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SLAMF4 is a Negative Regulator of Expansion of Cytotoxic Intra-epithelial CD8+ T Cells That Maintains Homeostasis in the Small Intestine

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Abstract

Background & Aims—Intra-epithelial T cells (IEL) are the first immune cells to respond to pathogens; they help maintain the integrity of the epithelial barrier. We studied the function of the glycoprotein SLAMF4 (encoded by *CD244*) on the surface of CD8αβ αβ T-cell receptor (TCR)$^+$ IELs, and the roles of these cells in homeostasis of the small intestine in mice.

Methods—SLAMF4$^-$ CD8$^+$ αβTCR$^+$ cells isolated from spleens of OT-I *Rag1*$^{-/-}$ mice were induced to express gut-homing receptors and transferred to C57BL/6J mice; levels of SLAMF4 cells were measured in small intestine tissues. After administration of anti-CD3 or antigen, with or without anti-SLAM4, to C57BL/6J and *Slamf4*$^{-/-}$ mice, CD8αβ αβTCR$^+$ IELs were collected; cytokine production and cytotoxicity were measured. Depletion of CX3CR1$^+$ phagocytes was assessed in mice by live-cell confocal imaging or by cytofluorometry; small intestine tissues were analyzed by histology and inflammation was quantified.

Results—Splenic CD8$^+$ αβTCR$^+$ cells began to express SLAMF4 only after migrating to the small intestine. Injection of C57BL/6J mice with anti-SLAMF4 and anti-CD3 increased levels of interleukin-10 and interferon-γ secretion by IEL, compared injection of only anti-CD3. Similarly, the number of granzyme B$^+$ cytotoxic CD8$^+$ αβTCR$^+$ IELs increased in *Slamf4*$^{-/-}$ mice following injection of anti-CD3 and anti-SLAMF4, administration of antigen, or injection of anti-CD3. Surprisingly, in vivo activation of CD8αβ$^+$ IELs with anti-CD3 or antigen caused transient depletion of CX3CR1$^+$ phagocytes, which was prolonged by co-injection with anti-SLAMF4 or in *Slamf4*$^{-/-}$ mice. Anti-CD3 aggravated inflammation in the small intestines of *Slamf4*$^{-/-}$ mice and *Eat2a*$^{-/-}$ *Eat2b*$^{-/-}$ mice, indicated by flattened villi and crypt hyperplasia.

Conclusions—In mice, the intestinal environment induces SLAMF4 expression and localization to the surface of CD8$^+$ αβTCR$^+$ IELs. Signaling via SLAMF4 controls expansion of cytotoxic CD8αβ$^+$ IELs, which regulate the reversible depletion of lamina propria phagocytes and inflammation in the small intestine.

Keywords

immune regulation; intestinal epithelium; T-cell development; immunity

Introduction

The intestinal immune system is requisite for the eradication of invading pathogens, while maintaining tolerance to commensal microbial and food antigens. Its dysregulation causes chronic or episodic inflammation in diseases such as IBD and celiac disease[1, 2] Both $CD4^+$ and $CD8^+$ T cells play a central role in regulating intestinal immune responses and inflammation[1–4]. The role of intraepithelial T lymphocytes (IELs) in intestinal inflammation is relatively poorly understood. Most αβTCR and γδTCR IELs, whether expressing CD8αα or CD8αβ, are predominantly antigen-experienced cells and are inactive under homeostatic conditions[3]. Recent studies show that αβTCR IELs not only partake in immune responses to luminal antigens in the small intestine, but they also respond to circulatory antigens[5, 6].

Here we report that the environment of the small intestine induces expression by $CD8^+$ IELs of the receptor SLAMF4 (CD244, 2B4)[7, 8], which controls the functions of IEL in the small intestine. SLAMF4 is member of the Signaling Lymphocyte Activation Molecule-family receptors (SLAMF1-9) that are differentially expressed on the surface of hematopoietic cells[9]. Mouse SLAMF4 has a high-affinity ligand SLAMF2 (CD48) and binds to CD2 with low-affinity[7]. In conjunction with the SLAMF-specific adapters SAP, EAT-2A and EAT-2B[7–12], Slamf4 governs signaling networks that positively or negatively modulate cytotoxic activity and cytokine production of NK cells[11–15]. Based on studies of mice with chronic viral infections[16], which contain a subset of exhausted $Slamf4^+CD8^+$ T cells, we hypothesize that Slamf4 is a negative regulator of the function of $CD8^+$ IELs. We focus on the *in vivo* and *in vitro* responses of αβTCR CD8αβ IELs upon co-administration of αCD3 or antigen in the presence or absence of αSLAMF4 or by comparison of IEL responses in *wt* and $Slamf4^{-/-}$ mice. Surprisingly, CD8αβ IELs that were activated *in vivo* by αCD3 or antigen caused a transient depletion of $CX3CR1^+$ phagocytes, which is prolonged by αSlamf4 or in $Slamf4^{-/-}$ mice. Furthermore, αCD3 induced small-intestinal inflammation in $Slamf4^{-/-}$ and $Eat2a^{-/-}Eat2b^{-/-}$ mice manifested as flattened villi and crypt hyperplasia.

Materials and Methods

Mice

C57BL/6J, $Rag1^{-/-}$ B6, CD45.1 congenic (Cg) mice, IL-10-GFP mice and CX3CR1-GFP mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6J, $Rag1^{-/-}$ B6, and OT-I $Rag1^{-/-}$ mice were purchased from Taconic Labs (Hudson, NY). $Slamf4^{-/-}$ *B6*, $SAP^{-/-}$ *B6*, and $Eat2a^{-/-}Eat2b^{-/-}$ B6 were generated as previously described [11, 17, 18]. All mice were housed in the BIDMC Center for Life Sciences animal facility. Experiments were executed in accordance with the guidelines of the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

IEL and LP DC/macrophage Isolation

Cells were isolated as described [5, 19].

Antibodies

Anti-γδTCR-PE, αTCRβ-APC-eFluor780, αCD11c-FITC, αCD45.1-BV421, αSlamf4-PE, αSlamf4-FITC and αGranzymeB-FITC (eBioscience, San Diego, CA); αCD4-FITC and αTCRβ-FITC (BD Biosciences, San Jose, CA). αCD3-PB, αCD3-BV421, αCD8α-APC, αCD8α-PE-Cy7, αCD8β-PE, αCD8β-PerCP/Cy5.5, αCD4-BV650, αSlamf2-PE, αCD45.2-AlexaFluor 647, αCD45.2 FITC, αCD11b-PECy7, αCD11c-APC, αI-A/I-E-PB, αCCR9-PE, and ααα4β7-APC were purchased from Biolegend (San Diego, CA). Data were collected using a BD LSRII and analyzed with FlowJo software (Tree Star, Ashland, OR).

Gut Homing T cells

OT-I *Rag1*$^{-/-}$ CD8^{+} T cells were activated *in vitro* and labeled with CMTMR (Molecular Probes, Carlsbad, CA) before transferring 2–5 million cells into CD45.1^{+} congenic mice[20]. After 48 h, CD8^{+} T cells from the spleen, MLN, and small intestinal IEL compartment were analyzed for Slamf4 expression.

Anti-CD3/αSlamf4 injections

Purified αCD3ε (145-2C11) hamster IgG (Biolegend, San Diego, CA), αSLAMF4 mouse IgG2b (hybridoma donated by Dr. Vinay Kumar, Un. Chicago or purchased from Biolegend) or Mouse IgG2b (Biolegend, San Diego, CA) were injected *i.p.*, as described [21]. Briefly, 20μg of antibody was injected 1, 2, or 3 times at 48 h intervals with the last injection occurring 4 h prior to euthanizing the mice. *Slamf4*$^{-/-}$ mice were euthanized 16 hours after the second injection.

Intracellular Staining and Cytokine Assay

CytoFix/CytoPerm Kit (BD Biosciences, CA) was used for cytoplasmic staining. Cytokines were analyzed in the supernatant fluid of 72 hour IEL cultures using the Mouse Cytokine Assay Kit (Affymetrix, CA) or the Mouse Inflammatory Cytokine CBA Kit (BD Biosciences, CA).

Activation of IELs by ovalbumin

OT-I *Rag1*$^{-/-}$ mice were fed ovalbumin in drinking water (1mg/ml). After one week, isolated IELs or LP DC/macrophages were used for *in vitro* cytotoxicity assays or flow cytometry, respectively. In some experiments, 20μg of αSlamf4 was injected, as indicated.

In vitro Cytotoxicity Assay

CD8αβ IELs were purified by FACS from mice injected with αCD3 and used in a redirected cytotoxicity assay with 5μg αCD3 and P815 target cells [11] [E:T= 5:1] for 4 hours. Alternatively, purified CD8αβ IELs from OT-I *Rag1*$^{-/-}$ mice used EL-4 target cells pulsed with SIINFEKL peptide in the presence of 5μg αSlamf4 or Mouse IgG2b for 4 hours. Target cell lysis was determined using a Nonradioactive Cytotoxicity Kit (Promega, Madison, WI).

Confocal Microscopy

Small intestine sections of IL-10-GFP or CX3CR1-GFP mice were prepared, imaged and analyzed using an A1R-A1 confocal microscope (Nikon, Melville, NY) as previously

described[5]. 3D reconstructions and quantification of GFP$^+$ cells were made using Volocity software (PerkinElmer, Waltham, MA).

Histology Scoring

Proximal, medial, and distal sections of the small intestine were stained with H&E. Histology scores were assigned as follows: 0 = normal intestine, 1 = limited to mild basal infiltration around crypts, no inflammation in villi, 2 = massive basal infiltration around crypts and localized infiltration into villi, 3 = widespread infiltration into villi with alterations in villus architecture.

Statistics

Statistical analyses were executed using GraphPad Prism Software. The Student *t* test was used to compare groups; results are represented as Mean ± SD. A p value < 0.05 was considered significant.

Results

Induction of SLAMF4 expression by intraepithelial T lymphocytes in the small intestine

Cytofluorometric analyses detected SLAMF4 on the surface of CD8αα, CD8αβ and CD8α$^+$CD4$^+$ IELs, but not of CD4$^+$ IELs of the small or large intestine (Fig. 1A). Slightly higher levels of Slamf4 were observed on the surface of CD8αα αβTCR and γδTCR cells than on CD8αβ αβTCR IELs (Supplement Fig. S1A–B). The long and the short isoforms of Slamf4 found in NK cells[15] are also expressed by IELs, albeit at different levels (Supplement Fig S2A). CD8$^+$ IEL subsets of the small intestine contain the Slamf-specific adapter SAP, as well as the adapter molecules EAT-2A, EAT2B, which are not present normally in T lymphocytes, but are found in NK cells (Supplement Fig. S2B–C).

To determine whether the environment of the small intestine induces expression of the receptor, Slamf4− CD8$^+$ T cells (CD45.2$^+$) were isolated from the spleen of OT-I x *Rag*$^{-/-}$ mice. After 4 days in culture with αCD3/αCD28 and retinoic acid (Fig. 1B), all CD45.2$^+$ CD8$^+$ T cells carried the gut homing receptors integrin α4β7 and CCR9, but remained SLAMF4− (Fig. 1C). Approximately 20% of these CD45.2$^+$ CD8$^+$ T cells expressed SLAMF4 two days after their adoptive transfer into congenic (CD45.1$^+$) *wt* recipient mice [20] and migration to the IEL compartment of the small intestine (Fig. 1D, *left* and 1E). By contrast, donor CD8$^+$ cells in the MLN and spleen remained SLAMF4−, while the endogenous IEL of the recipient mice were all SLAMF4$^+$ (Fig. 1D, **right panel**). Together the data demonstrate that SLAMF4 is expressed by all CD8$^+$ intraepithelial T lymphocytes in the small intestine and that the intestinal milieu induces expression of Slamf4 by CD8$^+$ IELs.

Anti-SLAMF4 alters secretion of the cytokines by CD8αβ IELs that are induced by αCD3

Regulatory CD8$^+$ IEL of the small intestine produce IL-10 in response to triggering the TCR/CD3 complex[5, 21]. To determine whether Slamf4 regulates IL-10 production by IELs, IL-10-GFP reporter mice received 3 injections of αSLAMF4, αCD3, or both antibodies [5, 21]. Confocal microscopy showed that αSlamf4 augmented the number of IL-10-secreting cells

in the villi of the small intestine induced by CD3 (Fig. 2A). Importantly, in IEL cultures more IL-10 and IL-13, but not IL-9, was secreted by cells isolated from mice that had received αSLAMF4 and αCD3 as compared to IELs isolated from αCD3 injected mice (Fig. 2B). While IELs isolated from αCD3 injected mice secreted IFNγ, IL-17, and TNFα (Fig. 2C), upon administering αCD3/αSLAMF4 IELs secreted only more IFNγ. These observations suggest that Slamf4 suppresses cytokine secretion by both effector and regulatory CD8 IELs.

SLAMF4 regulates αCD3 or antigen-induced expansion of cytotoxic CD8αβ IELs

Because IELs are maintained in an activated yet restrained state [3], we next tested the influence of Slamf4 on expansion and function of cytotoxic αβTCR CD8αβ IELs. Whereas the proportion of αβTCR CD8αβ IELs increased after αCD3 injections into *wt* mice, co-injection of αSlamf4 and αCD3 further increased this population (Fig. 3A–B). While αCD3 decreased the proportion of γδTCR CD8αα IELs, expression of Slamf4 by all CD8α$^+$ IELs remained the same (Supplement Fig. S3A–C). CD8αβ IELs isolated from mice injected with αCD3 or αCD3/αSLAMF4 expressed high levels of Granzyme B (> 80%) (Fig. 3C) suggesting that αCD3 increased IEL cytotoxicity to a maximum level and that co-administering αSLAMF4 did not substantially influence cytotoxic activity. Indeed, re-directed killing assays in which FACS-purified CD8αβ IELs from αCD3-injected mice were incubated with P815 target cells in the presence of αCD3 or αCD3/αSLAMF4 indicated that addition of αSLAMF4 did not further increase target cell lysis (Fig. 3D). However, in antigen specific killing assays employing FACS-purified OT-I CD8αβ$^+$ IELs, αSlamf4 caused a moderate increase in the lysis of SIINFEKL peptide-pulsed EL-4 target cells (Fig. 3E). We conclude that co-injections of αCD3 or antigen and αSlamf4 induce signals in IELs that synergize to increase the proportion of Granzyme B$^+$ CD8αβ IELs, which have a slightly increased cytotoxic activity.

The notion that αSLAMF4 lifts a negative signal in CD8αβ IELs is consistent with the observation that the proportion of Granzyme B$^+$ CD8αβ IELs was dramatically increased after administering αCD3 to *Slamf4*$^{-/-}$ mice, as compared to *wt* mice (Fig. 4A–B). Whereas αCD3 induced a very high level of Granzyme B in almost all CD8αβ IELs (Fig. 4C), FACS-purified *Slamf4*$^{-/-}$ CD8αβ IELs had a slightly higher cytotoxic activity in redirected killing assays compared to their *wt* counterparts (Fig. 4D). This redirected killing assay most likely does not permit an evaluation of the role of co-stimulatory molecules due to the massive activation of the TCR/CD3 complex. Together, the data indicate that Slamf4 acts as a negative regulator of αCD3 or antigen induced expansion and to a lesser extent of the cytotoxic activity of αβTCR CD8αβ IELs.

SLAMF4 negatively regulates IEL-induced depletion of CX3CR1$^+$ DCs in the lamina propria

To assess whether the increased number of cytotoxic IELs induced by αCD3/αSLAMF4 affected the homeostasis of the small intestine, we analyzed CX3CR1-GFP reporter mice by live imaging confocal microscopy [5, 22]. Surprisingly, three injections of αCD3/αSLAMF4 over four days completely depleted CD11c$^+$CX3CR1$^+$ phagocytes, while injection of αSlamf4 or αCD3 alone only caused a marginal reduction of the CX3CR1$^+$ phagocytes (~20%) (Fig. 5A). Importantly, depletion of the SLAMF2$^+$ CX3CR1$^+$ phagocytes by αCD3/

αSLAMF4 injections did not significantly change the overall structure of the villi or the lamina propria vasculature (Fig. 5A and Supplement Figure S4A). Removal of $CX3CR1^+$ phagocytes was specific to the small intestine, as the spleen, MLN, and Peyer's patches all contained $CX3CR1^+$ cells after αCD3/αSLAMF4 administration (Fig. 5B). Injecting αCD3, αSLAMF4 or αCD3/αSLAMF4 did not induce a depletion of myeloid cells in the intestine of *Rag*$^{-/-}$ mice, which lack T and B cells (Supplement Fig. S4B), supporting the notion that the effect of αSLAMF4 requires initial activation of the TCR/CD3 complex on $SLAMF4^+$ $CD8^+$ T cells and not NK cells. As judged by cytofluorometry, a partial elimination of the $CD11c^+$MHC-II^+ myeloid cells in the lamina propria was induced by three injections of αSLAMF4 into of *OT-1 x Rag*$^{-/-}$ mice that were kept on ovalbumin-containing drinking water (Fig. 5C). Thus, both antigen and αCD3 caused the same effect in conjunction with αSLAMF4.

Administering αCD3 caused a significant reduction of $CD11b^+CD11c^+$MHC II^+ myeloid cells, which include $CX3CR1^+$ phagocytes [23] in *Slamf4*$^{-/-}$ mice, but not in *wt* mice (Fig. 5D). Taken together the data show that αβTCR $CD8\alpha\beta^+$ IELs, which were *in vivo* activated by αCD3 or by antigen, depleted $CX3CR1^+$ phagocytes residing in the lamina propria of the small intestine. This depletion was aggravated in *Slamf4*$^{-/-}$ mice or by co-injecting αSLAMF4 into *wt* mice.

The αSLAMF4− and αCD3-induced depletion of myeloid cells is reversible

The $CX3CR1^+$ phagocyte depletion was reversible, as judged by live imaging at different time points after injecting αCD3 or αCD3/αSLAMF4 (Fig. 6A). After injection of αCD3 depleted $CX3CR1^+$ phagocytes by day 2, these cells re-populated the villi by day 4 (Fig. 6B–C). By contrast, after three co-injections of αCD3/αSLAMF4 $CX3CR1^+$ phagocytes were absent on day 4 and repopulation of the villi was detectable by day 7 and was complete by day 9 (Fig. 6D–E).

The reversible depletion of $CD11c^+CX3CR1\text{-}GFP^+$ phagocytes on day 4 of administering αCD3/αSLAMF4 was confirmed by cytofluorometry (Fig. 6A, F). Repopulation of $CD11c^+CX3CR1\text{-}GFP^+$ phagocytes in the villi in mice injected with αCD3/αSLAMF4 was delayed, as compared to αCD3-injected mice (Fig. 6F). In general, the effect of αCD3 was more profound by the cytofluorometric analyses than by confocal imaging. For instance, $CD11c^+CX3CR1\text{-}GFP^-$ cells had also been removed by both injection protocols (Fig. 6A, F). Interestingly, a subpopulation of $CD11c^-CX3CR1\text{-}GFP^{int}$ cells was present only after injecting αCD3 on day 4 (Fig. 6A, F).

We conclude that αCD3 induces a reversible depletion of $CD11c^+$ myeloid cells in the lamina propria of the small intestine, which is aggravated and extended by the presence of αSlamf4. The data show that destruction of myeloid cells is caused by activated cytotoxic IELs most likely in conjunction with TNFα/IFNγ secreting IELs. The notion that SLAMF4-induced negative signals prevent destruction of the myeloid cells is also supported by the observations made with the αCD3 injected *Slamf4*$^{-/-}$ mice (Fig. 5C).

Anti-CD3 induces inflammation in the small intestine of mice that are deficient in SLAMF4 or in its adapters EAT-2A and EAT-2B

Because administering αCD3 in *Slamf4*$^{-/-}$ mice expanded cytotoxic CD8αβ IELs, which in turn caused depletion of myeloid cells in the lamina propria, we reasoned that these events could cause an inflammation in the small intestine. Indeed, upon injecting αCD3 into *Slamf4*$^{-/-}$ mice a severe inflammation was indicated by diarrhea and hunching and confirmed by histology manifested as flattened villi and crypt hyperplasia (Fig. 7A–B). By contrast, administering αCD3 induced a mild reversible inflammation in *wt* mice, as found previously [6]. As IELs are unique T cells that express all three SLAMF adapters, SAP, EAT-2A and EAT-2B, mice deficient in SAP or EAT-2A and EAT-2B were analyzed for small intestine inflammation after 3 injections of αCD3. Whereas *Eat2a*$^{-/-}$*Eat2b*$^{-/-}$ mice developed a more severe inflammation with villi blunting and crypt hyperplasia in the small intestine as compared to the *wt* mice, *SAP*$^{-/-}$ mice responded similar to *wt* animals (Fig. 7C–D). Thus, EAT-2A and EAT-2B, not SAP, modulates the IEL functions that instigate inflammation. Co-injection of αCD3/αSLAMF4 into *wt* mice did not further aggravate the αCD3-induced inflammation (Fig. 7E–F). The data support the overall concept that SLAMF4 and its adaptors EAT-2A and EAT-2B, which are uniquely expressed in IELs, negatively control expansion of cytotoxic IELs that are poised to eliminate myeloid cells in the lamina propria, which in turn controls inflammation in the small intestine.

Discussion

The outcomes of the current studies provide evidence in support of the concept that SLAMF4 (CD244, 2B4) expression is induced on the surface of IELs in the small intestine, presumably by antigen in conjunction with TGF-β, IL-15 and CD27 and unknown factors [22–27]. SLAMF4 is a negative regulator of expansion of cytotoxic αβTCR IELs after *in vivo* triggering the TCR/CD3 complex by antigen or αCD3 whilst co-administering αSLAMF4. In addition, injection of αSlamf4 altered the balance between cytotoxic IELs and a regulatory subset of CD8^{+} IELs, as IL-10 production by IELs increased [5, 21]. The notion that SLAMF4 is an inhibitory receptor of cytotoxic IEL expansion and functions is supported by our studies with *Slamf4*$^{-/-}$ mice. Similar conclusions have been reached in studies with exhausted mouse virus-specific SLAMF4^{+} cytotoxic T cells [16].

More recently SLAMF4 has been shown to function on the surface of virus-specific human CD8^{+} T lymphocytes [28, 29]. Surprisingly, the Class I-MHC–peptide complex on the target cell can regulate the level of expression of SLAMF4 on virus specific cytotoxic T cells by an internalization mechanism [28]. Functional involvement on the surface of human CD8^{+} T cells is further indicated by the finding that in patients with systemic lupus erythomatosis the number of SLAMF4^{+} CD8 cells is dramatically increased suggesting that exhausted memory CD8^{+} cells partake in this complex autoimmune disease states [30–32]. Because functional single nucleotide polymorphism of the human Slamf4 gene are related to the onset of SLE and rheumatoid arthritis [30–32], the data suggest that variations in Slamf4 gene may affect human inflammatory bowel diseases.

Antigen- or αCD3− activated αβTCR CD8αβ IELs induce a reversible depletion of CX3CR1^{+} phagocytes from the intact villi of the small intestine, which is prolonged and

exacerbated by co-injection of αSLAMF4 or in *Slamf4*$^{-/-}$ mice. The increase of cytotoxic IELs is the likely cause the depletion as the CD8^{+} IELs infiltrate the lamina propria where they can contact myeloid cells. Suppression of the killing of CX3CR1^{+} and CX3CR1^{-} myeloid cells by Slamf4 on the surface of IELs, is critical for the homeostasis in the small intestine [33, 34]. The replenishment of the CX3CR1^{+} phagocytes is likely to be from newly arrived, recently divided Ly6C^{hi} CCR2^{+} monocytes [35–37].

Consistent with a negative regulatory effect of SLAMF4 on the surface of IELs is the observation that αCD3 induced aggravated inflammation in the small intestine of *Slamf4*$^{-/-}$ and *Eat2a*$^{-/-}$*Eat2b*$^{-/-}$ mice. In *wt* mice the *i.p.* injection of αCD3 induced a mild and reversible inflammation. We propose a mechanism by which in the absence of SLAMF4 signaling CD8αβ IEL-mediated depletion of myeloid cells in the villi leads to inflammation in the small intestine. A direct connection between CD8αβ T cell induced depletion of intestinal CX3CR1^{+} and CX3CR1^{-} phagocytes and inflammation cannot easily be made without the use of novel tissue-specific gene knockouts in conjunction with adoptive transfer experiments. However, strong arguments in favor of a direct connection between the inflammation in the small intestine and the cell death of phagocytes exist. These include, *i)* removal of CX3CR1^{+} and CX3CR1^{-} cells in the small intestine could generate both an infectious or a non-infectious inflammation, *ii)* impaired expansion of CD4^{+}FoxP3^{+} Treg [24, 25] and immunosuppressive CD8αβ T cells [5], which are dependent upon CX3CR1^{+} phagocytes, and *iii)* progressive infiltration of cytotoxic CD8αβ IELs into the villi of the small intestine.

In part due to the ability to induce immunosuppressive CD4^{+} and CD8^{+} T cells monoclonal antibodies directed against CD3ε have been used as treatment of autoimmune diseases including IBD [21, 38–40]. Variable successes of αCD3 therapy of immune related diseases including IBD[38–41], was caused by a transient "cytokine storm" or mild inflammation in the small intestine has been reported. The outcomes of our studies strongly suggest that genetic predisposition like functional single nucleotide polymorphisms (SNP) in the SLAMF4 gene [31, 32] may affect the efficacy of αCD3 therapy. Furthermore, a brief treatment with anti-CD3 and anti-SLAMF4 might reset the homeostasis of myeloid cells in the lamina propria of the small intestine, which could potentially be used for therapeutic purposes. Lastly, αCD3− or αCD3/αSLAMF4− induced inflammation in the small intestine may be used to model human celiac disease [2]. Thus, the findings reported here provide several [pre-] translational applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

Slamf	Signaling Lymphocyte Activation Molecule-family receptors
SAP	signaling lymphocyte activation molecule (Slam)-associated protein
EAT-2	Ewing's sarcoma-associated transcript-2
IEL	Intraepithelial lymphocytes
PP	Peyer's patches
DC	dendritic cell
CX3CR1	CX3CL chemokine receptor 1
IFN	interferon
IL	interleukin
WT	wild type
BV	Brilliant Violet
PB	Pacific Blue

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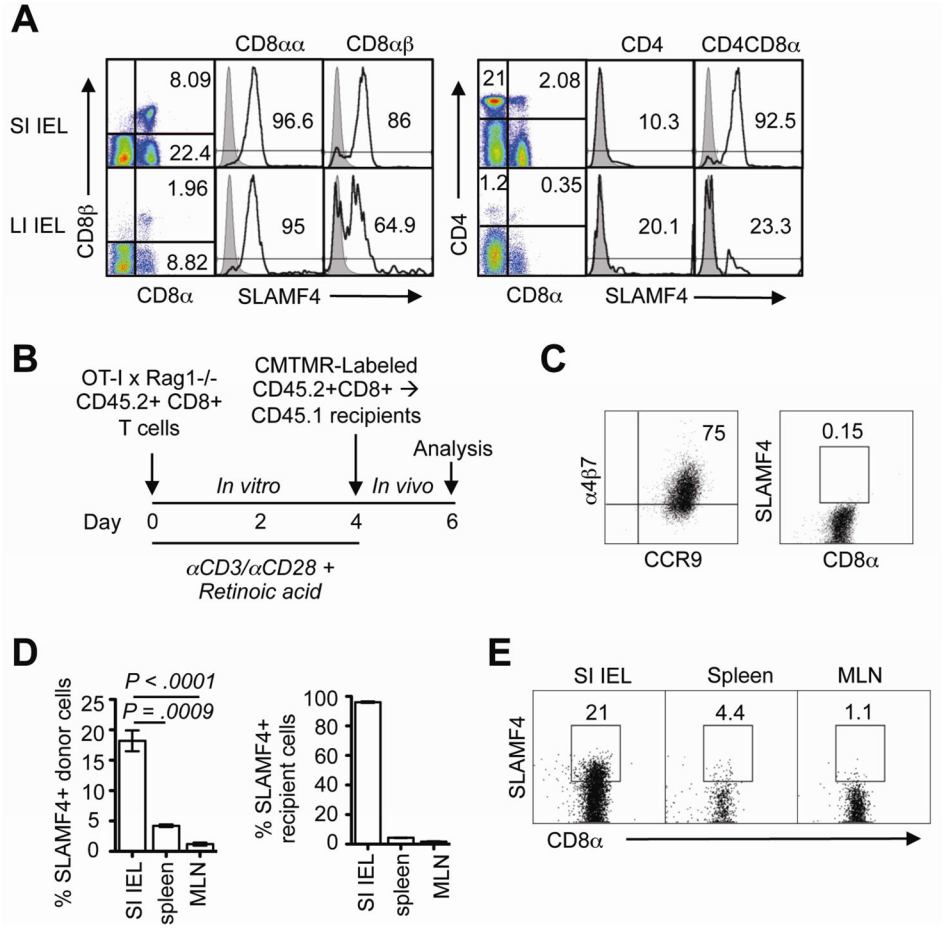

Figure 1. Expression of SLAMF4 by CD8$^+$ intraepithelial lymphocytes is induced in the small intestine

(A) SLAMF4 expression on intraepithelial T cell subsets (gated on CD3$^+$ cells) isolated from the small (SI) and large intestine (LI) from *wt* B6 mice. Bold line – SLAMF4, Gray histogram – isotype control.

(B) Outline of the adoptive transfer of splenic OT-I CD8$^+$ T cells, which had been cultured *in vitro* with αCD3/αCD28 and retinoic acid to generate gut homing CD8$^+$ T cells. After 4 days, CD8$^+$ T cells were transferred into CD45.1 congenic *B6* recipients. Tissues were analyzed 48h post transfer.

(C) CD8$^+$ T cells generated *in vitro*, as outlined **in 1B**, express gut homing markers Integrin α4β7 and CCR9, but not SLAMF4.

(D) Percentages of donor (CD45.2$^+$) (**Left**) or recipient (CD45.1$^+$) (**Right**) derived SLAMF4$^+$ CD8α$^+$ cells among SI IEL, MLN, and spleen 48 h after transfer.

(E) Representative expression of SLAMF4 on the surface of CD8α$^+$CD45.2$^+$ donor cells from small intestine IEL, MLN, and spleen.

Results in A are representative of at least 4 experiments. Percentages of SLAMF4$^+$ cells are shown as mean ± SEM and are representative of at least 3 independent transfer experiments.

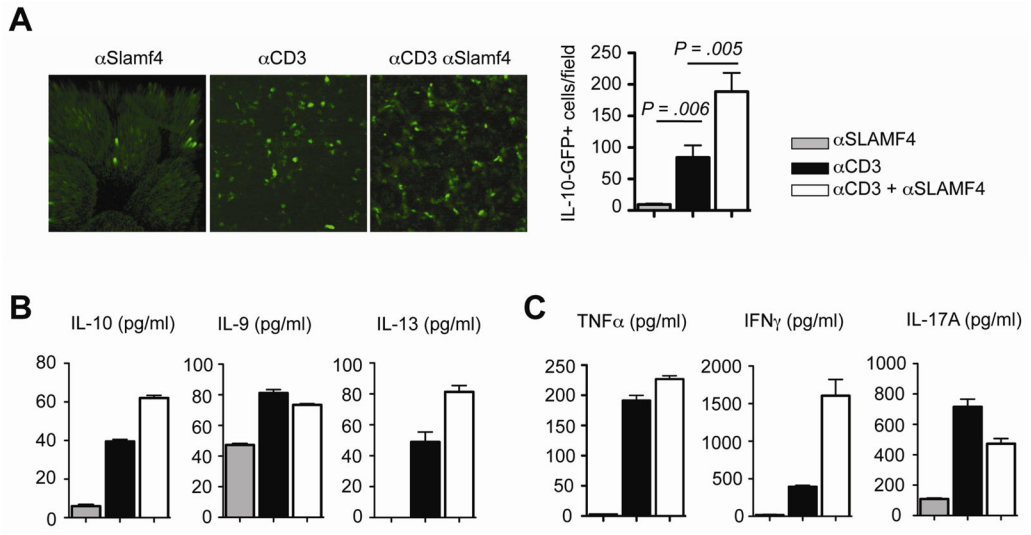

Figure 2. Secretion of key cytokines by IELs in the small intestine after co-administering αSlamf4 and/or αCD3

WT B6 or IL-10-GFP mice were injected with 20μg αSlamf4, αCD3, or αCD3/αSLAMF4 at 0, 48 and 96 h. Four hours after the last injection, mice were euthanized and purified IELs were cultured for 72 h before analysis.

(A) Live imaging of small intestine tissue by confocal microscopy (**left**). Number of GFP^+ cells was quantified using Volocity software (right).

(**B–C**) IL-10, IL-9, and IL-13 (**B**) and IFNγ, IL-17A and TNFα (**C**) in the supernatant fluids of IEL were determined, as in **Materials and Methods**.

Results are representative of 2 independent experiments.

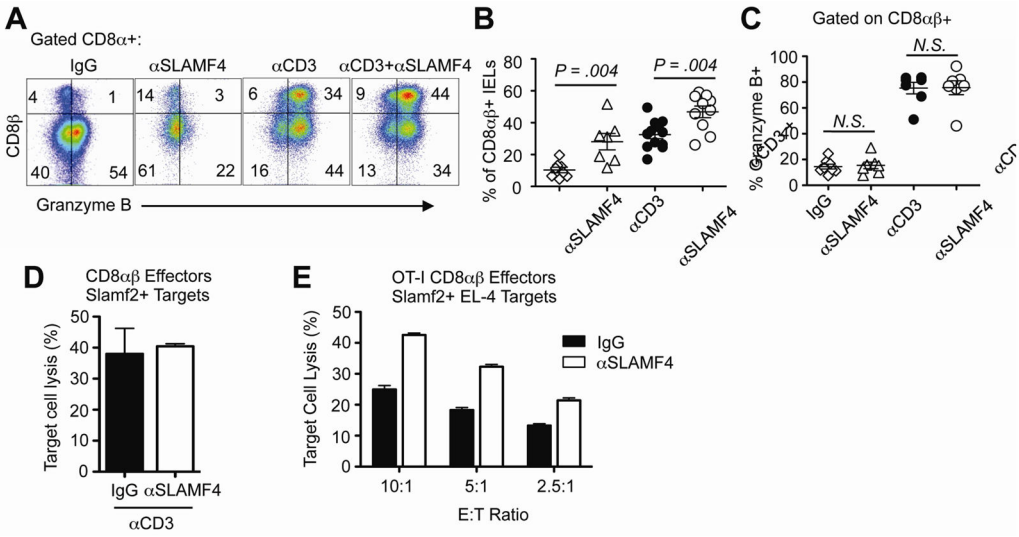

Figure 3. αSLAMF4 increases αCD3-induced expansion of cytotoxic CD8αβ IELs and ovalbumin specific killing by OT-I CD8αβ IELs

Wild type B6 mice (Fig. 3A–C) were injected with 20μg αSLAMF4, αCD3, or αCD3/αSLAMF4 at 0, 48 and 96 h after which IELs were isolated for flow cytometry.

(A) Granzyme B expression, as judged by intracellular staining.

(B) Percentage of $CD8\alpha\beta^+$ IELs on pre-gated $CD8\alpha^+$ cells.

(C) Percentage of Granzyme B^+ CD8αβ IELs.

(D) Re-directed cytotoxicity assay employing FACS-purified CD8αβ IELs from αCD3-injected mice and SLAMF2+ P815 target cells (E:T= 5:1) in the presence of αCD3 with αSLAMF4 or MouseIgG2b. Target cell lysis was assessed by the Promega Cytotoxicity kit.

(E) *OT-I x Rag−/−* mice were kept on drinking water containing 1mg/ml ovalbumin. FACS-purified $TCR\alpha\beta^+$ $CD8\alpha\beta^+$ IELs were incubated SIINFEKL OVA-peptide-pulsed EL-4 target cells in the presence of αSlamf4 or an isotype control, as in **D**.

Results are shown as mean ± SEM. Figures are representative of (**A, C–E**) or pooled from at least 3 (**B**) independent experiments. Points represent individual mice.

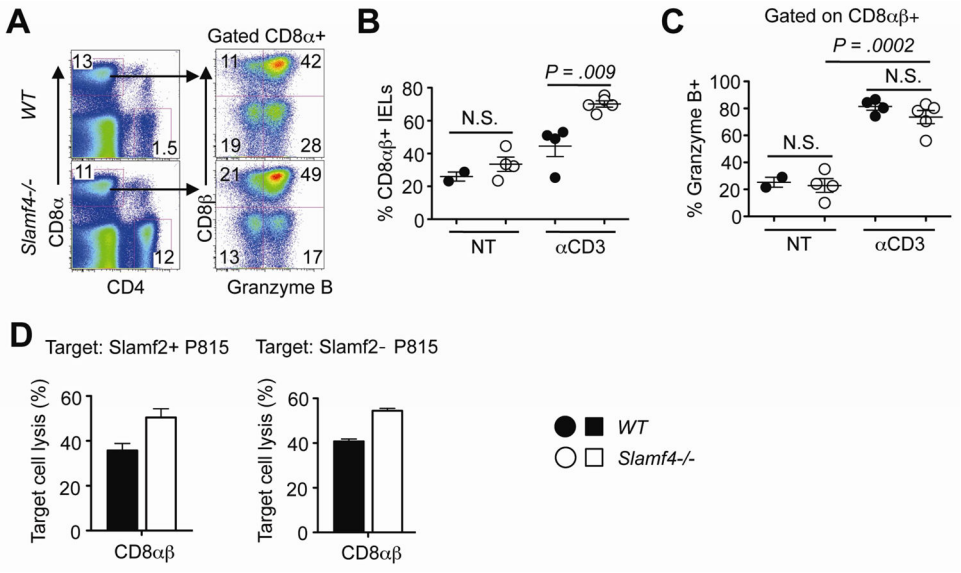

Figure 4. Increased numbers of cytotoxic intraepithelial T cells in the small intestine of *Slamf4*$^{-/-}$ mice

Slamf4$^{-/-}$ B6 mice were injected with 20μg αCD3 at 0 and 48 h. Sixteen hours after the last injection IELs were isolated.

(A) Representative staining of IEL subsets as indicated.

(B) Percentage of CD8αβ IELs as a proportion of total CD8α^{+} IELs.

(C) Percentage of Granzyme B producing CD8αβ IELs.

(D) CD8αβ IELs were purified by FACS from αCD3-injected mice and cytotoxicity was determined using re-directed lysis in the presence of αCD3 with SLAMF2^{+} (**left**) or SLAMF2^{-} (**right**) P815 target cells [E:T = 5:1].

Results are shown as mean ± SEM and are representative of at least 3 independent experiments. Dots represent individual mice.

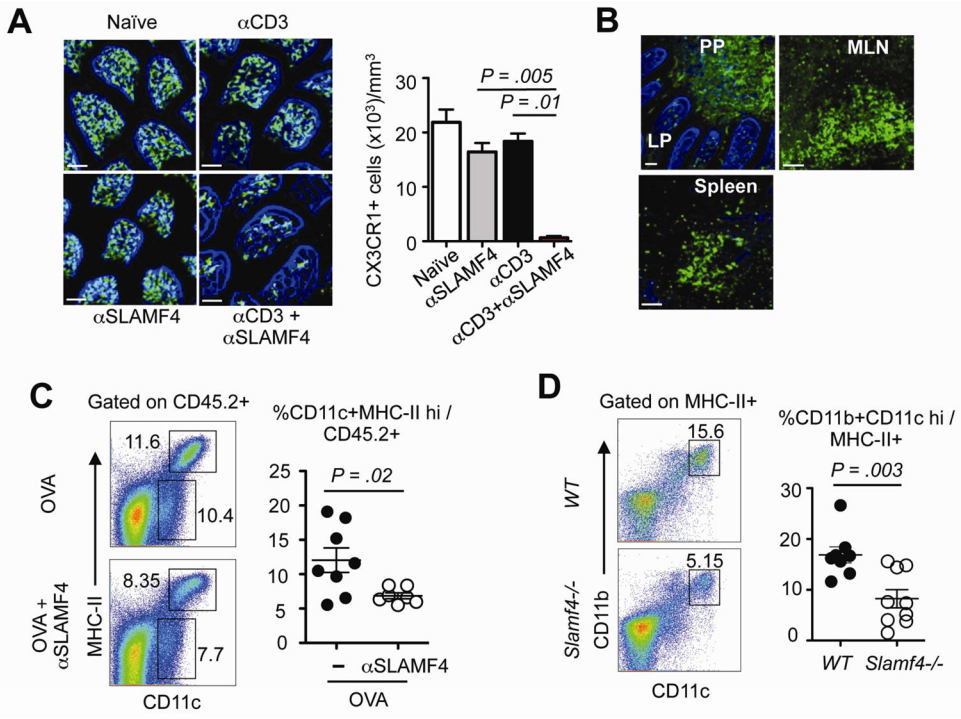

Figure 5. Slamf4 negatively regulates the αCD3− or antigen-mediated depletion of CX3CR1$^+$ phagocytes by IELs

(A) CX3CR1.GFP mice were injected as in Fig. 2 with αSLAMF4, αCD3, or αCD3/αSLAMF4. Small intestine sections were analyzed by live imaging with a confocal microscope Identification of CX3CR1.GFP$^+$ cell images employed Volocity software with cell count normalized to the same volume for all conditions. 3D reconstruction of confocal microscopic images of small intestine villi (**left**) and number of CX3CR1$^+$ cells per field (**right**).

(B) 3D reconstruction of confocal microscopic images of Peyer's patches, MLN, and spleen from αSlamf4/αCD3-injected mice.

(C) Lamina propria cells were isolated from *OT-I x Rag*$^{-/-}$ mice that had been kept on OVA-containing drinking water and had been injected three times with αSLAMF4. Cells were analyzed by flow cytometry. Representative staining (left) and percentage (right) of CD11c$^+$MHC-II$^+$ cells (right).

(D) Lamina propria cells were isolated from *Slamf4*$^{-/-}$ and *wt B6* mice 96 h after one injection of αCD3 and analyzed by flow cytometry. Representative staining (left) and percentage (right) of CD11b$^+$CD11c$^+$ MHC-II$^+$ cells.

Results are shown as mean ± SEM. Data are representative of 4 independent experiments (A–B) or pooled from 2 experiments (C–D). Points represent individual mice. Images in A and B were taken at 20x original magnification with scale bars (60μm); Green – CX3CR1$^+$ cells; Blue – AlexaFluor647-WGA staining of the microvasculature.

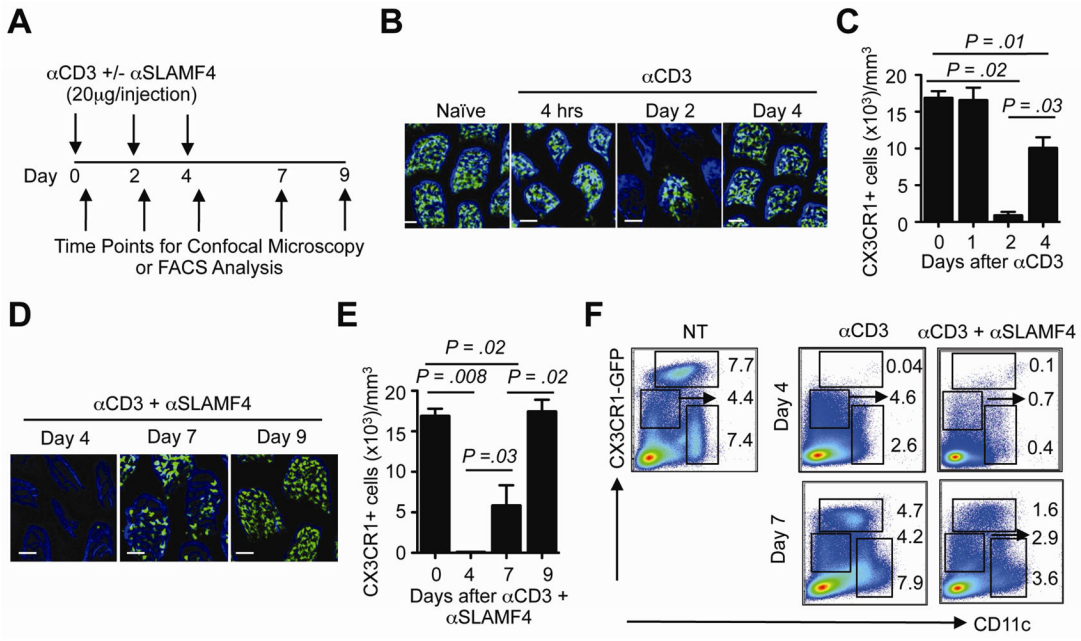

Figure 6. Co-injection of αSLAMF4 prolongs αCD3-induced depletion of CX3CR1$^+$ cells from the small intestine of CX3CR1-GFP mice

CX3CR1-GFP mice were injected with 20μg αCD3, or αCD3/αSLAMF4 at the indicated time points. The small intestine was analyzed by live-imaging confocal microscopy or by cytofluorometry at the indicated time points.

(A) Schematic outline of antibody injections used to assess kinetics of CX3CR1 $^+$ cell depletion and repopulation.

(B–E) 3D reconstruction of confocal microscopic images of the small intestine and quantitation of CX3CR1$^+$ cells at each time point after αCD3 **(B–C)** or αCD3/αSLAMF4 **(D–E)** injections.

(F) Analysis of the percentage of CX3CR1$^+$ populations by flow cytometry on Days 4 and 7. Lamina propria myeloid cells were isolated, as described in Fig. 5

Results are shown as mean ± SEM and are representative of 3 independent experiments. Images in **B** and **D** were taken at 20x original magnification with scale bars (60μm); Green – CX3CR1$^+$ cells; Blue – AlexaFluor647-WGA staining of the microvasculature.

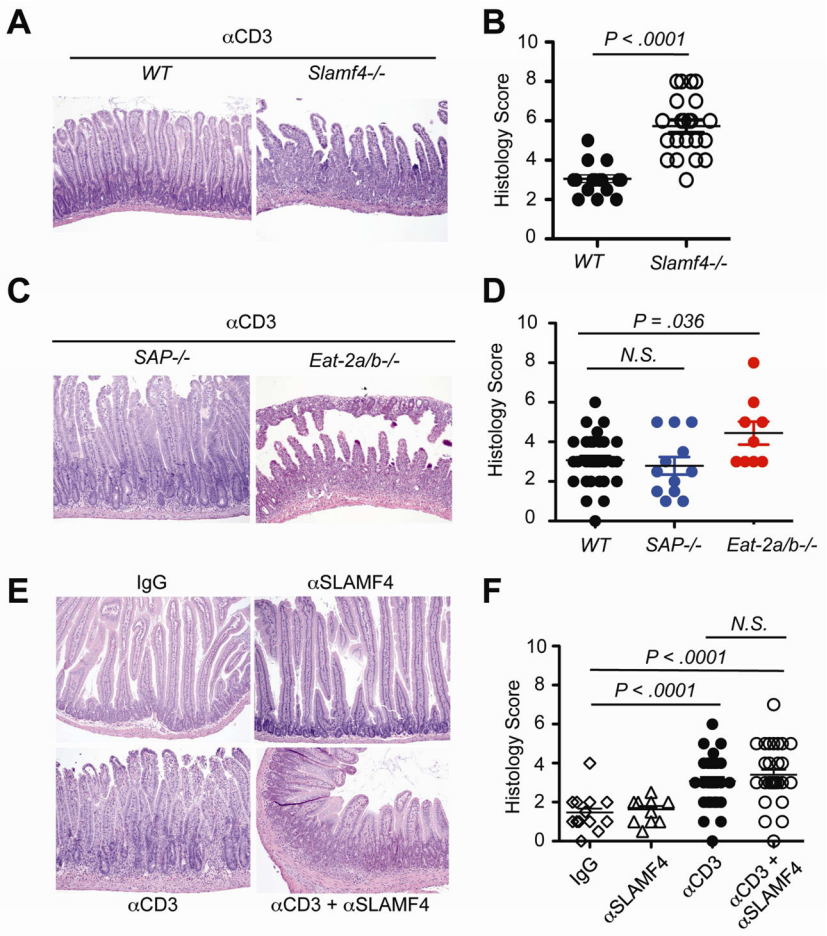

Figure 7. Inflammation of the small intestine was induced in *Slamf4*$^{-/-}$ or *Eat2a*$^{-/-}$*Eat2b*$^{-/-}$ by αCD3 or in *wt* mice by co-injection of αCD3/αSLAMF4

Mice were injected with 20μg αCD3 at 0 and 48 h (**A–D**), or with combination of αCD3 and αSLAMF4 at 0, 48 and 96 h (**E–F**). Inflammation was assessed after H&E staining of the proximal small intestine. Histology scores are pooled from 4 experiments (**B** and **F**) or at least 2 independent experiments (**D**). Points represent individual mice.

(A–B) Microscopic images (10x magnification) and histology scores were obtained from *Slamf4*$^{-/-}$ B6 mice that had been injected twice with αCD3

(C–D) Microscopic images (10x magnification) and histology scores were obtained from *SAP*$^{-/-}$ and *Eat2a*$^{-/-}$*Eat2b*$^{-/-}$ (B6) mice that had been injected twice with αCD3.

(E–F) Microscopic images (10x magnification) and histology scores were obtained from *WT B6* mice were injected three times with combinations of αCD3 and αSLAMF4.