Slamf receptors

Modulators of Phagocyte Immune Responses

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Colophon

Slamf receptors: modulators of phagocyte immune responses

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Modulators of Phagocyte Immune Responses

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Modulatoren van Immuun reacties door Fagocyten
(met een samenvatting in het Nederlands)

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Content

Chapter 1

Responses to microbial challenges by Slamf receptors

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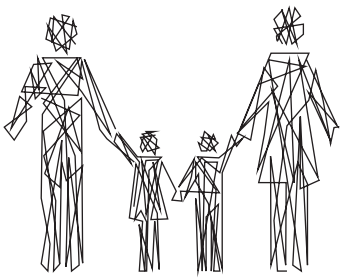
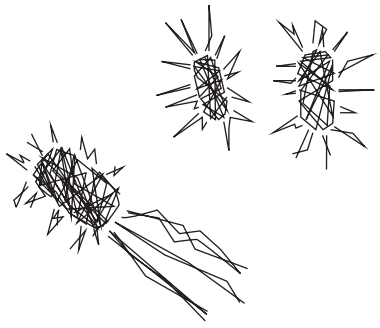


Abstract

The Slamf Family (Slamf) of immune cell surface receptors has nine members six of which are self-ligands. Their homophilic interactions induce signals *in trans*, thereby shaping immune cell-cell communications. Collectively, these receptors modulate a wide range of functions by immune cells, such as lymphocyte development, humoral responses and cytotoxicity. Interestingly, several Slamf receptors serve as microbial sensors and fight microbial challenges by modulating the function of macrophages, dendritic cells, neutrophils, and NK cells. The Slamf receptor-microbe interactions lead to distinct innate immune mechanisms, which contribute to microbicidal activity of the innate immune cells. In this review, we describe the current knowledge on how the Slamf receptors and their adapters SAP and Eat-2 regulate anti-microbial innate and adaptive immune responses.



SLAM family Receptors and adaptors SAP and Eat-2

The Slamf gene family

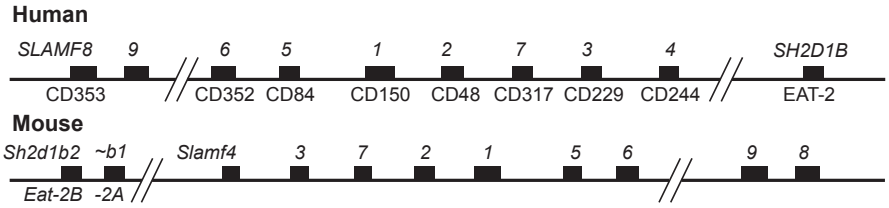
Seven of the nine members of the Signaling Lymphocytic Activation Molecule (SLAM) gene Family (Slamf1-7), a subfamily of the immunoglobulin superfamily, cluster on chromosome 1[1] and the other two (Slamf8 and Slamf9) as well as the SLAM-associated signaling adaptor Eat-2 (*Sh2d1b*) are located in close proximity to the 'core' Slamf locus (shown in Figure 1 and reviewed in [2,3]). The nine Slamf genes encode cell surface receptors, which expression is constrained to hematopoietic cells (Figure 1). A wide range of these cells expresses at least one member (Table 1). The activation state, presence of the adaptor molecules SAP (*Sh2d1a*) and Eat-2, and the location of immune cells dictate Slamf receptor expression and function. While Slamf receptors share intracellular interaction partners and display overlapping features, the individual members of this family all have a unique signature of functions.

The consensus structure of Slamf receptors exists of an extracellular distal IgV domain linked to a proximal IgC2 domain, a transmembrane region, and an intracellular signaling domain that contains several intracellular tyrosine-based switch motives (ITSM). Notable exceptions to the consensus structure are Slamf2, which lacks the intracellular and transmembrane region and instead harbors a glycosyl-phosphatidylinositol membrane anchor; Slamf3, which has a duplication of the IgV-IgC2 domains; and Slamf8 and Slamf9, which only have ~30 intracellular amino acid residues and therefore lack ITSMs.

Most Slamf receptors are homophilic

Slamf receptors are best described in cell-cell communication because they are self-ligands with signaling motives. Exceptions to this homotypic engagement are Slamf2 and Slamf4, which are co-ligands [4-6]. It remains elusive whether Slamf9 is a self-ligand. Crystal structures of both Slamf5 and Slamf6 have revealed an angled engagement of the IgV domains *in trans* [7,8] (Figure 2). Ligation of Slamf receptors leads to signaling events that can be inhibitory or activating, often depending on the presence of SAP and Eat-2. Approximately 15 years ago a different, xenophilic mode of Slamf interaction was described for Slamf1. Measles virus utilizes Slamf1 as one of two entry receptor, the other being CD46 [9,10]. Importantly, other studies also revealed cognate interactions between bacteria and Slamf1, Slamf2, and Slamf6, which represent a third mode of interaction (Table 2) [11-13].

Chromosome 1: Signaling lymphocytic activating molecule gene family



SLAMF molecules are predominantly homophilic receptors

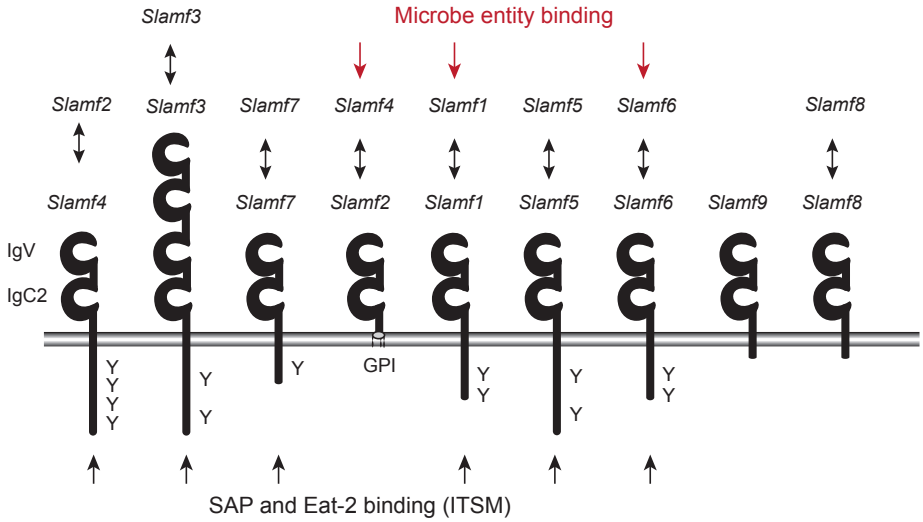


Figure 1 | Signaling Lymphocytic Activating Molecule gene Family (Slamf receptors family) and proteins. Organizational overview of the Slam family cluster on chromosome 1 in both human and mice. EAT-2 is also located proximal to this gene cluster and is duplicated in mice, encoding Eat-2a and Eat-2b. The Slamf receptors are part of the Ig-superfamily and they have an IgV and an IgC2 domain. Seven of the Slamf receptors are homophilic ligands. Slamf2 and Slamf4 are co-ligands that bind each other. Three SLAM genes have been shown to posess bacterial binding capacity. Six of the Slamf receptors have docking domains for SAP (and EAT-2) represented by Y (tyrosine in ITSM). Slamf2 is achored to the plasma membrane by a GPI-anchor.

Slamf specific adaptor proteins

A little under two decades ago, three independent research groups discovered an association between mutations in *SH2D1A*, the gene that encodes the intracellular adaptor pro-

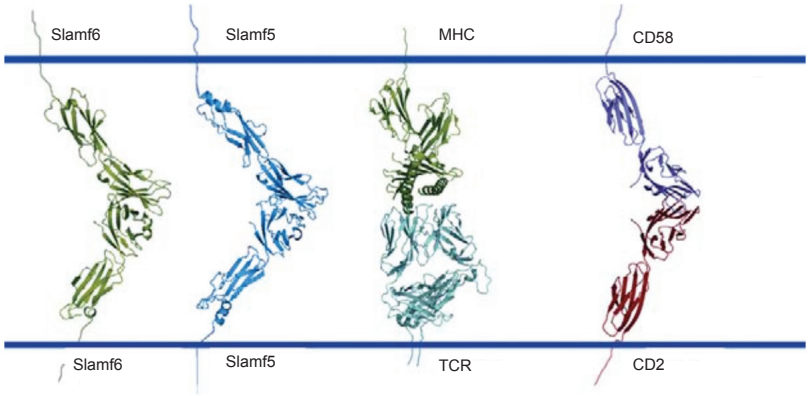


Figure 2 | Ribbon representation of Slamf6 and Slamf5 structures. Homophilic interactions of Slamf6 and Slamf5 as well as heterophilic interactions between two other Ig-superfamily receptors CD58 and CD2. MHC interacting with TCR functions as a reference for the molecular dimensions. ***Image adopted from Calpe S, et al. 2008 (Reference 2).***

tein SLAM Associated Protein (SAP) and X-linked lymphoproliferative syndrome (XLP) [14,15]. One of these groups showed that SAP is an intracellular binding partner of Slamf1 and proved the causative relation between SAP mutations and XLP [16]. SAP encodes a small adaptor protein (14kDa) that consists almost entirely of a Src homology 2 (SH2) domain. SAP can bind to specific tyrosine-phosphorylated ITSMs in the six Slamf receptors that harbor this motive (Figure 1) [16-18]. An interesting exception to this phosphorylation-induced SAP binding is the Slamf1-SAP interaction, which can occur independently of tyrosine phosphorylation of the receptor [19]. Two signaling modules have been postulated for SAP; competitive inhibition of the binding of other adaptors to ITSMs of Slamf receptors and recruitment of kinases and phosphatases after binding to an ITSM [3,16,20-22]. Mice that are deficient for the gene that encodes SAP (*Sh2d1a*$^{-/-}$) have a range of specific immune malfunctions, some of which manifest in the development and maturation of immune cells and others only after a microbial challenge. Although SAP expression by T-cells, NK cells and NKT-cells is well established, B-cells express SAP only under certain specific conditions [23,24]. EBV-transformed B-cells, Hodgkin's lymphomas and germinal center (GC) B-cells where shown to express SAP. The second Slamf associated adaptor, which was designated Eat-2, exhibits distinct functional features and is not associated with primary human immune deficiencies [25]. Eat-2 binds different ITSMs in Slamf receptors and is involved in the activation of antigen presenting cells and cytotoxicity of NK cells [25,26]. The expression profile of this adaptor also differs from SAP, as it is not found in T-cells. NK cells express Eat-2 as well as a range of antigen presenting cells, including monocytes [25,27].

Table 1 | Slam receptor expression, associated effector molecules, and functions.

		Effector functions			
	Expression	**Effectors**	**SAP-dependent**	**Eat2-dependent**	**other/ unknown**
Slamf1, SLAM, CD150	act T, act B, mono, Mø, DC, plat, HSC	Fyn, Lck, SHIP-1, Src, Shp-1/2, PKCθ, Bcl-10, Beclin-1,PI3K, Nf-κB, Ras-GAP, Akt, JNK1/2, Dok-1/2	T: (+) IL-4, IL-13, proliferation, Th2 / Th17 polarization, NKT: development (with Slamf6)	unknown	T: (+) IFNγ, B: (+)proliferation and activation, (+) apoptosis, Mø: (+) ROS, IL-12, TNFα, NO, (-) IL-6, (+) myeloid cell migration, (+) platelet aggregation, (+) phagocytosis
Slamf2, CD48	Pan-lymphocyte	Lck, Fyn, RhoA	N/A	N/A	T: (+) IL-2, proliferaton, B: (+) activation, (-) apoptosis Mast: (+) TNFα, eo: (+) activation, mobilization, Mø: (+) TNFα, IL-12, (+)phagocytosis, DC: (+)survival
Slamf3, Ly-9, CD229	T, B, iCD8, NKT, mono, Mø, HSC	AP-2, Grb-2, ERK, PLZF, NFAT	unknown	unknown	T: (-) IFNγ, (+) proliferation, IL-2, IL-4, iCD8+ T-cells, iNKT (-) development
Slamf4, 2B4, CD244	NK, NKT, T, γδ, CD8, DC, eo, mast, mono	LAT, PI3K, Vav-1, SHIP, c-Cbl, ERK, Shp-1/2, PLC-γ, 3BP2, Csk	T: (-) IFNγ, NK / CD8+: (+) Cytotoxicity, proliferation	NK: (-) Cytotoxicity of Slamf2-neg target cells, (-) IFNγ	eo: (+) adhesion, chemotaxis, peroxidase, (+) IFNγ, IL-4
Slamf5, CD84	Pan-lymphocyte, plat, mast, eo	Dok-1, c-Cbl, ERK, JNK, Fes, Shp-1, Nf-κB	T-B: (+) GC response	NK: (+) Cytotoxicity Mast: (+) Degranulation	plat: (+) spreading
Slamf6, NTB-A, Ly-108	NK, NKT, T, B, Mø, pDC	PLC-γ, SHIP, Shp-1/2, PI3K, PLZF, Lck, PKCθ, NFAT	T-B: (+) GC response, NK: (+) IFNγ, NKT: development (with Slamf1)	NK: (+) Cytotoxicity	T-B: (-) GC response, Neutro: (+) ROS, (+) IL-6, TNFα
Slamf7, CRACC, CS1, CD319	T, B, mono, DC, NK	PLC-γ, c-Cbl, SHIP, Akt, Vav-1, Shp-1/2	unknown / NA	NK: (+) Cytotoxicity	NK: without Eat2 (-) Cytotoxicity, B: (+) proliferation
Slamf8, BLAME,	iCD8, mono, DC, Mø, Neu, endo, FRC	PKC, p40(phox)	N/A	N/A	(-) myeloid cell migration, (-) ROS, iCD8+ T-cells, iNKT (+) development
Slamf9, SF2001	mono, DC	ND	N/A	N/A	unknown

T: T cells, B: B cells: act: activated, Mø: macrophage, DC: dendritic cell, plat: platelet, HSC: hematopoietic stem cell, mono: monocyte, NKT: natural killer T cell: eo: eosinophil, γδ: γδ receptor-expressing T cell, mast: mast cell, endo: endothelial cell, FRC: fibroblastic reticular cell, ROS: reactive oxygen species. Expression data is based on murine expression.

Structural basis of the interactions of Slamf receptors with SAP / Fyn and Eat-2

A set of functions of SAP in T-cells is dependent on the Src kinase Fyn, which is intricately involved in T-cell receptor (TCR) signaling (Figure 3). Fyn is crucial for GATA-3 transcription and contributes to IFNγ, IL-2, IL-4, and IL-13 production upon TCR stimulation. Slamf-Slamf homophilic ligation leads to the recruitment of SAP to their ITSMs, which can induce a subsequent SAP-Fyn interaction. Binding of Fyn to Slamf1-associated-SAP enhances only IL-4 and IL-13 production [21]. Structural analyses have shown that Arg78 of SAP is crucial to this interaction [20,21]. Indeed, SAP^{R78A} mice showed a lack of IL-4 production, similar to that of *Sh2d1a*$^{-/-}$ mice [21]. Eat-2 lacks this arginine and therefore does not signal through Fyn [20]. Eat-2 does associate with a variety of different Src kinases [27]. Similar to *Sh2d1a*$^{-/-}$ T-cells, *Slamf1*$^{-/-}$ $CD4^+$ T-cells are also less prone to TCR mediated IL-4 production [28]. It was therefore concluded that Slamf1 contributes to Th2 polarization. Subsequent studies showed a signaling cascade involving SAP and Fyn as well as GATA-3 transcriptional promotion by Nf-κB to be responsible for this phenotype [29-31]. This pathway in T-follicular helper cells effectively contributes to GC B-cell maintenance and optimal humoral responses [32].

SAP inhibits the production of IFNγ by T-cells under TCR-stimulating conditions. *Ex vivo* examined T-cells from virus-infected *Sh2d1a*$^{-/-}$ mice produced higher amounts of IFNγ compared to WT littermates [30,33]. Peptide-pulsed DCs were able to induce higher amounts of IFNγ in SAP-deficient T-cells. In addition, T-cells from mice with the SAP^{R78A} single amino acid mutation also overproduced IFNγ, showing that Fyn recruitment to SAP negatively affects the signals that result in the transcription of *ifng* [21,29]. Subsequent reports showed that in the presence of SAP only tyrosine-competent Slamf1, but not Slamf1 with signaling-null ITSMs abolishes IFNγ production, demonstrating that SAP binding to Slamf1 is a contributing step in this pathway [34]. However, there appears to be some redundancy within the Slamf receptor family on the inhibition of IFNγ production, because *Slamf1*$^{-/-}$ $CD4^+$ T-cells produce only slightly more IFNγ [28]. Indeed, IFNγ production is also enhanced when T-cells are incubated with anti-Slamf5 or anti-Slamf6 antibodies [35,36]. However, Slamf3 has little impact on IFNγ production [37].

Overall, these studies have demonstrated that Slamf receptors and SAP have a complex involvement in mechanisms that fight intracellular infections, via their effect on cytokine

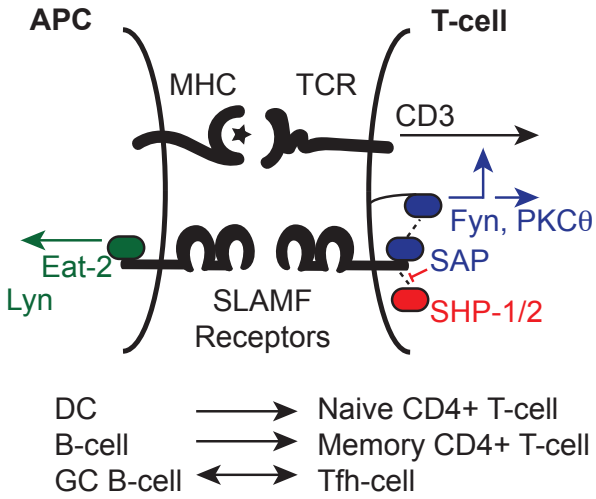


Figure 3 | Slamf receptors modulate the cellular communication between antigen presenting cells (APC) and T cells. Binding of SLAM-family members to their ligands induces the phosphorylation of their cytoplasmic tails and the subsequent binding of SLAM-associated protein (SAP) or EAT2 through a tyrosine-containing motif (ITSM). SAP is widely expressed by T cells and EAT2 is expressed by APCs. These two molecules can recruit and activate several Src kinases (including Fyn) that modulate cell activation by signals generated through the T cell receptor (TCR) and co-stimulatory proteins such as CD28. Signals mediated by the SLAM receptors can also affect the function of APCs. SLAM receptors recruit various SH2-domain-containing proteins giving rise to different signals that determine distinct and, in some cases, divergent biological outcomes.

production. Together, SAP – with and without Fyn – and Eat-2 dictate the major part of the Slamf signaling. However, distinct mediators dictate a set of Slamf receptor functions (Table 1).

Several Slamf receptors interact with bacteria

Slamf1 and Slamf6 interactions with Gram$^-$ bacteria

The importance of Slamf receptors in phagocytes was shown by experimental infections of *Rag*$^{-/-}$ mice that were crossed with Slamf-deficient mice and by infections that rely on macrophages for clearance. Per example, *in vivo* responses to Gram$^-$ bacteria are affected by Slamf1. This was first shown by a study in which *Slamf1*$^{-/-}$ mice are infected with *S. typhimurium* SseB$^-$, which lack a pathogenicity island rendering them incapable of phagosome escape [11]. *Slamf1*$^{-/-}$ mice as well as *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ mice have a defect in

Table 2 | Slamf receptors and their adaptor SAP modulate susceptibility to microbes.

	Deficiency: resistant	Deficiency: suseptable	Slamf ligand	Microbial ligand
Slamf1	*T. cruzi*	Gram⁻ bacteria, *L. major*	Slamf1	Measles virus *E. coli* (OmpC/F+) *S. typhimurium*
Slamf2	*S. aureus*	FimH+ enterobacterae,	Slamf4, CD2	*E.coli* (FimH+)
Slamf3		MCMV	Slamf3	
Slamf4		LCMV, γHV-68,	Slamf2	
Slamf5			Slamf5	
Slamf6	*L. mexicana,* *C. rodentium*	*S. typhimurium*	Slamf6	*E. coli,* *C. rodentium*
Slamf7			Slamf7	
Slamf8			Slamf8	
Slamf9			???	
SAP		Mouse: γHV-68, LCMV, Influenza, Human: EBV, some other viruses	Slamf1, 3, 4, 5, 6 Human: Slamf7	N/A

SAP (Sh2d1a, Slam associated protein), LCMV (Lymphocytic choriomengitis virus), Omp (Outer membrane porine), EBV (Epstein-Barr virus), FimH (bacterial lectin), MCMV (murine cytomegalovirus) γHV-68 (murine gamma-herpes virus 68). Deficiency: resistant and Deficiency: susceptible refer to observations made in Slamf deficient mice; **resistant** indicates that knock out animals have milder disease, **susceptible** indicates that knock out animals have stronger disease manifestations.

the clearance of this bacterium after peritoneal infection. In the pursuit of the mechanistic underpinnings of such phenotypes, key observations have shown that certain Slamf receptors engage in cognate interactions with bacterial entities [11,12,38]. Direct cognate interactions with microbial components modulate Slamf functions in phagocytes.

Evidence for direct interactions of Slamf1 and Slamf6 with *E. coli* outer membrane porines C (OmpC) and OmpF was shown in a cell-based luciferase reporter assay [11]. The specificity of these interactions extent to different Gram⁻ bacteria, but not Gram⁺ bacteria; Slamf1 interacts with *S. typhimurium* [11]; Slamf6 interacts with *S. typhimurium* and to some degree with *C. rodentium* [38]. Subsequent analyses demonstrated that this interaction depends on the IgV domain of Slamf1 and Slamf6. The structure of Slamf1 has proven difficult to unravel due to the flexible (non-rigid) nature and high degree of glycosylation of Slamf1. By a combination of techniques, several amino acid residues have been implicated in Slamf1 homophilic engagement as well as Slamf1 engagement with Measles virus protein

MV-H [10]. The FCC` beta-sheet of Slamf1 and the CC loop contain several conserved residues and substitution of Val63, Thr65, Ala67, Lys77, and Glu123 within these regions, all resulted in a reduction in the binding of Slamf1 to Slamf1 as well as to MV-H. Single mutations of equivalent residues in mouse Slamf1 resulted in little difference in the binding of OmpC/F containing *E. coli*. In line with this, Slamf6 engagement with *E. coli* structures does not require amino acid residues in the Slamf6 IgV domain that are crucial for Slamf6-Slamf6 homophilic ligation [38]. However, general masking of interaction domains by mAbs directed against epitopes in the IgV domains of Slamf1 or Slamf6 blocked their interactions with bacteria [11,38]. Thus, whereas there is overlap in the Slamf1 residues that are essential for Slamf1-Slamf1 ligation with the residues involved in MV-H binding to Slamf1, it is likely that OmpC/F binding involves a separate set of interacting Slamf1 residues. This would suggest that the interaction of Slamf1 with bacteria is of a separate origin, distinct of the Slamf1-Slamf1 interaction domain, and hence may represent a Slamf1 function of separate evolutionally significance. Structural analyses of Slamf1 or Slamf6 and *E. coli* outer membrane porines should provide conclusive insights in the mode of these interactions.

Slamf1 enhances phagocyte effector functions

The interaction of Slamf1 with OmpC/F+ *E. coli* results in a more effective phagocytosis of these bacteria by macrophages [11]. Clusters of Slamf1 bound to OmpC/F remain proximal to the bacterium during phagocytosis, thus co-localizing to intracellular phagosomes. A signaling complex is recruited to the intracellular domain of Slamf1 either directly upon bacterial ligation or shortly thereafter during internalization. The transient recruitment of the autophagy scaffold protein Beclin-1 is the initial event that leads to the formation of a functional complex that also contain Vps34, Vps15, and UVRAG (Figure 4) [13]. This novel Slamf1 signaling module is enhanced by, but not prerequisite of the presence of Eat-2 [13]. Vps34 supported by its co-enzyme Vps15 is the sole Class III phosphatidyl inositol kinase and produces the docking lipid phosphatidylinositol-3'-phosphate (PI_3P) [39]. This Slamf1-enhanced production of PI_3P affects two important phagosomal processes. First, formation and activation of the classical phagocytic NADPH oxidase (Nox2) complex is a tightly regulated process that involves assembly of the membrane bound catalytic $gp91^{phox}$ and $p22^{phox}$ with at least four cytosolic subunits $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, Rac1/2 [40]. By recruiting the $p40^{phox}$ subunit to the maturing phagosome, PI_3P initiates the formation of this superoxide-producing complex [39]. Second, PI_3P enables the recruitment of the teth-

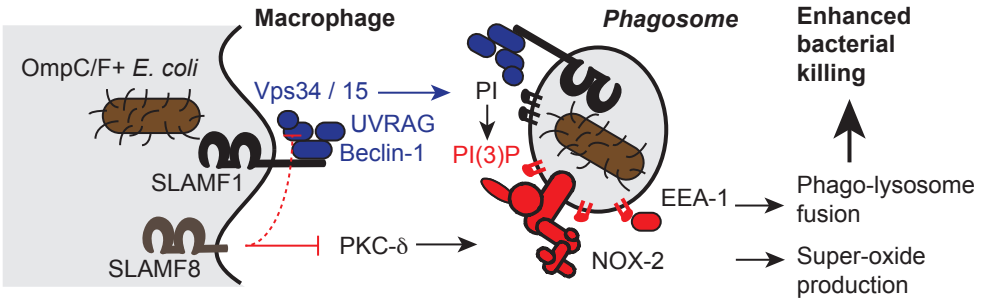


Figure 4 | Slamf1 affects phagosome functions in two ways, after binding to *E. coli*. OmpC/F+ *E. coli* can be bound by Slamf1. Subsequently, Slamf1 is internalized into the progressing phagosome. The Vps34/15>UVRAG>Beclin-1 complex is formed. PI is converted to PI_3P, which is the docking lipid for subunits of the Nox2 complex as well as the tethering molecule EEA-1. The result of the docking of these proteins is the progression of phagosomes toward bactericidal phagolysosomes that are able to kill the internalized bacteria. The positive modulation of Nox2 complex formation by PKC-delta is inhibited by Slamf8. There is preliminary evidence for an inhibition by Slamf8 of Vps34/15>UVRAG>Beclin-1 complex recruitment to Slamf1.

ering molecule EEA1, which is critically involved in phago-lysosomal fusion. Thus, in the absence of Slamf1 from phagocytes, the phagocytic process of specific Gram$^-$ bacteria is compromised.

Slamf2 interactions with Gram$^-$ bacteria

Slamf2 is implicated in the recognition of non-opsonized *E.coli* via surface type-1 fimbriae, which contain the lectin FimH [12]. Microscopy and genetic analysis suggest that Slamf2 binds to FimH, which is dependent on the presence of mannose on Slamf2 [41]. Uptake of FimH$^-$ *E. coli* is not mediated by Slamf2 [42].

Slamf2 internalizes with FimH upon phagocytosis of FimH+ *E. coli* by mast cells and macrophages (Figure 5), which can be inhibited by mAb directed against Slamf2. The 'force catch' interactions between Slamf2 and FimH are strengthened by the motility that is implicit to fimbriae and therefore represents a unique mode of interaction between phagocytes and *E. coli* [43]. Studies utilizing mast cells show that the Slamf2-FimH mediated phagocytosis, which results in cholesterol-dense *E. coli*+ caveolae [44], has a distinct outcome compared to phagocytosis of opsonized *E. coli*. Slamf2-aided uptake results in the expulsion of the bacterium rather than its intracellular killing [42]. Thus, Slamf2 mediates uptake of FimH+ *E. coli* via the formation of caveolin+ phagocytes that represent recycling vesicles that release their content to the extracellular milieu within several hours.

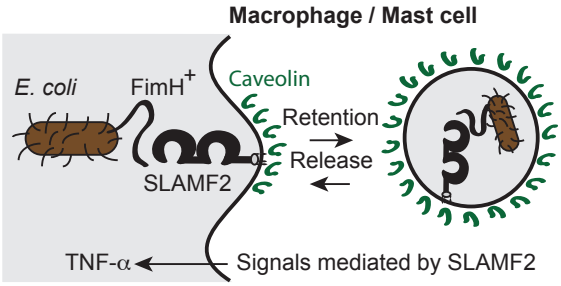


Figure 5 | Slamf2 mediated the temporary retention of FimH+ *E. coli* in phagocytes. Slamf2 can associate with the bacterial lectin FimH on the flagella of *E. coli*. The bacteria are internalized into caveolin+ vesicles to subsequently be released. The presence of Slamf2 on macrophages and mast cells induced an LPS or bacteria mediated enhanced burst of TNF-α production.

Slamf receptors alter cytokine production by phagocytes

Beside the delayed phagocytosis of *E. coli*, *Slamf1*$^{-/-}$ macrophages display impaired responses to crude LPS (bacterial homogenate) [11,13,28]. Stimulation with IFNγ and LPS, but not GpC or PGN, induced an ameliorated production of IL-12, TNF-α, and nitric oxide in *Slamf1*$^{-/-}$ macrophages [28]. Conversely, human DCs that were stimulated with CD40-L expressing cells produced less IL-12 and TNFc when Slamf1 co-stimulation was induced, even in the presence of IFNγ and LPS [45]. This discrepancy could suggest that Slamf1 plays distinct roles on cytokine production in phagocytes depending on whether Slamf1 engages in homophilic interactions and/or bacterial interactions (***i.e.*** OmpC/F). Although Slamf2 has no intracellular signaling domain, Slamf2 induces signaling events in human brain microvascular endothelial cells that involve an influx of intracellular Ca^{2+} and the phosphorylation of RhoA [46]. In mast cells, Slamf2 engagement results in an increase in their TNF-α production and histamine release [41,47,48]. Stimulation of *Slamf2*$^{-/-}$ macrophages with LPS results in reduced induction of TNF-α and IL-12 production [49]. No specific interactions of Slamf5 with bacterial entities have currently been reported, yet Slamf5 also affects phagocyte functions. Transfection studies in mast cells and macrophages have shown that Slamf5 signaling enhances phagocyte activation. Slamf5 engagement induces FcεRI mediated mast cell degranulation, which depends on Dok1 phosphorylation [50]. Interestingly, LPS stimulation of macrophages results in phosphorylation of Slamf5 at the second ITSM domain (Y300), which enhances the production of MCP-1 and TNF-α in an Nf-κB dependent fashion [51]. These observations provide the first direct evidence for Slamf receptor signaling through ITSM phosphorylation in phagocytic cells.

Whether these reports on cytokine modulation by different Slamf receptors represents a common mechanism is unlikely. Yet, Eat-2 may be a common mediator of some of these cytokine phenotypes. Indeed, a recent study suggested that Eat-2 mediates the production of TNF-α through several Slamf receptors in human DCs, whereas this study does not report IL-12 production [52]. Although specific mechanisms need to be further identified, it is clear that Slamf receptors modulate inflammatory effector functions of phagocytes in the presence of bacteria or LPS.

Slamf8 inhibits Nox2 activity in bacterial phagosomes

Slamf8 is a member of the Slamf receptor family that exhibits unique characteristics, as *Slamf8*$^{-/-}$ macrophages appear over-activated. The presence of Slamf8 in phagocytes inhibits the maturation of phagosomes, irrespective whether the cargo are Gram+ or Gram^{-} bacteria [53]. It was shown that Slamf8 negatively regulates the activity of PKC-δ, which phosphorylates the p40^{phox} subunit of the Nox2 complex [53]. The presence of Slamf8 therefore negatively regulates the production of superoxide. However, the molecular intermediates that facilitate this Slamf8 function have yet to be determined. Because Slamf8 does not contain an intracellular domain with known signaling motives, it is unlikely that Slamf8 recruits adaptor molecules that in turn inhibit PKC. Speculatively, competitive inhibition of Slamf1 by Slamf8 represents a possible mechanism. Although interactions *in trans* between Slamf1 and Slamf8 did not occur [54], the Slamf1-Beclin1-Vps34/15-UVRAG complex is more readily formed in the absence of Slamf8. This preliminary finding elutes to a functional interplay between these two Slamf receptors.

Slamf1 and Slamf8 regulate migration of myeloid cells to sites of inflammation

Differential expression of Slamf1 and Slamf8 by phagocytes

Several Slamf receptors are highly expressed by phagocytes after activation by inflammatory signals, suggesting a time-sensitive functional significance of Slamf receptor surface expression in these cells. Slamf1 expression is induced by stimulation with either LPS or IL-1β and in phagocytes during active colitis [28,55,56]. Resting blood leukocytes are virtually

devoid of Slamf8 transcripts and protein [57]. LPS only marginally induce Slamf8 expression, rather its expression in phagocytes is mainly dependent on IFNγ signals, which result in a strong up-regulation of Slamf8 [53,54,57]. Thus, during an ongoing infectious inflammation, phagocytes initially increase Slamf1 surface expression and subsequently induce Slamf8 expression.

Slamf1 and Slamf8 modulate myeloid cell motility

Phagocyte-expressed Slamf1 positively affects cell migration to sites of ongoing inflammation. Our study that focused on cell motility during inflammation revealed that phagocyte-intrinsic functions of Slamf1 enhance the capacity to migrate into sites of inflammation [54]. Inflammatory phagocytes are required to infiltrate the lamina propria of the colon to establish persisting colitis after transfer of $CD45RB^{hi}$ $CD4^{+}$ T-cell into *Rag*$^{-/-}$ mice. The impairment of inflammatory phagocytes in *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ mice to migrate to the lamina propria therefore resulted in ameliorated colitis [55]. The poor outcome in Slamf1-deficient mice of experimental infections with *Leishmania mayor,* which rely on macrophages for effective clearance, may also be partly explained by impaired migration of macrophage-forming monocytes [28]. Opposed to the positive effect that Slamf1 has on myeloid migration, Slamf8 has a phagocyte-intrinsic negative effect on cell motility [54]. Given the timing of the surface expression of Slamf1 and Slamf8 and their opposite effect on phagocyte activation, we hypothesize that these two Slamf molecules represent a rheostat mechanism that modulates the extent of infections inflammation at different stages.

The opposite effects on reactive oxygen production displayed by these two Slamf receptors were shown to influence cell motility. Specific inhibition of Nox2 activity canceled the *in vitro* migration phenotypes of both *Slamf1*$^{-/-}$ and *Slamf8*$^{-/-}$ phagocytes [54]. These two phenomena can be linked by the mounting evidence that hydrogen peroxide, which is the more stable intermediate of superoxide, can act as a 'second messenger' by oxidizing phosphatases and – as such – modulate cell motility [40,58,59].

Slamf1, 2, and 6 regulate enterocolitis

Thus far, we have discussed several features of Slamf1, Slamf2, and Slamf6 in phagocytes. All three of these Slamf receptors also affect the pathogenesis of murine models of colitis, which are complex, multi-facetted immune events. Part of the requirements of

colitis development in most murine models is an activation of the mucosal immune system by microbes. As discussed above, our group and others have collected evidence for a roll of Slamf receptors in cognate interactions with bacteria. The infiltration of pro-inflammatory phagocyte into the lamina propria of the colon is also prerequisite of the pathogenesis of colitis and some Slamf receptors affect the extent of the colitis by influencing this process. Additionally, modulation of cytokine production may also contribute to these colitis phenotypes.

Slamf6 enhances *C. rodentium* colitis

Citrobacter rodentium are attaching bacteria that harbor a pathogenicity island, which renders them capable of colonizing the colonic epithelia of mice. By doing so, *C. rodentium* causes lesions that result in a compromised mucosal barrier. Colitis induced by oral infection with *C. rodentium* is remarkably reduced in mice lacking both the *Rag1* and the *Slamf6* genes, but not in *Slamf6*$^{-/-}$ (single knock out) mice, showing an involvement of Slamf6 in innate responses to the mucosal infections with specific enterobacteriae [38]. Specific interactions between *E. coli* or *C. rodentium* and Slamf6 have also been reported. The interactions between Slamf6 and these bacteria may drive this phenotype, as the reduced pathology appears to manifest downstream of the phagocytes that first detect the effacing *C. rodentium* bacteria [38].

Phagocyte functions of Slamf1 contribute to colitis

A study employing transfer of CD45RB^{hi} CD4^{+} T-cells into *Rag*$^{-/-}$ mice demonstrated that only Slamf1 expression by innate cells, and not T-cells, is required for the full induction of experimental colitis [55]. This study showed that activation of macrophages and DCs via CD40-stimulation alone was not sufficient to overcome the reduced inflammation in *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ mice, further establishing a phagocyte-intrinsic cause of this phenotype. Functionally, Slamf1 likely causes enhanced colitis by three mechanisms. The hampered migratory capacity of Slamf1-deficient inflammatory phagocytes was shown to be the primary cause of this phenotype [55]. The enhanced phagosomal maturation and ROS production that results from the interaction of Slamf1 with *E. coli* could represent an additional mechanism if these Slamf1-mediated functions lead to a higher activation state of the lamina propria phagocytes. The production of pro-inflammatory cytokines that are implicated in colitis development are also impaired by Slamf1-deficiency [55].

Slamf2 is enhances colitis and Slamf4 is a negative regulator of cytotoxic IELs, which control inflammation of the small intestine

Comparable CD45RB^{hi} CD4^{+} T-cells transfer experiments showed that Slamf2 expression by both innate cells as well as transferred T-cells contribute for the development of colitis [49]. *Slamf2*$^{-/-}$ T-cells induced colitis in *Rag*$^{-/-}$ mice, but not in *Slamf2*$^{-/-}$ *Rag*$^{-/-}$ mice. Indeed, Slamf2-deficient mice were shown to have severely impaired CD4^{+} T-cell activation and Slamf2 expression is required on both T-cells and antigen presenting cells for proper activation [60]. Beside T-cell activation, which is a prerequisite for the development of colitis in this model, macrophage-expressed Slamf2 could contribute to colitis by inducing TNF-α production, as suggested by *in vitro* experiments [41,49]. Whether both Slamf2 interactions with Slamf4 and bacteria drive this *in vivo* remains to be determined.

Slamf4 also affects gut-mucosal immune responses. CD8^{+} T-cell transfer experiments showed that Slamf4 expression specifically correlated with localization to the intestinal lamina propria. Here, Slamf4 modulates homeostasis by negative regulation of the expansion of cytotoxic CD8^{+} IELs [61]. Slamf2 expression in myeloid cells especially the CX3CR1+ and CX3CR1- phagocytes in the lamina propria of the small intestine facilitate this negative regulation [61]. Vise versa, under specific conditions these cytotoxic IELs are capable of controlling the phagocyte population [61].

Innate effector functions of Slamf receptors affect the development of mucosal immune responses

No strong intestinal inflammation phenotype has been ascribed to XLP [62], thus SAP-independent functions of Slamf receptors likely modulate mucosal immune processes. Indeed, the current knowledge on the modes by which Slamf receptors modulate phagocyte functions describes three main features. Slamf engagement of microbial components results in an altered phagocytic response; Slamf1 and Slamf2 interact with bacteria, albeit in a different fashion, and enhance distinct phagocytic processes. Slamf6 interacts with bacteria, but an altered phagocytic response has to date not been described. Slamf8 also affects phagocytosis, but whether Slamf8 interacts directly with bacteria remains elusive. At least Slamf1 and Slamf8 also affect *in vivo* and *in vitro* phagocyte migration. Thirdly, cytokine production in response to inflammatory cues is modulated by Slamf receptors, often

promoting inflammation. How interactions between individual Slamf receptors influence the function of phagocytes and what adaptor molecules are involved in specific functions, either induced by Slamf-Slamf or by Slamf-microbe interactions needs further investigation. Studies that focus on the differences in Slamf signaling and function between these two modes of interaction will be instrumental in our understanding of Slamf functions in phagocytic cells.

SAP and Slamf receptors protect us from EBV and other viruses

Whereas Slamf receptor mediated immune responses to bacteria are mostly mediated by Slamf-bacteria interactions, the involvement of Slamf receptors in anti-viral immunity relies mostly on Slamf-Slamf homophilic interactions. To understand the immune implication of Slamf receptors and the adaptors SAP and Eat-2, we consider specific phenotypic observation of virus infections.

XLP and Epstein-Barr Virus

X-linked lymphoproliferative disease finds its primary cause in dysfunctional SAP [14-16]. Often, but not always [63], patients develop fulminant infectious mononucleosis with a fatal outcome upon the first encounter with Epstein-Barr Virus (EBV). Although SAP-deficient patients who survive EBV infections or never encounter EBV will develop aberrant B-cell response such as disgammaglobulinemia and B-cell lymphomas as well as a lack of innate type lymphocytes such as NKT-cells, the most prominent manifestations of this genetic defect arise in the context of EBV infections. Excellent reviews about EBV-independent immunologic manifestations of the aberrant response in SAP-deficient patients are published elsewhere [3,64-66]. In sum, in the absence of functional SAP, EBV-infected B-cells are not cleared and massive B- and T-lymphocytic expansion is found in most organs. The extent of our knowledge of SAP functions implement $CD4^+$ T-cells, $CD8^+$ CTLs, NKT cells, and NK cells in the defective immune mechanisms that result in uncontrolled or ineffective immune responses to EBV infections in XLP-patients. The phenotypic manifestations of non-EBV viral infections in XLP-patients are less striking, yet often more severe than in SAP-proficient individuals.

SAP, EAT and CD8+ T-cell expansion and cytotoxic responses

TCR signals in naïve T-cells induce a proliferative burst. SAP and Slamf receptors control both the extent of the CD8+ T-cell expansion as well as the cytotoxicity of these cells, thereby influencing the effectiveness of the immune response to viruses as well as potential immunopathology.

In an effort to delineate the complex phenotypes of EBV infections of XLP-patients, *Sh2d1a*$^{-/-}$ mice were generated and infected with γHV-68 [67] or LCMV [30,68]. The murine virus γHV-68 is, like EBV and Karposi's sarcoma-associated herpes virus, a gamma-herpes virus but has coevolved with rodents and therefore does not infect humans. In addition to B-cells, γHV-68 also infects macrophages and DCs, which should be noted when comparing EBV infections of XLP patients with γHV-68 in *Sh2d1a*$^{-/-}$ mice. Characterization of *Sh2d1a*$^{-/-}$ mice after infection with γHV-68 revealed that these mice have an expanded population of CD8+ T-cells [69,70]. These virus-activated CD8+ T-cells produce IFNγ. As a consequence of the expanded CD8+ T-cell population, IFNγ levels are higher in SAP-deficient mice [70]. This higher amount of IFNγ controls γHV-68 in macrophages in the peritoneum, but not in the B-cell reservoir [71]. In accordance with reports on γHV-68 infected *Sh2d1a*$^{-/-}$ mice, LCMV-Armstrong infections induce a stronger expansion of CD4+ and CD8+ IFNγ-producing T-cells [30,68]. However, the exacerbated immune pathology that is caused by the over-expansion of CD8+ T-cells in this infection results in a higher mortality [30,68].

One of the mechanisms that drive the massive expansion of T-cells is the deregulation of (re)activation induced cell death (RICD). In some expanding T-cells a second TCR activation leads to pro-apoptotic signals, thereby controlling the extent of the response. XLP-patients that suffer fulminant mononucleosis typically lack this T-cell restricting phase of the response to EBV, which is also not observed in virus-infected *Sh2d1a*$^{-/-}$ mice. SAP expression was shown to correlate with the extent of RICD in several cell lines and a lack of cell cycle arrest was found in irradiated lymphocytes from XLP-patients [72]. The observation that SAP immuno-precipitates with the pro-apoptotic valosin-containing protein (VCP) alludes to a potential mechanism. A later study showed that Slamf6 recruitment of SAP and Lck rather than Fyn in these re-stimulated T-cells results in a pro-apoptotic signal, which was not observed in T-cells obtained from XLP-patients [73].

The expanded population of γHV-68 specific CD8+ CTLs in *Sh2d1a*$^{-/-}$ mice does reduce

the amount of infected B-cells [69,70]. However, cytotoxicity *per* cell appears to not be affected by SAP [69]. In contrast to these murine T-cells, $CD8^+$ T-cells from XLP-patients are selectively impaired in their cytotoxic response to B-cells [74]. These human CTLs showed similar cytokine production and proliferation when they are stimulated *in vitro* with anti-CD3 and anti-CD28 or anti-Slamf1 mAbs [75,76]. However, incubation with anti-Slamf4 mAb markedly reduces cytotoxicity of the EBV-specific $CD8^+$ CTLs and lowered IFNγ production [76]. Because this defect is associated with aberrant lipid rafts, perforin release, and SAP recruitment to the cytolytic synapse, it can be concluded that Slamf4-SAP pathway plays a critical role in the cytotoxic response of $CD8^+$ T-cells to EBV-infected autologous B-cells [75]. Indeed, whereas virtually all EBV-specific $CD8^+$ T-cells in SAP-proficient individuals are SAP+, other viruses induce a mixed pool of SAP+ and SAP– virus-specific CTLs [77]. The dependence of EBV-specific $CD8^+$ T-cells on the Slamf4-SAP pathway to target infected B-cells together with the narrow B-cells tropism of EBV may represent two of the underlining principles for the strong susceptibility of XLP-patients to this virus.

SAP and $CD4^+$ T-cell responses and germinal centers

Like XLP patients, γHV-68 infected *Sh2d1a*$^{-/-}$ mice had a strong reduction in the amount of germinal center B-cells [69]. These mice also displayed the typical hypogammagobulinemia [67,69]. Whereas SAP-deficient mice develop normal acute IgG responses upon infection with LCMV, they lack a humoral memory response [78]. When the (chronic-infectious) $LCMV_{c113}$ strain was used, germinal centers were grossly absent from *Sh2d1a*$^{-/-}$ mice [68]. Although more $CD8^+$ cytotoxic cells were present, this is not sufficient to clear the virus and the lack of an adequate humoral response in addition to the enhanced cytotoxic environment increased the pathogenicity of this virus [68]. Protection against secondary influenza infections is best established by $CD4^+$ T-cell mediated humoral responses through the generation of memory B-cells and long-lived plasma cells. Experimental exposure of *Sh2d1a*$^{-/-}$ mice to a second influenza challenge established the observation that these mice have a severely impaired IgG antibody response and therefore succumb to this infection [79]. Thus, in the late stages of infections with LCMV, γHV-68, and influenza virus profound defects in humoral immunity become apparent in *Sh2d1a*$^{-/-}$ mice.

SAP is critical for the development of germinal centers (GC), the anatomical site for B-T-cell cooperation. The observation that T-cell-independent humoral responses are unaffected by SAP-deficiency, showed that this phenotype depends on T-cell interactions with B-cells

[80]. Whereas a B-cell intrinsic SAP component in IgG antibody production was reported in some transfer experiments but not in others, SAP expression by helper T-cells is indispensible for early GC responses [81-84]. The contact time of T-B-cell interactions is reduced in SAP-deficient mice, which is the likely underlining mechanism of the impaired GC response [85]. Slamf5 and Slamf6 have a crucial role in this phenotype. Sustained adhesion of T-cells to B-cells is dependent on Slamf5 [86]. An additional study showed that Slamf6, in the absence of SAP, conveys a negative signal resulting in an insufficient contact-time between B-cells and T-cells [87]. This negative signal is mediated by Slamf6 as *Slamf6*$^{-/-}$ *Sh2d1a*$^{-/-}$ mice have normal developing germinal centers. The authors argue that SHP-1 recruitment to Slamf6 is the signaling event that is responsible for the impaired cognate B/T-cell interaction [87]. Although Slamf1 signaling contributes to GC IL-4 production [32], Slamf1 and Fyn are not involved in proper GC formation [88]. Slamf3-deficiency does not notably affect germinal center formation either [37].

NKT cell development depends on SAP, Slamf1, and Slamf6

NKT-cells are implicated in responses to a wide range of microbes and are reactive to lipid antigens. Positive selection of NKT cells is meditated by semi-invariant TCR interactions with lipid-antigens in the MHC-I like CD1d molecule from one double positive (DP) thymocyte to a neighboring DP cell. Thus, commitment of NKT cells, which takes place in the thymus, is dependent on CD1d stimulation from proximal lymphocytes instead of stromal cells. A secondary signal is required to induce differentiation and expansion. Either Slamf1 or Slamf6 homophilic ligation is required for this second signal that induces SAP recruitment to their ITSM [89]. SAP mediated signals are crucial for the development of NKT cells as *Sh2d1a*$^{-/-}$ mice completely lack these cells [90]. Upon SAP recruitment to either Slamf1 or Slamf6, Fyn binds to the Slamf-SAP complex to induce signals that facilitate the requirements for differentiation and expansion. An in-depth review of Slamf receptors in NKT-cells and other innate lymphocyte has recently been published [91].

Role for SAP, Slamf4 and other Slamf receptors in NK cells

The capacity of chronic infections with lymphotropic viruses to transform their host cells makes targeted killing of infected cells an important requirement in the immunity to such

viruses. Slamf4 is the major Slamf receptor to mediate cytotoxicity in both NK cells as well as $CD8^+$ CTLs. Initial studies have shown that Slamf4 interactions with Slamf2 on target cells induced perforin-mediated killing, which is dependent of SAP [35,92-95]. Slamf4 phosphorylation is dependent on its sub-location in lipid rafts [96]. Within these rafts, association with linker for activation of T-cells (LAT) is prerequisite of Slamf4 phosphorylation and hence Slamf4-mediated killing of target cells [97]. Slamf4 has four ITSM domains and the membrane proximal ITSM recruits SAP to the cytotoxic immune synapse upon phosphorylation [98]. This Slamf4-SAP complex inhibits the recruitment of inhibitory phosphatases and hence is required for a sustained interaction between the NK cell and the target cells [99]. However, Slamf4 can also mediate inhibitory signals in cytotoxic cells [100,101]. The levels of Slamf4 surface expression on NK cells as well as the abundance of SAP appear to critically dictate whether signals induce or inhibit targeted killing [95,102,103]. Naïve human NK cells do not express SAP and activation with IL-2 or IL-12 induces SAP expression. Only NK cells that express SAP had the potential to kill target cells by Slamf4 ligation [104]. A recent review describes the intricacies of the dual function of Slamf4 on cytotoxicity in more detail [103].

Several NK cell studies have implicated Slamf5, Slamf6, and Slamf7 in Eat-2-mediated modulation of cytotoxicity. Analysis of Eat-2 mediated signals revealed that Eat-2 induces calcium fluxes and ERK phosphorylation, which results in exocytosis of cytotoxic granules [105]. Whereas Slamf4 appears to be dominated by SAP, other Slamf receptors have a stronger dependence of Eat-2. Slamf6 ligation was shown to induce a cytotoxicity signal by recruiting Eat-2 to its second phosphorylated ITSM, which does not bind to SAP [106]. In addition, Eat-2-deficient mice were incapable of Slamf5 or Slamf6 mediated targeted killing of Slamf2+ tumors [107]. Thus, Slamf6 signaling through Eat-2 in addition to SAP enhances the cytotoxicity of NK cells. Slamf7 expression on target cells enhanced NK cell cytotoxicity, which was solely dependent on Eat-2, as *Eat-2*$^{-/-}$ NK cells conveyed a signal that inhibits cytotoxicity through Slamf7 [26].

Negative effects of Slamf4 during chronic Hepatitis infection

Lysis of non-MHC HCV-infected cells by activated $CD8^+$ T-cells is mediated by Slamf4 [108]. However, during chronic HCV-infections, Slamf4 predominates as an inhibitor of cytotoxic functions in $CD8^+$ T-cells [95]. In line with this notion, recombinant IFN-α therapy of HCV-infected patients induces NK cell mediated enhanced immunity but reduces Slamf4 expres-

sion of these cells [109]. Slamf4 expression by $CD8^+$ T-cells also correlated with poor clinical outcomes in HBV-infected patients [110]. Blockade of Slamf4 signaling effectively enhanced IFNγ production and virus-specific $CD8^+$ T-cell proliferation in approximately one-third of HCV+ patients [111]. Overall, Slamf4 expression correlates with the T-cell exhaustion that is typically observed during HCV infections. However, functionally exhausted T-cells are not universally revived by blockade of Slamf4 alone, but other CTL inhibitory receptors are involved [111]. Thus, these β-herpes virus infections cause the expression and function of specific Slamf receptors to be detrimental to the immune outcome.

Viruses utilize Slamf receptors for their benefit

Thus far, we have discussed how Slamf receptors perform functions by interactions with bacterial entities and by interaction with Slamf receptors. Slamf receptors are also actively targeted by pathogens that seek to use or to alter functions of Slamf receptors for their benefit. Three such modes of interaction have been postulated to date. First, Morbilliviruses (most prominently Measles virus) utilize Slamf1 as entry receptors. Second, certain cytomegaloviruses express molecules that closely resemble the structure of Slamf receptor, potentially representing (negative) competitors of endogenous Slamf receptors to modulate their functions. Third, several other viruses encode molecules that interfere with cell surface expression of Slamf receptors and inhibit their functions.

Slamf1 on the surface of myeloid cells binds to the Measles virus H protein and is involved in virus entry

The human pathogenic Measles virus belongs to the lymphotropic Morbillivirus genus. Measles virus and other Morbilliviruses utilize Slamf1 as one of two entry receptors [9,112]. Crystal structures of Slamf1 and Measles virus protein MV-H reveal four binding domains that are conserved between marmoset and human but not between mice and human, which explains the tropism of Measles virus [10]. Mechanistically, the interaction between Slamf1 and MV-H reduces the distance between the membranes of the target cell and the virus. The subsequent release of the viral protein MV-F enables fusion of the membranes and hence facilitates infection.

Measles virus has evolved a mechanism to induce Slamf1 surface expression, thereby gaining access to its entry receptor (Figure 6) [113,114]. Acidic Sphingomyelinase (AS-

Mase)-containing vesicles, which are also Slamf1+, play an interesting role in this process. ASMases convert sphingolipids into ceramide, creating a lipid environment that favors endocytosis or internalization of small membrane fractures. Thus, under non-infectious conditions, the recruitment of these vesicles to the surface of cells provides a membrane repair mechanism. Activation of the lectin receptor DC-SIGN by Measles virus induces a signaling cascade that involves Raf-1 and ERK [113]. This signal relies on the expression of ASM and results in the relocation of ASM+ vesicles to the surface of DCs [113]. Thus, by activating DC-SIGN, Measles virus induces surface expression of its entry receptor [114]. This observation thus provides evidence of a coupling between Slamf1 localization and membrane dynamics and shows that Slamf1 resides in intracellular membranes, suggesting that Slamf1 has distinct intracellular location with putative intracellular functions. These functions may represent events that are similar to the functions that were described for Slamf1 in *E. coli*+ phagosomes.

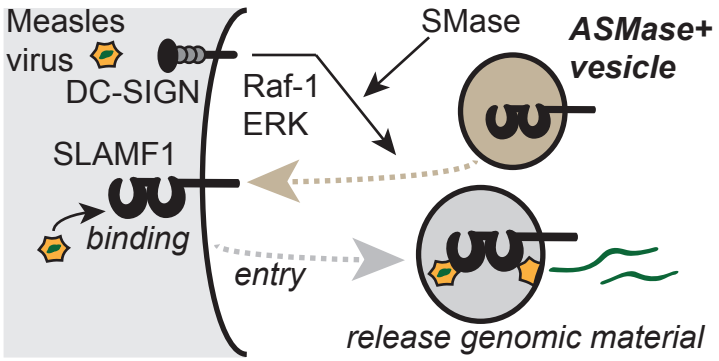


Figure 6 | Measles virus actively recruits its entry receptors Slamf1 to the cell surface. Binding of the lectin receptor DC-SIGN to a Measles virus particle induces a signaling cascade that involves Raf-1 and ERK and requires the activation of acidic SMase to induce a membrane trafficking event. Slamf1+ intracellular vesicles are recruited to the plasma membrane and fuse. This releases Slamf1 to the plasma membrane where Measles viral MV-H protein can bind to it to induce a fusion event between the viral membrane and the cellular membrane, consequently resulting in the delivery of the viral genomic material to the cytosol.

Viral expression of homologues of Slamf receptors

Remarkably, *Slamf3* has stronger sequence homology with the human cytomegalovirus (CMV) protein UL7 than with other human Slamf receptors [115]. Only one other CMV, which infects chimpanzees, bears a similar gene, suggesting that this gene was hijacked relatively late during the evolutionary arms race between mammals and β-herpes viruses. While no binding of UL7 to Slamf3 could be detected, this viral protein has been shown to be secreted from infected cells and to reduce the production of TNFα, IL-8, and IL-6 in DCs[115].

HIV-1 protein Vpu and CMV m154 modulate Slamf expression

Assessment of Slamf expression in HIV-1 infected cells showed a negative correlation between Slamf4 expression by NK cells and viral load, suggesting a positive role for Slamf4 in the killing of HIV-1 infected cells [116]. Indeed, NK cell treatment with specific antibodies for Slamf4 or Slamf6 decreased their *in vitro* killing potential of infected T-cells [117]. Surface expression of both of these Slamf receptors is actively down-modulated by HIV-1. $CD8^+$ CTLs of patients required both Slamf2-to-Slamf4 signaling and TCR stimulation for the down-modulation of Slamf4 surface expression [118]. HIV-1 infection also down-modulates the expression of Slamf2 and Slamf6 in infected $CD4^+$ T-cells suggesting active modulation of cytotoxicity by the virus. The HIV-1 protein Vpu associates with Slamf6 by interacting at the transmembrane regions. This interaction interferes with the glycosylation of Slamf6 and results in retention in the golgi-complex [119,120]. Slamf6 down-modulation leads to insufficient degranulation, and hence impaired targeted killing of HIV-1 infected cells [119].

A different viral protein that interferes with NK cell cytotoxicity is encoded by murine CMV. During infection, CMV m154 expression leads to proteolytic degradation of SLAMF2 resulting in a reduced capacity of NK cells to kill infected cells [121].

Concluding remarks

Slamf receptors and their adaptors are intricately involved in the responses to microbial challenges. Modulation of immune responses as a result of Slamf receptor homophilic interactions represents an important category of functions for these receptors. We can also observe an emerging theme that places Slamf receptors in a possibly underappreciated

category of functions; they can engage microbial ligands. SLAMF receptors are direct microbial sensors and are part of functional anti-microbial mechanisms. Thus, SLAMF receptors fulfill a unique role within the immune system, as they are both microbial sensors and cell-cell communicators of immunologic conditions. Additionally, we can distinguish a category of micro-encoded genes that directly interfere with Slamf functions. Interestingly, some of these genes have strong homology with endogenous Slamf receptors.

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Chapter 2

Migration of Myeloid Cells during Inflammation Is Differentially Regulated by the Cell Surface Receptors Slamf1 and Slamf8

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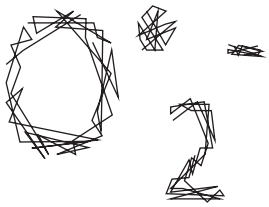
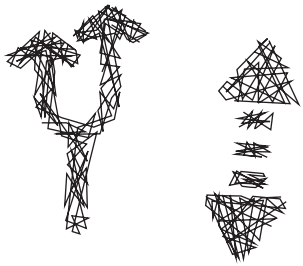


Abstract

Previous studies demonstrated that the receptor Slamf1 (CD150) enhances NADPH-oxidase dependent reactive oxygen species [ROS] production by phagocytes in response to Gram-negative bacteria. By contrast, Slamf8 (CD353) is a negative regulator of ROS in response to bacteria. This study explored the implications of this altered Nox2-mediated ROS production by *Slamf1*$^{-/-}$ and *Slamf8*$^{-/-}$ phagocytes beyond this bactericidal activity, as ROS can positively affect signaling processes that ultimately enhance cell migration. The results presented in this study show that migration of *Slamf1*$^{-/-}$ cells is reduced and migration of *Slamf8*$^{-/-}$ cells is enhanced in three distinct *in vivo* migration models, i.e. skin sensitization, peritonitis, and re-population of the small intestine. These opposing effects on myeloid cell migration appear to be cell-intrinsic and at least in part driven by the altered ROS production of *Slamf1*$^{-/-}$ and *Slamf8*$^{-/-}$ cells. Combined, these findings suggest that Slamf1 and Slamf8 dictate the magnitude of innate immune responses by modulating migration of several myeloid cell types, which process seems to be dependent on an altered production of ROS.



Introduction

Slamf1 (CD150, SLAM) and Slamf8 (CD353, BLAME) belong to the nine member Signaling Lymphocyte Activating Molecule (SLAM) family of hematopoietic cell surface receptors, which regulate a variety of immune responses, including T-cell activation, antibody generation, cytokine production, and natural killer T cell (NKT) development[1]. Slamf1, 3, 5, 6, and 7 are homophilic receptors and Slamf4 and Slamf2 are co-ligands[1-4]. The T cell co-stimulatory molecule Slamf1 signals in part through a specific association with the SLAM associated protein (SAP)[5]. In addition, in macrophages Slamf1 acts as a microbial sensor, which in response to *Escherichia coli* interacts with a Beclin-1>UVRAG>Vps34 complex. The Slamf1>Beclin-1>UVRAG>Vps34 complex converts phosphatidyl-inositol to phosphatidyl-inositol-3'phosphate (PI_3P), which activates the reactive oxygen producing enzyme complex Nox2 and promotes phagosome maturation[6,7]. As both of these bactericidal processes are involved in the killing of Gram$^-$ bacteria, removal of the attenuated *S. Typhumurium* Sseb$^-$ in *Slamf1*$^{-/-}$ mice is impaired[6]. Slamf1 and human SLAM are also expressed by neutrophils, monocytes, and activated dendritic cells (DCs)[8].

The cell surface glycoprotein Slamf8, as well as human Slamf8 (BLAME), is expressed by a variety of myeloid cells such as neutrophils, macrophages, monocytes and DCs upon encountering Gram$^-$ or Gram$^+$ bacteria, LPS or IFNγ[9,10]. In contrast to Slamf1, Slamf8 negatively regulates ROS production by inhibiting Nox2 activity and conversely Nox2 activity of *Slamf8*$^{-/-}$ macrophages is increased upon exposure to bacteria. One explanation could be that Slamf8 regulate protein kinase C activity, which is increased in *Slamf8*$^{-/-}$ macrophages causing an enhanced phosphorylation of $p40^{phox}$, in turn leading to greater Nox2 activity[10].

The classic paradigm that production of ROS by phagocytes is instrumental in their bactericidal activity is complemented by more recent studies that have established a role in the activation of many signaling pathways and the regulation of antigen cross-presentation[11,12]. Perhaps more importantly, ROS also affects cell adhesion and migration[13,14]. Recently two other groups independently reported that Nox2 regulates CSF-1 mediated macrophage and microglial chemotaxis[15,16]. In support of this notion are our observations that infiltration of *Slamf1*$^{-/-}$ macrophages and monocytes to the sites of inflammation is markedly reduced in two models of enterocolitis and in peritonitis[17].

The present study evaluates the respective roles of Slamf1 and Slamf8 in migration of dendritic cells (DCs), macrophages, and neutrophils by employing *in vivo* and *in vitro* as-

says. Not only does a correlation exist between the level of ROS production and altered migration in a cell-intrinsic fashion, the use of a Nox2 inhibitor prevents *in vitro* migration. As Slamf8 is a homophilic cell surface receptor, which is expressed on endothelial cells, we postulate that Slamf8 may control migration by adhesion to the lymphatic capillaries. Similarly, expression of Slamf8 by fibroblastic reticular cells (FRC)[18] suggests that this receptor may play a role in migration of DCs along FRC-containing conduits inside the lymph nodes.

Results

Slamf8 negatively regulates *in vivo* migration of DCs, whilst Slamf1 is a positive regulator

Innate immune cell trafficking is a critical process in triggering specific immune responses. For instance, inflammation of the skin induced by the hapten FITC leads to activation of skin dendritic cells (DCs) and their subsequent migration to the draining lymph nodes, which is primarily mediated by CC chemokine receptor 7 (CCR7) signaling[19]. A skin contact sensitization model was employed to assess whether migration of *Slamf1*$^{-/-}$, *Slamf8*$^{-/-}$ and wt DCs differed. To this end, the dorsal skin of mice was shaved and painted with 4mg FITC overnight and cells from the draining lymph nodes were isolated. Migratory DCs were quantitated by flow cytometry by gating for CD11c^{+}MHC^{hi} FITC-positive cells (Figure 1A)[20]. Compared to wt mice, the percentage and total number of FITC^{+} migratory DCs were significantly higher in *Slamf8*$^{-/-}$ mice (Figure 1B and 1C). As a difference in the number of dermal DCs may be responsible for the increased number of migrating DCs, the numbers of DCs in the skin of wt and *Slamf8*$^{-/-}$ mice were compared. The number of CD11c^{+} cells in the skin of the ear were comparable in wt and *Slamf8*$^{-/-}$ mice (Supplemental Figure 1A), showing that there is no numerical difference in the dermal DC population between wt and *Slamf8*$^{-/-}$ mice[21]. By contrast, fewer FITC^{+}-migratory DCs were detected in the draining lymph nodes of *Slamf1*$^{-/-}$ mice (Figure 1D), which was consistent with our previous finding that infiltration of *Slamf1*$^{-/-}$ monocyte-derived mononuclear phagocytes into the inflamed lamina propria of colitic mice is impaired[17]. As there was no obvious difference in expression of CCR7 on the surface of skin DCs before or after FITC painting of wt and *Slamf8*$^{-/-}$ mice (Supplemental Figure 1B and 1C), the altered DC migration is caused by a mechanism independent and/or downstream of CCR7 signaling — the major contributor

of DC migration to lymph nodes. Taken together, the outcomes of these experiments indicate that the cell surface receptors Slamf1 and Slamf8 control *in vivo* migration of DCs in an opposite manner.

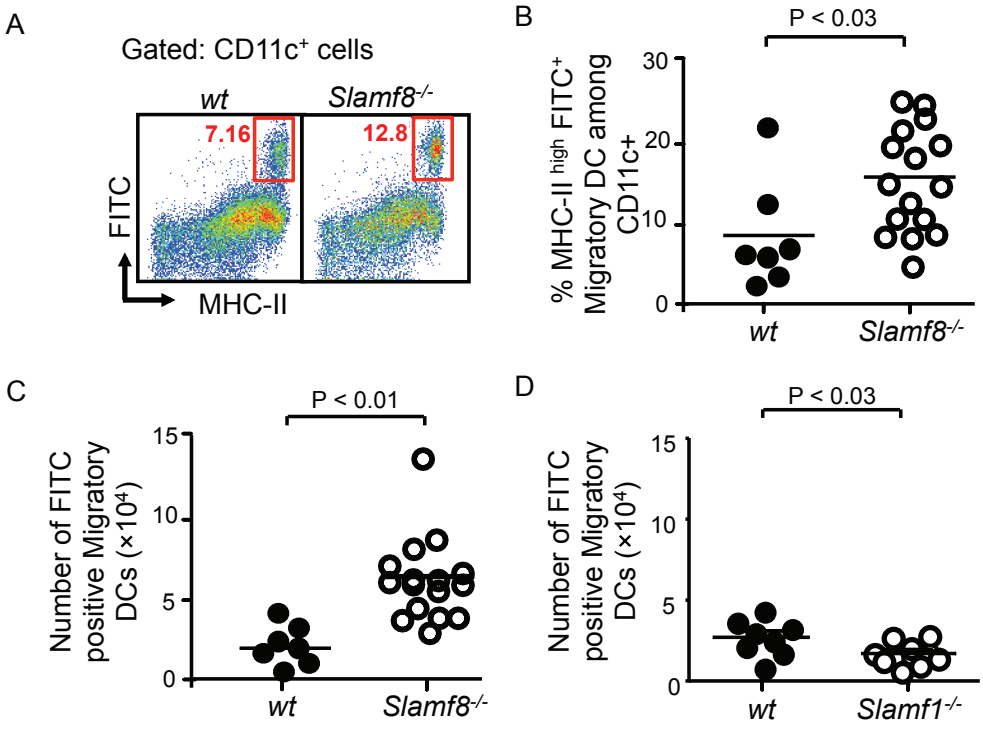


Figure 1 | Slamf8 negatively and Slamf1 positively affects DC migration from the skin to draining lymph nodes after Hapten-FITC sensitization. (**A**) Representative dot plots (gated on CD11c+ cells) and (**B**) percentages of migratory MHC-IIhi FITC+ DCs in the draining LNs of wt and *Slamf8*$^{-/-}$ mice, 24 h after painting dorsal skin with Hapten-FITC. Total number of migratory (CD11c^{+}MHC-IIhiFITC^{+}) DCs in the draining Lymph Nodes (LNs) of (**C**) *Slamf8*$^{-/-}$ mice or (**D**) *Slamf1*$^{-/-}$ mice, 24 h after FITC painting. Values represent mean. The data are representative of three or more independent experiments.

Slamf8 negatively regulates *in vivo* migration of macrophages to the inflamed peritoneal cavity

Intraperitoneal [i.p.] injection of thioglycollate broth induces peritonitis in mice resulting in infiltration of different inflammatory cells in a time-dependent fashion[22]. Whereas neutrophils are the major infiltrating cells in the peritoneal cavity after 4 hours, macrophages begin to

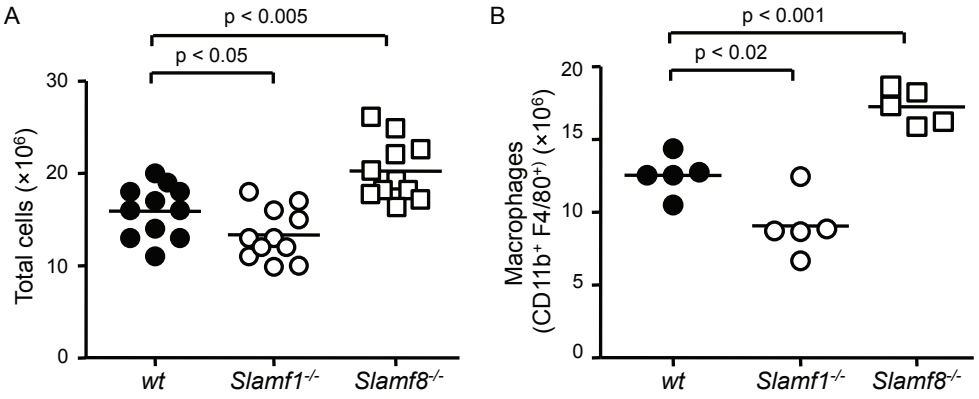


Figure 2 | Slamf8 negatively and Slamf1 positively affects macrophage migration upon peritonitis induction. (**A**) Total number of peritoneal cells from wt, *Slamf1*$^{-/-}$ and *Slamf8*$^{-/-}$ Balb/c mice four days after i.p. injection of 4% thioglycollate. (**B**) Number of macrophages (CD11b^{+} F4/80^{+}) among collected peritoneal cells. The data are representative of 3 independent experiments, each consisting of at least 3 mice per experimental condition.

appear after 24 hours and become the major constituents after 48 hours. As shown in Figure 2A, the total number of cells in the peritoneal cavity of *Slamf1*$^{-/-}$ Balb/c mice was lower than the number of cells in wt Balb/c mice four days after induction of peritonitis. However, the number of cells in the peritoneal cavity of *Slamf8*$^{-/-}$ Balb/c mice was increased as compared to wt mice (Figure 2A). Consistent with the latter observation, four days after the injecting thioglycollate the number of CD11b^{+} F4/80^{+} macrophages in the peritoneum of *Slamf8*$^{-/-}$ mice was higher than in the cavity of wt Balb/c mice (Figure 2B). The number of macrophages in the peritoneal cavity of *Slamf1*$^{-/-}$ Balb/c mice was lower than in wt Balb/c mice (Figure 2B), confirming our previous observation assessing macrophages in *Slamf1*$^{-/-}$ C57BL/6J mice[17]. We conclude that *Slamf8*$^{-/-}$ macrophages display a stronger migration response to the induction of peritonitis. This supports the concept that Slamf8 plays an inhibitory role in migration of both macrophages and dendritic cells.

Accelerated migration of macrophages and monocytes in the villi of the small intestine of *Slamf8*$^{-/-}$ mice

To confirm the notion of an accelerated *in vivo* migration of *Slamf8*$^{-/-}$ myeloid derived phagocytes we used a novel method by which repopulation of CX_3CR1^{+} phagocytes in the villi of the small intestine is evaluated. Administration of αCD3 mAb to C57BL/6J mice

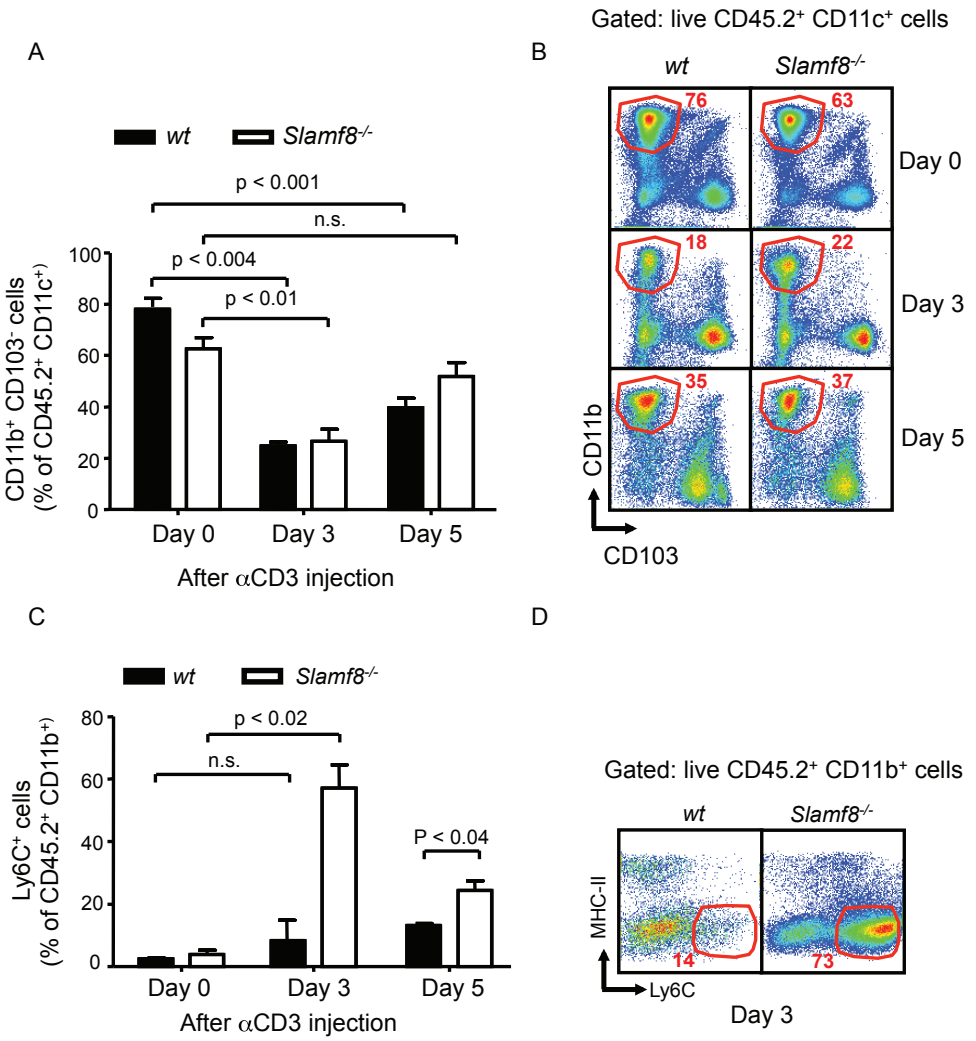


Figure 3 | Slamf8 deficient mice show expedited macrophage repopulation of small intestine lamina propria after anti-CD3 mediated depletion. Depletion of lamina propria macrophages 3 and 5 days after administration of αCD3. (**A**) The percentage of CD11b^{+} CD103^{-} of total CD45.2^{+} CD11c^{+} Ly6G^{-} lamina propria cells. (**B**) Representative dot plots (gated on CD45.2^{+} CD11c^{+} Ly6G^{-}) showing macrophages in lamina propria isolated cells from wt (left) and *Slamf8*$^{-/-}$ (right) naïve mice (day 0) or injected with αCD3 3 days prior (middle), or 5 days prior (bottom). (**C**) Percentages of monocytes (MHC-II$^{low/-}$ Ly6C^{+}) of total CD11b^{+}CD45.2^{+} that were isolated from the lamina propria on day 0, 3, or 5 after αCD3 administration. (D) Representative dot plots showing monocytes in the lamina propria of the small intestine of wt and *Slamf8*$^{-/-}$ mice on day 3. The data are representative of 2 independent experiments, each consisting of at least 3 mice per experimental condition.

activates cytotoxic CD8 T cells in the small intestine, which results in a transient depletion of CX_3CR1^+ phagocytes. Migratory monocytes then repopulate the villi of the small intestine[23]. . As shown in Figure 3A and 3B, three days after administering αCD3 to Balb/c mice a significant depletion of the number and percentage of $CD11b^+CD11c^+CD45.2^+CD103^-$ mononuclear phagocytes in the small intestine was observed. The majority of these cells are CX_3CR1^+ macrophages of monocytic origin that reside in the small intestine[24,25]. While *Slamf8*$^{-/-}$ mice appear to have a slightly reduced percentage of intestinal macrophages in homeostatic conditions, their repopulation is significantly faster than that of wt mice. Three and five days post αCD3 injection, the $CD11b^+CD11c^+CD45.2^+CD103^-$ cells begin to re-appear, more rapidly in *Slamf8*$^{-/-}$ than in Slamf8$^{+/+}$ mice. *Slamf8*$^{-/-}$ mice show no statistical difference in the percentage between day 0 and day 5, but Slamf8$^{+/+}$ mice have a lower percentage of these cells at day 5 (Figure 3A). Surprisingly, a steep increase in $CD11b^+Ly6C^+$ cells was observed in *Slamf8*$^{-/-}$ mice three days after the last αCD3 injection compared to wt mice (Figure 3C and 3D). This population, which was reduced in size at 5 days post injection, is known to represent newly arrived monocytes in the small intestine, which quickly transition to mature macrophages[26]. The results indicate that *Slamf8*$^{-/-}$ mononuclear phagocyte precursors infiltrate the lamina propria at a faster rate than their wt counterparts supporting the notion that Slamf8 negatively regulates the migratory capacity of myeloid cells to immunologically active sites.

Slamf8 negatively regulates Nox2 activity and *in vivo* migration of neutrophils

Neutrophils, which migrate from the blood to a site of inflammation, represent the first line of defense against bacteria. Because we found a very high Nox2 activity in *Slamf8*$^{-/-}$ macrophages and DCs (Supplemental Figure 2)[8], we tested the ROS production by *Slamf8*$^{-/-}$ neutrophils. As judged by a lucigenin-based assay, *Slamf8*$^{-/-}$ neutrophils produce indeed significantly more ROS than wt Balb/c neutrophils in response in response to *E. coli* or to PMA (Figure 4A).

We next evaluated the role of Slamf8 in neutrophil migration into the peritoneal cavity of wt and *Slamf8*$^{-/-}$ mice 4 hours after i.p. injection of thioglycollate. Consistent with the macrophage results, obtained 4 days after administering thioglycollate (Figure 2), the total number of cells obtained from the peritoneal cavity of *Slamf8*$^{-/-}$ mice was greater than the number of cells obtained from wt mice (Figure 4B). Importantly, the number of $CD11b^+Ly6G^+$

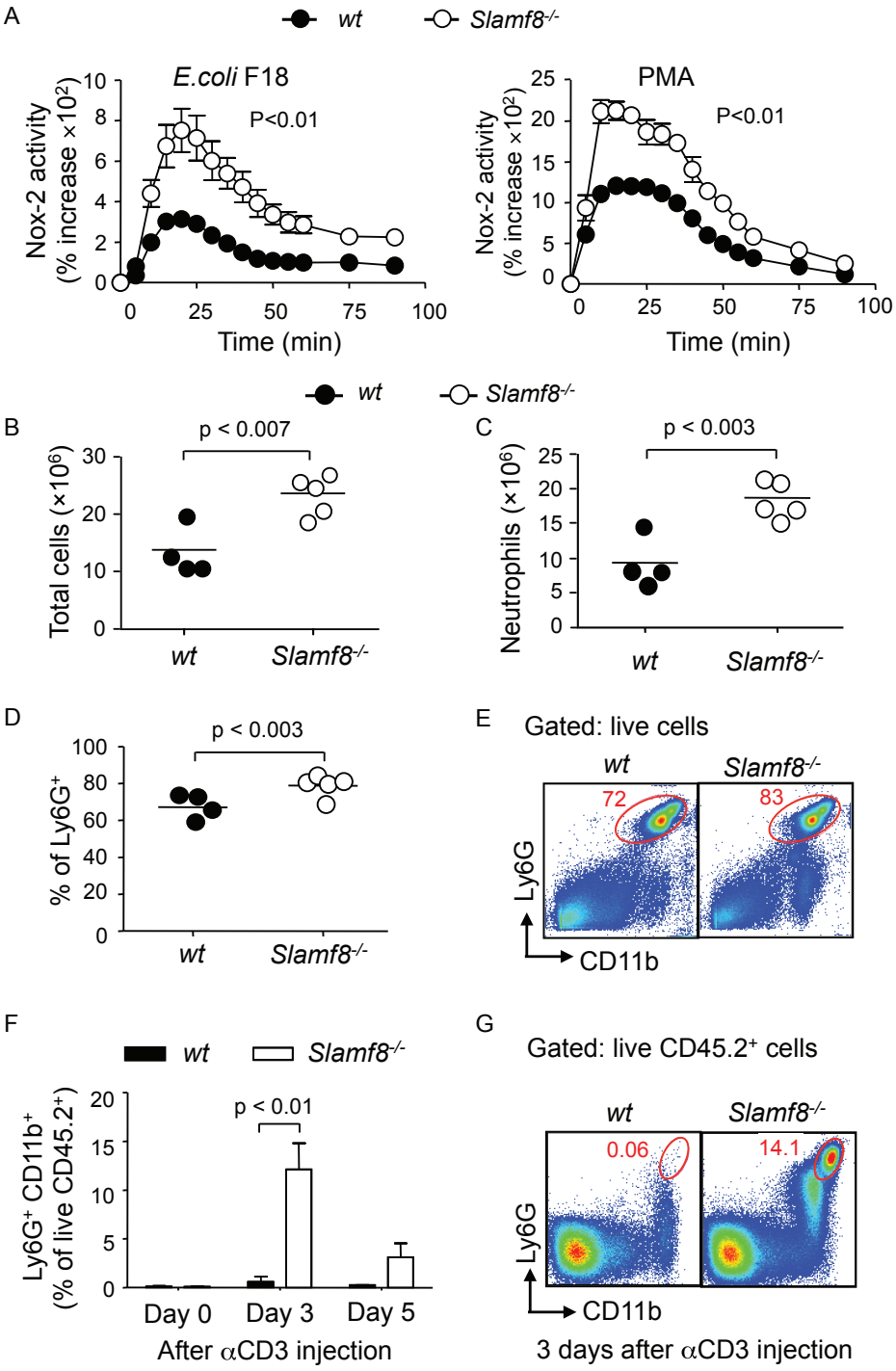

A
wt
Slamf8-/-
E.coli F18
P<0.01
Nox-2 activity (% increase ×10²)
Time (min)
PMA
P<0.01
Nox-2 activity (% increase ×10²)
Time (min)
B
wt
Slamf8-/-
p < 0.007
Total cells (×10⁶)
C
p < 0.003
Neutrophils (×10⁶)
D
p < 0.003
% of Ly6G+
E
Gated: live cells
wt
Slamf8-/-
72
83
Ly6G
CD11b
F
wt
Slamf8-/-
p < 0.01
Ly6G+ CD11b+ (% of live CD45.2+)
Day 0
Day 3
Day 5
After αCD3 injection
G
Gated: live CD45.2+ cells
wt
Slamf8-/-
0.06
14.1
Ly6G
CD11b
3 days after αCD3 injection

Figure 4 | *Slamf8*$^{-/-}$ neutrophils produce higher ROS, and Slamf8 negatively regulates neutrophil migration. (**A**) ROS production as measured by luminescence in wt and *Slamf8*$^{-/-}$ mice thio-neutrophils in response to heat inactivated E.coli F18 and PMA. (**B**) Total number of peritoneal cells obtained from wt and *Slamf8*$^{-/-}$ mice after 4 hours i.p. injection of 4% thioglycollate. (**C**) Number and (**D**) percentage of neutrophil (CD11b^{+} Ly6G^{+}) among peritoneal cells in wt and *Slamf8*$^{-/-}$ mice. (**E**) Representative dot plots (gated on DAPI^{-} cells) showing CD11b^{+} Ly6G^{+} neutrophil in the peritoneal cavity of wt and *Slamf8*$^{-/-}$ mice. (**F**) *Slamf8*$^{-/-}$ and wt mice were injected with αCD3. Percentage of neutrophils (CD11b^{+} Ly6G^{high}) from the small intestine lamina propria 0, 3 and 5 days after αCD3 injection. (G) Representative dot plots (gated on CD45.2^{+}) showing neutrophils. The data are representative of 2 independent experiments, each consisting of at least 3 mice per experimental condition.

peritoneal neutrophils in *Slamf8*$^{-/-}$ mice was 2-fold higher that of wt mice (Figure 4C-4E).

We also evaluated migration of neutrophils in the villi of the small intestine on day 3 and 5 after administering αCD3 to *Slamf8*$^{-/-}$ and wt mice (Figure 4F-4G). Whilst the percentage of neutrophils in the small intestine comprised ~14% of the CD45.2^{+} cells in *Slamf8*$^{-/-}$ mice on day 3 post injection, the percentage of neutrophils was ~5% on day 5. By contrast, on day 3 after administering αCD3 neutrophils comprised ~1% of the CD45.2^{+} cells in the lamina propria of wt mice, whereas no neutrophils were detected on day 5. In conclusion, Slamf8 negatively affects migration of neutrophils in two *in vivo* models consistent with the altered migration of DCs and monocyte-derived macrophages in *Slamf8*$^{-/-}$ mice.

Altered *in vitro* migration of Slamf8- and Slamf1-deficient DCs

To test the hypothesis that cell intrinsic functions of Slamf1 and Slamf8 give rise to an altered migratory capacity, chemotaxis was examined in a transwell migration assay were. We focused on CCR7 and its two major ligands, CCL21 and CCL19, which play an important role in facilitating migration of DCs in the lymphatic system and within lymph nodes[27]. DCs that were obtained by CD11c positive selection from the skin of the ear were allowed to migrate through 5μm pores in a transwell system toward CCL21 [0–400nM]. *Slamf8*$^{-/-}$ migrated much more efficiently toward CCL21 than wt Balb/c DCs (Figure 5A). *In vitro* trafficking of skin-derived *Slamf8*$^{-/-}$ DCs in response to CCL19 was similarly increased (Supplemental Figure 3). In contrast to *Slamf8*$^{-/-}$ cells, migration of *Slamf1*$^{-/-}$ skin-derived DCs toward CCL21 was markedly reduced (Figure 5B). These differences in *in vitro* migration indicate that this process is DC-intrinsic. As the surface expression of CCR7 is similar between *Slamf8*$^{-/-}$ and wt DCs the differences between wt and deficient DCs could not

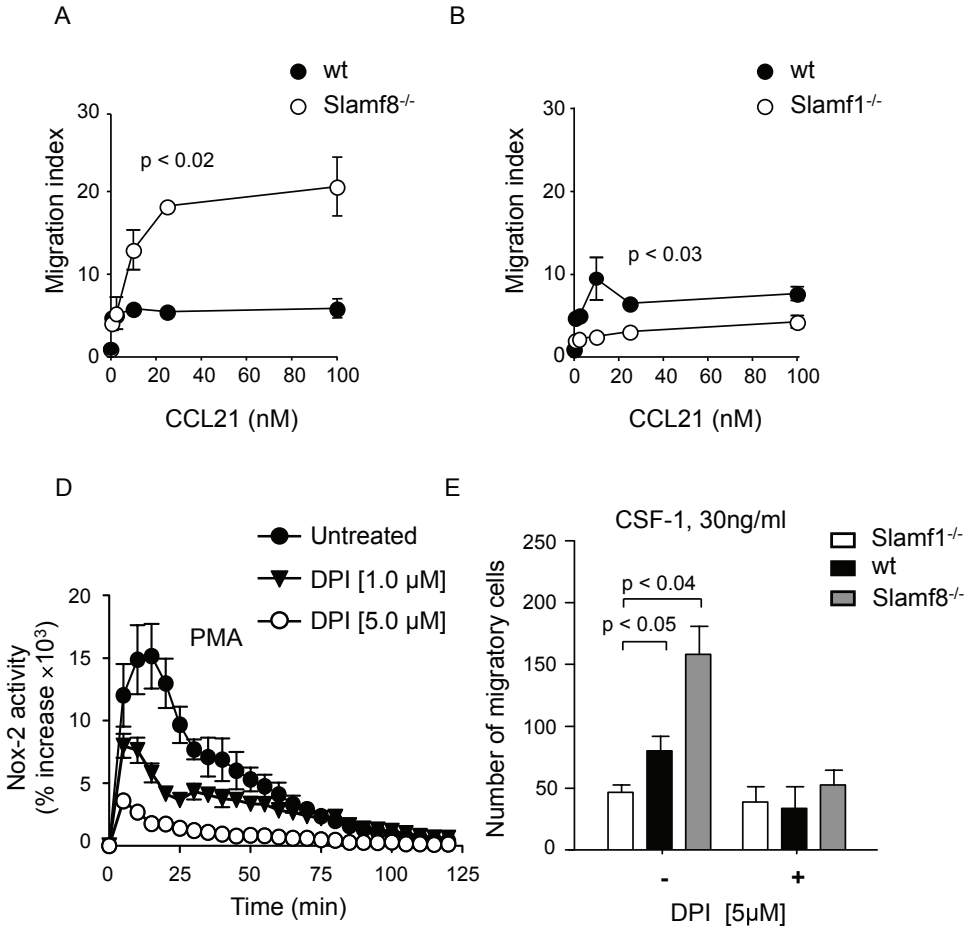


Figure 5 | Slamf8 negatively regulates *in vitro* migration of DCs and of macrophages, while Slamf1 is a positive regulator of the same process. Wt, *Slamf1*$^{-/-}$, and *Slamf8*$^{-/-}$ skin DCs were isolated and column-purified before they were allowed to migrate toward a concentration range of CCL21 [0–400nM]. The migration index of (**A**) *Slamf8*$^{-/-}$ vs. wt DCs and (**B**) wt vs. *Slamf1*$^{-/-}$ DCs are plotted. (**C**) Macrophages from wt mice were incubated with different concentrations of DPI (1µM and 5µM) for 15 minutes, and the Nox2 activity in macrophages was quantified upon PMA (1mg/mL) stimulation. The data are representative of two independent experiments. (**D**) Wt, *Slamf1*$^{-/-}$, and *Slamf8*$^{-/-}$ thio-macrophages migration in the presence of CSF-1, with and without pre- incubation with DPI. All the data are representative of three independent experiments.

be due to altered expression of CCR7 (S1B and S1C Fig.).

A role for Nox2-dependent production of ROS in migration of bone marrow derived macrophages in response to the chemo-attractant CSF-1 was suggested by experiments in gp91$^{-/-}$ mice, which lack an active Nox2 complex[15,28]. To test the hypothesis that cell intrinsic functions of Slamf1 and Slamf8 affect cell migration in response to CSF-1 in a ROS-mediated fashion an *in vitro* transwell system was used in the absence and presence of the Nox2 inhibitor diphenyleneiodonium chloride (DPI), which effectively inhibits human neutrophil migration[13]. First, we determined that 5μM of DPI inhibited PMA induced Nox2 activity of peritoneal macrophages without affecting cell viability (Figure 5C and Supplemental Figure 4). In response to CSF-1, *in vitro* migration of *Slamf1*$^{-/-}$ peritoneal macrophages was impaired, while an enhanced migration of *Slamf8*$^{-/-}$ macrophages was observed (Figure 5D). This observation is dependent on ROS as incubation with DPI for 15 minutes before the cells were allowed to migrate toward CSF-1 dramatically reduced the number of migrating cells. Thus, cell intrinsic migration towards CSF-1 is altered in *Slamf1*$^{-/-}$ and *Slamf8*$^{-/-}$ macrophages. Additionally, this cannot be due to altered expression of the CSF-1 receptor CSF-1R (CD115), as shown in Supplemental Figure 5. Taken together, these data suggest the signaling of two key cell surface receptors CCR7 and CSF-1R is altered in the Slamf1- and Slamf8-deficient cells in a ROS-dependent fashion.

The self-ligand Slamf8 affects the *in vivo* migration of DCs

The notion that majority of human and mouse Slamf adhesion molecules are homophilic prompted us to evaluate whether Slamf8 would be a self-ligand receptor. To this end, a soluble fusion protein comprised of the mouse Slamf8 ectodomain and the human IgG1 Fc domain (Slamf8-Fc) was generated. Next, 293 cells transiently transfected with Slamf8 or Slamf1 were incubated with the Slamf8-Fc protein and stained with a fluorescent antibody directed against the Fc portion of human IgG[29]. As predicted, Slamf8-Fc associated with Slamf8 transfected 293 cells, but not with Slamf1 transfectants. In the reciprocal experiment Slamf1-Fc associated with Slamf1 transfected cells but did not bind to Slamf8 transfected 293 cells (Figure 6A-6C). Thus, Slamf8 is a homophilic receptor, which does not ligate in trans with Slamf1.

Next, the effect of Slamf8-Fc in the skin contact sensitization model was evaluated. To this

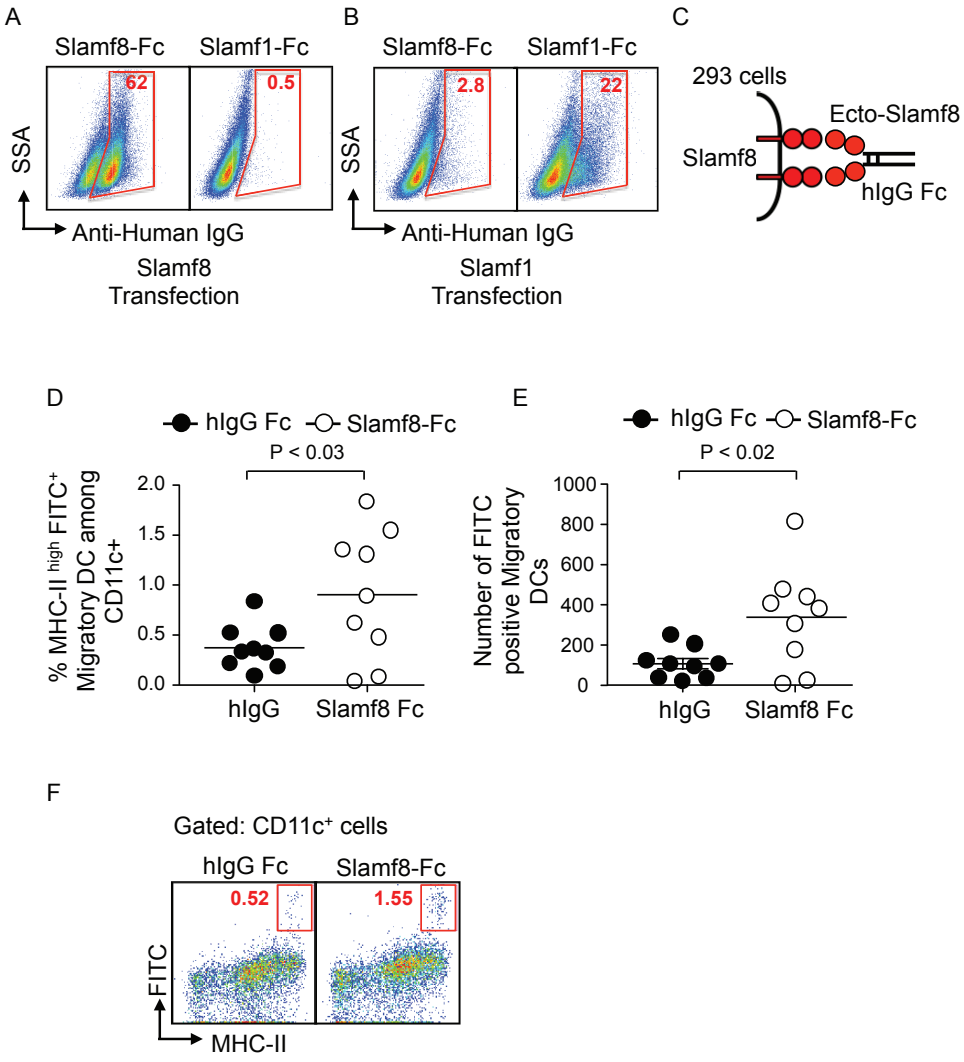


Figure 6 | Slamf8 is a homophilic receptor, and Slamf8-Fc enhances DCs migration in vivo. Slamf8 receptor interacts in a homophilic manner. HEK 293 cells were transfected with Slamf8 (**A**) or Slamf1 (**B**), and the binding of Slamf1-Fc and Slamf8-Fc to these transfected cells was determined by flow cytometry. (**C**) Scheme for the Slamf8-Fc binding assay. The data are representative of three independent experiments. Slamf8-Fc enhances DCs migration in vivo. Wt Balb/c mice were i.p. injected with Slamf8-Fc or human IgG1. Three hours later, FITC painting assay was performed. (**D**) Percentages among CD11c^{+} cells (**E**) total number of migratory (MHC-II^{hi} FITC^{+}) DCs in the draining LNs. (**F**) Representative dot plots (gated on CD11c^{+} cells) showing migratory MHC-II^{hi} FITC^{+} DCs in the draining lymph nodes. The data are representative of two independent experiments.

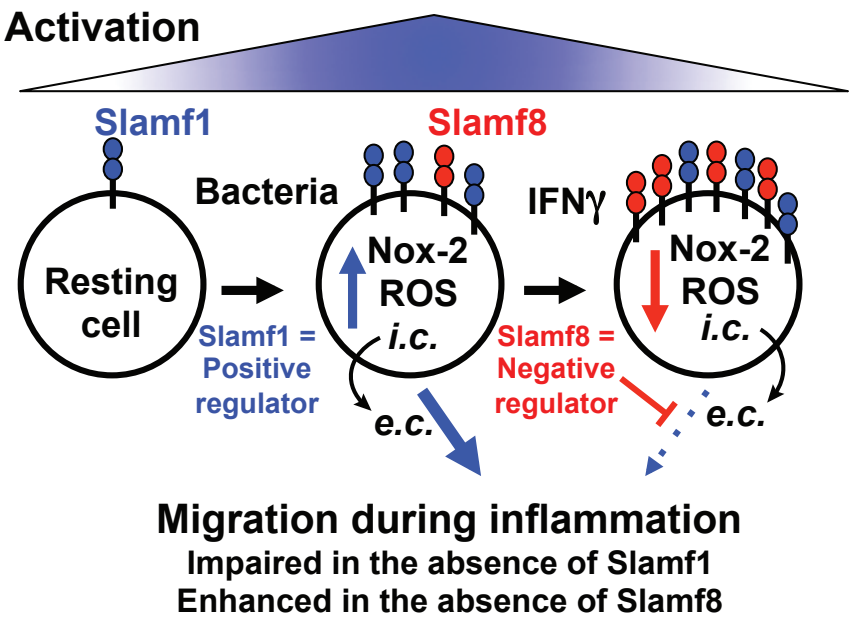
end wt mice were injected with the Slamf8-Fc fusion protein 3 hours prior to painting 2mg FITC onto the shaved skin. Mice that had received the Slamf8-Fc fusion protein both the percentage and the number of migratory DCs were significantly elevated compared to the mice injected with human IgG1 (Figure 6D-6F). This suggests that Slamf8-Fc interrupted the homophilic Slamf8 interactions that suppress DC migration (Figure 1).

Discussion

Three distinct *in vivo* models, i.e. skin sensitization by painting with the hapten FITC, thioglycollate induced peritonitis and migration in the lamina propria of the small intestine, were employed to demonstrate that migration of Slamf8-deficient DCs, macrophages and neutrophils is increased. This indicates that Slamf8 negatively regulates this process in wt mice. *In vitro* transwell experiments showed an enhanced migration of *Slamf8*$^{-/-}$ macrophages in response to CSF-1 and dendritic cells in response to the chemokines CCL19 and CCL21, which bind to the CCR7 receptor[27]. The increased migration of *Slamf8*$^{-/-}$ macrophages and DCs is cell intrinsic and macrophage migration is inhibited by the Nox2 inhibitor DPI. We conclude that Nox2-dependent ROS production, which raises intracellular and extracellular levels of H_2O_2 causes accelerated migration of *Slamf8*$^{-/-}$ phagocytes. To our knowledge, Slamf8-deficient mice are one of two mutant mice with an inducible increased ROS production, the other being the recently discovered Negative Regulator of ROS (NRROS)[30], which was studied in the context of autoimmune disease. In wt cells Slamf8 is therefore a negative regulator of signal transduction events in myeloid cells, which affect their migration by interacting with its self-ligand on endothelial cells or fibroblastic reticular cells. By contrast, Slamf1 appears to enhance migration of phagocytes by stimulating Nox2-dependent events. Hence, myeloid cell migration is consistently impaired in *Slamf1*$^{-/-}$ mice, which generate low levels of ROS in response to several bacteria, LPS and other inflammation related biologicals[6,17].

The classical phagocytic NADPH oxidase (Nox2) produces reactive oxygen upon assembly of the membrane bound catalytic gp91^{phox} and p22 ^{phox} with at least four cytosolic subunits p40^{phox}, p47^{phox}, p67^{phox}, Rac1/2[11]. Nox2 complex assembly is a tightly regulated process that involves phosphorylation of its cytosolic subunits or binding of p40^{phox} to the lipid phosphatidyl-3'-phosphate (PI_3P), which is phosphorylated by the Class III phosphatidyl-inositol kinase Vps34[31]. The latter enzyme can be recruited by the intracellular tail of

Figure 7 | Proposed model showing Slamf1 and Slamf8 are counterparts for the regulation of Nox2 generated ROS and cell migration. In migratory phagocytes, the early actor Slamf1 enhances ROS-mediated migration signals; subsequently Slamf8 reduces ROS-mediated migration signals. Inflammatory signals enhance the activity of Slamf1, which induces Nox-2 mediated ROS production. Inflammatory mediators, such as IFN-γ subsequently increase Slamf8 expression and function. This leads to the suppression of ROS production resulting in the reduction in infiltration of phagocytes. Thus, Slamf1 and Slamf8 together, balance the extent of infiltration of inflammatory cells. (i.c.; intracellular, e.c.; extracellular)



Slamf1 and in the absence of Slamf1 macrophages and dendritic cells produce reduced amounts of ROS and consequently H_2O_2[6,7]. ROS in phagocytes, which plays a role in killing of some bacteria, is also involved in other aspects of innate immune responses, such as NF-κB signaling through TRAF6 recruitment[32]. Perhaps the best-known signaling effects of ROS are in oxidation of specific Cys residues of the protein tyrosine phosphatases SHP-1 and SHP-2, which impair their enzymatic function thus allowing for enhanced phosphorylation by tyrosine kinases[33,34]. Another example of these phosphatases is the lipid phosphatase PTEN, which impact directional mobility[35]. Effects on cytoskeletal functions is exemplified by β-actin Cys374 that can be S-glutathionylated by ROS thereby promoting cell spreading, which is a necessary step in adequate migration[36]. DC migration is affected through the function of oxidative stress response kinase 1 (OSR-1)[37]. Direct

evidence of the involvement of Nox2 in cell migration and chemotaxis comes from studies that used mice with genetic ablations of Nox2 components. For instance, Nox2 knock out (gp91$^{phox-/-}$) bone marrow macrophages completely lost their chemotactic response toward a CSF-1 gradient, which is likely due to a reduction of ERK1/2 phosphorylation leading to defected cell migration[15] In the murine macrophage cell line RAW264.7, Nox2 is required for LPS induced matrix metalloproteinase MMP-9 expression, which eventually influences cell migration[38]. In sum, ROS signaling has implications on a variety processes including migration, adhesion , and chemotaxis.

Using a small-molecule screen for drugs capable of inhibiting neutrophil chemotaxis, Hattori and colleagues found that the Nox2 inhibitor diphenyleneiodonium chloride (DPI), can effectively inhibit human neutrophil directionality during migration. Furthermore, they found that both gp91$^{-/-}$ murine neutrophil and neutrophil from Chronic granulomatous disease (CGD) patients lost their directional migration, establishing the notion that Nox2 activity influences cell migration[13].

Our previous study shows that expression of murine Slamf8 is not only significantly induced in macrophages by bacteria and bacterial components, e.g. *E. coli* and LPS, but more dramatically by IFN-γ[10]. As shown in Supplemental Figure 6A–D, IFN-γ also induces Slamf8 transcripts in bone marrow derived DCs, peritoneal neutrophils, human monocytes and macrophages and protein expression in peritoneal macrophages. Perhaps more importantly, Slamf8 expression can be induced by TNF-α treatment of mouse heart endothelial cells (Supplemental Figure 6E), consistent with the notion that the homophilic interaction might play a role in cell migration. That concept is also supported by the observation that Slamf8 is expressed in skin and mesenteric lymph nodes by fibroblastic reticular cells (FRC)[18,39], which function as a scaffold for the support and migration of hematopoietic cells[40].

Based upon this study and published work employing Slamf1 as well as Slamf8 deficient mice and mutant macrophage cell lines[5-7,10,17], we propose a model in which Slamf1 and Slamf8 help shape innate immune responses (Figure 7). This includes differential expression and induction of Nox2 activity in response to innate stimuli[6,10]. Furthermore, the model graphically represents how migration of various myeloid cells is dependent upon the two receptors. Initial inflammatory queues may trigger Slamf1-meditated Nox2 activation resulting in a spike in ROS, which is rapidly converted into hydrogen peroxide that contributes

to the migratory activity of Slamf1-expressing cells. Subsequent inflammatory mediators (IFN-γ) induce Slamf8 expression, which suppresses the production of ROS and hence reduces additional migratory infiltration.

Taken together, the outcomes of our experiments provide the first evidence that Slamf8 is a novel homophilic receptor, and that Slamf1 and Slamf8 oppositely regulate macrophage migration in a Nox2 dependent manner. The similarity of the *in vivo* migration and *in vitro* ROS production phenotypes suggest that Nox2 has a similar involvement in the migration of DCs and neutrophils as macrophages. These findings point out that Slamf1 and Slamf8 are potential therapeutic targets to modulate DC, macrophage and neutrophils migration and function, thus therapeutic strategies based on Slamf1 and Slamf8 can be pursued to regulate cellular immune response. As *Slamf8*$^{-/-}$ mice show an enhanced migration of antigen presenting cells and Slamf8-Fc approximates the *Slamf8*$^{-/-}$ phenotype, Slamf8 may be of interest for potential therapeutic applications in vaccine development.

Experimental procedures

Mice

Slamf1$^{-/-}$ and *Slamf8*$^{-/-}$ BALB/c mice were described previously[5,10]. Age and sex matched wt BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All animals were maintained under specific pathogen-free conditions at the Center for Life Science animal facility of Beth Israel Deaconess Medical Center (BIDMC) and were used at 8–12 weeks of age. The experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at BIDMC.

In vivo assay for migration of skin DC-FITC painting assay

Experiments were performed as described[41]. In brief, the dorsal skin of individual mice that were anesthetized with isoflurane was shaved, followed by application of 400μL of 10mg/mL FITC dissolved in 1:1 acetone/dibutylphthalate (Sigma-Aldrich). After 24 hours, mouse inguinal and axillary lymph nodes were isolated by digestion at 37°C for 1h with a cocktail of 100U/ml DNase I (fraction IX; Sigma-Aldrich) and 1.6 mg/mL collagenase (CLS4; Worthington Biochemical). In the experiment using Slamf8-Fc fusion, 100μg of

Slamf8-Fc fusion protein or human IgG-Fc (Jackson ImmunoResearch Laboratories) was intraperitoneally injected 3 hours before 200µL FITC application. Single-cell suspensions were stained and analyzed by flow cytometry.

In vivo Thioglycollate broth-induced peritonitis

Wt, *Slamf1*$^{-/-}$ and *Slamf8*$^{-/-}$ mice were intraperitoneally injected with 2mL of sterile 4% thioglycollate broth. Four hours or four days later, mice were euthanized. The cells from the peritoneal cavity were washed out with 10mL of RPMI medium and stained for FACS.

In vivo Anti-CD3 induced myeloid cell migration model

20µg of purified αCD3ε (145–2C11) (Biolegend, San Diego, CA) or Mouse IgG2b (Biolegend, San Diego, CA) were intraperitoneally injected. Three or five days later the mice were euthanized. Lamina propria cells were isolated and analyzed by flow cytometry[42].

Nox2 lucigenin assays

Nox2 assays using lucigenin (Sigma-Aldrich, St. Louis, MO) were done using a standard Glo- max luminometer (Promega, Madison, WI) after exposure to heat inactivated E.coli F18 bacteria (multiplicity of infection [MOI] 100) or phorbol myristate acetate (PMA) (1µg/ml)[6].

Intracellular ROS detection

Intracellular ROS was quantified by using CM-H_2DCFDA (Life Technologies, C-6827) based on the manufacturer's protocol. CM-H_2DCFDA was added to RPMI 1640 medium at a final concentration of 10µM. Thioglycollate-elicited macrophages were incubated with CM-H_2DCFDA at 37°C for 1 hour. After incubation, the macrophages were incubated with heat inactivated *E. coli* F18 bacteria (multiplicity of infection [MOI] 100). Fluorescence was measured every 5 minutes by flow cytometry for 2 hours.

In vitro Chemotaxis assay

Ear skin was collected and digested for 2 hours with DNase (15µl from 10 mg/ml stock, Sigma) and 500µL Liberase (Roche) in RPMI 1640 at 37°C in a shaking incubator. After filtration through a 70µm cell strainer, single cell suspensions were obtained. CD11c$^+$ cells

were further isolated using CD11c MicroBeads (Miltenyi Biotec) in a positive selection column. Chemotaxis of skin DCs was measured using a polycarbonate filter with 5μm pores in 96-well transwell chambers (Neuro Probe, Gaithersburg MD). 30μL chemotaxis media was added to the lower chamber, and 2.5×10^4 DCs were added to the upper chamber and incubated for 8 hour at 37°C with 5% CO_2.

In vitro transwell (5μm pore) migration analysis for macrophages was performed using a 96-well migration chamber (NeuroProbe, Gaithersburg MD). The lower wells contained recombinant murine CSF-1 (30ng/mL), and 5.0×10^4 Thio-macrophages were added to the upper well to migrate for 150 minutes in a humidified chamber (5% CO_2, 37°C). In some experiments diphenyleneiodonium chloride (DPI) (5μM) was added to cell suspension 15 min- utes prior to migration. Migrating cells were counted according to the manufacturer's protocol by flow cytometry, using counting beads.

Generation of mouse Slamf8-Fc and Slamf1-Fc recombinant soluble protein

The murine Slamf8-Fc and Slamf1-Fc fusion protein containing the Fc region of human IgG1 was obtained by inserting the sequence corresponding to the extracellular domain (including the signal peptides and two extracellular domains) of Slamf8 or Slamf1 into the mammalian expression vector pcDNA4/myc-HisC (Invitrogen). The construct expressing Slamf8-Fc was transfected in NS-1 myeloma cells and the construct expressing Slamf1-Fc was transfected in 293F cells. Stable Slamf8-Fc or Slamf1-Fc expressing cells were selected with zeocin and ex- panded for large-scale culture. The supernatants containing the fusion proteins were purified with a protein G agarose bead column from the supernatant.

Slamf8-Fc fusion protein binding assay

Mouse Slamf8 cDNA were cloned into pcDNA4.0 Myc/his vector (Life Technology, Carlsbad, CA, USA). HEK293 cells were transfected with mouse Slamf1 or Slamf8 cDNAs using FuGENE6 Transfection Reagent (Roche). 24 hours after transfection, 10μg of Fc fusion proteins Slamf1-Fc or Slamf8-Fc were incubated with 1×10^6 transfected HEK293 cells on ice for 60 minutes, followed by incubation with biotin-conjugated goat anti-human IgG Fc (Life Technologies) and then streptavidin-PE. Binding was assessed by flow cytometry.

Flow Cytometry

Macrophages, DCs and neutrophils were incubated with Fc-blocker at 4°C for 20 minutes. Samples were stained with; MHC class II (Biolegend), CD11c (BD Bioscience), CD11b (BD Bioscience), F4/80 (eBioscience), Ly6G (Biolegend), CD115 (Biolegend) and CCR7 (Biolegend), CD103 (Biolegend), Ly6C (eBioscience), CD45.2 (Biolegend) on ice for 30 min. Dead cells were visually excluded by DAPI (Roche). The cells were acquired on a BD LSRII flow cytometer and the data analysis was performed by FlowJo software (Trees Star Inc. Ashland, OR).

Preparation of Mouse Heart Endothelial Cells

Mouse heart endothelial cells (MHEC) were prepared from the heart of newborn mice (7–9 days old) as described[43]. 100ng/ml of TNFα was used to stimulate endothelial cells for 24 hours, and the cell RNA was isolated.

RNA isolation and real-time PCR (qPCR)

Bone marrow derived DCs and peritoneal Thio-neutrophils total RNA was isolated using TRIzol (Invitrogen). qPCR was performed and analyzed on the 7500 FAST Real-Time PCR System (Applied Biosystems). Slamf8 and the Eukaryotic 18S ribosomal RNA Endogenous Control TaqMan probes were purchased from Life Technologies (Carlsbad, CA, USA). Slamf8: For- ward: 5'-CCTGGCTGGTCTCTTTGGG-3'; Reverse 5'-CGTCAGTGCAAG-CATCCTTC-3'; Probe: 5'-CACCATGGCCTCTGCTCAGGGAAG-3'. Human Slamf8: For-ward: 5'- GCTTCCAAGTCCGTGAGGC-3'; Reverse 5'-AAAACGTGGCCAGGAGCTC-3'. The relative gene-specific fold change, normalized to 18S rRNA, was calculated using the 2–ΔΔct method and expressed relative to untreated wt levels.

Western Blot

Thio-Macrophages were stimulated with IFN-γ (10ng/ml) plus LPS (100ng/ml) overnight. Slamf8 protein expression was detected with Sheep anti-mouse Slamf8 Polyclonal anti-body (R&D System, AF4156) by Western Blot as described [7].

Statistical analysis

The Prism 5.0 software (GraphPad, San Diego, CA, USA) was used to analyze the data.

Results are reported as mean ± SEM. All the statistical comparisons were performed using the 2-tailed Student's t test. Values of $p < 0.05$ were considered to be statistically significant.

Ethics statement

The murine protocols used in this study were approved by the BIDMC IACUC (Protocol 020– 2012), which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All efforts were made to minimize animal suffering, including the use of isoflurane when appropriate, i.e., in the skin sensitization assay. Euthanasia was performed by CO_2 in an IACUC-approved setup. The use of PBMCs was approved by the Institutional Review Board of Beth Israel Deaconess Medical Center.

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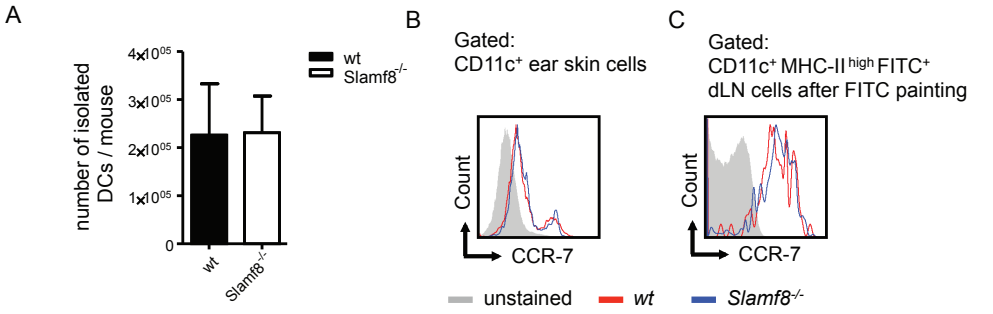
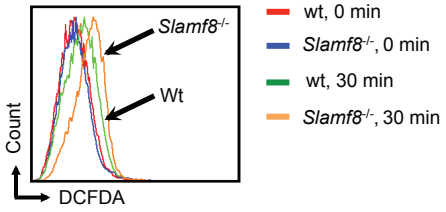
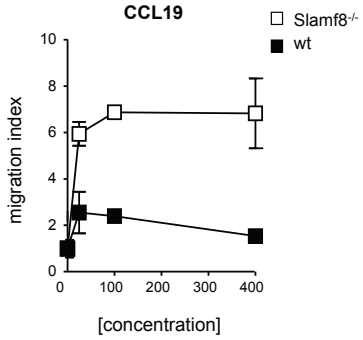
Supporting Information



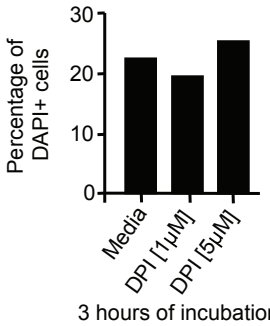
Supplemental Figure 1 | Comparable skin DC numbers and CCR7 expression in wt and *Slamf8*$^{-/-}$ DCs. (**A**) Both Ears were collected from wt and *Slamf8*$^{-/-}$ mice, the ear skin was digested with DNase and Liberase, the single cell suspensions were obtained. The CD11c^{+} cells were further isolated using CD11c positive selection column MicroBeads (Miltenyi Biotec) and the individual num- ber of CD11c+ cells from each mouse was quantified. The data are representative of 5 independent experiments, each consisting of at least 5 mice per experimental condition. (**B**) Flow cytometric representation of wt and *Slamf8*$^{-/-}$ CCR7 expression in CD11c^{+} cells isolated from naïve mouse ear skin. (**C**) 24 hours after administration of FITC on the mouse dorsal skin, wt and *Slamf8*$^{-/-}$ CCR7 expression in the migratory DCs (CD11c^{+} MHC-II^{high} FITC^{+}) in skin draining lymph nodes.



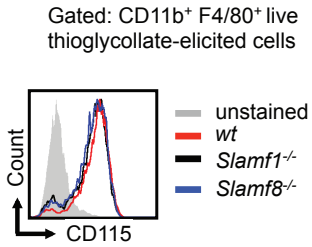
Supplemental Figure 2 | *Slamf8*$^{-/-}$ macrophages produce more intracellular ROS than wt macrophages. Wt and *Slamf8*$^{-/-}$ mice thio-macrophages were incubated with CM-H_2DCFDA for 1 hour, and then stimulated with heat inactivated *E.coli* F18 for 2 hours. The intracellular ROS generation was quantified by flow cytometry. Representative histogram shows an enhanced intracellular ROS production in Slamf8$^{-/-}$ macrophages at the 30-minute time point after stimulation.



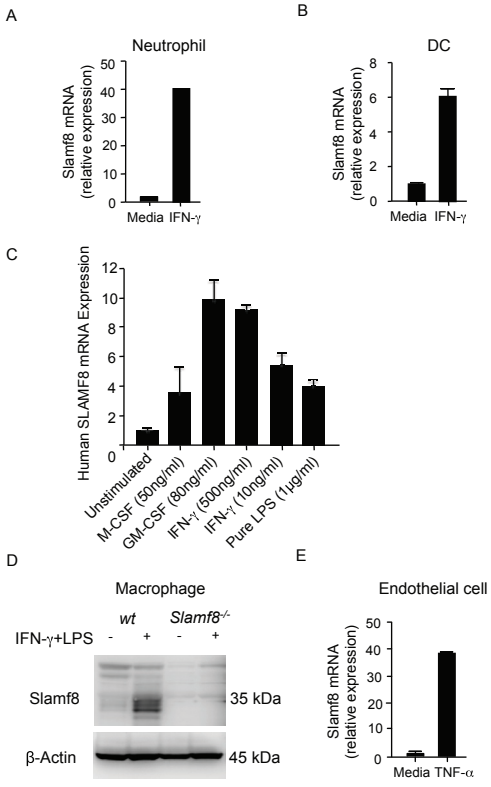
Supplemental Figure 3 | *Slamf8*$^{-/-}$ DCs migrate more efficiently toward CCL19. Wt and *Slamf8*$^{-/-}$ skin DCs were isolated and column-purified before they were allowed to migrate toward a concentration range of CCL19 [0–400nM]. The relative migration of wt and Slamf8$^{-/-}$ DCs is plotted as the migration index.



Supplemental Figure 4 | DPI (5μM) incubation does not affect cell viability. Thio-macrophages were incubat- ed without or with DPI [1 and 5μM] for 3 hours in complete RPMI. The cell viability was deter- mined by staining with DAPI. The percentage of DAPI+ (dead) cells was quantified by flow cytometry.



Supplemental Figure 5 | Comparable CD115 expression in wt, *Slamf1*$^{-/-}$, and *Slamf8*$^{-/-}$ macrophages. CSF-1R (CD115) expression in wt, *Slamf1*$^{-/-}$, and *Slamf8*$^{-/-}$ thio-macrophages (CD11b^{+} F4/80^{+}) assessed by flow cytometry.



Supplemental Figure 6 | Slamf8 expression can be induced by inflammatory stimulation. Slamf8 mRNA expression in (**A**) thio-neutrophils and (**B**) bone marrow DCs upon overnight IFNγ (10ng/mL) activation was quantified by Taqman. (**C**) Expression of human SLAMF8 by purified PBMC monocytes, before and after differentiation into macrophages (M-CSF, GM-CSF) and after stimulation with various inflammatory mediators (IFNγ, LPS). (**D**) Thio-macrophages from wt and *Slamf8*$^{-/-}$ mice were stimulated with IFNγ (10ng/ml) plus LPS (100ng/ml) overnight. Slamf8 protein expression was detected with anti-Slamf8 polyclonal antibody (R&D System) by Western Blot. (**E**) Mouse vascular endothelial cells were isolated from new born mice heart and treated with TNFα (100ng/mL) for 24 hours. Slamf8 transcripts were quantified by Taqman. The results were normalized to the expression of the house keeping gene 18Sr-RNA and presented relative to untreated cells. The data are representative of 2 independent experiments.

Chapter 3

Signaling Lymphocyte Activation Molecule Regulates Development of Colitis in Mice

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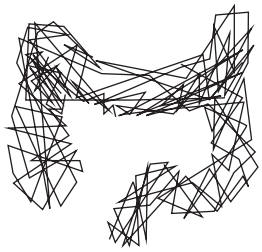


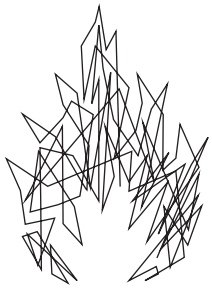
Abstract

BACKGROUND & AIMS: Signaling lymphocyte activation molecule (Slamf)1 is a co-stimulatory receptor on T cells and regulates cytokine production by macrophages and dendritic cells. Slamf1 regulates microbicidal mechanisms in macrophages. Therefore, we investigated whether the receptor affects development of colitis in mice.

METHODS: We transferred CD45RB^{hi}CD4^{+} T cells into or *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ mice to induce colitis. We also induced colitis by injecting mice with an antibody that activates CD40. We determined the severity of enterocolitis based on disease activity index, histology scores, and levels of cytokine production, and assessed the effects of antibodies against Slamf1 on colitis induction. We quantified migration of monocytes and macrophage to inflamed tissues upon induction of colitis or thioglycollate-induced peritonitis and in response to tumor necrosis factor-α in an air-pouch model of leukocyte migration.

RESULTS: Colitis was reduced in Slamf1$^{-/-}$*Rag*$^{-/-}$ mice, compared with *Rag*$^{-/-}$ mice, after transfer of CD45RB^{hi}CD4^{+} T cells or administration of the CD40 agonist. The numbers of monocytes and macrophages were reduced in inflamed tissues of Slamf1$^{-/-}$*Rag*$^{-/-}$ mice, compared with *Rag*$^{-/-}$ that inhibited Slamf1 reduced the level of enterocolitis in *Rag*$^{-/-}$ mice.

CONCLUSION: Slamf1 contributes to the development of colitis in mice. It appears to indirectly regulate the appearance of monocytes and macrophages in inflamed intestinal tissues. Antibodies that inhibit Slamf1 reduce colitis in mice so human Slamf1 might be a therapeutic target for inflammatory bowel disease.



Introduction

Signaling lymphocyte activation molecule family (Slamf) receptors play a role in adaptive as well as in innate immune responses and human Slamf1 (CD150) and mouse Slamf1 serve several distinct roles in macrophages[1]. A variety of CD150 functions are well characterized in adaptive immune processes, including signaling in the immune synapse of T cells, cytokine production, and natural killer–T-cell development[2]. Although we are starting to comprehend the functions of Slamf1 on innate cells, little is known about the *in vivo* implications of Slamf1 on macrophages, monocytes, and dendritic cells. Because Slamf1 positively regulates microbicidal mechanisms directed at some bacteria in macrophages, we evaluated whether Slamf1 would affect disease in enterocolitis models, which are reminiscent of human inflammatory bowel diseases (i.e., ulcerative colitis and Crohn's disease)[3].

Although both innate and adaptive immunity are involved in managing the commensal bacteria in the lumen of the colon, the adaptive immune system responds most aggressively to luminal antigens or bacterially induced host antigens by $CD4^+$ T helper (Th)1, Th2, or Th17 cell expansion. For instance, colitis is induced when $CD4^+$ T cells are not counterbalanced by immune-suppressive mechanisms[4-7]. However, mice without an adaptive immune system (e.g., *Rag*$^{-/-}$ mice), are perfectly capable of coping with the intestinal bacteria through their innate immune defenses. Key players in this defense are macrophages, which show remarkable functional plasticity in response to environmental cues. Under steady-state conditions, in the subendothelial lamina propria, $Ly6C^{hi}$ monocytes can differentiate into tolerogenic $F4/80^{hi}CX3CR1^{hi}CD11b^+$ macrophages, which eradicate commensal microbes without eliciting an immune response. By contrast, at the onset of colitis, $Ly6C^{hi}$ monocytes are thought to infiltrate into the colon, where they differentiate into $F4/80^+CX3CR1^{int}CD11b^+$ inflammatory phagocytes, and produce tumor necrosis factor-α (TNFα) and inducible nitric oxide synthase[8,9].

Because Slamf1 is expressed on both lymphoid and myeloid cells, we assessed the role of Slamf1 in murine chronic enterocolitis using the transfer of wild-type (wt) and *Slamf1*$^{-/-}$ naive and memory $CD4^+$ T cells into *Rag*$^{-/-}$ or *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice to induce experimental colitis[7]. To focus on the role of Slamf1 on myeloid cells, we induced disease in *Rag*$^{-/-}$ or *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice with an agonistic αCD40 monoclonal antibody, as described by Uhlig et al[7]. We found that only the absence of Slamf1 in the recipient mice mitigated disease.

This appears to be independent of the Slam family–specific adaptors EWS/FLI1 activated transcript 2 (Eat-2)a and Eat-2b. Because the homeostasis of monocytes/monocyte-derived macrophages in the colitic lamina propria is affected by the absence of Slamf1, the receptor may regulate macrophage infiltration into, or retention in, inflamed tissues. Because monoclonal αSlamf1 antibodies also ameliorate colitis in both models, we conclude that Slamf1 partakes in the pathogenesis of experimental enterocolitis.

Results

The Presence of the Cell Surface Receptor Slamf1 on Nonlymphoid Cells Is Requisite for the Development of Chronic Enterocolitis

To study the role of Slamf1 in chronic enterocoli- tis, *Slamf1*$^{-/-}$ or wt disease-inducing CD45RB^{hi}CD4^{+} T cells were transferred into either *Rag*$^{-/-}$ or *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice (Figure 1A). Surprisingly, upon transfer of wt CD45RB^{hi}CD4^{+} T cells into *Slamf1*$^{-/-}$*Rag*$^{-/-}$ recipients, mice failed to develop colitis as judged by the DAI, histology score, and interferon-γ production by CD4^{+} T cells from the mesenteric lymph nodes, suggesting a role for Slamf1 in the pathogenesis of the disease (Figure 1B–D).

Slamf1 is expressed on the surface of activated APCs as well as on the surface of memory and recently activated CD4^{+} T cells[2,10]. We therefore evaluated whether the homophilic interactions between Slamf1 on the surface of the donor CD45RB^{hi}CD4^{+} T cells and the APCs in the *Slamf1*$^{-/-}$*Rag*$^{-/-}$ recipients might play a role in ameliorating colitis. As shown in Figure 1E, *Slamf1*$^{-/-}$ and wt CD45RB^{hi}CD4^{+} T cells induced disease with the same efficiency in *Rag*$^{-/-}$ recipient mice. Thus, the absence of Slamf1 from the surface of CD45RB^{hi}CD4^{+} T cells did not impact the severity of colitis. Furthermore, when *Rag*$^{-/-}$ mice are reconstituted with both wt CD45RB^{hi}CD4^{+} and *Slamf1*$^{-/-}$ CD45RB^{low}CD25^{+}CD4^{+} regulatory T cells (Tregs), no colitis developed (Figure 1F). Thus, the homophilic interaction between Slamf1 on the surface of Treg cells and that on the surface of CD45RB^{hi}CD4^{+} T cells or APCs is not critical for maintaining their suppressive capability.

Consequently, the outcomes of these experiments indicate that the presence of Slamf1 on the surface of nonlymphoid cells, rather than on CD4^{+} T cells, is required for the onset of the pathogenesis of chronic enterocolitis.

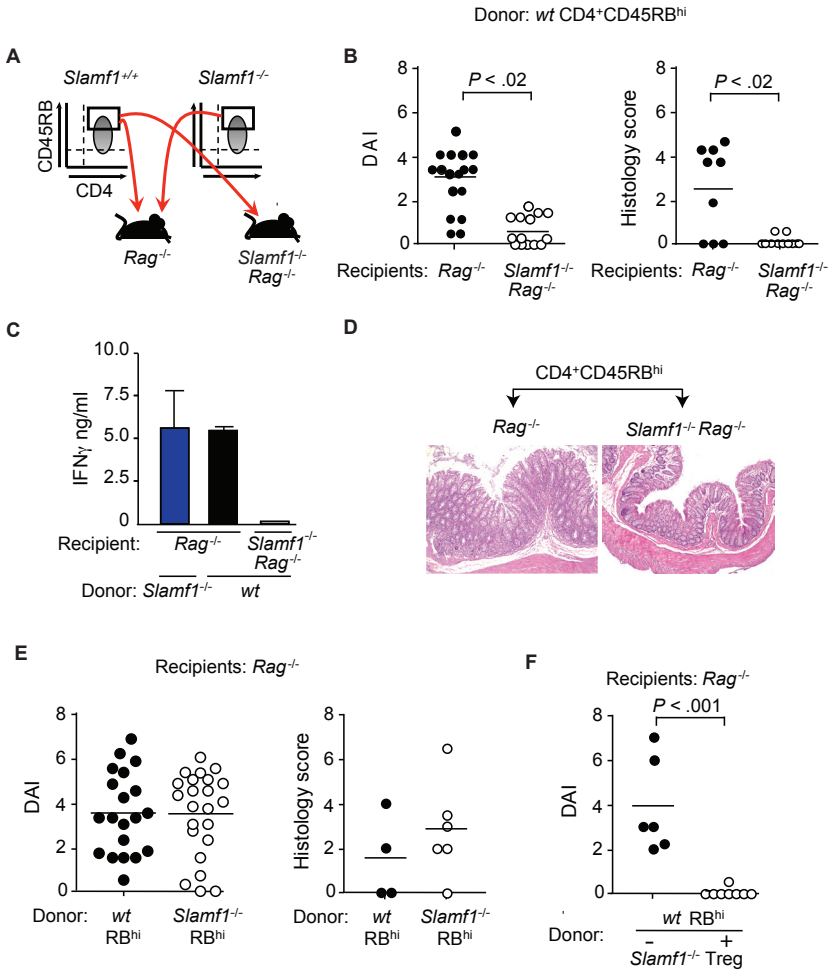


Figure 1 Reduced chronic enterocolitis in the absence of Slamf1.

(**A**) Outline of the CD45RBhiCD4+ T cell transfers into either *Slamf1*$^{-/-}$*Rag*$^{-/-}$ or *Rag*$^{-/-}$ mice. CD45RB^{hi}CD4^{+} T cells obtained from wt spleens by FACS were intraperitoneally injected into *Rag*$^{-/-}$ or *Slamf1*$^{-/-}$*Rag*$^{-/-}$ hosts (5 x 10^{5} cells /mouse)[20]. Alternatively, CD45RB^{hi}CD4^{+} T cells from *Slamf1*$^{-/-}$ donors were injected into *Rag*$^{-/-}$ hosts. Each open circle and filled circle represents one mouse. (**B**) DAI of 3 pooled experiments and histology scores. Mean and individual values of each group are indicated. (**C**) IFNγ production in mesenteric lymph node (MLN) CD4+ T cell culture. IFNγ secretion by CD4^{+} T cells from mesenteric lymph nodes of wt into *Rag*$^{-/-}$ (n = 5), *Slamf1*$^{-/-}$ into *Rag*$^{-/-}$ (n = 5) and wt into *Slamf1*$^{-/-}$*Rag*$^{-/-}$ (n = 5) mice. Cells were activated with 10µg/mL of plate-bound aCD3 for 36 hours. Supernatant was analyzed for IFNγ by standard ELISA. (**D**) Representative histology of colon tissue from wt into *Rag*$^{-/-}$ and wt into *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice. Original magnification 10X. (**E**) DAI and histology scores of *Rag*$^{-/-}$ mice injected with wt CD45RB^{hi}CD4^{+} or *Slamf1*$^{-/-}$ CD45RB^{hi}CD4^{+} T cells. Mean and individual values of each group are indicated. (**F**) DAI of *Rag*$^{-/-}$ hosts that received an injection with wt CD45RB^{hi}CD4^{+} T cells or a mix of wt CD45RBhiCD4^{+} and *Slamf1*$^{-/-}$ CD45RB^{low}CD25^{+}CD4^{+}. Means and independent values of each group are indicated. P values are shown.

Anti-CD40-Induced Colitis Is Ameliorated in *Slamf1*$^{-/-}$ *Rag*$^{-/-}$, But Not in Eat-2a/b$^{-/-}$ *Rag*$^{-/-}$ Mice

To directly determine that expression of Slamf1 on the surface of innate immune cells (e.g., dendritic cells and monocyte/macrophages), is important in the pathogenesis of experimental colitis, we used an agonistic αCD40 antibody (FGK45) to induce disease in *Rag*$^{-/-}$ mice. In the absence of T and B lymphocytes, agonistic αCD40 primarily activates myeloid CD40-expressing cells by mimicking the CD40 ligand, which is induced on the surface of activated CD4^{+} T cells. When *Rag*$^{-/-}$ mice are administered αCD40, they develop acute colitis that is driven primarily by macrophages and dendritic cells within 1 week[7]. Reminiscent of the result after CD45RB^{hi}CD4^{+} T-cell induction, *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice developed considerably milder colitis compared with the *Rag*$^{-/-}$ mice as judged by DAI, histology score, weight loss (Figure 2A and B, Supplementary Figure 1), and splenomegaly (data not shown). This was not caused by altered CD40 expression on Slamf1-deficient macrophages[2]. Immunohistochemistry staining of the colon tissues with monoclonal antibodies directed against the macrophage markers F4/80 and CD11b indicated a reduced infiltration of CD11b^{+}F4/80^{+} macrophages in *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice than in *Rag*$^{-/-}$ mice after αCD40 induction. The numbers of CD11c^{+} cells were comparable between these mice (Figure 2C and D).

Signal transduction by engagement of 6 of the 9 SLAM family receptors in a variety of hematopoietic cells is modulated by the specific adaptors SAP (SH2D1A) and/or Eat-2 (SH2D1B)[1]. Because both Eat-2a and Eat-2b regulate Slamf1-initiated signal transduction and they are found in myeloid cells, we reasoned that their absence might affect signaling of Slamf1 in colitis-driving phagocytes and hence the development of colitis. To test this, we generated triple-knockout Eat-2a/b$^{-/-}$*Rag*$^{-/-}$ mice, which responded similarly to the αCD40 antibody as judged by DAI, histology score, and end weight (Figure 2E and F, Supplementary Figure 2). This result indicates that the absence of Slamf1 from the surface of myeloid cells ameliorates colitis, but that this process does not involve signaling through the adaptor proteins Eat-2a/b.

Reduced Numbers of Infiltrating CD11b^{+} F4/ 80^{+} Macrophages in Colitic *Slamf1*$^{-/-}$*Rag*$^{-/-}$ Mice

To correlate the reduced number of CD11b^{+}F4/80^{+} cells with the ameliorated colitis in

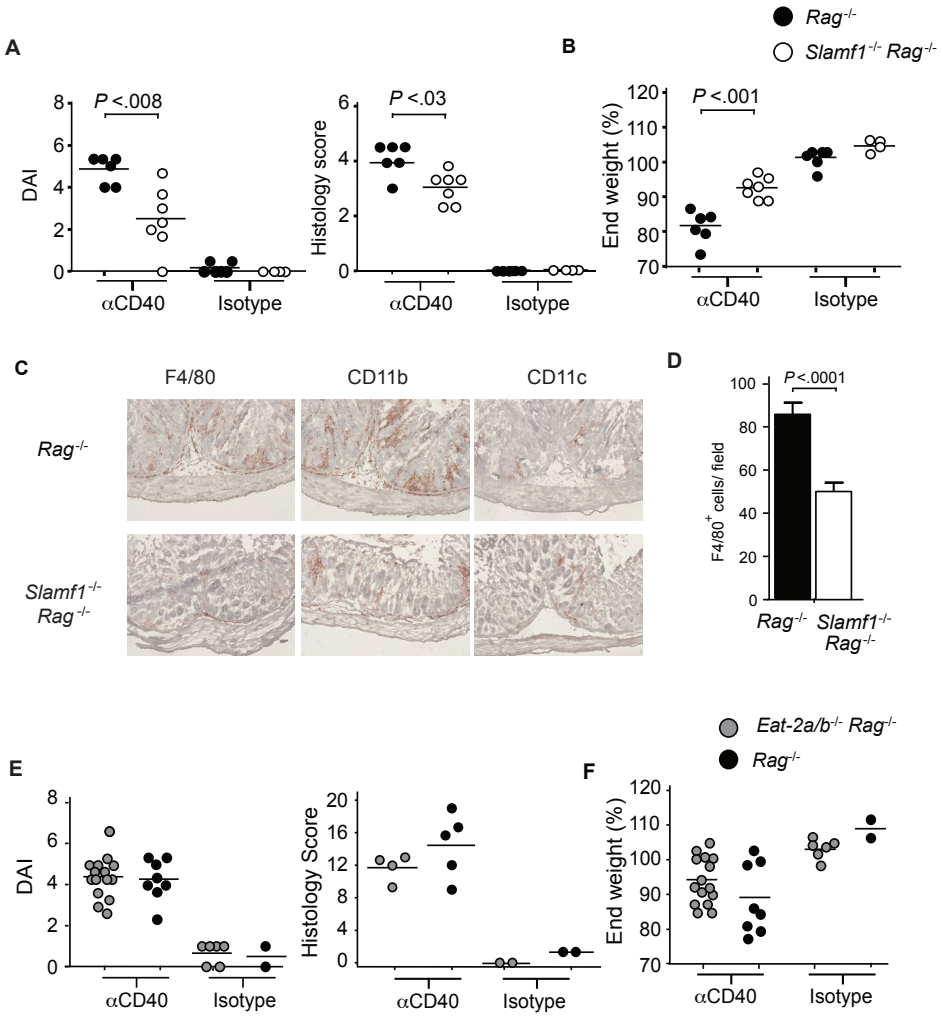


Figure 2 | Anti-CD40 antibody induced colitis is reduced in *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice but not in *Eat-2a/b*$^{-/-}$ *Rag*$^{-/-}$. Anti-CD40 was intraperitoneally injected into *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice or into *Rag*$^{-/-}$ and *Eat-2a/b*$^{-/-}$*Rag*$^{-/-}$ mice (200µg) or with rat IgG2a control. Each open circle and filled circle represents one mouse. The data are representative of three separate experiments. (**A**) DAI and histology score. Mean and individual values of each group are indicated. (**B**) Weight loss as a percentage of the initial weight. (**C**) Representative immunohistochemistry colon sections prepared from *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice at day seven after injection with anti-CD40. Colon samples were stained with antibodies directed against F4/80, CD11b or CD11c and counterstained with hematoxylin. Original magnification 40X. (**D**) Graphic representation of the number of F4/80^{+} macrophages per 1-mm^{2} high-power field in sections depicted in (**C**). Sixty fields were counted for each mouse. Statistical significance was determined by 2-tailed Student t test. (**E**) DAI from three separate experiments and histology score. Each grey circle and filled circle represents one mouse. (**F**) Weight loss.

Slamf1$^{-/-}$*Rag*$^{-/-}$ mice in more detail, we isolated lamina propria cells from *Rag*$^{-/-}$ mice in which colitis had been induced. Cytofluorometric analyses using multiple macrophage markers confirmed that after the transfer of CD45RB^{hi}CD4^{+} T cells (Figure 3A) or after administering agonistic αCD40 (Figure 3B), the percentage of CD11b^{+}F4/80^{+} macrophages in the lamina propria of the *Slamf1*$^{-/-}$*Rag*$^{-/-}$ recipients was reduced significantly as compared with *Rag*$^{-/-}$ recipients. Most CD11b^{+} cells isolated from the inflamed colonic lamina propria express Ly6C on their surface, suggesting they are of monocytic origin. The expression of F4/80, TLR2, MHCII, and CD86 on the surface of these cells indicates a proinflammatory M1 macrophage phenotype[11,12] (Figure 3C). The expression levels of these functionally relevant molecules are comparable between wt and *Slamf1*$^{-/-}$ cells (Figure 3C), indicating that Slamf1 deficiency does not affect the development of this cell population, which confirmed our previous report[2]. However, the absolute number of the CD11b^{+}F4/80^{+} macrophages isolated from the lamina propria of *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice was reduced significantly as compared with that from the *Rag*$^{-/-}$ mice after αCD40 induction (Figure 3D–F). These CD11b^{+}F4/80^{+} macrophages that infiltrated the colonic lamina propria after αCD40 induction express Slamf1 as judged by reverse-transcription polymerase chain reaction as well as flow cytometry (Supplementary Figure 3). To exclude the possibility that a Slamf1 polymorphism might affect APC-mediated colitis[1], we also induced αCD40 colitis in BALB/c *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice. This is possibly ruled out because αCD40 antibody also induced milder colitis in *Slamf1*$^{-/-}$*Rag*$^{-/-}$ BALB/c mice as compared with the *Rag*$^{-/-}$ BALB/c mice (Figure 3B, Supplementary Figure 4).

It was possible that the reduced macrophage levels in the colon of *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice might be caused by an increased apoptosis as a result of the absence of Slamf1. To test this, the colon tissues of αCD40-treated *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice were co-stained with αCD11b-APC and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL). As shown in Supplementary Figure 5, the percentage of TUNEL-positive, CD11b^{+} macrophages is comparable between *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice after αCD40 induction. Therefore, a reduced number of macrophages in the colon of αCD40-induced *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice is not likely the result of increased apoptosis of macrophages lacking Slamf1.

These outcomes show that Slamf1 governs functions of macrophages that are responsible for the development of enterocolitis. We find fewer M1 macrophages in the inflamed colon of *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice. Because it is well accepted that inflammatory M1 macro-

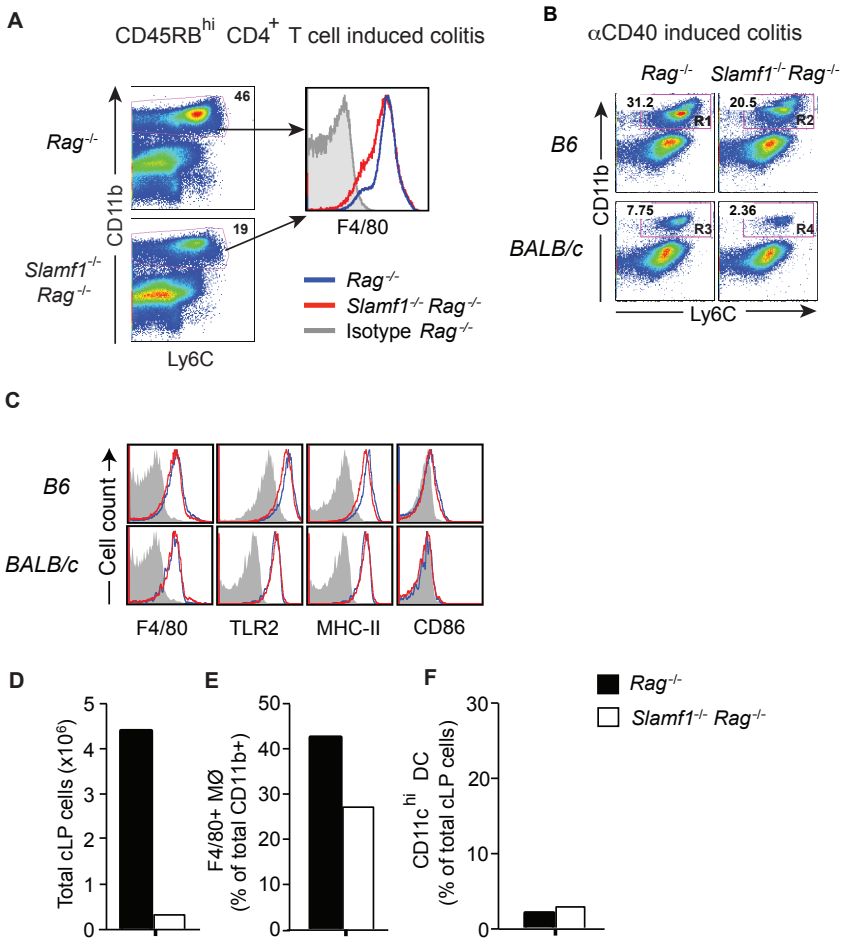


Figure 3 | *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice have decreased numbers of infiltrating inflammatory-type phagocytes, in the lamina propria. (**A**) Flow cytometry analysis of isolated lamina propria cells of *Rag*$^{-/-}$ or *Slamf1*$^{-/-}$*Rag*$^{-/-}$ C57BL/6 mice, in which colitis was induced by CD45RBhiCD4+ T cell transfer. The CD11b+ population is gated and the gate numbers represent the percentage of total isolated cells and depicted in a histology graph representing F4/80 expression. Each plot represents a pool of five mice. The figure is representative of 2 separate experiments. (**B**) Flow cytometry analysis of isolated lamina propria cells of *Rag*$^{-/-}$ or *Slamf1*$^{-/-}$*Rag*$^{-/-}$ C57BL/6 or BALB/c mice, in which colitis was induced by injection of αCD40. The CD11b^{+} population is gated and the gate numbers represent the percentage of total isolated cells. Each plot represents a pool of five mice. The figure is representative of 3 separate experiments. (**C**) Flow cytometry analysis of inflammatory CD11b^{+} phagocytes, as represented in R1 and R2 of C57BL/6 mice or R3 and R4 of BALB/c mice described in (**B**) were stained with monoclonal antibodies directed against F4/80, TLR2, MHC-II (I-Ab) and CD86. (**D**) Bars represent the total number of infiltrated cells after percoll isolation. The total cells were further analyzed by FACS analysis. (**E**) CD11b^{+}F4/80^{+} phagocyte or (**F**) CD11chi dendritic cells percentages of the total pool of isolated cells. Each bar represents pooled samples obtained from five mice.

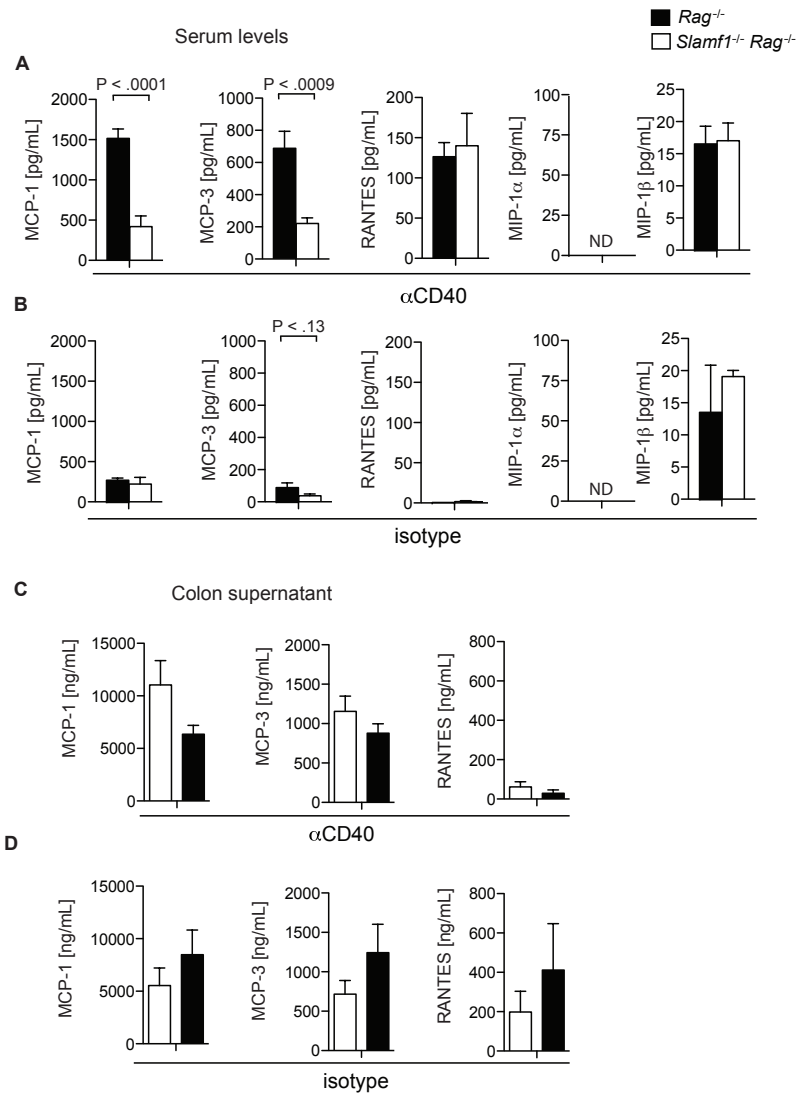


Figure 4 | MCP-1 and MCP-3 levels in the serum of *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice are lower than in *Rag*$^{-/-}$ mice in which colitis had been induced by αCD40. Serum (**A & B**) and colon culture supernatant (**C & D**) was collected from *Slamf1*$^{-/-}$*Rag*$^{-/-}$ and *Rag–/–* mice (n = 8) that were either injected with anti-CD40 (**A & C**) or isotype (**B & D**), 7 days prior to harvest. Bars represent the amount of chemokines (i.e. MCP-1, MCP-3, MIP-1α, MIP-1β and GM-CSF) that was measured in the respective samples. Statistical significance was determined by student t test, mean ±SEM. Experiments were done twice. (**E**) *In vitro* transwell migration analysis of bone marrow derived CD115^{+} monocytes obtained from wt and *Slamf1*$^{-/-}$ mice. The lower wells contained MCP-1, TNF-α, or MIP-1α. A 1:1 mix of red (CMPTX) wt and green (CFSE) *Slamf1*$^{-/-}$ cells, and vise versa, was added to the upper well and left to migrate.

phages play a role in colitis[8,9,13,14], it is likely that the reduced numbers of these macrophages in the *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice during inflammation is causative of ameliorated colitis.

MCP-1 and MCP-3 Serum Levels Are Reduced Upon Induction of Colitis by αCD40 in *Slamf1*$^{-/-}$*Rag*$^{-/-}$ Mice as Compared With *Rag*$^{-/-}$ Mice

It is well known that chemoattractant molecules play an important role in regulating migration of monocytes to the sites inflammation. To further assess whether the reduced macrophage infiltration in the absence of Slamf1 is caused by an impaired migration of

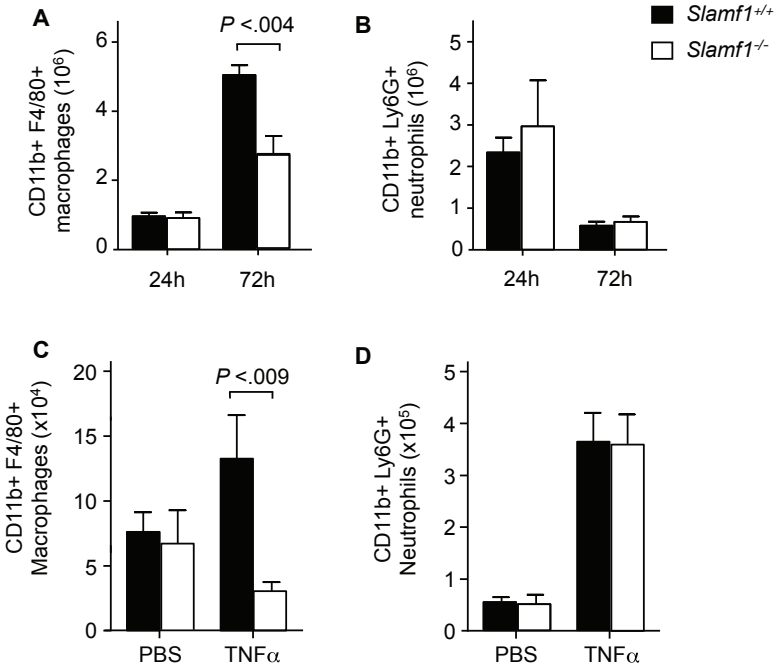


Figure 5 | Fewer *Slamf1*$^{-/-}$ macrophages accumulate at the site of inflammation than Slamf1$^{+/+}$ macrophages in two in vivo models. *Slamf1*$^{-/-}$ and wt mice (n = 5) were intraperitoneally injected with thioglycollate broth (2mL, 4%). The number of macrophages and neutrophils in the peritoneal lavage at 24 and 72 hours after injection of thioglycollate were analyzed by FACS. Bars represent the total number of CD11b^{+}F4/80^{+} macrophages (**A**) or CD11b^{+}Ly6G^{+} neutrophils (**B**) in the peritoneal lavage. *Slamf1*$^{-/-}$ and wt mice (n = 5) were subcutaneously injected twice with sterile air. A third injection consisted of sterile air and TNFα (500ng/mouse). Macrophages and neutrophils that migrated into the subcutanous air pouch 4 hours after injection of TNFα were characterized by FACS and quantified. Bars represent the total number of CD11b^{+} F4/80^{+} macrophages (**C**) or CD11b^{+}Ly6G^{+} neutrophils (**D**) that infiltrated the air pouch.

monocytes, we evaluated MCP-1 (Chemokine (C-C motif) ligand 2 (CCL2)) and MCP-3 (CCL-7), which both bind to Chemokine (C-C motif) receptor (CCR)2, a receptor that is expressed on Ly6C^{hi} monocytes,[22] RANTES (CCL-5), MIP- 1α, and MIP-1β in the serum of *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice in which colitis had been induced by agonistic αCD40.

We find that the serum levels of MCP-1 and MCP-3, but not the levels of RANTES, MIP-1α, and MIP-1β, are significantly lower in *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice compared with *Rag*$^{-/-}$ mice at day 7 after induction of colitis by αCD40 (Figure 4A), whereas no difference was detected after isotype treatment (Figure 4B). Because these chemokines are markedly up-regulated in inflammation, this finding con- firms the notion that inflammation is more severe in *Rag*$^{-/-}$ mice than in *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice. Whether this lower production of chemokines is caused by the Slamf1 mutation is unknown because these chemokines are secreted by a variety of cell types[15]. Furthermore, MCP-1 or MCP-3 were secreted equally well by colon cultures of *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice after treatment with αCD40 (Figure 4C and D). Thus, the increased levels of chemokines in the serum of colitic *Rag*$^{-/-}$ mice compared with *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice might be caused indirectly by the Slamf1 mutation.

We next used an *in vitro* approach to assess whether migration of monocytes in response to relevant chemokines is impaired as a result of Slamf1 deficiency. To this end we used CD115^{+}Ly6C^{hi} monocytes isolated from bone marrow and allowed them to migrate in response to MCP-1, MIP-1α, or TNF-α across the membrane of a transwell chamber[16]. Both wt and *Slamf1*$^{-/-}$ monocytes migrated equally well in response to the two chemokines and to TNF-α (Figure 4E). These data indicate that our *in vivo* observations most likely arose through a Slamf1-dependent mechanism that is not dictated directly by CCR1, CCR2, or CCR5 signaling.

Altered Homeostasis of *Slamf1*$^{-/-}$ Macrophages During Peritonitis and Subcutaneous Inflammation

The altered homeostasis of intestinal macrophages suggested the possibility of an impaired migration of monocytes to the site of inflammation. Because the impaired migration of *Slamf1*$^{-/-}$ monocytes is not mimicked by our *in vitro* transwell migration assay, we then tested this possibility in two *in vivo* models. First, we induced peritonitis in wt and Slamf1-deficient mice by administering thioglycollate[17] and characterized the infiltrating cells (Supplementary Figure 6A). Seventy-two hours after injection of thioglycollate, we

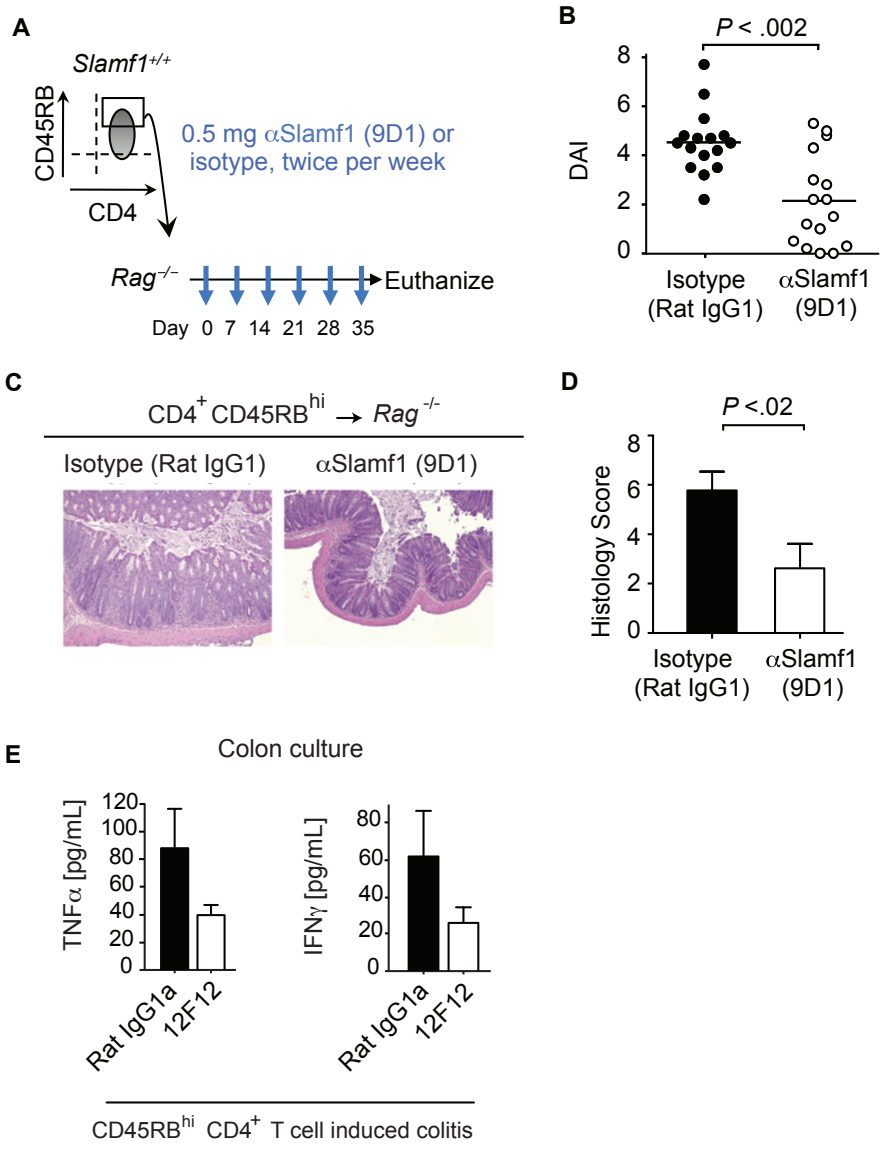


Figure 6 | Anti-Slamf1 antibody ameliorates chronic T cell transfer colitis. (**A**) Outline of the αSlamf1 injection protocol. The CD45RB^{hi}CD4^{+} T cells were transferred to *Rag*$^{-/-}$ recipients and intraperitoneally injected with αSlamf1 (9D1) or an immunoglobulin G isotype twice weekly starting at the day of the cell transfer (500 µg/ mouse/ injection). Mice were euthanized eight weeks after T cell transfer. Each open circle and filled circle represents one mouse. (**B**) DAI. (**C**) Representative histology. Colon sections prepared from *Rag*$^{-/-}$ after treatment and (**D**) histology scores. Statistical significance was determined by the Mann–Whitney test. P values are shown.

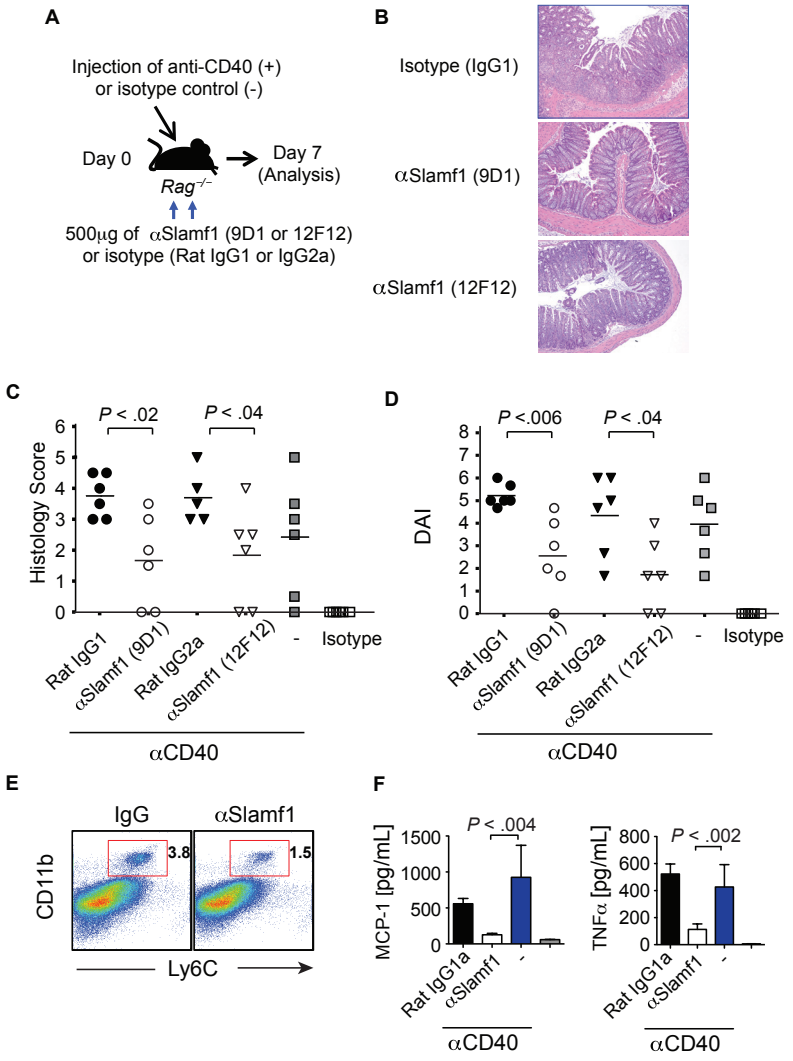


Figure 7 | Anti-Slamf1 antibody ameliorates αCD40-induced colitis. (**A**) Outline of the αSlamf1 injection protocol. Anti-CD40 or rat IgG2a were intraperitoneally injected into *Rag*$^{-/-}$ mice (200µg/mouse). αSlamf1 (9D1 and 12F12) or IgG isotype control were injected intraperitoneally on the same day as αCD40 injection and one day later (500µg/mouse). (**B**) Representative histological analysis of colon tissues from mice injected with αCD40 and treated with 9D1, 12F12, or immunoglobulin isotypes. Original magnification 10X. (**C & D**) DAI and histology score. Mean and individual values of each group are indicated. P values are shown. The data is representative of three separate experiments. (**E**) Flow cytometry analysis of lamina propria cells isolated from *Rag*$^{-/-}$ mice were injected with αCD40 and co-injected on days 0 and 1 with either αSlamf1 (9D1) or an isotype. The Ly6C^{+}CD11b^{+} population is gated, and the gate numbers represent the percentage of total isolated leukocytes. Each plot represents five pooled mice. (F) MCP-1 and TNFα levels in the serum that was collected at day seven after colitis induction. Experiment was done twice.

found that fewer CD11b^{+}F4/80^{+} macrophages were present in the peritoneal cavity of *Slamf1*$^{-/-}$ mice as compared with wt mice (Figure 5A). In contrast, the number of CD-11b^{+}Ly6G^{hi} neutrophils, which peaked at 24 hours after injection as expected[18], did not differ between wt and mutant mice at either time point, as judged by flow cytometry (Figure 5B) and myeloperoxidase (MPO) production (Supplementary Figure 6B). The latter also indicated that no major phenotypic differences existed between wt and *Slamf1*$^{-/-}$ neutrophils.

Although many factors determine recruitment of macrophages to sites of inflammation, TNFα is a major component, which is produced by lymphoid and nonlymphoid cells in both murine enterocolitis and Crohn's disease[4]. To test the hypothesis that monocytes might migrate to a TNFα-primed environment, we used a second *in vivo* assay using cytofluorometric analysis. Recruitment of leukocytes to a subcutaneous air pouch in which TNFα has been injected was assessed[19]. The number of macrophages recruited by TNFα was reduced in *Slamf1*$^{-/-}$ mice compared with wt animals, yet the number of neutrophils remained constant (Figure 5C and D). Together with the colitis experiments, the peritonitis and *in vivo* migration assays indicated that *Slamf1*$^{-/-}$ monocytes may have a general diminished ability to migrate to sites of inflammation.

Administration of Anti-Slamf1 Monoclonal Antibody Ameliorates Colitis

To investigate whether blocking the Slamf1/ Slamf1 homophilic interaction would ameliorate the development of colitis, we used the transfer of wt CD45RB^{hi}CD4^{+} T cells into *Rag*$^{-/-}$ mice in the presence of an IgG1 preparation of αSlamf1 (9D1) (Figure 6A). As judged by DAI (Figure 6B), histology scores (Figure 6C and D), αSlamf1-antibody treatment ameliorated CD45RB^{hi}CD4^{+} T-cell–induced colitis as compared with an isotype control (rat IgG1).

Next, two monoclonal antibodies directed against Slamf1 (i.e., 9D1 and 12F12) were tested in αCD40-induced colitis in *Rag*$^{-/-}$ mice (Figure 7A). Injection of each of the αSlamf1 antibodies mitigated disease as evidenced by histology (Figure 7B and C), DAI (Figure 7D), and TNFα serum levels (Figure 7F). Flow cytometry analyses of the lamina propria cells isolated from the *Rag*$^{-/-}$ mice treated with αSlamf1 or its isotype showed that αSlamf1 treatment reduces the number of CD11b^{+}Ly6C^{hi} inflammatory macrophages in the lamina

propria (Figure 7E). Similar to the experiments with *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice (Figure 5), MCP-1 levels were reduced significantly in the serum of αSlamf1-treated mice (Figure 7E). The studies with the monoclonal antibodies directed against mouse Slamf1 show not only that treatment mitigates colitis, but they also support the notion that Slamf1 influences the homeostasis of macrophages in the lamina propria of the colon.

Discussion

The co-stimulatory molecule Slamf1, a homophilic adhesion molecule, is known to regulate several functions in T cells, macrophages, and dendritic cells[1]. To assess the role of Slamf1 in regulating immune responses of T-cell subsets *in vivo*, we adopted a well-known colitis model, namely the transfer of wt or *Slamf1*$^{-/-}$ disease inducing CD45RB^{hi}CD4^{+} T cells or memory CD4^{+} T cells into *Rag*$^{-/-}$ mice. Upon an evaluation of the ensuing chronic enterocolitis, we found that the homophilic interaction between Slamf1 on CD45RB^{hi}CD4^{+} T cells with Slamf1 on APCs is not required for inducing colitis. In addition, the interactions between Slamf1 adhesion molecule on the surface of Tregs and effector T-cells or Tregs and APCs are not required for maintaining the suppressive function of the CD25^{+}CD4^{+} Tregs.

Surprisingly, when wt CD45RB^{hi}CD4^{+} T cells were transferred into *Slamf1*$^{-/-}$*Rag*$^{-/-}$ recipients, colitis was ameliorated. Consistent with the milder disease, the number of monocyte-derived macrophages in the colon of *Slamf1*$^{-/-}$*Rag*$^{-/-}$ recipients was reduced as compared with *Rag*$^{-/-}$ recipients. This was not caused by a lack of the ability of naive CD4^{+} T cells to skew toward a pathogenic Th1 response by the Slamf1-deficient APCs[10] because this phenotype was recapitulated by the induction with an agonistic αCD40 antibody. Furthermore, because the αCD40-induced colitis was not dependent on the presence of the Slam family–specific adaptors Eat-2a and Eat-2b and as neutralizing antibodies to Slamf1-mitigated disease, the Slamf1/Slamf1 interactions on the cell surface of macrophages/monocytes are likely to play a role in the development of colitis.

Perhaps more importantly, the outcomes of these studies support the interpretation that Slamf1 regulates the number of macrophages in the inflamed colon. A characteristic of an early inflammatory landscape in the colon is an increased recruitment of monocytes, which are skewed to become inflammatory mononuclear phagocytes instead of hyporesponsive resident macrophages. This monocyte population is defined as CD11b^{+}Ly6C^{hi}CX3CR1^{+}

and differentiates to $CD11b^{+}F4/80^{+}CX3CR1^{int}$ phagocytes in situ, perpetuating a preliminary response and driving inflammation through secretion of TNFα and a number of other effector mechanisms[9,20,21]. We find reduced numbers of inflammatory (M1) macrophages in the colon of the *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice, which received either $CD45RB^{hi}CD4^{+}$ T cells or αCD40. Furthermore, administering monoclonal anti- bodies directed against Slamf1 ameliorates colitis in both models. These results were unexpected because Slamf1 plays a role in phagosomes of macrophages in the killing of several gram-negative bacteria (i.e., Escherichia coli or attenuated Salmonella typhimurium)[2]. Although it would be expected that an impaired clearance of a subset of commensal colonic gram-negative bacteria by *Slamf1*$^{-/-}$ macrophages exacerbates colitis, the opposite is the case. Furthermore, although in the absence of Slamf1 the phagosomal Nox2 (p40phox) activity is reduced in response to *E. coli*, a recent publication describes increased colitis in the absence of p40phox[22].

The possible explanations for how Slamf1 might regulate the homeostasis of macrophages during chronic or acute enterocolitis can be grouped as follows: (1) Slamf1 controls production proliferation, and/or survival of $CD11b^{+}$ macrophages, or (2) Slamf1 is requisite for migration to, or retention in, the inflamed tissues.

It is unlikely that the reduced number of macrophages in the lamina propria of the colitic mice would be caused by a defective bone marrow production of the precursors of $CD11b^{+}$ macrophages[23]. Although Slamf1 is one of the markers on the surface of hematopoietic stem cells, the numbers of myeloid cells in Slamf1-deficient mice were not altered[10,23]. The possibility that the Slamf1 deficiency may change the survival of macrophages in the gut is not supported by our TUNEL-staining experiments. In addition, there is no indication that the proliferation of *Slamf1*$^{-/-}$ macrophages is impaired when *Slamf1*$^{-/-}$ bone marrow was used to generate macrophages and dendritic cells *in vitro* under stimulation with macrophage colony-stimulating factor or granulocyte-macrophage colony-stimulating factor and interleukin-4 (unpublished observations).

The notion of a reduced migration of Slamf1-deficient monocytes into inflammatory sites is suggested by the outcomes of two *in vivo* migration experiments. The number of $CD11b^{+}F4/80^{+}$ cells that were recovered from both the inflamed peritoneum as well as from the subcutaneous cavity in the air pouch experiments was reduced in the *Slamf1*$^{-/-}$ mice. Furthermore, we do find reduced levels of key factors that are involved in monocyte migration, namely MCP-1 and MCP-3 in the serum of *Slamf1*$^{-/-}$ mice. Although the latter result

confirms that the state of inflammation in *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice is less than in *Rag*$^{-/-}$ mice upon induction of colitis, the result only indirectly supports the notion of an impaired migration in the absence of Slamf1.

A number of experiments were conducted to explain how the absence of a homophilic adhesion molecule, Slamf1, could be involved directly in the proper infiltration into an inflamed colon. First, the results of our *in vitro* transwell experiments did not support the concept that in the absence of the self-ligand adhesion molecule Slamf1 monocytes migrate less well. The intrinsic propensity of Slamf1-deficient monocytes to respond to several inflammatory chemokines is unaltered. It is unlikely that a difference in migration could be established in an assay using monocyte migration across the endothelial lining because Slamf1 is not expressed on the surface of endothelial cells[1]. Second, bone marrow-derived CD115^{+} monocytes were transferred into *Rag*$^{-/-}$ mice after induction of colitis by either αCD40 or by the transfer of CD45RB^{hi}CD4^{+} T cells. Although a modest number of bone marrow-derived *Slamf1*$^{-/-}$ monocytes repopulated the splenic Ly6C^{hi} monocyte reservoir with the same efficacy as wt monocytes, no donor cells were found in the lamina propria (data not shown).

Taken together, the presence of the self-ligand adhesion molecule Slamf1 is instrumental in the development of enterocolitis in the mouse. Interestingly, because αSlamf1 mitigates the pathogenesis of colitis, monoclonal antibodies directed against human Slamf1 could represent a potential therapeutic target for treating inflammatory bowel diseases.

Experimental procedures

Mice

Slamf1$^{-/-}$ BALB/c or C57BL/6 FITC mice were used to generate *Slamf1*$^{-/-}$Rag-1$^{-/-}$ BALB/c or *Slamf1*$^{-/-}$Rag-2$^{-/-}$ C57BL/6 mice. Rag-1$^{-/-}$ and Rag-2$^{-/-}$ mice were from Jackson Labs (Bar Harbor, ME) and Taconic (Hudson, NY), respectively[10]. Similarly, the previously described Eat-2a/b$^{-/-}$ C57BL/6 mice[24] were crossed with Rag-2$^{-/-}$ C57BL/6. All mice were kept under specific pathogen–free conditions at the Animal Research Facility with approval by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Antibodies

CD11b–FITC, Ly6C-PerCP-Cy5, F4/80-PE, CD11c–APC, Ly6G-PE, and CD115-biotin were from Biolegend (San Diego, CA). TLR2-PE, F4/80-PacificBlue, CD86-PacificBlue, and I-A^{b} (MHC-II)-APC were from eBioscience (San Jose, CA).

Adoptive transfer of CD45RB^{hi}CD4^{+} and CD45RB^{low}-CD4^{+} CD25^{+} T cells

Adoptive transfer into *Rag*$^{-/-}$ or *Slamf1*$^{-/-}$*Rag*$^{-/-}$ recipients was described previously[25]. The Disease Activity Index (DAI) and histology scores were determined as previously described[25,26].

Induction of colitis by agonistic αCD40.

Mice were injected intraperitoneally with 200 µg of rat anti-mouse-CD40 (FGK45, IgG2a, generously donated by Professor Ton Rolink, Basel, Switzerland) or with rat IgG2a (BioXcell, West Lebanon, New Hampshire)[7].

Antibody treatment

A total of 250µg per mouse of αSlamf1 (9D1 or 12F12 clones) or rat IgG isotypes (IgG1 and IgG2a, respectively) (BioXcell) was injected twice weekly in the CD45RB^{hi}CD4^{+} transfer model. In the αCD40-induced colitis model, 1mg of αSlamf1 (9D1 or 12F12) and their respective isotype (rat IgG1 or rat IgG2a) were administered on days 0 and 1.

In vivo migration experiments

Peritonitis was induced by one intraperitoneal injection with 2mL of 4% thioglycollate broth, and 24 or 72 hours later the mice were sacrificed. Cells were harvested with a peritoneal lavage, as described[17].

Recipient mice were anesthetized by isoflurane inhalation and subcutaneously injected into the lower back with an air bubble on days 0 and 3 and with 500ng TNFα in 200µL phosphate-buffered saline on day 7. At 4 hours after injection with TNFα, exudate cells were analyzed by fluorescence-activated cell sorter (FACS)[19].

Transwell Migration Assay

In vitro transwell (5-µm^2 pores) migration analysis was performed using a 48-well migration chamber (NeuroProbe, Gaithersburg MD). The lower wells contained monocyte-chemoattractant protein (MCP)-1 (20ng/mL), TNFα (50ng/mL), or macrophage inflammatory molecule (MIP-1α) (50ng/mL). A 1:1 mixture of Cell Tracker red CMPTX-stained wt and carboxyfluorescein diacetate, succinimidyl ester (CFSE)-stained (Life Technologies, Grand Island, NY) *Slamf1*$^{-/-}$ cells, and vice versa, was added to the upper well to migrate for 70 minutes in a humidified chamber (5% CO_2, 37°C).

Cell Preparation, and Cytokine and Chemokine Analyses

Cells from the lamina propria were obtained as described[27]. Interferon-γ and TNFα were detected in supernatants of 100 mg colonic tissue cultures (36 hours) by enzyme-linked immunosorbent assay (BD PharMingen, San Diego, CA). Chemokine analyses were performed using supernatant or serum by a mouse chemokine Flowcytomix kit (eBioscience, Vienna, Austria).

Flow Cytometry

All samples for flow cytometric analysis were washed with FACS buffer (phosphate-buffered saline, 2% fetal bovine serum), and Fc-receptors were blocked with anti-CD16/32 antibody at 4°C for 20 minutes. Cells were stained using directly conjugated antibodies on ice. Cells were analyzed on a BD LSRII using the FlowJo analysis package (Trees Star,

Inc, Ashland, OR).

Immunohistochemistry

Fresh tissue samples from the colon were frozen in optimal cutting temperature compound (Ames Company, Elkhart, IN). Frozen tissue sections (4-µm thick) were stained by the avidin-biotin complex method as previously described[17]. Sixty fields were counted per condition.

Statistical Analysis

Parametric data are shown as the mean ± standard deviation. Nonparametric data were analyzed using the Mann-Whitney test, as described by Liao et al.[16] The median ± standard error of the mean was determined. The statistical analyses were performed with Prism 5 software (GraphPad, San Diego, CA).

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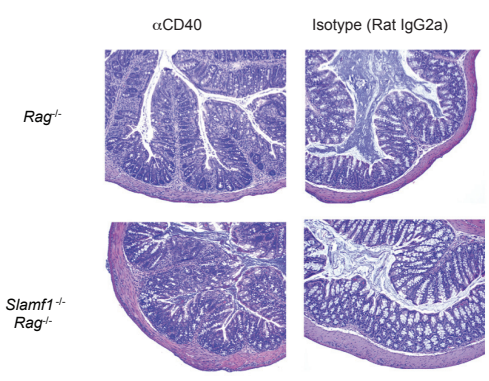
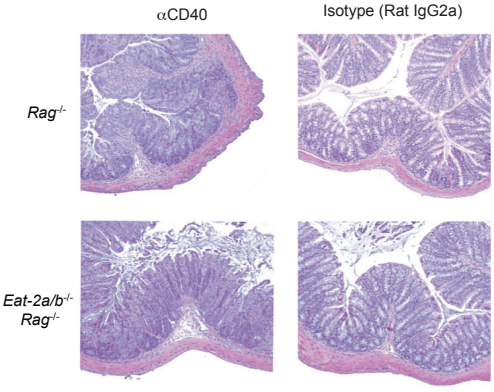
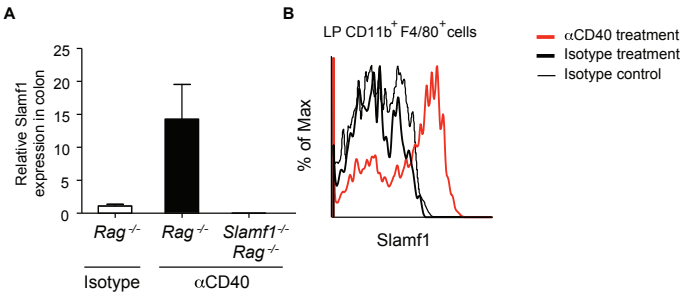
Supporting Figures



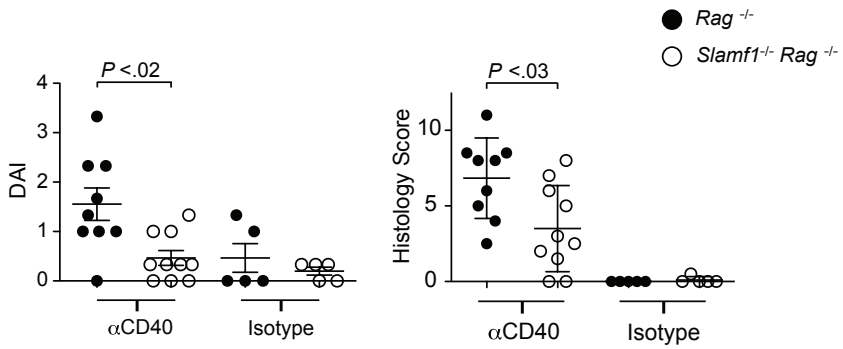
Supplemental Figure 1 | Representative histology of colon tissue. Representative histology colon sections prepared from *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ mice at day seven after injection with αCD40, as described in Figure 2.



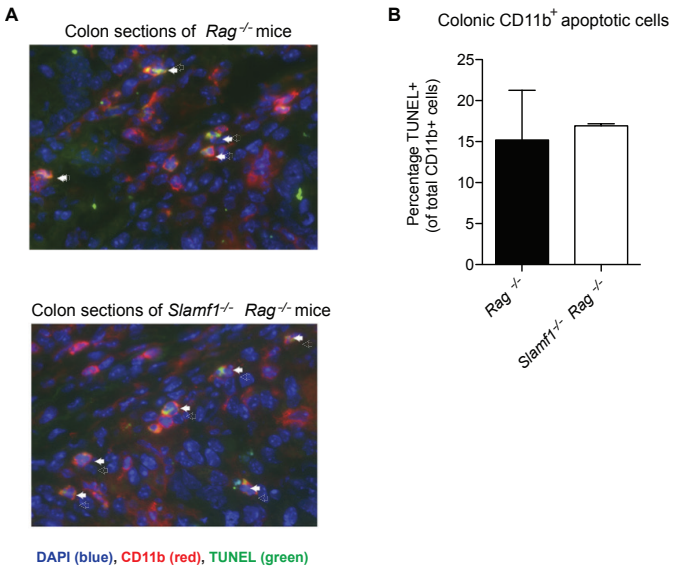
Supplemental Figure 2 | Representative histology of colons from *Rag*$^{-/-}$ and *Rag*$^{-/-}$ *Eat2a/b*$^{-/-}$ mice. Representative histology colon sections prepared from *Rag*$^{-/-}$ and *Rag*$^{-/-}$*Eat2a/b*$^{-/-}$ mice at day seven after injection with αCD40, as described in Figure 2.



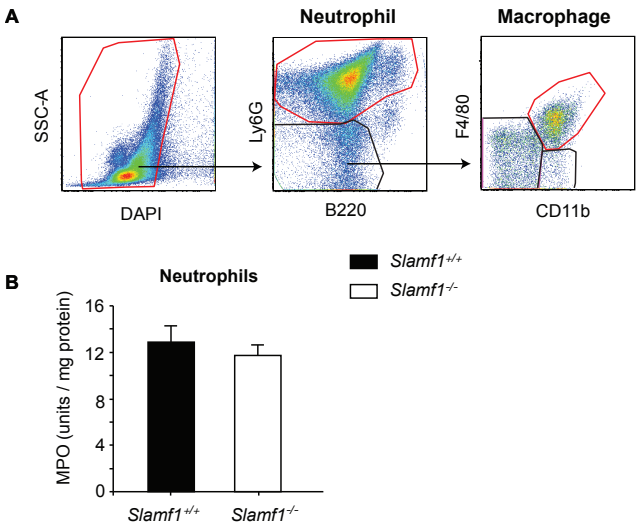
Supplemental Figure 3 | Slamf1 is expressed in inflamed colons. (**A**) The bars represent colonic Slamf1 mRNA expression levels before and seven days after injection of FGK45 (αCD40) in *Rag*$^{-/-}$ mice. Injected *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ mice are also shown. (**B**) Lamina propria cells were isolated from *Rag*$^{-/-}$ mice treated with αCD40 or its control isotype rat IgG2a. After staining with CD11b, F4/80 and Slamf1 antibody, CD11b^{+}F4/80^{+} cells were gated for comparing Slamf1 expression on the cell surface. Bold and thin black lines represent cells from αCD40 or its isotype induced *Rag*$^{-/-}$ mice stained with isotype (for Slamf1) control, respectively. Bold red line represents cells from αCD40 induced *Rag*$^{-/-}$ mice stained with Slamf1.



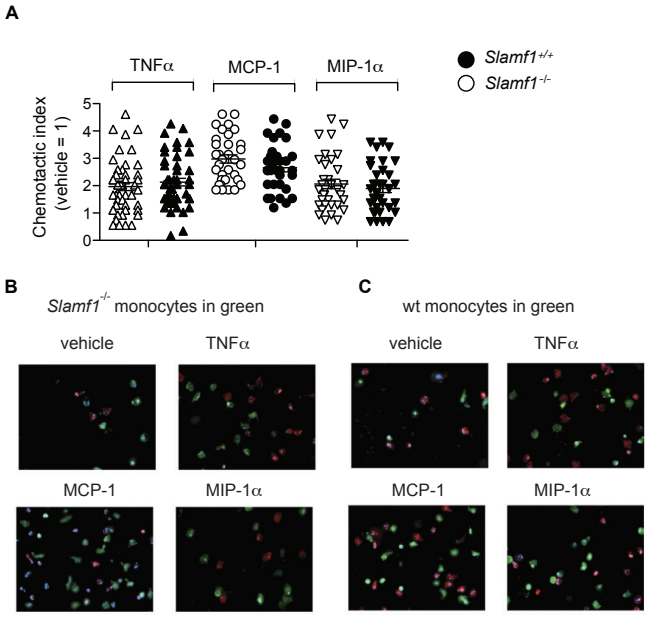
Supplemental Figure 4 | BALB/c mice show a similar colitis phenotype as B6 mice. DAI and histology score. The agonistic antibody αCD40 (FGK45, 200μg/mouse, one injection) were intraperitoneally injected into *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ mice in the BALB/c background (200μg). Mice that were injected with a matching isotype for FGK45 (rat IgG2a) served as controls for colitis. Mice were euthanized after seven days. Each open circle and filled circle represents one mouse. Statistical significance was determined by the Mann-Whitney test. Mean and individual values of each group are indicated.



Supplemental Figure 5 | Apoptosis of CD11b⁺ leukocytes is not altered by Slamf1. Representative immunofluorescence staining of colon sections that were obtained from *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ mice, seven days after αCD40-mediated colitis induction. Samples where stained with TUNEL staining (green), anti-CD11b-APC (red) and counterstained with DAPI (blue). The arrows indicate cells that are double positive for TUNEL and CD11b staining. At least 100 cells were counted per mouse (n = 3). Statistical significance was determined by 2-tailed Student t test.



Supplemental Figure 6 | Gating of thioglycollate elicited macrophages and neutrophils and MPO activity assay. (**A**) Gating strategy to identify peritoneal lavage cells for Figure 5A & B. Peritoneal lavage cells 24 or 72 hours after thioglycollate injection were stained with Ly6G, B220, CD11b and F4/80. Red gates indicate in live cells in the left panel, neutrophils in the middle panel, and macrophages in the right panel. (**B**) Myeloperoxidase activity in peritoneal lavage cells that were obtained from wt or *Slamf1*$^{-/-}$ mice, 6 hours after injections with thioglycollate.



Supplemental Figure 7 | *Slamf1*$^{-/-}$ CD115^{+} monocytes respond similar to several chemokines as wt monocytes. *In vitro* transwell migration analysis of bone marrow derived CD115^{+} monocytes obtained from wt and *Slamf1*$^{-/-}$ mice. The lower wells contained MCP-1, TNFα, or MIP-1α. A 1:1 mix of red (CMPTX) wt and green (CFSE) *Slamf1*$^{-/-}$cells, and vise versa, was added to the upper well and left to migrate. (**A**) Representative fields of migrated cells in response to the respective stimuli, were *Slamf1*$^{-/-}$ monocytes that were stained with CFSE and wt monocytes that were stained with CMPTX or (**B**) vise versa. At least 30 fields were counted per condition. Statistical significance was determined by student t test, mean ± SEM. The figure is representative of 4 experiments.

Supporting Methods

Colon RNA Isolation and Real-Time PCR

Colons were homogenized manually by using a mortar and pestle, at –80°C. Trizol® (1ml) was added to 100 mg of thoroughly smashed colon tissue and total RNA was isolated according to the manufacturers manual (Invitrogen).

The mouse slamf1 mRNA expression was measured by quantivtive RT-PCR (SYBR Green) based on the instruction of Roche LightCycler® 480 real-time PCR system.

The Slamf1 primer sequences are: Forward TCTGCGATTGCTGGCTAA, Reverse CGAGGATGCGGACACTTT, the product size is 210bp. The primers for GAPDH are: Forward TGAGGACCAGGTTGTCTCCT, Reverse CCCTGTTGCTGTAGCCGTAT, the product size is 140bp.

MPO Enzymatic Assay

A modified protocol was used to determine MPO activity in neutrophils as described1. Briefly, peritoneal lavage was obtained from mice that were i.p. injected with thio-glycolate, 6 hours prior to harvesting. Cells were homogenized in HETAB (Sigma, St. Louis, MO) and equal volumes were incubated with peroxide [3.5mM] and O-dianisidine · 2HCl [10mM] (Sigma). Then, samples are incubated at 37°C for 10 minutes and the reaction is stopped by adding sodium azide. Activity is calculated from the absorbance, measured at 460nm.

Transwell Migration Assay

In vitro transwell [5m^2 pores] migration analysis was done using a 48-well migration chamber (NeuroProbe, Gaithersburg MD). The lower wells con- tained MCP-1 [20ng/mL], TNFα [50 ng/mL], or MIP-1α [50ng/mL]. A 1:1 mixture of red (CMPTX) wt and green (CFSE) *Slamf1*$^{-/-}$ cells, and vise versa, was added to the upper well and left to migrate for 70 minutes in a humified chamber (5% CO2, 37°C).

Immunofluorescence TUNEL Staining

Apoptosis of CD11b$^+$ cells in the colon of αCD40-treated *Rag*$^{-/-}$ and *Slamf1*$^{-/-}$*Rag*$^{-/-}$ mice. Frozen colon section of the mice that were described in Figure 2D were stained for TUNEL (In situ cell death detection kit, Roche scientific), CD11b-PE (eBioscience) and 4-,6-diamidino-2-phenylindole (DAPI, Invitrogen). A minimum of 100 cells were analyzed per mouse (n = 3). TUNEL is depicted in green, PE in red, and DAPI in blue.

Supplemental References

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Chapter 4

The cell surface receptor Slamf6 modulates innate immune responses during *Citrobacter rodentium* induced colitis

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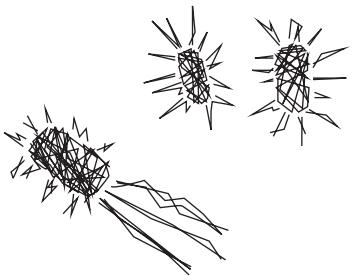
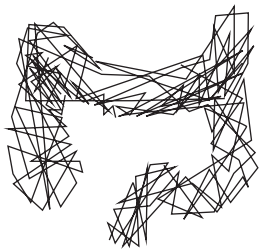


Abstract

The homophilic cell surface receptors CD150 (Slamf1) and CD352 (Slamf6) are known to modulate adaptive immune responses. Here we examine whether Slamf6, like Slamf1, is also involved in responses to bacteria and regulates peripheral inflammation. Using a reporter-based binding assay, we show that Slamf6 can engage structures on the outer cell membrane of several Gram$^-$ bacteria. Although the Th17 response was enhanced in *Slamf6*$^{-/-}$ C57BL/6 mice upon oral infection with *Citrobacter rodentium*, the pathologic consequences are indistinguishable from an infection of WT C57BL/6 mice. Surprisingly, the pathology and immune responses in the lamina propria of *C. rodentium* infected *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice were markedly reduced as compared to those of *Rag*$^{-/-}$ mice. Furthermore, administering a monoclonal antibody [mAb 330] directed against Slamf6 to *Rag*$^{-/-}$ mice ameliorated the infection compared to a control antibody. During *C. rodentium* infections, infiltration of inflammatory phagocytes into the lamina propria was consistently lower in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice than in *Rag*$^{-/-}$ animals. Concomitant with the reduced systemic translocation of the bacteria was an enhanced production of IL-22. We conclude that Slamf6-mediated interactions of colonic innate immune cells with specific Gram$^-$ bacteria reduce mucosal protection and enhance inflammation, contributing to lethal colitis that is caused by *C. rodentium* infections in *Rag*$^{-/-}$ mice.



Introduction

Most hematopoietic cells express Slamf6, which is one the of nine Signaling Lymphocyte Activating Molecule (SLAM) family receptors. Slamf6 expression is highest in activated T cells and B cells, whereas expression in DCs and macrophages can be induced by inflammatory signals [1]. Although Slamf6 signaling is extensively studied in adaptive immunity [2,3], less is known about the function of Slamf6 in innate responses.

Several Slam family glycoproteins serve as receptors for certain pathogens (Table 1). For instance, human Slamf1 is one of the receptors of Measles Virus [4]. SLAM (CDw150. Slamf2 is a receptor for the lectin present on pili of certain enterobacteriaceae [5]. Recently, we found that Slamf1 can recognize the outer membrane porins OmpC and OmpF of *E. coli* [6,7]. Furthermore, most of the Slamf receptors modulate mechanisms that protect against microbial challenges mediated by signals that are induced by Slamf-Slamf homophilic ligation. Per example in T cells and B cells, Slamf6 recruits SH-2-containing signal-transducing molecules to its intracellular ITSM domains following homophilic ligation and receptor clustering, which is critically involved in germinal center reactions [3,8]. Table 2 summarizes the susceptibility of Slam receptor-deficient mice to various infectious agents that have been used in to study Slamf functions.

Here we assess whether Slamf6, like Slamf1, might be a microbial sensor for bacteria, especially *Eschericia coli* and *Citrobacter rodentium*, which both reside in the gastrointestinal tract. Furthermore, enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are attaching bacteria that harbor a pathogenicity island that renders them capable of colonizing colonic epithelia and causing lesions resulting in a compromised mucosal barrier [9]. They represent a major threat to global health, as they are responsible for a large number of cases of diarrhea that can be life-threatening for infants and children. The closely related bacterium *C. rodentium* is a natural Gram$^-$ murine pathogen, and oral infection with this bacterium results in an infectious colitis characterized by local Th1 responses, neutrophil and macrophage recruitment and epithelial hyperplasia. T cells and B cells are necessary for sterilizing immunity to *C. rodentium* and mice that lack CD4$^+$ T cells have systemic dissemination of bacteria [9-11].

To study the role of Slamf6 in innate immune responses we employ *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ and *Rag*$^{-/-}$ mice, which solely rely on innate mechanisms to combat *C. rodentium* infections, because they lack T cells and B cells. The absence of T cells and B cells renders *Rag*$^{-/-}$

mice unable to mount an effective immune response to *C. rodentium* and the infection results in severe and ultimately fatal colitis [12].

Roles for a range of innate cells have been implicated in the immunity against *C. rodentium*. Lamina propria CX_3CR1^+ macrophages are key in the early detection of the bacterium and specific depletion of these macrophages leads to enhanced pathologic inflammation and more systemic translocation [13,14]. Upon activation, CX_3CR1^+ macrophages produce IL-1β, IL-12, and IL-23 as well as other inflammatory mediators [14,15]. The role of innate lymphocytes manifests predominantly through their production of IL-22, which contributes to mucosal protection by inducing the production of antimicrobial peptides and accelerating mucosal healing [16,17]. Here we report that, surprisingly, Slamf6-deficiency renders *Rag*$^{-/-}$ mice resistant to *C. rodentium* induced colitis, which suggests that Slamf6 negatively affects mucosal protection during colonic *C. rodentium* infection.

Results

Slamf6 engages in cognate interactions with several Gram- bacteria

To investigate a possible role of Slamf6 in innate immune responses during bacterial infections, we first assessed potential interactions between Slamf6 and Gram$^-$ *E. coli*, S. typhumiurium (SseB$^-$), *C. rodentium* and the Gram$^+$ bacterium S. aureus. To this end, we used a cell based reporter assay based on co-transfection of a DNA segment encoding a chimeric Slamf6 / CD3ζ together with an IL-2 promoter-driven luciferase gene into Jurkat cells [6]. Cognate interactions between the Ig-domain of Slamf6 and bacterial surface entities result in increased luciferase activity. Using this assay, specific interactions between Slamf6 and *E. coli* and *S. typhumiurium* (SseB$^-$), but not between Slamf6 or S. aureus, were detected (Figure 1A). *C. rodentium* displayed binding to the ecto-domain of Slamf6 (Figure 1B), albeit to a far lesser extent. Mutated *E. coli* that lack Outer Membrane Porin C (OmpC), OmpF, or both lost the ability to interact with the ecto-domain of Slamf6, suggesting that these bacterial proteins contain both the Slamf6- and Slamf1-interacting structures (Figure 1C) [6]. Further analysis of the interaction between Slamf6 and *E. coli* revealed that anti-Slamf6 mAb blocked this interaction (Figure 1D).

Structural studies that assessed the formation of human Slamf6-Slamf6 homo-dimers

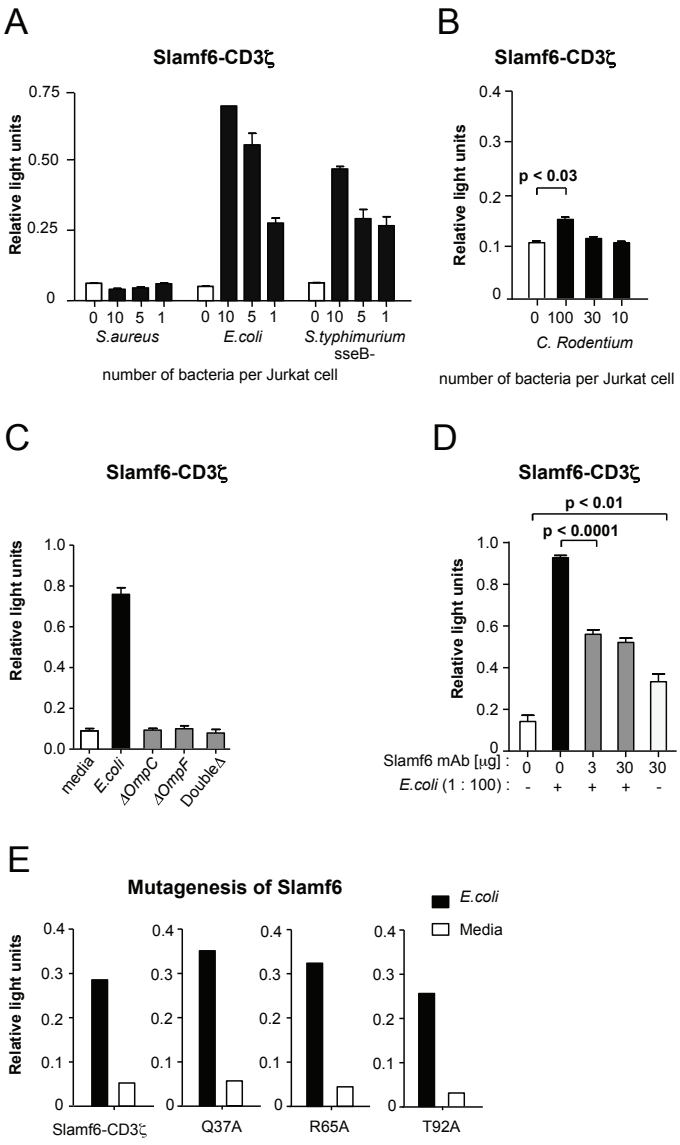


Figure 1 | Slamf6 interacts with *E. coli* and *C. rodentium*. Relative luminescence measured in Jurkat cells that were transfected with a fusion construct of Slamf6 and CD3ζ and a Renilla luciferase reporter. (**A**) The luciferase activity of Jurkat cells after o/n stimulation with serial dilutions of heat inactivated S. aureus, *E. coli* (F18) or *S. typhimurium* and (**B**) *C. rodentium* (DS100). (**C**) The luciferase activity of Jurkat cells after o/n stimulation with *E. coli*, *E. coli* ΔOmpC, *E. coli* ΔOmpF, or double deficient *E.coli*. (**D**) The luciferase activity of Jurkat cells after o/n stimulation in the absence and presence of anti-Slamf6 mAb (330). (**E**) The luciferase activity of Jurkat cells in which single mutations were made, after o/n stimulation.

revealed thirteen amino acid residues that are critical for this interaction [18]. To evaluate whether these residues are also involved in bacterial interactions, we constructed three mutant Slamf6-CD3ζ chimeras at positions that affect homo-dimer formation (Figure 1E). These mutant Slamf6 molecules were capable of inducing a signal upon exposure to *E. coli*, suggesting that bacterial ligation does not involve amino acid residues that are essential in homo-dimer formation. Similar conclusions were made based upon the same type of analyses with mutants of human Slamf6 and mouse Slamf1 (data not shown). Thus, the amino acids in the ecto-domain of Slamf6 and Slamf1 that interact with structures in the outer cell wall of several Gram$^-$ bacteria are different from the amino acid residues, which are requisite for formation of the homo-dimers.

Stronger Th17 response in *Slamf6*$^{-/-}$ mice during *Citrobacter rodentium* infection

To evaluate the effect of Slamf6 on immune responses to bacteria, we selected *C. rodentium*, which is a well-studied model organism that induces intestinal inflammation. First, *Slamf6*$^{-/-}$ and wild-type (wt) C57BL/6 mice were orally infected with 2x10^9 *C. rodentium* bacteria and analyzed for progression of colitis. Upon infection, both colonic macrophages and a set of CD11c$^-$ cells express Slamf6 (Supplemental Figure 1A-B). *Slamf6*$^{-/-}$ mice, like

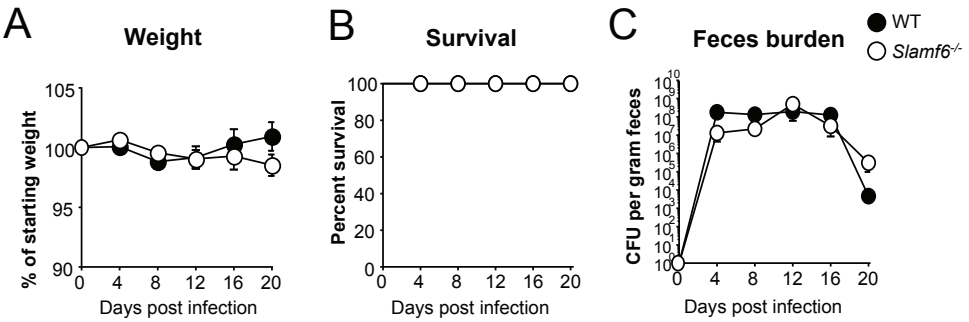


Figure 2 | *Slamf6*$^{-/-}$ mice are equally resistant to *C. rodentium* induced colitis as WT mice. WT and *Slamf6*$^{-/-}$ mice were infected by oral gavage of 2x10^9 *C. rodentium* bacteria. The animals were checked daily and sacrificed when their weight dropped below 80% of their starting weight. (**A**) The weight of individual mice was measured every four days and represented as a percentage of their weight on the day of infection. (**B**) Survival of infected WT and *Slamf6*$^{-/-}$ mice. (**C**) *C. rodentium* counts of fecal pellets that were obtained directly from mice every three days. Serial dilutions were made in PBS and plated on MacConkey plates. Counted bacterial colonies are represented as CFU g^{-1} feces.

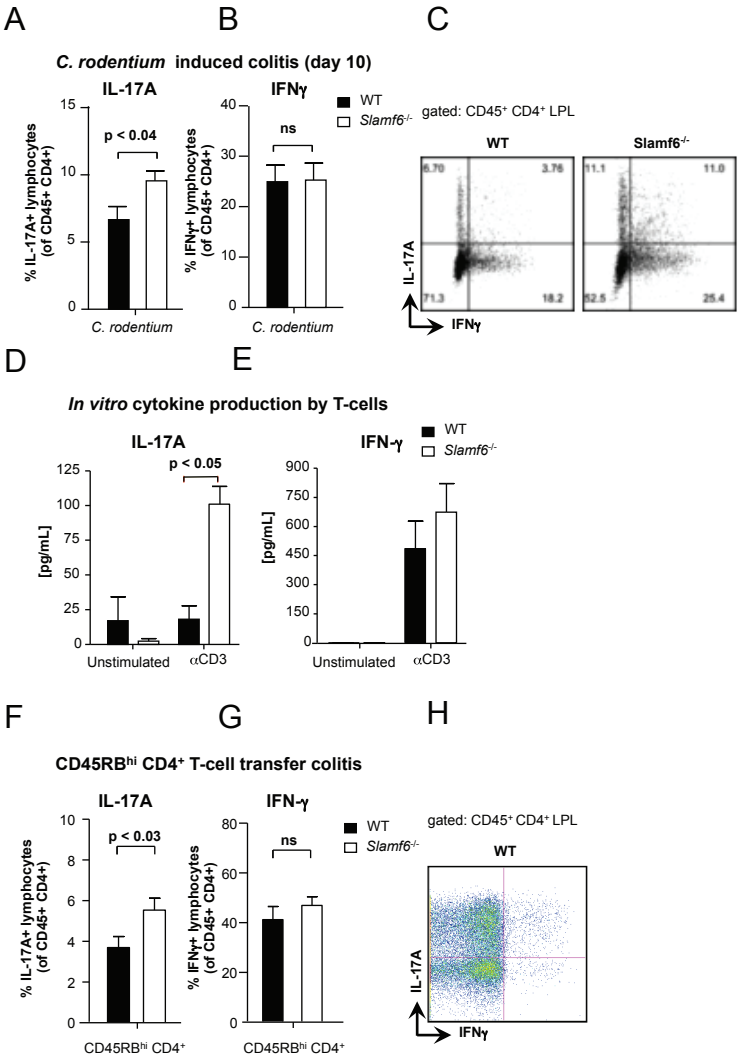


Figure 3 | Enhanced Th17 response in *Slamf6*$^{-/-}$ mice during *C. rodentium* induced colitis. WT and *Slamf6*$^{-/-}$ mice were infected by oral gavage of 2x10^9 *C. rodentium* bacteria. At day 10 post-infection, leukocytes were isolated from the colon lamina propria. The percentage of (**A**) IL-17A$^+$ and (**B**) IFNγ$^+$ lymphocytes is represented as a percentage of total CD45$^+$ CD4$^+$ lymphocytes. (**C**) Representative dot plots of intracellular staining for IL-17A and IFNγ obtained from WT and *Slamf6*$^{-/-}$ mice. Isolated CD4$^+$ splenocytes were cultured o/n in the presence of plate-bound anti-CD3 antibody. The amount of (**D**) IL-17A and (**E**) IFNγ cytokines in the supernatant of these cultures is represented. WT and *Slamf6*$^{-/-}$ CD45RB^{hi} CD4$^+$ splenocytes were transferred into *Rag*$^{-/-}$ mice. The percentage of (**F**) IL-17A$^+$ and (**G**) IFNγ$^+$ lymphocytes is represented as a percentage of total CD45$^+$ CD4$^+$ lymphocytes. (**H**) Representative dot plots of intracellular staining for IL-17A and IFNγ obtained from *Rag*$^{-/-}$ mice in which WT T-cells were transferred.

wt mice, showed a slight reduction in their body weight on day 12 post-infection (Figure 2A) with a fecal *C. rodentium* burden of ~10^7 colony forming units (CFU) per mg feces. The fecal burden steadily declined after day 16 and both wt and *Slamf6*$^{-/-}$ mice appeared to recover from the infection (Figure 2B-C). Thus, Slamf6-deficiency has little impact on the pathogenesis of *C. rodentium*-induced colitis in the presence of an adaptive immune system.

As both Th1 and Th17 cells have been implicated in inflammation and mucosal protection during *C. rodentium* infections [19,20], isolated T-cells from infected wt and *Slamf6*$^{-/-}$ mice were assessed for the production of IFNγ and IL-17A. Intracellular staining of these cells revealed that Th17 cells are more prevalent in the lamina propria of *Slamf6*$^{-/-}$ mice, compared to wt mice (Figure 3A). However, an equal percentage of CD4$^+$ INFγ$^+$ T-cells were found in colonic the lamina propria of wt and *Slamf6*$^{-/-}$ mice (Figure 3B-C), suggesting that Th1 development is unaffected by Slamf6. To assess whether the enhanced percentage of Th17 cells in the lamina propria of infected *Slamf6*$^{-/-}$ mice is a T-cell intrinsic phenomenon, wt and *Slamf6*$^{-/-}$ T-cells were stimulated *in vitro*. The culture supernatant of *Slamf6*$^{-/-}$ T-cells contained significantly more IL-17A upon stimulation with αCD3 mAb compared to wt culture supernatant, while IFNγ production was similar (Figure 3D-E). Experiments in which colitis-inducing CD45RB^{hi}CD4$^+$ T-cells were transferred to *Rag*$^{-/-}$ mice also showed that *Slamf6*$^{-/-}$ T-cells are more prone to IL-17A production (Figure 3F) [21], as the percentage of IL17A$^+$ lymphocytes was significantly higher in *Rag*$^{-/-}$ mice that had received *Slamf6*$^{-/-}$ T-cells, compared to wt T-cells. Again, equal percentages of Th1 cells were detected in the lamina propria of *Rag*$^{-/-}$ mice 6 weeks after transfer of wt or *Slamf6*$^{-/-}$ T-cells (Figure 3G-H). Taken together the data suggest that Slamf6 inhibits the production of IL-17A by CD4$^+$ T-cells.

Slamf6$^{-/-}$ *Rag*$^{-/-}$ mice are resistant to *C. rodentium*

To focus on the role of Slamf6 in the surface of innate immune cells, *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ and *Rag*$^{-/-}$ mice were infected with *C. rodentium*. *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice have a mucosal homeostasis that is similar to *Rag*$^{-/-}$ mice before and after co-housing. However, after oral infection with 2x10^9 *C. rodentium*, *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice are resistant to the typical lethal colitis that develops in *Rag*$^{-/-}$ mice (Figure 4) [10,17,20]. The loss in weight that is caused by a progressing colitis in infected *Rag*$^{-/-}$ mice, which starts two weeks after the oral gavage with *C. rodentium*, was not observed in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice (Figure 4A). Ultimately,

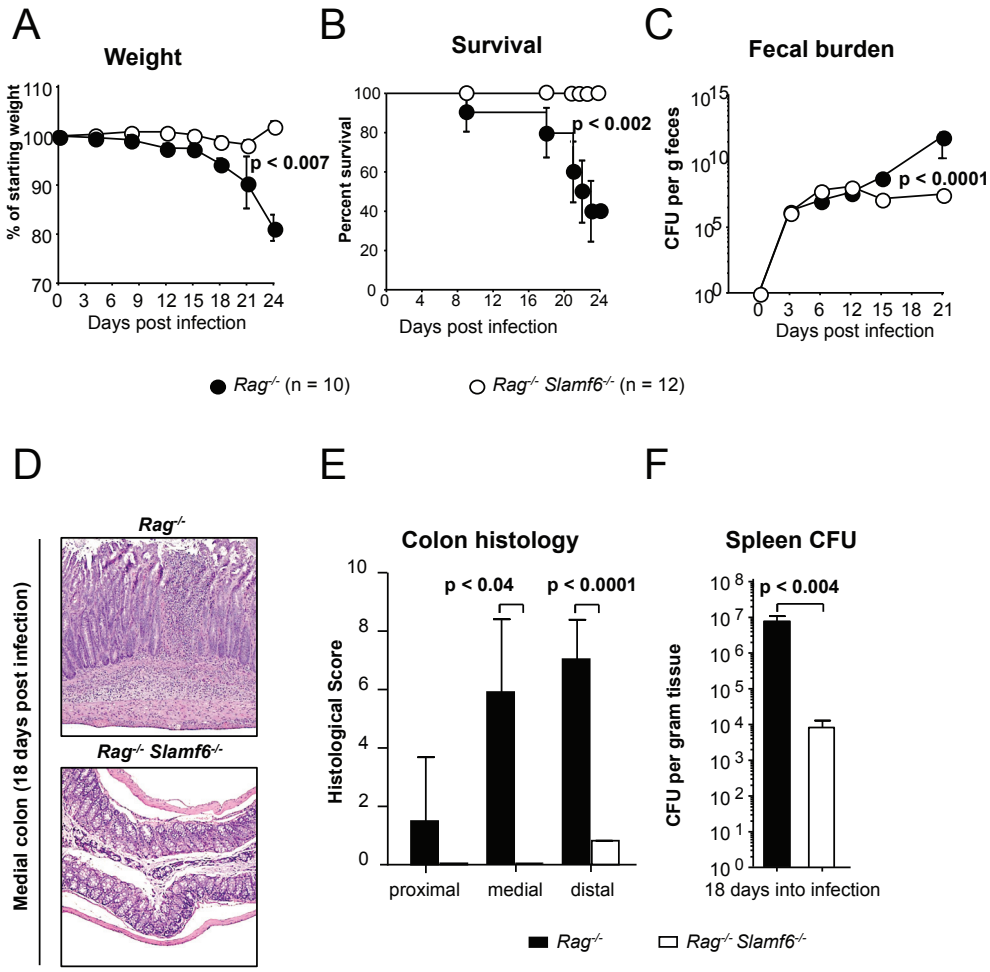


Figure 4 | *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice are resistant to *C. rodentium* induced colitis, whilst *Rag*$^{-/-}$ mice are not. *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice were infected by oral gavage of 2x10^{9} *C. rodentium* bacteria. The animals were checked daily and sacrificed when their weight dropped below 80% of their starting weight. (**A**) The weight of individual mice was measured every three days and represented as a percentage of their weight on the day of infection. (**B**) Survival of infected *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice. (**C**) *C. rodentium* counts of fecal pellets that were obtained directly from mice every three days. Serial dilutions were made in PBS and plated on MacConkey plates. Counted bacterial colonies are represented as CFU g^{-1} feces. (**D**) H&E staining of representative longitudinal sections of the medial colon of *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice, 18 days after infection. (**E**) Histological score of proximal, medial, and distal colon longitudinal sections, 18 days after infection. An independent pathologist performed scoring blind. Maximal scores were 13, judged by mononuclear cell infiltration (3), epithelial integrity (4), hyperplasia (4), and edema (2). (**F**) *C. rodentium* counts of spleen homogenates. Serial dilutions were made in PBS and plated on MacConkey plates. Counted bacterial colonies are represented as CFU g^{-1} spleen tissue.

Rag$^{-/-}$ mice are unable to manage the infection, leading to diarrhea and fatal colitis (~60% at day 20), whereas all of the *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice survived past day 24 (Figure 4B).

Fecal cultures of infected mice reveal an expansion of the colonic bacterial burden over the first ~14 days after infection in both *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice. As expected, *Rag*$^{-/-}$ mice were unable to control the *C. rodentium* burden after day 14, reaching >10^{10} CFU per mg feces [11]. In contrast, *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice stabilize the bacterial burden at ~10^{7} CFU per mg feces (Figure 4C). *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice were incapable of sterile clearance as late as day 40 post-infection (data not shown). Thus, *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice can harbor *C. rodentium* without developing lethal colitis.

At day 18 post-infection when most *Rag*$^{-/-}$ mice were moribund, *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice showed a strongly reduced pathology of the colon (Figure 4D-E). A loss in epithelial integrity as well as some inflammatory infiltration of mononuclear phagocytes was observed in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice. In contrast, *Rag*$^{-/-}$ mice showed a strong reduction of epithelial integrity and more cellular infiltration, paired with submucosal edema (Figure 4D). This indicates that mucosal barrier damage and inflammation as a consequence of *C. rodentium*-induced colitis is reduced in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice.

C. rodentium translocates to the spleen of both *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice

One of the major differences in the early response to *C. rodentium* between *Rag*$^{-/-}$ and wt mice is the presence of mucosal CD4^{+} T-cells. Th1 cells localize at sites of bacterial lesions, produce IFNγ, and aid in an early inflammatory response. However, Th1 cells also contribute to immune pathology [10-12,22]. Th17 cells are required to contain *C. rodentium* and to prevent aberrant intestinal pathology [23]. Overall, the lack of T-cells causes *Rag*$^{-/-}$ mice to develop a systemic multi-bacterial infection past the second week after oral infection due to this loss of colon barrier integrity [12]. Although pathologic features were significantly lower in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice (Figure 4D-E), *C. rodentium* was detected in splenic homogenates of these mice when cultured on MacConkey agar plates. However, more *C. rodentium* CFUs were cultured from *Rag*$^{-/-}$ spleens (Figure 4F). *C. rodentium* systemic translocation manifested in mice with both genetic backgrounds, albeit to a lesser extent in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice. This indicates that the mucosal barrier is less compromised in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice.

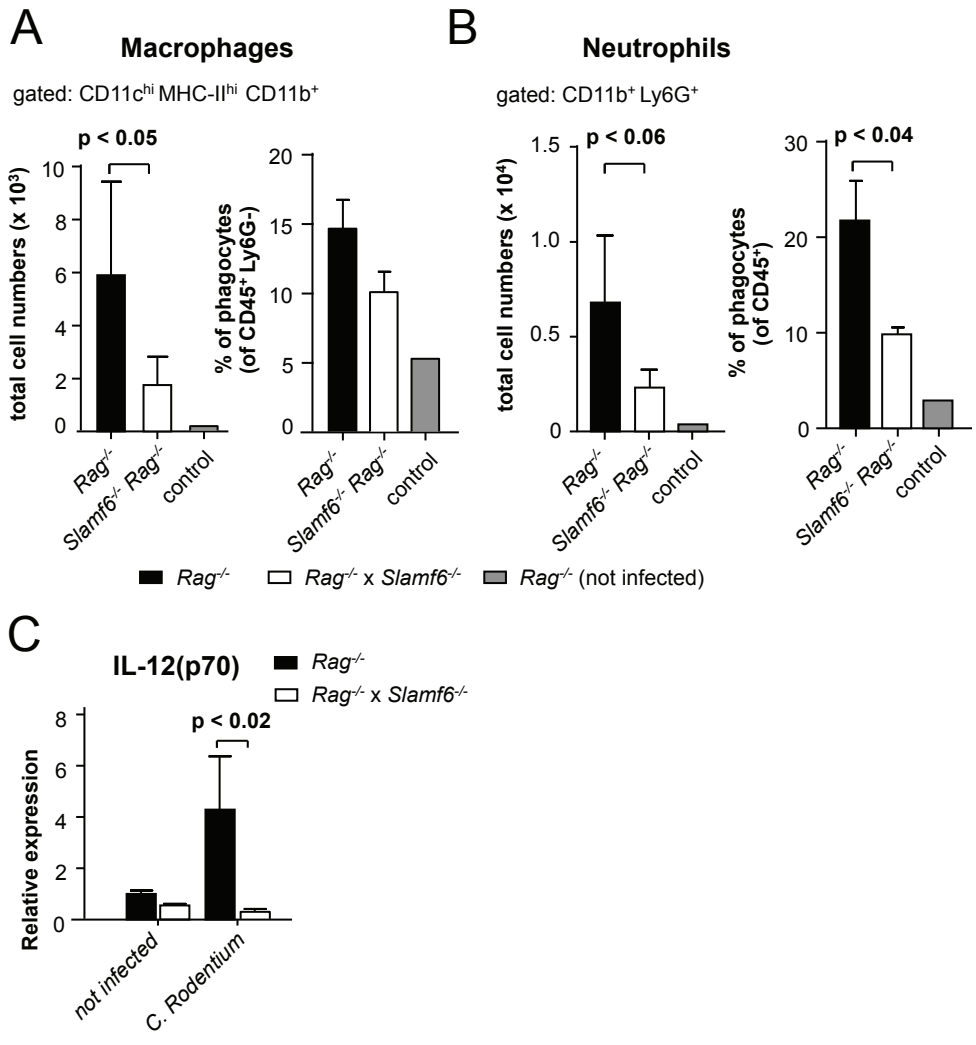


Figure 5 | Reduced lamina propria inflammation during *C. rodentium* induced colitis of *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice. *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice were infected by oral gavage of 2x10^9 *C. rodentium* bacteria. Uninfected *Rag*$^{-/-}$ mice are represented as controls. Animals were sacrificed on day 6 post-infection for analysis. (**A**) Quantification of flow cytometric analysis of isolated lamina propria macrophages. The total number of CD11c^{hi} MHC-II$^+$ CD11b$^+$ CD103$^-$ Ly6G$^-$ CD45$^+$ cells and the percentage of CD11c^{hi} MHC-II$^+$ CD11b$^+$ CD103$^-$ cells relative to the total pool of Ly6G$^-$ CD45$^+$ lamina propria cells are represented. (**B**) Quantification of flow cytometric analysis of isolated lamina propria neutrophils. The total number of CD11b$^+$ Ly6G$^+$ CD45+ cells and the percentage of CD11b$^+$ Ly6G$^+$ relative to the total pool of CD45$^+$ lamina propria cells are represented. (**C**) Quantitative PCR relative expression of IL-12(p70) in colon homogenates of uninfected *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice and 6 days after infection with *C. rodentium*.

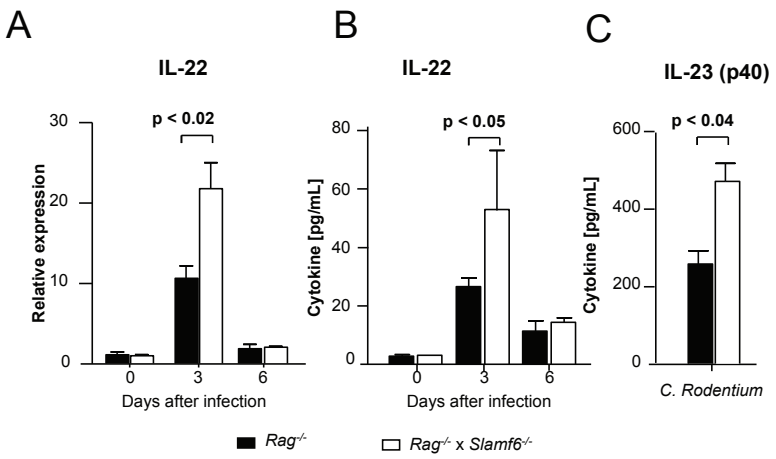


Figure 6 | Enhanced IL-22 production in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice during *C. rodentium* induced colitis. *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice were infected by oral gavage of 2x10^{9} *C. rodentium* bacteria. Uninfected *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice are represented as controls (day 0). Animals were sacrificed on day 3 or day 6 post-infection for analysis. (**A**) Quantitative PCR relative expression of IL-22 in colon homogenates. (**B**) IL-22 production of ex vivo cultured colon tissue. (**C**) Ex vivo IL-23(p40) production by 3-day infected colon tissue cultures.

Reduced inflammation in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice during *C. rodentium* infection

Having established that the absence of Slamf6 in T-cell and B-cell deficient mice improves the health of infected animals, the immune activation in the colonic lamina propria was assessed. The colonic lamina propria of *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice showed an influx of phagocytes in the early progressive stage of a *C. rodentium* infection. As predicted from our histopathology analysis (Figure 4D-E), smaller numbers of CD11b^{+} CD103^{-} macrophages and Ly6G^{+} neutrophils were detected in the lamina propria of *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice compared to *Rag*$^{-/-}$ mice 6 days post-infection (Figure 5A-B). As judged by quantitative PCR of colon homogenates, a reduced production of the inflammatory cytokine IL-12(p70) was observed in the colons of *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice (Figure 5C). This indicates that fewer inflammatory phagocytes migrate into the lamina propria of *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice. These inflammatory cells contribute to the clearance of the infection, but can also drive mucosal damage. Without an effective adaptive response, these inflammatory infiltrates contribute to local immune pathology. Therefore, the reduced pathology in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice could perhaps be explained by impaired monocyte and neutrophil recruitment.

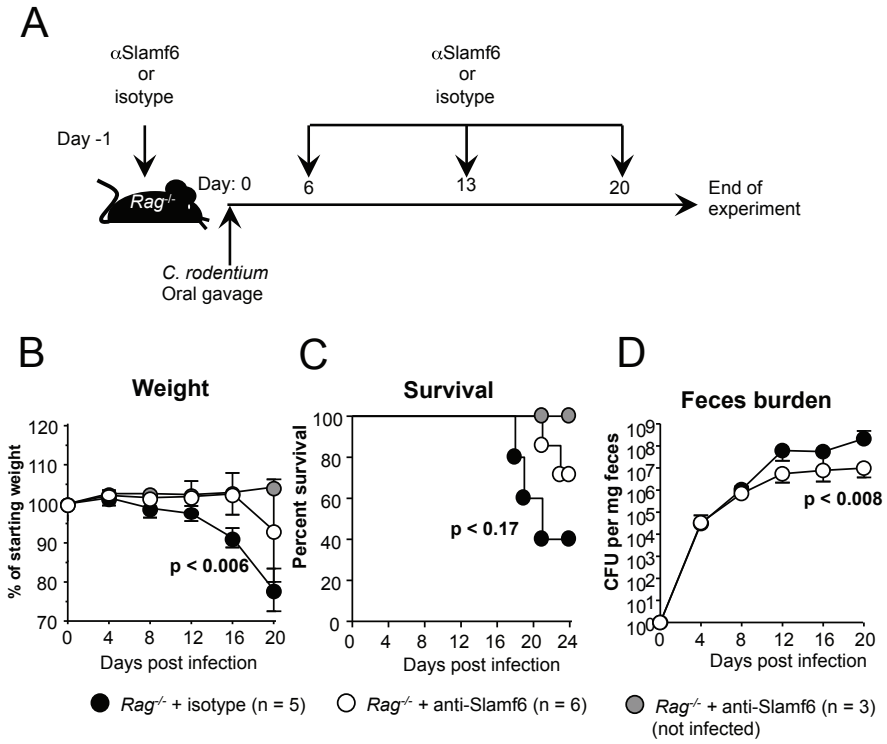


Figure 7 | Administering a monoclonal antibody directed against Slamf6 ameliorates *C. rodentium* induced colitis in *Rag*$^{-/-}$ mice. (**A**) Infection and treatment diagram for *Rag*$^{-/-}$ mice that were intra-peritoneally injected with 100µg anti-Slamf6 mAb (330) one day prior to infection and subsequently every seventh day after the first injection. Control mice were injected with 100µg mouse IgG2a in the same regimen. (**B**) The weight of individual mice was measured every four days and represented as a percentage of their weight on the day of infection. (**C**) Survival of infected anti-Slamf6 mAb treated and isotype treated *Rag*$^{-/-}$ mice. (**D**) *C. rodentium* counts in fecal pellets that were obtained directly from mice every three days. Serial dilutions were made in PBS and plated on MacConkey plates. Counted bacterial colonies are represented as CFU g^{-1} feces.

The presence of Slamf6 reduces the production of IL-22 in the colon of infected *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice

IL-22 and the cells that produce it are a key component of protective immunity to *C. rodentium* in the early stages of infection [16,17]. IL-22 production was assessed in infected colons of *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice at days 0, 3, and 6 after infection. As judged by qPCR of mRNA that was isolated from whole colon homogenates and by cytokine analysis of colon cultures supernatant, the peak of IL-22 production in both *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice is at day 3 post-infection. At this time, higher amounts of IL-22 were

detected in the colon of *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice. This difference reduced to a non-significant level at day 6 post-infection, when IL-22 levels had dropped to 3-fold higher than those of uninfected mice (Figure 6A-B). As IL-22 is one of the key protective cytokines during *C. rodentium* infections, this observation may contribute to the reduced pathology and lower number of translocated bacteria in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice (Figure 4F). As IL-22 production is driven primarily by IL-23, colon cultures were assessed for the production of this cytokine after *C. rodentium* infection. In contrast to the IL-12(p70) subunit of the IL-12 cytokine, *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ colons produced more IL-12/IL-23(p40) (Figure 6C), suggesting an enhanced production of IL-23 in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice. Interestingly, Slamf6 expression was detected in a small set of CD45.2^{+} CD90.2^{+} CD127^{+} RORγt^{+} (ILC3) cells, which represent the main producers of IL-22 in *Rag*$^{-/-}$ mice (Supplementary Figure S1C) [14,17].

Administering a monoclonal antibody directed against Slamf6 reduces *C. rodentium* colitis in *Rag*$^{-/-}$ mice

In order to test whether the anti-Slamf6 mAb, which inhibits the cognate interactions of Slamf6 with bacteria in the recognition assay (Figure 1), affects the bacterial colitis pathology, *Rag*$^{-/-}$ mice were i.p. injected with anti-Slamf6 or isotype (100 µg) one day before and every seven days after *C. rodentium* infection (Figure 7A). *Rag*$^{-/-}$ mice that received the anti-Slamf6 mAb showed a delay in their weight loss, a reduction of fecal *C. rodentium* burden at the peak of infection, and an increased survival rate (Figure 7B-D). These findings suggest that blocking the interaction of Slamf6 with bacteria reduces the pathology of *C. rodentium* induced colitis.

Discussion

In this report we provide evidence that *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice are markedly less susceptible to *C. rodentium* induced colitis than *Rag*$^{-/-}$ mice. Through the course of infection, *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice show a reduction in the inflammatory response in the lamina propria of the colon. Fewer bacteria translocate to the spleen of *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice and ultimately the infection is fatal in a significantly lower percentage of infected mice when compared to co-housed control *Rag*$^{-/-}$ mice. Moreover, anti-Slamf6 mAb treatment of infected *Rag*$^{-/-}$ mice improves the disease outcome associated with less weight loss, an improved survival rate, and a reduction in the fecal *C. rodentium* burden.

Our *in vitro* observation that Slamf6 can interact with certain Gram$^-$ bacteria suggests that the reduced colitis displayed by *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice in the context of *C. rodentium* infections might be mediated by the absence of the binding of Slamf6 to this bacterium. The observations that anti-Slamf6 mAb inhibited bacterial binding and that *in vivo* administration of this antibody reduced the pathology of infected *Rag*$^{-/-}$ mice further corroborate this notion.

The balance between immune activation and mucosal protection is one of the defining factors in the pathogenic outcome of a *C. rodentium* infection. Whereas a range of key regulators of innate immune responses is indispensible for an effective response to *C. rodentium*, not all innate mechanisms are beneficial to the host. Several recent review articles discuss how innate receptors are involved in detection of *C. rodentium* [19,24-26]. Two major receptor families mediate inflammatory signaling in response to *C. rodentium*: Toll-like receptors and Nod-like receptors, both of which utilize the NF-κB pathway. Interestingly, TLR4 signaling does not appreciably contribute to host protection against *C. rodentium*, but rather enhances mucosal pathologic immune responses [27]. Contrary to this, TLR2 signaling promotes inflammatory responses as well as mucosal integrity [28,29]. The authors of these studies argue that TLR4-mediated immune activation drives immune pathology during *C. rodentium* infections, which can be reduced by the protective function of TLR2 [25]. Along similar lines, an excess of IL-1β is detrimental to infected mice, whereas normal levels are required for an appropriate response to *C. rodentium* [30]. From our data in *Rag*$^{-/-}$ mice, we conclude that Slamf6 also breaks tolerance and enhances inflammatory responses.

An interesting notion is that Slamf6 appears to affect the development of colitis only in *Rag*$^{-/-}$ mice. T cells and B cells play key roles in the development of an adequate immune response to *C. rodentium*. Mice that are impaired in their ability to mount a specific humoral response to the bacterium are unable to generate sterilizing immunity [11]. However, the protective role of CD4$^+$ T cells appears to be two-fold: the direct protective effects of lesion-proximal T cells and eventually T cell help to induce a sterilizing humoral immune response. μMT mice, which lack B-cells, but have T cells, develop a milder colitis than *Rag*$^{-/-}$ mice [12]. Additionally, *Rag*$^{-/-}$ mice in which CD4$^+$ T cells were adoptively transferred show more inflammation, but also better mucosal protection [20]. Both Th1 and Th17 cells have been implicated in the immune response to *C. rodentium* [19]. The lesions at sites of *C. rodentium* infections are associated with IFNγ-producing Th1 clusters that contribute

to the local response to the infection [10,22]. Th17 development contributes to a protective response and CD4$^+$ T cell depletion leads to an exacerbated pathology and increased loss of barrier function. Although Slamf6 negatively affects IL-17A production by CD4$^+$ T cells, this suppressed Th17 response does not appear to significantly affect the pathology that is caused by *C. rodentium* infections. The differences in immune activation between *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice precede the time when a humoral response would arise in wt mice. Therefore, the observations that Slamf6 contributes to the pathology in *Rag*$^{-/-}$ mice, but not wt mice, are likely due to the absence of CD4$^+$ T cells from these mice that play a protective role in mucosal integrity.

Ample studies have addressed functions of Slam receptors in lymphocyte and phagocyte functions. However, little is known about their functions in mucosal phagocytes and innate lymphocytes. Here, we show that Slamf6 affects the cytokine production of these cells, thereby affecting a critical component of the immune response to attaching bacteria. In wt as well as *Rag*$^{-/-}$ mice interactions of CD103$^-$CX$_3$CR1$^+$ colonic macrophages with *C. rodentium* result in the phagocytosis of the bacterium [14,15,31]. CX$_3$CR1-deficient macrophages are impaired in their ability to probe the lumen of the intestine, which in wt as well as *Rag*$^{-/-}$ mice results in decreased IL-22 production and hence increased pathology of *C. rodentium* infection. Thus, interactions of colonic phagocytes with *C. rodentium* result in the production of IL-22 by phagocyte-proximal innate lymphocytes in *Rag*$^{-/-}$ mice [31]. In another study, the role of IL-22 in the innate immunity to *C. rodentium* infection was clearly demonstrated utilizing an IL-22–specific antibody. Administration of this antibody to *Rag*$^{-/-}$ mice led to rapid weight loss, more severe colitis and expedited mortality [17]. Conversely, vitamin D receptor deficient mice have higher numbers of ILC3 and produce more IL-22. Consequently, these mice have a reduced bacterial burden and lower immune activation [32].

Since Slamf6 affects *C. rodentium* binding *in vitro* and IL-22 production *in vivo*, we propose a role of Slamf6 in the immune mechanisms that involves the recognition of bacteria by CX$_3$CR1$^+$ macrophages and the subsequent production of IL-22 by ILC3 cells. Higher levels of IL-22 result in stronger mucosal protection, thereby lessening epithelial damage [19]. We therefore speculate that engagement of *C. rodentium* by Slamf6 negatively affects phagocyte-induced signals that promote IL-22 production. Longman et al. have shown that IL-23 and IL-1β production by phagocytes plays a role in the induction of IL-22 production by ILC3 cells [14]. We report that infected colons of *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice produce

less IL-12(p70) and more IL-12/23(p40). Thus, Slamf6 has important implications in the development of a colonic innate immune response to pathogenic Gram$^-$ bacterial challenge. Slamf6-deficiency in innate cells renders the outcome of *C. rodentium* mediated immune activation more inflammatory and less protective, which profoundly affects the pathology and survival of *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice.

Experimental procedures

Mice

Slamf6$^{-/-}$ C57BL/6J mice were described previously [33]. These mice were interbred with Rag1$^{-/-}$ mice that were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Age and sex matched wt and Rag1$^{-/-}$ C57BL/6J mice were bred in-house and originally purchased from Jackson Laboratory. Mice were co-housed for at least 10 days prior experimental use. All animals were maintained under specific pathogen-free conditions at the Center for Life Science animal facility of the BIDMC and were used at 8-13 weeks of age. The experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee at BIDMC.

Evaluating bacterial binding by the IL-2 luciferase assay[6]

Chimeric constructs, consisting of extracellular Ig-domains of the Slamf6 receptor fused with the signaling competent cytoplasmic domain of CD3ζ, together with an IL-2 promoter-driven Firefly luciferase gene and Renilla luciferase under a mammalian promoter, are transfected into the Jurkat human T-cell line. Six hours after transfection, heat-inactivated bacteria are added as stimulation and incubated overnight. Cells are washed and lysed according to the manufacturers protocol (Dual Luciferase Reporter Assay, Promega, Madison, WI). Substrates for Firefly luciferase and subsequently Renilla luciferase are added to 10 µL of the cell lysates and luminescence is measured using a standard Glomax luminometer (Promega). Values represent the ratio of Firefly and Renilla luminescence.

In vivo bacterial infection by oral gavage

C. rodentium (DBS100) (ATCC#51459) was cultured in LB-broth for four hours and washed in PBS prior to inoculation via oral gavage. Bacteria (2×10^9) were resuspended in

200 µL PBS, which was used for the inoculation of one mouse. The mice were monitored daily for morbidity and their weight was recorded every 3 or 4 days. Mice were sacrificed for analysis when their weight dropped below 80% of their starting weight or at the end of the experiment. Fresh stool pellets were collected in Eppendorf tubes for serial dilution in PBS and plating on MacConkey agar plates for quantification of CFUs.

Treatment with an antibody directed against Slamf6

Rag$^{-/-}$ mice were intra-peritoneally injected with 100 µg anti-Slamf6 mAb (330) one day prior to infection and subsequently every seventh day after the first injection. Control mice were injected with 100 µg mouse IgG2a in the same regimen.

Adoptive CD45RB^{hi} CD4^{+} T cell transfer colitis

Adoptive transfer of CD45RB^{hi} CD4^{+} T-cells into *Rag*$^{-/-}$ or *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ recipients was described previously [34].

Histology

Fresh colon tissue was harvested and fixed in paraformaldehyde (10%). H&E staining was performed on slices of the proximal, medial, and distal part of the colon. Scoring was performed by an independent pathologist (A.K.B.) assessing: (1) mononuclear cell infiltration, (2) epithelial integrity, (3) hyperplasia, and (4) edema.

Flow Cytometry

Macrophages and DCs were incubated with anti-CD16/32 antibody to block Fc receptors at 4°C for 20 minutes. All samples were stained with relevant antibodies on ice for 30 min. Dead cells were excluded by DAPI (Roche, Indianapolis, IN, USA). The cells were acquired on a BD LSRII flow cytometer and the data analysis was performed using the FlowJo analysis package (Tree Star Inc. Ashland, OR, USA).

Cytokine analysis

Colons were harvested, washed in PBS with gentamycin (100 µg/mL) and 100 mg tissue was cultured in 1 mL complete DMEM for 24 hours. The amount of cytokines in the supernatant was analyzed using LEGENDplex reagents (Biolegend, San Diego, CA, USA)

or CBA reagents (BD biosciences, San Jose, CA, USA) according to the manufacture's instructions.

Taqman qPCR

Fresh colon tissue was collected, washed in PBS and homogenized by sonication in TRIzol reagent (Life Technologies). The vendor's protocol was followed for RNA purification. FAM/MGB 16sRNA probes were used as control housekeeping genes. Commercially available FAM/MGB probes (Mm04203745_mH) and (Hs01011518_m1) were used for Iltifb (IL-22) and Il12b (IL-12(p70)), respectively (Life Technologies, Grand Island, NY, USA). Analysis was performed using the 7500 FAST Real Time PCR.

Statistical analysis

The Prism 5.0 software (GraphPad, San Diego, CA, USA) was used for results analysis. Results are reported as mean ± SEM. Most of the statistical comparisons were performed using the 2-tailed Student's t test. Values of $p < 0.05$ are considered to be statistically significant.

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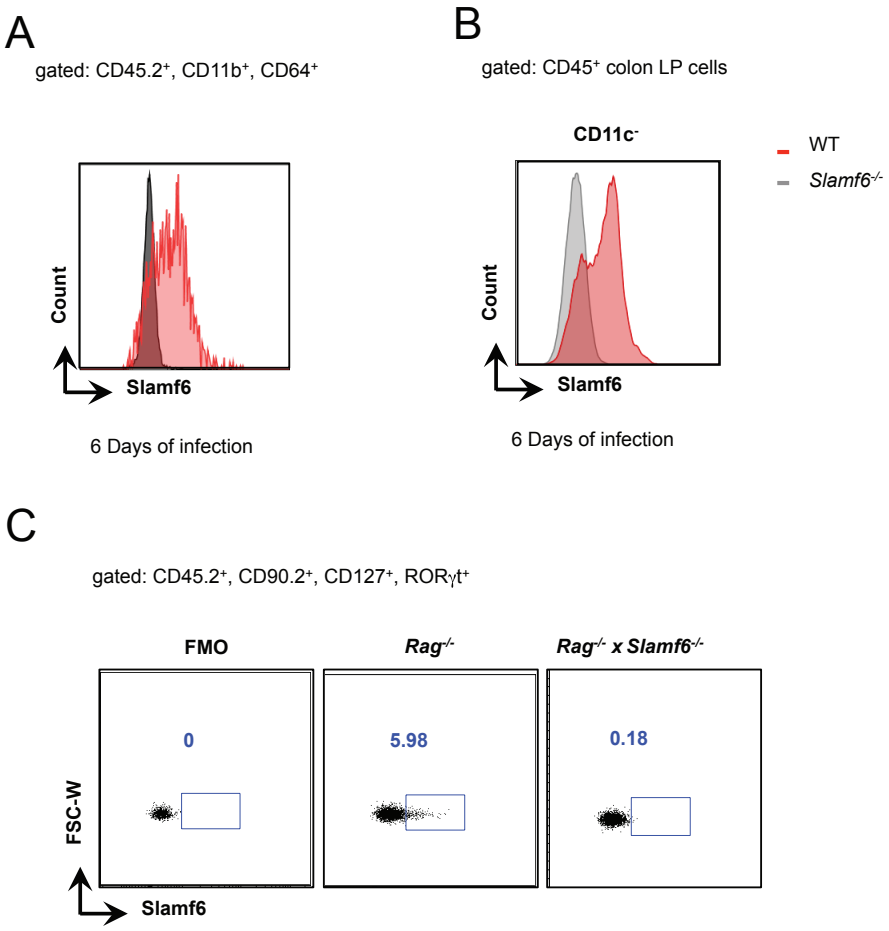
Supporting Information



Supplemental Figure 1 | Expression of Slamf6 in infected colons. (**A**) Slamf6 expression in isolated colon lamina propria CD11c- leukocytes, on day 6 post-infection. (**B**) Slamf6 expression in CD11b$^+$ CD64$^+$ colon lamina propria macrophages, 6 days after infection. (**C**) Expression of Slamf6 on colon lamina propria isolated CD45$^+$ CD90.2$^+$ RORγt$^+$ CD127$^+$ CD3ε$^-$ leukocytes, obtained from *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice by flow cytometric analysis. From left to right: *Rag*$^{-/-}$ fluorescence minus one (no Slamf6 staining) (FMO), *Rag*$^{-/-}$ and *Slamf6*$^{-/-}$ *Rag*$^{-/-}$.

Chapter 5

Summary

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Signaling Lymphocyte Activation Molecule family (Slamf) receptors can operate in three distinct modes. As an immunological voice of nuance, Slamf receptors can dictate the extent of immune responses, thereby maneuvering immunity to the optimal zone between immunopathology or autoimmunity and weak, ineffective immune responses. An example of this is the modulation of cytotoxic responses by Slamf4, Slamf6, or Slamf7. This mode is mostly mediated by the adaptors SAP and by Eat-2(a/b) and hence involves signaling and transcriptional regulation. A second mode of action, which is more strictly correlated with SAP binding to Slamf signaling tyrosines, affects the development of leukocytes. The prime example here is the total absence of NKT cells from SAP- or Slamf1 x Slamf6-deficient mice. Slamf receptors and SAP signaling have recently been ascribed to the development of other innate-like lymphocytes as well. Lastly, Slamf receptors can have direct functional involvement in immune effector mechanisms. Slamf1 or Slamf6 cognate interaction with specific Gram$^-$ bacteria represents one of these direct modes of operation. Adaptor molecules may not be for the highest importance for these functions.

The seminal theory postulated by Metchnikoff that gave birth to cellular immunology was based on the observation of phagocytes in various simple organisms. Phagocytes are typically considered to be primordial immune cells. They represent a collection of developmentally distinct cells that are unified by their capacity to engage foreign entities and engulf them into specialized vesicles called phagosomes. Macrophages, dendritic cells, and neutrophils represent major classes of phagocytic cells, all of which are extensively plastic. Depending on the environmental context, they scale between immune-suppressive to pro-inflammatory.

The differences between macrophages and DCs, specifically in the lamina propria of the colon, have been a topic of debate. Traditional cell surface markers were proven insufficient to distinguish between them. Rather, functional features – combined with devel-

opmental origin – should be utilized to categorize these cell types. Typically, DCs are excellent antigen presenters to T-cells while macrophages are not. What is more important during colonic inflammation is that the influx of neutrophils and monocytes is responsible for the increase in numbers and change in features of phagocytes. Under these conditions, monocytes give rise to pro-inflammatory mononuclear phagocytes that have been deemed macrophages in some reports and DCs in others.

Phagocytes use innate receptors to sense their environments. The concept of germline-encoded receptors that engage in cognate interactions with microbial entities was proposed by Janeway and subsequently championed by Medzhitov. This theorem has proven to be one of the cornerstones of innate immunity to microbial pathogens. Toll-like receptors (TLRs) are the most widely studied of these pattern recognition receptors (PPRs) and collectively TLRs are specific for a range of pathogen-associated molecular pattern (PAMPs), including LPS, nucleotides, and non-essential molecules such as flagellin. An ever-growing slew of PRRs are being described, all with their own specificity and tightly regulated implications on the development of immune responses. Generally, these implications arise due to the assembly of downstream signaling platforms. Research from the Terhorst lab and other groups have shown that Slamf receptors can also be considered PRRs.

Depending on the specific type of phagocyte, these cells have an extensive arsenal of effector mechanisms to effectively process pathogens. The generation of reactive oxygen species (ROS) and nitric oxide (NO) represent a set of these mechanisms. Slamf1 clustering, caused by interactions with bacterial entities or by other means, results in the formation of the NADPH-oxidase 2 enzyme-complex, which produces ROS. Specifically, when NADPH-oxidase 2 is activated it generates superoxide, which is quickly converted to peroxide. In activated phagosomes, peroxide can react with NO to form peroxynitrate, which is bactericidal even *in vitro*.

The classic paradigm that production of ROS by phagocytes is instrumental in their bactericidal activity is complemented by more recent studies that have established a role in cell adhesion and migration. Slamf1 acts in phagocytic cells as an enhancer of cell motility as is shown in Chapter 2. Subsequent experiments showed that the effect on phagocyte motility of Slamf8, which is the receptors that appears to – in effect – antagonize Slamf1's enhancement of reactive oxygen species production, is also reversibly upheld. These

phenotypic parallels appear to be linked, as inhibitions of reactive oxygen species production resulted in a loss of the migration phenotypes.

Because Slamf receptors have well-described functions in cell-cell communication between T and B lymphocytes, we eliminated the interference of Slamf receptor functions in these cells by using Slamf1$^{-/-}$*Rag*$^{-/-}$ in Chapter 3 and Slamf1$^{-/-}$*Rag*$^{-/-}$ mice in Chapter 4 to induce experimental colitis. This allowed us to test the effect of Slamf receptor deficiencies on phagocytes.

In assessment of the *in vivo* implications of the effects of Slamf1 on bactericidal activity and cell motility, murine models of intestinal inflammation are described in Chapter 3. Slamf1 contributes to the development of colitis in mice. It regulates the appearance of pro-inflammatory monocytes in inflamed intestinal tissues. The killing of bacteria is an important feature of mucosal immunity, especially in the context of inflammation. Slamf1 expedites the killing of specific Gram^{-} bacteria by phagocytes. However, the effect of Slamf1 on this process appears to be subordinate to the cell migration phenotype. Speculatively, alternative explanations arise when we consider that bacterial processing in the presence of Slamf1 may render phagocytes more 'active' and hence contribute to inflammation.

Citrobacter rodentium infections in mice resemble enteropathogenic *Escherichia coli* in humans, causing diarrhea. This murine model that approximates human bacterial colitis furthered the understanding of the interplay between Slamf receptors in phagocytes and Gram^{-} bacteria. Chapter 4 describes how Slamf6, which is the closest functional relative of Slamf1, also engages Gram^{-} bacteria, including entities on *Escherichia coli* and to some extent *Citrobacter rodentium*. Immunity to these bacterial infections relies on adaptive responses; *Rag*$^{-/-}$ animals succumb to a *Citrobacter rodentium* infection. *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ mice survive this colitis, develop a very mild immune response, and uphold a better mucosal barrier function in the face of this bacterium. Concomitant with the reduced systemic translocation of the bacteria is an enhanced production of IL-22, which is a key cytokine for the maintenance of the mucosal integrity. The production of IL-22 depends on the detection of *Citrobacter rodentium* by lumen-probing phagocytes. We have therefore hypothesized that Slamf6 is involved in this pathway by engaging the bacterium.

Of importance, antibodies that block Slamf1 or Slamf6 reduce colitis in mice. We observe consistent parallels between the phenotypes of mice deficient for Slamf1, Slamf8, or Slamf6 and mice that received the respective monoclonal antibodies during the exper-

iments. This suggests that engagement with a ligand is required for the *in vivo* functions of these receptors, regardless whether the ligands are homophilic or of bacterial origin. Effects of the Slamf-specific antibodies are promising in the light of therapeutic approaches. Manipulation of phagocyte mobility may prove of interest for vaccine applications, especially when an antibody (i.e. anti-Slamf8) can enhance migration.

In conclusion, Slamf1 and Slamf6 operate alongside of the well-described B and T lymphocytes also in phagocytic cells. When they do, these receptors tip the scales toward inflammation. Slamf1 enhances phagosome progression, but also utilizes aspects of this mechanism to influence cell motility. Slamf8 puts restrictions on these processes. Slamf6 can engage bacteria and – during bacterial colitis – effectively enhance pro-inflammatory processes. How interactions of individual Slamf receptors influence the function of phagocytes and what adaptor molecules are involved in specific functions, either induced by Slamf-Slamf or by Slamf-microbe interactions, needs further investigation. Additional studies that focus on the differences in downstream effects of Slamf receptor engagement between these two modes of interaction will be instrumental in our understanding of Slamf functions in phagocytic cells.

Hoofdstuk 6

Samenvatting

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Signalering Lymfocyt Activatie Molecuul (Slamf) familie van receptoren kunnen in drie modules actief zijn. Als 'stem der nuance' kunnen ze de mate van de immuun reactiviteit bepalen. Hierdoor dragen ze bij aan het bereiken van de optimale effectiviteit, tussen immuun pathologie of auto-immuniteit en een te zwakke reactie. Een voorbeeld hiervan vinden we in het moduleren van Slamf4, Slamf6, en Slamf7 van de cytotoxiciteit van killer cellen. SAP en Eat-2 – twee adaptoren van Slamf receptoren – spelen hierin een voorname rol. Deze module leidt dan ook tot intracellulaire signalen en transcriptie regulatie. De tweede module beïnvloed de ontwikkeling van leukocyten en stoelt nog sterker op de functies van SAP. Het beste voorbeeld hiervan is de volledige afwezigheid van NKT cellen van SAP-deficiënte muizen of Slamf1 x Slamf6 dubbel deficiënte muizen. Recentelijk is de betrokkenheid van SAP in de ontwikkeling van andere aangeboren immuniteit-achtige lymfocyten aan het licht gebracht. Tot slot kunnen Slamf receptoren een directe invloed hebben op functionele immuun mechanismen. De herkenning van Slamf1 en Slamf6 van bepaalde bacteriële structuren leidt tot één van deze modules. Adaptor molekulen zijn wellicht minder van belang in deze functies van Slamf receptoren.

De theorie die Metchnikoff postuleerde, waardoor hij later werd beschouwd als grondlegger van de cellulaire immunologie, is gebaseerd op zijn bevindingen in fagocyten uit verscheidene kleine organismen. Deze cellen worden vaak gezien als oer-immuun cellen. Het collectief van deze cellen met uiteenlopende voorouders worden verenigd door hun capaciteit om lichaamsvreemde (en lichaamseigen) microstructuren op te nemen in speciale vacuoles, genaamd fagosomen. De even flexibele macrofagen, dendritische cellen (DCs), en neutrofielen representeren de grootste klassen van fagocyten. Afhankelijk van hun omgeving zijn deze cellen immuun-suppressief of inflammatie bevorderend, of daartussen in.

Het verschil tussen macrofagen en DCs, in het bijzonder in de lamina propria van de colon, is een onderwerp van discussie. De traditionele manieren om deze cellen te onder-

scheiden (middels cel oppervlakte kleuringen), zijn niet toereikend gebleken. Functionele aspecten – gecombineerd met kennis over hun voorouders – leiden tot een betere onderscheiding. Het belangrijkste daarbij is dat DCs goed zijn in het presenteren van antigeen aan T cellen en macrofagen niet. Hetgeen van grotere relevantie is, is het gegeven dat neutrofielen en monocyten na mobilisatie naar ontstekende darmen worden getrokken en op die manier de samenstelling en de hoeveelheid van fagocyten in de lamina propria veranderen. Als dit gebeurt, zijn deze monocyten de precursors van inflammatoire fagocyten die soms macrofagen worden genoemd en soms DCs.

Fagocyten gebruiken kiem lijn gecodeerde receptoren om hun omgeving waar te nemen. Het concept van deze aangeboren receptoren die een interactie zoeken met microbiële entiteiten is naar voren gebracht door Janeway en vervolgens verder uitgedragen door Medzhitov. Deze theorie is één van de hoekstenen gebleken van aangeboren immuun reactie op microbiële pathogenen. Het meest bekende voorbeeld van deze patroon herkenning receptoren (PRRs) zijn de toll-like receptoren (TLRs). Samen dekken zij een scala aan pathogeen-geassocieerde moleculaire patronen (PAMPs), waaronder LPS, nucleotieden, en niet-essentiële molekulen zoals flagellin. De groep van PRRs waar we kennis van hebben is nog altijd aan het uitbreiden. Allen hebben zij hun eigen specificiteit en nauw gereguleerde implicaties op de ontwikkeling van een immuun reactie. Over het algemeen volgen deze implicaties op de vorming van een signaleringsplatform aan de geactiveerde receptoren. Onderzoek in het Terhorst laboratorium en onderzoek in andere laboratoria hebben laten zien dat sommige Slamf receptoren ook kunnen vallen onder PPRs.

Afhankelijk van het type fagocyt, heeft deze cel een groot arsenaal aan antimicrobiële effector mechanismen. De productie van reactief zuurstof (ROS) en stikstof oxide (NO) is onderdeel van dit arsenaal. Slamf1 clustering resulteert in de formatie van een enzymcomplex (NADPH-oxidase 2), wat ROS produceert. Dit product wordt snel omgezet in waterstofperoxide wat weer kan reageren met NO om peroxinitraat te vormen. Peroxinitraat is zelfs buiten cellen erg antibacterieel.

Het klassieke paradigma dat ROS productie door fagocyten een belangrijke bijdrage heeft in antibacteriële werkingen is gecomplementeerd met recentere vindingen dat het ook cel adhesie en celmigratie beïnvloedt. Slamf1 fungeert in fagocyten door hun mobiliteit aan te wakkeren, zoals beschreven in Chapter 2. Aansluitende experimenten lieten zien dat het effect van Slamf8 op zowel ROS productie als op fagocyt mobiliteit tegengesteld is aan dat van Slamf1. Deze beide fenotypische tegenstellingen zijn gelinkt, want onderdrukking

van de ROS productie onderdrukt ook het effect van Slamf1 en Slamf8 op celmobiliteit.

Omdat Slamf receptoren bekende en duidelijke functies hebben op de communicatie tussen T en B lymfocyten, hebben wij de meeste muizenexperimenten gedaan met *Slamf1*$^{-/-}$ *Rag*$^{-/-}$ muizen zoals beschreven in Chapter 3 en *Slamf6*$^{-/-}$*Rag*$^{-/-}$ muizen zoals beschreven in Chapter 4. Hierdoor konden we het effect van deze receptoren op de functies van fagocyten beter isoleren.

In een bijdrage aan de kennis van de invloed van Slamf1 op fagocyten, beschrijft Chapter 3 muismodellen voor colitis. Slamf1 draagt actief bij aan de ontwikkeling van colitis. Het reguleert het verschijnen van inflammatoire monocyten in ontstoken darmweefsel. Het doden van bacteriën is een relevante functie van het mucosale immuun systeem, in het bijzonder in de context van inflammatie. Slamf1 versneld het verwerken van Gram^{-} bacteriën door fagocyten. Echter, dit effect blijkt ondergeschikt aan het positieve effect op cel migratie. Speculatief kunnen alternatieve verklaringen ontstaan als Slamf1-afhankelijke antibacteriële processen leiden tot een verhoogde activatie van de betreffende fagocyt, en derhalve een bijdrage leveren aan de ontstekingsreactie.

Citrobacter rodentium infecties in muizen zijn vergelijkbaar met enterophatologische *Escherichia coli* infecties in mensen en veroorzaakt diarree. Dit murale model heeft bijgedragen aan het begrip van de relatie tussen Slamf receptoren en Gram^{-} bacteriën. Zoals beschreven in Chapter 4 bindt Slamf6 (meest gelijkende familielid van Slamf1) ook aan Gram^{-} bacteriën, waaronder *Escherichia coli* en tot op zekere mate *Citrobacter rodentium*. Immuniteit tegen deze bacteriële infecties is gestoeld op adaptieve reacties zoals het maken van specifieke antilichamen; *Rag*$^{-/-}$ dieren sterven aan de gevolgen van een *Citrobacter rodentium* infectie. *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ muizen overleven echter deze colitis, ontwikkelen een sterk afgezwakte reactie en onderhouden de mucosale barrière beter. Evenredig aan een verminderde disseminatie van deze bacterie, is de productie van IL-22 (een essentieel cytokine in het bevechten van *Citrobacter rodentium*) verhoogd in *Slamf6*$^{-/-}$ *Rag*$^{-/-}$ muizen. Slamf6 heeft dus een rol in het proces tussen bacteriële herkenning en de beschermende functie van de productie van IL-22.

Van belang is dat antilichamen die Slamf1 of Slamf6 blokken colitis reduceren. We hebben consistent parallellen gevonden tussen de fenotypen van Slamf-deficiënte muizen en muizen die zijn behandeld met deze antilichamen. Het lijkt er dus op dat expressie én interactie van Slamf receptoren noodzakelijk zijn voor het effect op hun *in vivo* functies, ongeacht of

het ligand de receptor zelf is of van bacteriële origine. De implicatie van de observaties met Slamf-specifieke antilichamen zijn veelbelovend in het licht van therapeutische applicaties. Manipulatie van fagocyt mobiliteit kan bijdragen aan het verbeteren van vaccins, zeker als fagocyt migratie kan worden geboost door een (anti-Slamf8) antilichaam.

Concluderend, Slamf1 en Slamf6 opereren buiten hun uitgebreid beschreven functies in B en T lympocyten ook in fagocyten. Zodra deze receptoren werken in fagocyten, verschuiven ze het homeostatisch evenwicht ten gunste van inflammatie. Slamf1 stuwt de progressie van fagosomen, maar gebuikt aspecten van dit proces ook om cel mobiliteit positief te beïnvloeden. Slamf8 remt deze processen. Slamf6 bindt aan bacteriën en – tijdens bacteriële colitis – bevordert inflammatoire processen. Hoe individuele Slamf receptoren invloed uitoefenen op de functies van fagocyten, geïnduceerd door Slamf-Slamf interacties of door Slamf-bacteriële interacties, behoeft verder onderzoek. Vergelijkend onderzoek tussen de gevolgen van Slamf interacties met Slamf receptoren ten opzichte van Slamf interacties met microben zullen ons preciezere inzichten geven in dit onderwerp.

Addendum

Acknowledgements

The alternative story of S.

Prologue

The genesis of this molecule does not share any space with the rest of its story here, though it was familiar ground to all parties involved. He started his journey on the projector in the blue lecture hall in the UMCU[1], brought into my world by prof. dr. Terhorst[2].

Flash forward past construction of the administrative foundation on which his story rests[3-5].

Genesis

Somewhat shaken up by his the jetlag it stares at the mere two buildings that constitute the skyline. The world interacts differently with you on this side of the ocean. "Excellence!"[6]. Here, the interaction generated a more infectious environment, which turned out to be liberating in that way. The air is thicker also. Crisp, yet pregnant with academic thoughts. He was ready! Then it thinks of his siblings. Will they visit[7]? Will they be equally excited[8]!?

I would argue that the start of love between women and men holds a similar ontology for molecules as well[9]. The journey had been a long time coming. In fact, the end of the winning streak of the local hoop-shooting sports team had signified it[10]. Tears of sorrow or tears of joy – who knows – tears nonetheless.

The first mission

Perhaps not unique to this kind of stories, the journey was full of encounters with the most interesting of people. In the general vicinity, clarity was provided by knocking off a dimension of the real world without losing complexity[11,12]. The thought of the significance of one's own motility started to appeal to S after meeting these figures. Under the same roof, the young one was bunkered into the German *grundlichheid* providing a rock-solid base[13], while planting the seeds for the solution to the mission to the dark side. Modest and insightful words came from across the street[14]. These words encouraged finishing the mission, because it had been completed.

The second mission – or the void

What then!? Next up!? It tried to make friends in all corners of the living world. The mice provided a home. That was good. But their furriness was a disguise for their sharp teeth and unpredictability. The temperament of one of the bugs was reflected by the paradigms about their original place of decent. Lots of movement, yet progression remained absent[15]. Aaaahh!? "Ever tried. Ever failed. No matter. Try again. Fail again. Fail better[16]." Dark at times, but one must convince oneself that the thing he does at any given time, is of utmost importance. This was the most impactful message that was mostly nonverbally communicated many a time[17]. They kept on moving. We did realize that the air we breathe contains the oxygen we use for destruction. We needed to breathe, and destructive it felt at times. Uncle had known this[18]. Now this insight moved us both.

The third mission

The earlier realization that the indivisible unit is not the relevant one again had promptness. It started with the realization that Friday does actually rhymes with Wine. This truth ascended from the tightknit network, the interactions that created things [19-22]. Critically, trough this truth arose the blueprints to the solution to the third question. The other great influences were oriental[23,24]. Like many sound and tranquil masters, they provided just that – tranquility and insight. Albeit from opposite ends.

Epilogue

The alpha and the cradle had both the courage to let go and the strength to hold on[25,26]. Scientifically, the progenitor is important for classification, but ultimately environmental cues determine one's role[27,28]. As immunology is the most holistic biomedical discipline – all of it has to do with everything – S felt at home in this universe[29]. S had known that this story (about the discipline of immunology) is about blending an organism into their environment, not about shielding it from the world. This fact justified S's colorful and complex personality.

Conclusions

Lesson #1_There are immune receptors (the Slamf's) that modulate inflammatory functions of phagocytes; they alter their motility and the way phagocytes interact with bacteria by altering intracellular ROS production.

(Alternative) Lesson #2_If you fail to love and share this world with your environment, you will be alone and incomplete – the significance of living is found in the interactions, with our own and with others.

List of thanks

1 Romero, X. You provide guidance and friendship, as well as Xavisms, that made the initial time so much fun. *Terhorst Lab* **1**, 12 (2010).

2 Terhorst, C. You have been an inspiring mentor. In addition, you deeply care for (your) people. I have tried to make every day a day of learning – from you and from the work you enabled me to do. . *Smart people I know / Promoter* **1961** (2011).

3 Lefort, M. Solving visa-, mouse-, employment-, and other administrative (and humane) issues. *Administrative Superpowers inc.* **BIDMC**, 9th (2011).

4 Eldik-Bonouvrie, J. v. Provided fast and good solutions and sorted out the process as well as the subject (me) of the discertation. *Administrative Superpowers inc.* **Hubrecht Institiute** (2014).

5 Steverink, J. Thanks for providing your expertise (again). *Creative Superpower inc.* **Blick** (2015).

6 Leescommissie. quote after reading *Origin of Species*. *Dr. M. L. Boes, Prof. dr. J. Borst, Prof. dr. L. Koenderman, Prof. dr. F. Miedema, Prof. dr. C. E. Hack* **My gratitude for taking the time to read and evaluate my thesis.** (2015).

7 Justin, Mexime, Emi, Xem van Driel. The indivisible unit is often not the relevant one. *Family* **later additions** (1987, 1999, 2000, 2001).

8 Slamf6, Slamf8. Gratitute for sharing the excitement about Slamf6 and Slamf8 to all of the Terhorst lab members who worked on these two bad boys. *Terhorst Lab* **6, 8** (2013).

9 Kundera, M. The love of a man for a woman begins when she places her first words into his poetic memory. *The Unbearable lightness of Being* **Wedding words to Carlien Frijlink**, 2011 (1988).

10 Flipse, J. You were there. Thank you for staying in touch. . *Good Men (PhD)* (2010).

11 Bahn, A. The long conversation you demanded of the scientists that visit you with slides before you allowed them to get to the scientific work, was what kept me coming back with more slides. (Our talks about Indian migration come to mind). *Smart people I know* **MGH** (2012).

12 Mizoguchi, E. You are among the kindest people in this world. . *Kind people I know* **MGH** (2012).

13 Reinecker, H.-C. You have provided feedback on every aspect of my work. Thank you. *Smart people I know* **MGH** (2012).

14 Luscinskas, F. B. Also thanks to Vero. *NRB* (2012).

15 Burleigh, B. Although the outcomes of our collaboration have not been fruitful in a scientific way, I did learn a lot from our interactions. *Swimmers and Sinkers* (2013).

16 Beckett, S. Quoted by Cox may a times, sometimes to my frustration, but more often it provided a source of inspiration. *Quotes* **2** (~1970).

17 Heesters, B. Thank you for your friendship and all the rest. *Let's continue the conversation* **oneven** (2008).

18 Wang, G. For all of our different traits, we understand each other remarkably well, thanks. *Good Men (PhD)* **F8** (2013-2015).

19 Detre, C. Knowing you is an experience all by itself. *Weird people I know (said with the most respect and appreciation)* **Californian Hippy** (2011).

20 Yigit, B. Little girl, we share a history. I feel you understand me very well. (miss you). *Turkish Activists* **keep going!** (2012).

21 Keszei, M. Representative for the true scientists of our generation. *Optimists I know* **1** (2010).

22 O'Keeffe, M. Gut brothers are the smelly kind of blood brothers. *Wine drinkers* **Republican** (2011).

23 Liao, G. Experimental guru, thanks for teaching me. *Ninjas* **1** (2012).

24 Wang, N. Mouse guru, thanks for teaching me. *Ninjas* **2** (2014).

25 Driel, P. v. Your presence kept us sound and safe during our big enterprise. *Parents* **Love, respect, and gratitude** (1984).

26 Jobse, M. Your warmth kept us grounded *Parents* **Love, respect, and gratitude** (1984).

27 van der Lee-van Driel, A. Thanks most for your love, guidance and debate. *Family* (1995).

28 Frijlink, H.C. Thank you for letting go of your daugther, to let us embark on our great journey. We will be here for you on your current journey. *Family* **2** (2010).

29 Clevers, H. Your presence without being present provided the safety that I needed at times - to continue. Thank you. *Promoter* **1** (2015).

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Curriculum vitae

On 28 November 1984, Boaz Job van Driel was born in Breda, the Netherlands. There he finished high school in six years, obtaining a diploma from Graaff Engelbrecht College at age 18. In the summer of 2003 he ventured to Utrecht enrolled in the Biomedical Sciences Bachelor program at Utrecht University. Five years later he received his Bachelor degree, while having spent one fulltime academic year in the board of his fraternity as Aedilis Internus, as well as having invested some time in courses at the faculty of philosophy. In the same year Boaz enrolled in the Master program of Immunity and Infectious Diseases at his alma mater. During this two-year program, he interned at the faculty of Veterinary Medicine, department of Virology, in the research group of Prof. Dr. Xander de Haan under the supervision of Dr. Marne Hagemeijer. He worked on methods for the detection of viral (MHV) protein interactions. After the completion of this internship, Boaz continued his research career at the lab of Prof. Dr. Cox Terhorst at Beth Isreal Deaconess Medical Center, Harvard Medical School in Boston, USA. This second internship, under the supervision Dr. Xavier Romero, was focused on the functions of SLAM family receptors in phagocytes. That topic was of particular interest to Boaz as he decided, upon the gracious invite by Prof. Dr. Cox Terhorst, to pursue a PhD in this laboratory to continue the research on SLAM receptors in the context of colitis, in February 2011. The result of this work from then up to the summer of 2015 is described in this dissertational thesis. On 19 October 2015, Boaz will defend this work, before the University of Utrecht, to obtain the degree of PhD with Prof. Dr. Hans Clevers and Prof. Dr. Cox Terhorst. Boaz continues his professional journey and personal mission to understand scientific progress as well as societal needs and facilitate the required connection.

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