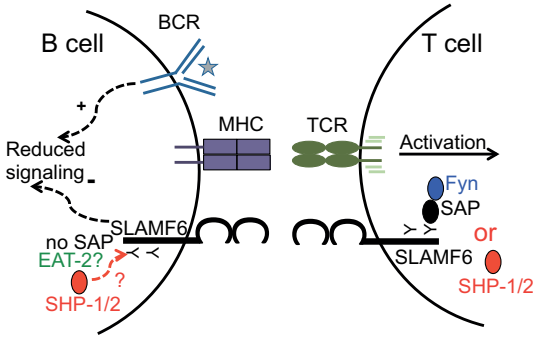

Figure 5. SLAMF6 localizes at the immune synapse. Ligation of SLAMF6 on a T cell recruits binding of SAP to the ITSM on the cytoplasmic tail. SAP recruits Fyn and induces activation. SAP has the highest affinity for cytoplasmic tail of SLAMs and blocks binding of SHP-1/2. In the absence of SAP, SHP-1/2 binds to cytoplasmic tail of SLAMF6 and induces negative signaling on T cells [126]. B cells do not express SAP. Whether EAT-2 is expressed or it blocks recruitment of SHP-1/2 is not known. However, ligation of SLAMF6 with an antibody in B cells appear to induce negative signals, which may be due to binding of SHP-1/2.

3.3.4 SLAMF6 is an activating receptor on human NK cells

Activating signals induced upon ligation of NTB-A on an NK cell induces phosphorylation of its cytoplasmic tail, which is Src kinase dependent, and leads to recruitment of SAP, EAT-2 and SHP1/2 [127, 128]. Full SLAMF6 dependent activation of NK cell cytotoxicity depends on simultaneous binding of SAP and EAT-2 to the phosphorylated tyrosine residues on the cytoplasmic tail [128]. Crosslinking of human SLAMF6 with antibodies stimulate NK cells for target cell killing in cytotoxicity assays, and homophilic interaction of ligands induces NK cell cytotoxicity against target cells with subsequent IFNγ and TNFα secretion [103, 127]. However, if the SLAMF6 homophilic interaction between an NK cell and a target cell is blocked using an anti-SLAMF6 antibody, NK cell cytotoxicity is inhibited [106]. Interestingly, blocking the homophilic interaction between neighboring NK cells has no effect on proliferation or cytotoxicity [129]. Instead, homophilic interaction with a MHC-I negative target cell induces potent cytotoxicity of NK cells. This selective mechanism is probably in place to ensure NK cells do not end up killing each other.

While expression of SLAMF receptors is restricted to hematopoietic cells, a recent study demonstrated that SLAMF6 enhanced activation of NK cells against a non-hematopoietic target cell [130]. This was regulated by SAP, which uncoupled SHP-1 binding from the cytoplasmic tail of SLAMF6, diminishing the effect of

the receptor on NK cell responsiveness to non-hematopoietic cells. This proved that SLAM-SAP pathways could also influence NK cell education [130].

3.3.5 SLAMF6 regulates NKT cell development

Natural killer T (NKT) cells represent a subset of T lymphocytes that develop from double positive (DP) $CD4^+CD8^+$ precursor cells in the thymus [131]. While positive selection of conventional T cells is mediated by interactions with thymic epithelial cells, NKT cells are selected by lipid antigens presented by CD1d on other DP thymocytes [132, 133]. These cells express an invariant TCR and can rapidly secrete cytokines following infections [134].

With the severe lack of NKT cells in XLP patients, SAP was hypothesized to be involved in NKT cell development [1]. Indeed, mouse studies showed that SAP expression in NKT cells is necessary for cognate help to B cells[135]. As binding of SAP and recruitment of Fyn is induced by signaling through SLAMF receptors, SLAMF1 and SLAMF6, which are expressed on DP thymocytes, their possible role in NKT cell development was investigated. Mouse studies from single knockouts ($SLAMF1^{-/-}$, $SLAMF6^{-/-}$) revealed only a modest effect on NKT cell development, probably due to overlapping functions of these two receptors [62, 115]. Supporting this hypothesis, mice reconstituted with double mutant ($SLAMF1^{-/-}SLAMF6^{-/-}$) bone marrow chimeras had severely impaired NKT cell development [105]. Furthermore, expression of promyelocytic zinc finger (PLZF), a transcription factor required for development of invariant NKT (iNKT) and other innate like T lymphocytes, was demonstrated to be modulated by co-stimulation through SLAMF6 [136-138]. These studies together underline the importance of SLAMF6 in NKT cell development. Other SLAMF members were also found to regulate NKT cell development in a SAP dependent and independent manner [139].

It has also been determined that SLAMF6 acts as a SAP-dependent on and off switch for stable T-B cell interactions [126]. In the absence of SAP, SLAMF6 recruited the negative regulator SHP-1 at the T-B cell synapse limiting adhesion. Interestingly, when both SAP and SLAMF6 were removed ($SAP^{-/-}SLAMF6^{-/-}$), both $CD4^+$ T cell function as well as NKT cell differentiation was restored. Therefore, SLAMF6 also serves an important role in sending positive and negative signals depending on the competition between SAP and SHP-1 binding, which in turn regulates T cell help and NKT cell development.

3.3.6 SLAMF6 as a susceptibility gene for Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that mainly affects females and is characterized by the production of autoantibodies against self antigens [140]. Generation of these antibodies results in clinical manifestations including arthritis, kidney damage, skin disease and blood cell abnormalities. Both genetic and environmental factors are known to contribute to disease manifestation [140, 141].

Analysis of the SLAM/CD2 gene cluster revealed extensive polymorphisms, and among 35 inbred mouse strains two stable haplotypes were identified [97, 142]. Haplotype 1 is found in C57BL/6J (B6) and related strains while haplotype 2 is found in autoimmune-prone mouse strains, i.e. NZW[97]. It appeared that autoimmunity was induced only when the haplotype 2 SLAM locus was expressed in B6 background [97]. Sle1 was identified on murine chromosome 1 as a cause of loss of tolerance and autoantibody production. Fine mapping of the *Sle1* locus identified 4 loci contributing to the disease manifestation, one of which was the most potent: *Sle1b* [97]. The congenic B6.Sle1b mouse strain presented with spontaneous autoimmunity and production of antinuclear antibodies (ANAs), indicating a failure to maintain central and peripheral tolerance [97, 143, 144]. Among the SLAMF genes, SLAMF6 was the strongest candidate for lupus due to the expression of alternatively spliced variants: Ly108-1 and Ly108-2. Of the two, Ly108-1 had a greater tendency to be phosphorylated at the cytoplasmic tail for SAP, Fyn recruitment and signaling than Ly108-2 isoform [145]. Kumar et al. and colleagues demonstrated that the Ly108-1 isoform was more abundant in B6.Sle1b mice compared to B6 mice, which was found to sensitize immature B cells to deletion and RAG re-expression [146]. This study provided the idea that SLAMF6 serves as a regulatory checkpoint for self-reactive B cells to protect from autoimmunity.

A later study identified a new isoform of Ly108 present in haplotype 1 but not haplotype 2: Ly108-H1 [98]. This isoform was found to regulate SLE in a $CD4^+$ T cell dependent manner and expression of this isoform in lupus-prone mice significantly suppressed autoimmunity. Deletion of SLAMF6 in Chr1b also disrupted the autoimmune phenotype. In addition, autoimmunity in B6.Sle1b mice correlated with expansion of an osteopontin-expressing TFH cell subset, which was suppressed when the Ly108-H1 isoform was expressed in these mice [147]. Overall, these studies pointed to the importance of alternative splicing in pathogenesis of SLE and that both B and T cell tolerance is compromised.

3.4 SLAMF2, 4, 5, 7 and 8

SLAMF2 (CD48) has multiple binding partners besides SLAMF4, including CD2, CD58 and bacterial lectin FimH [11, 148, 149]. Roles of SLAMF2 in regulating immunity and tolerance are reviewed in detail excellently elsewhere [148]. Briefly, SLAMF2 is constitutively expressed nearly on all hematopoietic cells and is upregulated upon activation on some subsets [148, 150, 151]. SLAMF2 interacts with protein tyrosine kinase Lck in T cells and its crosslinking induces TCR activation and IL-2 production [152, 153]. SLAMF2 interaction *in cis* with CD2 also facilitates TCR signaling by bringing Lck and linker for activation of T cells (LAT) to the TCR/CD3 complex. Furthermore, SLAMF2/CD2 interactions contribute to APC-T cell adhesion and synapse organization as well as regulate effector functions of $CD8^+$ T cells, highlighting that SLAMF2 is important in immune cell responses at different levels [154, 155]. However, SLAMF4 (2B4) also can contribute to these various responses by being the ligand for SLAMF2. Originally identified as an activating NK cell receptor, SLAMF4 is also expressed on $CD8^+$ T cells, $\gamma\delta^+$T cells and monocytes [156, 157]. Crosslinking with monoclonal antibodies against SLAMF4 and interactions of SLAMF4 on NK cells and SLAMF2 on target cells induces NK cell mediated cytotoxicity [156]. This perforin-mediated killing by NK cells is governed by phosphorylation of SLAMF4 by associating with LAT in the lipid rafts and subsequent recruitment of SAP to the immune synapse [158, 159]. However, in the absence of SAP, phosphatases SHP-1/2 bind to the phosphorylated tyrosine residues inducing inhibitory signals on NK cell cytotoxicity [160]. This helps explain why NK cell cytotoxicity is impaired in XLP patients. This duality of SLAMF4 signaling in NK cell functions is reviewed in detail elsewhere [161].

SLAMF5 is expressed on T cells, B cells, monocytes, DCs and platelets [61, 63, 86, 162-164]. High and low expression of SLAMF5 on B cells dictates their subset. Human memory B cells express high SLAMF5 ($CD84^{hi}$) along with the memory marker CD27, somatically mutated Ig variable genes, and exhibit increased proliferation compared to other $CD84^{lo}$ subsets[165]. High expression of SLAMF5 in memory B cells may be the consequence of signals induced upon binding of SAP and EAT-2 to the cytoplasmic tail of SLAMF5.

Activation of T cells is also dependent on SLAMF5, similar to that observed in other SLAMF receptors. Crosslinking of the TCR and ligation of SLAMF5 induces T cell activation and IFNγ secretion [163]. Activation of SLAMF5 leads to phosphorylation of its cytoplasmic tail by Lck, which recruits SAP [166]. Mouse studies involving GC – T_{FH} interactions revealed that SLAMF5 interaction is im-

portant for prolonged cell-cell contact and optimal T_{FH} and GC formation [104], stressing the relevance and importance of this receptor at the immune synapses and regulating immune cell functions.

In addition to its role in regulating T and B cells, SLAMF5 is shown to modulate downstream signaling of TLR4 upon LPS stimulation in macrophages and regulate effector function and cell fate decisions [167].

SLAMF7 is expressed on the surface of NK cells, NKT cells, B cells, DCs, macrophages and activated T cells in mice, while in humans its expression is induced after maturation of DCs [168, 169]. SLAMF7 binds EAT-2, but not SAP [169-171], and two isoforms of SLAMF7 have been identified: CS-1 long (CS1-L) and CS-1 short (CS1-S) [172]. While CS1-S does not bind EAT-2, CS1-L binds EAT-2 and induces NK cell mediated cytotoxicity. Presence or absence of EAT-2 dictates whether SLAMF7 induces positive or negative signals on NK cell activation [169, 171]. As EAT-2 is not expressed in T cells, signaling through SLAMF7 induces inhibitory signals in T cell responses [169]. SLAMF7 also acts as a negative regulator of proinflammatory responses in activated human monocytes [173]. Additionally, expression and stimulation of SLAMF7 isoform CS1-L on B cells induces B cell proliferation and autocrine signaling [174]. SLAMF7 expression on multiple myeloma cells has emerged as a therapeutic target, which will be discussed later.

SLAMF8 (CD353, BLAME) is expressed on myeloid cells, including monocytes, macrophages, DCs and neutrophils upon activation with bacteria or IFNγ [175, 176]. Studies using SLAMF8 deficient mice revealed that SLAMF8 may regulate protein kinase C (PKC) activity in macrophages, which phosphorylates p40phox leading to enhanced NADPH Oxidase (NOX2) responses upon exposure to bacteria [176]. In addition, migration of macrophages and neutrophils are accelerated in SLAMF8-/- mice and inhibition of NOX2 responses by diphenyleneiodonium (DPI) inhibited this migration [177]. These studies suggest SLAMF8 regulates inflammatory responses of myeloid cells in a NOX2 dependent manner.

While SLAMF8 signaling is SAP independent, de Calisto et al. demonstrated the lack of SLAMF8 led to the expansion of innate $CD8^+$ T cells that expressed the transcription factor Eomes and produced IFNγ upon stimulation [139]. These mice also had increased proportion of $PLZF^{hi}$ NKT cells in the thymus.

In conclusion, different SLAMF receptors play distinct roles in regulating immune responses and lymphocyte development.

4 SLAMF receptors in Hematological Malignancies

Hematologic malignancies are cancers that affect the blood, lymphatic system and the bone marrow. Malignant cells arise from blood cells of common lymphoid and myeloid progenitor origin and are categorized under three main subsets: leukemias (acute and chronic), lymphomas and myelomas [178]. Chromosomal translocations are a common cause of these diseases unlike solid tumors, and thus are commonly used as diagnostic factors.

As SLAMF members are expressed on a variety of normal immune cells that form the tumor microenvironment as well as cells that become malignant, it is plausible to hypothesize that SLAMFs may play a role in all these complex interactions. In fact, in recent years many investigators have demonstrated relevant roles for SLAMFs, specifically SLAMF6 in chronic lymphocytic leukemia and SLAMF7 in multiple myeloma (MM), both of which will be discussed in further detail.

4.1 The Pathogenesis of Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common leukemia in western countries accounting for 40% of all leukemias. It is a malignancy of mature $CD5^+$ B cells that accumulate in the blood, bone marrow and secondary lymphoid organs [179]. Phenotypically they are similar to antigen-experienced B cells expressing CD19, CD5, CD23, CD25, CD69 and CD71, and the memory B cell marker CD27 [180-182]. CLL is broadly divided into 2 subsets depending on the immunoglobulin (Ig) heavy chain mutation status (IGHV). CLL B cells with unmutated IGHV (U-CLL) derive from mature $CD5^+$ B cells, whereas CLL cells with mutated IGHV (M-CLL) derive from $CD5^+CD27^+$ post-germinal center B cell subsets [183].

CLL patients can have varying clinical outcomes depending on the aggressiveness of the disease. While some patients are stable and are only observed over time without any need for treatment, some experience more aggressive disease and require immediate treatment. Multiple factors play role in determining the course of the disease. Mutational status of CLL B cells is an important prognostic factor and patients with U-CLL show a more aggressive disease and shorter survival time compared to M-CLL patients [184, 185]. Chromosomal alterations are another parameter for disease outcome. Patients with 13q deletions that include

the miRNAs miR15a and miR16-1 are usually associated with favorable disease outcome [186]. Mouse models with deletion of the miR15a and miR16-1 locus mimic many features of human CLL [187]. Chromosomal deletion of 17p and 11q harbor the p53 and ataxia telangiectasia mutated (ATM) genes and are associated with poor disease outcome [186, 188]. Trisomy 12 is found in ~ 15% of CLL patients and signifies an intermediate prognosis. In addition to chromosomal aberrations, high expression of CD38 and ZAP70 in CLL B cells is associated with a poor outcome and shorter time to treatment [184, 189, 190]. Besides these clinical markers that are associated with disease outcome of CLL patient, studies using the application of whole exome sequencing have identified recurrent somatic mutations that are involved in DNA damage, mRNA processing, WNT and Notch signaling and chromatin modifications that can affect B cell signal transductions [191-194].

4.1.1 CLL Microenvironment

Cells of the immune system coevolve with the tumor and provide the tumor a friendly microenvironment for survival of CLL cells. Culturing CLL B cells *in vitro* after isolation from peripheral blood mononuclear cells (PBMCs) leads to the induction of spontaneous apoptosis indicating that CLL B cells are highly dependent on the microenvironment for survival signals [195, 196]. Only when cultured with bone marrow stromal cells, CLL B cells survived *in vitro*. This suggests that the tumor microenvironment *in vivo* has a profound effect on survival and expansion of CLL leukemic cells [179]. Some of the key interactions between CLL B cells and the tumor microenvironment are highlighted in **Figure 6**.

One of the CLL supporting cells is the 'nurse like cells' (NLCs), which are of monocyte origin [197]. These cells were found to differentiate *in vitro* from PBMCs of CLL patients and secrete chemokines such as CXCL12 and CXCL13 for their survival *in vitro* [197, 198]. These chemokines induce chemotaxis for migration of CLL cells in and out of secondary lymphoid organs *in vivo*. CXCL12 mediated signaling is increased by CD38 expressed on CLL cells further promoting survival [199, 200]. NLCs also activate the B cell receptor signaling and NF-κB pathway for survival [201].

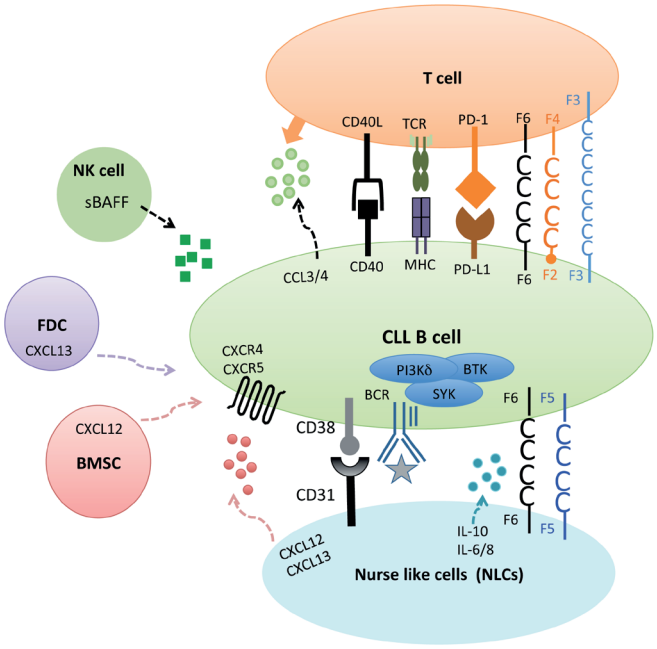

Figure 6. The CLL Microenvironment. Contact between CLL cells and nurse like cells (NLCs) is established by chemokine receptors and adhesion molecules expressed on CLL cells and ligands on NLCs. The CD38-CD31 axis promotes CLL survival. CXCR4/CXCL12 chemokine gradient allows shuffling of CLL cells between circulation and secondary lymphoid organs to receive survival and proliferation signals. SLAMF5 and SLAMF6 are expressed on the surface of NLCs (Yigit, unpublished data). The relevant contribution of these receptors to CLL survival requires further investigation. T cells are another major contributor to CLL survival. Secretion of chemokines CCL3/4 by CLL cells attracts T cells nearby. CD40/CD40L interaction promotes survival and PD-1/PD-L1 pathway favors immune evasion of CLL cells. NK cells, bone marrow stromal cells (BMSCs) and follicular dendritic cells (FDCs) also contribute to CLL survival.

The B cell receptor (BCR) signaling pathway is a key survival factor for CLL [202]. The BCR is composed of an antigen-specific surface membrane Ig (smIg) along with Igα/Igβ heterodimers. Engagement of the BCR triggers phosphorylation of Igα/Igβ and recruitment of Lyn, which in turn activates kinases SYK, BTK and PI3K. This leads to the activation of downstream signaling cascades including phospholipase C gamma 2 (PLCγ2), calcium signaling, NF-κB and mitogen-activated protein kinase (MAPK) pathways. These signaling events promote survival and proliferation of B cells [203]. IGHV mutation status of CLL cells determines the responsiveness of BCR engagement. While CLL B cells from U-CLL patients are more responsive to BCR stimulation and mostly recognize autoantigens [204, 205], M-CLL cells show constitutive phosphorylation of ERK kinase along with

reduced surface BCR leading to an "anergic" phenotype [206, 207]. In addition to activated BCR signaling, ligation of CD40/CD40L on malignant B cells and T cells also promote survival of CLL cells [208].

Another hallmark of CLL is dysfunctional T cells in the tumor microenvironment. Although there is an overall expansion in the T cell compartment, the normal CD4/CD8 ratio appears to be inverted due to differential sensitivity of $CD4^+$ and $CD8^+$ T cells to Fas/Fas Ligand induced cell death [209-213]. Within CD4 T cells, frequency of regulatory T cells (Treg) is increased further supporting leukemic expansion [214-216]. $CD4^+$ and $CD8^+$ T cells show many defects in CLL including T cell exhaustion, inability to form immune synapses and impaired cytotoxic function [217-219]. Exhausted $CD8^+$ T cell state correlates with increased expression of exhaustion markers such as programmed death-1 (PD-1), CD160, SLAMF4 and KLRG1[217, 220-222]. Several studies hypothesized one factor for exhaustion may be cytomegalovirus (CMV) infection, which influences and expands the CMV-specific $CD4^+$ and $CD8^+$ T cell subsets in healthy individuals and is also expanded in CMV-seropositive CLL patients [223-225]. However, within the exhausted T cell pool, CMV-specific T cells showed reduced expression of exhaustion markers as well as retained cytotoxic capacity and cytokine production compared to other exhausted T cells [226, 227]. These data indicated that there might be other factors influencing changes in the T cell compartment in CLL.

PD-1/PD-L1 is a major pathway contributing to the known T cell defects in CLL [228]. PD-L1 is overexpressed on CLL cells and myeloid derived suppressor cells (MDSCs), which further up-regulates PD-1 on T cells [229, 230]. Within CD4 and CD8 T cell populations, naïve T cells are reduced while effector memory $CD4^+$ T cells and terminally differentiated $CD8^+$ T cells are increased, which clinically corresponds with disease aggressiveness [228]. Up-regulated PD-1 inhibits IFNγ secretion, skewing the immune responses to a dysregulated T_H2 response [228]. These T cell defects are recapitulated in the TCL1 transgenic (Eμ-TCL1) mouse model of CLL [231-233], allowing a useful platform to study and understand the contribution of PD1/PD-L1 pathway in CLL pathogenesis and how this pathway can be targeted therapeutically.

4.1.2 Therapeutic options in CLL

Allogeneic hematopoietic stem cell transplantation (HSCT) represents one of the oldest treatments for hematological malignancies which set the foundation for the development of cancer immunotherapy [234]. Performed for the first time in 1968, high doses of radiation and chemotherapy were given to the patient that

wiped out the entire immune system followed by donor HSC transplantation for repopulation of the hematopoietic system. Extensive studies over the years provided great insight into the efficiency of the ability of donor immune cells to eliminate recipient tumor cells. This is known as 'graft versus leukemia' (GVL) effect. GVL potency was further appreciated by the finding that post- HSCT, donor lymphocyte infusions (DLI) induced remarkable responses and remissions without radiation or chemotherapy, in leukemias [235, 236].

Targeting BCR signaling using small inhibitor molecules has dramatically improved treatment options for CLL patients [202]. The BTK inhibitor, ibrutinib (PCI-32765) binds irreversibly to a cysteine residue (Cys-481) in the BTK kinase domain and inhibits its phosphorylation and enzymatic activity [237]. Ibrutinib inhibited proliferation and stromal cell contact of CLL cells and reduced their viability *in vitro* [238]. Ibrutinib also prevented tissue homing in response to chemokines CXCL12 and CXCL13 *in vitro* and in a mouse model of CLL [239]. This inhibition of tissue homing chemokines directly correlated with a transient lymphocytosis in CLL patients undergoing ibrutinib treatment, which allowed CLL cells to move from secondary lymphoid organs into the circulation and induced cell death [240]. Because ibrutinib not only binds to BTK in CLL B cells but also to ITK in T cells, its immunomodulatory role was also investigated in CLL patients [241]. In patients treated with ibrutinib, the increased T cell numbers normalized and production of inflammatory cytokines were reduced, and the T cell repertoire diversity increased [242, 243]. Ibrutinib also reduced Treg numbers [244]. Expression of PD-1 and PD-L1 upon ibrutinib treatment markedly decreased, improving activated effector T cell functions [245, 246].

While ibrutinib targeted CLL B and T cells, a second-generation highly selective BTK inhibitor, acalabrutinib, was produced [247]. Pharmacodynamics and proteomic analysis appeared to be similar on leukemic cells compared to ibrutinib, while off target effects on T cells was more pronounced using ibrutinib than acalabrutinib [248, 249].

Rituximab, a monoclonal antibody against the B cell surface antigen CD20, is widely used in the treatment of B cell malignancies including CLL [250-252]. The primary mode of action of rituximab includes ADCC and complement dependent cytotoxicity (CDC) as well as direct anti-proliferative and pro-apoptotic effects [253, 254]. While major advances were brought by rituximab, relapse and resistance to treatment are eventually seen in patients. One potential reason is the removal of bound CD20 complexes from the surface of CLL cells by trogo-

cytosis [255, 256]. Administering rituximab together with ibrutinib led to better responses, but follow up studies identified that ibrutinib interferes with the effect of rituximab by downregulating CD20 on the cell surface of CLL cells [257, 258]. More efficient combinations with rituximab may be required for better and durable effects of this immunotherapy agent.

Lenalidomide is an immunomodulatory agent that affects the tumor microenvironment and the immune system. In particular, it corrects CLL B cell - T cell immunological synapse formation and down-regulates PD-1 on T cells [218, 229, 259]. Lenalidomide normalizes total T cell and Treg numbers, similar to ibrutinib, *in* vivo [260]. Combining lenalidomide with αCD20 improved ADCC activity of NK cells *in vitro*, and this combination also demonstrated efficacy in clinical trials [261, 262]. Other small inhibitor molecules targeting BCR signaling, monoclonal antibodies and immunomodulatory drugs used in treatment of CLL patients are discussed in detail elsewhere [202, 263].

4.2 SLAMF Receptors in CLL

The importance of SLAMF receptors in regulating innate and adaptive immune responses makes them relevant candidates in context of various diseases including chronic lymphocytic leukemia. SLAMF receptors, particularly SLAMF1, SLAMF5 and SLAMF6, are expressed on both human and mouse CLL cells [264]. Two separate studies have identified surface expression of SLAMF1 with longer time to treatment and overall increased survival in CLL patient cohorts using either cluster analysis or multivariate prognostic models [265, 266]. Kaplan Meyer curves predicted longer treatment free survival rates in $SLAMF1^+$ CLL patients (6% cut-off applied) compared to $SLAMF1^-$ patients [267]. SLAMF1 surface expression appeared to be important for an intact autophagy pathway. As demonstrated in previous studies evaluating the role of SLAMF1 in recruiting NOX2/Vps34/Beclin/UVRAG complex in phagosomes [20], ligation of SLAMF1 using an agonistic antibody triggered ROS accumulation and induced formation of this macrocomplex in a CLL cell line, MEC-1. When SLAMF1 was silenced in primary CLL cells, they became resistant to autophagy inducing agents demonstrating that this may be the underlying reason for unfavorable outcome in SLAMF1 low CLL patients as they may be more resistant to various drugs targeting this pathway.

An additional study revealed a link between SLAMF1 and CD180 in modulating transcriptional program in CLL cells by regulating expression levels of some transcription factors [268]. Furthermore, an isoform of SLAMF1 that lacks the

transmembrane domain was identified and found to co-localize with CD180 on the cell surface [269]. Co-ligation of SLAMF1 and CD180 on CLL cells led to inhibition of the Akt and MAPK pathways that disrupts survival signals in CLL B cells. These studies suggest that SLAMF1 may be important as a diagnostic marker as well as an interesting therapeutic target.

The observation that SLAMF5 was overexpressed on CLL B cells compared to healthy B cells suggested that SLAMF5 might be involved in survival of CLL cells in the tumor microenvironment [270, 271]. Blocking SLAMF5 *in vitro* and *in vivo* disrupted the tumor – tumor microenvironment interactions inducing cell death of CLL cells [272]. It would be relevant to study the SLAMF5 interaction between CLL B and T cells in immune synapse formation and cytotoxicity as studies using WT mice indicated SLAMF5 is required for optimal T-B cell interaction in germinal centers [104].

SLAMF6 expression is high on normal T and B cells and studies have already indicated its importance in B-T cell signaling. Therefore it was plausible to hypothesize that monoclonal antibodies targeting SLAMF6 may be of therapeutic interest in CLL [264]. An adoptive transfer model of an aggressive TCL1 clone, TCL1-192 [273], into SCID (severe combined immune deficiency) mice, which lack T and B cells, was injected with anti-SLAMF6 upon leukemic expansion. This led to significantly reduced leukemic burden by inducing antibody dependent cellular cytotoxicity (ADCC) and reduced proximal B cell receptor signaling [264]. Interestingly, the antibody was unable induce ADCC in peritoneal cavity (PerC) of mice, due to possibly different microenvironments and signaling compared to blood or spleen [264, 274]. One such finding was the elevated reactive oxygen species (ROS) production in PerC CLL B and normal B1a B cells compared to the cells residing in spleen [274]. While inhibition of ROS limited leukemic expansion in this niche, the finding that ROS is reduced upon BTK inhibitor, ibrutinib and leukemic infiltration to the blood, prompted us to do a combination therapy. By combining anti-SLAMF6 with ibrutinib, leukemic cells were pushed out of this niche into the circulation making them targetable by the antibody. This led to an overall greater reduction in leukemic burden than either regimen alone. Exploration of SLAMF6 as a therapeutic target would be of great interest in CLL and other B cell malignancies.

4.3 Targeting SLAMF7 in Multiple Myeloma

Multiple myeloma (MM) is a malignancy of plasma cells characterized by monoclonal expansion in bone marrow and accumulation of monoclonal antibodies produced by malignant plasma cells that result in organ damage, including bone le-

sions, renal diseases and anemia [275, 276]. In the past decade, the front line therapy for MM included proteasome inhibitors (i.e. bortezomib) and immunomodulatory agents that resulted in overall survival of 9 months emphasizing a need for additional treatment options [277, 278]. Use of monoclonal antibodies in treatment of MM opened a new era in treatment for this disease [279-281]. One of these antibodies (elotuzumab) targets SLAMF7, which is highly expressed on MM cells [168, 282]. In plasma cells and MM cells, EAT-2 is normally not expressed. However, in approximately ~30-50% of MM patients, amplification of the long arm of chromosome 1q leads to expression of EAT-2, which is actually associated with poor prognosis and unresponsiveness to therapy [283, 284]. In addition, SLAMF7 is implicated to have a tumor-supporting effect in MM, which is further induced upon EAT-2 expression. When SLAMF7 expression was inhibited on MM cells, adhesion to bone marrow stromal cells was reduced [282]. A similar reduction was observed when elotuzumab was used making it an ideal candidate for use in clinic with MM patients.

Several mechanisms of action have been identified for elotuzumab. Primarily the antibody induces ADCC [168], activates NK cell cytotoxicity by binding to SLAMF7 on NK cells [285], and inhibits tumor cell stromal cell interaction [282]. While elotuzumab alone had no clinical effect in advanced MM patients, combination with proteasome inhibitors or immunomodulatory drugs improved progression free survival rates [286, 287]. Blocking PD-1 further increased efficacy of elotuzumab and enhanced NK and CD8 T cell activity in mouse models of MM [288]. In addition, a SLAMF7 specific peptide inducing antigen-specific cytotoxic T cells was identified, which may be used as a novel immunotherapy for MM [289]. A recent study demonstrated the effectiveness of SLAMF7-CAR-T cells to eliminate relapsed/refractory MM cells [290] . Overall, all these approaches underline the importance of targeting SLAMF7 in MM.

5 Conclusions

SLAMF receptors and their adaptors play vital roles in maintaining a balanced immune response and their interrupted functions are associated with various diseases. Ability of SLAMF receptors to play activating and inhibitory roles depending on the signals they receive or the cell-cell interactions they encounter makes them an important target when thinking about designing therapeutics. Monoclonal antibodies that can block or engage SLAMF-SLAMF interactions are being targeted in diseases such as CLL and MM. Further understanding the fundamentals on how SLAMF members play a role in the bigger picture will make them one step closer to being targets in many more diseases.

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CHAPTER 2

A combination of an anti-SLAMF6 antibody and ibrutinib efficiently abrogates expansion of chronic lymphocytic leukemia cells

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ABSTRACT

The signaling lymphocyte activation molecule family [SLAMF] of cell surface receptors partakes in both the development of several immunocyte lineages and innate and adaptive immune responses in humans and mice. For instance, the homophilic molecule SLAMF6 (CD352) is in part involved in natural killer T cell development, but also modulates T follicular helper cell and germinal B cell interactions. Here we report that upon transplantation of a well-defined aggressive murine B220^{+}CD5^{+} Chronic Lymphocytic Leukemia (CLL) cell clone, TCL1-192, into SCID mice one injection of a monoclonal antibody directed against SLAMF6 (αSlamf6) abrogates tumor progression in the spleen, bone marrow and blood. Similarly, progression of a murine B cell lymphoma, LMP2A/λMyc, was also eliminated by αSlamf6. But, surprisingly, αSLAMF6 neither eliminated TCL1-192 nor LMP2A/λMyc cells, which resided in the peritoneal cavity or omentum. This appeared to be dependent upon the tumor environment, which affected the frequency of sub-populations of the TCL1-192 clone or the inability of peritoneal macrophages to induce Antibody Dependent Cellular Cytotoxicity (ADCC). However, co-administering αSlamf6 with the Bruton tyrosine kinase (Btk) inhibitor, ibrutinib, synergized to efficiently eliminate the tumor cells in the spleen, bone marrow, liver and the peritoneal cavity. Because an anti-human SLAMF6 mAb efficiently killed human CLL cells *in vitro* and *in vivo*, we propose that a combination of αSlamf6 with ibrutinib should be considered as a novel therapeutic approach for CLL and other B cell tumors.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common B cell leukemia in adults and is characterized by the accumulation of $CD19^+CD5^+CD23^+$ B cells in the bone marrow, blood and secondary lymphoid organs [1]. Survival and proliferation of these tumors depend on two main factors: the tumor microenvironment and B cell receptor (BCR) signaling [2, 3]. Within CLL "proliferation centers", tumor cells interact with monocyte derived nurse-like cells (NLCs) [2–6], which provide survival signals via secretion of chemokines and cytokines [7, 8], or interacting cell surface receptor-ligand structures. The latter include CD74/MIF, CD84 (SLAMF5) and CD150 (SLAMF1) [9–11].

Here we evaluate the efficacy of monoclonal antibodies directed against mouse and human SLAMF6 (CD352) in CLL prognosis. SLAMF6 [12] is a homophilic SLAMF receptor [13–22], which plays a key role in the interactions between T follicular helper (TFH) cells and Germinal Center B (GCB) cells [19, 23–32]. To study the effect of anti-SLAMF6 on tumor progression we use the aggressive transplantable murine CLL clone TCL1-192 and the B cell lymphoma LMP2A/λMyc [33–35] into SCID or $Rag1^{-/-}$ mice, respectively. The murine TCL1-192 clone expresses a B cell receptor with a single IGHV-D-J arrangement, which is specific for phosphatidyl choline [PtC], has many characteristics in common with human CLL cells [33]. As the transfer of TCL1-192 into SCID mice leads to an aggressive disease progression within 5-6 weeks, this offers a very useful platform to identify relevant potential therapeutic targets [33].

The data indicate that removal of the tumor cells by a mouse anti- mouse Slamf6 (αSlamf6) antibody (13G3) [26], see Materials and Methods) relies on antibody dependent cell-mediated cytotoxicity (ADCC) and co-stimulation of B cell receptor (BCR) signaling, which is of importance to progression of CLL. Co-administering αSlamf6 with the Bruton tyrosine kinase (Btk) inhibitor ibrutinib has a synergistic effect on treatment of the tumors. Furthermore, we provide evidence that mouse anti-human SLAMF6 (αhSLAMF6) antibody is efficient in *in vitro* and *in vivo* killing of two CLL cell lines MEC-1 and OSU-CLL [36, 37].

RESULTS

Administering αSlamf6 prevents expansion of TCL1-192 cells in the spleen and blood, but not in the peritoneal cavity

We first determined that surface expression of SLAMF receptors by TCL1-192 cells [33] is comparable to SLAMF surface expression by patient-derived human CLL cells and the CLL cell lines MEC1 and OSU-CLL (Supplementary Figure S1 and S2). Consistent with its high level of expression by B lineage cells [38], this SLAMF6 is found on the surface of freshly isolated human CLL cells (Supplementary Figure S1C) or frozen patient cells (Supplementary Figure S2). Whereas SLAMF6 expression varies somewhat between CLL cells from different patients, SLAMF1 and SLAMF7 expression differs more between individual patients (Supplementary Figure S2). Similar to its relative expression by mouse B cells, (www.immgen.org) [26], Slamf6 is highly expressed on the surface of TCL1-192 cells. Surprisingly, the level of expression of Slamf6 on the surface of TCL1-192 cells in the peritoneal cavity was twice that on cells isolated from the blood or spleen (MFI P: 23739, B: 13279, S: 14384) (Supplementary Figure S1).

To assess the efficacy of αSlamf6 in preventing expansion of the mouse CLL cells, αSlamf6 IgG2a was administered on day 7, 14 and 21 post-transplant of the TCL1-192 cells into SCID mice (Figure 1A). Prior to these experiments we had determined that one week after injecting 0.5×10^6 TCL1-192 cells *i.p.* into a SCID mouse, the cells primarily reside in the peritoneal cavity, but that at day 28, the tumor cells have expanded and are found in the peritoneal cavity [$\sim 1 \times 10^8$], spleen [$\sim 4 \times 10^8$], and blood [$\sim 10^5$/μl] (data not shown). Importantly, in a previous study a similar distribution of TCL1-192 cells was found regardless of whether the tumor cells were injected *i.v.* or *i.p.* [33].

At day 28 the spleen size of αSlamf6-treated mice was 20% of the spleen size of recipients of isotype-control mice or of mice that had not received antibody (Figure 1B). More importantly, the number of leukemic cells in the spleen of recipients of αSlamf6 injected mice was 26 fold reduced (Figure 1C). TCL1-192 cells were virtually absent in the blood of αSlamf6-injected mice compared to the control mice (Figure 1D). Surprisingly, αSlamf6 did not affect the number of tumor cells in the peritoneal cavity (Figure 1E) or in the omentum, a well-known reservoir for B1a cells [39] (Figure 1F). On day 28 expression of Slamf6 by the leukemic cells in the peritoneal cavity, blood and spleen from all groups was comparable (Supplementary Figure S3A).

Together the data show that, three injections of αSlamf6 eliminated TCL1-192 cells in the spleen and blood of the recipient mice, but not in the peritoneal cavity.

Administering αSlamf6 reduced the number of LMP2A/λMyc B cell lymphomas in Rag-$1^{-/-}$ mice

To evaluate whether αSlamf6 would also effectively remove an unrelated $CD19^+B220^+$ murine B cell lymphoma, LMP2A/λMyc [35], which expresses Slamf6 (Figure 2A), on day 7 and 14 after *i.p* injection of LMP2A/λMyc [1×10^6 cells/mouse] into *Rag1*$^{-/-}$ mice, 200μg/mouse αSlamf6 or isotype control was administered (Figure 2B). On day 19 post-transplant αSlamf6 treated mice had significantly smaller spleens (Figure 2C) and less tumor cells than did control mice (Figure 2C and 2D). Thus, like in the case of the TCL1-192 CLL cells, αSlamf6 also reduces the number LMP2A/λMyc lymphoma cells.

Treatment with αSlamf6 after expansion of TCL1-192 cells in SCID mice

Next, we employed a "treatment protocol" to assess whether αSlamf6 would affect survival of SCID mice in which TCL1-192 cells had expanded for three weeks after transplant and antibodies were subsequently administered *i.p.* once a week (Figure 3A). While after 6 injections of αSlamf6 recipient mice were alive at day 60, the mice that had received control mIgG2a died between 35-42 days (Figure 3A).

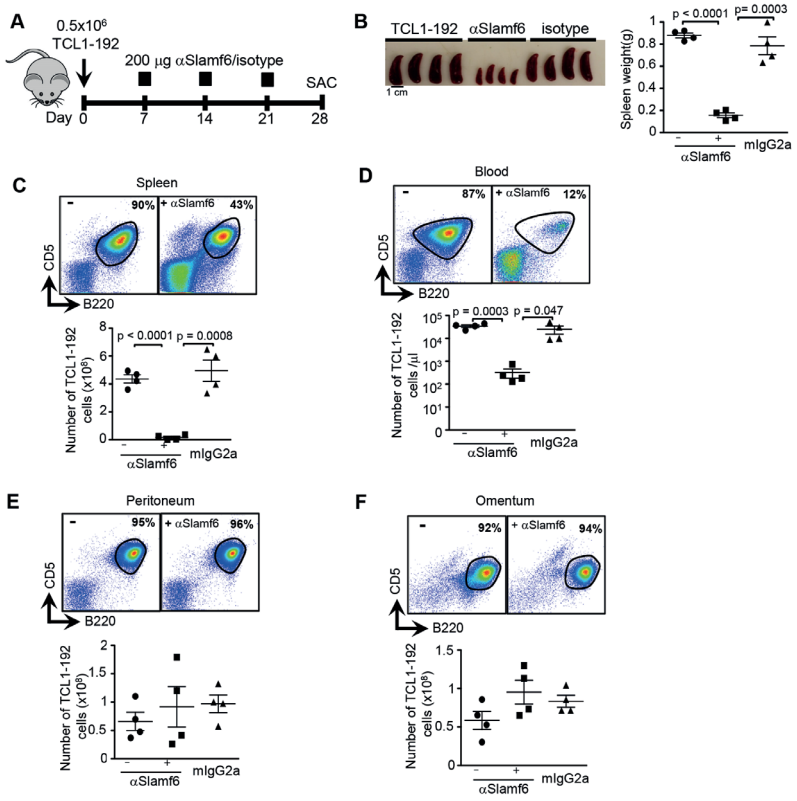

Figure 1: Anti-Slamf6 prevents TCL1-192 expansion in the spleen and blood, but not in the peritoneal cavity, of SCID mice. A. Schematic outline of the prevention experiment. TCL1-192 cells were injected on d0 and 200µg mouse αSlamf6 (13G3) or a mouse IgG2a isotype control was injected *i.p.* into SCID mice on day 7, 14 and 21. Mice were sacrificed on day 28. **B.** Spleen size and weight at day 28. Administering αSlamf6 vs IgG2a isotype caused a 5.0- fold reduction (0.15 ± 0.02 vs. 0.78 ± 0.08 g; $p = 0.0003$) or 5.8-fold reductions comparing αSlamf6 *vs.* no antibody (0.15 ± 0.02 vs. 0.87 ± 0.02 g; $p < 0.0001$). **C.** A 26-fold reduction of the number of TCL1-192 cells was detected in the spleen of αSlamf6-injected vs. non-injected ($1.7 \pm 0.8 \times 10^7$ vs. $4.4 \pm 0.3 \times 10^8$ $p < 0.0001$); or 29-fold in αSlamf6-injected vs. isotype-injected ($1.7 \pm 0.8 \times 10^7$ vs. $4.9 \pm 0.7 \times 10^8$; $p = 0.0008$) SCID mice. Cells were gated on viable, $DAPI^-$, $B220^+CD5^+$ cells. Counting beads were used to determine the number of TCL1-192 cells per µl of blood. **D.** A 113-fold reduction of TCL1-192 cells in the blood of αSlamf6-injected vs. non-injected mice was found ($0.3 \pm 0.1 \times 10^3$ *vs.* $3.4 \pm 0.4 \times 10^4$ per µl blood; $p = 0.0003$); 100-fold in αSlamf6-injected vs. isotype-injected mice ($0.3 \pm 0.1 \times 10^3$ *vs.* $3 \pm 1.1 \times 10^4$ per µl blood; $p = 0.047$). **E.** Number of TCL1-192 cells in the peritoneal cavity: αSlamf6-injected vs. non-injected ($9.38 \pm 3.6 \times 10^6$ *vs.* $5.8 \pm 2.3 \times 10^6$) or αSlamf6-injected vs. isotype-injected ($9.38 \pm 3.6 \times 10^6$*vs.* $1 \pm 0.1 \times 10^7$). **F.** Number of TCL1-192 cells in the omentum: αSlamf6-injected vs. non-injected ($9.5 \pm 1.55 \times 10^6$ *vs.* $5.9 \pm 1.2 \times 10^6$ or αSlamf6-injected *vs.* isotype-injected ($9.5 \pm 1.55 \times 10^6$ *vs.* $8.3 \pm 0.7 \times 10^6$). Results are representative of at least 3 independent experiments.

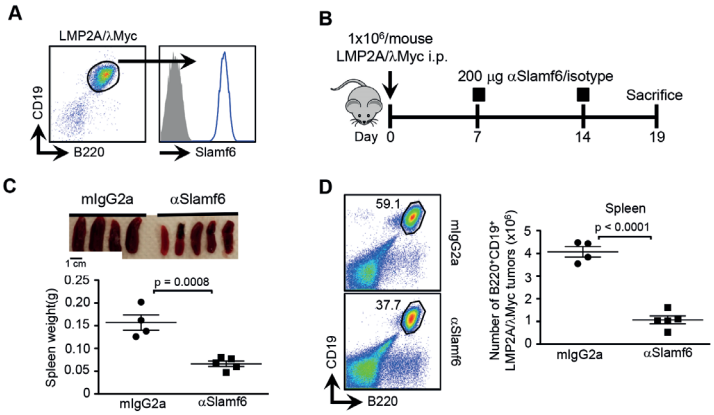

Figure 2: Anti-Slamf6 reduces tumor burden in LMP2A/λMyc bearing Rag1$^{-/-}$ mice. A. Expression of Slamf6 on CD19^{+}CD20^{+} LMP2A/lMyc cells. **B.** Schematic outline of the experiment. Rag1$^{-/-}$ mice were i.p. injected with 1 × 10^{6} cells and injected *i.p.* with 200 μg αSlamf6 or isotype control on day 7 and 14. Mice were sacrificed on day 19. **C.** Differences in spleen size and weight on day 19. A 3-fold reduction was observed in spleen weight of mice injected with αSlamf6 compared to isotype-injected group (0.066 ± 0.005 vs. 0.15 ± 0.01; p = 0.0008). **D.** Percentage of CD19^{+}CD20^{-} LMP2A/λMyc tumors and absolute cell numbers in the spleen are shown. A 4-fold reduction in tumor burden was observed in αSlamf6-injected group (1.05 ± 0.17 × 10^{6} vs. 4.07 ± 0.22 × 10^{6}; p < 0.0001). Representative of 2 independent experiments.

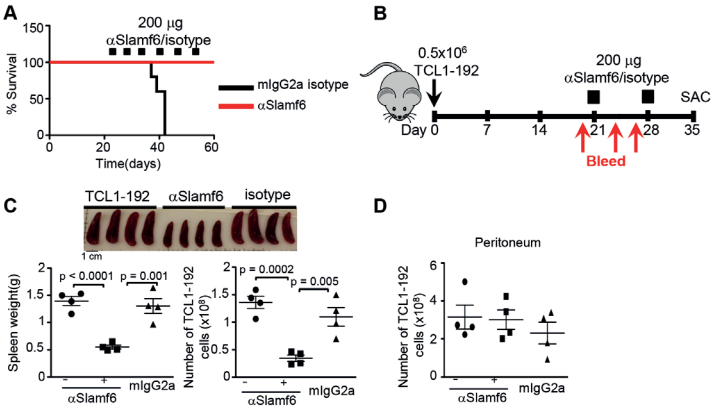

Figure 3: Treatment with αSlamf6 of TCL1-192 in the spleen, but not in the peritoneal cavity of SCID mice. A. Three weeks after transplanting TCL1-192 cells, SCID mice were injected with αSlamf6 or isotype mIgG2a once a week (Squares indicate the number of injections). Mice were monitored everyday. Whereas isotype treated mice died between 35-42 days, αSlamf6 injected mice were still alive on day 60, when the experiment ended (n = 5 mice each group). **B.** Schematic outline of the experiment indicating the timeline of i.p. injection of TCL1-192 cells and *i.p.* injections of 200 μg αSlamf6 (13G3) or the IgG2a isotype control on day 21 and 28. Mice were monitored by bleeding on days 21, 24 and 27 and were sacrificed on day 35. **C.** A 2.5-fold reduction in spleen size was caused in αSlamf6 vs. isotype treated mice (0.55 ± 0.03 vs. 1.4 ± 0.08g; *p = 0.0018)* or 2.4-fold vs. untreated mice (1.303 ± 0.13 g; *p < 0.0001*) on day 35. The number of TCL1-192 cells was 3-fold reduced in αSlamf6 vs. isotype treated mice (3.4 ± 0.6 × 10^{8} vs. 1.1 ± 0.1 × 10^{9}; *p = 0.005*) or 4-fold vs. untreated mice (3.4 ± 0.6 × 10^{8} vs. 1.36 ± 0.1 × 10^{9} *p = 0.0002*). **D.** The number of TCL1-192 cells in the peritoneum of αSlamf6 injected and isotype or non-injected SCID mice (3 ± 0.5 × 10^{8} vs. 2.3 ± 0.5 × 10^{8} and 3.1 ± 0.6 × 10^{8} *p = 0.8* and *p = 0.4*). The data are representative of 3 independent experiments. Results are representative of 4 independent experiments.

In order to analyze the difference in leukemic burden between αSlamf6 and mIgG2a injected mice, αSlamf6 was administered only twice, *i.e.* on day 21 and day 28 after transplanting the TCL1-192 cells (outlined in Figure 3B). Mice were sacrificed on day 35 when the control group was moribund. On day 35, the spleen size of αSlamf6 treated mice was reduced 2.5 fold (Figure 3C) and the massive tumor infiltrate in the liver was absent in the αSlamf6 treated group compared to the two control groups (Supplementary Figure S4A). The total number of TCL1-192 cells in the spleen of αSlamf6 treated mice was significantly less than that of the control mice (Figure 3C). As in the prevention experiments, the leukemic cells from the peritoneal cavity were not affected by the monoclonal antibody (Figure 3D). The outcomes of these experiments indicate that treatment of existing tumors with two injections of αSlamf6 effectively eliminates TCL1-192 cells in the spleen, but not in the peritoneum.

Anti-Slamf6 eliminates TCL1-192 cells in the blood of transplanted SCID mice

One week after the first injection of αSlamf6, *i.e.* on day 28, the number of $B220^{+}CD5^{+}$ TCL1-192 cells in the blood (Figure 4A Right panel and 4B) was lower than in control mice. However, at day 35, a week after the second injection, the number of TCL1-192 cells in the blood had increased in αSlamf6-injected mice, although it remained significantly lower than in the control mice (Figure 4A–4C). This raises the possibility that on day 28 before the second αSlamf6 injection, αSlamf6-IgG from the first injection still occupied the receptor, which rendered the second injection of αSlamf6 ineffective (Figure 3B). This is indeed the case, because the Slamf6 receptor on the surface of the blood TCL1-192 cells was not accessible by a PE-conjugated αSlamf6 on 3 days after the first antibody injection [on day 24] and was only partially accessible on day 27, six days after the first injection (Supplementary Figure S3B). This is consistent with the half-life of mouse IgG2a of 6-8 days [40]. The data indicate that only the first injection of αSlamf6 was effective.

Distinct responses to αSlamf6 by the $B220^{hi}CD5^{hi}$ and $B220^{int}CD5^{int}$ TCL1-192 cell subsets

The dependence of the αSlamf6 antibody treatment on the location of the tumor cells prompted us to compare the key properties of the TCL1-192 cells in peritoneum, blood and spleen. Thirty-five days after transplant TCL1-192 cells in the peritoneum and spleen consisted primarily of a $B220^{hi}CD5^{hi}$ sub-population, whereas both $B220^{int}CD5^{int}$ and $B220^{hi}CD5^{hi}$ cells were found in the blood (Figure 4A Left Panel). As the $B220^{int}$ $CD5^{int}$ TCL1-192 cells are absent from the blood of αSlamf6 treated mice, this sub-population appears to have been eliminated by the antibody. By contrast, the $B220^{hi}CD5^{hi}$ TCL1-192 subpopulation appears resistant to the αSlamf6 treatment (Figure 4A and 4D).

These results prompted us to compare the state of activation, viability and signaling of the subpopulations in the blood of the treated and non-treated mice. Approximately half of the $B220^{hi}CD5^{hi}$ TCL1-192 subpopulation appears to be early apoptotic, as judged by Annexin-V/7-AAD staining (Figure 5A and Supplementary Figure S5A). By contrast, the $B220^{int}CD5^{int}$ population, which is eliminated by αSlamf6, is Annexin-V/7-AAD negative (Figure 5A).

The resistance of the $B220^{hi}CD5^{hi}$ TCL1-192 sub-population in the blood to αSlamf6 treatment also coincides with high expression of the BCR (Figure 5B). BCR proximal signaling, as judged by pSyk, pBtk and pPLCγ2, is also higher in the $B220^{hi}CD5^{hi}$ population than in the $B220^{int}CD5^{int}$ population of TCL1-192 cells (Figure 5C). Expression of the chemokine receptor CXCR4 was significantly higher in the $B220^{hi}CD5^{hi}$ subset than in the $B220^{int}CD5^{int}$ subset (Figure 5B, Right Panel). Overall, the $B220^{hi}CD5^{hi}$ population presents a mixture of cells, while some are undergoing apoptosis, remaining cells are on their way to migrating to secondary lymphoid organs, as suggested by their high BCR and CXCR4 expression levels [41].

The levels of pSyk, pBtk and pPLCγ2 were significantly lower in splenic TCL1-192 cells of αSlamf6 treated mice compared to those in non-injected SCID mice (Figure 5D–5F and Supplementary Figure S5B). Interestingly, BCR signaling by peritoneal cavity TCL1-192 cells differed from signaling by splenic tumor cells. Together, these findings suggest that the αSlamf6 antibody impacts the leukemic cells differently; most likely depending on the microenvironment in which the cells reside.

Administering αSlamf6 down-regulates proximal BCR signaling and induces ADCC

It is likely that the removal of leukemic cells in the blood involves αSlamf6-induced signaling in the TCL1-192 cells, as well as cell mediated cytotoxicity (ADCC) by macrophages or NK cells [42]. To further assess the possibility that administering αSlamf6 instigates co-stimulatory signaling networks in TCL1-192 cells, six doses of 200 μg/mouse of $F(ab')_2$ αSlamf6 were injected (outlined in Figure 6A). Although the leukemic burden was not affected (Figure 6B–6E), probably due to the short half life of the $F(ab')_2$ fragments *in vivo*, BCR signaling in spleen and peritoneum was observed (Figure 6F–6H). In sum, the BCR signaling of TCL1-192 cells, which is induced by endogenous PtC [33], is affected by treatment of the CLL bearing SCID mice with αSlamf6.It would therefore appear that αSlamf6 regulates both BCR signaling through the Slamf6 receptor and efficiently induces ADCC.

Absence of CLL cells in the peritoneal cavity after co-administering the BTK inhibitor ibrutinib

Although αSlamf6 antibody by itself is very effective in targeting the leukemic cells in blood, spleen and liver, it did not affect cells that reside in niches, e.g. the peritoneal cavity and omentum. Because macrophages and monocytes of the mouse peritoneal cavity are often found in lymphoid aggregates with T cells and $CD5^{+}B220^{+}$ B1 cells, which are almost absent in lymphoid tissues, we reasoned that the CLL cells resided in niches, which prevent efficient killing by αSlamf6 or other monoclonal antibodies [42]. Because in CLL patients the BTK inhibitor, ibrutinib, is known to release the tumor cells from their niches into the blood [43], we combined αSlamf6 treatment with administering ibrutinib.

Using the treatment protocol (Figure 3B), SCID mice injected with TCL1-192 were given 200 μg/mouse αSlamf6 and 25 mg/kg/day ibrutinib in the drinking water on day 21 (Figure 7A). Ibrutinib was kept in the drinking water until sacrifice and αSlamf6 was injected again on day 28. The number of leukemic cells in the blood was determined on day 20 and 27 (Figure 7A).

The spleen size and number of TCL1-192 cells was reduced 2-fold in αSlamf6 / ibrutinib-treated mice as compared to the number of splenocytes in animals that had been treated with αSlamf6 alone (Figure 7B and 7C). As predicted, ibrutinib alone or αSlamf6 / ibrutinib efficiently eliminated TCL1-192 cells in the peritoneal cavity (Figure 7D).

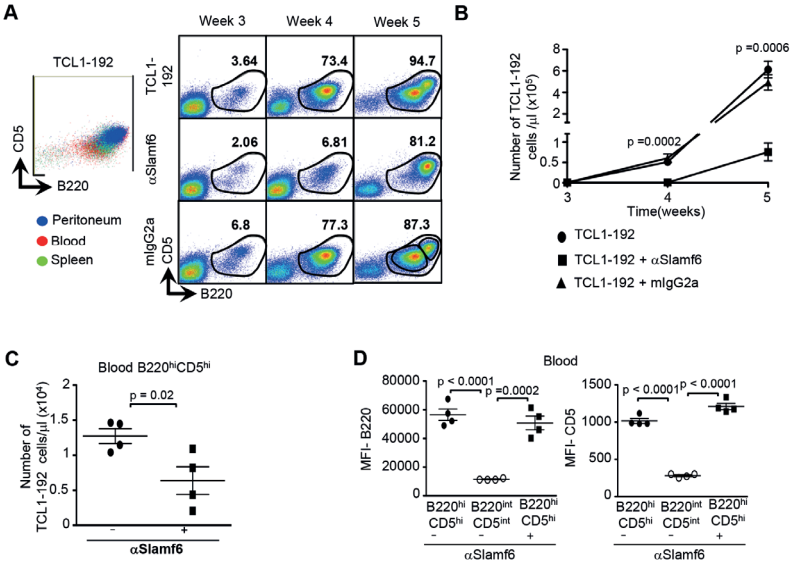

Figure 4: The number of TCL1-192 cells is reduced in the blood of SCID mice upon treatment with αSlamf6. Schematic outline of the experiment is described in Figure 3B. **A. Left Panel:** Representative flow cytometry plot showing the overlay of $B220^{+}CD5^{+}$ cells from the peritoneum, blood and spleen. While TCL1-192 cells from the peritoneum, spleen and blood express comparable levels of high B220 and CD5; the blood contains a second population that expresses lower levels of B220 and CD5 (named $B220^{int}CD5^{int}$). **Right Panel:** Percentage of $B220^{+}CD5^{+}$ TCL1-192 cells in the blood of SCID mice at weeks 3, 4 and 5 in non-injected and αSlamf6 or mIgG2a injected groups. Representative gating for the two sub-populations ($B220^{int}CD5^{int}$ and $B220^{hi}CD5^{hi}$) is depicted in the lower right corner for the isotype control group. **B.** On day 28, the total $B220^{+}CD5^{+}$ cell number per μl blood is a 100-fold less in αSlamf6-injected vs. mIgG2a injected mice (per μl blood: $0.6 \pm 0.1 \times 10^{3}$ vs. $6 \pm 1 \times 10^{4}$; *p = 0.001*); or 85-fold less as compared to the non-injected group ($0.6 \pm 0.1 \times 10^{3}$ vs $5.1 \pm 0.6 \times 10^{4}$; p = *0.0002*). Although cells keep expanding by day 35, αSlamf6 injected group still has significantly less leukemic burden compared to mIgG2a injected (per μl blood: $7.5 \pm 2.1 \times 10^{4}$ vs. $4.9 \pm 0.7 \times 10^{5}$; *p = 0.0015*) vs. non-injected group ($6.1 \pm 0.7 \times 10^{5}$; *p = 0.0006*). The data are representative of 3 independent experiments. **C.** Number of $B220^{hi}CD5^{hi}$ leukemic cells in the blood of αSlamf6 treated mice is less than that in non-treated mice ($0.63 \pm 0.19 \times 10^{5}$ vs. $1.2 \pm 0.1 \times 10^{5}$; p = 0.02). **D.** Mean Fluorescence Intensity (MFI) values of B220 and CD5 ex pression on $B220^{hi}CD5^{hi}$ and $B220^{int}CD5^{int}$ populations of the non-treated group at week 5. Results are representative of 4 independent experiments.

Treatment with αSlamf6 / ibrutinib or αSlamf6 reduced the number of TCL1-192 cells by 95% and 90%, respectively. Ibrutinib-treatment alone reduced the number of TCL1-192 cells by 70% (Figure 7E). As in Figure 4A, the Slamf6 resulted in the loss of B220^{int}CD5^{int} tumor cells, while ibrutinib did not differentially affect either sub-population.

The reduction of tumor cells in the bone marrow of αSlamf6 / ibrutinib treated animals was ten-fold, while αSlamf6 reduced the percentage of TCL1-192 cells only 4.6 fold (Figure 7F). H&E staining indicated a complete absence of leukemic cells in the liver of αSlamf6 / ibrutinib-treated mice as compared to either αSlamf6 or ibrutinib alone (Supplementary Figure S4B).

To further show the efficacy of αSlamf6 and ibrutinib treatment on TCL1-192 cells, we employed *in vitro* assays using both splenic and peritoneal cells and measured viability (AnnexinV/7AAD negative) after 72 hours of culturing with ibrutinib, αSlamf6 or both (Supplementary Figure S4C). In splenic TCL1-192 cells, ibrutinib or αSlamf6 alone led to a significant reduction in viability, and it was further reduced when the two compounds were combined. Interestingly, peritoneal cells were not affected by either treatment *in vitro*, suggesting

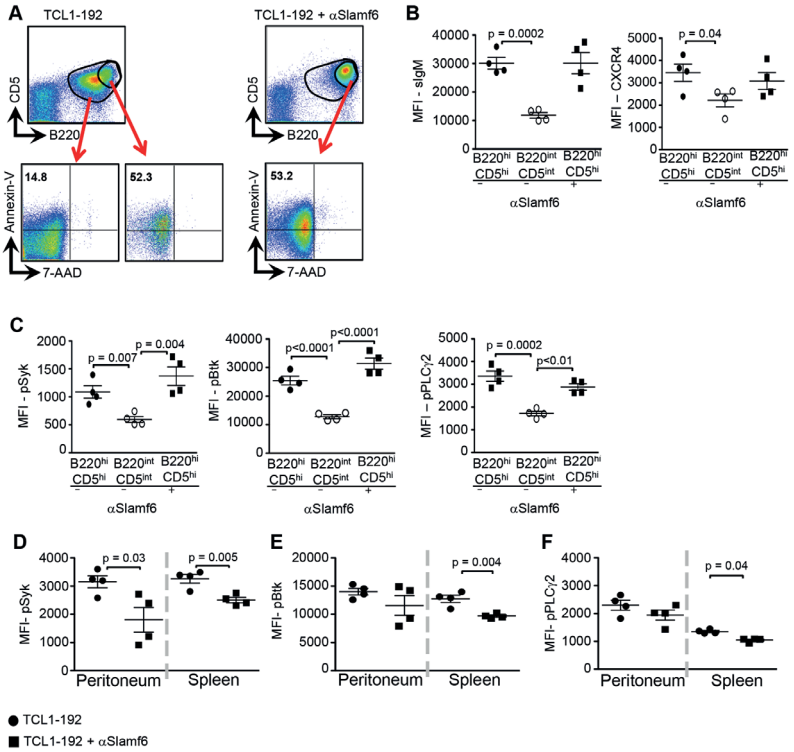

Figure 5: Differential αSlamf6-dependent signaling in B220^{hi}CD5^{hi} and B220^{int}CD5^{int} TCL1-192 CLL cells in the blood, spleen and peritoneum. Schematic outline of the experiment is described in Figure 3B. **A.** Comparison of total B220^{+}CD5^{+} cells in blood of the non-treated group (−) to its B220^{hi}CD5^{hi} subpopulation and B220^{hi}CD5^{hi} TCL1-192 cells in the αSlamf6-treated (+) mice by Annexin-V/7-AAD staining. **B.** MFI values of surface IgM (sIgM) and CXCR4 expression on B220^{hi}CD5^{hi} and B220^{int}CD5^{int} TCL1-192 cells in the blood of αSlamf6-treated (+) and non-treated (−) mice. **C.** Proximal BCR signaling in B220^{hi}CD5^{hi} and B220^{int}CD5^{int} populations in the blood of αSlamf6-treated (+) and non-treated (−) mice was assessed by intracellular staining of pSyk, pBtk and pPLCγ2 and measured by flow cytometry. Representative of three experiments. **D-F.** Expression of pSyk, pBtk and pPLCγ2 in spleen and peritoneum. Results are representative of 4 independent experiments.

that they are indeed more resistant to killing compared to splenic cells. This also indirectly suggests that *in vivo*, ibrutinib does remove the cells from the niche, rather than affecting their survival within the niche.

Taken together, the outcomes of these studies indicate that αSlamf6 and ibrutinib synergize in decreasing the overall leukemic burden.

Anti-human SLAMF6 reduces the number of human CLL cells

In order to support the findings with the murine TCL-1 model, we assessed responses to αhSLAMF6 of the human MEC-1 and OSU-CLL cell lines, which highly express SLAMF6 (Supplementary Figure S1). First, we transplanted 10^7 MEC-1 cells subcutaneously into [Rag x γc]$^{-/-}$ mice [44] before administering αhSLAMF6 or an isotype control (mIgG2b) on days 7 and 14 (Figure 8A).

Twenty-one days after transplanting the MEC-1 cells, the volume of subcutaneous tumors in αhSLAMF6-injected mice was 3.5 times smaller than that in isotype control mice (Figure 8A). This difference was already indicated on day 18 without reaching statistical significance. Because the [Rag x γc]$^{-/-}$ mice do not have NK cells, killing of MEC-1 tumors might be caused by macrophages via ADCC and possibly by αSLAMF6-induced signaling.

We also tested the effect of the αhSLAMF6 antibody in combination with ibrutinib on apoptosis of *in vitro* cultured human OSU-CLL cells [37]. The combination of ibrutinib with αhSLAMF6 resulted in a higher percentage of Annexin-V$^+$ cells (Figure 8B and 8C), once again

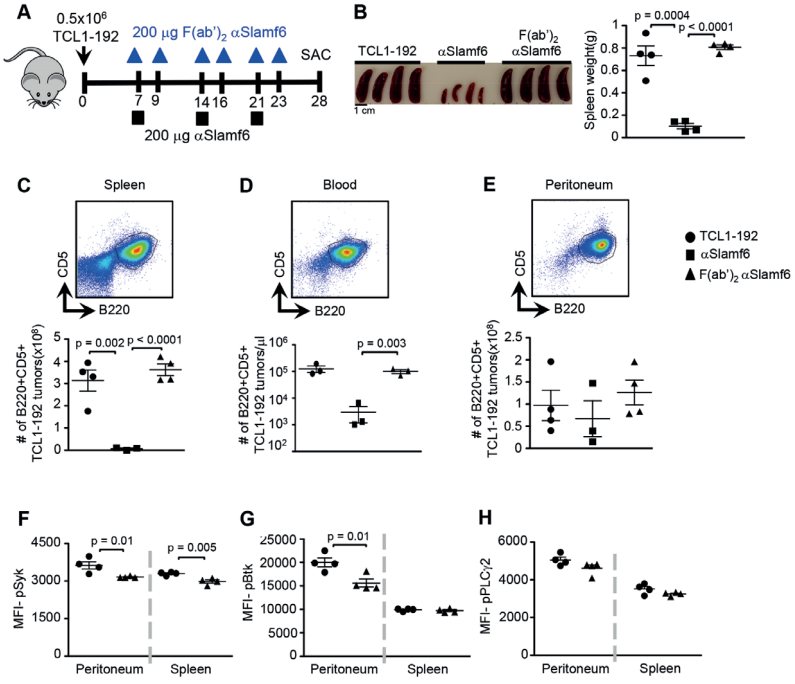

Figure 6: Administering αSlamf6 F(ab')$_2$ fragments does not reduce the number of TCL1-192 cells. F(ab')$_2$ αSlamf6 was generated using Pierce F(ab')$_2$ preparation kit. **A.** Outline of the experiment. TCL1-192 cells were i.p. injected on d0. On d7, d14 and d21, 200 µg αSlamf6 (13G3) was i.p. injected, or on d7, d9, d14, d16, d21 and d23, 200 µg F(ab')$_2$ αSlamf6 was injected. Mice were sacrificed on d28. **B.** Differences in spleen size and weight in non-injected, αSlamf6 or F(ab')$_2$ αSlamf6 injected SCID mice on d28. **C-E.** Representative staining of B220+CD5+ from non-injected mice and number of B220+CD5+ cells in SCID mice after αSlamf6, F(ab')$_2$ αSlamf6 injected were compared to those of non-injected mice in spleen, blood and peritoneum. Next, levels of pSyk **F.**, pBtk **G.** and pPLCγ2 **H.** in TCL1-192 cells isolated on d28 from non-injected and F(ab')$_2$ αSlamf6 injected mice were compared. Representative of 2 independent experiments with n = 9 mice total for F(ab')$_2$ αSlamf6 injected group. P values are as shown.

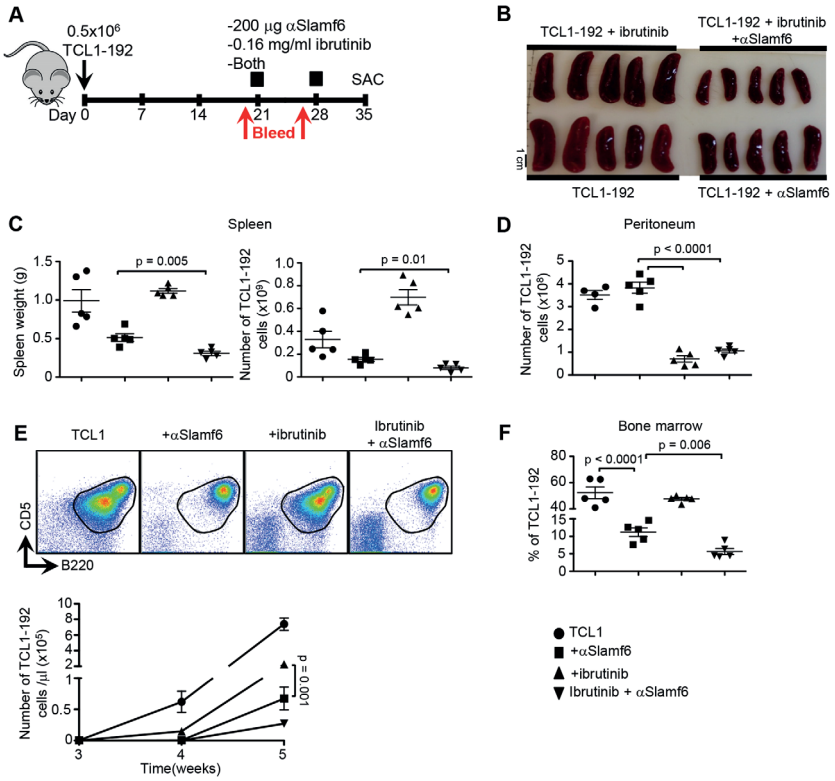

Figure 7: Co-administering αSlamf6 with ibrutinib significantly reduces the number of TCL1-192 cells in the peritoneal cavity. A. Outline of the experiment. TCL1-192 cells were *i.p.* injected on d0. On d21, group of mice were either *i.p.* injected 200 µg αSlamf6 (13G3), given 0.16 mg/ml ibrutinib in drinking water or was given both. Mice on ibrutinib received the drug until sacrifice. Mice received another injection of αSlamf6 on d28. Mice were sacrificed on d28. **B.** Differences in spleen size is as shown. **C.** Mice treated with a combination of αSlamf6 and ibrutinib had significantly smaller spleens and leukemic burden compared to αSlamf6-treated mice (weight: 0.31 ± 0.02 vs. 0.51 ± 0.04 g; $p = 0.005$ and a two-fold smaller number of TCL1-192 cells: 0.78 x10^8 ± 1.54 × 10^7 vs. 1.53 × 10^8 ± 1.76 × 10^7; $p = 0.01$) on day 35. **D.** Relative numbers of TCL1-192 cells in the peritoneal cavity. BTK inhibitor resulted in 5.4 fold reduction in leukemic burden in peritoneal cavity compared to the control or αSlamf6 treated group alone (7.07 ± 1.43 × 10^7 vs. 3.82 ± 0.24 × 10^8; $p < 0.0001$). **E.** Representative FACS plots of $B220^+CD5^+$ in different groups and the exact cell numbers are as shown in the graph below. Ibrutinib alone treated mice resulted in 3-fold lower leukemic burden in blood compared to non-treated mice (per µl blood: 2.2 × 10^5 ± 2.9 × 10^4 vs. 7.3 × 10^5 ± 7.7 × 10^4; $p = 0.0003$). However, αSlamf6 alone treated mice had significantly less TCL1-192 burden compared to ibrutinib alone group (0.67 × 10^5 ± 1.8 × 10^4; $p = 0.0001$). Difference in leukemic burden between αSlamf6 alone and ibrutinib/αSlamf6 treated group did not reach statistical significance (0.67 × 10^5 ± 1.8 × 10^4 vs. 0.27 × 10^5 ± 0.5 × 10^4; $p = 0.06$). **F.** Percentage of TCL1-192 cells in the bone marrow. Anti-Slamf6 resulted in 4.6- fold reduction in percentage of TCL1-192 cells in bone marrow compared to non-treated group (52.2 ± 4.4 vs. 11.2 ± 1.2%; $p < 0.0001$). When compared, αSlamf6 in combination with ibrutinib resulted in a further reduction (2 fold) compared to αSlamf6 treatment alone (11.2 ± 1.2 vs. 5.6 ± 0.8%; $p = 0.006$). Representative of 2 independent experiments is shown. P values are as shown.

suggesting a synergistic effect of the two agents on human CLL cell survival.

DISCUSSION

The pathogenesis of CLL is in part driven by signaling of the BCR in response to a restricted set of auto-antigens, which might function both during precursor cell initiation and clonal progression. Disease aggressiveness has been correlated with the cell surface density and the kinetics of membrane microdomain formation of the BCR and its signaling networks [45, 46]. Whereas clinical trials with small molecules that target BCR initiated signaling, *e.g.* inhibitors of Syk [47], PI3K [48] and Btk [49], have been successful, monoclonal antibodies remain promising as therapeutic targets. Here we show that αSlamf6 efficiently eliminates the murine CLL clone TCL1-192, which after transplantation into SCID mice resides in the spleen and blood. We have chosen this TCL1-192 clone, because increased binding of its specific ligand PtC correlates with enhanced BCR signaling and cell proliferation in the spleen and lymph nodes, which correlates with observations in patients [33].

While αSlamf6 treatment causes a significant loss of leukemic cells in the blood and spleen of TCL1-192-bearing SCID mice, as well as LMP2A/λMyc bearing Rag1$^{-/-}$ mice, both tumor cells remain in the peritoneal cavity of the recipient animals. The Chiorazzi lab discovered that the tumor microenvironments, *e.g.* spleen vs. peritoneal cavity, influences the way TCL1-192 cells respond to antigenic stimuli and BCR signaling [33]. It is therefore plausible that the αSlamf6 antibody does not affect peritoneal TCL1-192 cells, because of the protective microenvironment and altered signaling of the leukemic cells in the peritoneal cavity. In support of this concept is that administering Rituximab (αCD20) does not remove B220^{+}CD5^{+} B1a cells from the peritoneal cavity, while it is very efficient in the killing of B cells in most tissues [42]. Impaired Fc-receptor functions have been implicated [42]. Similarly, CD5^{+} B1 cells

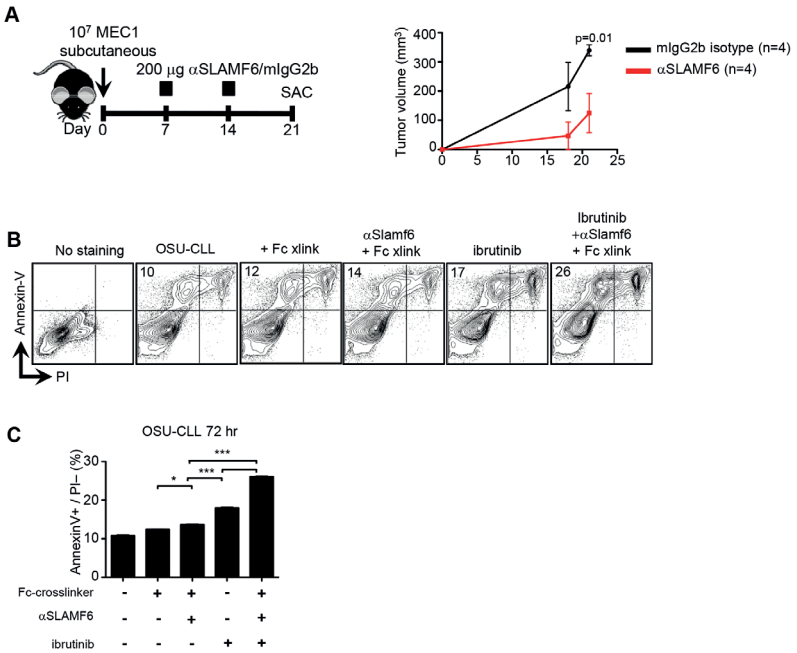

Figure 8: Anti-human SLAMF6 affects progression of human MEC-1 and OSU CLL cells *in vitro* and *in vivo*. A. 10^7 MEC-1 cells were subcutaneously injected into Rag2$^{-/-}\gamma c^{-/-}$ mice. 200µg/ml mouse αhuman-SLAMF6 or isotype mIgG2b was injected *i.p.* on day 7 and 14 (**Left Panel**). Tumor volume was determined by measuring 3 diameters at indicated time points (**Right Panel**). **B-C.** OSU-CLL cells were cultured *in vitro* in the presence of 50 µg/ml Fc-crosslinker alone, 10 µg/ml mouse αhSLAMF6 and Fc-crosslinker, 0.5 µM ibrutinib or in combination for 72 hours and apoptosis was measured by Annexin-V+/PI-. **B.** The plots demonstrate the representative gating strategy for AnnexinV/PI staining. Results are representative of 3 independent experiments. (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$)

respond differently to BCR stimuli in the peritoneum of WT mice than that in the spleen due to expression of the Src kinase Lck, which renders peritoneal B1 cells hypo-responsive [50–52]. Interestingly, most human CLL cells express Lck albeit at varying levels [53]. Importantly, the outcomes of several studies indicate that some CLL patients have massive ascites, suggesting that the tumor cells in the peritoneal cavity may not respond to some treatment protocols [54].

We find that in the blood two sub-populations of mouse TCL1-192 CLL cells exist, *i.e.* IgM^{hi} / $CXCR4^{hi}$ / $B220^{hi}CD5^{hi}$ and IgM^{low} / $CXCR4^{low}$ / $B220^{int}CD5^{int}$ TCL1-192 cells. Coelho et al. [41] suggest that IgM^{low} patient-derived CLL cells have just entered the circulation, as the low levels of BCR are caused by antigenic exposure within tissues. By contrast, cells that have been in circulation longer display a higher BCR expression [41]. A plausible interpretation of our observations is therefore that IgM^{low} / $CXCR4^{low}$ / $B220^{int}CD5^{int}$ TCL1-192 cells are entering into the circulation from the peritoneal cavity, spleen or other secondary lymphoid organs. Administering αSlamf6 eliminates this IgM^{low} / $CXCR4^{low}$ / $B220^{int}CD5^{int}$ TCL1 subset in the blood. While half of the IgM^{hi} / $CXCR4^{hi}$ / $B220^{hi}CD5^{hi}$ cells are in the process of apoptosis as judged by the Annexin-V staining, the other half of this sub-population is possibly on its way to migrating to other tissues for antigenic stimulation [41]. Alternatively, the pro-apoptotic $B220^{hi}CD5^{hi}$ population may already have undergone cell membrane damage, thus not allowing proper binding of the antibody for efficient targeting. Expression levels of CXCR4 support the concept, as this chemokine receptor is not only a migration marker, but is also involved in apoptosis of CLL and acute myeloid leukemia cells [55, 56].

Administering a combination of αSlamf6 and ibrutinib eliminated TCL1-192 tumors in the recipient SCID mice due to a synergistic effect, which was greater than caused by each agent alone. The most likely explanation is that ibrutinib down-regulates BCR signaling and causes apoptosis of the tumor cells within the peritoneum and elsewhere in the body. Second, as ibrutinib is known to move CLL cells out of their various niches into the circulation and removal of leukemic cells from the peritoneal cavity could have resulted in this synergistic reduction of the overall tumor burden. Our use of αhSLAMF6 in *in vitro* and *in vivo* systems using CLL cell lines support the potential of the antibody as a therapeutic target and deserves further investigation using primary CLL cells. Thus, the outcomes of this study suggest that αSLAMF6 and ibrutinib should be considered as a combination therapy for CLL and possibly other SLAMF6 expressing B cell tumors.

MATERIALS AND METHODS

Mice

CB17 SCID and $Rag2^{-/-}\gamma c^{-/-}$ mice from Taconic (Hudson, NY) and Rag1−/− mice from the Jackson Laboratory are maintained under specific pathogen-free conditions at the Beth Israel Deaconess Medical Center (BIDMC) animal facility. Experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at BIDMC.

CLL cells

Peripheral blood samples were obtained after informed consent from CLL patients at BIDMC, Department of Hematology/Oncology. Patient consent for samples used in this study was obtained in accordance with the Declaration of Helsinki on protocols that were approved by the Institutional Review Board at BIDMC. Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium (Corning, Manassas, VA).

The human CLL MEC-1 cell line [36] was a gift from Dr. Silvia Deaglio (University of Turin, Italy). Cells were cultured in RPMI 1640 medium, as described [57]. The human OSU-CLL cell line [37], which was generously donated by Dr. John C. Byrd (Ohio State University), was cultured as described.

The leukemic TCL1-192 clone (B6xC3H) was generated as described [33]; it's BCR recognizes phosphatidylcholine (PtC) with a single IgHV-D-J rearrangement.

Flow cytometry

PBMCs from CLL patients, the MEC-1 and OSU-CLL cell lines were stained with: PE, FITC or APC conjugated anti-human monoclonal antibodies were purchased from Biolegend (San Diego, CA): CD3 (HIT3a), CD19 (HIB 19), CD5 (UCHT2), SLAMF1 (A12), SLAMF2 (BJ40), SLAMF3 (Hly-9.1.25), SLAMF4 (C1.7), SLAMF5 (CD84.1.21), SLAMF6 (NT-7), SLAMF7 (162.1), PE-anti-mouse mIgG1 (MOPC-21), anti-mIgG2a (MOPC-173) and anti-mIgG2b isotype controls (MPC-11). Anti-human SLAMF8 (250014) was from R&D Systems (Minneapolis, MN). SLAMF surface expression on CLL cells from 57 patients was determined with antibodies provided by the "Ninth International Workshop on Leukocyte Antigens" [22].

Dead cells were excluded by DAPI staining and cell count per µl of blood was determined by CountBright absolute counting beads (Life Technologies, Carlsbad, CA).

PE, FITC, APC, APC/Cy7, PerCP/Cy5.5, PE/Cy7 or Pacific blue conjugated anti-mouse antibodies were purchased from Biolegend: B220 (RA3-6B2), Slamf1 (TC15-12F12.2), Slamf2 (HM48-1), Slamf3 (Ly9ab3), Slamf5 (mCD84.7), Slamf6 (330-AJ), IgM (RMM-1) and CXCR4 (L276F12). Anti-mouse CD5 (53-7.3) and Slamf4 (eBio244F4) antibodies were purchased from eBioscience (San Diego, CA). Anti-mouse Slamf7 (520914) antibody was purchased from R&D Systems (Minneapolis, MN).

For intracellular staining with the BD Cytofix/Cytoperm Kit, the following antibodies were used: pZAP70/pSyk(Y319/Y352) (n3kobu5) and pBtk(Y551/Y511) (M4G3LN) from eBioscience and pPLCγ2(Y759) (K86-689.37) from BD Biosciences (San Jose, CA). The procedure described in the manufacturer's manual was followed.

The Annexin-V Apoptosis Kit was purchased from eBioscience.

The cells were acquired on a BD LSRII flow cytometer and data analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR).

LMP2A/λMyc tumor cells

Peripheral lymph node tumor cells from LMP2A/λMyc (Tg6/λMyc) mice [35] were *i.p.* injected into $Rag1^{-/-}$ mice.

Histology

Liver sections were fixed in 10% formalin and stained with H&E at the BIDMC histology core.

Reagents

The mouse monoclonal αSlamf6 (clone 13G3) hybridoma was generated from spleens of $Slamf6^{-/-}$ mice immunized with WT thymocytes [26]. Hybridomas were used to produce IgG2a by Harlan Laboratories (South Easton, MA). Mouse IgG2a (C1.18) isotype control was purchased from BioXcell, Inc. (West Lebanon, NH). Mouse anti-human SLAMF6 antibody (994.1) was obtained from ARCA Biopharma (Westminster, CO).

$F(ab')_2$ goat anti-mouse IgG was purchased from Jackson Immunoresearch.

The BTK inhibitor, ibrutinib, was purchased from ChemieTek (Indianapolis, IN). Ibrutinib was dissolved in DMSO and prepared as described elsewhere [49].

In vitro culturing of OSU-CLL

5×10^4 OSU-CLL cells were seeded in 96-well flat bottom plate. Relevant wells received 50 μg/ml Fc-crosslinker, 10 μg/ml αSLAMF6 or 0.5 μM ibrutinib. Plate was incubated for 72 hours at 37C and 5% CO_2. Viability was measured by Annexin-V/PI staining.

Injection of MEC-1 in [Rag x γc]$^{-/-}$ mice

10^7 MEC-1 cells were injected subcutaneously into $[Rag \times \gamma c]^{-/-}$ (Taconic) mice and monitored as previously described [44].

Statistics

Statistical analyses were calculated using GraphPad Prism software (GraphPad, La Jolla, CA). The Student t test or 2-way ANOVA was used to compare groups; results are represented as mean ± SD. $P < 0.05$ was considered significant.

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AUTHORSHIP AND CONFLICTS OF INTEREST

B.Y performed the experiments with the help of NW, PJH, MSO; the manuscript was prepared by BY, PE and CT; DA and JA provided the fresh CLL samples. OC and RL generated the LMP2A/lMyc tumors. SSC and NC provided TCL1-192. AB did the pathology analyses. VG generated the SLAMF expression data from 57 patients. All the authors contributed to the writing of the manuscript.

None of the authors declares a conflict of interest.

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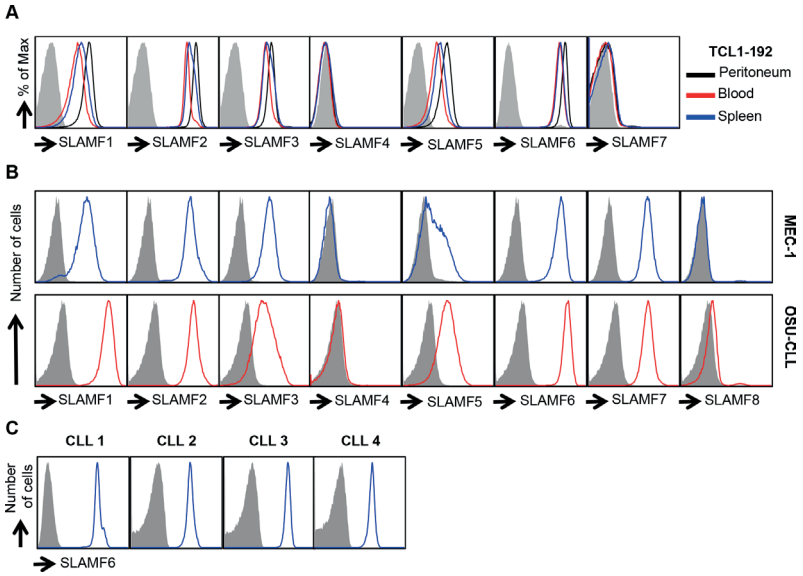
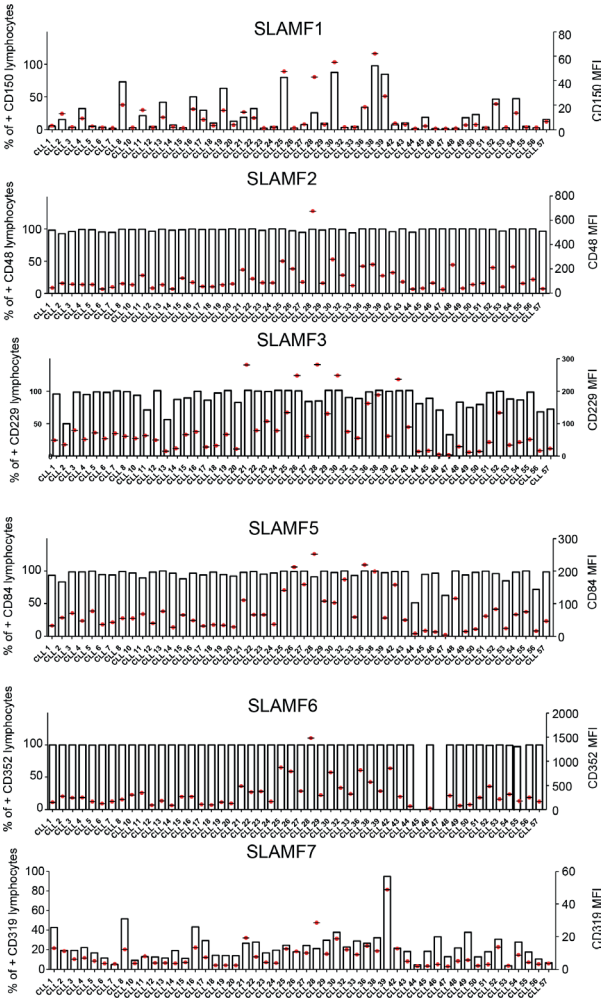
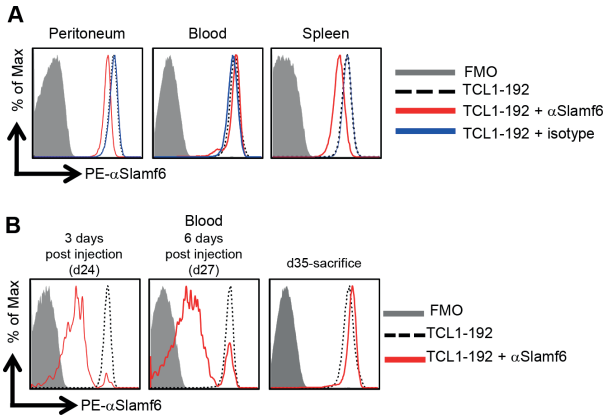
SUPPLEMENTARY FIGURES



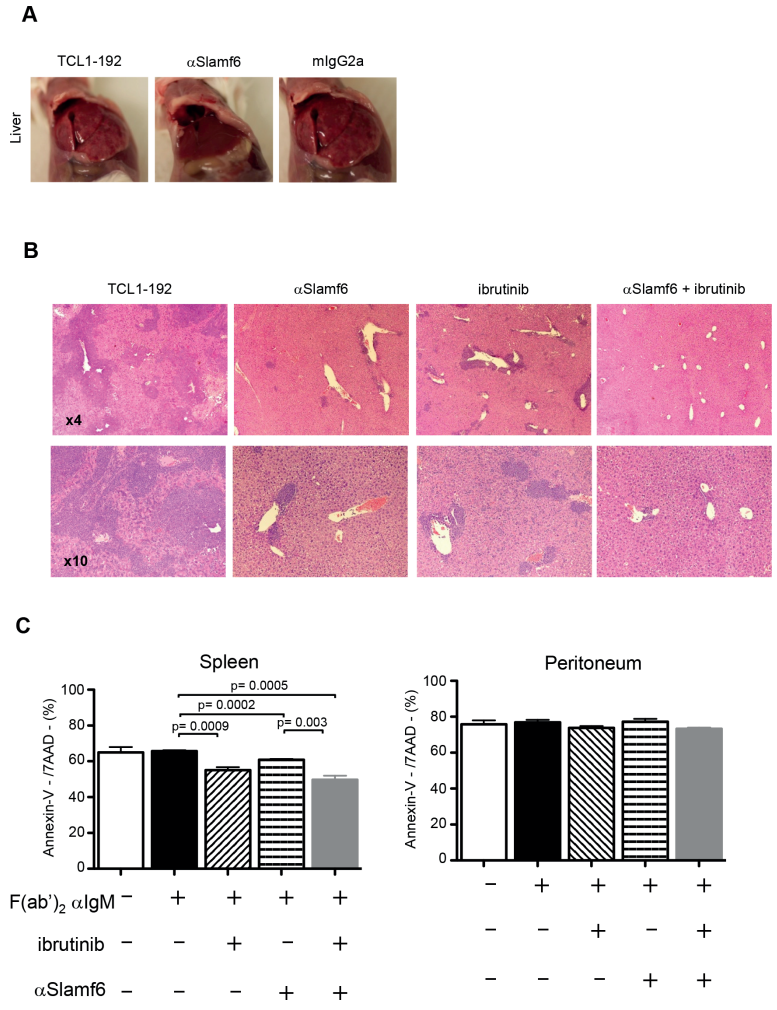
Supplementary Figure S1: Expression of SLAMF receptors on the surface of CLL cells. A. Representative histograms of Slamf receptor expression on the surface of TCL1-192 cells. Four weeks after *i.p.* injection into SCID mice (0.5×10^6 cells/mouse) TCL1-192 cells were isolated from the peritoneal cavity (referred to as peritoneum throughout the figures), blood or spleen. Gray bars indicate isotype controls or FMO (Fluorescence minus one) in all histograms. TCL1-192 cells in the peritoneal cavity also express the highest levels of Slamf1 (MFI P: 3952, B: 1218, S:1827), Slamf2 (MFI P:22850, B:8906, S:13810), Slamf3 (MFI P:3637, B:2139, S:2852) and Slamf5 (MFI P:1488, B:553, S:780). **B.** Human CLL cell lines MEC-1 and OSU-CLL were cultured *in vitro*, and expression of SLAMF receptors was determined by flow cytometry. **C.** PBMCs were obtained from the blood of 4 CLL patients. SLAMF6 expression was determined on $CD3^-/CD19^+CD5^+$ CLL cells using flow cytometry.



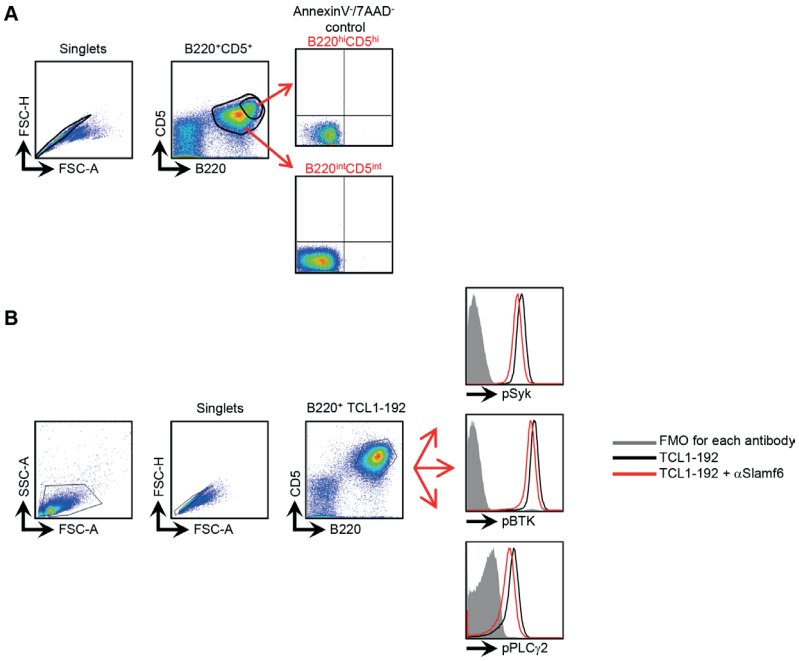
Supplementary Figure S2: Expression of SLAMF receptors on the surface of CLL cells isolated from 57 patients. Cell surface expression of SLAMF molecules from PBMCs of 57 CLL patients was measured using flow cytometry. The left y-axis represents the percentage of expression and the right y-axis represents the MFI values for each molecule. Representative flow cytometry plot showing the overlay of $B220^{+}CD5^{+}$ cells from the peritoneum, blood and spleen.



Supplementary Figure S3: Slamf6 expression on the surface of TCL1-192 cells after injection of αSlamf6. A. Expression of Slamf6 on TCL1-192 cells from non-injected, αSlamf6 or isotype control injected mice was measured. Cells in the spleen, blood and peritoneal cavity at d28 (from experiment outlined in Figure 1A) were stained for Slamf6 receptor availability by PE-conjugated anti-mouse Slamf6 antibody (details in Materials and Methods), and determined by flow cytometry on $B220^{+}CD5^{+}$ cells. Histograms are representative from each group. **B.** Occupancy of the Slamf6 receptor by the injected αSlamf6 on the surface of $B220^{+}CD5^{+}$ cells in the blood was assessed 3 and 6 days after the first αSlamf6 injection (days 24 and 27) and at d35. Representative histograms of *in vitro* staining with a PE-conjugated αSlamf6 FACS antibody comparing unoccupied Slamf6 receptor expression in αSlamf6 injected and non-injected groups.



Supplementary Figure S4: Anti-Slamf6 + BTK inhibitor, ibrutinib, has synergistic effect on liver and in *in vitro* apoptosis of TCL1-192 cells. A. Representative figure indicating TCL1-192 leukemic infiltration in the liver of SCID mice on d35, as indicated in Figure 2B. **B.** Representative H&E staining of liver from experiment in Figure 6. Double treated mice show no sign of leukemic infiltration compared to single treated mice. **C.** *In vitro* stimulation and treatment of splenic TCL1-192 cells in the presence of both αSlamf6 and ibrutinib, results in significantly higher Annexin-V+ apoptotic cells compared to either alone (Left Panel). Viability of peritoneal TCL1-192 cells were not affected from treatment with either regimen.



Supplementary Figure S5: Gating strategy of AnnexinV/PI and phosphoflow staining. A. Representative plots of B220+CD5+ gating in blood (Figure 5A). Corresponding $B220^{hi}CD5^{hi}$ or $B220^{int}CD5^{int}$ populations were gated based on AnnexinV/PI negative control group. **B.** Representative gating for pSyk, pBtk and pPLCγ2 antibodies (Figure 5C-F). Singlet cells were determined and B220+CD5+ TCL1-192 cells were gated. Fluorescence minus one (FMO) was used as a negative control for each antibody staining.

CHAPTER 3

Inhibition of reactive oxygen species limits expansion of chronic lymphocytic leukemia cells

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A key physiological role of reactive oxygen species (ROS) in biological processes and diseases has gained more attention over the past years. ROS produced by cells of the immune system, for example, neutrophils, macrophages and B cells, consist primarily of hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radicals (OH^-) that facilitate cell processes such as homeostasis and antimicrobial responses.[1]

Several studies have indicated a relationship between ROS and chronic lymphocytic leukemia (CLL) cell survival.[2–4] Upon cross-linking and activation of the B-cell receptor (BCR) on the surface of B cells, particularly in B2 B cells, NOX2 produces ROS that subsequently inhibits tyrosine phosphatases.[5] This inhibition then promotes BCR signal amplification.[5] Unlike B2 B cells, $CD5^+$ B1a B cells, which are already detected during early fetal development and which reside primarily in the peritoneal cavity (PerC), are autoreactive and spontaneously secrete IgM.[6] These B1a B cells show distinct characteristics compared with that of B2 B cells in response to BCR ligation.[6]

The human equivalent of $CD5^+$ B1a B cells in mice are thought to be the origin of CLL cells.[7] Cell-autonomous BCR ligation and low-affinity autoantigens, such as phosphatidylcholine (PtC) and Sm, drive CLL development.[8] As there are many similarities with human CLL, murine CLL cells provide useful tools for *in vitro* and *in vivo* study of the biology of the disease and for testing therapeutic interventions. Monoclonal antibody treatments, for example, with anti-CD20 or anti-SLAMF6, can efficiently remove CLL cells in peripheral blood and lymphoid organs, in part by antibody-dependent cell-mediated cytotoxicity (ADCC).[9,10] However, these reagents are not effective in removing CLL cells in PerC,[9,10] even though coating of B cells with anti-CD20 antibody and duration of binding to the surface of B cells residing in the PerC and in blood or lymphoid organs is similar.[9] This suggests that there could be a mechanism provided by the microenvironment that offers protection to B cells in the PerC. Given the importance of ROS in tumor microenvironments, we hypothesized that ROS plays a niche-specific role in CLL. To test this idea, we used severe combined immunodeficient (SCID) mice transplanted with murine TCL1-192 leukemic cells, clonal VH11/Vk14 BCR and reactive with PtC, derived from TCL1-bearing mice.[11] Expansion of TCL1-192 cells in SCID mice results in aggressive disease progression, similar to observations in CLL patients with unmutated IGHV.

First, we determined extracellular O_2^- levels in TCL1-192 cells by luminol-based chemiluminescent reagent, as an indicator of basal ROS production. Cells are freshly isolated and processed from spleen, blood and PerC of TCL1-192 transplanted SCID mice 5 to 6 weeks after transfer. Interestingly, TCL1-192 cells from PerC had significantly higher ROS levels compared with blood or spleen (Figure 1a). However, when stimulated with phorbol 12-myristate 13-acetate, TCL1-192 cells from all compartments produced comparable levels of ROS, suggesting a microenvironmental influence on basal ROS levels (Supplementary Figure 1a). We then tested whether this high level of basal ROS also occurred in normal B1a B cells from the spleen and PerC of B6.C3H wild-type (WT) mice, and used naive splenic B2 B cells as controls. As shown in Figure 1a, PerC B1a B cells have elevated levels of basal ROS as compared with splenic naive B2 and B1a B cells. When we determined the extent that ROS was further induced in TCL1-192 cells in PerC upon BCR stimulation, no significant difference was detected as compared with nonstimulated cells (Figure 1a). To exclude the possibility that this induction of ROS was due to cell death, we incubated TCL1-192 cells *in vitro* for 1.5 h at 37 °C, the time point when ROS production peaks, and observed no significant apoptosis by Annexin V/propidium iodide (Supplementary Figure 1b). To test whether the isolation procedure had an effect on this induction, TCL1-192 cells from the PerC were also tested for ROS production without washing. This did not make a difference in the response of cells from PerC (Supplementary Figure 1c). Taken together, the data demonstrate that there is a PerC-specific induction of ROS in leukemic and B cells in this compartment that is independent of BCR stimulation.

We next assessed whether the ROS produced by PerC TCL1-192 cells was generated by mitochondria, as suggested by some studies with patient-derived CLL cells.[4] Indeed, TCL1-192 cells freshly obtained from the PerC had mitochondrial ROS production, as judged by MitoSOX, which was inhibited by the antioxidant *N*-acetylcysteine (NAC) (Figure 1b). Similarly, extracellular ROS was also inhibited by NAC (Figure 1b), demonstrating that indeed mitochondria are one of the sources of spontaneous ROS in PerC TCL1-192 leukemic cells.

Previously, we demonstrated that ibrutinib treatment significantly reduced the leukemic burden in the PerC of SCID mice that had been transplanted with TCL1-192.[10] We therefore first determined ROS levels from PerC TCL1-192 cells after *in vitro* incubation with 1 μM ibrutinib and found that basal ROS levels as well as spontaneous ROS was significantly reduced as compared with control cells (Figure 1c). To test whether ibrutinib induced migration, we intraperitoneally (i.p.) transplanted TCL1-192 cells into SCID mice and 10 days later, when the cells were still in the PerC, a single dose of 25 mg/kg ibrutinib was given by gavage. After 4 and 24 h, the number of TCL1-192 cells had significantly increased in the blood of ibrutinib-treated mice as compared with control mice (Figure 1c). At this time point, no leukemic cells were detected in the spleen of either group ($< 1\%$, data not shown). Taken together, ibrutinib reduces ROS production in PerC TCL1-192 cells and induces migration out of PerC. Together with our previous findings,[10] these data suggest a causal relationship between reduced ROS production by TCL1-192 and their exit from the PerC, consistent with ibrutinib inducing an exit from CLL cell niches in patients.[12]

As ROS can have prosurvival effects, we also tested whether inhibition of ROS affected engraftment and growth of TCL1-192 cells in PerC after i.p. injection. At 3 days after transfer of TCL1-192 cells into SCID mice, we administered i.p. a dose of 75 mg/kg/day NAC or PBS as control daily until mice were killed on day 10, a time at which TCL1-192 cells had not yet left the PerC (Figure 2a).

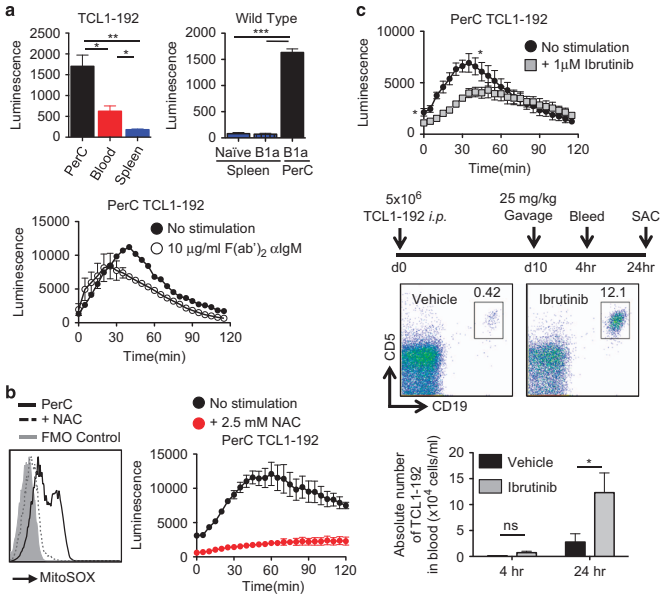

Figure 1. Spontaneous ROS release is observed in PerC TCL1-192 cells but not in spleen or blood. (**a**, left panel) TCL1-192 cells isolated from PerC of TCL1-192-bearing SCID mice had significantly higher spontaneous ROS levels as compared with ones from spleen or blood, measured by a cell-impermeable, luminol-based, chemiluminescent reagent that measure levels of O_2^-. (**a**, right panel) Similarly, WT control of TCL1-192 cells, B1a B cells, isolated from PerC and spleen of B6.C3H WT mice as well as naive splenic B2 B cells were measured for spontaneous release of ROS. B1a B cells from PerC had elevated levels of ROS as compared with either splenic B-cell populations. $^*P < 0.05$, $^{**}P < 0.01$. Data are representative of 3 independent experiments with $n \geqslant 3$ mice in each group. (**a**, lower panel) BCR stimulation of TCL1-192 cells from PerC with 10 μg/ml $F(ab')_2$ αIgM had no significant effect on ROS levels. ROS levels upon stimulation were measured at 5 min intervals using a luminometer. (**b**, left panel) Representative histogram showing levels of mitochondrial ROS, using MitoSOX, in PerC TCL1-192 cells. Pretreatment with NAC (2.5 mM) inhibited mitochondrial ROS. (**b**, right panel) NAC pretreatment also inhibited extracellular ROS release in PerC TCL1-192 cells. (**c**) *In vitro* incubation of PerC TCL1-192 cells with 1 μM ibrutinib significantly reduced ROS levels. $^*P < 0.05$. TCL1-192 cells were i.p. transplanted into SCID mice and 10 days later were gavaged 25 mg/kg ibrutinib. Mice were bled 4 h later and killed after 24 h. Representative plots from blood at 24 h after treatment. Absolute numbers of TCL1-192 significantly increased in blood after 24 h. $^*P < 0.05$ ($n = 4$ vehicle, $n = 3$ ibrutinib).

NAC-injected SCID mice had a significantly lower number of total PerC cells, as well as significantly lower numbers of $B220^+CD5^+$ TCL1-192 cells (Figure 2a).

Then, we tested whether NAC administration would have an effect on leukemic cells after they had already expanded in SCID mice. To this end, we injected 250 mg/kg/day NAC i.p. for 4 consecutive days, beginning 21 days post transplant when TCL1-192 leukemic cells had begun expanding in the spleen and mice were euthanized on day 25 (Figure 2b). Administering NAC lowered the numbers of TCL1-192 leukemic cells in both spleen and PerC (Figure 2b). These findings suggest that inhibition of ROS by NAC might slow down leukemic progression and limit its expansion. Overall, our data with CLL cells suggest the possibility that varying ROS levels might differentially induce reduced proliferation, cell death and/or migration in distinct tumor microenvironments.[13] The specific role of ROS in this context requires further investigation.

In this study, we documented differential ROS production by mouse CLL cells and normal B1a B cells residing in PerC that is independent of BCR stimulation and is not found in spleen or blood. Furthermore, inhibition of this ROS by NAC at varying doses slowed down leukemic progression *in vivo*. These findings suggest that increased spontaneous ROS production by autoreactive CLL B cells or normal B1a B cells is important for their expansion and/or maintenance in niches like PerC. It is plausible that this extracellular ROS in PerC may affect ADCC function of monoclonal antibodies and possibly prevent Fc receptors on other immune cells from recognizing, making it an interesting niche to study to understand biology of current and developing therapies better.

Although small inhibitor molecules targeting BCR signaling in CLL, like phosphatidylinositol 3-kinase-δ inhibitor idelalisib, have shown efficacy in patients, various side effects occur,[14] including diarrhea, colitis and hepatotoxicity.[14] Administration of NAC can protect against hepatotoxicity.[15] Besides the biological effects of ROS, exploring the effect of antioxidants and/or anti-ROS reagents in combination with current treatments might improve the overall health of CLL patients.

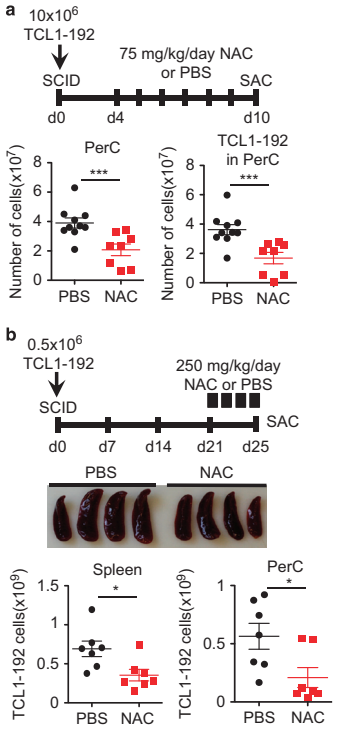

Figure 2. *In vivo* injection of NAC reduces leukemic burden in TCL1-192 transferred SCID mice. (**a**) Schematic outline of injection. On day 0, 10×106 TCL1-192 cells i.p. injected into SCID mice. On day 4, 75 mg/kg/day NAC administered daily and mice killed on day 10. Numbers of total cells and TCL1-192 cells ($B220^+CD5^+$) are significantly reduced in PerC of NAC-injected mice. $^{***}P < 0.001$ (phosphate-buffered saline (PBS): $n = 10$, NAC: $n = 8$ mice). (**b**) Schematic outline of injection. At 21 days after transfer and expansion of TCL1-192 cells in SCID mice, 250 mg/kg NAC administered i.p. for 4 consecutive days and mice killed on day 25. Spleens are as shown. Numbers of TCL1-192 cells are significantly reduced in spleen of NAC-injected mice. Numbers of TCL1-192 cells are significantly reduced in PerC of NAC-injected mice. $^*P < 0.05$, $n = 7$ mice each group.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

BY designed and performed the experiments. S-SC, NC and NW provided input into the experiments. BY and CT wrote the manuscript. All authors read and approved the manuscript.

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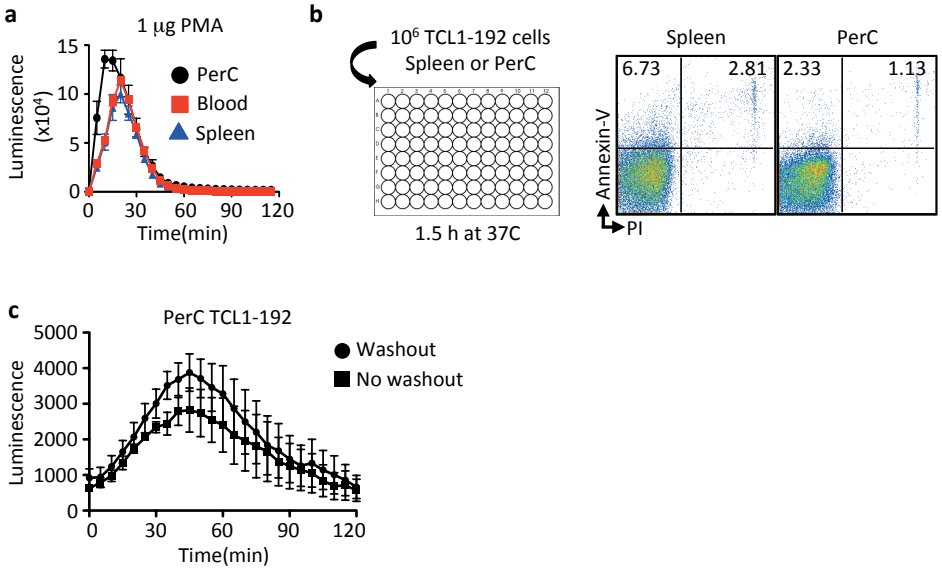
Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)

Supplementary Material



Supplementary Figure 1. (a) TCL1-192 cells from PerC, blood and spleen of tumor bearing SCID mice were stimulated with 1 μg PMA and ROS levels, mainly O^{2-}, were measured at 5 minute intervals using a Promega GloMax luminometer. TCL1-192 cells from all compartments can produce similar levels of ROS. (b) Splenic and PerC TCL1-192 cells were put in a 96-well flat bottom plate at 10^6 cells per well concentration, which is the experimental setup for measuring ROS. Cells incubated at 37^0 in an incubator for 1.5 hours, which is the time point where PerC ROS production peaks. Cell viability was measured with Annexin V/PI staining and revealed that increase in ROS is not due to cell death. (c) Cells from the PerC were washed out in the assay reagent HBSS + 5% FBS. Cells were left without washing or were washed according to the ROS measurement protocol. Washing and no washing prior to the assay did not have a significant effect on ROS production.

Supplementary Material

Materials and Methods

Mice

CB17 SCID mice from Taconic (Hudson, NY) and B6.C3H WT mice from The Jackson Laboratory (Bar Harbor, ME) are maintained under specific pathogen-free conditions at the Beth Israel Deaconess Medical Center (BIDMC) animal facility. Experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at BIDMC.

CLL cells

The leukemic TCL1-192 clone (B6.C3H) was generated as described [14]. The BCR of TCL1-192 recognizes phosphatidylcholine (PtC) with a single IgHV-D-J rearrangement.

TCL1-192 transplantation

SCID mice were *i.p.* transplanted with TCL1-192 cells and were subject to injection with NAC, vehicle, or ibrutinib (details in manuscript). No statistical method was used for sample number estimate in these experiments. No randomization or blinding used in the studies. All mice were included in the study, unless the tumor had not engrafted and were removed from the analysis.

Flow Cytometry

Following fluorescently labeled monoclonal antibodies were purchased from Biolegend (San Diego, CA): anti-mouse B220 (RA3-6B2), F4/80 (BM8) and CD11b (M1/70). Anti-mouse CD5 (53-7.3) was purchased from eBioscience.

Dead cells were excluded by DAPI staining. Cells were acquired on a BD LSRII flow cytometer and data analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR).

Reagents

$F(ab')_2$ goat anti-mouse IgM was purchased from Jackson Immunoresearch (West Grove, PA).

Diogenes Cellular Luminescence Enhancement System was purchased from National Diagnostics (Atlanta, Georgia) and used according to manufacturer's protocol.

N-Acetylcysteine (NAC) was purchased from Sigma-Aldrich.

ROS Assays

Diogenes, chemiluminescent reagent

Cells were washed twice with PBS and resuspended in HBSS + 5% FBS. 1x106 cells plated on 96 well plate and Diogenes reagent added and incubated at 37C for 45 minutes in Promega GloMAx Multi Detection system to measure basal levels. Then the cells were stimulated with 10 μg/ml $F(ab')_2$ αIgM and chemiluminescence was measured automatically every 5 minutes.

MitoSOX, for mitochondrial ROS

Cells (1-1.5x10^6 per tube) were put in FACS tubes and washed with pre-warmed HBSS + 1% FBS. 5 μM MitoSOX solution was prepared in HBSS + 1% FBS and cells were incubated for 10 minutes at 37 Celcius. Cells then washed 2 times with warm buffer and resuspended in FACS buffer (PBS + 2% FBS) and measured using BD LSR II flow cytometer.

Statistics

Statistical analyses were calculated using GraphPad Prism software (GraphPad, La Jolla, CA). Results are represented as mean ± SD. $P < 0.05$ was considered significant.

CHAPTER 4

SLAMF6 is a checkpoint inhibitor of CD8^{+} T cell exhaustion in Chronic Lymphocytic Leukemia.

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Abstract

Chronic lymphocytic leukemia (CLL) cells induce a shift of activated $CD8^+$ T cells to an "exhausted" state, characterized by loss of proliferative capacity and impaired immunologic synapse formation. Efficient strategies and targets need to be identified to overcome T cell exhaustion and further improve overall responses in clinic. Here we employ the transfer of mouse Eμ-TCL1 CLL cells into WT and SLAMF6-/- mice to assess the role of the homophilic receptor SLAMF6 as an immune checkpoint regulator. As judged by the analysis of Eμ-TCL1-bearing SLAMF6-/- mice, SLAMF6 affects the interactions of CLL cells with the tumor microenvironment. The transfer of $SLAMF6^+$ Eμ-TCL1 cells into SLAMF6-/- recipients, in contrast to WT recipients, significantly induced expansion of a $PD\text{-}1^+$ subpopulation among $CD3^+CD44^+CD8^+$ T cells, which had impaired cytotoxic functions. Administering αSLAMF6 significantly reduced the leukemic burden in Eμ-TCL1 recipient WT mice concomitantly with a loss of $PD1^+$ $CD3^+CD44^+CD8^+$ T cells with significantly increased effector functions. When αSLAMF6 was co-administered with ibrutinib, Eμ-TCL1 cell engraftment was further reduced. Consistent with the observations in mice, αSLAMF6 together with ibrutinib reduced viability of CLL cells in cultures with peripheral blood mononuclear cells from CLL patients. Taken together, αSLAMF6 both effectively corrects $CD8^+$ T-cell dysfunctions and has a direct effect on CLL progression and the outcomes of these studies directly suggest therapeutic strategies.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by accumulation of clonal $CD5^+$ mature B cells in the bone marrow, peripheral blood and secondary lymphoid organs[1]. CLL cells can induce a tumor supportive microenvironment comprised of myeloid, stromal cells and largely dysfunctional T cells, which results in failure of the host to mount proper anti-tumor immune responses [2-4]. In addition, $CD8^+$ T cell dysfunctions contribute to the pathogenesis of CLL, which include defective cytoskeleton formation and impaired cytotoxicity in concert with an increased expression of inhibitory receptors, including the programmed cell death 1 protein (PD-1, CD279) [5-9]. PD-1 expression under physiological conditions controls the magnitude of T cell effector functions upon activation [10]. Within the tumor microenvironment, however, overexpression of the PD1 ligand, PD-L1, on the surface of tumor cells increases levels of PD1 on T cells, which in turn suppresses these effector functions [10,11]. This is associated with an "exhausted state" of T cells. Blocking PD-1/PD-L1 axis has been demonstrated to be an effective way to remove the break on these otherwise suppressed T cells, restoring their cytotoxic capacity in setting of advanced solid tumors as well as in leukemia [12,13].

Signaling Lymphocyte Activation Molecule Family 6, SLAMF6 (CD352, Ly108, NTB-A), is a homophilic cell surface receptor, belonging to the immunoglobulin superfamily[14,15]. SLAMF6 is a type I transmembrane protein with two extracellular immunoglobin (Ig) like domains and three cytoplasmic tyrosine based signaling motifs one of which is immunoreceptor tyrosine-based switch motif (ITSM)[14,16].

The SLAMF6 receptor is expressed on the surface of a wide variety of hematopoietic cells, *e.g.* T, B and NK cells (expression restricted to human), and interactions on different cell types allow for diverse immune-modulatory functions, some of which include, adhesion, innate T lymphocyte development, neutrophil function, NK and $CD8^+$ T cell mediated cytotoxicity[17-26]. Upon phosphorylation of the immunoreceptor the two tyrosine-based switch motifs (ITSM), the SH2 domain containing T and NK cell adaptor SLAM-associated protein (SAP) is recruited to the SLAMF6 cytoplasmic tail[16,25,27]. Upon engagement of SLAMF6 the ensuing signaling induces both cooperation between T follicular helper cells and germinal center B cells[22] as well as interactions between cytotoxic T cell and B cell targets[27]. This process is dependent upon the presence of SAP. By contrast, in the absence of SAP, SLAMF6 negatively regulates both processes by recruiting the tyrosine phosphatases SHP1 or SHP2 to its cytoplasmic tail.

As B cells do not express SAP, we hypothesized that triggering SLAMF6 would negatively regulate B cell responses. Indeed, monoclonal antibodies directed at mouse Slamf6 (αSLAMF6, Clone 13G3 and 330)[28] reacted with TCL1-192 cells, a $CD5^+$ CLL B cell clone, which had been transferred into SCID mice, which lack B and T cells[29,30]. Perhaps more importantly, one injection of the antibody limited expansion of TCL1-192 due to antibody-dependent cytotoxicity (ADCC) and down-regulation of B cell receptor (BCR) signaling[30]. Based on these observations we hypothesized that antibodies directed against SLAMF6 should also impact the immunomodulatory action of cytotoxic T cells that respond to Eμ-TCL1 CLL cells. Here we show that αSLAMF6 monoclonal antibody (mAb) alters the CD8 T cell responses to CLL resulting in a significantly reduced proportion of exhausted cytotoxic T lymphocytes (CTL). Thus, αSLAMF6 both directly affects CLL expansion and increases CTL responses to the leukemic cells.

Materials and Methods

Mice

C57BL/6J (B6) WT mice were obtained from the Jackson Laboratory. Eμ-TCL1 mice were kindly provided by Dr. Amy Johnson (Ohio State University, Columbus, OH). SLAMF6-/- mice generated from Bruce4 ES cells[31]. All animals are maintained under specific pathogen-free conditions at the Beth Israel Deaconess Medical Center (BIDMC) animal facility. Experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at BIDMC.

CLL cells

Patient consent for samples used in this study was obtained in accordance with the Declaration of Helsinki on protocols that were approved by the Institutional Review Board at BIDMC. Frozen peripheral blood mononuclear cells (PBMCs) were kindly provided by Dr. Jan Burger (University of Texas MD Anderson Cancer Center, Houston, TX). Information on patient samples is provided in Supplementary Table II.

In vitro stimulation of human CLL cells

Briefly, frozen CLL PBMCs were thawed washed and re-suspended in RPMI1640 + 10% fetal bovine serum (FBS) + Penicillin/Streptomycin + L-Glutamine. Cells were either left unstimulated, or stimulated with 20 μg/ml goat anti-human $F(ab')_2$ IgM (MP Biomedical, Santa Ana, CA) alone, together with 1 μM ibruti-

nib (ChemieTek, Indianapolis, IN), 10 μg/ml humanized anti-human SLAMF6 (ARCA, Westminster, CO) or in combination with both. Cells were cultured for 24 and 48 hours and viability was determined by AnnexinV/Propidium Iodide (Biolegend, San Diego, CA) staining by flow cytometry.

The *in vivo* Em-TCL1 adoptive transfer model

Fresh or frozen splenocytes (15-20 x10^6 per mouse) from 12-14 months old leukemic Eμ-TCL1 mice were injected intraperitoneally (*i.p.*) to 8-12 weeks old B6 WT or SLAMF6-/- recipients (Mice were irradiated at 400Rad prior to leukemic transplantation). For experiments involving αSLAMF6/isotype injections, mice were bled bi-weekly 4 weeks after transfer. When leukemic burden reached 20-40% in blood, mice were randomized and injected *i.p.* with 200 μg/mouse anti-mouse SLAMF6[22] or mIgG2a isotype control (Clone C1.8, BioXCell, West Lebanon, NH) (n=16 for each group). Mice were injected a total of 3 times, bi-weekly and were euthanized one week after the third injection. Cells from the peritoneal cavity (PerC) were collected by flushing 10 ml PBS + 2%FBS and subsequent drawing of the fluid from the PerC. Bone marrow cells were flushed from the femurs with 5 ml PBS + 2%FBS. Single cell suspensions were prepared from spleen and red blood cells (RBC) were removed by RBC lysis buffer (Sigma, St. Louis, MO). Serum was obtained by centrifugation at 8,000xg for 10 minutes at RT.

NP-OVA Immunization

WT mice were immunized intraperitoneally with 50μg of NP-ovalbumin (NP-OVA) (Biosearch Technology) precipitated with complete Freund's adjuvant (CFA) (Difco). One day post immunization, mice were randomized and injected *i.p.* with either 200 μg/mouse isotype control or αSLAMF6 antibody. Mice were analyzed on day 9 post-immunization.

Flow Cytometry

Antibodies used for flow cytometry are listed in Supplementary Table I. Surface staining was done by first incubation with 20% rabbit serum + Fc block for 10 minutes at RT and the prepared antibody cocktails were incubated for 30 minutes at 4°C. Samples were washed twice with FACS buffer (PBS+ 2%FBS) and analyzed on a five-laser BD LSRII analyzer (BD Biosciences, Oxford, UK). Viable cells were gated as Diaminidino-2-phenylindole (DAPI) negative and doublets were excluded. Results were analyzed by FlowJo software (Ashland, Oregon).

Serum Cytokine Quantification

Interleukin 10 (IL-10) levels were measured by Biolegend (San Diego, CA) LEDENDPLEX bead based immunoassay, according to manufacturer's protocol.

Identification of cytotoxic $CD8^+$ T cells

Fresh splenocytes isolated from mice were cultured with 50ng/ml phorbol myristate acetate (PMA) and 1μg/ml ionomycin for 4 hours in the presence of brefeldin A (1μl/ml) at 37°C with 5% CO_2. After 4 hours, cells were washed twice with PBS and cells were stained for CD3 and CD8, as mentioned in flow cytometry section. Next, cells were fixed and permeabilized using BD Cytofix/Cytoperm kit, according to manufacturer's protocol and intracellular staining for Granzyme B, IFNγ, IL-2 were performed. For CD107a staining, the antibody was added to the culture in the beginning for cell surface staining of CD107a upon degranulation.

Histology

Liver sections were fixed in 10% formalin and stained with H&E at the BIDMC histology core.

Statistical Analysis

Statistics for normally distributed mouse data sets were done by unpaired Student t test, whereas for non-normally distributed sets, two-sided Mann Whitney U test was used. For human CLL data, nonparametric Wilcoxon signed-rank test was used. Values reported as mean ± SD and analyses were carried out using GraphPad Prism (La Jolla, CA).

Results

Absence of SLAMF6 increases the $PD1^+$ subpopulation of $CD3^+CD44^+CD8^+$ T cells in response to murine CLL cells.

We first assessed whether expansion of the Eμ-TCL1 CLL cells would be affected upon transferring into irradiated $SLAMF6^{-/-}$ mice instead of WT mice. When Eμ-TCL1 mice recipients were euthanized 35 days after the transfer of tumor containing splenocytes, we found no significant difference in spleen size or total number of splenocytes **(Supplementary Figure 1a and 1b**). As judged by the percentage of $CD19^+CD5^+$ TCL1 cells among lymphocytes in spleen, blood, peritoneal cavity

(PerC) and bone marrow leukemic infiltration was unaffected by the absence of SLAMF6 interactions in the tumor microenvironment (**Supplementary Figure 1c**). We then assessed SLAMF6 expression on the surface of $CD4^+$ and $CD8^+$ T cells from WT recipients and levels were comparable (**Supplementary Figure 2a and 2b**).

While there was no difference in the percentages of $CD3^+$ T cells (CD3: 6.1±0.2 vs. 7.5±0.6%), we observed a decrease in CD4/CD8 ratios in SLAMF6-/- mice as compared to the WT recipients (CD4/CD8 ratio 1.7±0.12 vs. 2.2±0.18, p=0.04). As shown in **Figure 1a**, a significant decrease in $CD3^+CD4^+$ concomitantly with an increase in $CD3^+CD8^+$ T cell percentage was observed in the spleen. Within CD4 and CD8 T cell subsets, there was no significant shift in naïve ($CD62L^+CD44^-$) and effector/memory ($CD62L^-CD44^+$/$CD62L^+CD44^+$) subsets (**Figure 1b and Supplementary Figure 1d**). However, among the activated antigen experienced $CD3^+CD44^+CD8^+$ T cells, we found significantly increased proportions of a $PD1^+$ subpopulation in $SLAMF6^{-/-}$ recipients compared to WT mice (4.5±0.7 vs. 13.1±3 p=0.0019) (**Figure 1c, left panel**). Furthermore, a tenfold increase in numbers of $PD1^+$ $CD3^+CD44^+CD8^+$ cells was detected in $SLAMF6^{-/-}$ *vs.* WT recipients ($1.2±0.4 \times 10^6$ vs. $1.7±0.2 \times 10^5$, p<0.0001) (**Figure 1c, right panel**), which suggested that the presence of SLAMF6 negatively regulates T cell exhaustion.

Next, we determined whether major alterations in T cell effector functions had occurred in SLAMF6-/- recipient mice. To this end, splenocytes were stimulated *in vitro* with phorbol myristate acetate (PMA) and ionomycin for 4 hours. As judged by surface staining of CD107a (23.2±3.3 vs. 18.5±0.7%, ns), and intracellular Granzyme B (41.9±2.5 vs. 28±1.7% *p=0.002*) and IFNγ (46.5±0.9 vs. 38.2±1.8% *p=0.003*), the CTL effector functions of CD8+ T cells isolated from SLAMF6-/- recipients were impaired, while IL-2 production remained unchanged (**Figure 1d and e**).

Taken together, these findings suggest that expression and signaling through SLAMF6 negatively controls expansion of $PD\text{-}1^+$ $CD44^+CD8^+$ T cells and subsequent T cell exhaustion in recipients of Eμ-TCL1 CLL cells.

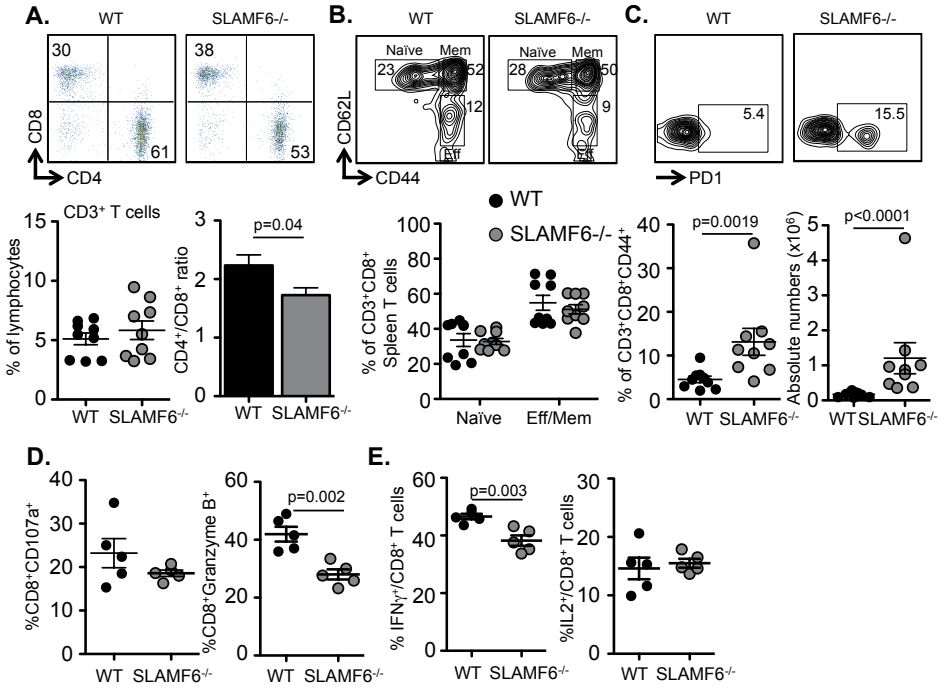

Figure 1. Expansion of TCL1 cells upon transfer into SLAMF6-/- mice coincides with reduced effector functions and increased exhaustion of CD8$^+$ T cells.

Splenocytes from Eμ-TCL1 mice were transferred into irradiated (4 Gy) WT or SLAMF6-/- mice and after 35 days the recipient mice were sacrificed for analysis. (a) Cells from WT (n=5) or αSLAMF6 (n=5) injected mice were stained for CD3, CD4 and CD8, CD44 and CD62L; dead cells were excluded as DAPI negative. CD3$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ T cell percentages in spleen compared between WT and SLAMF6-/- mice. (b) Naïve (CD62L$^+$CD44$^-$), Effector/Memory (CD62L$^-$CD44$^+$, CD62L$^+$CD44$^+$) subsets as percentage of CD3$^+$CD8$^+$ T cells in spleen of WT and SLAMF6-/- mice. (c) Percentage of PD1+ among CD3$^+$CD44$^+$CD8$^+$ T cells from spleen. (d) Fresh total splenocytes from WT and SLAMF6-/- injected mice were cultured with PMA/Ionomycin for 4 hours in the presence of Brefeldin A. After cell surface staining with CD3 and CD8, cells were fixed and permeabilized for intracellular staining. CD107a antibody was added to the culture in the beginning. CD107a and Granzyme B levels as a measure of cytotoxic capacity of CD8$^+$ T cells from WT and SLAMF6-/- are compared. (e) Levels of IFNγ and IL-2 as a measure of effector function of CD8$^+$ T cells in WT vs. SLAMF6-/- group. All graphs depict mean ± SD. P values are as shown.

Administering α-mouse-SLAMF6 limits expansion of adoptively transferred Eμ-TCL1 CLL cells in WT mice.

To evaluate whether αSLAMF6 antibody induced signaling would have an effect on T cell responses and leukemic expansion, we adoptively transferred tumor bearing total splenocytes from Eμ-TCL1 mice (15-20x10^6 / mouse) into WT

mice. When TCL1 cell infiltration in the blood approached 20-40%, mice were randomized into two groups; one of which received once a week *i.p.* αSLAMF6 200μg/mouse and the other mouse IgG2a isotype control (**Figure 2a**). One week after the third injection, the recipient mice were sacrificed and analyzed for expansion of the transferred Eμ-TCL1 CLL cells. Spleen weight and total number of splenocytes of αSLAMF6-injected recipient mice were significantly reduced compared to isotype control mice (**Figure 2a)** (spleen weight 0.95±0.04 vs. 0.38±0.1g p=0.003, total splenocytes 6.1±1.03x10^8 vs. 0.74±0.16x10^8 p<0.0001). Leukemic cell infiltration in the spleen was also significantly reduced as judged by the percentage of $CD19^+CD5^+$ lymphocytes (79.7±1.7 vs. 37.3±5.1% p<0.0001) (**Figure 2a right panel**). Consistent with this, αSLAMF6 treated recipient mice exhibited a significantly reduced number of TCL1 cells in the blood (73.4±2.3 vs. 38.5±3.8% p<0.0001) and infiltrating the bone marrow (5.5±1.3 vs. 0.9±0.1% p<0.0001) (**Figure 2b**). As judged by H&E staining, tumor infiltration in the liver was also diminished by administering αSLAMF6 (**Figure 2c**).

Previously we had shown that αSLAMF6 failed to reduce the leukemic burden in the PerC of SCID mice into which the aggressive CLL clone TCL1-192 had been transferred[30]. Surprisingly, upon administering αSLAMF6 a significantly reduced number of leukemic cells (3.1±0.68 vs. 1.1±0.26 x10^7 p=0.01) as well as a significantly reduced percentage of leukemic engraftment (91.4±1.1 vs. 79.8±2.7% p=0.001) was found in the PerC of WT recipients of Eμ-TCL1 cells (**Figure 2d**). This suggests that αSLAMF6 might induce T cell- signaling in the PerC, but not ADCC, which is known to be impaired in the PerC[32].

Eμ-TCL1 CLL cells in the spleen, blood and PerC of αSLAMF6-injected mice appeared significantly more pro-apoptotic, as measured by percentage of $AnnexinV^+$ (**Figure 2e**). Furthermore, IL-10, a cytokine that promotes CLL cell survival, was significantly reduced in serum of αSLAMF6-injected mice (**Figure 2f).** We conclude that leukemic cell expansion and viability are significantly reduced by administering αSLAMF6 into WT mice in which Eμ-TCL1 CLL cells have been adoptively transferred.

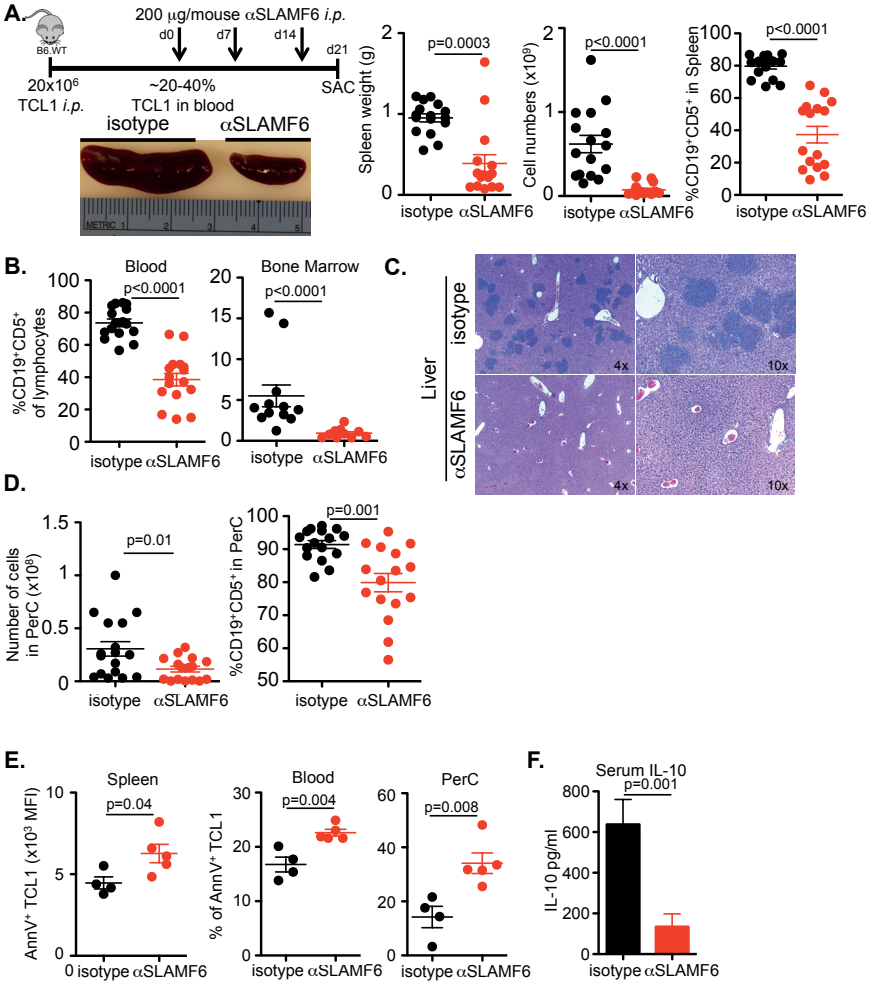

Figure 2. *In vivo* injection of mouse αSLAMF6 effectively limits expansion of TCL1 CLL cells in WT mice.

8-12 weeks old WT mice transplanted with 15-20x10^6 total splenocytes from Eμ-TCL1 mice, was randomized and injected 200 μg/mouse αSLAMF6 (n=16) or isotype control (n=16) when leukemic burden reached 20-40% in blood. After three biweekly injections, mice were euthanized and analyzed. (a) Schematic outline of injection and representative spleen pictures. Spleen weight, total number of splenocytes and % CD19$^+$CD5$^+$ TCL1 significantly reduced in αSLAMF6-injected mice compared to isotype-injected group. (b) Percentages of TCL1 (CD19$^+$CD5$^+$) in blood and bone marrow compared between isotype and αSLAMF6 injected group. (c) Representative H&E staining from liver and spleen of isotype and αSLAMF6 injected mice. (d) Numbers and percentage of TCL1 engraftment to PerC (e) TCL1 cells isolated from spleen, blood and PerC of αSLAMF6-injected group (n=5) compared for pro-apoptosis by % of AnnexinV$^+$ to TCL1 cells from isotype injected group (n=4). (f) Blood was collected from mice and serum levels of IL-10 (pg/ml) was compared between isotype (n=12) and αSLAMF6 (n=11) injected mice. All graphs depict mean ± SD. P values are as shown.

Anti-SLAMF6 reduces the number of exhausted CD8$^+$ T cells, which correlates with an increase in effector functions.

Surprisingly, the percentage of CD3$^+$ T cells was increased in the spleen and blood (Spleen: 6.6±0.7 vs. 15.3±2.3% p=0.0002, Blood: 7.6±0.9 vs. 17.4% p=0.01) as a consequence of administering αSLAMF6 into Eμ-TCL1 CLL-bearing WT mice (**Figure 3a**). The CD4/CD8 ratio among CD3$^+$ T cells was significantly reduced in the spleen of αSLAMF6-injected mice, but not in the blood (Spleen: 1.18±0.1 vs. 0.74±0.17 p=0.04, Blood: 0.68±0.06 *vs.* 0.61±0.2) (**Figure 3b**). Indeed, there was a shift among splenic CD8$^+$ T cells from naïve (CD62L$^+$CD44$^-$) to antigen-experienced effector memory (CD62L$^+$CD44$^+$) phenotype (Naïve 30.5±4.1 vs. 14.5±3.1% *p=0.02*, Eff/mem 45±5 vs. 64.8±4.3% *p=0.02*) (**Figure 3c**). This shift in CD8$^+$ T cell subsets was also observed in the blood (Naïve 30.6±3.7 *vs.* 8.9±2.6%, *p=0.0008*, Eff/mem 54.9±4.5 *vs.* 80.8±3.9%, *p=0.001*) and the PerC (Naïve 38.7±4.1 vs. 11.8±2.04% *p=0.0004*, Eff/mem 52.5±4 vs. 75.8±1.9,

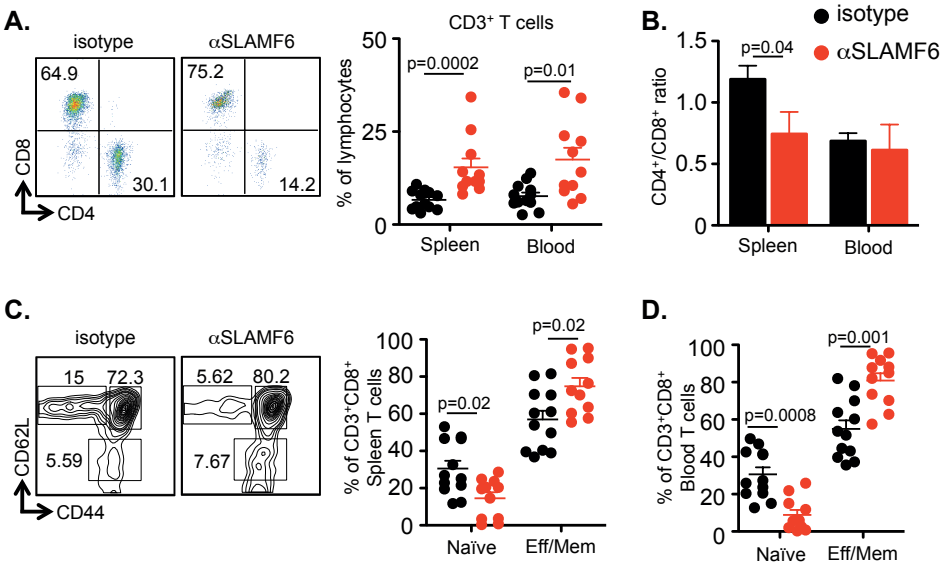

Figure 3. Anti-SLAMF6 causes a skewing of CD8 T cells toward a memory phenotype.

Cells from isotype (n=12) or αSLAMF6 (n=11) injected mice were stained for CD3, CD4 and CD8, CD44 and CD62L; dead cells were excluded as DAPI negative. (a) Representative FACS plots for CD4/CD8 T cells pre-gated on CD3$^+$ samples are as shown. CD3$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ T cell percentages in spleen compared between isotype and αSLAMF6 injected group. (b) CD3$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ T cell percentages in blood. (c) Representative FACS plots for CD8$^+$ T cell subsets: Naïve (CD62L$^+$CD44$^-$), Effector/Memory (CD62L$^-$CD44$^+$, CD62L$^+$CD44$^+$). Subsets presented as percentage of CD3$^+$CD8$^+$ T cells in spleen of isotype vs. αSLAMF6 injected. (d) Subsets presented as percentage of CD3$^+$CD8$^+$ T cells in blood. All graphs depict mean ± SD. P values are as shown.

p=0.0009) (**Figure 3d** and **Supplementary Figure 3a-b**). By contrast, $CD4^+$ T cell subsets, *i.e.* $CD62L^+$ $CD44^-$ or $CD62L^+CD44^+$, were not affected by the αSLAMF6 antibody (**Supplementary Figure 3c**).

Because exhausted $CD8^+$ T cells display a loss of effector function in CLL patients[6,7,33], we further analyzed exhausted $CD8^+$ T cells after injecting αSLAMF6. Remarkably, the percentage of activated $CD3^+CD44^+CD8^+$ T cells in the spleen that expressed the well-known marker PD-1 in the αSLAMF6 injected group was significantly lower compared to that in isotype control mice (3.1±0.5% vs. 11.7±1.6 *p=0.0002*) (**Figure 4a, left panel**). The absolute number of $PD1^+$ $CD3^+CD44^+CD8^+$ T cells was also markedly reduced in the spleen (0.1±0.03 vs. 0.6±0.1 $x10^6$, *p=0.0003*) (**Figure 4a, right panel**).

This was confirmed in the blood by a reduction of the percentage of $PD1^+$ $CD3^+$ $CD44^+$ $CD8^+$ T cells in αSLAMF6-injected mice as compared to isotype controls (5±1 vs. 11.1±1.2 % *p=0.001*) (**Figure 4b**). In contrast, in the PerC of αSLAMF6-injected mice the percentage of $PD\text{-}1^+$ cells among $CD3^+CD44^+CD8^+$T cells was increased (9.5±1.1 vs. 3.6±1.9 % *p=0.03*) (**Figure 4c, left panel**). However, the increase in absolute numbers did not reach statistical significance possibly due to the smaller group sizes (1.7±0.4 vs. 0.5±0.3 $x10^4$, *p=0.09*) (**Figure 4c, right panel**).

The proportion of $CD3^+CD44^+CD8^+$ T cells expressing the exhaustion markers CD160, LAG3 and KLRG1, was also significantly reduced in the spleen after injection of αSLAMF6 (**Supplementary Figure 4**).

To test whether the phenotypical changes in the exhausted $CD8^+$ T cell compartment of αSLAMF6-injected correlated with an increase in effector functions, splenocytes isolated from αSLAMF6 injected mice were *in vitro* stimulated with PMA/Ionomycin and Brefeldin A for 4 hours. Intracellular staining of $CD8^+$ T cells from αSLAMF6-injected mice showed significantly increased lysosomal CD107a (6.7±0.3 vs. 8.8±0.50% *p=0.003*) and Granzyme B (14.3±0.6 *vs.* 21.8±1.5% *p=0.001*) (**Figure 4d**). Additionally, IFNγ (35.5±2.9 *vs.* 46.8±1.7% *p=0.003*) and IL-2 (8.7±0.9 *vs.* 12±0.5% *p=0.009*) expressing $CD8^+$ T cells were significantly increased (**Figure 4e**).

We conclude that SLAMF6 is a negative checkpoint inhibitor, which restricts $CD8^+$ T cell exhaustion in response to murine CLL cells. Consequently, signaling

induced by αSLAMF6 reduces the number of exhausted CD8⁺ T cells, which empowers CTL responses to the leukemic cells.

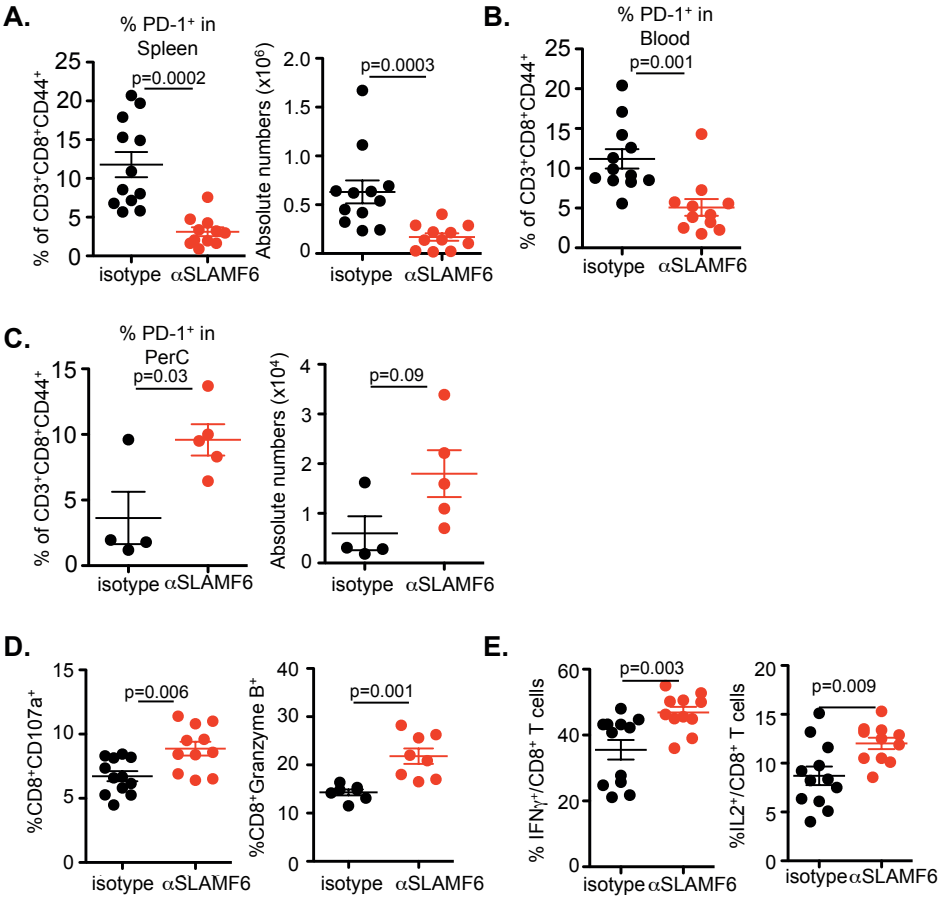

Figure 4. ***In vivo*** **αSLAMF6 injections reduce PD1⁺ CD3⁺CD44⁺CD8⁺T cells and improve effector functions**

(a) Percentage and absolute numbers of PD1⁺ T cells among antigen-experienced CD8 T cells (CD3⁺CD44⁺CD8⁺) in spleen (b) percentages in blood, and (c) percentage and absolute numbers in PerC in isotype vs. αSLAMF6 injected mice. (d) Fresh total splenoytes from isotype and αSLAMF6 injected mice were cultured with phorbol myristate acetate (PMA)/Ionomycin for 4 hours in the presence of Brefeldin A. After cell surface staining with CD3 and CD8, cells were fixed and permeabilized for intracellular staining. CD107a antibody was added to the culture in the beginning. CD107a and Granzyme B levels as a measure of cytotoxic capacity of CD8⁺ T cells from isotype and αSLAMF6 are compared. (e) Levels of IFNγ and IL-2 as a measure of effector function of CD8⁺ T cells in isotype vs. αSLAMF6 group. All graphs depict mean ± SD. P values are as shown.

Anti-SLAMF6 antibody exerts its function on CD8$^+$ T cells in mice immunized with hapten antigen.

To test whether this effect we see on T cells after αSLAMF6 injection into TCL1 bearing WT mice applies to other systems as well, we used WT mice immunized with NP-OVA in Complete Freund's adjuvant (CFA). Nine days after αSLAMF6/ isotype injection mice were sacrificed and spleen was analyzed for T cell compartment (**Supplementary Figure 5a**). While total % of CD3$^+$ T cells were decreased, proportion of CD4 and CD8 T cell subsets remained unchanged (**Supplementary Figure 5b**). We found an increase in percentage of effector/memory CD4$^+$ T cells (**Supplementary Figure 5c**). In line with our finding in the CLL model, injection of αSLAMF6 decreased naïve and increased effector/memory CD8$^+$ T cells with reduced percentage of PD1$^+$ CD3$^+$CD44$^+$ CD8$^+$ T cell subpopulation (**Supplementary Figure 5d**).

Cooperation of the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib and aSLAMF6 in CLL therapy.

Thus far the outcomes of our studies indicate that in addition to its ability to attack the B cell leukemia by ADCC and reduce BCR signaling[30], αSLAMF6 also restores the CTL functions of exhausted CD3$^+$CD44$^+$CD8$^+$ T cells. Given that αSLAMF6 is capable of reducing BCR signaling alone, we evaluated whether combining ibrutinib with αSLAMF6 would cooperate in the *in vivo* clearance of mouse CLL cells. To this end, we transplanted WT mice with Eμ-TCL1 CLL cells and when the transplanted cell numbers reached a 20-40% level in the blood, mice were randomly divided into 4 groups for treatment as indicated in **Figure 5a**. Mice were given either 200μg/mouse αSLAMF6 weekly injections 3 times, ibrutinib daily 25mg/kg ibrutinib, or combination of both. Mice that received αSLAMF6 or ibrutinib alone had significantly smaller spleen sizes (Control: 0.67±0.03g, αSlamf6: 0.22±0.01g, Ibrutinib: 0.48±0.04g, Combo: 0.14±0.01g), accompanied by significantly reduced absolute numbers of CD19$^+$CD5$^+$ TCL1 cells as compared to control group (Control: 6.5±0.49x10^8, αSlamf6: 1±0.09x10^8, Ibrutinib: 3.5±0.3x10^8, p values shown on figure) (**Figure 5b**). This was further reduced in mice that received combination of αSLAMF6 and ibrutinib (Combo: 0.65±0.08x10^8). These findings suggested that combining ibrutinib with αSLAMF6 further improved potency of the antibody in Eμ-TCL1 transfer model.

To begin to apply the outcomes of our studies for therapeutic purposes, we set up *in vitro* experiments with a humanized anti-human-SLAMF6 monoclonal antibody (αhSLAMF6). This allowed us to determine viability of human CLL cells with αhSLAMF6 in the presence of and in combination with ibrutinib. We

used peripheral blood mononuclear cells (PBMCs) from IGHV unmutated CLL patients with varying levels of IgM surface expression (**Supplementary Table**

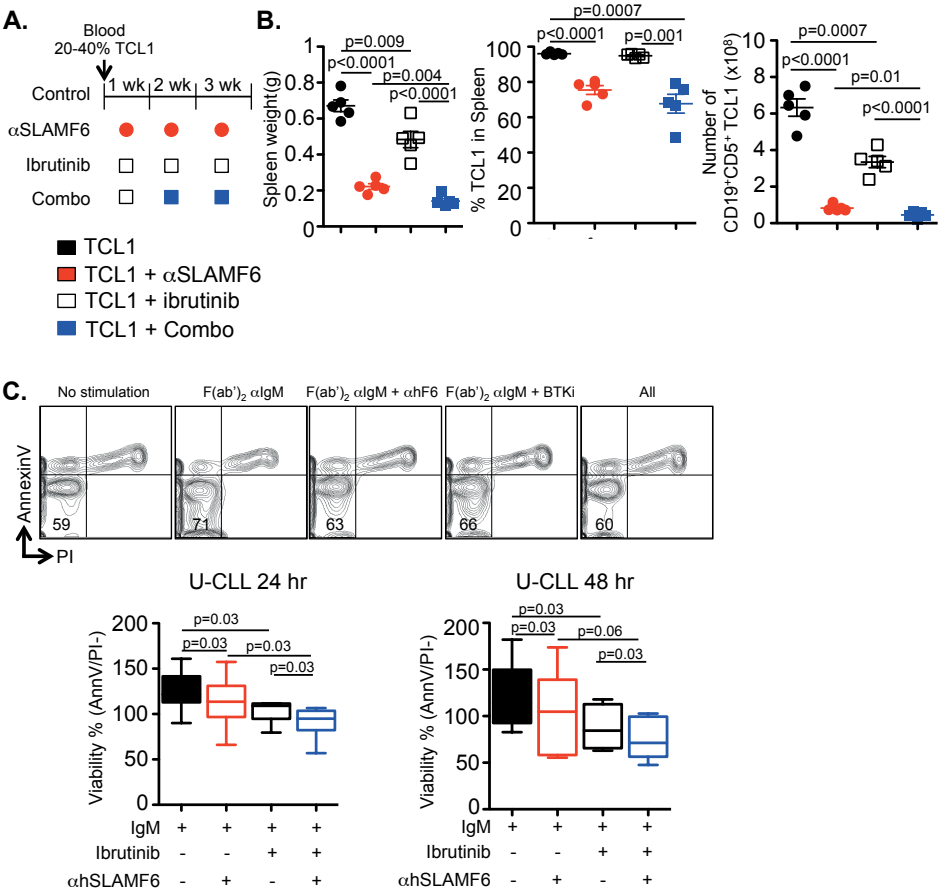

Figure 5. Anti-SLAMF6 in combination with ibrutinib further reduces the leukemic burden in TCL1 bearing mice

(a) Schematic Outline. Mice bearing 20-40% TCL1 in blood after transfer were randomized to 4 groups and received either 200 μg/mouse αSLAMF6 weekly injections, 3 times, one group was oral-gavaged daily with 25 mg/kg ibrutinib for 3 weeks and another group was initially started with daily ibrutinib for 1 week and then received 2 injections of αSLAMF6 along with ibrutinib. Mice were sacrificed and analyzed after 3.5 weeks (n=5 each group). (b) Spleen weight, % of $CD19^{+}CD5^{+}$ TCL1 in spleen and absolute numbers in spleen are as depicted, with relative p-values. (c) PBMCs from IGHV unmutated CLL patients (n=6) were cultured in presence of anti-human $F(ab')_2$ IgM, ibrutinib, and αhSLAMF6 or in combinations for 24 and 48 hours. Viability of cells were measured by AnnexinV/PI staining. Representative gating is as shown. Viability of nonstimulated wells was set to 100% as a baseline and percentage of viability was calculated accordingly. All graphs depict mean ± SD. P values are as shown.

II). Ibrutinib alone served as a positive control for the system, as its interference with BCR-induced survival *in vitro* is well established[34]. CLL cells were either stimulated with anti-human IgM or left unstimulated. In addition, ibrutinib, αhSLAMF6, or both were added to the cells and CLL viability was expressed as percentage of AnnexinV/PI negative cells 24 or 48 hours after stimulation (**Figure 5c**). We found that upon BCR stimulation, addition of αhSLAMF6 alone reduced viability to comparable levels as those of ibrutinib and this was enhanced if the two were combined, suggesting that the improved effect of combining αSLAMF6 and ibrutinib we observed *in vivo* also applies to human CLL cells, in an *in vitro* setting.

Discussion

Overcoming immune evasion, a phenomenon by which pathogens and cancer cells escape the host immune system, is being actively pursued to identify immune checkpoints for therapeutic targeting. Chief among the successfully exploited mechanisms is the well-characterized PD1/PD-L1 axis; blockade of this pathway has proven to be effective in solid tumors as well as hematologic malignancies[12,13].

CLL is well known to generate impaired immune responses in the host, with the malignant clone residing in well-vascularized tissues and circulating in peripheral blood but also in close proximity to effector cells that are capable, if activated appropriately, of carrying out a cytotoxic response. Defective T cell responses have been observed in CLL patients, including imbalance of T cell subsets, inability to form immune synapses between CLL B cells and T cells, increased expression of inhibitory receptors (e.g. PD-1, CD160, LAG3), and loss in proliferation and cytotoxic capacity[6,7,11,33,35]. These in turn result in ineffective antitumor responses. Thus, treatment protocols are based on agents with the ability to generate an immune response *e.g.*, anti-CD20 monoclonal antibody (mAb), checkpoint inhibitors or cellular therapies[36].

Currently, the most effective method of studying the impact of a tumor microenvironment on a host immune system is through the use of murine models[37]. In this study, we employ the Eμ-TCL1 adoptive transfer model to assess the relevance of SLAMF6 in the murine CLL microenvironment and the usefulness of targeting this receptor with a monoclonal antibody αSLAMF6 to improve its therapeutic action on leukemic cell expansion and enhance $CD8^+$ T cell functions. When Eμ-TCL1 leukemic cells were made to reside in a tumor microenvironment that lacks SLAMF6, *i.e.* the SLAMF6-/- mouse, we observed an expansion of a $PD1^+$ subset

of $CD3^+CD44^+CD8^+$ T cells with reduced cytotoxic functions. This supports the concept that SLAMF6-SLAMF6 interactions, and possibly intracellular signaling initiated from this interaction, are important in the development of $CD8^+$ T cell functions.

This concept was further supported by the outcomes of our experiments with αSLAMF6 mAb, which *in vivo* reduced the leukemic burden in the Eμ-TCL1 adoptive transfer model. Administering αSLAMF6 reduced the number and proportion of $PD1^+$ $CD3^+CD44^+CD8^+$ T cells concomitantly with an increase in cytotoxicity, as determined by expression of CD107a and Granzyme B. Possible reasons for reduction in exhausted $PD1^+$ $CD8^+$ T cells include induction of ADCC and/or down-regulation of PD1 from the cell surface after SLAMF6 ligation. SLAMF6 serves as a co-stimulatory receptor in T cells and recruits SAP to its cytoplasmic tail. We found no effect of SAP deficiency in this system when murine CLL cells were transferred into SAP-/- mice (**Supplementary Figure 6)**. This suggests that our findings are independent of SAP and whether there is a direct link between SLAMF6 and PD1 signaling needs further investigation.

Thus, empowering $CD8^+$ T cell effector functions adds to the ability of αSLAMF6 to control disease by removing the CLL cells by ADCC and downregulating of BCR signaling. This dual activity of αSLAMF6 is in contrast with the mechanism by which αCD20 and ibrutinib control CLL in patients [36] and in a CLL mouse model (BY & CT, unpublished data). The mechanisms of actions of αSLAMF6 antibody in CLL are summarized in **Figure 6**. Primarily, αSLAMF6 induces activation of ADCC mediated by engagement of antibody Fc portion with Fc receptors on macrophages and NK cells[30]. Secondly, signaling of SLAMF6 in CLL B cells to reduce proximal BCR signaling and survival in both murine and human models. Lastly, antibody binding to SLAMF6 on the surface of T cells causes reduction in the number of $PD1^+$ $CD3^+CD44^+CD8^+$ T cells and results in increased effector functions of remaining $CD8^+$ T cells. This finding of the αSLAMF6 antibody applies to other models as well. Previously, our lab had demonstrated that injections of αSLAMF6 inhibited antibody responses in an immunization model[28]. Inhibition of antibody production coincides with our finding that αSLAMF6 reduces BCR signaling, thus survival of B cells. Similarly, analyzing the CD8 T cell compartment in this model, we again found reduced percentage of $PD1^+$ $CD3^+CD44^+CD8^+$ T cells (**Supplementary Figure 5d**). This suggests that there is a selection by the antibody towards $PD1^+$ subpopulation in activated $CD8^+$ T cells and this is irrespective of the disease model used.

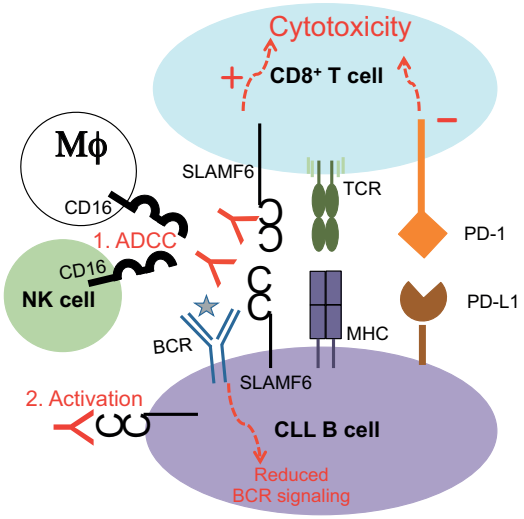

Figure 6. Mechanism of action of αSLAMF6 in mouse model of CLL.

One mechanism involves activation of antibody dependent cellular cytotoxicity (ADCC) mediated by engagement of antibody Fc portion with Fc receptors on NK cells. A second mechanism involves signaling of SLAMF6 in CLL B cells to reduce proximal BCR signaling and survival. Other mechanism constitutes of reduction of PD1⁺ CD3⁺CD44⁺CD8⁺ T cells upon αSLAMF6 triggering (Anti-SLAMF6 antibody and its targets are indicated in red).

Nicely complementing our findings in CD8⁺ T cells, SLAMF6 was reported to play a role in trogocytosis, the transfer of membrane patches from target to immune effector cells[38]. Trogocytosis and cytotoxic capacity of tumor specific CD8⁺ T cell clones from melanoma patients correlated with signaling of SLAMF6. Blocking antibodies against SLAMF6 reduced cytotoxic effector functions, suggesting a co-stimulatory role for SLAMF6 in T cell functional diversity[38]. It would be of interest to test whether our αSLAMF6 antibody enhances trogocytosis.

One of the intriguing outcomes of the current study was that the leukemic burden and infiltration of Eμ-TCL1 cells in the PerC was lowered by αSLAMF6 (**Figure 2d**). Leukemic cells, as well normal B cells show different characteristics when they are in the PerC or in spleen and one idea is that the PerC is a hypoxic niche that favors adhesion and growth of tumors[39-42]. We had previously demonstrated no ADCC effect of αSLAMF6 in PerC in a T-cell independent system, similar to that observed with αCD20 injections[30,32]. While the ADCC function of an antibody is eliminated in this niche due to the microenvironment, signaling through SLAMF6 in T cells was able to reduce tumor infiltration in this niche. Interestingly, αSLAMF6 antibody was selectively targeting PD1⁺ CD3⁺CD44⁺CD8⁺ T

cells in spleen and blood and possibly induced their killing by ADCC. However, as the antibody was unable to induce ADCC in the PerC, it enriched for $PD1^+$ $CD8^+$ T cells. This suggests that there is a correlation between PD1 and SLAMF6 signaling and understanding the mechanisms behind this may be relevant therapeutically. PD-1 contains a cytoplasmic tail with an ITSM motif that binds SHP-1 and SHP2, similar to that in SLAMF6 [43-45]. There may be a competition for binding of SHP1/2 that in turn dictates the responses from T cells. Furthermore, understanding these niche dependent changes to tumor killing is important, as there may be niches in the human body that are not accessible for certain type of treatments.

The change in CD4/CD8 T cell ratio appears to be associated with CLL progression[5,9,46,47]. This association is complemented by the findings that both CD4 and CD8 T cells acquire a $PD1^+$ phenotype that is associated with their inability to perform effector functions[9,11,46-48]. While the shift in CD4/CD8 ratio appears to be inverted in our system, αSLAMF6 is able to restore the phenotype and functions of exhausted CD8 T cells, which would support the notion that exhausted T cells need to be re-activated within suppressive tumor microenvironments for anti-tumor immunity.

Recently Ayers and colleagues[49] identified IFNγ – related gene expression profiles that would predict response to PD-1 checkpoint blockade in variety of tumor types. Starting with 19 and validating in 62 melanoma patients, a "preliminary expanded immune" 28-gene set correlating with IFNγ signatures was identified. One of these genes was SLAMF6. IFNγ signaling is known to associate with a T-cell inflamed microenvironment that responds to anti-PD1 therapy. This may be a platform to test combination of αSLAMF6 together with αPD1 for a better T cell response against various tumors.

In this study we chose to combine αSLAMF6 together with ibrutinib. Besides ibrutinib's ability to induce migration out of niches and reduce BCR signaling[34], it is also known to improve T cell function and responses in patients with CLL[50-52]. Although ibrutinib has been combined in clinic with rituximab, studies showed that ibrutinib interferes with ADCC function of rituximab by down-regulating CD20 from surface of CLL B cells[53]. This suggests that there is still need for better combinations. In contrast to rituximab, besides αSLAMF6 antibody's ability to induce ADCC, improvement in effector $CD8^+$ T cell responses makes αSLAMF6 an intriguing candidate for therapy. We propose that the combination of ibrutinib

and αSLAMF6 of should further be explored in B cell leukemias and lymphomas a clinical setting.

Author contributions: BY, NW and CT planned the experiments, which were conducted and analyzed by BY and NW. BY, NW, PE and CT wrote the manuscript. ETH co-analyzed the experiments and edited the manuscript. JAB provided fresh CLL samples. AKB generated and interpreted the histology images. SSC and NC provided input to the experiments and edited the manuscript. All authors were actively involved in discussion of the project. All authors read and commented on the manuscript.

Conflict of interest: Authors declare no conflict of interest.

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Supplementary Material

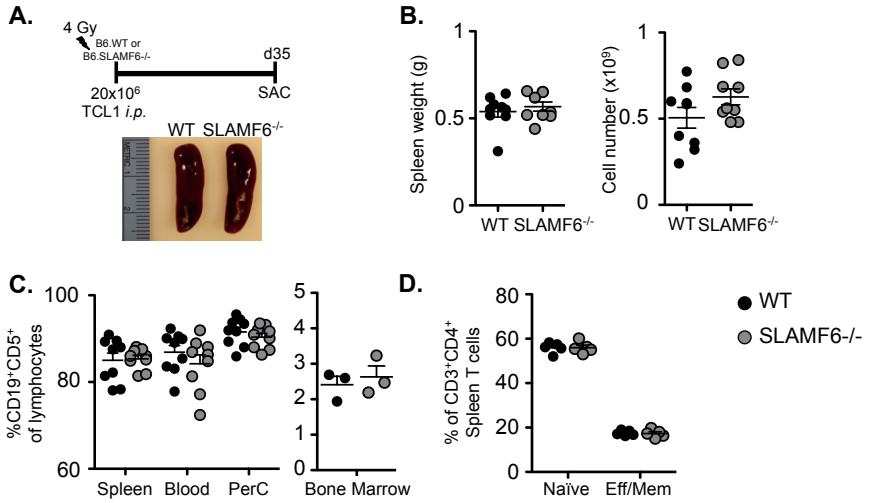
Supplementary Table I – Antibody Panel used for Flow Cytometry

TCL1 tumors		T cell markers		Exhaustion markers		Intracellular staining	
Antibody	Clone	Antibody	Clone	Antibody	Clone	Antibody	Clone
CD19	eBio1D3	CD3	17A2	CD160	eBioCNX46-3	CD107a	1D4B
CD5	53-7.3	CD4	RM4-5	LAG3	eBioC9B7W	Granzyme B	16G6
PD-L1	MIH5	CD8	53-6.7	Slamf4	eBio244F4	IFNγ	XMG1.2
IgK	RMK-45	CD44	1M7	KLRG1	2F1/KLRG1		
		CD62L	MEL-14	PD1	RMP1-30		
		SLAMF6	330-AJ				

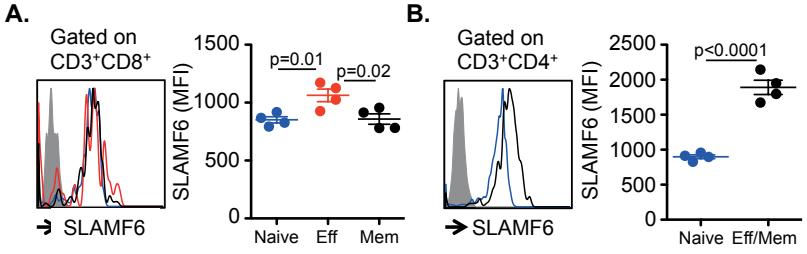
Supplementary Table II – CLL patient information

CLL #	Rai Stage	Age	Sex	CD38	ZAP70	Cytogenetics
1	I	68	M	Negative	Positive	Trisomy 12
2	II	63	F	Negative	Positive	del(17p)
3	I	54	M	Negative	Positive	del(13q)
4	0	55	F	Positive	Positive	del(17p), del(13q)
5	I	74	F	Negative	Negative	Trisomy 12
6	0	72	M	Negative	Negative	del(11q), del(13q)

Supplementary Figure 1. Leukemic expansion in WT and SLAMF6$^{-/-}$ mice.
(a) WT and SLAMF6$^{-/-}$ mice were irradiated at 4 Gy prior to tumor injection. 20x10^6 TCL1 splenocytes were transplanted *i.p.* and leukemic cells were expanded for 35 days and mice were sacrificed. Representative spleen pictures as shown. (b) Spleen weight (left panel), total number of splenocytes (right panel) from WT and SLAMF6$^{-/-}$ mice are shown. (c) Leukemic infiltration by percentage of CD19$^+$CD5$^+$ TCL1 cells in spleen, blood, peritoneal cavity and bone marrow of WT and SLAMF6$^{-/-}$ mice. (d) Percentage of naïve (CD62L$^+$CD44$^-$) and effector/memory (CD62L$^-$CD44$^+$), pre gated on CD3$^+$CD4$^+$ T cells from spleen of TCL1 expanded WT and SLAMF6$^{-/-}$ mice.

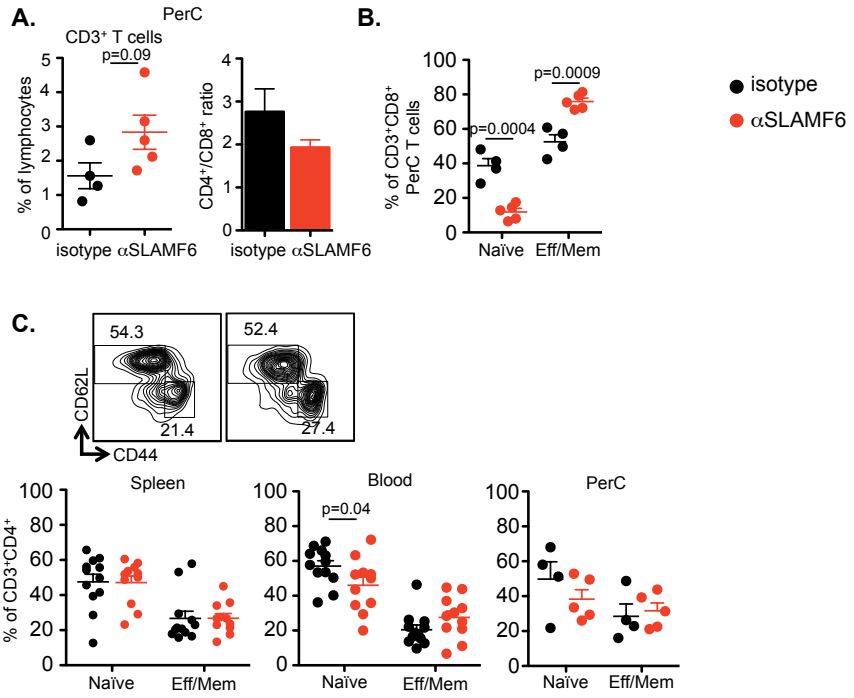


Supplementary Figure 2. SLAMF6 is expressed on subsets of T cells from TCL1 bearing WT mice.
WT mice transplanted with TCL1 splenocytes were checked for SLAMF6 expression after full leukemic expansion. (a) Representative histogram for SLAMF6 expression on Naïve (CD62L$^+$CD44$^-$), Effector/Memory (CD62L$^-$CD44$^+$, CD62L$^+$CD44$^+$), pre gated on CD3$^+$CD8$^+$ T cells FMO: gray. Right panel shows respective MFI values. P values as shown. (b) Representative histogram for SLAMF6 expression on Naïve (CD62L$^+$CD44$^-$), Effector/Memory (CD62L$^-$CD44$^+$), pre gated on CD3$^+$CD4$^+$ T cells FMO: gray. Right panel shows respective MFI values. P values as shown.



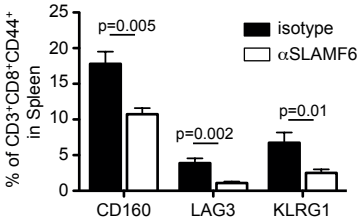
Supplementary Figure 3. CD4 T cell subsets are not affected by administering αSLAMF6 in TCL1 bearing WT mice.

(a) Percentage of CD3+ T cells from PerC of isotype and αSLAMF6 injected TCL1 bearing mice and CD4/CD8 ratios, pre gated on $CD3^+$ T cells are shown. (b) Percentage of naïve ($CD62L^+CD44^-$), Effector/Memory ($CD62L^-CD44^+$, $CD62L^+CD44^+$) of $CD3^+CD8$+ T cell subsets from PerC of isotype and αSLAMF6 injected mice. (c) Representative FACS plots for CD4 T cell subsets; pre gated on $CD3^+CD4^+$ T cells, naïve ($CD62L^+CD44^-$) and effector/memory ($CD62L^-CD44^+$) subsets. Percentage of naïve and effector/memory $CD4^+$ T cells from spleen, blood and PerC of isotype and αSLAMF6 injected mice are shown.



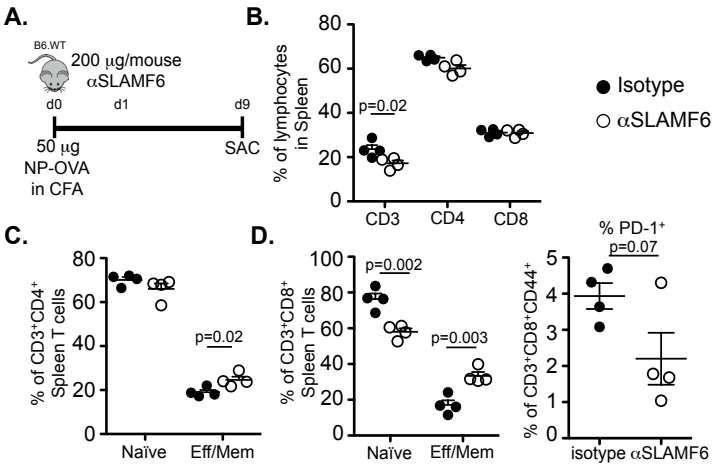
Supplementary Figure 4. The percentage of exhausted CD8$^+$ T cells in TCL1 transplanted WT mice are reduced upon administering αSLAMF6.

The percentage of CD3$^+$CD8$^+$CD44$^+$ T cells expressing CD160, LAG3 and KLRG1 in spleen of αSLAMF6 and isotype injected TCL1 bearing WT mice. P values are as shown (n=5 mice each group)

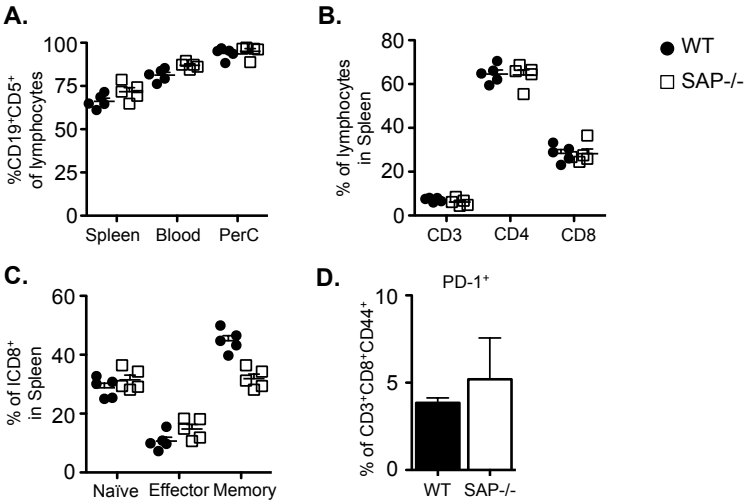


Supplementary Figure 5. Injection of αSLAMF6 into NP-OVA immunized mice reduces percentage of PD1$^+$ of CD8$^+$CD44$^+$ T cells.

(a) Schematic outline. WT mice were immunized with 50 μg NP-OVA in CFA on day 0. Next day later mice were injected either with 200 μg/mouse isotype or αSLAMF6 and mice were sacrificed on day 9. (n=4 mice each group) (b) Percentage of CD3$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ of lymphocytes in spleen are shown. (c) Among the CD3$^+$CD4$^+$ T cells, percentages of naïve and effector/memory subsets are shown. (d) Percentage of naïve and effector/memory of CD3$^+$CD8$^+$ T cell subsets (left panel) and Percentage of PD1$^+$ among CD3$^+$CD8$^+$CD44$^+$ T cells from isotype and αSLAMF6 injected mice (right panel).



Supplementary Figure 6. Leukemic expansion in WT and SAP$^{-/-}$ mice.
WT and SAP$^{-/-}$ mice were irradiated at 4 Gy prior to tumor injection. 20x10^6 TCL1 splenocytes were transplanted *i.p.* and leukemic cells were expanded for 35 days and mice were sacrificed. (a) Leukemic infiltration by percentage of CD19$^+$CD5$^+$ TCL1 cells in spleen, blood and peritoneal cavity of WT and SAP$^{-/-}$ mice. (b) Percentage of CD3$^+$, CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cell subsets in spleen. (c) Percentage of naïve (CD62L$^+$CD44$^-$) and effector and memory (CD62L$^-$CD44$^+$), pre gated on CD3$^+$CD8$^+$ T cells from spleen of TCL1 expanded WT and SAP$^{-/-}$ mice. (d) Percentage of PD1$^+$ activated CD8$^+$ T cells in spleen.



CHAPTER 5

The checkpoint regulator SLAMF3 preferentially prevents expansion of auto-reactive B cells generated by Graft-versus-Host Disease.

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Abstract

Absence of the mouse cell surface receptor SLAMF3 in SLAMF3-/- mice suggested that this receptor negatively regulates B cell homeostasis by modulating activation thresholds of B cell subsets. Here, we examine whether anti-SLAMF3 affects both B and T cell subsets during immune responses to haptenated ovalbumin [NP-OVA] and in the setting of graft versus host disease (cGVHD) induced by transferring B6.C-*H2*^{bm12}/ KhEg (bm12) CD4^{+} T cells into B6 WT mice. We find that administering one dose of αSLAMF3 to NP-OVA immunized B6 mice primarily impairs antibody responses and Germinal center B cell [GC B] numbers, whilst CXCR5+, PD-1+, and ICOS+ T follicular helper (TFH) cells are not significantly affected. By contrast, two injections of αSLAMF3 markedly enhanced autoantibody production upon induction of cGVHD by the transfer of bm12 CD4^{+} T cells into B6 recipients. Surprisingly, αSLAMF3 accelerated both the differentiation of GC B and donor-derived TFH cells initiated by cGVHD. The latter appeared to be induced by expansion of donor-derived Treg and T follicular regulatory (TFR) cells. Collectively, these data show that control of anti-SLAMF3-induced signaling is requisite to prevent autoantibody responses during cGVHD, but reduces responses to foreign antigens.

Introduction

The signaling lymphocyte activation molecule family (SLAMF) of cell surface receptors, which consists of nine trans-membrane proteins (SLAMF1-9) serve as co-stimulatory molecules at immune synapses, are involved in viral and bacterial recognition and modulate myeloid and lymphocyte development[1]. We previously found that the homophilic receptor SLAMF6 (Ly108, NTB-A) is implicated in the Germinal Center Reaction and that monoclonal antibodies directed against SLAMF6 reduce antibody responses to foreign antigens (Ags) and affect the number of auto-reactive B cells [2, 3]. SLAMF3 (CD229. Ly9) is also localized at the interface between the immune synapse, suggesting a role for SLAMF3 in regulating humoral immune responses [1, 4, 5].

Our studies with SLAMF3 deficient mice demonstrated that transitional 1, MZ, and B1a B cells were markedly expanded, whereas the development of conventional B-lymphocytes was unaltered [6]. As MZ and B1 B cells respond to foreign Ags more rapidly than conventional B cells, elevated levels of IgG3 natural Abs were found in the serum of SLAMF3-deficient mice. Furthermore, a striking increase of T-independent Abs after immunization with 2,4,6-trinitrophenyl-Ficoll was found [6]. Administering a mouse monoclonal antibody (mAb) directed against murine SLAMF3 (αSLAMF3) selectively eliminated splenic MZ B cells and significantly reduced the numbers of B1 and transitional 1 B cells in wild-type mice. Surprisingly, administering αSLAMF3 mAb or its $(Fab')_2$ fragments thereof diminished both T cell-dependent and –independent antibody responses indicating a role for SLAMF3 dependent signaling in negative regulation of humoral immune responses. The concept of SLAMF3 as negative regulator of antibody responses was further supported by the finding that aged SLAMF3-deficient mice developed spontaneous autoantibodies against nuclear antigens [6, 7].

Here, we examine whether αSLAMF3 affects both B and T cell subsets during immune responses to haptenated ovalbumin [NP-OVA] and in the setting of chronic graft versus host disease (cGVHD) induced by transferring B6.C-$H2^{bm12}$/KhEg (bm12) $CD4^+$ T cells into B6 WT mice [8-10]. We find that administering a single dose of αSLAMF3 to NP-OVA immunized B6 mice primarily impairs antibody responses and Germinal center B (GC B) cell numbers, whilst $CXCR5^+PD\text{-}1^+$, and $ICOS^+$ T follicular helper (TFH) cells are not significantly affected. By contrast, two injections of αSLAMF3 markedly enhanced autoantibody production upon induction of cGVHD by the transfer of bm12 $CD4^+$ T cells into B6 recipients [9, 10]. Surprisingly, αSLAMF3 accelerated both the differentiation of GCB and donor-derived TFHs cell initiated by cGVHD. The

latter appeared to be induced by expansion of donor-derived regulatory T (Treg) and T follicular regulatory (TFR) cells. Collectively, these data show that control of anti-SLAMF3-induced signaling is requisite to prevent autoantibody responses during cGVHD, but reduces responses to foreign antigens.

Materials and Methods

Mice

C57BL/6 (B6) WT and B6.C-2^{bm12}/KhEg (*bm12*) mice were obtained from the Jackson Laboratory. B6.C-2^{bm12}/KhEg (*bm12*) x CD45.1 mice were generated by crossing B6.C-2^{bm12}/KhEg (*bm12*) with CD45.1.B6. Experiments were conducted using age-matched 8-10 weeks old female mice. All animals are maintained under specific pathogen-free conditions at the Beth Israel Deaconess Medical Center (BIDMC) animal facility. Experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at BIDMC.

Mouse anti-SLAMF3 antibody

Mouse anti-mouse SLAMF3 (SLAMF3.7.144) (IgG1 isotype) monoclonal antibody was generated as described elsewhere [11].

NP-OVA immunizations

NP-OVA immunizations and measurement of NP-specific antibodies are described elsewhere [2]. In short, WT mice were immunized with 50μg NP-OVA in Complete Freund's Adjuvant (CFA). At the same time, mice were injected with 250μg/mouse αSLAMF3 or mIgG1 isotype control. Immunized mice were sacrificed on day 9. Cell subsets analyzed from splenocytes: TFH cells: CD4$^+$CXCR5$^+$PD1$^+$; GC B cells: B220$^+$GL-7$^+$FAS$^+$.

cGVHD induction by transferring *bm12* CD4+ T cells into B6 mice.

We adapted the *bm12* transfer model, as originally described by Morris *et al.*[10]. Eight to ten weeks old B6 WT mice were injected *i.p.* with 6x10^6 purified CD4$^+$ T cells from *bm12* or *bm12*xCD45.1 mice.

For *in vivo* anti-SLAMF3 injections, recipients were injected *i.p.* with 200 μg of anti-SLAMF3 antibody or IgG1 isotype control on days -1 and 14 after transfer of 3x10^6 *bm12* CD4$^+$ T cells into B6.WT mice. Mice were sacrificed and analyzed on day 28.

Flow Cytometry

Single-cell suspensions were prepared from spleens using standard procedures. After red blood cell (RBC) lysis (Sigma, St. Louis, MO), single cell suspensions were obtained. Cells were blocked with anti-CD16/32 Ab (2.4G2, Biolegend) and stained in FACS staining buffer (2.5% FBS, 0.05% sodium azide in PBS). The following antibodies were used: CD4 (L3T4), CD44 (IM7), CD62L (MEL-14, CD69 (H1.2F3), CD86 (GL-1), CD138 (281-1), B220 (RA3-6B2), CD19 (6D5), FAS (Jo2), T-and B-cell activation antigen (GL-7), CXCR5 (2G8), and PD-1 (29F, 1A12) were purchased from eBioscience (ThermoFisher, Cambridge, MA), BD Biosciences (Woburn, MA), or Biolegend (San Diego, CA). TFH cells were stained as previously described[2]. Dead cells were excluded with 4,6-Di-amidino-2-phenylindole (DAPI). Data were acquired on a BD LSR II cytometer and analyzed using FlowJo software (Tree Star, Ashland, Oregon).

Intracellular Cytokine Staining

Cytokine production was assessed with BD Cytofix/Cytoperm containing BD Golgi-Plug (BD Biosciences). Cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma), Ionomycin (1µg/ml, Sigma), and GolgiStop (1µl/ml, BD Biosciences) at 37^0C in 5% CO_2 for 4 hr. After surface staining, cells were fixed, permeabilized, and stained for IFN-γ (PE-anti-mouse IFN-γ, Biolegend), IL-4 (PE-anti-mouse IL-4, Biolegend) and IL-17 (PE-anti-mouse IL-17A, Biolegend). For intracellular staining IL-21, permeabilized cells were incubated with IL-21R/Fc chimera (R&D systems) for 1 h at 4^0C. Cells were then washed and stained with PE-conjugated affinity-purified $F(ab')_2$ fragment of goat anti-human Fc γ antibody (Jackson ImmunoResearch Laboratories) for 30 min at 4^0C. Viability was assessed using LIVE/DEAD Cell Viability Assays (Life Technologies).

ELISA

Titers of anti-nucleosome antibodies in the serum were determined by ELISA as described previously [12, 13]. In brief, met-BSA-precoated Immunolon plated were coated overnight with double stranded DNA (dsDNA) and then with total histone solution. Samples were incubated on plates in various dilutions between 1:600 and 1:1200, and then washed, and autoantibodies were detected with anti-mouse IgG-HRPO (GE Healthcare).

Autoantibody titer was expressed as ELISA unit, comparing OD values of samples with a standard curve prepared with serial dilutions of ANA-positive NZM2410 serum pool. Anti-chromatin and anti-dsDNA titers were determined as for the

anti-nucleosome levels. UV-irradiated Immunolon plates were incubated overnight with 3g/ml chicken chromatin [14] or mung bean nuclease (New England Biolabs, Ins.)-treated dsDNA (Sigma-Aldrich. Anti-single-stranded DNA (ssDNA) was determined as describe previously [15].

Statistical analysis

Statistical significance was determined by unpaired *t*-test (two-tailed with equal SD) using Prism software (GraphPad, San Diego, CA, USA). The p value <0.05 was considered statistically significant.

Results

Administering αSLAMF3 to NP-ovalbumin immunized B6 mice reduces GC B cell maturation and antibody responses

We first assessed which cell types are affected from SLAMF3 signaling when mice are immunized with foreign antigen. Previously, in response to T- independent and dependent antigens (TNP-KLH or TNP-Ficoll), upon αSLAMF3 injection significantly reduced antibody responses were reported [6]. Taking a similar approach, we immunized B6. WT mice with NP-OVA in conjunction with αSLAMF3 or isotype control injection. Mice were euthanized and analyzed on day 9. We found no difference in total spleen weight or total number of splenocytes between isotype and αSLAMF3 injected groups (**Fig. S1**). By contrast, in the serum of αSLAMF3-injected mice, we found significantly reduced levels of NP-specific antibodies as compared to isotype-injected mice (**Fig. 1A**). Further analysis revealed a significant reduction in total B cells and MZ B cells (**Fig. 1B and S1**), but more importantly dramatically reduced percentage and numbers of GC B cells in spleen of αSLAMF3 injected mice (**Fig. 1C**). We did not find, however, a difference in total $CD4^+$ T cells or TFH cells (**Fig. 1D and S1**), suggesting that the antibody primarily affects B cells in this system. While this was in the case of co-injection of αSLAMF3 together with NP-OVA immunization, injection of antibody at a later time point (day 4) showed similar results (**Fig. S2**), demonstrating that our findings are independent of time of injection.

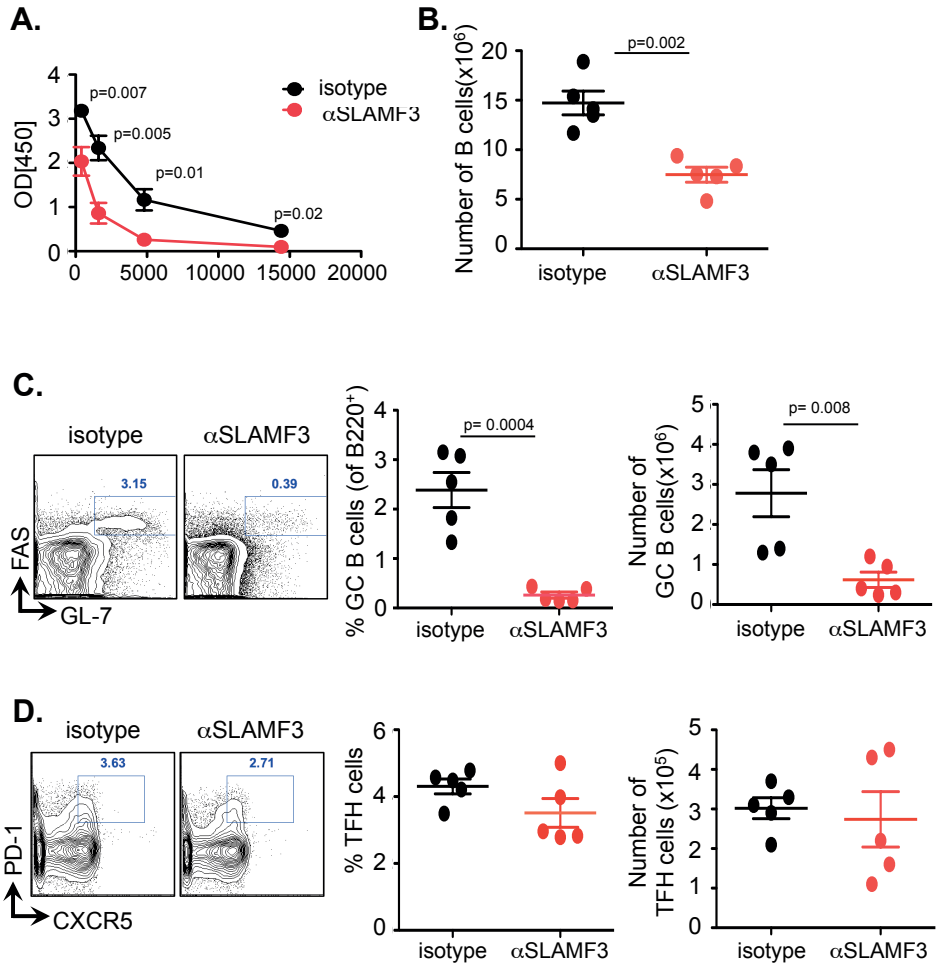

Figure 1. Administering αSLAMF3 to NP-OVA immunized B6 WT mice reduces B cell numbers and antibody responses

WT mice were immunized with NP-OVA in CFA along with 200μg/mouse αSLAMF3 or isotype IgG1. Nine days later mice were euthanized and spleens were analyzed. **A.** NP-specific antibody titers from serum of αSLAMF3 and isotype injected mice are as shown. **B.** Total number of splenocytes from αSLAMF3 and isotype injected mice. **C.** Representative Flow cytometry plots for GC staining: $CD19^+GL\text{-}7^+FAS^+$ B cells (left), percentage and numbers of GC B cells (right). **D.** Representative Flow cytometry plot showing gating strategy for TFH cells: $CD4^+PD\text{-}1^+CXCR5^+$ (left panel) Percentages and numbers of TFH cells in spleen of αSLAMF3 and isotype injected mice (right panel). Data representative of three independent experiments. P values are as shown.

Administering αSLAMF3 enhances autoantibody production upon induction of cGVHD by the transfer of bm12 CD4+ T cells into B6 recipients.

Our previous studies demonstrated that spontaneous anti-nuclear antibody (ANA) production in SLAMF3-/- mice was independent of the background (B6.129 or Balb/c.129), suggesting that SLAMF3 may play an important role in regulating autoimmunity[7]. These findings led us to investigate the consequences of modulating SLAMF3 during autoimmune responses using a monoclonal mouse anti-SLAMF3 antibody (αSLAMF3). In addition to initiating cGVHD, 200μg/mouse αSLAMF3 or isotype control was *i.p.* injected into *bm12* mice -1 and 14 days after transfer of B6.WT donor $CD4^+$ T cells. Mice were euthanized and analyzed 28 days after transfer. Mice injected with αSLAMF3 had significantly bigger spleen size, weight and total number of splenocytes compared to isotype control (**Fig. S3**). The levels of anti-chromatin, anti-single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) were substantially increased in the serum of mice that had received αSLAMF3 as compared to isotype control (**Fig.2A-C**). Percentage and numbers of marginal zone B cells decreased while total number of B cells in the spleen remained unchanged (**Fig. S3**). Consistent with the increase in autoantibody production, the percentage of plasma cells was significantly expanded in the spleen of αSLAMF3-injected mice (**Fig. 2D**). To further validate these observations, lymphoid follicles and GCs of recipient mice were measured with immunofluorescence staining from frozen spleen sections. B cell zones were identified by B220 and GC area was marked by $GL7^+$ zone surrounded by IgD^+ naive B cells. This showed that the size of Germinal Centers was increased in B6 recipients that had received two injections of anti-SLAMF3 (**Fig 2E**). Similar results were obtained with 4 injections of αSLAMF3 in bm12 $CD4^+$ T cell transfers and αSLAMF1 was used as a control (See Fig. S5). Anti-SLAMF3 injected group had significantly higher percentages of GC B cells and increased autoantibody production, whereas αSLAMF1 did not promote autoantibody production (**Fig. S5-S6**).

Anti-SLAMF3 accelerates GC B cell differentiation initiated by cGVHD.

Selection, isotype switching and expansion of GC B cells require critical signals from T follicular helper (TFH) cells. During chronic GVHD autoantibodies are produced due to the generation of autoimmune TFH and GC B cells in response to the donor $CD4^+$ T cells [10]. To assess whether the increased autoantibody production in mice injected with αSLAMF3 was the consequence of modulating the GC reaction, we first examined the presence of GC B cells in the spleen of recipient mice, 14 days post transfer of bm12 $CD4^+$ T cells. In keeping with enhanced GC formation, the expression of CD86 and the proportion of CD69

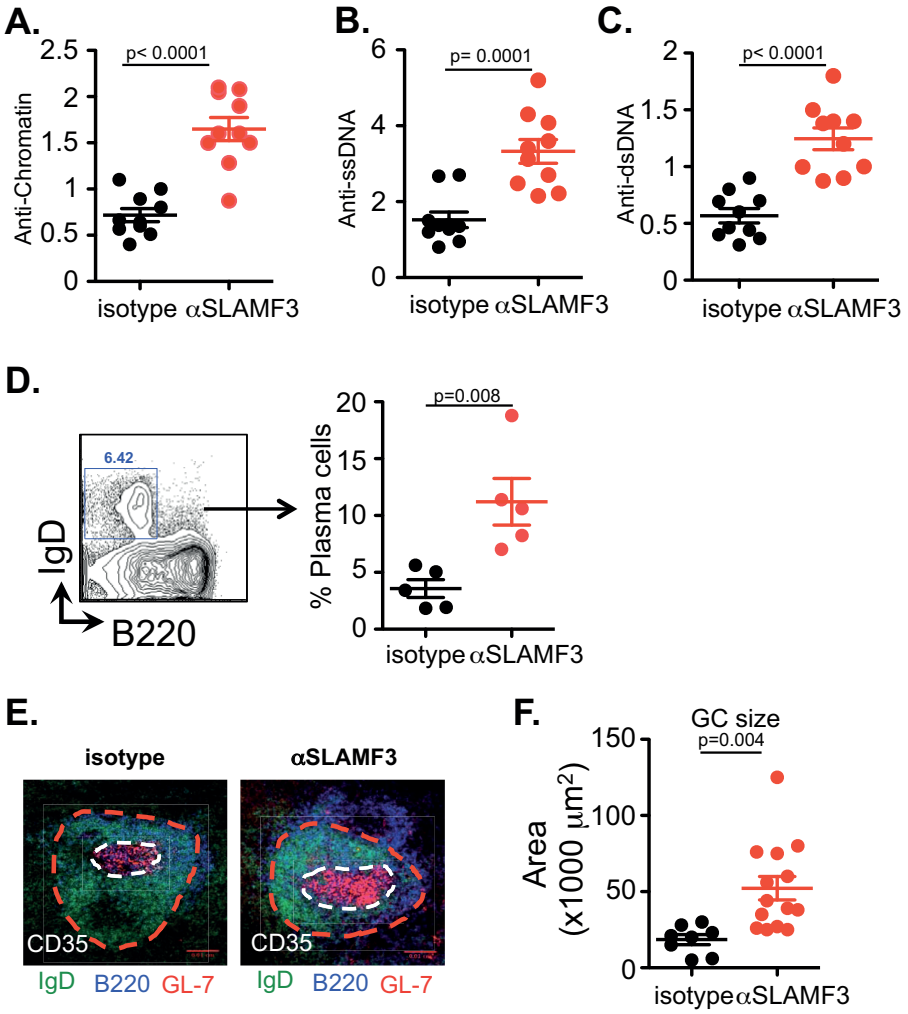

Figure 2. Administration of αSLAMF3 induces lupus-related autoantibody responses in the recipients of bm12 CD4⁺ T cells.

6x10⁶ CD4⁺ T cells isolated from *bm12* female mice were transferred into B6 WT recipients by *i.p.* injection. The recipients of bm12 CD4⁺ T cells were injected with anti-SLAMF3 or Isotype IgG1 (200μg/mouse) on days -1 and 14. Mice were euthanized on day 28. Spleens and serums were analyzed. **A-C.** Anti-Chromatin, anti-ssDNA and anti-dsDNA in the serum of recipient mice were determined by ELISA. **D.** Representative FACS plots showing B220⁺IgD⁻CD138⁺ plasma cells from the spleens (Left panel). Percentage of plasma cells in spleen of isotype and αSLAMF3 injected mice (Right panel). **E-F.** The indicated recipient mice were sacrificed and spleen were embedded in optimal cutting temperature compound (OCT) tissue media and frozen on dry ice. Seven-micrometer thick frozen sections were fixed to slides in ice-cold acetone for 15 min. The sections were stained with B220 FITC, GL-7 PE and CD35 Pacific Blue. Representative confocal images and quantification of the germinal center sizes are shown. Data represent at least four independent experiments, p values are as shown.

activated B cells were markedly increased in anti-SLAMF3 injected recipients (**Fig. 3A-B**). Surprisingly, the expression of FAS was not only increased on the surface of GC B cells, but also on all B cells (**Fig. 3C**). As expected, flow cytometry analyses revealed a significant expansion of the percentage and number of $CD19^+FAS^+GL\text{-}7^+$ GC B cells in αSLAMF3-injected recipients as compared to isotype control (**Fig. 3D-E**).

Previous studies have indicated a key role for the IFN-γ receptor (IFN-γR) in development of autoantibody production in lupus-prone mice, *e.g.* MRL/Lpr, NZB/W, B6.Sle1b and Roquin san/san [16-19]. Deletion of IFN-γR on B cells abrogates formation of autoimmune GCs and autoimmunity [20]. Based on these observations, we examined the surface expression of IFN-γR on GC B cells from recipients of bm12 $CD4^+$ T cells. In parallel with the enhanced GC formation, administration of αSLAMF3 markedly increased percentage of $IFN\text{-}\gamma R^+$ GC B cells, compared to recipients that received isotype control (**Fig. 3F-G**). These findings suggest that SLAMF3 indeed negatively regulates expansion of autoreactive GC B cells and that the αSLAMF3 antibody affects this cGVHD driven expansion.

Administering αSLAMF3 increases T cell activation and TFH cell differentiation initiated by cGVHD

As the cGVHD in B6 recipients is initiated and driven by the transfer of bm12 co-isogenic $CD4^+$ T cells we further analyzed the T cell compartment. As judged by expression of CD44, CD62 and CD69, the percentage and number of effector $CD4^+$ T cell were also higher in αSLAMF3 treated recipients (**Fig. 4A-B**). In accordance with the increase in total $CD4^+$ T cells, the percentage of $CD4^+CXCR5^+PD\text{-}1^+$ TFH cells were significantly increased in αSLAMF3-injected recipient mice (**Fig. 4C**). PD1 expression was not only increased on the surface of CD4 of TFH cells, we also found an increase in number and expression of PD1+ $CD4^+$ T cells in αSLAMF3-injected recipients (**Fig. S4**). Importantly, these activated $CD4^+$ T cells did not only secrete more IFNγ in response to administering, but also increased amounts of the key cytokines IL-4 and IL-21, while IL-17 remained unchanged (**Fig. 4E-H)**. These findings correlate with reports that SLE patients as well as lupus-prone mice have increased serum levels of IL-21, IFNγ and IL-17[21-24]. We conclude that the high levels of IL-4, IL-21 and IFNγ in cGVHD are further enhanced by triggering αSLAMF3, which drives the dysregulated GC reaction.

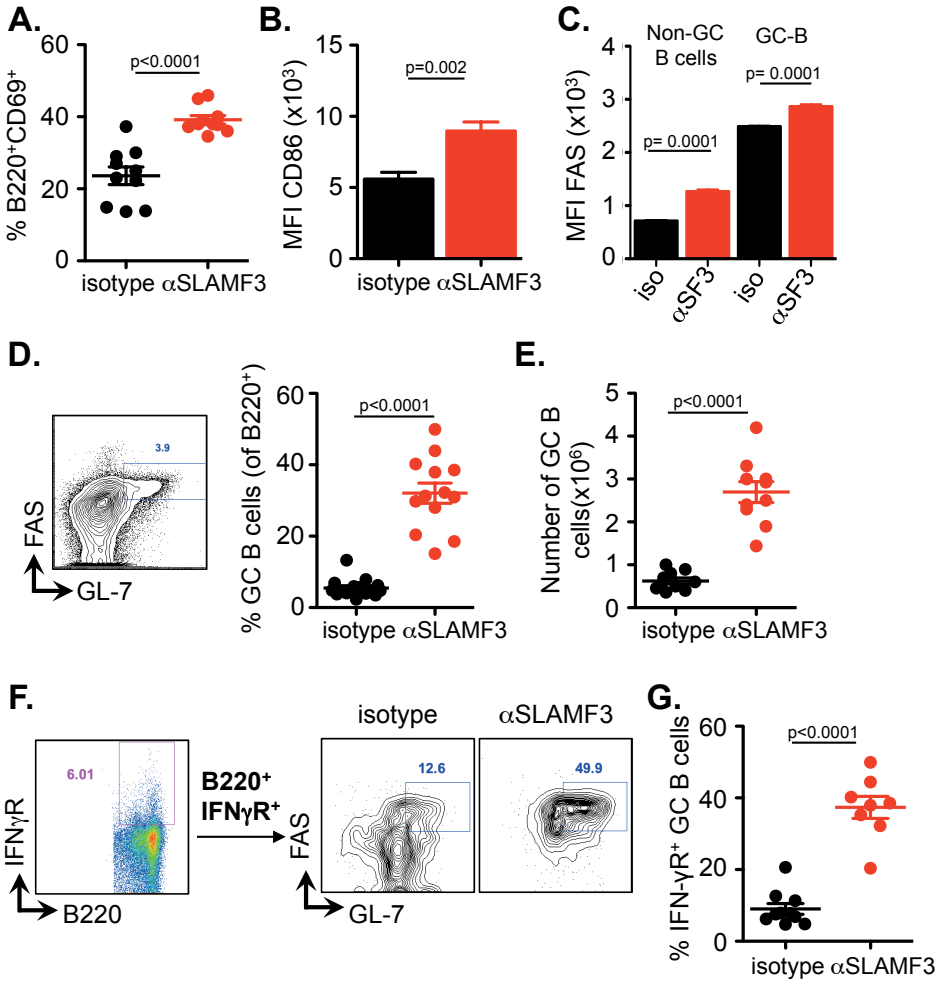

Figure 3. Anti-SLAMF3 accelerates GC B cell differentiation initiated by cGVHD.

Anti-SLAMF3 or isotype injected bm12 CD4⁺ T cell transferred WT recipient mice were analyzed on day 14 when GC reactions peaked. **A.** Percentages of B220⁺CD69⁺ B cells are as shown. **B.** Expression of CD86 on B cells. **C.** FAS expression in non-GC B and GC B cells from isotype and aSLAMF3 injected recipients. **D-E.** Representative Flow cytometry plot for GC staining: CD19+GL-7+FAS+ B cells (left panel), percentages of GC B (Right panel) and numbers of GC B cells in spleen of mice. **F.** Representative FACS plots of B220⁺IFN-γR⁺ GC B cells from isotype and αSLAMF3-injected recipients. **G.** The percentage of B220⁺IFN-γR⁺ GC B cells in the spleens of isotype and αSLAMF3-injected recipients. Data represent at least four independent experiments.

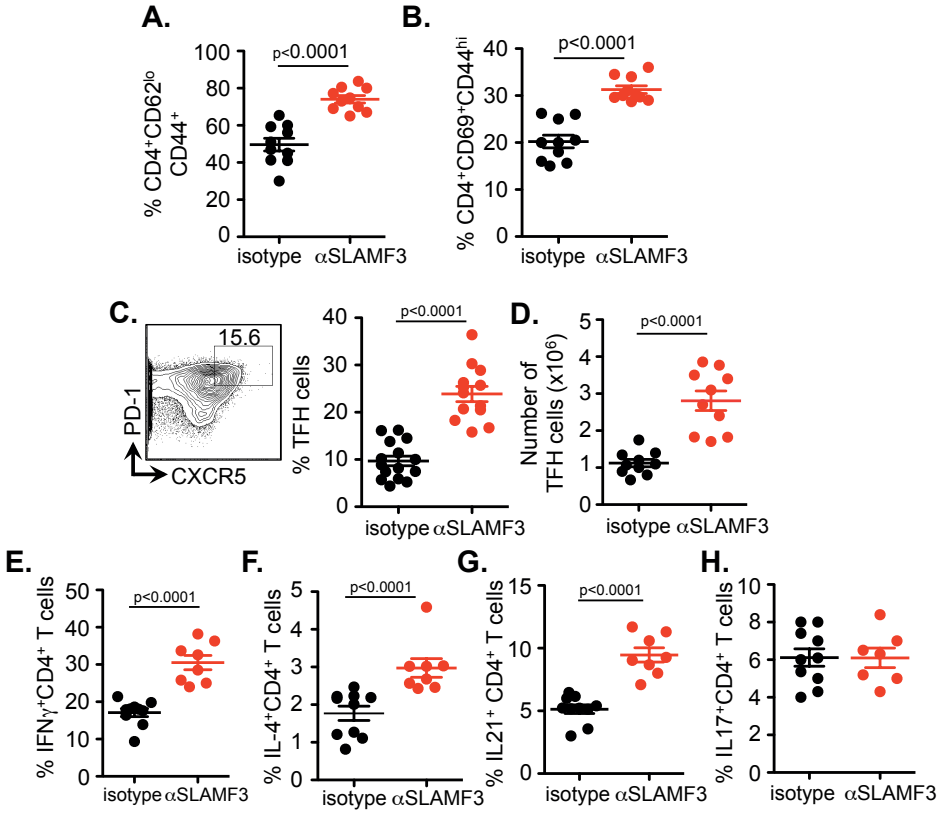

Figure 4. Administering αSLAMF3 increases T cell activation and TFH cell differentiation initiated by cGVHD.

Anti-SLAMF3 or isotype injected bm12 CD4+ T cell transferred B6 WT recipient mice were analyzed on day 14 when GC reactions peaked. **A-B.** Percentages of $CD4^+CD44^+CD62^{lo}$ memory T cells and $CD4^+CD69^+CD44^{hi}$ effector T cells in isotype and αSLAMF3 injected mice. **C.** Representative Flow cytometry plot showing gating strategy for TFH cells: $CD4^+PD\text{-}1^+CXCR5^+$ (left panel) Percentages of TFH cells in spleen of αSLAMF3 and isotype injected mice (right panel). **D.** Absolute numbers of TFH cells in isotype and aSLAMF3 injected mice. **E-H.** Expression of following cytokines was measured by intracellular staining in $CD4^+CD45.1^+$ splenocytes from isotype and αSLAMF3-injected recipients. Percentages of $CD4^+IFN\text{-}\gamma^+$ T cells, $CD4^+IL4^+$ T cells, $CD4^+$ IL-21^+ T cells and $CD4^+$ IL-17^+ T cells are shown.

Selective Increase of the number of donor $CD4^+CD45.1^+$ T cells upon administering αSLAMF3 during cGVHD

In order to distinguish between the role of donor and recipient T cells in the GC reaction, we crossed bm12 and CD45.1 mice and subsequently transferred bm12xCD45.1 donor $CD4^+$ T cells into recipient $CD45.2^+$ B6 WT mice. 14 days after transfer, mice were euthanized and $CD4^+$ T cells were analyzed. Significantly,

only the donor CD4$^+$ T cells (CD4$^+$CD45.1$^+$) had expanded in αSLAMF3-injected mice, while recipient CD4$^+$ T cells were unaffected (**Fig 5A-B**). More specifically, we identified significantly expanded CD45.1$^+$ donor TFH cells in the recipient mice injected with αSLAMF3 (**Fig. 5C**).

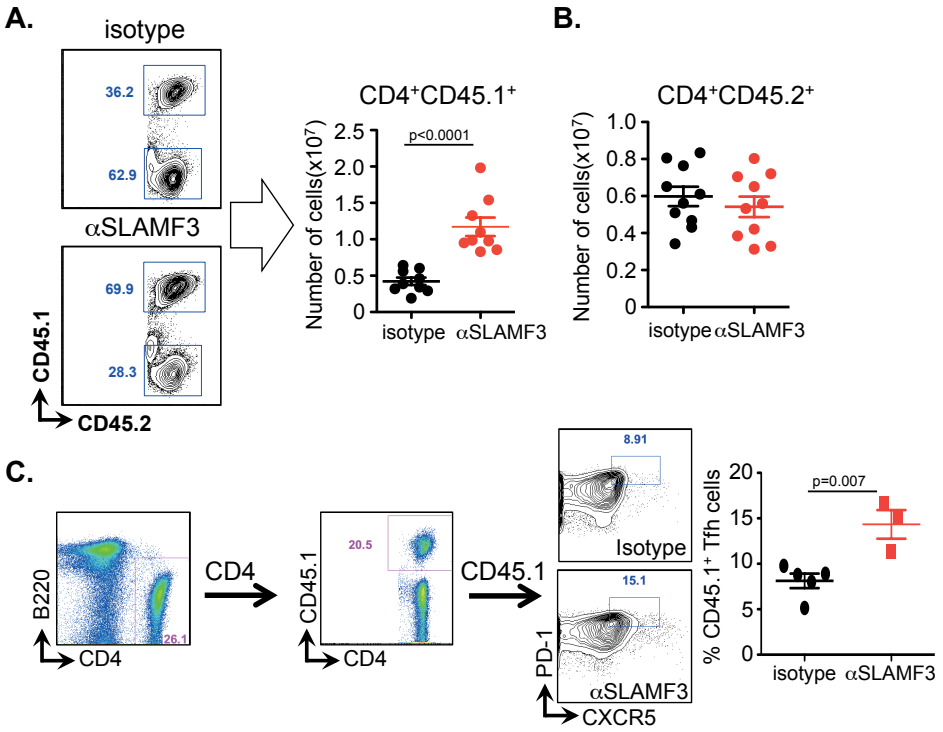

Figure 5. Selective increase of the number of donor CD4$^+$CD45.1$^+$ T cells upon administering αSLAMF3 during cGVHD.

A. Representative dot plots of CD4$^+$CD45.1$^+$ T cells from isotype and αSLAMF3-injected recipients (left panel). The number of CD4$^+$CD45.1$^+$ donor T cells in the spleens of isotype and αSLAMF3-injected recipients (right panel). **B.** The number of CD4$^+$CD45.2$^+$ recipient T cells in the spleens of isotype and αSLAMF3-injected recipients. **C.** Representative FACS plots of CD45.1$^+$ donor TFH cells (left) with percentages of CD45.1$^+$ TFH cells in isotype and αSLAMF3 (right). Data represent at least four independent experiments.

Upon induction of chronic GVHD by the transfer of bm12 CD4+ cells into B6 mice donor cell Treg and TFR development is selectively impaired by αSLAMF3

Regulatory T (Treg) cells have been demonstrated to play vital roles in suppressing cellular and humoral immune responses, i.e. by suppressing autoreactive B cell functions and subsequent autoantibody production [25-27]. Of interest, recent studies have identified a subset of regulatory T cells in the GCs, termed T fol-

licular regulatory (TFR) cells that suppress TFH and GC B cells [28]. Therefore we hypothesized that TFR cells may be affected from injections of αSLAMF3. 14 days after transfer of bm12 CD4$^+$ T cells into B6 WT recipients and subsequent 2 injections of αSLAMF3, mice were euthanized and analyzed for TFR cells at the peak of GC reactions. TFR cells, defined as CD4$^+$CXCR5$^+$PD-1$^+$FoxP3$^+$ were significantly reduced from spleen of αSLAMF3-injected mice as compared to isotype control (**Fig. 6A-B**). Furthermore, TFRs present in αSLAMF3-injected mice had significantly higher PD1 expression compared to isotype control (**Fig. 6C**). This suggests that negative regulation within the GCs is lost upon αSLAMF3 injections.

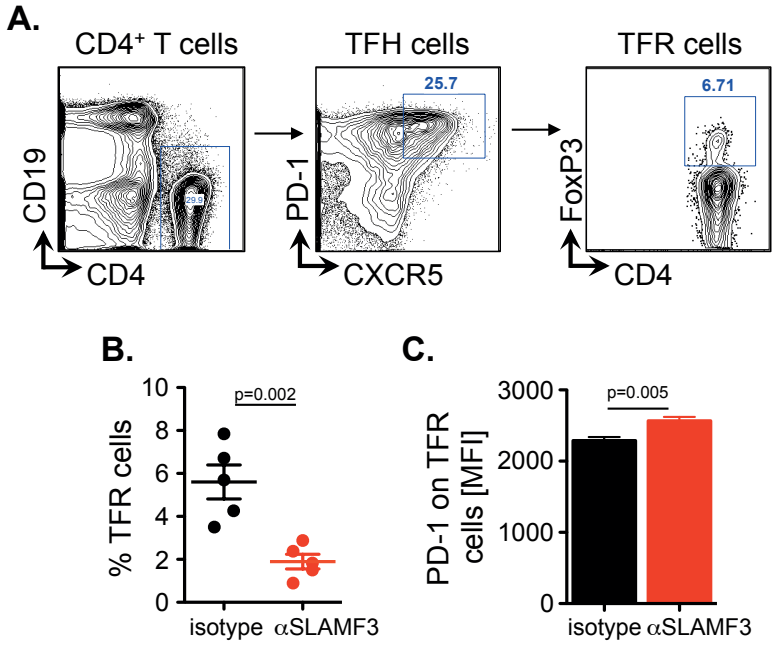

Figure 6. Anti-SLAMF3 inhibits TFR cell differentiation

A. Representative gating strategy of TFR cells from spleens of the recipients of bm12 CD4+ T cells: CD4$^+$CXCR5$^+$PD-1$^+$Foxp3$^+$ **B.** Percentages of TFR cells in the spleens of isotype and αSLAMF3-injected mice. **C.** Cell surface expression of PD-1 on TFR cells in the spleens of isotype and αSLAMF3-injected mice. Data represent at least four independent experiments.

As TFR cells originate from Treg precursors [28], we checked whether SLAMF3 signaling also has negative effect on Treg differentiation. Similar to the impact on TFR cells, αSLAMF3-injected recipients exhibited a substantial decrease in the frequency of CD4$^+$Foxp3$^+$CD25$^+$ T cells compared with isotype control injected mice **(Fig. 7A-B)**. Strikingly, the impact of anti-SLAMF3 was specific

to donor-derived Treg cells because the administration of αSLAMF3 exerted no effect on recipient-derived Treg cells (**Fig. 7C**). Together, these data demonstrate that SLAMF3 signaling not inhibited TFR cell differentiation but also of Treg cells, which may contribute to exacerbated autoimmune responses in αSLAMF3-injected recipients.

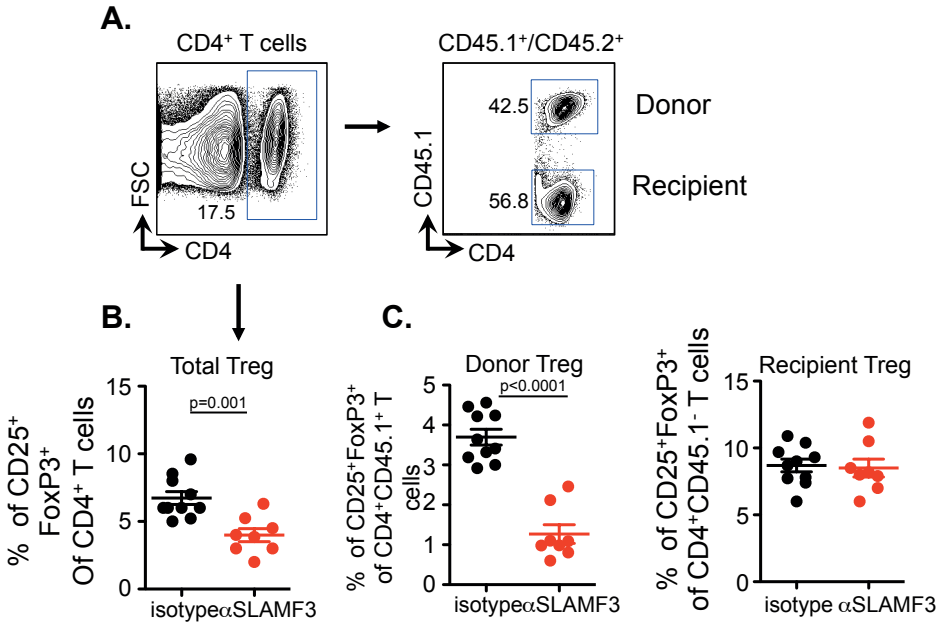

Figure 7. Upon induction of chronic GVHD by the transfer of bm12 CD4⁺ T cells into B6 mice donor cell Treg development is selectively impaired by aSLAMF3.

A. Representative FACS plots of total CD4⁺ T and subsequent donor/recipient CD4⁺CD45.1⁺ and CD4⁺CD45.2⁺, respectively. Of these subsets, Treg population was determined as CD25⁺FoxP3⁺. **B.** Percentage of total CD4⁺CD25⁺Fox3⁺Treg cells in the spleens of isotype and αSLAMF3-injected recipients. **C.** Percentages of CD4⁺CD45.1⁺CD25⁺Fox3⁺ donor Treg (left panel) and CD4⁺CD45.1⁻CD25⁺Fox3⁺ recipient Treg (right panel) cells in the spleens of isotype and αSLAMF3-injected recipients.

Discussion

In studies of murine autoimmune-like chronic graft versus-host disease (cGVHD), B cells are activated by donor CD4+ T cells to upregulate MHC II and costimulatory molecules. Acting as efficient APCs, donor B cells further augment donor the clonal expansion, differentiation, and survival of pathogenic CD4+ T cells, which drives autoreactivity. Because SLAMF3-deficient mice develop autoantibodies irrespective of their genetic background, and as development several B

cell subsets, *e.g.* Marginal zone [MZ] B cells are eliminated in these mice, we adopted a cGVHD model to investigate the importance of SLAMF3 signaling in this disease. To this end we examined the effect of a mouse anti-mouseSLAMF3 monoclonal antibody on hapten-induced humoral responses and on autoantibody production in the bm12 CD4+ > B6 transfer model of cGVHD.

The latter model was chosen because it mirrors a few common pathways of human disease, e.g. high levels of circulating anti-nuclear antibodies, concomitantly with large frequencies of T follicular helper cells (Tfh), germinal center (GC) B cells, and plasma cells. We previously had shown that by using lupus – prone B6 mutants in this cGVHD model the autoantibody production is accelerated. Conversely, specific monoclonal antibodies, which affect GC formation in mice that are immunized with foreign antigens, also ameliorate the generation of anti-nuclear antibodies in this bm12>B6 cGVHD model[12, 13]. As also shown by others, cGVHD can be induced both by the transfer of bm12 derived CD4+ cells into B6 mice or by B6-derived CD4+ cells into the co-isogenic bm12 recipients with equal efficiency [9, 10].

In our analyses of NP-OVA immunizations combines with administering αSLAMF3, we demonstrate that in response to foreign antigens, SLAMF3 primarily affects the maturation into GC B cells, leading to diminished antibody responses. By contrast, injections of αSLAMF6 in NP-OVA immunized mice, SLAMF6 signaling affected both B and T cells in the germinal centers [2].

Precise regulation of TFH cell numbers is critical for optimal humoral responses, and aberrant expansion of TFH cells is associated with autoimmune diseases, including lupus [29, 30]. In our system, we found that the administration of αSLAMF3 significantly enhanced proliferation of donor-derived $CD4^+$ T cells, which lead to profound TFH cell differentiation in GCs. As a consequence, we observed severe autoimmune phenotypes in αSLAMF3-injected recipients, which included increased serum levels of anti-dsDNA, anti-ssDNA and anti-chromatin autoantibodies (**Fig.1**). This was a consequence of enlarged germinal centers with massive accumulation of GC B cells in the spleens (**Fig. 2**). Although several molecules, including CD40L, ICOS, PD-1 and SAP, are known to be involved in TFH cell differentiation, the transcriptional repressor Bcl6 is found to be a lineage-defining factor for TFH cells. Bcl6 is necessary to specify the TFH cell program and overexpression of Bcl6 is sufficient to drive TFH cell differentiation [31]. Indeed, the expression of Bcl6 was markedly increased in αSLAMF3-injected recipients, but not in isotype control injected recipient mice (Unpublished obser-

vation). Beside BCR signaling, the survival and selection of GC B cells within GCs are dependent on survival signals from GC TFH cells. The experiments reported here are consistent with strong TFH cell differentiation with extensive GC B cell responses. Recent publications have suggested that B cells have a cell-intrinsic requirement for expression of CD80 and/or CD86 for differentiation into GC B cells [32, 33].

In accord, we found increased expression of CD86 in B cells and GC B cells in αSLAMF3-injected recipient mice. Interestingly, IFN-γR signals are shown to synergize with BCR, TLR and CD40 dependent signal to enhance expression of the GC master regulator transcription factor Bcl6 [20], which suggest IFN-γ facilitates autoimmune GC formation by initiating a GC transcriptional program. Based on these findings by others, we examined the expression Bcl6 and IFN-γR and found that GC B cells exhibited increased Bcl6 and IFN-γR. In addition, we observed higher expression of anti-apoptotic protein Bcl-2 in αSLAMF3-injected GC B cells (unpublished observation). This in turn may reduce overall apoptosis of GC B cells and ultimately result in increased numbers of GC B cells in the GCs. It will be of interest to determine whether the expression of other members of the Bcl-2 family, such as Mcl-1, are also increased in αSLAMF3-injected GC B cells. Thus, it appears that αSLAMF3 may act on both B and T cell sides during autoimmune GC reactions.

In addition, αSLAMF3 had a pronounced effect on key cytokines produced during GC reactions. IL-21, IL-4 and IFN-γ production were significantly elevated in donor-derived $CD4^+$ T cells of αSLAMF3-injected recipients. Although IL-21 production is restricted to activated CD4 T and NKT cells, IL-21 receptor is expressed on a variety of immune cells [34, 35]. IL-21 promotes the differentiation and expansion of TFH cells, regulates B cell proliferation and survival, GC formation, and plasma cell differentiation [36-38]. Furthermore, IL-21 has an important role in regulating T cell-dependent B cell responses, partly in cooperation with IL-4 [39]. IL-21 is markedly elevated in autoimmune-prone mice and lupus severity is diminished in the absence of IL-21 or IL-21R signaling [22, 40]. Besides IL-21, IFN-γ also contributes to lupus in both human and murine models. The increased serum IFN-γ levels are associated with disease activity and the inhibition of IFN-γ expression prevents the development of murine lupus [41, 42]. Thus, the combined alteration of IL-21, IFN-γ and IL-4 production in αSLAMF3-injected recipients may play an important role in higher GC formation and autoantibody production.

We were surprised to find that αSLAMF3 injections resulted in reduced numbers of Treg cells in the recipients of bm12 $CD4^+$ T cells. The pivotal roles of Treg cells in the development and maintenance of immune self-tolerance have been well documented [43]. It was thought that Treg cells functioned as immunosuppressors in allogeneic immune responses and in autoimmune responses. In this study, recipient Treg cells does not seem to play a major role in suppressing lupus-like phenotype as similar numbers of Treg cells were differentiated in both αSLAMF3 and isotype-injected recipient mice. In contrast, donor-derived Treg cells were significantly reduced after αSLAMF3 injections. Thus, these data suggest that αSLAMF3 specifically limits proliferation of donor-derived Treg cells, but have minimal effect on proliferation of host Treg cells. Perhaps the most striking finding in our studies was the reduced differentiation of donor-derived TFR cells. TFR cells are another subset of $CD4^+$ T cells in the GCs [44, 45]. TFR cells share phenotypic characteristics with TFH but are derived from suppressive $FoxP3^+$ Treg cells. Within the GC, TFR cells inhibit GC formation and restrict the autoimmune responses[46]. Our findings indicate that the reduced number of TFR cells that leads to reduced suppression on TFH cells in the GCs leads to increased autoantibody production in αSLAMF3-injected recipients. Although the suppressive capacity of TFR cells is not examined, the higher expression of PD-1 on TFR cells indirectly suggest there may be reduced suppressive functions in αSLAMF3-injected recipients. Further work is needed to understand how PD-1 expression is regulated *in vivo* by SLAMF3 signaling. However, the increased expression of PD-1 on TFR cells might partly contribute to the reduced numbers of TFR cells in αSLAMF3-injected recipients. This idea is supported by previous observations that mice deficient in PD-1 have increased numbers of TFR cells with enhanced suppressive capacity [47]. Collectively, impaired Treg and TFR compartments could enhance TFH activity, resulting in the expansion of autoreactive B cells and autoantibody production.

In the cGVHD model, the key cellular mechanism that results in the loss of B cell tolerance is the interaction of donor $CD4^+$ T cells with MHC class II on host B cell surface. During T-B cell interactions, allogeneic donor $CD4^+$ T cells provide the abnormal T cell help to host B cells that appears to have functional consequences different from what occurs in spontaneous model. We conclude that the checkpoint regulator SLAMF3 preferentially prevents expansion of autoreactive B cells generated by Graft-versus-Host Disease with aggravated autoantibody production. This is caused by αSLAMF3 induced hyperactivation of the activated donor $CD4^+$ T cells, which causes an imbalance of TFR/TFH ratio, i.e.

impaired development of TFR and Treg cells as well as the altered production of key cytokines.

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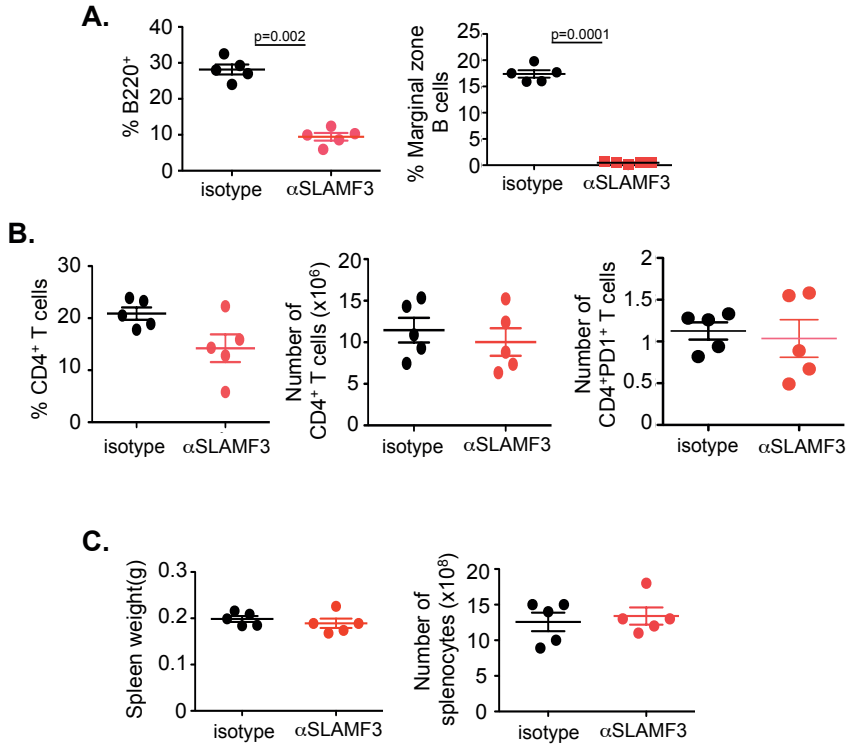
Supplementary Figure 1. Injection of αSLAMF3 in NP-OVA immunized mice reduces percentages of total and MZ B cells, while no effect on total CD4⁺ T cells.

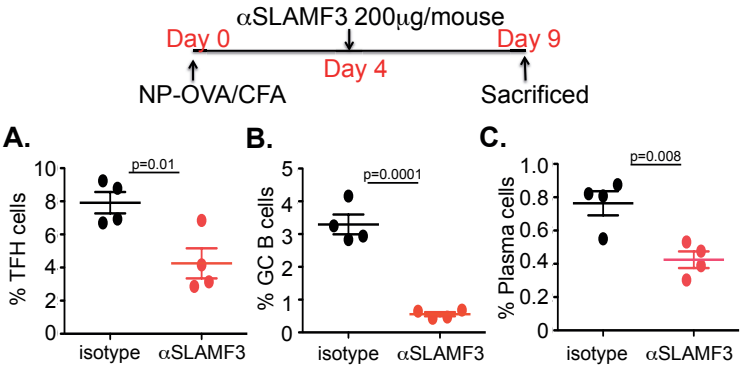
WT mice were immunized with NP-OVA in CFA along with 200μg/mouse αSLAMF3 or isotype IgG1. Nine days later mice were euthanized and spleens were analyzed.

A. Spleen weight and total number of splenocytes from isotype and αSLAMF3 injected mice. **B.** Percentages of total B cells (left) and marginal zone B cells (right) are as shown. **C.** Percentages and numbers of total CD4⁺ T cells from spleen of aSLAMF3 and isotype injected mice (left). Numbers of PD1⁺ CD4⁺ T cells (right) are shown.



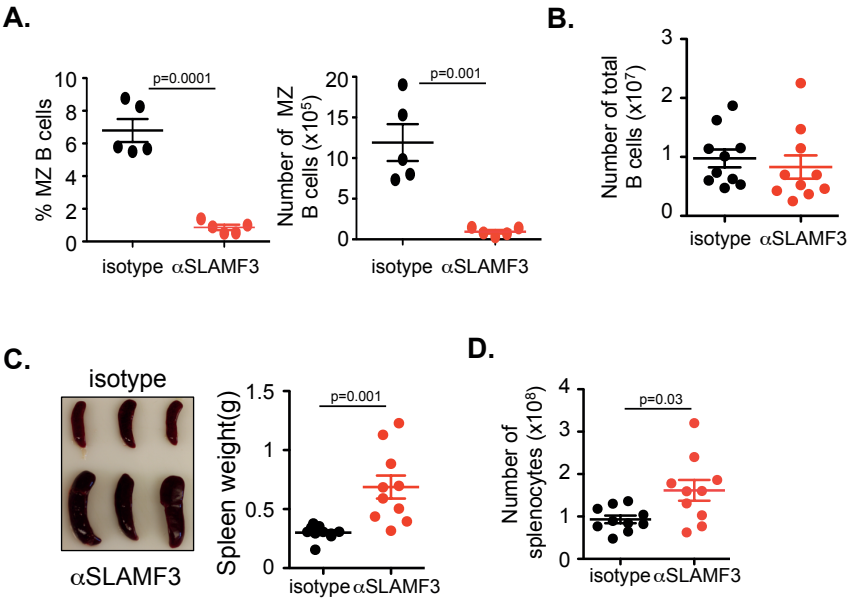
Supplementary Figure 2. Anti-SLAMF3 injection 4 days after NP-OVA immunization inhibits GC B cell responses.

WT mice were immunized with NP-OVA in CFA. Four days later 200μg/mouse αSLAMF3 or isotype IgG1 was injected. Mice were euthanized on day 9 and spleens were analyzed. Schematic outline is shown. Percentage of **A.** TFH cells, **B.** GC B cells, **C.** Plasma cells are as shown.



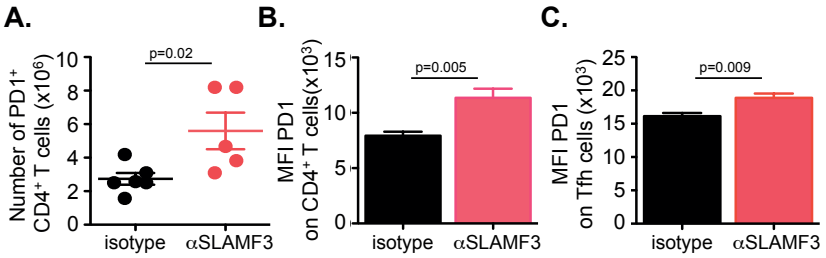
Supplementary Figure 3. Two injections of αSLAMF3 expand number of total splenocytes, while has no effect on total B cell numbers in bm12 cGVHD model.

$6x10^6$ $CD4^+$ T cells isolated from *bm12* female mice were transferred into B6 WT recipients by *i.p.* injection. The recipients of bm12 $CD4^+$ T cells were injected with anti-SLAMF3 or Isotype IgG1 (200μg/mouse) on days -1 and 14. Mice were euthanized on day 28. Spleens and serums were analyzed. **A.** Spleen size and weight from isotype and αSLAMF3 injected mice. **B.** Number of total splenocytes. **C.** Number of total B cells. **D.** Percentages and number of MZ B cells from isotype and αSLAMF3 injected mice.



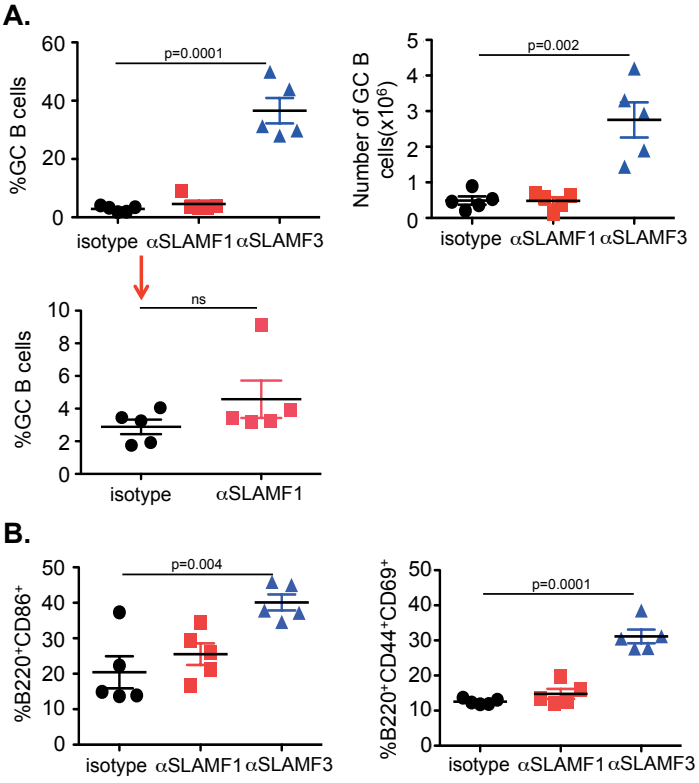
Supplementary Figure 4. Two injections of αSLAMF3 expand PD1$^+$ CD4$^+$ T cells and upregulates PD1 on TFH cells in bm12 cGVHD model.

Anti-SLAMF3 or isotype injected bm12 CD4$^+$ T cell transferred WT recipient mice were analyzed on day 14 when GC reactions peaked. **A.** Numbers of PD1$^+$CD4$^+$ T cells in WT recipients. **B.** Expression of PD1 on CD4$^+$ T cells and **C.** TFH cells are as shown.



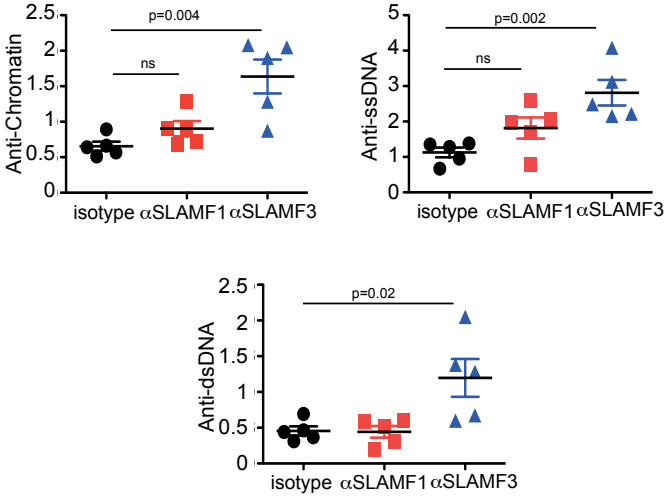
Supplementary Figure 5. No effect of αSLAMF1 in bm12 cGVHD model, serving as a control for the system.

$6x10^6$ $CD4^+$ T cells isolated from *bm12* female mice were transferred into B6 WT recipients by *i.p.* injection. The recipients of bm12 $CD4^+$ T cells were injected with anti-SLAMF3, anti-SLAMF1 or isotype IgG1 (200μg/mouse) on days -1, 7, 14 and 21 days. Mice were euthanized on day 28. Spleens and serums were analyzed. **A.** Percentages of GC B and numbers of GC B cells in spleen of mice injected with αSLAMF3, αSLAMF1 or isotype. **B.** Percentages of $B220^+CD86^+$ and $B220^+CD44^+CD69^+$ B cells are as shown.



Supplementary Figure 6. No effect of αSLAMF1 on autoantibody production in bm12 cGVHD model as compared to αSLAMF3 injected mice.

6x10^6 CD4$^+$ T cells isolated from *bm12* female mice were transferred into B6 WT recipients by *i.p.* injection. The recipients of bm12 CD4$^+$ T cells were injected with anti-SLAMF3, anti-SLAMF1 or isotype IgG1 (200μg/mouse) on days -1, 7, 14 and 21 days. Mice were euthanized on day 28. Anti-Chromatin, anti-ssDNA and anti-dsDNA in the serum of recipient mice were determined by ELISA is shown.



CHAPTER 6

Conclusion

There is an increasing understanding of how SLAMF receptors and its adaptors are able to fine-tune innate and adaptive immune responses[1]. However, as these receptors are underappreciated as therapeutic targets, we began to employ SLAMF receptors to design novel therapies for chronic lymphocytic leukemia (CLL), other B cell tumors and graft versus host disease. To this end, we focused on the use of monoclonal antibodies directed against SLAMF6 in CLL and SLAMF3 in graft versus host disease. We had previously found that anti-SLAMF6 and anti-SLAMF3 inhibits antibody responses following immunization with 4 Hydroxy-3-nitrophenylacetyl hapten conjugated ovalbumin (NP-OVA)[2, 3]. Here we determined that the mouse monoclonal antibodies directed against mouse SLAMF6 (αSLAMF6) in combination with small molecules provide novel and valuable immunotherapies for CLL and B cell lymphomas. Surprisingly, whilst αSLAMF6 reduces autoantibody production in a chronic Graft versus Host Disease (cGVHD) model[4], mouse monoclonal antibodies directed against SLAMF3 (αSLAMF3) exacerbate an existing autoantibody production in this model.

Chapter 2 examines the effect of αSLAMF6 in a mouse model of CLL in which an aggressive TCL1-192 leukemia clone proliferates in SCID (Severe combined immune deficiency) mice that lack T and B cells. Administering one or two injections of αSLAMF6 into these tumor-bearing mice showed two modes of action of the antibody. First, we observed induction of antibody dependent cell-mediated cytotoxicity (ADCC), similar to that of rituximab (anti-CD20) (unpublished). Second, we demonstrated that αSLAMF6 was also able to down-regulate proximal BCR signaling, which is a critical factor for CLL survival. Combining the current front line therapy in CLL, the small molecule inhibitor of Bruton's Tyrosine Kinase (BTK) ibrutinib, together with anti-SLAMF6 resulted in a better treatment than either regimen alone.

So far, the above-mentioned findings of Chapter 2 were based mainly on analyses of the spleen and blood, which are major reservoirs for CLL expansion. Further analyses indicated that αSLAMF6 removed leukemic cells effectively from all organs except the peritoneal cavity (PerC) and omentum. This led to the notion that the tumor microenvironment affected αSLAMF6-induced ADCC. This inhibition could however be overcome by co-administering ibrutinib, which appeared to drive TCL1-192 cells out of the PerC. It has been known for a long time that B1a B cells in the PerC, although similar in phenotype were functionally distinct from their counterparts in the spleen[5]. This in part explains why signaling intensity of CLL cells in the PerC is different than that in the spleen[6, 7].

Studies by the Tedder lab showed that like αSLAMF6 / CLL cells, αCD20 is ineffective in removing B1a B cells in the PerC by ADCC, although binding and duration of the antibody was similar to that in spleen or blood [8]. These investigators suggest that the abnormal macrophages in the PerC could be the cause of the impaired ADCC[8].

The analyses of the PerC in Chapter 2 paved the road for the findings in **Chapter 3**. Given the importance of reactive oxygen species (ROS) in tumor microenvironments, we tested whether CLL cells in these two niches would differ in their ROS production. Indeed, the basal mitochondrial ROS production by TCL1-192 cells in the PerC was significantly higher than in the spleen[9]. Interestingly, this was also the case for normal B1a B cells in the PerC and spleen of WT mice. The ROS production by TCL1-192 cells was independent of BCR stimulation and could be inhibited by the antioxidant N-acetylcysteine (NAC). Surprisingly, ibrutinib reduced ROS levels by CLL cells in the PerC and induced them to emigrate into the circulation. These findings led us to conclude that there may be niches in the body that hide and protect tumor cells from being targeted, possibly due to the production of ROS. This may in turn reduce the durability of response to a treatment of a patient. Combining multiple drug targets may help reduce these possibilities.

As key players in mounting proper immune responses against bacteria and pathogens, $CD8^+$ T cells suffer from "exhaustion" in case of chronic viral infections, exposure to bacteria and cancer[10, 11]. Re-activating these dysfunctional T cells is of high interest for durable responses against tumor cells. Having demonstrated the efficacy of αSLAMF6 in its ability to induce ADCC and modulate BCR signaling, we asked in **Chapter 4** whether SLAMF6 signaling and αSLAMF6 had an impact on $CD8^+$ T cells. In order to address this question, we used an adoptive transfer model of EμTCL1 cells into immuno-competent WT mice. As leukemic cells expand, $CD8^+$ T cells acquired a phenotypical and functional state of exhaustion, demonstrating the EμTCL1 mouse model a useful platform to study the role of T cells[12].

First, we investigated the role of SLAMF6 in the tumor microenvironment by transferring EμTCL1 cells into SLAMF6-/- mice. Strikingly, we identified an increased percentage and numbers of $PD1^+$ subpopulation of $CD3^+$ $CD44^+$ $CD8^+$ T cells compared to transfers into WT mice. These T cells had significantly reduced cytotoxic capacity measured by degranulation, granzyme B and IFNγ, which are

characteristics of effector $CD8^+$ T cell functions. These findings already suggested that SLAMF6 is a negative regulator of T cell exhaustion.

Then, we hypothesized that injections of αSLAMF6 would have a profound effect on T cells in this system and indeed found a remarkable effect. The exhausted $PD1^+$ subpopulation of $CD3^+$ $CD44^+$ $CD8^+$ T cells was significantly reduced in spleen and blood of these mice and the cytotoxic capacity of remaining $CD8^+$ T cells were significantly improved. In addition to the antibodies ability to induce ADCC and reduce BCR signaling, the finding that αSLAMF6 can also empower $CD8^+$ T cell responses makes it a favorable candidate for targeting. The selective reduction of $PD1^+$ $CD3^+CD44^+CD8^+$ cells by αSLAMF6 was independent of the presence of the T / NK cell adaptor SAP, as determined by the use of SAP-/- mice.

Distinct from the microenvironments analyzed in Chapter 2 and 3, analysis of PerC in **Chapter 4** revealed significantly reduced percentage of leukemic infiltration and number of cells in mice injected with αSLAMF6 compared to its isotype control injected mice. Surprisingly, αSLAMF6 induced signaling by $CD8^+$ T cells in this compartment, although ADCC is impaired in the PerC. Furthermore, the increase in the $PD1^+$ subpopulation of $CD3^+$ $CD44^+$ $CD8^+$ T cells suggests that the antibody selectively targets this population. The decrease in leukemic expansion indicates that the antibody likely reactivated some of the T cells in the PerC.

In aggregate, these findings show that initiation of SLAMF6 signaling shapes the immune response at multiple levels. Effects we have identified using the murine model of CLL and anti-SLAMF6 are summarized in **Figure 1.** Binding of antibody to SLAMF6 on the surface of CLL B cells induces ADCC by Fc receptor binding and activation of NK cells. Simultaneously, this binding on CLL cells reduces BCR signaling vital for their survival. This reduction is enhanced when used in combination with ibrutinib in both murine as well as human CLL cells, presented in **Chapters 2 and 4**. Furthermore, binding of αSLAMF6 to the co-stimulatory molecule SLAMF6 induces a reduction of exhausted $PD1^+$ $CD3^+CD44^+CD8^+$ T cells, thus potentiating the cytotoxicity of $CD8^+$ T cells. Several hypotheses may help explain the immunomodulatory action of αSLAMF6. First, recent studies demonstrated that SAP is a negative regulator of PD-1 function[13]. While SAP does not bind directly to PD-1 cytoplasmic tail or with SHP-2 binding, it inhibits their activity by shielding substrates of SHP-2 from its phosphatase activity. Therefore, it is possible that stimulation of SLAMF6 recruits SAP, which in turn inhibits SHP-2 activity at the immune synapse and thus disrupts PD-1 signaling and/or expression on the surface. Secondly, there may be a selective induction of

apoptosis of PD1$^+$ CD8$^+$ T cells by the antibody, thus leaving only the effector CD8 T cells with enhanced cytotoxic functions due to signaling. In fact, Shi and colleagues demonstrated that PD1 and PD-L1 upregulation induces apoptosis of CD8$^+$ T cells[14]. While we don't find upregulation of PD-L1 on the tumors (unpublished), whether αSLAMF6 can upregulate PD-1 on CD8$^+$ T cells, thus causing apoptosis, needs further investigation.

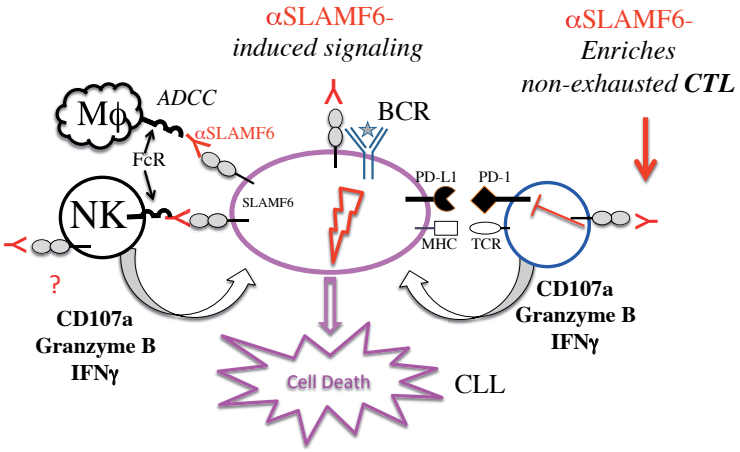


Figure 1. Mechanisms of action of aSLAMF6.

a. In the CLL mouse model, αSLAMF6 induces ADCC (1) mediated by engagement of antibody Fc portion with Fc receptors on macrophages and NK cells. Signaling of SLAMF6 in CLL B cells reduces proximal BCR signaling and survival (2). Anti-SLAMF6 reduces the number of PD1$^+$ CD3$^+$CD44$^+$CD8$^+$ T cells and results in increased effector functions. **b.** Anti-SLAMF6 also induces ADCC in a setting where B cells are primed with antigen (NP-OVA or auto-antigen) to inhibit T cell dependent responses and thus result in reduced antibody responses and inhibit autoimmunity[2, 4].

Taken together, the outcomes of our studies clearly identify αSLAMF6 as a useful tool to target the tumor and the tumor microenvironment at multiple points, by ADCC, reducing exhausted CD8$^+$ T cells and direct signaling. The data predict that αSLAMF6 will be more efficacious in the treatment of B-cell malignancies than rituximab (αCD20), which is well-accepted treatment of B cell tumors. Although promising responses have been made, patients eventually relapse or become refractory. When combined with ibrutinib, the leukemic cells appeared to down-regulate CD20 from its cell surface to escape ADCC and thus leave the treatment ineffective[15]. For all these reasons, SLAMF6 may represent to be a good target that could warrant more durable responses in clinic.

Chapter 5 of this thesis sheds light into the hypothesis that SLAMF3 is a negative checkpoint inhibitor of auto-reactive B cells that regulates cGVHD. Previous work by the Engel and Terhorst labs demonstrated that SLAMF3 deficient mice spontaneously produced autoantibodies regardless of their genetic background, suggesting a role for SLAMF3 in safeguarding mechanisms to prevent a breach of tolerance[16]. Injections of αSLAMF3 into WT mice eliminated marginal zone B cells, significantly reduced innate like B cells and modulated B cell activation thresholds[3]. In addition, αSLAMF3 reduced invariant natural killer T (iNKT) cell numbers with impaired IFNγ and IL-4 production[17]. Using the cGVHD model, we showed that injections of αSLAMF3 monoclonal antibody expanded formation of spontaneous germinal centers (GCs), which are seminal to autoantibody production. In accordance with increased GCs, T follicular helper (TFH) cells were also expanded with increased cytokines IL-21, IFNγ and IL-4. In contrast, abundance of regulatory T (Treg) and T follicular regulatory (TFR) cells were reduced. These results overall indicated that GC B – TFH cell interactions in the germinal centers are regulated by SLAMF3 signaling and blocking SLAMF3 signaling in context of autoreactive B cells may serve as a therapeutic target in autoimmunity. In contrast, injections of αSLAMF6 to WT mice immunized with NP-OVA or in a cGVHD model inhibited T and B cell responses in both of these distinct *in vivo* models[2, 4]. These finding are summarized in **Figure 2**.

In conclusion, SLAMF6 and SLAMF3 play vital roles in regulating T – B cell interactions and inducing or interrupting these signals appear to have impact in cellular responses (See Figure 1). While SLAMF6 is a negative checkpoint inhibitor of $CD8^+$ T cell exhaustion, targeting with monoclonal antibody provided therapeutic benefit to T cell functions and overall CLL expansion. In addition, we found that SLAMF3 is a negative regulator of autoreactive B cells and targeting receptor interactions with blocking antibodies can help autoimmunity related diseases. How these SLAMF members and others may be involved in other immune responses that can contribute to disease pathogenesis needs further investigation. Targeting SLAMF receptors will add to current treatments of B cell diseases.

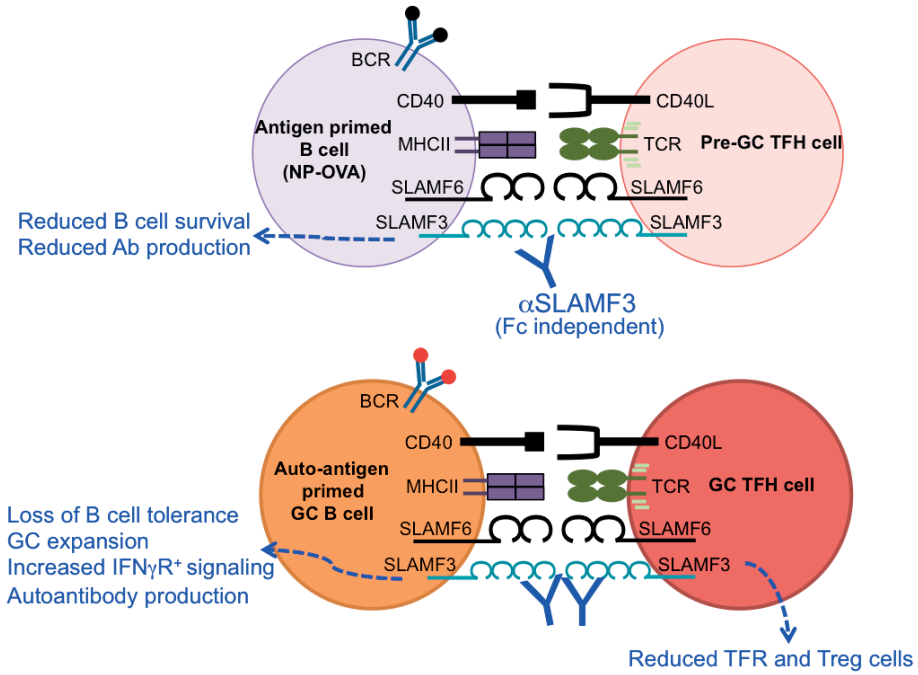


Figure 2. Mechanisms of action of aSLAMF3.

Anti-SLAMF3 used in these experiments work in an Fc independent manner. When B cells are primed with antigen, e.g. NP-OVA immunizations, injections of αSLAMF3 down-regulates the CD19 complex and inhibits activation and survival[3]. However, when αSLAMF3 is administered in an auto antigen primed GC B cell, it leads to loss of B cell tolerance and induces expansion of GC B cells and autoantibody production. In addition, reduced TFR and Treg cells in the GCs supplement this loss of tolerance.

Future Directions

Although single agent immunotherapies are effective in the initial phases, tumors eventually figure out a way to escape from the detrimental effects of treatment. Reasons of relapse in case of CLL include down-regulation of CD20 from the cell surface of CLL after rituximab, or upon acquired mutations at BTK at the binding site of ibrutinib. Therefore, targeting the tumor and tumor microenvironment by multiple approaches is essential for more durable responses. With this thesis, we have shown promising effects of αSLAMF6 in its ability to target the tumor directly and activate T cells in the microenvironment. While αSLAMF6 combinations with ibrutinib are more effective than either agent alone, identifying additional optimal combinations will be key. These include:

- **Combining αSLAMF3 with αSLAMF6** to test whether T cell responses are improved will be explored. In a preliminary set of experiments, we have tested

αSLAMF3 in our murine aggressive TCL1-192 CLL model and found lowered leukemic infiltration in bone marrow and blood (unpublished).

- **Combining MIF inhibitors with αSLAMF6** to further reduce the leukemic burden. Previous studies by the Shachar lab demonstrated that SLAMF5 is a survival receptor in CLL that gets upregulated in the presence of MIF[18]. MIF inhibitors are readily available that the Terhorst Lab [19]. It will also be of interest to study the effect of MIF inhibition in combination on macrophage functions.
- **Generating bispecific antibodies**. Generating and testing CD19-SLAMF6 or CD3-SLAMF6 bispecific antibodies in B cell malignancies can work to bring B and T cell together. Binding of SLAMF6 on a $CD8^+$ T cell can activate them, to induce more efficient killing.
- SLAMF6 can be an activating or inhibitory receptor on the surface of human NK cells[20, 21]. The Watzl lab has shown that binding of the SLAMF4, SLAMF6 and SLAMF7 ligands **enhance cytotoxicity of NK cells**. Understanding this mechanism should add to the repertoire of SLAMF6-based therapeutic approaches.
- Because SLAMF6 responds to **microbial proteins**, these entities could form a platform for novel immunotherapy approaches.
- With the understanding that there may be niches in the body that render therapies ineffective, activating immune cells or improving efficacy through combination therapy is of importance. **Targeting the hypoxic environment of the PerC** is one such possibility. Hypoxia inducible factor 1α (HIF-1α) induces immune suppression and promotes leukemic survival and expansion in CLL. Inhibiting HIF-1α or by inhibiting A2A adenosine receptor may reactivate the PerC environment and in combination trigger ADCC and induce cytotoxicity of T cells by αSLAMF6.

In conclusion, SLAMF6 is a key receptor for targeting and warrants further investigation for its use in clinic.

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CHAPTER 6

Nederlandse samenvatting en conclusies

Het wordt steeds evidenter dat SLAMF receptoren en hun signaaleiwitten in staat zijn om de aangeboren en adaptieve immuunreacties te beinvloeden[1]. Ondanks deze voortschrijdende kennis, blijven deze receptoren ondergewaardeerd als mogelijke therapeutische targets. In dit proefschrift beschrijven we een studie naar SLAMF receptoren als targets voor nieuwe therapieën tegen chronische lymphocytische leukemie (CLL), andere B cel tumoren en graft-vs-host ziekte. We hebben monoclonale antilichamen tegen SLAMF6 getest in CLL-modellen, en tegen SLAMF3 in graft-vs-host-ziekte modellen. Al eerder was gevonden dat anti-SLAMF6 en anti-SLAMF3 adaptieve humorale immuniteit tegen 4-hydroxy-3-nitrophenylacetyl hapten-ovalbumine conjugaten konden remmen (NP-OVA)[2, 3]. In dit proefschrift wordt beschreven hoe dit monoclonale antilichaam tegen SLAMF6 (αSLAMF6) in combinatie met kleine moleculen als nieuwe en waardevolle immuuntherapie tegen CLL en B cel lymfomen kan worden ingezet. Tot onze verdere verrassing ontdekten we dat in een model van chronische Graft-vs-Host ziekte (cGVHD)[4] αSLAMF6 de productie van auto-reactieve antilichamen kan remmen, maar dat anti-SLAMF3 antilichamen (αSLAMF3) de productie van auto-reactieve antilichamen juist verhogen in dit zelfde model.

In **Hoofdstuk 2** worden de effecten van αSLAMF6 in een CLL muis-model onderzocht; in dit model werd een agressieve TCL1-192 leukemie-kloon geïnjecteerd in een SCID (Severe combined immune deficiency) muis, die geen B en T-cellen aanmaakt. Het toedienen van één of twee injecties van αSLAMF6 in deze muizen lijkt twee effector functies te hebben. Allereerst zien we de inductie van antilichaam-afhankelijke cellulaire cytotoxiciteit (*antibody dependent cellular cytoxocity,* ADCC), vergelijkbaar met de ADCC die wordt gezien tijdens het toedienen van rituximab (anti-CD20) (ongepubliceerde waarneming). Ten tweede tonen we aan dat αSLAMF6 in staat is de proximale BCR signalering te dempen. Deze signalering is essentieel voor de overleving van CLL cellen. Verder gaf het combineren van twee experimentele therapieën tegen CLL: ibrutinib, een remmer van Brutons tyrosine kinase (BTK), en anti-SLAMF6, betere resultaten dan de twee individuele behandelmethodes los van elkaar.

De bevindingen beschreven in hoofdstuk 2 zijn vooral gebaseerd op analyses van de milt en het bloed, aangezien zich hier de meeste CLL-cellen bevinden. αSLAMF6 bleek echter ook in staat de leukemiecellen te verwijderen uit alle organen, behalve uit de buikholten en het omentum. Dit leidde tot de hypothese dat het micromilieu van de tumor effect had op de door αSLAMF6-geinduceerde ADCC. De remming van ADCC in deze niches kon worden omzeild door het toedienen van ibrutinib. Dit leek een reductie in het aantal TCL1-192 in de buikholte tot gevolg

te hebben. Het is al langer bekend dat B1a B-cellen in de buikholte een andere functie hebben dan de B-cellen die in de milt worden gevonden; ondanks een vergelijkbaar fenotype[5]. Dit kan verklaren waarom de hoogte van de signaaltransductie in CLL cellen in de buikholte anders is dan in de milt[6, 7].

Onderzoek uit het lab van Tedder heeft aangetoond dat, net als αSLAMF6 bij CLL cellen, ook, αCD20 therapie niet in staat is B1A B-celaantallen in de buikholte te verminderen d.m.v. ADCC; ook al bond het antilichaam even snel en even lang als in de milt of in het bloed[8]. Deze onderzoekers stellen dat het mogelijk atypische macrofagen in de buikholte zijn die verantwoordelijk zijn voor deze verminderde ADCC[8].

De analyses van de buikholte beschreven in hoofdstuk twee hebben geleid tot de bevindingen beschreven in **Hoofdstuk 3**. Aangezien reactieve zuurstofradicalen (*Reactive Oxygen Species,* ROS) een belangrijke rol spelen in het tumor micro-milieu, hebben we getest of CLL cellen andere ROS produceren in deze twee verschillende niches. Dit blijkt inderdaad het geval: de mitochondriale ROS productie door TCL1-192 cellen in de buikholte was significant hoger dan die in de milt[9]. Dit bleek ook het geval te zijn voor gewone B1a B-cellen in de buikholte en milt van wildtype muizen. De mate van ROS productie door TCL1-192 cellen was onafhankelijk van BCR stimulatie en kon worden geremd door antioxidant N-acetylcysteine (NAC). Verrassend genoeg kon ibrutinib de ROS productie door CLL cellen in de buikholte terugbrengen en deze cellen ertoe aanzetten uit de buikholte de bloedbaan in te migreren. Naar aanleiding van deze bevindingen hebben we geconcludeerd dat er mogelijk niches in het lichaam zijn waarin de tumorcellen beschermd worden tegen antibody-therapieën, mogelijk door de productie van ROS. Dit kan op zijn beurt het succes van de behandeling van een patiënt bepalen. Een combinatie van verschillende medicijnen kan dit ROS-effect weer terugdringen.

$CD8^+$ T-cellen zijn belangrijke spelers in de immuunrespons tegen bacteriën en andere pathogenen. Deze cellen kunnen echter aan 'uitputting' lijden tijdens chronische virale infecties, blootstelling aan bacteriën, en tijdens kanker[10, 11]. Er is zeer veel interesse in de reactivatie van deze niet-functionerende T-cellen als behandeling tegen tumoren. Nadat we in de voorgaande hoofdstukken hebben aangetoond dat αSLAMF6 ADCC kan opwekken en BCR-signalerings routes kan moduleren, stellen we ons in **Hoofdstuk 4** de vraag of SLAMF6 signalering en αSLAMF6 ook een impact hebben op $CD8^+$ T-celbiologie. Dit hebben we onderzocht met behulp van een model waarin EμTCL1 leukemiecellen worden

geplaatst in immuun-competente wildtype muizen. Wanneer deze cellen gaan groeien in de muis ontwikkelen de $CD8^+$ T-cellen een 'uitputtings'-fenotype. Dit maakt de EμTCL1 muis tot een nuttig model om T-celuitputting te bestuderen[12].

We hebben eerst de rol van SLAMF6 in het tumor micro-milieu onderzocht door EμTCL1 cellen in SLAMF6-/- muizen te spuiten. In dit systeem vonden we een verhoging van het percentage $PD1^+$ cellen binnen de $CD3^+$ $CD44^+$ $CD8^+$ T-cel populatie, vergeleken met wildtype muizen. Deze T-cellen hadden een significant lagere cytoxische capaciteit, bepaald aan de hand van T-cell effector parameters zoals de-granulatie en de aanwezigheid van granzyme B en IFN γ. Deze bevindingen doen vermoeden dat SLAMF6 een negatieve regulator van T-cel uitputting is.

Onze volgende hypothese was dat het injecteren van αSLAMF6 een drastisch effect zou hebben op T-cellen in dit systeem. Dit was inderdaad wat we vonden. De uitgeputte $PD1^+$ subpopulatie van de $CD3^+$ $CD44^+$ $CD8^+$ T cellen was significant minder in de milt en het bloed van deze muizen en de cytotoxiciteit van de overgebleven T-cellen was significant hoger. De bevinding dat anti-SLAMF6 $CD8^+$ T cellen kan activeren, naast het opwekken van ADCC en het afzwakken van BCR signalering, maakt deze receptor een belangrijke therapeutische target. Het terugdringen van het aantal $PD1^+$ $CD3^+CD44^+CD8^+$ T-cellen door αSLAMF6 bleek verder onafhankelijk van de aanwezigheid van de T / NK cel adapter SAP. Dit was onderzocht door ook $SAP^{-/-}$ muizen te gebruiken (ongepubliceerde data).

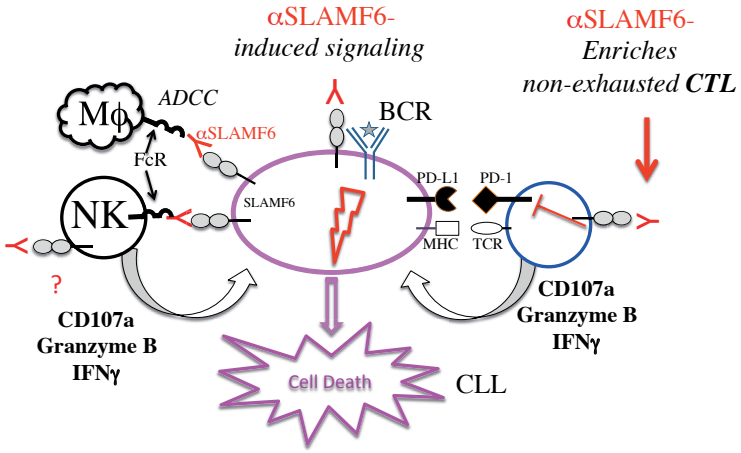
In tegenstelling tot het tumor micro-milieu beschreven in hoofdstukken 2 en 3 gaf de analyse in **hoofdstuk 4** van cellen uit de buikholte van muizen geïnjecteerd met αSLAMF6 een ander beeld, namelijk een significante verlaging van het aantal en percentage leukemie cellen, in vergelijking met controle muizen. Verrassend genoeg bracht αSLAMF6 hier juist de signaaltransductie door $CD8^+$ T cellen op gang, ondanks de remming van ADCC. De toename van de $PD1^+$ subpopulatie van $CD3^+$ $CD44^+$ $CD8^+$ T-cellen doet verder vermoeden dat het antilichaam deze populatie selectief target. De remming van de groei van de leukemie geeft aan dat het antilichaam waarschijnlijk sommige T-cellen reactiveert in de buikholte.

Deze bevindingen tonen aan dat het activeren van SLAMF6 signalering de immuunrespons op verschillende niveaus kan beïnvloeden. De effecten die we hebben aangetoond met behulp van het muismodel van CLL en anti-SLAMF6 zijn samengevat in **Figuur 1.** Binding van het antilichaam op het oppervlakte van B-cellen induceert ADCC via de Fc-Fc receptor interactie en de daaropvolgende activatie van NK cellen. Tegelijkertijd verzwakt deze binding de BCR signaal-

transductie, die essentieel is voor het overleven van de CLL. Ibrutinib versterkt deze signaalverzwakking in zowel muizen als menselijke CLL cellen. Dit staat beschreven in **hoofdstukken 2 en 4**. Verder activeert αSLAMF6 het co-stimulatoire eiwit SLAMF6 op T-cellen, wat een reductie in het percentage uitgeputte $PD1^+$ $CD3^+CD44^+CD8^+$ T-cellen tot gevolg heeft. Hiermee bevordert het de cytoxiciteit van $CD8^+$ T-cellen. Recent onderzoek heeft SAP aangewezen als negatieve regulator van PD-1[13]. SAP bindt niet direct aan de cytosolaire staart van PD-1, of aan het SHP-2-domain. De werking berust in plaats daarvan op het afschermen van de substraten van de fosfatase activiteit van dit molecuul.

Het is daarom mogelijk dat SLAMF6 SAP rekruteert, dat op zijn beurt weer SHP-2 remt in de synaps en daarmee de signaaltransductie van PD-1 verstoort. Deze data laten duidelijk zien dat αSLAMF6 een goed middel is om de tumor en het tumor micro-milieu op verschillende punten aan te pakken, o.a.door ADCC en door CD8 T-celuitputting te verminderen. De data voorspellen ook dat αSLAMF6 een betere behandeling zou kunnen zijn tegen B-cel tumoren dan de geaccepteerde behandeling met rituximab (αCD20). Tegen dit medicijn ontwikkelen tumoren vaak resistentie, wat tot terugval leidt. Wanneer het in combinatie met ibrutinib wordt gebruikt, verlagen de tumorcellen de expressie van CD20 op hun oppervlakte om zo de ADCC te ontwijken en daarmee de behandeling ineffectief te maken[14]. Om deze reden, zou SLAMF6 een potentieel klinische target kunnen zijn waarmee wel langdurige effecten kunnen worden bewerkstelligd.

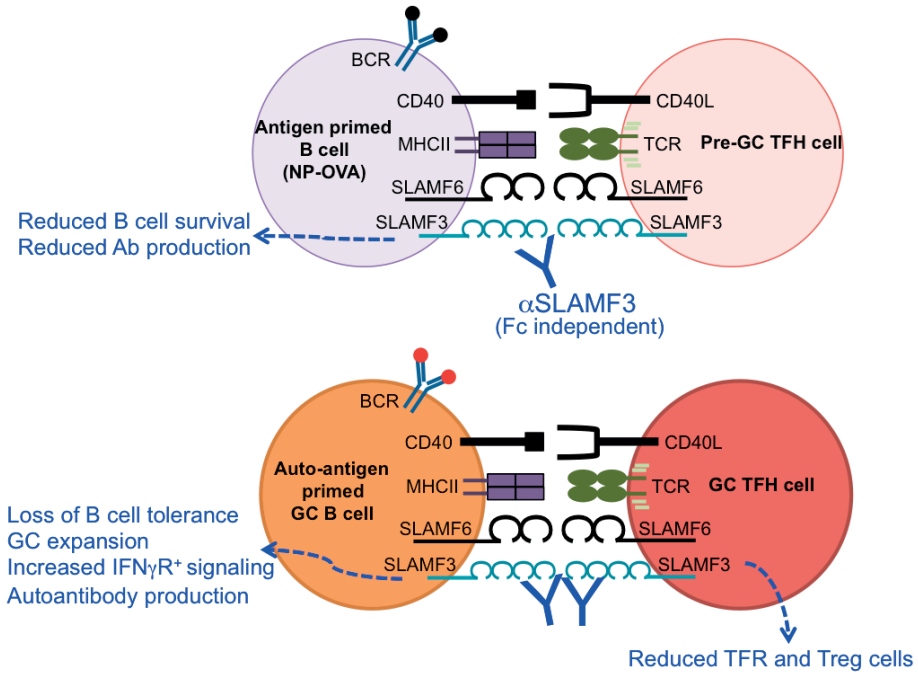
Hoofdstuk 5 van dit proefschrift werpt licht op de hypothese dat SLAMF3 een remmer is van auto-reactieve B-cellen die cGVHD reguleren. Eerder werk uit de laboratoria van Engel en Terhorst heeft aangetoond dat SLAMF3-deficiënte muizen spontaan auto-antilichamen produceren, ongeacht hun genetische achtergrond. Dit suggereert een rol voor SLAMF3 bij het beschermen van mechanismen die tolerantie-overschrijding voorkomen[15].



Figuur 1. Werkingsmechanismen van het antilichaam tegen SLAMF6.

In het CLL-muismodel induceert αSLAMF6 ADCC via de Fc-Fc-receptor interactie op macrofagen en NK-cellen. Het signaleren van SLAMF6 in CLL B-cellen verlaagt proximale BCR-signalering en daarmee het overleven van de B-cellen. Anti-SLAMF6 vermindert het aantal $PD1^+$ $CD3^+$ $CD44^+$ $CD8^+$ T-cellen, wat weer resulteert in verhoogde effectorfuncties.

Injecties met αSLAMF3 in wildtype muizen zorgde voor een eliminatie van B-cellen in de marginale zone, verminderde 'aangeboren' B-cellen en een modulatie van de B-cel activeringsdrempel[3]. Daarnaast verminderde αSLAMF3 de invariante natural killer T-cellen (iNKT) door verminderde IFNγ- en IL-4-productie[16]. In een cGVHD model hebben we aangetoond dat injecties met αSLAMF3 de spontane vorming van germinal centers (GCs) bevorderde. Deze zijn op hun beurt weer essentieel voor de productie van auto-reactieve antilichamen. In overeenstemming hiermee werd ook een groei in het aantal folliculaire T-helper (TFH) cellen gezien door hogere concentraties van IL-21, IFNγ en IL-4. In tegenstelling tot deze groei werd het aantal regulatoire T-cellen juist lager. Deze resultaten suggereren dat de GC B–TFH cel-interacties in germinale centers worden gereguleerd door SLAMF3 signalering en dat het blokkeren van SLAMF3 signalering bij auto-reactieve B-cellen een mogelijke therapeutische target voor de behandeling van auto-immuunziekten kan zijn. Hier staat echter wel tegenover dat het toedienen van injecties met αSLAMF6 aan wildtype muizen, die waren geïmmuniseerd met NP-OVA, of in het cGVHD model, de B- en T-celresponses werden geremd. Deze bevindingen zijn samengevat in **Figuur 2**.



Figuur 2. Werkingsmechanismen van αSLAMF3. De werking van αSLAMF3 is onafhankelijk van het Fc-domein: wanneer B-cellen worden geprimed met antigeen, door bijvoorbeeld NP-OVA-immunisaties, onderdrukken injecties van αSLAMF3 het CD19-complex, waarmee ze activering en overleving van de B-cel remmen[3]. Wanneer echter αSLAMF3 wordt toegediend in een met autoantigeen geprimede GC B-cel, leidt dit tot verlies van B-celtolerantie en induceert het de expansie van GC B-cellen en autoantilichaam-productie. Bovendien leidt de reductie in FHT en T_{reg} cellen in de GC tot een versterking van dit fenotype.

We kunnen dus concluderen dat SLAMF6 en SLAMF3 belangrijke rollen spelen in het reguleren van T- en B-cel interacties. Het induceren of onderbreken van deze signalen lijkt een impact te hebben op de cellulaire immuunrespons (zie Figuur 1). SLAMF6 is een negatieve regulator van checkpoint inhibitie tijdens CD8 T-celuitputting. Het targeten van deze receptor met een monoclonaal antilichaam heeft een therapeutische verbetering tot gevolg. Verder hebben we ontdekt dat SLAMF3 een negatieve regulator van auto-reactieve B cellen is en dat het targeten van deze receptor met antilichaam-agonisten zou kunnen bijdragen aan de behandeling van auto-immuunziektes. Deze en andere leden van de SLAM-familie kunnen echter ook een rol spelen bij de pathogenese van andere ziekten; verder onderzoek is dus nodig. Het targeten van SLAMF receptoren heeft de potentie een waardevolle toevoeging te zijn aan het huidige arsenaal van medicijnen tegen B cell–gerelateerde aandoeningen.

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Curriculum Vitae

On 17 December 1989, Burcu Yigit was born in Ankara, Turkey. There, she finished high school, obtaining a diploma from Ankara Ataturk Anatolian High School at age 16. In 2006, she started her undergraduate studies at Bilkent University in Ankara in Molecular Biology and Genetics. In fall of 2010, she joined the Molecular Cell Biology Master's Program at Free University in Amsterdam. During this two-year program, within the first year she did an internship at the Netherland Cancer Institute (NKI) under the supervision of Dr. Sander van Kasteren in Dr. Huib Ovaa's lab. This was her first real exposure to immunology, where she studied antigen cross presentation using a beta-galactosidase assay system. After the completion of this internship, she decided to pursue a career in the field of immunology and joined Prof. Dr. Cox Terhorst's lab at Beth Israel Deaconess Medical Center, Harvard Medical School for an internship in her second year. Under the supervision of Dr. Cynthia Detre, she focused on role of SAP in B cells in a genetic background dependent manner. Upon the kind invite from Prof. Terhorst to pursue her PhD in his laboratory, she started her journey in November 2012 to try and utilize SLAMF receptors as therapeutic targets in chronic lymphocytic leukemia. The result of this work is described in this dissertational thesis. On June 4, 2018, Burcu will defend this work, before the University of Utrecht, to obtain the degree of PhD with Prof. Dr. Emmanuel Wiertz and Prof. Dr. Cox Terhorst.

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