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Article

T Cell-Derived Protein S Engages TAM Receptor Signaling in Dendritic Cells to Control the Magnitude of the Immune Response

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SUMMARY

Dendritic cell (DC) activation is essential for the induction of immune defense against pathogens, yet needs to be tightly controlled to avoid chronic inflammation and exaggerated immune responses. Here, we identify a mechanism of immune homeostasis by which adaptive immunity, once triggered, tempers DC activation and prevents overreactive immune responses. T cells, once activated, produced Protein S (Pros1) that signaled through TAM receptor tyrosine kinases in DCs to limit the magnitude of DC activation. Genetic ablation of *Pros1* in mouse T cells led to increased expression of costimulatory molecules and cytokines in DCs and enhanced immune responses to T cell-dependent antigens, as well as increased colitis. Additionally, PROS1 was expressed in activated human T cells, and its ability to regulate DC activation was conserved. Our results identify a heretofore unrecognized, homeostatic negative feedback mechanism at the interface of adaptive and innate immunity that maintains the physiological magnitude of the immune response.

INTRODUCTION

The innate immune response functions as both the first line of defense against pathogens and also as the initiating trigger for adaptive immunity (Iwasaki and Medzhitov, 2010; Janeway, 1989; Medzhitov et al., 1997). Activation of dendritic cells (DCs), the professional antigen-presenting cells, drives T cell activation. These essential functions notwithstanding, the magnitude of DC activation must be precisely controlled. Unrestrained DC responses can lead to pathological conditions characterized by overreactive immune responses, such as allergy, autoimmunity, and chronic inflammatory diseases (Coombes and Powrie, 2008; Lambrecht and Hammad, 2010). A priori, the induction of T cell activation must be coupled to a homeostatic negative feedback mechanism that limits the function of DCs. Such a mechanism would still allow T cell activation and initiation of adaptive immunity by DCs, yet enable T cells, once activated, to signal back to DCs and restrain further stimulation of the immune response.

Receptor tyrosine kinases (RTKs) of the TAM family, Axl and Mertk, are pleiotropic negative regulators of Toll-like receptor (TLR) and cytokine receptor signaling in DCs (Rothlin et al., 2007). Two ligands for the TAM receptors, Protein S (Pros1) and Gas6, have been identified by in vitro approaches (Stitt et al., 1995). Although the source of the ligands that activate TAM receptors in DCs in vivo is unknown, T cell-dependent activation of TAM receptors would allow for an inflammatory response in DCs upon initial pathogen encounter, followed by downregulation of this response once antigen presentation and T cell activation have occurred. Therefore, we considered the possibility that T cells might be an important source of TAM ligands. Here, we show that Pros1 was expressed by mouse and human activated T cells and inhibited DC function. Although Pros1 is well known to function as an essential anticoagulant where its action is TAM-independent (Burstyn-Cohen et al., 2009; Dahlbäck, 2007), we reveal an anti-inflammatory function of T cell-derived Pros1 as the in vivo TAM ligand. Our results also reveal that this T cell-derived Pros1-DC TAM signaling axis is an indispensable, evolutionarily conserved, homeostatic feedback mechanism by which adaptive immunity controls the magnitude of the innate immune response.

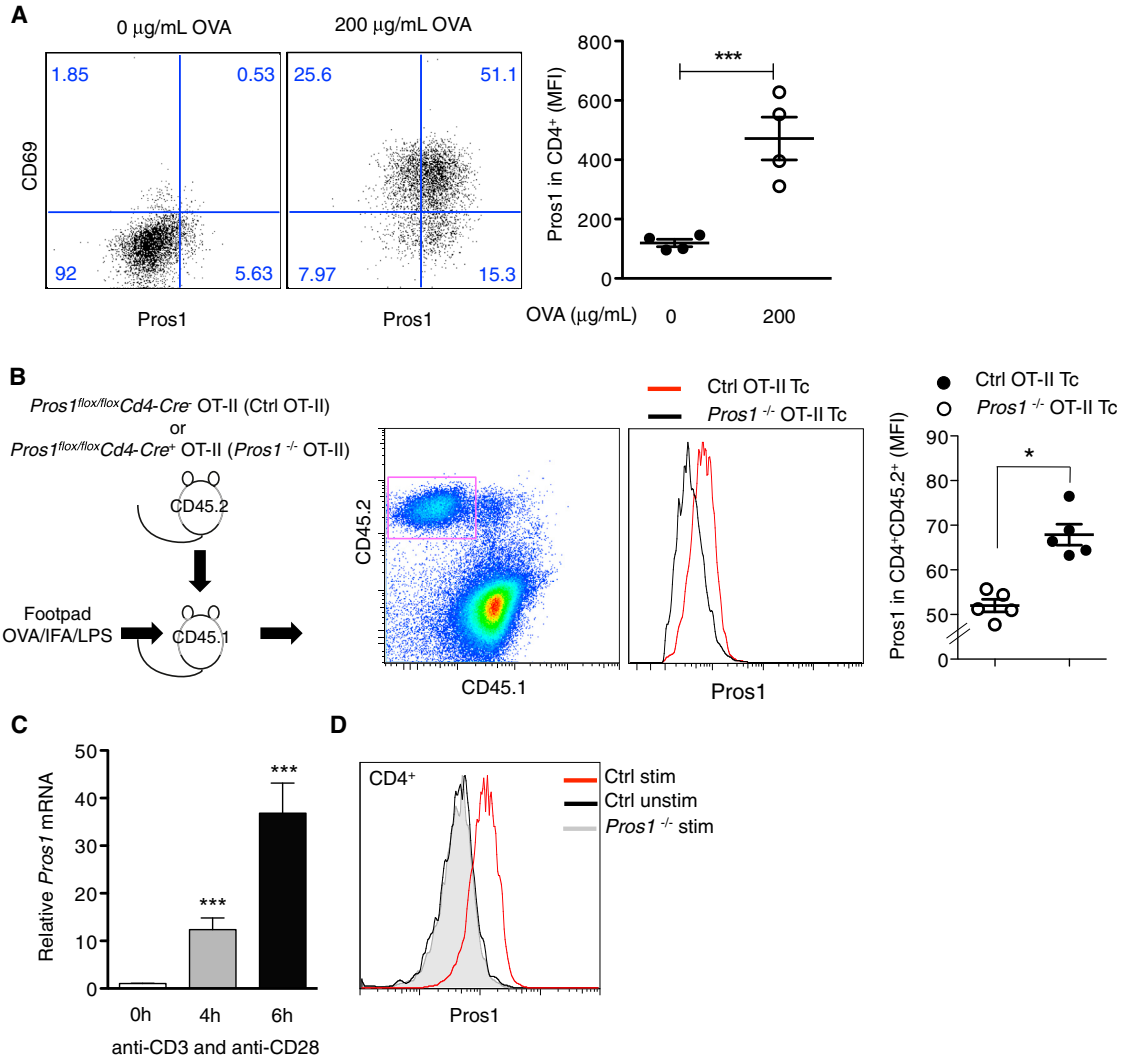


Figure 1. Activated T Cells Express Pros1

(A) Splenic CD4$^+$ T cells from OT-II mice were cocultured with BM-DC alone or in the presence of 200 μg/mL OVA for 24 hr. Pros1 induction was analyzed after antigen-specific activation of CD4$^+$ T cell (CD4$^+$CD69$^+$). Representative FACS plots (left) and MFI for Pros1 expression on CD4$^+$ T cells (right) are shown.

(B) CD45.2 T cells from indicated genotypes were transferred into CD45.1 recipients and 1 day after transfer recipient mice were injected into the rear footpad with 50 μg of OVA/IFA/LPS. Popliteal and inguinal LNs were collected 48 hr after immunization. Representative plots show CD45.2 transferred T cells and Pros1 expression in Ctrl OT-II T cells compared with *Pros1*$^{-/-}$ OT-II T cells.

(C) Splenic CD4$^+$ cells were isolated and activated in vitro with anti-CD3 and anti-CD28. *Pros1* mRNA expression was determined by qPCR and normalized to unstimulated cells.

(D) Representative FACS histograms of Pros1 expression on resting and activated CD4$^+$ T cells with anti-CD3 and anti-CD28 for 15 hr. Gray histogram represents activated CD4$^+$ cells from *Pros1*$^{flox/flox}$ *Cd4-Cre*$^+$ (*Pros1*$^{-/-}$) mice. Data are presented as representative individual samples or as mean ± SEM of at least 4 to 6 independent samples per group. *p < 0.05, ***p < 0.001. See also Figure S1.

RESULTS

Activated T Cells Express Pros1

To test the hypothesis that activated T cells constitute a relevant immunological source of Pros1, we first measured Pros1 expression upon antigen presentation in vitro. Activation of OT-II transgenic CD4$^+$ T cells upon incubation with bone marrow (BM)-DCs in the presence of ovalbumin (OVA) in vitro led to the detection of Pros1 on activated T cells (Figure 1A). Next, we generated a mouse where *Pros1* expression was genetically ablated specifically in T cells. Mice homozygous for "floxed" *Pros1* alleles (Burstyn-Cohen et al., 2009) were crossed with mice expressing CRE recombinase under the control of the *Cd4* promoter. Although complete *Pros1*-deficient mice die in utero due to fulminant coagulopathy (Burstyn-Cohen et al., 2009; Saller et al., 2009), *Pros1*$^{flox/flox}$*Cd4-Cre*$^+$ mice were viable. T cells do not substantially contribute to the physiological amounts of Pros1 in plasma because the amounts of Pros1 in the plasma of control and *Pros1*$^{flox/flox}$*Cd4-Cre*$^+$mice were comparable (see Figure S1 available online). Furthermore, no overt defects in thymic

development or on activation of splenic T cells were detected in *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ mice at 2–4 months of age (Figure S1). To directly test whether activated, antigen-specific T cells express Pros1 in vivo, we crossed *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ to OT-II transgenic mice. Next, we transferred control and *Pros1*$^{-/-}$ OT-II CD45.2^{+} CD4^{+} T cells into CD45.1^{+} recipient mice and immunized them with OVA-LPS-IFA in their footpads (Figure 1B). Pros1 expression was detected in activated antigen-specific T cells in vivo (Figure 1B). Finally, direct activation of isolated murine splenic CD4^{+} T cells via anti-CD3 and anti-CD28 stimulation led to the upregulation of *Pros1* messenger RNA (mRNA) (Figure 1C) and protein (Figure 1D). Consistent with the genetic ablation of *Pros1* in T cells, this upregulation was undetectable in in vitro activated T cells from *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ mice (Figure S1). Resting CD4^{+} T cells did not express Pros1 (Figure 1D). Similar results were obtained by using T cells from *Pros1*$^{flox/flox}$ *Lck-Cre*$^{+}$ mice. Neither resting nor activated T cells expressed Gas6 (data not shown). Taken together, these results indicate that T cells, activated in an antigen-specific manner, express Pros1.

Deficiency of Pros1 in T Cells Leads to Accelerated Disease Onset in a Model of Induced Colitis

The transfer of CD4^{+}CD25^{-}CD45RB^{hi} cells into *Rag1*$^{-/-}$ mice results in the induction of colitis, a disease dependent on microbiota and triggered by antigen-specific DCs (Coombes and Powrie, 2008; Feng et al., 2010). We hypothesized that whether T cell-derived Pros1 limits DC activation in vivo, transfer of *Pros1*$^{-/-}$CD4^{+}CD25^{-}CD45RB^{hi} cells into *Rag1*$^{-/-}$ mice would result in exacerbated colitis. Indeed, transfer of *Pros1*$^{-/-}$ naive T cells led to a substantial acceleration of disease onset, as indicated by higher colonoscopy scores (Figure 2A; Figure S2). Increased numbers of interferon-γ (IFN-γ) and interleukin-17A (IL-17A) expressing T cells were detected in the mesenteric lymph nodes of *Pros1*$^{-/-}$CD4^{+}CD25^{-}CD45RB^{hi} recipients, relative to the control CD4^{+}CD25^{-}CD45RB^{hi} recipients (Figure 2B–2D). Similarly, higher numbers of IL-17A expressing T cells were detected in the lamina propria of mice receiving *Pros1*$^{-/-}$ naive T cells (Figure 2E). These results indicate that Pros1 expression in T cells limits pathological inflammation.

T Cell-Derived Pros1 Limits DC Activation

Next, we directly tested whether T cell-derived Pros1 regulates DC activation in vivo. Control and *Pros1*-deficient OT-II CD45.2^{+}CD4^{+} T cells were transferred into CD45.1^{+} recipient mice, which were subsequently immunized with OVA-LPS-IFA in their footpads. Remarkably, the loss of Pros1 in antigen-specific T cells resulted in a significant increase in the population of activated DCs, as detected by the expression of the costimulatory molecules CD86 and CD40, in the draining lymph node (Figure 3A). Similarly, we detected an enhancement in the activation of DCs when *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ mice were directly immunized with OVA-LPS-IFA (Figure S2). Given these findings, we tested the ability of T cell-derived Pros1 to directly regulate the magnitude of DC activation upon antigen presentation in vitro. We cocultured CD4^{+}CD25^{-} cells obtained from either *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ OT-II or *Pros1*$^{flox/flox}$*Cd4-Cre*$^{-}$ OT-II mice with BM-DCs in the presence of OVA. In agreement with an immunomodulatory function of T cell-derived Pros1, we detected a significant increase in the percentage of CD86^{+} and CD40^{+} BM-DCs when cultured with Pros1-deficient as opposed to Pros1-expressing OT-II T cells (Figure 3B). Furthermore, tumor necrosis factor-α (TNF-α) and IL-6 production were also increased in the absence of T cell-derived Pros1 (Figures 3C and 3D). Importantly, Pros1 produced by activated T cells did not exert an autocrine effect: There were no significant differences in the expression of activation markers, production of cytokines, and proliferation between control (*Pros1*$^{flox/flox}$*Cd4-Cre*$^{-}$ or *Pros1*$^{wt/wt}$*Cd4-Cre*$^{+}$) and *Pros1*-deficient T cells when they were activated in vitro with anti-CD3 and anti-CD28 (Figure S3). In agreement with the increased activation of DCs in the absence of T cell-derived Pros1, *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ mice immunized with OVA-LPS-IFA developed larger lymph nodes in comparison to control mice, primarily due to an expansion of T cells (Figure S2). Furthermore, we detected an increase in the proliferation of *Pros1*$^{-/-}$ CD4^{+} T cells isolated 10 d.p.i. when they were restimulated with OVA in vitro, as well as higher amounts of IL-2 in the culture supernatant (Figure S2). Similarly, when control or *Pros1*$^{-/-}$ OT-II T cells were transferred into mice that were subsequently immunized with OVA-LPS-IFA, we detected significantly higher numbers of transferred OT-II T cells and increased cellularity of the draining lymph nodes in those mice that received Pros1 deficient antigen-specific T cells (Figure S2). These findings are consistent with an increase in the number of antigen-specific T cells generated in the absence of T cell-derived Pros1.

To further confirm whether T cells are a relevant source of Pros1 for immunomodulation, we compared the immune response of control and *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ mice to T-dependent and T-independent antigens. *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ mice developed significantly higher titers of antitrinitrophenyl (TNP) immunoglobulin G1 (IgG1) in comparison to control mice upon immunization with the hapten TNP linked to keyhole limpet haemocyanin (KLH), a T-dependent antigen (Figure 4A). Conversely, no differences between *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ and control mice were detected upon immunization with the T-independent antigen TNP-Ficoll (Figure 4B). Interestingly, by 9 months of age, *Pros1*$^{flox/flox}$*Cd4-Cre*$^{+}$ mice showed a spontaneous expansion of CD4^{+}CD44^{hi}CD62L^{-} cells (Figure S4). However, autoantibodies, such as anti-DNA antibodies, were not detectable in 6- to 9-month-old mice (Figure S4). Taken together, the above results indicate that Pros1 expression in activated antigen-specific T cells is required for the homeostatic regulation of the magnitude of DC activation, thus ensuring a physiological immune response.

T Cell-Derived Pros1 Acts Locally to Inhibit DC Activation through TAM Signaling

Pros1 carries an N-terminal γ-carboxyglutamic acid (GLA)-rich domain that allows it to bind to cell membranes containing the negatively charged phospholipid, phosphatidylserine (PtdSer). Binding of Pros1 to PtdSer is an essential feature of its biological activity (Nyberg et al., 1997). Interestingly, PtdSer is transiently exposed on the outer leaflet of the plasma membrane of activated T cells (Figure S5; Fischer et al., 2006). This feature prompted us to test whether T cell-derived Pros1 functioned locally, at the interface of DC-T cell interaction, by

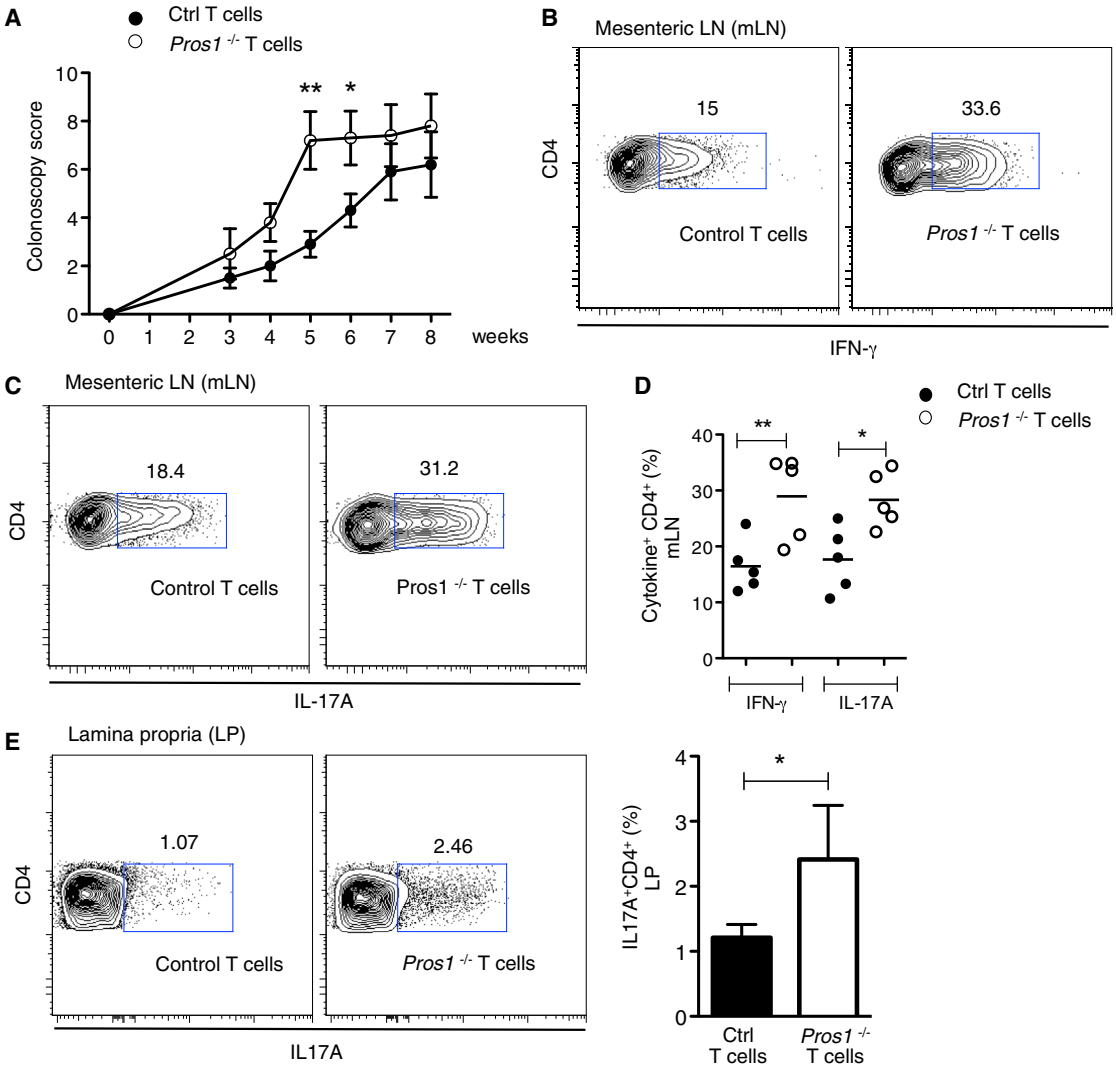


Figure 2. Deficiency of Pros1 in Transferred T Cells Leads to Accelerated Colitis in *Rag1*$^{-/-}$ Recipient Mice

Sorted CD4$^+$CD25$^-$CD45RB^{hi} T cells from *Pros1*$^{flox/flox}$ *Cd4-Cre*$^-$ (Ctrl) or *Cre*$^+$ (*Pros1*$^{-/-}$) were transferred i.p. into *Rag1*$^{-/-}$ mice and colitis development was monitored.

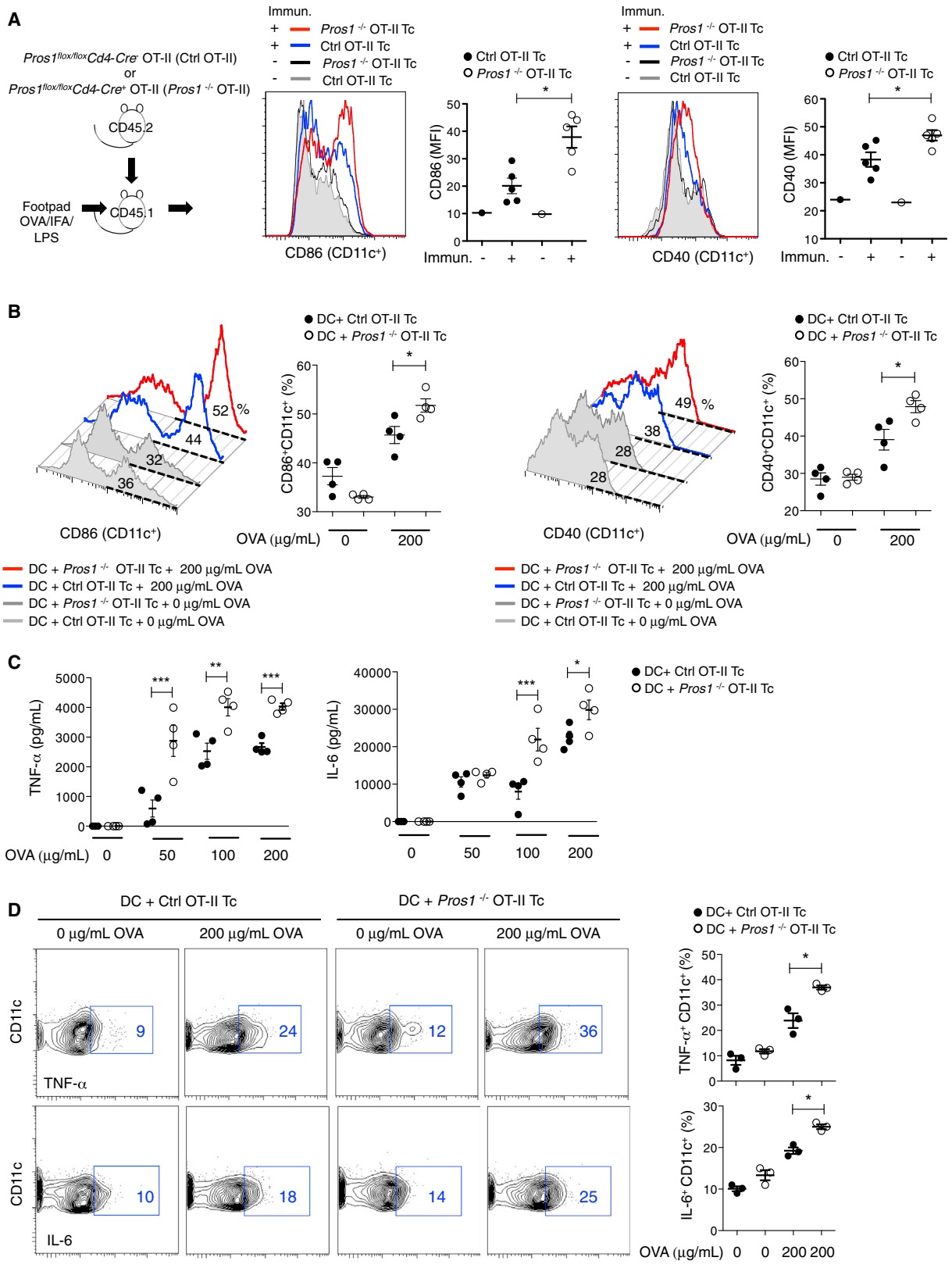
(A) Colonoscopy score of disease severity.

(B) Representative intracellular FACS staining for IFN-γ and (C) IL-17A production on CD4$^+$ cells from mesenteric LN.

(D) Percentage of IFN-γ$^+$ and IL-17A$^+$ CD4$^+$ cells in the mesenteric LN.

(E) Representative intracellular FACS staining for IL-17A (left) and percentage of IL-17A$^+$CD4$^+$ cells (right) in lamina propria leukocytes of large intestine. Data are presented as individual samples or as mean ± SEM and are representative of two independent experiments with at least five samples per group. *$p < 0.05$, **$p < 0.01$. See also Figure S2.

physically separating DC and T cells with a transwell system. To this end, we utilized T cells that had been prestimulated in vitro with anti-CD3 and anti-CD28 to bypass the need for DC-T cell contact to induce Pros1 expression in the T cells. Splenic CD4$^+$CD25$^-$ T cells were activated in vitro and were then cocultured with BM-DCs in the presence of several different agonists of pattern recognition receptors. BM-DCs expressed higher amounts of CD86 and CD40 in response to poly I:C when cocultured with Pros1-deficient versus control T cells (Figure S5). Furthermore, the production of many different cytokines (TNF-α, IL-6, IFN-β) was significantly higher when BM-DCs were activated in the presence of *Pros1*$^{-/-}$ T cells than control T cells (Figure S5). Similarly, the activation of BM-DCs with CpG-A was also higher when DCs were cocultured with *Pros1*-deficient T cells than with control T cells (Figure S5). Addition of recombinant Pros1 to the cocultures rescued the ability of activated *Pros1*$^{-/-}$ T cells to regulate DC function (Figure S6). Next, we utilized the transwell system to physically separate in vitro activated T cells from BM-DCs. Interestingly, no differences in the expression of activation markers (e.g., costimulatory molecules) were detected when BM-DCs were cultured across the transwell with either control or *Pros1*$^{-/-}$ T cells (Figure 5A). These results suggest that Pros1-TAM signaling is not engaged in the presence of a



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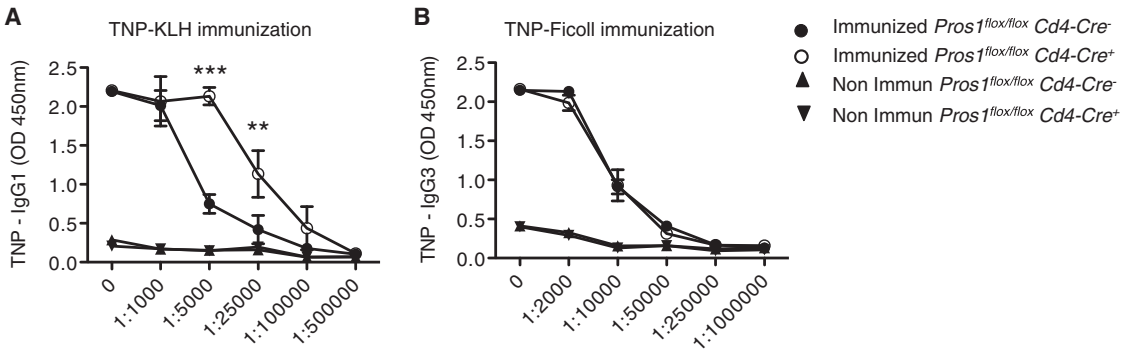

Figure 4. Deletion of Pros1 in T Cell Results in Increased Immune Responses upon Immunization with T-Dependent Antigens

(A) Serum TNP-specific IgG1 in TNP-KLH-immunized *Pros1*$^{flox/flox}$ *Cd4-Cre*$^{-}$ or *Pros1*$^{flox/flox}$ *Cd4-Cre*$^{+}$ mice.

(B) TNP-specific IgG3 in TNP-Ficoll-immunized mice. Data are presented as mean ± SEM and are representative of two independent experiments with at least four samples per group. **p < 0.01, ***p < 0.01. See also Figure S4.

permeable but physical barrier. Binding to exposed PtdSer on activated T cells might restrict Pros1 to the T cell surface, accounting for this localized function. Because Annexin V has been shown to efficiently compete with Pros1 for PtdSer binding (Anderson et al., 2003; Webb et al., 2002), we compared the activation of BM-DCs cocultured with activated T cells in the absence or presence of Annexin V. Addition of Annexin V to the coculture led to a significant enhancement of the expression of CD86 and CD40 by BM-DCs upon poly I:C stimulation in wild-type (WT) but not *Pros1*-deficient T cells (Figure 5B; Figure S6). These results indicate that Pros1 binding to PtdSer is necessary for its immunomodulatory effect.

We also tested whether the loss of Axl and Mertk signaling in DCs phenocopied the loss of Pros1 in T cells by using the in vitro antigen-presentation assay. When cocultured with Pros1-expressing OT-II T cells, BM-DCs lacking both *Axl* and *Mertk* (*Axl*$^{-/-}$*Mertk*$^{-/-}$) expressed substantially higher amounts of costimulatory molecules, relative to WT BM-DCs (Figure 5C). These results indicate that the loss of *Axl* and *Mertk* renders DCs refractory to T cell-derived Pros1-mediated regulation. TAM signaling regulates the degree of DC activation through the induction of Socs, which in turn leads to reduction of NF-κB activity (Rothlin et al., 2007). The use of *Pros1*-deficient T cells in the T cell:DC coculture system led to the reduced induction of Socs3, increased IκBα degradation, and increased p65 NF-κB phosphorylation in BM-DCs in comparison to control T cells (Figures 5D–5F). Together, all of these data demonstrate that T cell-derived Pros1 functions locally at the DC-T cell interface and engages TAM signaling within DCs to limit their activation.

PROS1 Expression and Function Are Conserved in Humans

To test whether human T cells express PROS1, human naive CD4^{+} T cells were isolated by negative selection from human peripheral blood mononuclear cells (PBMCs) and stimulated with anti-CD3 plus anti-CD28. In vitro activation of human CD4^{+} T cells led to a significant induction of *PROS1* mRNA as well as PROS1 expression on the cell surface (Figures 6A and 6B). We also compared PROS1 expression in the interfollicular T cell area of reactive versus nonreactive human lymph nodes. Sections from nonmalignant human lymph nodes classified by histopathological analyses as nonreactive (n = 6) or reactive (n = 6) were stained with anti-CD3 and anti-PROS1 antibodies and counterstained with hematoxylin. PROS1 staining was positive in the CD3^{+} T cell area of reactive lymph nodes with no expression detectable in nonreactive lymph nodes (Figure 6C).

To explore whether human T cell-derived PROS1 indeed functions in regulating the degree of DC activation, we performed mixed lymphocyte reactions (MLRs). Monocyte-derived DCs and naive T cells were isolated from different donors and cocultured for 5 days. Monocyte-derived DCs express both AXL and MERTK, whereas activated T cells express PROS1 in the MLR (data not shown). In agreement with a regulatory role for PROS1, the neutralization of PROS1 with an anti-PROS1 antibody led to a significant increase in CD86 and CD40 expression in human monocyte-derived DCs (Figures 6D and 6E).

To test whether the loss of function of PROS1 is associated with pathological inflammation in humans, we extended our studies to patients with Inflammatory Bowel Disease (IBD), including Ulcerative Colitis and Crohn's Disease. PROS1

Figure 3. T Cell-Derived Pros1 Limits DC Activation

(A) CD45.2 T cells from indicated genotypes were transferred into CD45.1 recipients and 1 day after transfer recipient mice were injected with 50 μg of OVA/IFA/LPS into the rear footpad. Popliteal and inguinal LNs were collected after 72 hr and CD86 and CD40 expression were measured on CD11c^{+} cells. Representative histograms for nonimmunized and OVA immunized mice and independent data are shown.

(B) Splenic CD4^{+}CD25^{-} T cells were isolated from *Pros1*$^{flox/flox}$ *Cd4-Cre*$^{-}$ OT-II (Ctrl OT-II Tc) and *Pros1*$^{flox/flox}$ *Cd4-Cre*$^{+}$ *OT-II* (*Pros1*$^{-/-}$OT-II Tc) mice and cocultured with BM-DCs in the presence of the indicated concentrations of OVA. Representative FACS histograms and percentage of CD86^{+} CD11c^{+} and CD40^{+} CD11c^{+} cells are shown.

(C) TNF-α and IL-6 production in the coculture supernatants after 72 hr, as measured by ELISA.

(D) Representative plots showing intracellular staining for TNF-α and IL-6 in CD11c^{+} (left) and individual samples (right) are shown. Data are presented as representative individual samples or as mean ± SEM of 3–5 independent samples per group. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figures S2 and S3.

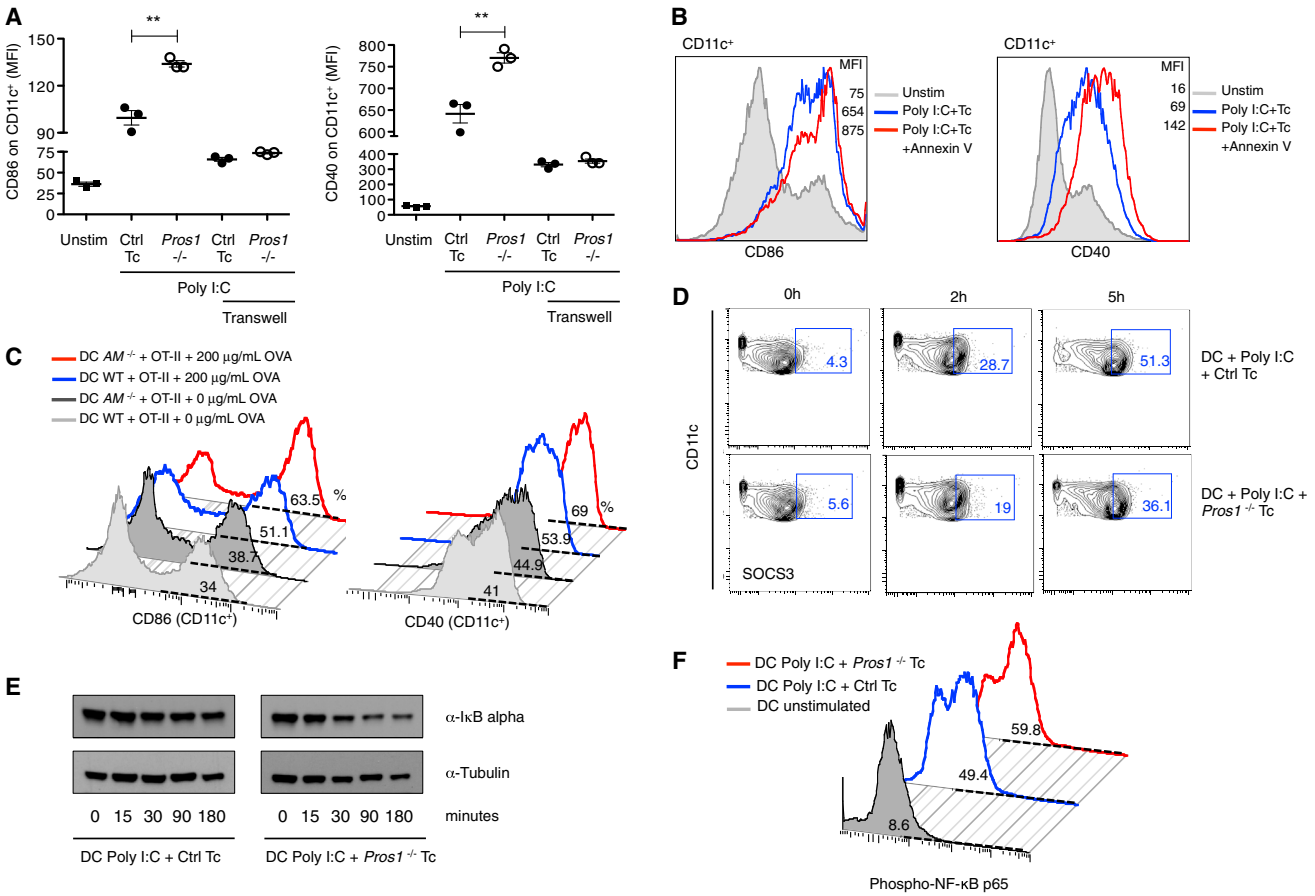

Figure 5. Negative Feedback of T Cell-Derived Pros1 Depends of Cell Proximity and Axl and Mertk Signaling on DCs

(A) Splenic CD4$^+$ CD25$^-$ T cells from *Pros1*$^{flox/flox}$ *Cd4-Cre*$^-$ (Ctrl Tc) or *Pros1*$^{flox/flox}$ *Cd4-Cre*$^+$ (*Pros1*$^{-/-}$) mice were activated in vitro with anti-CD3 and anti-CD28 for 4 hr. Activated T cells were then cocultured with BM-DCs in presence of 30 μg/mL of poly I:C for 15 hr. To prevent close contact between activated CD4$^+$ T cells and BM-DCs, we cultured BM-DCs on the upper chamber of a transwell system, whereas we cultivated activated T cells on the lower chamber. MFI of CD86 and CD40 expression on CD11c$^+$ cells in the indicated conditions are shown.

(B) Where indicated, 10 μg/mL of Annexin V (AnxV) was added to the T cell culture 1 hr prior to coculture with BM-DCs. Representative FACS histograms of CD86 and CD40 expression on CD11c$^+$ cells are shown.

(C) CD4$^+$CD25$^-$ OT-II Ctrl T cells were cocultured with WT or *Axl*$^{-/-}$*Mertk*$^{-/-}$ BM-DC in the presence of 200 μg/mL of OVA for 48 hr. Representative histograms for CD86 and CD40 expression on CD11c$^+$ cells are shown.

(D) Percentage of CD11c$^+$SOCS3$^+$ in activated BM-DCs upon coculture with Ctrl or *Pros1*$^{-/-}$ T cells.

(E) IkBα (top) and Tubulin (bottom) in the coculture were analyzed by immunoblot at the indicated time points.

(F) Phospho-NF-κB p65 (Ser536) subunit was measured on activated CD11c$^+$ cell cocultured with Ctrl or *Pros1*$^{-/-}$ T cells by FACS. Data are presented as percentage of phospho-NF-κB p65 on CD11c$^+$ cells, by histograms. Data are presented as individual samples or as mean ± SEM and are representative of at least two independent experiments with three to four independent samples per group. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figures S5 and S6.

amounts are measured routinely from patient plasma for the diagnosis of PROS1 deficiencies. Interestingly, patients with active IBD frequently showed lower amounts of plasma PROS1 relative to healthy controls (Figure 6F). More than 200 germline mutations in the *PROS1* gene have been described. Type I deficiencies, characterized by reduced PROS1 amounts, are associated with mutations within the coding region (Gandrille et al., 2000; García de Frutos et al., 2007). Therefore, for Type I deficiencies, the plasma concentration of PROS1 (produced by hepatocytes and endothelial cells) is likely to function as a surrogate for the amount of PROS1 produced by other cellular sources, including T cells. Future studies directly measuring T cell-derived PROS1 will further validate this hypothesis. Collectively, these results indicate that this anti-inflammatory pathway is present in humans and functions in maintaining immune homeostasis.

DISCUSSION

Our results reveal a hitherto unknown indispensable, negative feedback mechanism by which activated T cells restrain DC activation and, thereby, regulate the overall magnitude of the immune response. Following initial DC activation and antigen presentation, T cells are activated, leading to the induction of Pros1. Pros1 engages the anti-inflammatory TAM receptor tyrosine kinase signaling pathway in DCs. This enables activated T cells to report back to DCs and restrain further stimulation of the overall immune response. This feedback mechanism is

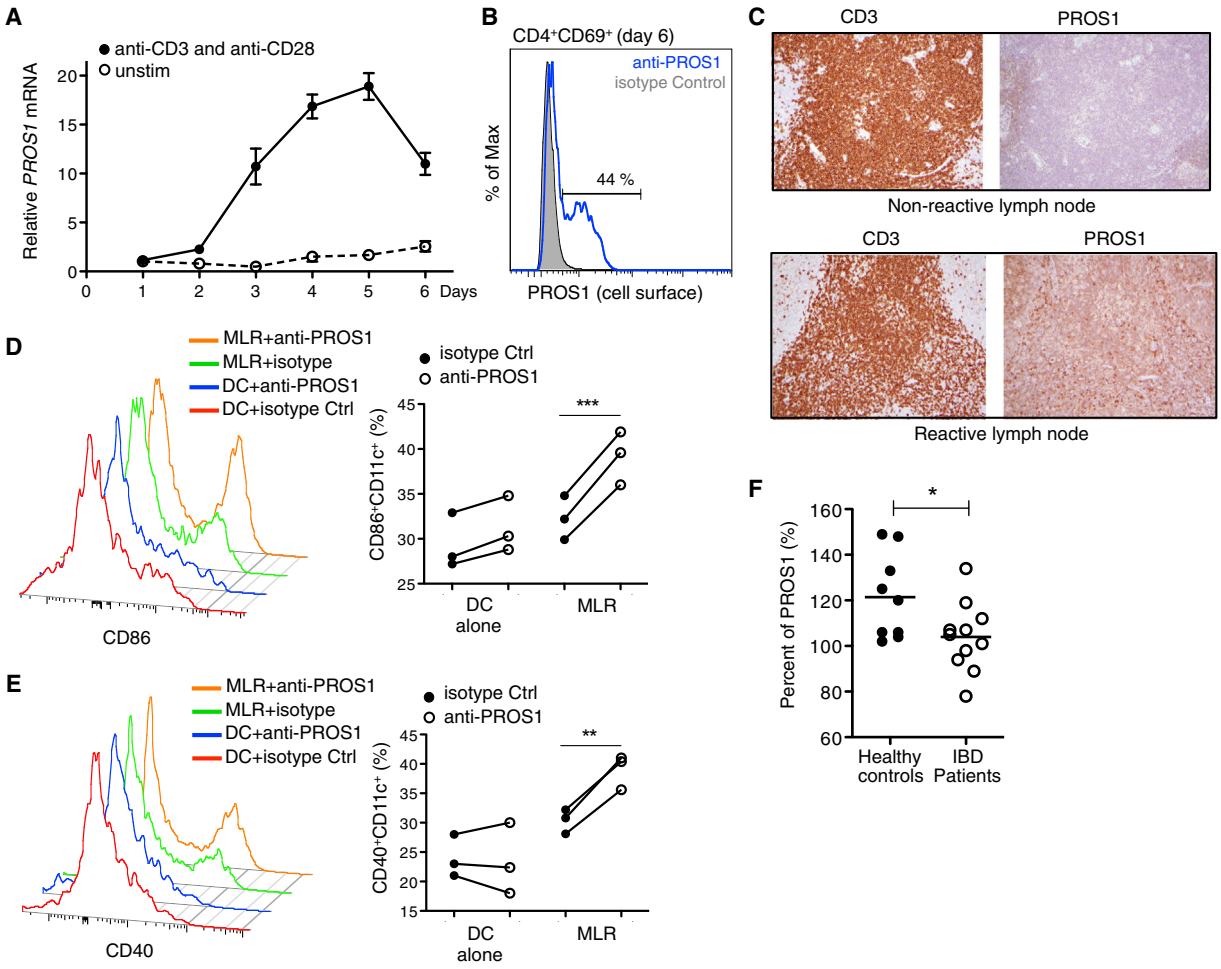

Figure 6. PROS1 Is Expressed in Activated Human CD4 T Cells and PROS1 Blockade Leads to Enhanced Activation of DCs in MLR

Human naive CD4 T cells were isolated from PBMCs by negative selection and stimulated with anti-CD3 and anti-CD28 for 6 days.

(A) Expression of PROS1 mRNA, normalized to unstimulated naive $CD4^+$ T cells. Data are presented as mean ± SEM of at least five samples per condition.

(B) Surface PROS1 expression on $CD4^+CD69^+$ cells after 6 days of stimulation with anti-CD3 and anti-CD28. Representative histogram from at least five independent donors is shown.

(C) Sections from nonreactive and reactive human lymph nodes (LNs) were stained with anti-CD3 and anti-PROS1. Representative PROS1 staining in the interfollicular $CD3^+$ T cell areas on nonreactive (n = 6) and reactive (n = 6) LNs are shown.

(D and E) CD86 and CD40 expression were measured on $CD11c^+$ cells at day 5. Representative histograms (left) and independent data with at least three independent samples per group (right) are shown. Each experiment was repeated at least twice employing three to five different donors.

(F) Percentage of total PROS1 in healthy controls (n = 9) and IBD patients (n = 11). Horizontal lines indicate the mean in each group. Total PROS1 was determined using automated latex immunoassay methodology and normalized to an average value of PROS1 from a pool of healthy donors. *$p < 0.05$, **$p < 0.01$, ***$p < 0.01$.

essential for maintaining the physiological balance of immune activation capable of host defense yet avoiding exacerbated inflammation. The absence of this "immunological brake" leads to pathological inflammation and chronic inflammatory diseases, such as colitis. Importantly, Pros1-TAM signaling is not redundant with respect to other negative regulators. Although Pros1 is a secreted protein, its immunoregulatory function is dependent on its binding to the T cell-surface associated PtdSer, thereby restricting Pros1 function to DC-T cell interface. Because cognate DC-T cell interactions are antigen-specific, this property of Pros1 presumably results in an antigen-specific negative regulatory function. Whether this feature makes Pros1 distinct from other known negative regulators of DC activation, such as IL-10 (Banchereau et al., 2012), and prevents compensation remains unknown. Future studies focused on Pros1 function in a variety of immunological settings and its integration with known regulatory pathways will provide a more comprehensive understanding of mechanisms through which the adaptive immune system, once engaged, regulates the magnitude of the innate immune response and how overall immune homeostasis is maintained.

Pros1, produced by hepatocytes and endothelial cells, is found in large amounts in the blood (Burstyn-Cohen et al., 2009). T cells do not substantially contribute to the physiological amounts of Pros1 in plasma because the concentrations of Pros1 in the plasma of control and $Pros1^{flox/flox}Cd4\text{-}Cre^{+}$mice were comparable. Conversely, our findings unambiguously demonstrate that Pros1 in the blood is unable to compensate

for the loss of Pros1 expression by activated T cells. The fact that T cell-derived Pros1 is necessary to control DC responses, despite a substantial amount of Pros1 being present in the circulation, is intriguing. Our results demonstrate that Pros1 acts locally as an immunomodulatory signal at the physical T cell-DC interface. The simultaneous production of Pros1 and the exposure of PtdSer on activated T cells allows for the localization and the bioactivity of Pros1 at the T cell membrane surface, engaging TAM signaling in DCs following T cell priming in lymphoid organs. Additionally, Pros1 in the plasma, an approximately 72 kDa molecule in its monomeric form, is likely to be excluded from the extravascular sites where DC-T cell interaction occurs. A local, circulation-independent production of Pros1 has also recently been observed for retinal pigment epithelial cells of the eye (Burstyn-Cohen et al., 2012).

There are two known ligands for the TAM receptors—Gas6 and Pros1. These ligands often function redundantly. For example, *Gas6*$^{-/-}$ mice do not recapitulate the phagocytosis defects in the retinal pigmental epithelial cells observed in *Mertk*$^{-/-}$ mice (Prasad et al., 2006). The combinatorial deletion of *Gas6* and *Pros1* in the retina is required to phenocopy the loss of *Mertk* (Burstyn-Cohen et al., 2012). Interestingly, neither resting nor activated T cells express Gas6. Consistent with this expression pattern, the loss of Pros1 alone in T cells is sufficient to account for the enhanced magnitude of the immune response. Albeit fundamental and nonredundant for controlling the magnitude of the immune response and preventing pathological inflammatory responses, loss of T cell-derived Pros1, by itself, is not enough to spontaneously break tolerance. TAM receptors have two major biological functions—the removal of apoptotic cells and the negative regulation of innate immune responses. The loss of these distinct functions likely accounts for the autoimmunity in TAM triple-deficient mice. Thus, the combinatorial deletion of the two ligands might be required to recapitulate the spontaneous autoimmunity characteristic of TAM triple-deficient mice.

Our results reveal the hitherto unknown function of Pros1 as an endogenous anti-inflammatory protein and a key regulator of immune homeostasis. The most well-established activity of Pros1 to date is TAM-independent, in which it functions as an anticoagulant (Gandrille et al., 2000). In fact, Smiley et al. reported the expression of Pros1 in activated T cells yet ascribed this to an anticoagulant role of T cells (Smiley et al., 1997). Is the duality of Pros1 function relevant in physiological or pathological settings? Chronic inflammatory and autoimmune diseases have been described anecdotally, yet repeatedly, in patients with low circulating amounts of PROS1 (Aadland et al., 1992; Koutroubakis et al., 2000; Suh et al., 2010; Zezos et al., 2007). Additionally, diseases, such as IBD, are often concurrent with thrombotic events (Grainge et al., 2010). The discovery of the novel immunomodulatory function of Pros1 might provide a unifying molecular explanation for these two clinical features of IBD. The association of reduced PROS1 with IBD supports the notion that there might be a causal link. However, thrombotic events can directly lead to inflammation, and our present results do not rule out an association between IBD and a subset of patients with PROS1 loss limited to the plasma. On the other hand, we are also likely to miss patients with T cell-specific loss of PROS1 because T cells do not contribute to the plasma concentration of PROS1. Nevertheless, the association is intriguing, and dedicated efforts focused on measuring T cell-derived PROS1 might causally link PROS1 deficiencies with the development and progression of autoimmune or chronic inflammatory diseases, as well as reveal PROS1 as a useful biomarker for minimally invasive diagnosis and prognosis of IBD in the future.

EXPERIMENTAL PROCEDURES

Mice

Pros1$^{flox/flox}$, *Axl*$^{-/-}$*Mertk*$^{-/-}$ mice have been described previously (Burstyn-Cohen et al., 2009; Lu et al., 1999). *Cd4-Cre*, *Lck-Cre*, B6.Cg-Tg (TcrαTcrβ) (OT-II) transgenic mice, *Rag1*$^{-/-}$, and B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (CD45.1) were obtained from Jackson Laboratory. All mice were bred at Yale University animal facility. All mice were specific-pathogen free, maintained under a strict 12 hr light cycle (lights on at 7:00 a.m. and off at 7:00 p.m.), and given a regular chow diet. All experimental procedures were approved by Yale IACUC.

Antibodies

The following anti-mouse antibodies were purchased from BD-Biosciences, eBioscience, Santa Cruz or BioLegend (USA), as conjugated to FITC, PE, PE-Cy5, PerCP-Cy5.5, APC-Cy7, Alexa Fluor 700, PE-Cy7, Pacific Blue, or APC: CD3 (145-2C11), CD4 (GK1.5, L3T4, RM4-5), CD8 (53-6.7), CD11b (MI/70), CD11c (N418, HL3), CD25 (PC61), CD40 (HM40), CD44 (IM7), CD45 (30-F11), CD45R (RA3-6B2), CD62L (MEL-14), CD69 (H1.2F3), CD86 (GL1), F4/80 (BM8), I-A/I-E (M5/114.15.2), IL-6 (MP5-20F3), IL-12p40 (C17.8), TNF-α (MP6-XT22), IFN-γ (XMG1.2), IL-17A (17B7). Unconjugated antibodies employed were CD3 (145-2C11), CD28 (37.51), IL-4 (11B11), IFN (XMG1.2), Protein S (H90): sc 25836, CD16/32 (2.4G2).

As a secondary antibody, DyLight 649 donkey anti-rabbit IgG (Poly4064) was used. Monoclonal antibody against human GLA residues (American Diagnostica) was used to immunoprecipitate GLA containing proteins from serum.

The following anti-human antibodies were purchased from BD-Biosciences or BioLegend (USA), conjugated to Alexa Fluor 488, FITC, APC-Cy7, APC, PE, Pacific Blue: CD2 (TS1/8), CD69 (FN50), CD3 (UCHT1), CD4 (RPA-T4), CD11c (3.9), CD11b (MI/70), CD40 (5C3), CD86 (IT2.2), and PROS1 (PS7).

Flow Cytometry Analysis

For standard stainings, cells were harvested and washed once with PBS and then blocked with Fc-block (BD- Bioscience) for at least 10 min in PBS-2% FBS. Cells were washed again and incubated with the respective cocktail of antibodies for 30 min on ice. Antibody cocktails were prepared in PBS-2% FBS. 7ADD was also included to gate on live cells. After the last wash, cells were acquired on a FACSCalibur, LSR-II (BD) or Stratedigm S1000EX (Stratedigm).

For Pros1 staining, after cells were collected and washed, they were fixed with cold acetone (−20°C) for 5 min and washed three times with PBS to discard all remaining acetone. After washing, cells were blocked with PBS-3% BSA for 30 min on ice. They were washed again and anti-Pros1 (H90) (Santa Cruz Biotechnology, USA) was added at 1 μg per 1 × 10^{6} of cells in PBS-3% BSA. Cells were incubated for 1 hr on ice and then washed twice. The secondary DyLight 649 donkey anti-rabbit (Biolegend, USA) was added and incubated for 30 min on ice. After the final wash, samples were acquired on a FACSCalibur or LSR-II (BD). All the directly conjugated antibodies used together with Pros1 staining were tested for acetone fixation.

RT- qPCR

At the indicated time points, cells were harvested and washed, and RNA was isolated with RNeasy mini kit (QIAGEN, USA) following the manufacturer's instructions. Reverse transcription was performed with RT Superscript III (Invitrogen) or iScript cDNA Synthesis kit (BIO-RAD). qPCR reactions were performed on Stratagene Mx3000 System with KAPA SYBR Fast qPCR kit (KAPABIOSYSTEM). The reactions were normalized to housekeeping gene and the specificity of the amplified products was checked by dissociation curves. Primers used in this paper are listed in Table S1.

ELISAs

TNF-α, IL-6, and IL-2 were measured by ELISA Ready-Set-Go (eBioscience, USA), and IFN-β was tested with Verikine mouse IFN-β ELISA kit (PBL Interferon Source). Culture supernatants were tested at different dilutions following the manufacturer's protocol.

T Cell Isolation

Mouse $CD4^+$ T cells were isolated from the spleen by negative selection with EasySep Mouse $CD4^+$ T cell enrichment kit and following the manufacturer's recommendation (STEMCELL Technologies, USA). $CD4^+CD25^-$ T cells were then obtained upon depletion of $CD25^+$ cells with EasySep mouse CD25 selection kit (STEMCELL Technologies). Human naive or total $CD4^+$ T cells were isolated from PBMCs with the EasySep human naive, CD4 T cell enrichment kit, according to manufacturer's protocol (STEMCELL Technologies).

BMDC Differentiation

Bone marrow dendritic cells (BMDC) were obtained from WT or *Axl*$^{-/-}$*Mertk*$^{-/-}$ (*AM*$^{-/-}$) mice following a standard protocol for differentiation. Briefly for BMDC, bone marrow progenitors were collected from the femurs of the indicated mice and 7–10 × 10^6 cells were cultured in 100 × 20 mm non-treated cell culture plates in 10 ml of enriched medium, RPMI 1640 plus 10% fetal bovine serum (FBS), 1% penicillin and streptomycin and supplemented with 20 ng/mL of recombinant GM-CSF (Peprotech, USA). At days 3 and 5 of cell culture, 10 and 5 ml of enriched media were added, respectively. On day 6, BMDCs were harvested and 90%, on average, of harvested cells were positive for CD11c.

Antigen Presentation Assays

For testing Pros1 expression upon antigen presentation, splenic $CD4^+$ T cells were purified from OT-II mice and cocultured with BMDC in presence of 200 μg/mL of OVA (A5503, SIGMA). After 24 hr, cells were collected and fixed with cold acetone (−20°C). Pros1 expression was analyzed on $CD4^+CD69^+$ or $CD4^+CD69^-$ cells. Pros1 staining was performed according to the protocol described above.

Splenic $CD4^+CD25^-$ T cells were purified from C57BL/6 (WT), *Pros1*$^{flox/flox}$ *Cd4-Cre*$^-$ OT-II (Ctrl OT-II) and *Pros1*$^{flox/flox}$ *Cd4-Cre*$^+$ *OT-II* (*Pros1*-deficient OT-II) mice and cocultured with WT or *AM*$^{-/-}$ BMDC at 1:1 ratio in presence of 50 to 200 μg/mL of OVA (SIGMA). After 24, 48, and 72 hr, cells were collected. CD86 and CD40 were measured on $CD11c^+$ cells by FACS. TNF-α, IL-6, and IL-12 were tested by intracellular staining and in the coculture supernatant by ELISA.

For T cell proliferation assays, $CD4^+$ T cell were isolated from inguinal and popliteal lymph nodes of immunized mice. 1 × 10^5 $CD4^+$ T cells stained with 5 μM CFSE were cultured with 3 × 10^5 irradiated splenocytes in presence of serial dilutions of OVA, starting at 900 μg/mL. Proliferating T cells were analyzed by FACS at day 4 by dilution of CFSE. Additionally, IL-2 production was measured in the coculture supernatant by ELISA.

T Cell—BMDC Coculture

$CD4^+CD25^-$ cells were isolated by negative selection of $CD4^+$ cells followed by depletion of $CD25^+$ cells following manufacturer's instructions (STEMCELL Technologies). $CD4^+CD25^-$ T cells were preactivated in serum-free medium (STEMCELL Technologies) with 10 μg/mL plate bound anti-CD3 and 2 μg/mL soluble anti-CD28 for 4 hr. BMDCs were then added onto preactivated T cells at a 1:1 ratio and stimulated with 30 μg/mL of poly I:C or 0.5 μM CpG-A (Invivogen, USA) for 12–15 hr. CD86 or CD40 were analyzed on $CD11c^+$. For experiments using recombinant Pros1, preactivated $CD4^+CD25^-$ T cells from *Pros1*$^{flox/flox}$ *Cd4-Cre*$^+$ were cocultured with BMDC at 1:1 ratio and stimulated with 30 μg/mL of Poly I:C or 0.5 μM CpG-A (Invivogen, USA) in serum-free medium supplemented with 1/10 of conditioned media from HEK293 expressing recombinant murine Protein S (Prasad et al., 2006).

Competitive Annexin V and Transwell Assay

T cell/BMDC coculture was performed as described above. For competitive Annexin V assay, 10 μg/mL of recombinant Annexin V (BD-Bioscience, USA) was added to the T cell culture 1 hr prior to the addition of BMDCs. BMDCs were added onto preactivated T cells that have been incubated with Annexin V and the protocol was continued as was previously described. For the transwell assay, T cells were preactivated on the bottom portion of the transwell for 4 hr and then DCs were added on top of transwell membrane (0.4 μm, Corning, USA). As a control, DCs were also added at the bottom, directly onto preactivated T cells. The activation of DCs was performed as described above.

Immunization Protocols

$CD4^+$ cells were isolated by negative selection as per manufacturer's instructions (STEMCELL Technologies) from the spleen and LN of CD45.2 OT-II *Pros1*$^{flox/flox}$*Cd4-Cre*$^-$ (Ctrl OT-II) or *Cre*$^+$ (*Pros1*$^{-/-}$ OT-II). 5 × 10^6 $CD4^+$ CD45.2 Ctrl OT-II or *Pros1*$^{-/-}$ OT-II were transferred into C57BL/6 CD45.1 recipient mice by retro-orbital injection. After 1 day, mice were challenged by injection of 50 μg of OVA/5 ng LPS/emulsified in incomplete Freund's adjuvant (OVA/LPS/IFA) in one of the rear footpads. Inguinal and popliteal LNs were removed, total number cells were counted, and Pros1 expression in CD45.2 and DC compartment in CD45.1 were analyzed by FACS 48 and 72 hr after immunization. *Pros1*$^{flox/flox}$ *Cd4-Cre*$^-$ (Ctrl) or *Cre*$^+$ (*Pros1*$^{-/-}$) mice, 8 to 10 weeks old, were immunized with 300 μg TNP-KLH (13:1) or 100 μg TNP-Ficoll (90:1) (both from Biosearch Technologies) in PBS on day 0 and 14. anti-TNP antibodies were analyzed by ELISA from serum at day 30 after immunization.

T Cell-Induced Colitis

$CD4^+CD45RB^{hi}CD25^-$ T cells were isolated from the spleen and lymph nodes of *Pros1*$^{flox/flox}$ *Cd4-Cre*$^-$ (Ctrl) or *Cre*$^+$ (*Pros1*$^{-/-}$) mice and transferred intraperitoneally (i.p.) into *Rag1*$^{-/-}$ mice (4 × 10^5 cells/mouse). Colonoscopy was performed in a blinded fashion for colitis scoring via the Coloview system (Karl Storz, Germany). In brief, colitis scoring was based on granularity of mucosal surface, stool consistence, vascular pattern, translucency of the colon, and fibrin visible (0–3 points for each). Mice were sacrificed about 6–8 weeks after the transfer, and cells isolated from the colon and mesenteric lymph nodes were analyzed for IFN-γ and IL-17A-producing CD4 cells.

Generation of Human Monocyte-Derived DC

We plated 1 × 10^8 PBMCs in 8 ml of RPMI 1640 plus 2% of human serum in 75 cm^2 cell culture flask and incubated at 37°C with 5% CO2 for 60 min for monocyte adherence. After the incubation period, the nonadherent cells were removed by washing four times with 6–10 ml of PBS. The adherent cells were cultured in 8 ml RPMI-1640 10% FCS containing 300 U/mL of IL-4 (R&D), 450 U/mL of GM-CSF (R&D) at 37°C, and 5% CO2. Cytokine supplementation was performed again on day 3 by adding 4 ml RPMI 1640 10% FCS containing 900 U/mL of IL-4, 1,350 U/mL GM-CSF. Monocyte-derived DCs were harvested on day 7.

PROS1 Expression in Human T Cells

Human naive CD4 T cells were obtained from PBMCs with EasySep human naive $CD4^+$ T cell enrichment kit (STEMCELL Technologies). We cultured 0.5 to 1 × 10^5 purified naive $CD4^+$ cells in presence (1:1 ratio) or absence of anti-human CD3/CD28 beads (Life Technologies) plus 3 ng/mL hIL-2 (Peprotech) in serum free medium (STEMCELL, USA) for 1 to 6 days. Cells were harvested daily and PROS1 expression was evaluated by qPCR with specific primers (Table S1) and anti-PROS1-FITC for surface staining by FACS.

Mixed Lymphocyte Reaction (MLR)

Human naive CD4 T cells were obtained by negative selection (STEMCELL Technologies) and cocultured with monocyte-derived DC, from a different donor, at 2:1 ratio for 5 days.

For PROS1 blocking experiment, anti-PROS1 (PS7) antibody was added at day 4, and cells were harvested at day 5. CD40 and CD86 activation markers were measured on $CD11c^+$ cells by FACS.

Protein S Total Antigen in IBD Patients

This study was approved by the Mayo Clinic Institutional Review Board. Patients at Mayo Clinic in Arizona with a confirmed diagnosis of active inflammatory bowel disease (Crohn's Disease or Ulcerative Colitis) not on steroids were enrolled. Exclusion criteria included patients with possible infectious gastroenteritis, ischemia, or drug-induced disease, as well as those treated with steroids. Prior to enrollment, all patients were provided informed consent. Blood was then collected from all patients as well as age-matched adult

healthy volunteers. Total PROS1 measurements were performed at the Mayo Clinic with the Diagnostica Stago LIATEST Protein S Kit on the Beckman Coulter ACL TOP (http://www.mayomedicallaboratories.com/test-catalog/Performance/83049).

Immunohistochemistry

Immunohistochemistry was performed with an automated Benchmark XT IHC system with ultraview universal DAB Detection Kit (Ventana, Tucson, AZ) and primary antibodies to PROS1 (Sigma; 1:50) and CD3 (Ventana, 0.45 μg/mL). In brief, paraffin-embedded formalin fixed tissue sections were processed according to mild CC1 (PROS1) and standard CC1 (CD3) programs including incubation with the primary antibodies for 30 min (CD3) or 1 hr (PROS1) at 37°C. Appropriate positive (liver) and negative (secondary antibody only) controls were stained in parallel for each round of immunohistochemistry.

Statistical Analysis

Differences between the means of experimental groups were analyzed with a two-tailed Student's t test or ANOVA (Prism GraphPad software). Bonferroni multiple comparison test was used following two-way ANOVA. p values $\leq$ 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.06.010.

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