Published in final edited form as:

J Immunol. 2022 March 15; 208(6): 1417–1423. doi:10.4049/jimmunol.2100819.

SLAMF1 is dispensable for vaccine induced T cell development but required for resistance to fungal infection

Elaine M. Kohn[*], **Lucas dos Santos Dias**[*], **Hannah E. Dobson**[*], **Xin He**[*], **Huafeng Wang**[*], **Bruce S. Klein**[*,†,‡], **Marcel Wüthrich**[*]

[*] Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, University of Wisconsin-Madison.

[†] Department of Internal Medicine, University of Wisconsin School of Medicine and Public Health, University of Wisconsin-Madison.

[‡] Department of Medical Microbiology and Immunology, University of Wisconsin School of Medicine and Public Health, University of Wisconsin-Madison.

Abstract

Homotypic SLAM receptor-ligand cell surface interactions between myeloid and lymphoid cells regulate innate and adaptive immune responses. Here, we report that SLAMF1 is indispensable for host resistance to primary and vaccine-induced protection against fungal infection. Since vaccine immunity is dependent on cell-mediated immunity, we investigated the development of Ag-specific T cells. We studied the T cell intrinsic and extrinsic role of SLAMF1. We generated SLAMF1$^{-/-}$ TCR transgenic mice and analyzed the responses of adoptively transferred T cells. We also tracked endogenous Ag-specific T cells by using a tetramer. Intrinsic and extrinsic SLAMF1 signaling was dispensable for the development of anti-fungal Th1 and Th17 cells, which are requisite for the acquisition of vaccine-induced immunity. Despite intact T cell development, vaccinated SLAMF1$^{-/-}$ mice failed to control fungal infection. Failed accumulation of antigen-specific T cells in the lung upon infection of vaccinated mice was due to uncontrolled early infection and inflammation, revealing a role for SLAMF1 in innate host immunity.

Keywords

SLAMF1; T cell; fungi; vaccine

INTRODUCTION

Innate and adaptive immune responses are orchestrated by interactions between cell surface molecules expressed on leukocytes. The induction of adaptive immune responses mediated by T cells requires the interaction between the T cell receptor (TCR) and the cognate antigen presented by the cognitive major histocompatibility complex (MHC) as a first signal. As a second signal, engagement of costimulatory molecules by their ligands strengthens

Reprints and correspondence to: Dr. Marcel Wüthrich, University of Wisconsin, Microbial Sciences Building, 1550 Linden Drive, Madison, WI, 53706, Telephone: 608-262-7703. Fax: 608-262-8418. mwuethri@wisc.edu.

cell-cell interactions between antigen presenting cells (APCs) and naïve T cells and triggers intracellular signaling networks that regulate T cell activation, differentiation, apoptosis, proliferation, and acquisition of effector function and tolerance induction (1, 2). The best characterized T cell costimulatory pathways are initiated by members of the B7 family of receptors, which interact with the CD28/CTLA-4 receptors expressed on T cells (3). In this report, we investigate the costimulatory role of recently discovered signaling lymphocyte activation molecule (SLAM) receptors in regulating adaptive immune responses.

SLAM family receptors are widely expressed in hematopoietic cells and control innate and adaptive immune responses (4, 5). Receptor-ligand interactions occur between members of the SLAM family in a homotypic fashion (6). Subsequent binding of SLAM-associated protein (SAP) or ETA2 to the cytoplasmic tail of the SLAM receptors through a tyrosine-containing motif regulates signal transduction (6). SAP is expressed by T cells and ETA2 is expressed by antigen presenting cells (APCs). These two molecules can recruit and activate several Src kinases that modulate cell activation by signals generated through the T cell receptor (TCR) and costimulatory molecules such as CD28. Signals mediated by the SLAM receptors can also affect the function of APCs (7). SLAM family receptor-ligand pairs regulate proliferation, cytotoxicity, and cytokine production of T cells; lytic activity and cytokine production of natural killer (NK) cells; B cell activation and memory generation; and regulation of neutrophil and macrophage killing (5).

Mouse and human SLAMF1 is rapidly up-regulated upon activation of T cells, macrophages and dendritic cells (DC) (8). Cannons et al. (9) found that SLAMF receptors use the CARD9-containing CBM complex to regulate NKT cell selection. Since Card9 is required for vaccine induced resistance to fungal infection and differentiation of Th1 and Th17 cells (10), we investigated whether SLAMF1 operates as an obligate costimulatory molecule on APCs and naïve T cells to promote vaccine-induced T cell immunity (5). We found that SLAMF1 expression is upregulated in DCs from wild type but not Card9$^{-/-}$ mice when stimulated *in vitro* with vaccine yeast. We also found that vaccinated SLAMF1$^{-/-}$ mice were unable to control fungal infection whereas the development of vaccine induced Th1 and Th17 cells were unimpaired. Thus, we conclude that SLAMF1 and Card9 are not part of the same T cell-intrinsic and -extrinsic signaling pathway and that SLAMF1 is crucial for the innate host response and control of infection.

MATERIALS AND METHODS:

Fungi

B. dermatitidis strains used were American Type Culture Collection (ATCC) 26199, a wild type virulent strain, and the isogenic, attenuated mutant lacking BAD1, designated strain 55 (11). Isolates of *B. dermatitidis* were maintained as yeast on Middlebrook 7H10 agar with oleic acidalbumin complex (Sigma Chemical Co., St. Louis, MO) at 39°C.

Mice

SLAMF1$^{-/-}$ mice were generated and obtained from Dr. Cox Terhorst at the Beth Israel Deaconess Medical Center at the Harvard Medical School in Boston (10). *C57BL/6* mice

were obtained from Jackson Laboratory and bred at our facility. *Blastomyces*-specific T-cell receptor (TCR) transgenic (Tg) 1807 mice were generated in our laboratory and were backcrossed to congenic CD90.1^{+} (Thy1.1^{+}) mice as described previously (12, 13). Mice were 7 to 8 weeks old at the initiation of these experiments. Mice were housed and cared for as per guidelines of the University of Wisconsin Animal Care Committee who approved all aspects of this work.

Vaccination and fungal infection

Mice were vaccinated once subcutaneously (SC) with 1–1.6×10^{7} *B. dermatitidis* yeast, (ATCC strain 26199 or 55), heat killed (65C for 30 minutes). Mice were challenged intratracheally 28 days post vaccination with 2×10^{4} wild-type virulent 26199 *B. dermatitidis* yeast and analyzed for lung T cell responses and lung CFU at day 4 post-infection. Fungal burdens were measured from homogenized tissue by serial dilutions on brain heart infusion agar plates with penicillin and streptomycin.

Tissue processing

Spleens and brachial lymph nodes were harvested either 7, 8, 14, or 28 days post vaccination, or 4 days post infection. Spleen and draining lymph nodes were then mashed through 40 μm filters and washed with 5 ml cold wash buffer (RPMI with 1% FBS, 1% penicillin and streptomycin). Splenocytes were subjected to red blood cell lysis with 3 ml ACK lysis buffer for 3 minutes at room temperature, stopped with 5 ml complete RPMI (RPMI with 10% FBS, 1% penicillin and streptomycin). Lungs were harvested after challenge and dissociated in Miltenyi MACS tubes and digested with collagenase (1 mg/ml) and DNase (1 μg/ml) for 25 minutes at 37°C. Digested lungs were resuspended in 5 ml of 40% Percoll in RPMI; 3 ml of 66% percoll in PBS was underlaid (GE healthcare 17–0891-01). Samples were spun for 20 min at 2000 rpm at room temperature. Lymphocytes were then harvested from the buffy coat layer and resuspended in complete RPMI.

Generation of bone marrow-derived dendritic cells (BMDC)

Bone marrow was harvested from the femurs of 6–8 weeks old mice. Bone marrow was harvested by centrifugation through nested microcentrifuge tubes as described by (14). Cells were plated at 10^{6}/ml in 6 ml of complete media (RPMI with 10% FBS, 1% penicillin and streptomycin) with 20 ng/ml GM-CSF and 5 ng/ml IL-4 (15). BMDCs were collected from the nonadherent cells in culture.

DC/yeast coculuture and Slamf1 expression by microarray and RT-PCR

3 × 10^{5} non-adherent BMDCs from wild-type C57BL6, Card9$^{-/-}$ and MyD88$^{-/-}$ mice were cocultured with 3 × 10^{5} *B. dermatitidis* vaccine yeast or medium alone in a 24-well plate for 24 hours. Total RNA from the BMDC cultures described above was purified using the Qiagen RNeasy kit (Cat. # 74106) according to the manufacturer's protocol. Genomic DNA was removed using Turbo DNase (Ambion Cat.#AM2238). Following DNase treatment, the RNA was cleaned up using Qiagen RNeasy columns according to the manufacturer's protocol. RNA was reverse transcribed and Slam1 expression analyzed by Affymetrix mouse gene 2.0 ST microarray at the Biotech Center at UW-Madison and RT-PCR. The n-fold

change of gene expression for yeast stimulation vs. non-stimulation was calculated using the comparative C_T method (16).

Quantitative RT-PCR

RNA was isolated and reversely transcribed using the RNeasy Kit (Qiagen, Cat# 74106) and the Invitrogen SuperScript IV VILO Master Mix (ThermoFisher Scientific, Cat# 11756050). qPCR was performed on a Rotor-Gene Q system (Qiagen) using TaqMan Gene Expression Assays (Slamf1 Mm00443316_m1; GAPDH Mm99999915_g1) and Rotor-Gene Multiplex PCR Kit (Qiagen, Cat# 204774). Relative quantification was performed by the ΔΔCT method with GAPDH as a reference gene.

Flow cytometry

All FACs samples were stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen L34975) and F_c block for 10 minutes at room temperature. Tetramer staining was conducted for 1 hour at room temperature, followed by surface and intracellular target staining for 20 minutes at 4°C. All panels included a dump channel to decrease background in $CD4^+$ T cells. 50 μL of AccuCheck Counting Beads (Invitrogen PCB100) were added to all samples to determine absolute cell counts. Samples were acquired on an LSR Fortessa at the University of Wisconsin Carbone Cancer Center Flow Lab. All flow plots presented are concatenates of groups.

For cell surface analysis, several antibody cocktails were used. For adoptive cell transfer experiments (Fig. 2+3): CD90.1 PerCP-Cy5.5 (OX-7 BioLegend 202516), CD8a PE-Cy7 (53–6.7 BD A15385), CD44 BV650 (IM7 BioLegend cat#103049), CD90.2 BV785 (30-H12 BioLegend 105331), CD11b APC (M1/70 BioLegend 101212), CD11c APC (N418 BioLegend cat#117310), NK1.1 APC (PK136 BioLegend 108710), B220 APC (RA3–62B BioLegend 103212), CD4 BUV737 (RM4–5 BD 565246). For endogenous surface-stained experiments (Fig. 4+5): CD8 PerCP-Cy5.5 (53–6.7 BioLegend 100734), CD44 BV650 (IM7 BioLegend cat#103049), CD90.2 BV785 (30-H12 BioLegend 105331), CD11b APC (M1/70 BioLegend 101212), CD11c APC (N418 BioLegend 117310), NK1.1 APC (PK136 BioLegend 108710), B220 APC (RA3–62B BioLegend 103212), CD4 BUV737 (RM4–5 BD 565246). For intracellular staining (Fig. 5) the following antibodies were used: IFNγ PE-Cy7 (XMG1.2 BD 557649), IL-17A BUV395 (TC11–18H10 BD 565246), IL-5 BV421 (TRFK5 BioLegend 504311), and IL-13 (eBio13A eBioscience 48–7133-82). Some mice (Fig. 5) received 2mg BrdU by intraperitoneal injection for three consecutive days starting on the day of challenge. BrdU uptake was analyzed by flow cytometry via FITC conjugated anti-BrdU antibody (BD 559619).

Tetramer enrichment and positive selection for $CD4^+$ T cells

Miltenyi LS columns on a quadroMACS magnet were used to enrich $tetramer^+$ cells from secondary lymphoid organs (SLOs) before challenge (Fig. 4A+B). After processing, samples were washed with 15 mL and resuspended in cold sorter buffer (PBS with 2% FBS) to a volume twice the size of the pellet. 2 μL of Fc block was added to each sample and incubated for 5 minutes before adding Bl-Eng2 tetramer at a concentration of 5–25 nM. The tetramer stain was done for 1 hr at room temperature in the dark. Samples were washed

and keep on ice for the remainder of the protocol. 100 μL of Miltenyi anti-PE microbeads (Miltenyi 130–048-801) were added to each sample and incubated for 30 minutes on ice; samples were then washed and resuspended in 3 mL sort buffer. LS columns were wet and samples were filtered through 40 μm filters into columns. Columns were washed with 3 mL cold sorter buffer three times before eluting bound fractions. Bound fractions were then surface stained and analyzed as described above.

Adoptive transfer of transgenic 1807 T cells

Single cell suspensions of 10^6 magnetic bead purified $CD4^+$ cells from $SLAMF1^{+/+}$ 1807 Tg $Thy1.1^+$ and $SLAMF1^{-/-}$ 1807 Tg $Thy1.1^+/Thy1.2^+$ mice were injected intravenously into $Thy1.2^+$ wild-type C57BL/6 recipients.

Ex vivo stimulated cytokine production by splenocytes

10^6 splenocytes and draining lymph node cells harvested from vaccinated mice were harvested at day 4 post-infection and stimulated with media (containing 10% FBS, 1% penicillin and streptomycin in RPMI), Bl-Eng2 (5 ug/ul), cell wall membrane extract (CW/M, 10 ug/ml), or heat killed yeast (at a ratio of 1 yeast per 2 lymphocytes). Protein concentration was analyzed by Magpix (MCYTOMAG-70K).

Staining of apoptotic and necrotic cells—Annexin-V/propidium iodide staining (A-V/PI) is a common flow cytometric method to analyze cells in apoptosis. However, A-V/PI staining does not permit fixation and/or permeabilization of cells making it impossible to evaluate intracellular markers and study pathogen-infected cells due to biosafety concerns. Instead, we used a fixed apoptotic/necrotic (FAN) stain (17). FAN uses a combination of anti-phosphatidylserine antibody (Millipore, Cat#16–256, clone 1H6) and fluorescent amine-binding dyes (live/dead fixable near-IR dead staining kit Invitrogen L34975).

Statistics—All statistics were calculated in Prism 7 for Mac OS X, version 7.0d. An unpaired two-tailed T test with Welch's correction was used to calculate significance between groups in absolute number of cells (Figures 2,3,4,5,6). A Mann-Whitney test used for analysis of all CFU data. A p value of <0.05 was considered statistically significant (* indicates $p<0.05$, ** <0.005, *** <0.001, and **** <0.0001). Whiskers represent the geometric mean and geometric standard deviation unless stated otherwise. For survival, significance was set at $p=0.0083$ after Bonferroni's correction for multiple comparisons. For ex vivo cytokine production, significance was determined by unpaired T test with Welch's correction (* = $p<0.05$).

RESULTS

SLAMF1 is required for innate and adaptive immunity to *B. dermatitidis*.

Both Card9 and Myd88 signaling are required for the acquisition of vaccine induced immunity to fungi (10, 18). Here, we sought to uncover CARD-9 and Myd88 dependent signaling components involved. We stimulated bone marrow derived dendritic cells (BMDCs) from wild-type, $MyD88^{-/-}$ and $Card9^{-/-}$ mice with heat killed yeast and analyzed differential gene expression by Affymetrix microarray analysis. SLAMF1 was

among the most differently upregulated genes in cells from wild-type vs. knockout mice (Supplementary Table 1). Stimulated BMDCs from wild-type mice upregulated SLAMF1 by 12.014-fold relative to unstimulated cells, cells from MyD88$^{-/-}$ and Card9$^{-/-}$ mice only upregulated SLAMF1 by 1.807 and 1.133, respectively. We validated these results by RT-qPCR. By RT-qPCR, stimulated BMDCs from wild-type mice upregulated SLAMF1 by 27.0-fold relative to unstimulated cells, cells from MyD88$^{-/-}$ and Card9$^{-/-}$ mice only upregulated SLAMF1 5.8 and 1.64, respectively (Fig. 1A).

To test whether SLAMF1 is required for vaccine-induced protection, we performed a survival analysis and also measured lung CFU. The majority of naïve and vaccinated SLAMF1$^{-/-}$ mice succumbed to infection within one week, and thus failed to acquire protection after vaccination (Fig. 1B). All vaccinated wild-type mice survived more than 30 days post-infection and nine of 14 vaccinated mice acquired sterilizing immunity. The CFU of the remaining five had a mean and SEM of 30 ±21.15 (data not shown). At day 4 post infection, vaccination decreased lung CFU by 172-fold in wild-type mice, but only 11.3-fold in SLAMF1$^{-/-}$ mice (Fig 1C). In addition, unvaccinated SLAMF1$^{-/-}$ mice had 36-fold higher lung CFU than unvaccinated wild-type controls (Fig 1C), and the SLAMF1$^{-/-}$ mice succumbed to infection a week before the unvaccinated wild-type mice. These data indicate that SLAMF1 is required for resistance to fungal infection.

Splenocytes from vaccinated SLAMF1$^{-/-}$ mice produce less IL-17 and IFN-γ after stimulation with fungal antigen (Fig. 1D+E), compared to SLAMF1$^{+/+}$ mice, indicating that SLAMF1 is required for the production of protective type 1 and 17 cytokines in vaccinated mice. Since we previously reported that Card9 and Myd88 are required for the acquisition of T cell mediated resistance to fungal infection (10, 18), we sought to investigate the role of SLAMF1 in the development of Ag-specific T cells.

T cell intrinsic contribution of SLAMF1 to CD4 T cell activation, expansion and contraction.

We hypothesized that SLAMF1 is required for the development of Th1 and Th17 cells. To test this, we studied Ag-specific CD4^{+} T cells during activation, expansion, and contraction. SLAMF1 is expressed on both T cells and APCs (5, 6), To investigate the role of SLAMF1 expressed on T cells, we generated SLAMF1-deficient, anti-fungal TCR transgenice 1807 mice by crossing SLAMF1$^{-/-}$ mice with congenic (CD90.1^{+}) calnexin-specific TCR Tg 1807 mice (19). (Fig. 2A). We co-transferred equal numbers of naive SLAMF1-deficient (CD90.1^{+}/CD90.2^{+}) and sufficient (CD90.1^{+}) 1807 T cells into a wild-type (CD90.2^{+}) recipient mice prior to vaccination to assess the effect of T cell intrinsic SLAMF1 on T cell development. At day 7 post-vaccination, the expansion of SLAMF1$^{-/-}$ 1807 T cells was actually enhanced compared to SLAMF1$^{+/+}$ 1807 cells. Comparable numbers were detected at day 14, and enhanced contraction of SLAMF1$^{-/-}$ 1807 T cells was found by day 28 (Fig. 2B+C). These data suggest that SLAMF1 expression on CD4 T cells is required for their survival during contraction, but not for their activation and expansion.

T cell extrinsic contribution of SLAMF1 on T cell expansion, contraction, and recall after vaccination.

To assess the role of SLAMF1 expressed on APCs (T cell extrinsic role) we adoptively transferred naïve SLAMF1$^{+/+}$ 1807 T cells into SLAMF1-sufficient or deficient recipient mice (Fig. 3A) and compared T cell expansion, contraction, and the recall response upon challenge. At day 8 post-vaccination, wild type 1807 T cells expanded comparably in the skin draining lymph nodes (sdLN) and spleen in SLAMF1$^{-/-}$ and SLAMF1$^{+/+}$ recipients. However, at day 28 post-vaccination, activated 1807 T cells underwent enhanced contraction in SLAMF1$^{-/-}$ recipients compared to SLAMF1$^{+/+}$ controls. Consequently, hardly any 1807 T cells migrated to the lungs upon recall and the burden of lung infection was not reduced in SLAMF1$^{-/-}$ mice compared to SLAMF1$^{+/+}$ mice (Fig. 3B–D). These data imply that T cell extrinsic SLAMF1 is required for the survival of activated T cells during the contraction phase. Below, we explore the possible mechanisms.

Passenger gene retention in SLAMF1$^{-/-}$ mice potentially confound adoptive transfer experiments.

We observed enhanced contraction of activated CD4 T cells in the adoptive transfer experiments studying the T cell intrinsic and extrinsic roles of SLAMF1 (Figs. 2+3). A “trivial” explanation for the almost complete loss of T cells after contraction in the absence of SLAMF1 could be slow rejection of transferred cells due to insufficiently backcrossed recipient mice or donor T cells. SLAMF1$^{-/-}$ mice were generated with 129 ES cell clones, which were injected into C57BL/6 blastocytes and founder mice were backcrossed to C57BL/6 mice at least 12 generations (20, 21). To investigate whether retention of 129 genes in the SLAMF1$^{-/-}$ mice could have caused the slow rejection of adoptively transferred cells, a strain-based SNP testing panel was performed. Eight SNPs out of 120 tested were 129 associated—one located on chromosome 16, one on chromosome 17, and 6 on chromosome 1, yielding an overall homology rate between the SLAMF1−/− and C57BL/6 mice of 93% (n=2; Supplementary Table 2). All MHC genes on chromosome 17 are implied to be C57BL/6 associated, making a rapid rejection of transferred cells unlikely. However, SLAMF1$^{-/-}$ mice likely retained at least 110MB of 129 passenger genes on chromosome 1 (Fig. 4). The gradual rejection of transferred T cells could have occured due to the expression of 129 background related proteins either on SLAMF1$^{-/-}$ T cells (Fig. 2) or SLAMF1$^{-/-}$ recipient mice (Fig. 3). Therefore, slow rejection of transferred cells due to insufficient backcrossing could be a potential confounding factor for the results generated with the adoptive cell transfer models.

SLAMF1 is required for the recall of endogenous Bl-Eng2-specific T cells to the lung upon challenge.

Despite the potentially confounding findings from the adoptive transfer experiments, vaccinated SLAMF1$^{-/-}$ mice fail to acquire vaccine-induced resistance and have reduced amounts of protective cytokines compared to wild-type controls (Fig. 1). Thus, we hypothesized that the development of endogenous antigen (Ag)-specific T cells is impaired in the absence of intrinsic and/or extrinsic SLAMF1. To test this hypothesis, we used a MCHII tetramer specific for the fungal antigen Bl-Eng2 (22) to track the expansion and

contraction of endogenous Ag-specific $CD4^+$ T cells following vaccination and recall to the lung upon challenge. The expansion (days 7 and 14 post-vaccination) and contraction (day 28) of $tetramer^+$ T cells was comparable in $SLAMF1^{-/-}$ and $SLAMF1^{+/+}$ mice (Fig. 5A+B). However, upon recall, significantly fewer $tetramer^+$ cells migrated to the lungs of $SLAMF1^{-/-}$ mice compared to $SLAMF1^{+/+}$ controls (Fig. 5C+D). Among $tetramer^+$ T cells a lower frequency produced IL-17 and IFN-γ in vaccinated SLAMF1 deficient mice that in wild type mice and consequently fewer cytokine producing T cells were found in the lung for the former group (Fig. 5E+F). Reduced numbers and function of $tetramer^+$ T cells coincided with increased lung CFU and inflammation in vaccinated SLAMF1-deficient mice compared to wild type mice, as evidenced by elevated number of total $CD45^+$ leukocytes in the lung including neutrophils, monocytes, eosinophils and B cells (SFig. 1). These data imply that SLAMF1 is required for innate host control of lung CFU and inflammation and for either the migration and cytokine production of Ag-specific $CD4^+$ T cells to the lung or for secondary proliferation after recall.

Loss of SLAMF1 KO T cell phenotype when inflammatory conditions are equivalent.

Elevated lung CFU typically leads to increased inflammation. Since $SLAMF1^{-/-}$ mice exhibit higher lung CFU at day 4 post-infection (Fig. 1C), we wondered whether increased inflammation could undermine the recruitment or function of primed T-cells in the lung. To circumvent the confounding effect of increased inflammation due to increased lung CFU, we challenged vaccinated mice with heat killed *B. dermatitidis* yeast. At day 4 post-infection, vaccinated $SLAMF1^{-/-}$ and $SLAMF1^{+/+}$ mice showed no difference in the frequency (Fig. 6A) or absolute number (Fig 6B) of $tetramer^+$ CD4 T cells in the lung, whereas challenge with live yeast resulted in reduced numbers of $tetramer^+$ cells. We wondered whether this phenotype is due to reduced secondary proliferation of T cells in $SLAMF1^{-/-}$ vs. $SLAMF1^{+/+}$ mice. To address this question, we administered BrdU to the mice at the time of challenge and daily thereafter. After challenge with live yeast, the incorporation of BrdU in lung $tetramer^+$ T cells was comparable in $SLAMF1^{-/-}$ and $SLAMF1^{+/+}$ mice (Fig. 6C), as indicated by the frequency of $BrdU^+$ cells among $tetramer^+$ T cells. However, the absolute number of $BrdU^+$ $tetramer^+$ cells in lungs was reduced in $SLAMF1^{-/-}$ mice compared to $SLAMF1^{+/+}$ controls (Fig. 6D). We investigated whether increased cell death of $tetramer^+$ cells occurred in the absence of SLAMF1. The frequencies and numbers of live $tetramer^+$ cells were reduced and frequencies and numbers of early and late apoptotic and necrotic cells were increased in $SLAMF1^{-/-}$ mice compared to $SLAMF1^{+/+}$ mice (SFig. 2). These data imply that increased lung CFU and associated inflammation reduced the migration of $tetramer^+$ cells and increased their cell death but did not alter the proliferation of the cells in $SLAMF1^{-/-}$ mice compared to $SLAMF1^{+/+}$ controls.

Since SLAMF1 has been shown to affect T cell polarization via its intracellular adaptor SAP (9, 21, 23, 24), we wondered whether T cell differentiation is impaired its absence. To investigate this question, we again challenged vaccinated mice with heat-killed yeast to circumvent the potentially confounding issue of differential fungal burden and inflammation. The frequencies (Fig. 6E) and absolute numbers (Fig. 6F) of cytokine (IFN-γ, IL-17 and IL5/IL-13) producing lung T cells were comparable in vaccinated in $SLAMF1^{-/-}$ and $SLAMF1^{+/+}$ mice. These data indicate that SLAMF1 is not required for the differentiation

of Th1, Th2 and Th17 cells. In summary, SLAMF1 is dispensable for the development and recruitment of Ag-specific Th1 and Th17 cells to the lungs of Bl-Eng2 vaccinated mice upon fungal infection.

DISCUSSION

SLAMF1 is required for primary and vaccine-induced resistance to fungal infection. Vaccinated SLAMF1$^{-/-}$ mice succumbed at the same time as unvaccinated littermates, whereas vaccinated wild-type mice survived a lethal pulmonary infection and most surviving mice acquired sterilizing immunity. Since vaccine-induced immunity is predominantly mediated by T cells (25), we sought to investigate whether SLAMF1 regulates intrinsic or extrinsic T cell development. To our surprise, enumeration of endogenous Ag-specific T cells by tetramer revealed no deficit in T cell differentiation or recruitment to the lungs. A rigorous assessment of this question was accomplished upon challenge with heat-killed yeast to control for differential fungal burden and inflammation in the lung following infection with live organisms. Thus, SLAMF1 does not regulate T cell development (intrinsically or extrinsically). However, in the absence of SLAMF1 the fungal burden is significantly higher during a primary infection with a live virulent strain. Hence, we postulate that SLAMF1 is required for the innate killing of the yeast. Consequently, in vaccinated mice the failure to control the fungal burden yields increased inflammation and blunts the recruitment and function of primed T cells in the lung.

We studied the role of SLAMF1 for the development of vaccine-induced T cell immunity because SLAMF1 was highly upregulated in wild-type DCs, but not Card9$^{-/-}$ and Myd88$^{-/-}$ DCs that were stimulated with vaccine yeast *in vitro*. We postulated that SLAMF1 could be part of (e.g. directly downstream of) the Card9 or Myd88 signaling pathways. Dectin-2/Card9 signaling is required for the differentiation of naïve T cells into Th1 and Th17 cells (10) and Myd88 signaling regulates the survival of vaccine induced T cells (18). Since SLAMF1 does not have a known role in regulating T cell development, it is unlikely that SLAMF1 is downstream of the Card9 and/or Myd88 signaling pathway.

Our adoptive cell transfer experiments pointed to both an intrinsic and extrinsic deficit in the maintenance of CD4 T cells after vaccination. However, this result is potentially confounded by the rejection of cells due to the presence of 129 passenger genes in the SLAMF1$^{-/-}$ mice. Allogenic cells are first recognized and rejected by differences in major histocompatibility (MHC) proteins. In the SLAMF1$^{-/-}$ mice, all MHC genes on chromosome 17 are likely BL6 associated, making the immediate rejection of transferred cells unlikely. However, minor histocompatibility remains an issue. Any genetic differences could produce proteins that could be recognized as foreign antigens and cause transferred cell rejection. Berghe et al. (26) analyzed the presence of passenger genes in 129-derived BL6 mutants, and found that nearly all congenic mice maintain passenger genes, even after extensive backcrossing. There is essentially no adequate level of backcrossing to ensure the survival of transferred cells in a mouse with a competent immune system. Passenger genes may also have functional consequences beyond cell transfer experiments: phenotypes attributed to the lack of SLAMF1 could result instead from polymorphisms in genes passenger to it. Strain-associated allelic differences have been discovered in the SLAMF1$^{-/-}$ mice. Keszei et al.

(27) found that SLAMF1$^{-/-}$ mice crossed to C57BL/6 mice but not to BALBc background developed autoimmunity.

In summary, we cannot rule out the possibility that despite more than 12 backcross generations to the C57BL/6 background, our results of adoptive transfer experiments could be confounded by slow rejection due to minor histocompatibility. Nevertheless, our findings are important because this observed experimental pitfall could be applicable to any other study that uses knockout mice that have been generated with a genetically disparate mouse strain that was then backcrossed more than 10 times to the wild type control strain.

Since SLAMF1$^{-/-}$ mice are more susceptible to primary infection, our results imply that SLAMF1 expressed on innate immune cells plays a significant role in the initial, innate response to fungal infection. SLAMF1 has yet to be described in a model of fungal infection, however, its contribution to resistance in bacterial and viral models of infection has been described. Surface SLAMF1 senses *E. coli* by binding the porins OmpC and OmpF, and mediating phagocytosis (28). Intracellular SLAMF1 regulates TLR4 mediated TRAM-TRIF killing of Gram-negative bacteria in macrophages in a MyD88 independent manner (29). SLAMF1 on the surface of myeloid cells is used as an entry receptor for measles and other morbilliviruses (30, 31).

We hypothesize that SLAMF1$^{-/-}$ myeloid cells fail to kill yeast early after infection, allowing fungal burden to rapidly overwhelm mice and blunt the ensuing adaptive immune response. In summary, SLAMF1 is dispensable for the acquisition of vaccine-induced immunity and is likely required for robust innate immune responses and early restraint of fungal infection in the lungs. The mechanisms by which an overwhelming fungal infection and corresponding inflammation reduces the recruitment, viability and function of primed T cells to the lung will require further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants R01 AI093553 (MW).

REFERENCES:

1. Barclay AN 2003. Membrane proteins with immunoglobulin-like domains--a master superfamily of interaction molecules. Semin Immunol 15: 215–223. [PubMed: 14690046]
2. Reichardt P, Dornbach B, and Gunzer M. 2007. The molecular makeup and function of regulatory and effector synapses. Immunol Rev 218: 165–177. [PubMed: 17624952]
3. Greenwald RJ, Freeman GJ, and Sharpe AH. 2005. The B7 family revisited. Annu Rev Immunol 23: 515–548. [PubMed: 15771580]
4. van Driel BJ, Liao G, Engel P, and Terhorst C. 2016. Responses to Microbial Challenges by SLAMF Receptors. Front Immunol 7: 4. [PubMed: 26834746]
5. Calpe S, Wang N, Romero X, Berger SB, Lanyi A, Engel P, and Terhorst C. 2008. The SLAM and SAP gene families control innate and adaptive immune responses. Adv Immunol 97: 177–250. [PubMed: 18501771]

6. Engel P, Eck MJ, and Terhorst C. 2003. The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. Nat Rev Immunol 3: 813–821. [PubMed: 14523387]
7. Sever L, Radomir L, Stirm K, Wiener A, Schottlender N, Lewinsky H, Barak AF, Friedlander G, Ben-Dor S, Becker-Herman S, and Shachar I. 2019. SLAMF9 regulates pDC homeostasis and function in health and disease. Proc Natl Acad Sci U S A 116: 16489–16496. [PubMed: 31346085]
8. Aversa G, Carballido J, Punnonen J, Chang CC, Hauser T, Cocks BG, and De Vries JE. 1997. SLAM and its role in T cell activation and Th cell responses. Immunol Cell Biol 75: 202–205. [PubMed: 9107577]
9. Cannons JL, Yu LJ, Hill B, Mijares LA, Dombroski D, Nichols KE, Antonellis A, Koretzky GA, Gardner K, and Schwartzberg PL. 2004. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kappaB1. Immunity 21: 693–706. [PubMed: 15539155]
10. Wang H, Lebert V, Hung CY, Galles K, Saijo S, Lin X, Cole GT, Klein BS, and Wüthrich M. 2014. C-type lectin receptors differentially induce th17 cells and vaccine immunity to the endemic mycosis of north america. J Immunol 192: 1107–1119. [PubMed: 24391211]
11. Brandhorst TT, Wüthrich M, Warner T, and Klein B. 1999. Targeted gene disruption reveals an adhesin indispensable for pathogenicity of Blastomyces dermatitidis. J Exp Med 189: 1207–1216. [PubMed: 10209038]
12. Wüthrich M, Ersland K, Sullivan T, Galles K, and Klein BS. 2012. Fungi subvert vaccine T cell priming at the respiratory mucosa by preventing chemokine-induced influx of inflammatory monocytes. Immunity 36: 680–692. [PubMed: 22483803]
13. Wüthrich M, Hung CY, Gern BH, Pick-Jacobs JC, Galles KJ, Filutowicz HI, Cole GT, and Klein BS. 2011. A TCR Transgenic Mouse Reactive with Multiple Systemic Dimorphic Fungi. J Immunol 187: 1421–1431. [PubMed: 21705621]
14. Amend SR, Valkenburg KC, and Pienta KJ. 2016. Murine Hind Limb Long Bone Dissection and Bone Marrow Isolation. J Vis Exp.
15. Fites JS, Gui M, Kernien JF, Negoro P, Dagher Z, Sykes DB, Nett JE, Mansour MK, and Klein BS. 2018. An unappreciated role for neutrophil-DC hybrids in immunity to invasive fungal infections. PLoS Pathog 14: e1007073. [PubMed: 29782541]
16. Johnson MR, Wang K, Smith JB, Heslin MJ, and Diasio RB. 2000. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. Anal Biochem 278: 175–184. [PubMed: 10660460]
17. Mariotti S, Pardini M, Teloni R, Gagliardi MC, Fraziano M, and Nisini R. 2017. A method permissive to fixation and permeabilization for the multiparametric analysis of apoptotic and necrotic cell phenotype by flow cytometry. Cytometry A 91: 1115–1124. [PubMed: 29072808]
18. Wang H, Li M, Hung CY, Sinha M, Lee LM, Wiesner DL, LeBert V, Lerksuthirat T, Galles K, Suresh M, DeFranco AL, Lowell CA, Klein BS, and Wüthrich M. 2016. MyD88 Shapes Vaccine Immunity by Extrinsically Regulating Survival of CD4+ T Cells during the Contraction Phase. PLoS Pathog 12: e1005787. [PubMed: 27542117]
19. Wüthrich M, Brandhorst TT, Sullivan TD, Filutowicz H, Sterkel A, Stewart D, Li M, Lerksuthirat T, LeBert V, Shen ZT, Ostroff G, Deepe GS Jr., Hung CY, Cole G, Walter JA, Jenkins MK, and Klein B. 2015. Calnexin induces expansion of antigen-specific CD4(+) T cells that confer immunity to fungal ascomycetes via conserved epitopes. Cell Host Microbe 17: 452–465. [PubMed: 25800545]
20. Wang N, Morra M, Wu C, Gullo C, Howie D, Coyle T, Engel P, and Terhorst C. 2001. CD150 is a member of a family of genes that encode glycoproteins on the surface of hematopoietic cells. Immunogenetics 53: 382–394. [PubMed: 11486275]
21. Wang N, Satoskar A, Faubion W, Howie D, Okamoto S, Feske S, Gullo C, Clarke K, Sosa MR, Sharpe AH, and Terhorst C. 2004. The cell surface receptor SLAM controls T cell and macrophage functions. J Exp Med 199: 1255–1264. [PubMed: 15123745]
22. Dobson HE, Dias LDS, Kohn EM, Fites S, Wiesner DL, Dileepan T, Kujoth GC, Abraham A, Ostroff GR, Klein BS, and Wuthrich M. 2020. Antigen discovery unveils resident memory and migratory cell roles in antifungal resistance. Mucosal Immunol 13: 518–529. [PubMed: 31900406]

23. Davidson D, Shi X, Zhang S, Wang H, Nemer M, Ono N, Ohno S, Yanagi Y, and Veillette A. 2004. Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine regulation. Immunity 21: 707–717. [PubMed: 15539156]

24. Mikhalkevich N, Becknell B, Caligiuri MA, Bates MD, Harvey R, and Zheng WP. 2006. Responsiveness of naive CD4 T cells to polarizing cytokine determines the ratio of Th1 and Th2 cell differentiation. J Immunol 176: 1553–1560. [PubMed: 16424184]

25. Wüthrich M, Filutowicz HI, Warner T, and Klein BS. 2002. Requisite elements in vaccine immunity to Blastomyces dermatitidis: plasticity uncovers vaccine potential in immune-deficient hosts. J Immunol 169: 6969–6976. [PubMed: 12471131]

26. Vanden Berghe T, Hulpiau P, Martens L, Vandenbroucke RE, Van Wonterghem E, Perry SW, Bruggeman I, Divert T, Choi SM, Vuylsteke M, Shestopalov VI, Libert C, and Vandenabeele P. 2015. Passenger Mutations Confound Interpretation of All Genetically Modified Congenic Mice. Immunity 43: 200–209. [PubMed: 26163370]

27. Keszei M, Latchman YE, Vanguri VK, Brown DR, Detre C, Morra M, Arancibia-Carcamo CV, Paul E, Calpe S, Castro W, Wang N, Terhorst C, and Sharpe AH. 2011. Auto-antibody production and glomerulonephritis in congenic Slamf1−/− and Slamf2−/− [B6.129] but not in Slamf1−/− and Slamf2−/− [BALB/c.129] mice. Int Immunol 23: 149–158. [PubMed: 21278219]

28. Berger SB, Romero X, Ma C, Wang G, Faubion WA, Liao G, Compeer E, Keszei M, Rameh L, Wang N, Boes M, Regueiro JR, Reinecker HC, and Terhorst C. 2010. SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages. Nat Immunol 11: 920–927. [PubMed: 20818396]

29. Yurchenko M, Skjesol A, Ryan L, Richard GM, Kandasamy RK, Wang N, Terhorst C, Husebye H, and Espevik T. 2018. SLAMF1 is required for TLR4-mediated TRAM-TRIF-dependent signaling in human macrophages. J Cell Biol 217: 1411–1429. [PubMed: 29440514]

30. Tatsuo H, Ono N, Tanaka K, and Yanagi Y. 2000. SLAM (CDw150) is a cellular receptor for measles virus. Nature 406: 893–897. [PubMed: 10972291]

31. Tatsuo H, Ono N, and Yanagi Y. 2001. Morbilliviruses use signaling lymphocyte activation molecules (CD150) as cellular receptors. J Virol 75: 5842–5850. [PubMed: 11390585]

Key points:

1. Unvaccinated and vaccinated SLAMF1$^{-/-}$ mice fail to control fungal infection
2. SLAMF1 signaling is dispensable for the development of anti-fungal T cells
3. Lack of SLAMF1 signaling results in uncontrolled early infection and inflammation

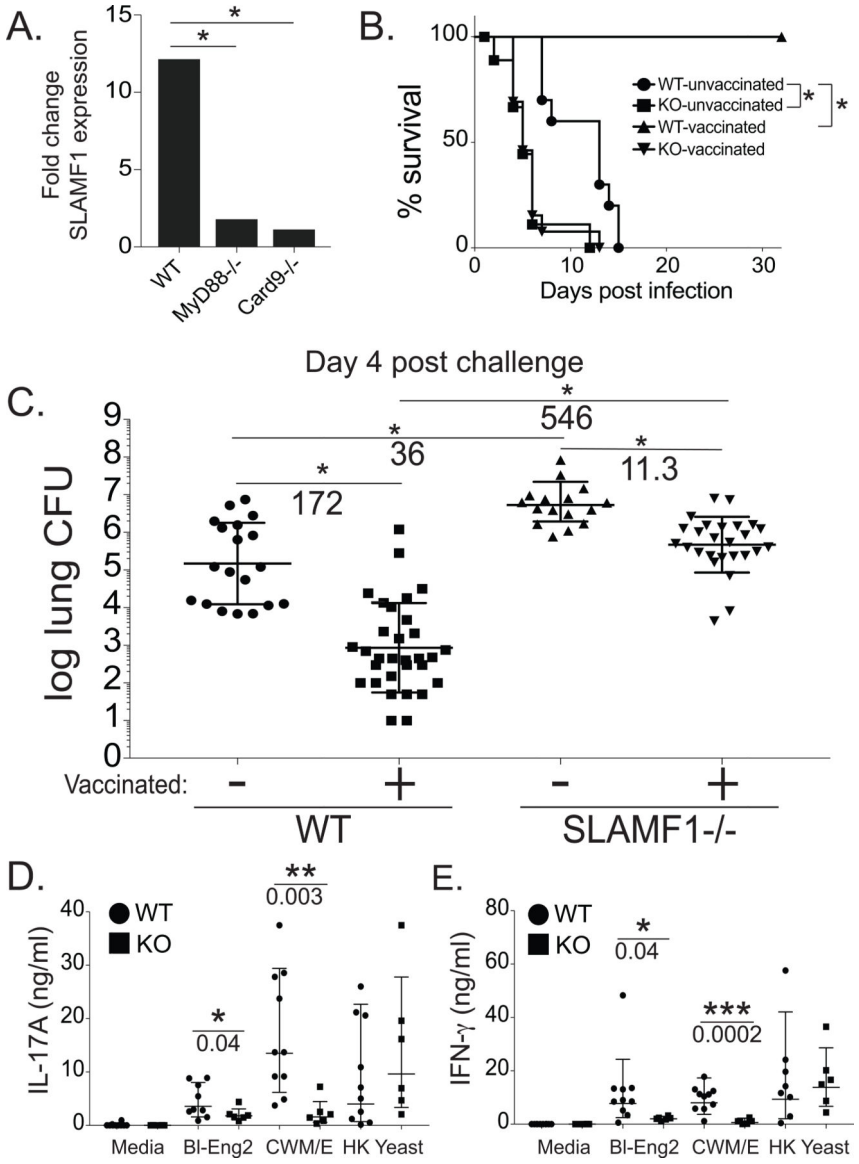


Figure 1. SLAMF1 is required for protection against *B. dermatitidis*.

A. SLAMF1 expression by RT-PCR of BMDC stimulated with vaccine yeast vs. not. **B.** Survival curve of SLAMF1$^{-/-}$ and wild type mice (n=10–15 per group). Survival was recorded when mice were moribund. *, p<0.0001. **C.** CFU analysis at day 4 post challenge. Data aggregated from four independent experiments (n=16–31 per group). N-fold change of geometric means displayed under bars (*, p<0.0001). **D+E.** Ex vivo cytokine production by splenocytes from vaccinated mice. Splenoyctes were stimulated *ex vivo* with medium alone, Bl-Eng2, or cell wall membrane extract (CWM/E).

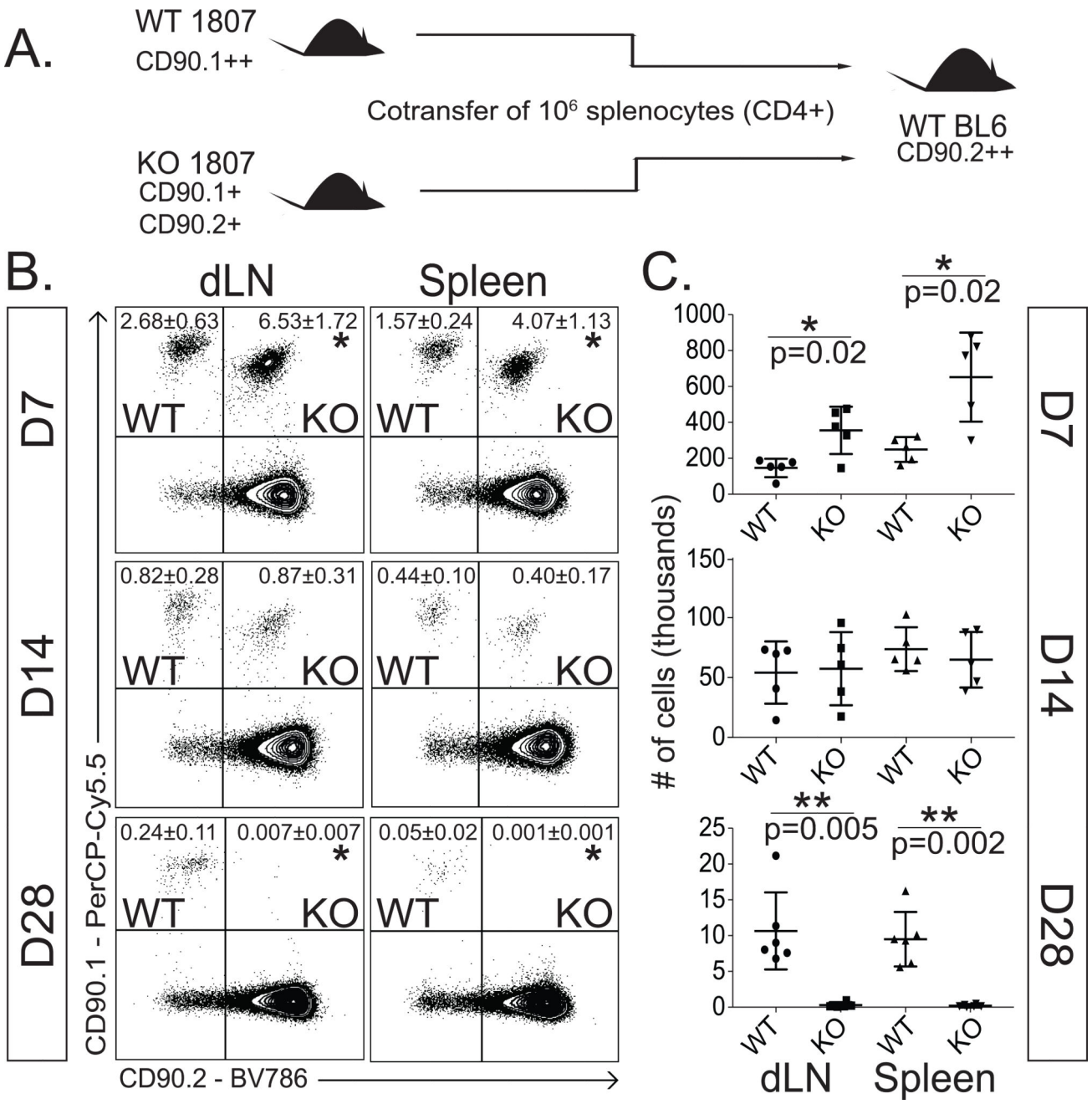


Figure 2. T cell intrinsic SLAMF1 is required for T cell survival.

Kinetic analysis of co-transferred splenocytes from TCR transgenic 1807 mice into WT BL6 recipients one, two, and four weeks post cell transfer and vaccination. **A.** Schematic of congenic adoptive transfer model to study the T cell intrinsic role of SLAMF1. **B.** Flow cytometry analysis of transferred CD4 T cells by CD90 variant phenotype (previous gates: singlets, live, dump-, CD4+CD8-). **C.** Absolute number of transferred cells and statistical analysis of flow cytometry in B (* = $p<0.05$). Whisker plots represent mean with standard deviation. Data representative of three independent experiments (n=5 per group).

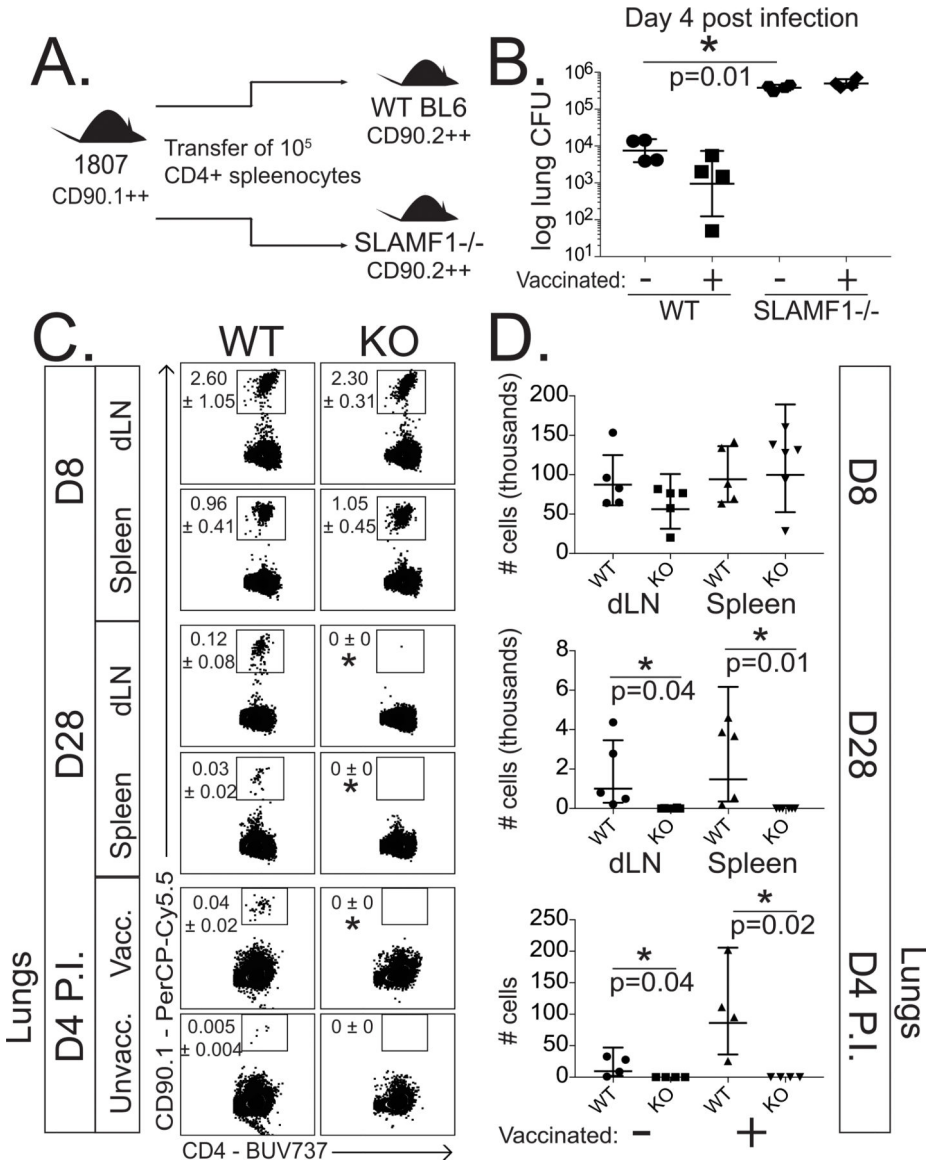
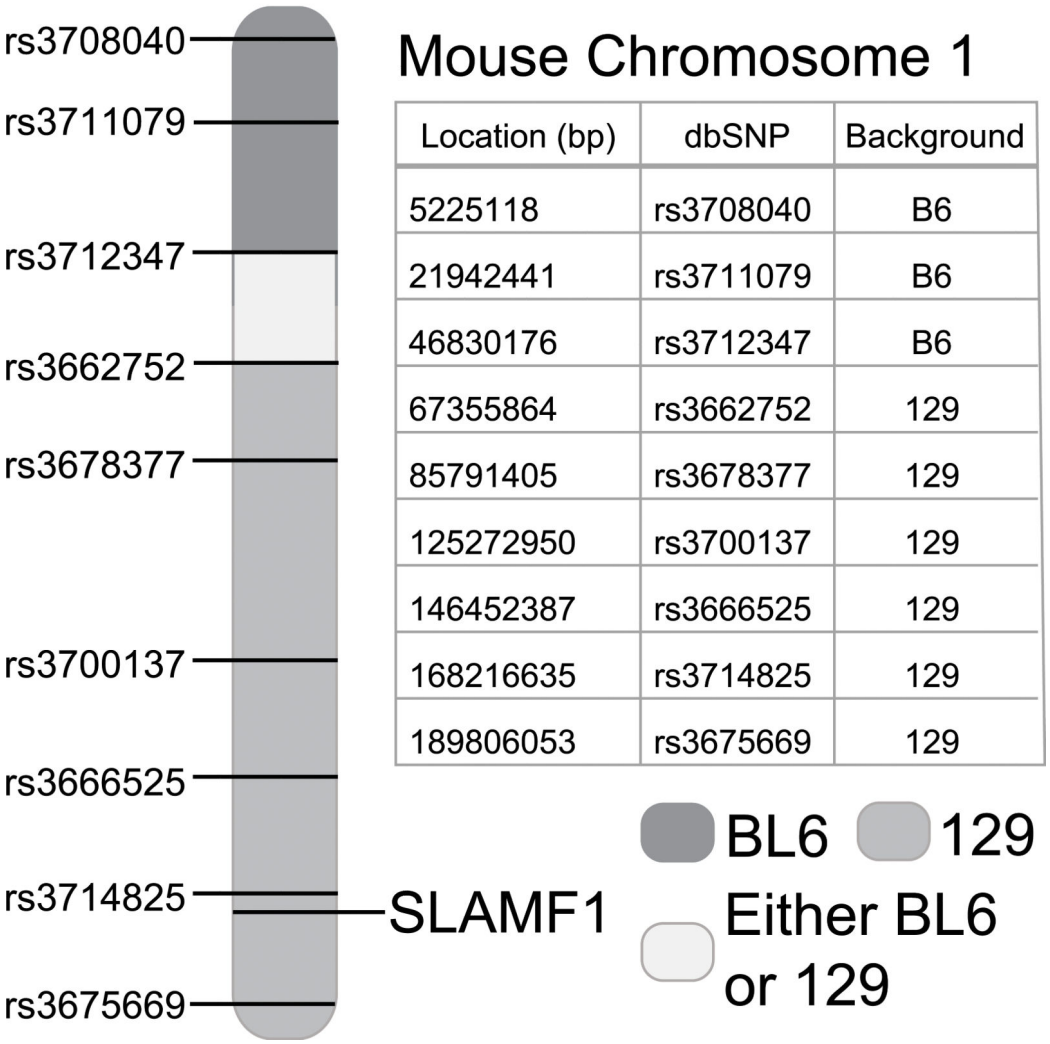


Figure 3. T cell extrinsic SLAMF1 is required for T cell survival.

Kinetic analysis of transferred TCR transgenic 1807 splenocytes one and four weeks after vaccination. Mice were challenged 28 days post cell transfer and vaccination and harvested four days later. **A.** Schematic of trackable cell transfer model. **B.** CFU analysis from D4 post infection (* = p<.05). **C.** Flow cytometry analysis of transferred 1807 CD4 T cells (previous gates: singlets, live, dump-, CD4+CD8-). **D.** Absolute numbers of transferred 1807 CD4 T cells and statistical analysis of flow cytometry in C (* = p<0.05). Data representative of two independent sets of experiments (n=5 per group).



Location (bp)	dbSNP	Background
5225118	rs3708040	B6
21942441	rs3711079	B6
46830176	rs3712347	B6
67355864	rs3662752	129
85791405	rs3678377	129
125272950	rs3700137	129
146452387	rs3666525	129
168216635	rs3714825	129
189806053	rs3675669	129

Figure 4. Passenger genes associated with the knockout of SLAMF1.

A. Schematic of mouse chromosome 1 with a label for each SNP tested. Implied B6 background and 129 background sections labeled. **B.** Table of each tested SNP at chromosome 1 and the background associated with each SNP. Tested by Transnetyx® using 129 vs. C67/BL6 strain test. Total number of SNPs tested = 120, number of mice tested = 2. Total SNP testing results in Supplemental Table 1.

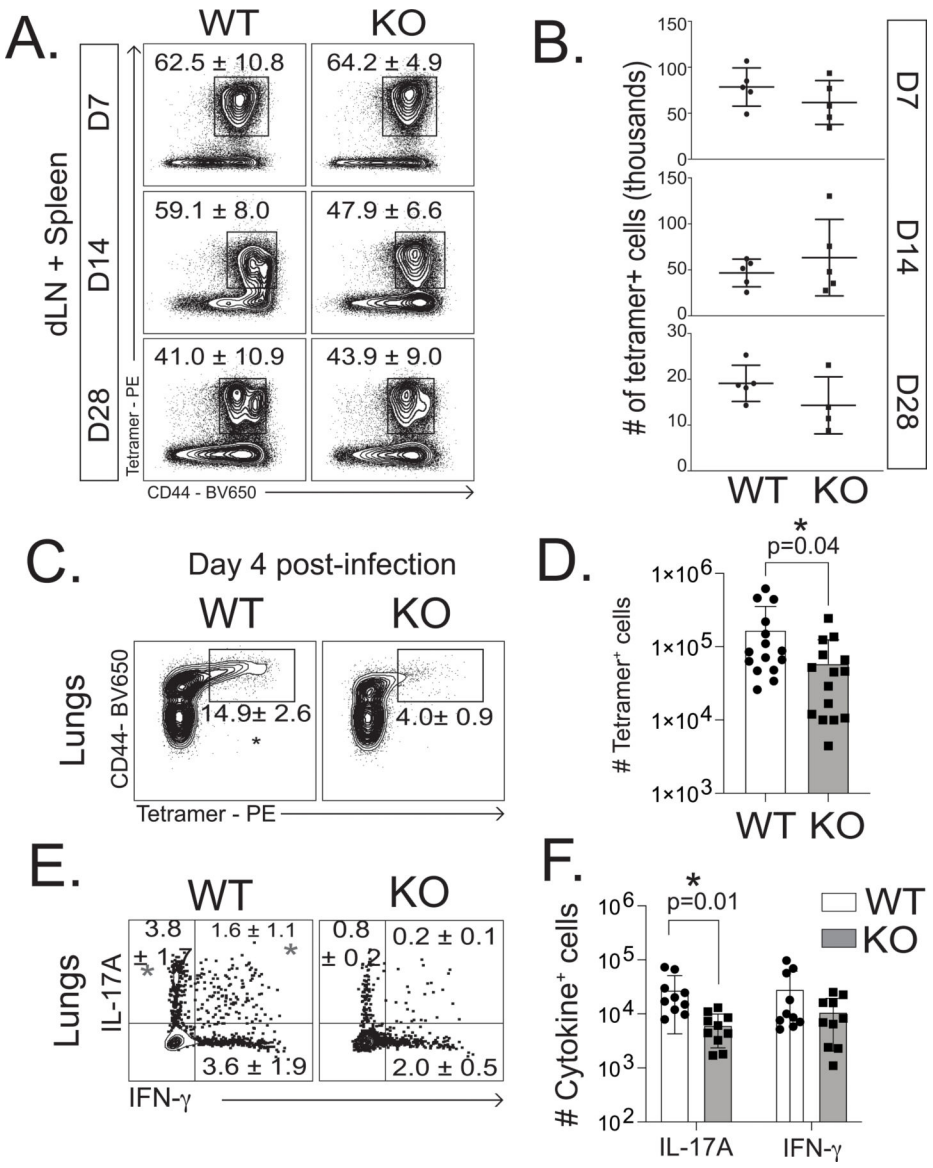


Figure 5. SLAMF1 is dispensable for T cell development, but required for the recruitment or expansion of Bl-Eng2 specific T cells to the lung upon recall.

Kinetic analysis of endogenous antigen specific T cells. **A.** Flow cytometry analysis of $CD4^+$ $tetramer^+$ $CD44^{hi}$ splenocytes and draining lymph node cells collected 7, 14, and 28 days post (previous gates: singlets, live, dump, CD4+CD8-). **B.** Absolute number of $CD4^+$ $tetramer^+$ $CD44^{hi}$ T cells at each time point (n=4–5 per group). No significant differences were found by Welch's T test. **C-D.** Frequencies and numbers of $CD4^+$ $tetramer^+$ $CD44^{hi}$ cells collected from the lung four days post infection. **E-F.** Frequencies and numbers of cytokine producing $CD4^+CD44^+$ T cells upon stimulation with Bl-Eng2 peptide. Data is representative from three independent experiments (n=15 per group) (* = $p<0.01$).

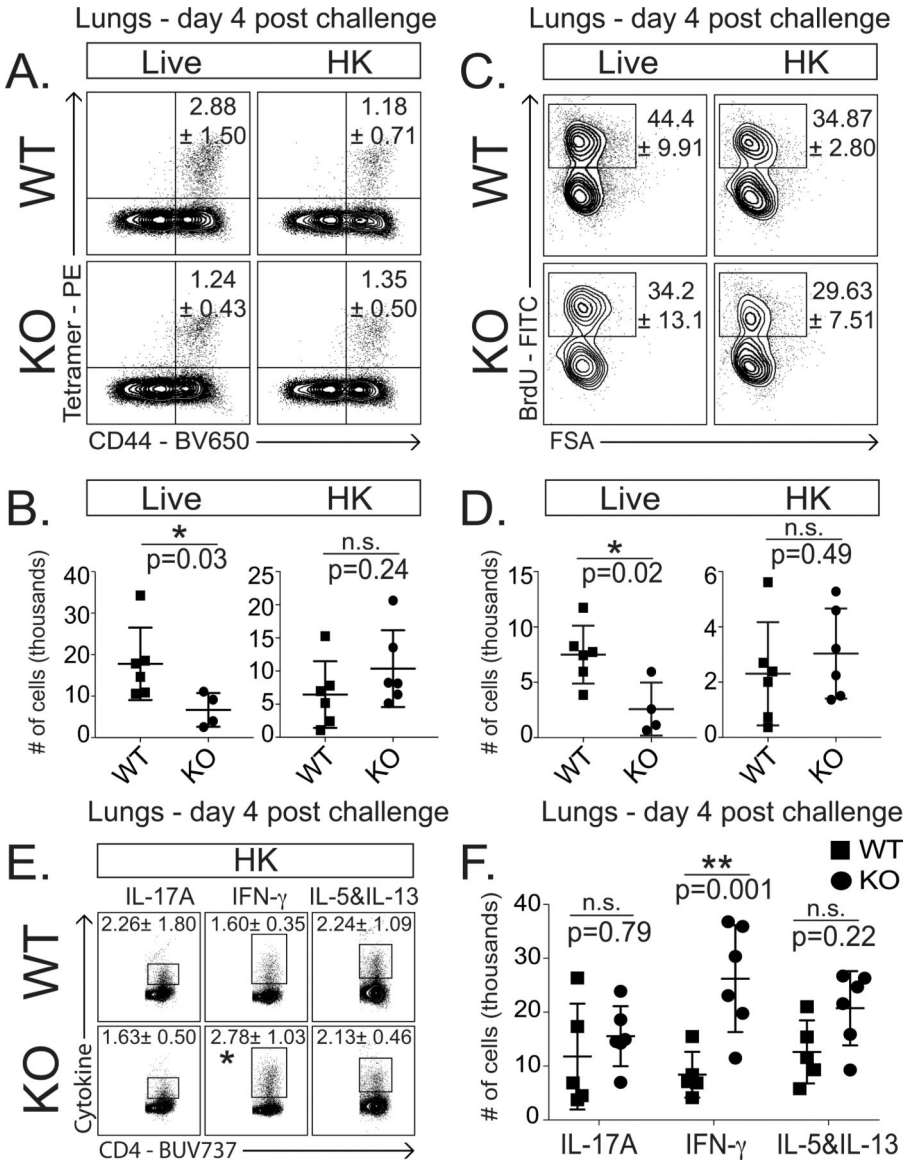


Figure 6. SLAMF1−/− T cell phenotype is lost with equivalent inflammatory conditions.

WT and KO mice harvested four days post challenge with either live or heat killed yeast. **A.** Flow cytometry analysis of antigen specific CD4 T cell populations in either live or heat killed challenged mice. **B.** Absolute number of tetramer+ CD4 T cells as determined by flow analysis in A (*=p<.05). **C.** Proliferation of CD4+tetramer+ cells measured by BrdU uptake and analyzed by flow cytometry. **D.** Absolute number of BrdU+tetramer+ CD4 T cells as determined by flow analysis in C (n=4–6 per group; *=p<.05). **E.** Flow analysis of cytokine producing CD4 T cells from heat killed yeast challenged mice. Three-quarters of total lung cells harvested from a Percoll gradient were stimulated for 5 hours with a-CD3 and a-CD28, and for 4 hours with Golgi stop. **F.** Quantification of flow data from E (*=p<.05, **=p<.01).