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**ORIGINAL PAPER**

# P-selectin glycoprotein ligand-1 modulates immune inflammatory responses in the enteric lamina propria

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## Abstract

**P-selectin glycoprotein ligand-1 (PSGL-1), a leukocyte adhesion receptor that interacts with selectins, induces a tolerogenic programme in bone marrow-derived dendritic cells (DCs), which in turn promotes the generation of T regulatory (Treg) lymphocytes. In the present study, we have used a mouse model of dextran sulphate sodium (DSS)-induced colitis and studied the characteristics of the inflammatory cell infiltrate in the lamina propria (LP), mesenteric lymph nodes (mLNs) and Peyer's patches (PPs) to assess the possible role of PSGL-1 in the modulation of the enteric immune response. We have found that untreated PSGL-1-deficient mice showed an altered proportion of innate and adaptive immune cells in mLNs and PPs as well as an activated phenotype of macrophages and DCs in the colonic LP that mainly produced pro-inflammatory cytokines. Administration of an anti-PSGL-1 antibody also reduced the total numbers of macrophages, DCs and B cells in the colonic LP, and induced a lower expression of MHC-II by DCs and macrophages. After DSS treatment, PSGL-1$^{-/-}$ mice developed colitis earlier and with higher severity than wild-type (WT) mice. Accordingly, the colonic LP of these animals showed an enhanced number of Th1 and Th17 lymphocytes, with enhanced synthesis of IL-1α, IL-6 and IL-22, and increased activation of LP macrophages. Together, our data indicate that PSGL-1 has a relevant homeostatic role in the gut-associated lymphoid tissue under steady-state conditions, and that this adhesion receptor is able to down-regulate the inflammatory phenomenon in DSS-induced colitis.**


**Keywords:** PSGL-1; chemical colitis; dendritic cells; macrophages; immune responses

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*No conflicts of interest were declared.*

## Introduction

Innate immune cells—including macrophages, dendritic cells (DC) and natural killer (NK) cells—reside in the intestinal mucosa and secrete cytokines and chemokines that are crucial for the regulation of the innate and the adaptive immune responses under steady-state conditions and during inflammation [1]. Inflammatory bowel diseases (IBDs) are chronic inflammatory diseases of the intestine that develop as a result of a deregulated immune response to commensal bacteria. Crohn's disease (CD) and ulcerative colitis (UC) are chronic autoimmune inflammatory disorders, of unknown aetiology, that affect the intestinal mucosa. Current therapies for IBD employ a sequence of treatments, aimed initially at treating the acute disease and subsequently at maintaining remission [2]. While some biological agents have been used to inhibit the aberrant immune response in the gut [3], there is a need to identify new and more effective targets. Animal models of IBD are invaluable tools for achieving this goal because they permit investigation into the underlying disease mechanisms and the testing of possible treatments [4].

Inflammatory processes are dependent on the recruitment and activation of immune cells which in turn require the formation of intercellular contacts involving cell adhesion molecules [5]. P-selectin glycoprotein ligand-1 (PSGL-1) is a ligand of P-, E- and L-selectins, and is able to mediate the tethering and rolling of circulating leukocytes on the activated endothelium prior to their extravasation [6]. It has been shown that PSGL-1 engagement initiates intracellular signals in leukocytes that promote their firm adhesion to the endothelial cells and induce the transcriptional activation of transcription factors, such as cFos, thereby altering the leukocyte activation state [7–9]. In addition, we have previously demonstrated that the engagement of PSGL-1 in DC induces a tolerogenic programme that enables

them to trigger the differentiation of naive T lymphocytes into regulatory T cells (Treg) [10]. On the other hand, it has been reported that the interaction of PSGL-1 with P-selectin suppresses the proliferation of human $CD34^+$ haematopoietic progenitor cells [11]. Consistently, it has been recently reported that T cells from PSGL-1-deficient mice proliferate more than wild-type (WT) cells [12]. Furthermore, PSGL-1 is involved in the recruitment of leukocytes to the ileum, in an animal model of CD [13,14], and also to the intestinal lamina propria (LP) [15,16]. In addition, PSGL-1 deficiency increases the severity of acute gastroenteritis induced by *Salmonella typhimurium* infection, with an enhanced synthesis of pro-inflammatory cytokines [17]. All these data suggest a role of PSGL-1 in the regulation of the immune responses in the gut.

Since PSGL-1 signalling modifies the activation state of myeloid leukocytes [8,18], in this work we used a model of dextran sulphate sodium (DSS)-induced colitis [19] to explore the involvement of PSGL-1 in the disease's pathogenesis. We provide evidence that PSGL-1 exerts a homeostatic role in the gut-associated lymphoid tissue, and that this adhesion receptor is involved in the pathogenesis of DSS-induced colitis.

## Materials and methods

### Reagents and monoclonal antibodies

The rat 4RA-10 anti-mouse CD162 monoclonal antibody and the following conjugated antibodies (Abs) were obtained from BD Pharmingen (San Jose, CA, USA): anti-CD11c-PECy7, F4/80-biotin, MHC-FITC, CD4-PE, CD8-APC, CD25-biotin, B220-FITC, Gr1-APC, DX5α-biotin, CD3-FITC, IL-12-PE, IL-10-PE, IFNγ-FITC, IL-4-APC, IL-17-PE and streptavidin–PerCP. DSS was from MP Biomedicals, LLC (Illkirch, France). Collagenase IA, dispase, DNAse I and the TRI® reagent were from Sigma-Aldrich (St. Louis, MO, USA). The mouse Th1/Th2 10plex Kit was from Bender Medsystems GmbH (Vienna, Austria). Tissue proteins were extracted with T-PER® tissue protein extraction buffer from Thermo Scientific (Rockford, IL, USA) and quantified with the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA).

### Mice

C57Bl/6 PSGL-$1^{-/-}$ mice were kindly provided by Dr MK Wild and Dr D Vestweber (Max Planck Institute for Molecular Biomedicine, Münster, Germany). C57Bl/6 WT mice were obtained from the Jackson Laboratory. Mice were bred in our animal facilities for 3–4 months before being used for experiments, and all animal experiments were performed in accordance with national and institutional guidelines for animal care.

### Cell preparation from tissues and analysis

Cell suspensions were obtained from mesenteric and popliteal lymph nodes (mLNs and pLNs), spleen and Peyer's patches (PPs) by frictional disaggregation between two microscope slides. Cell suspensions were processed for cell counting, antibody staining and FACS analysis. Peripheral blood leukocytes were analysed with an ABACUS Junior Vet haematological automatic analyser or stained for FACS analysis.

To prepare cell suspensions from LP, colons were extracted, washed with PBS, cut into 1 mm pieces and incubated in RPMI medium supplemented with 4% fetal calf serum and 1 mg/ml collagenase IA, 1 mg/ml dispase and 40 µg/ml DNAse I in a shaking bath at 37 °C for 1 h. Then the cells were washed, suspended in PBS containing 1% BSA, 5 µM EDTA, and immunostained as follows: biotinylated-antibody was added first and samples incubated at 4 °C for 15 min. The cells were then fixed, permeabilized and simultaneously labelled with streptavidin–PerCP and the indicated conjugated antibodies. Labelled cells were analysed in a FACSCalibur flow cytometer. To measure cytokines, colons and mLNs were frozen in liquid nitrogen, powdered and suspended in protein extraction lysis buffer. Protein content was quantified with the BCA™ kit and cytokine levels were determined with the cytokine Mouse 10plex Kit, following the manufacturer's instructions.

### Induction of colitis and disease evaluation

Colitis was induced by daily administration of 4% (for 5 days) or 1% DSS (for 10 days) ($M_w$ 30 000–40 000) dissolved in drinking water. The clinical parameters used to score the disease (disease activity index or DAI) were weight loss, loose stools/diarrhoea and presence of occult/gross bleeding [19]. The animals were weighed before starting the treatment (initial weight) and every day until the end of the treatment. Animals were also checked daily for stool consistency and for the presence of blood in the stools. At the end of treatment, colons were processed for histological analysis, to check for the presence of infiltrate in the LP, or disaggregated and processed to study the immune cells present in the colonic LP infiltrate.

### *In vivo* blockade of PSGL-1

The rat 4RA-10 anti-mouse PSGL-1 mAb (BD) or normal rat IgG (Sigma) were injected intravenously (i.v.) three times (200 µg) every other day into WT C57Bl/6 mice. Eighteen hours after the last dose, the animals were sacrificed. The colons were extracted and disaggregated and the cell suspensions were analysed by flow cytometry.

### Statistical analysis

Statistical significance was estimated by applying the Mann–Whitney U test. Differences were considered significant at $^*p < 0.05$.

## Results

### PSGL-1 deficiency alters immune system homeostasis

Although selectins and their ligands are involved in the homing of leukocytes to different tissues [6,20,21], the exact role of PSGL-1 in the establishment of resident immune cell populations in the gut-associated lymphoid tissue has not been described. We therefore analysed the cellularity of lymphoid organs and the distribution of leukocyte populations in lymphoid tissues from PSGL-1-deficient mice. We found that spleens from these animals contained an enhanced number of cells compared to WT spleens (Figure 1A). In contrast, the number of cells in pLNs and mLNs as well as PPs from PSGL-$1^{-/-}$ animals was significantly diminished (Figure 1A). In addition, blood from PSGL-$1^{-/-}$ mice showed a significant increase in the number of leukocytes/ml, with enhanced levels of granulocytes, DC and monocytes, and similar numbers of lymphocytes. The percentage of circulating granulocytes, DC and monocytes was significantly increased in PSGL-$1^{-/-}$ mice, whereas the percentage of circulating lymphocytes was significantly reduced in these animals (Figure 1B). Furthermore, mLNs from PSGL-1-deficient mice contained a reduced number of $CD4^+$ T lymphocytes and $CD8^+$ NKT cells (Figure 1C). Finally, PPs from these animals showed a significant reduction in the number of $CD8^+$ or $CD4^+$ NKT cells and pDCs (Figure 1D). These results suggest that PSGL-1 exerts an important regulatory role in the tissue distribution of different leukocyte subsets.

### PSGL-1 is implicated in the distribution and activation state of LP leukocytes

Given the altered frequencies of immune cells in mLNs and PPs of PSGL-$1^{-/-}$ mice under steady-state conditions, we next analysed the phenotype of the leukocytes in the colonic LP. We found relative increased numbers of granulocytes and $CD8^+$ cells (Figure 2A) and, in contrast, a diminished percentage of macrophages ($F4/80^+$), DC (lineage-negative, $CD11c^+$) and NK lymphocytes ($DX5\alpha^+$) in the LP of the knockout (KO) mice. In addition, whereas in WT mice two subsets of macrophages and DCs were detected, with high and low expressions of class II MHC molecules ($MHC^{high}$ and $MHC^{dim}$), in the LP of PSGL-1-deficient mice, only MHC-$II^{med}$ cells were observed (Figure 2B). Furthermore, while both WT and PSGL-$1^{-/-}$ DCs had the same expression level of the activation marker CD86, all PSGL-$1^{-/-}$ macrophages expressed the co-stimulatory molecule CD86, a marker of activation also in macrophages [22,23], whereas only 20% of WT macrophages were positive for this molecule (Figure 2B). These data suggest that in the colonic LP of PSGL-$1^{-/-}$ mice

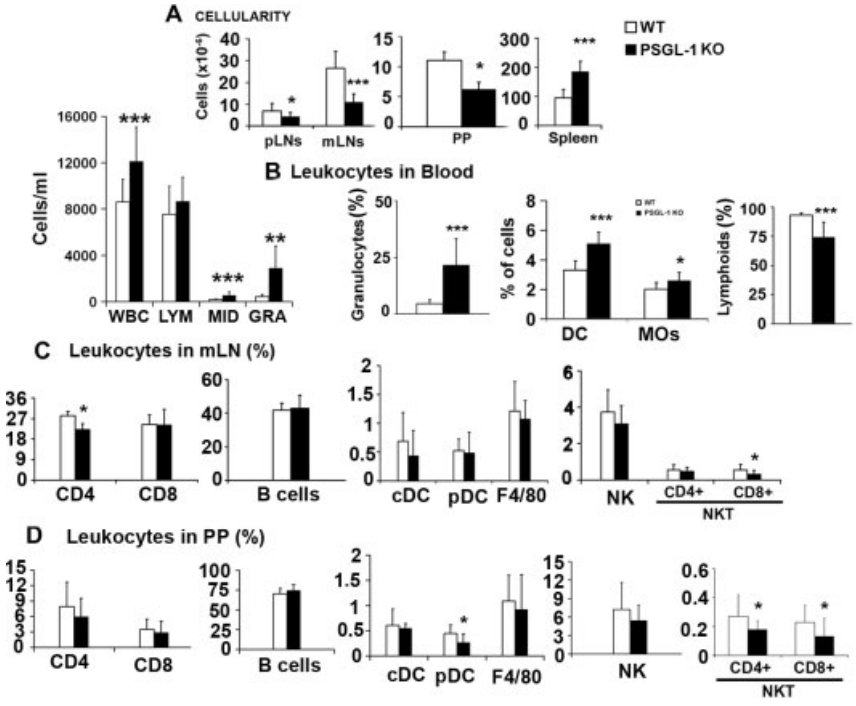


**Figure 1.** Lymphoid organ cellularity and leukocyte populations resident in lymphoid organs of PSGL-$1^{-/-}$ mice. (A) Popliteal lymph nodes (pLN), mesenteric lymph nodes (mLNs), Peyer's patches (PPs), thymus and spleen were obtained from WT and PSGL-1 KO mice, disaggregated and processed to obtain cell suspensions. Total cell numbers in each organ were counted in an automatic cell counter ($n = 7$). (B) The absolute number and the percentage of circulating leukocytes was measured in an automatic haematology analyser ($n = 21$). Circulating DCs ($CD11c^+$) and monocytes (MOs, $F4/80^+$) were analysed by FACS after staining with the appropriate antibody set ($n = 10$). (C, D) Percentage leukocyte populations present in (C) mLNs and (D) PPs. Cells were stained with the appropriate antibodies and analysed by FACS: $CD4^+$ and $CD8^+$ T lymphocytes, B cells ($B220^+$), DCs ($CD11c^+$), pDCs ($CD11c^+$ $B220^+$), macrophages ($F4/80^+$) and NK cells ($DX5\alpha^+$). Data are mean ± standard deviation (SD); $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

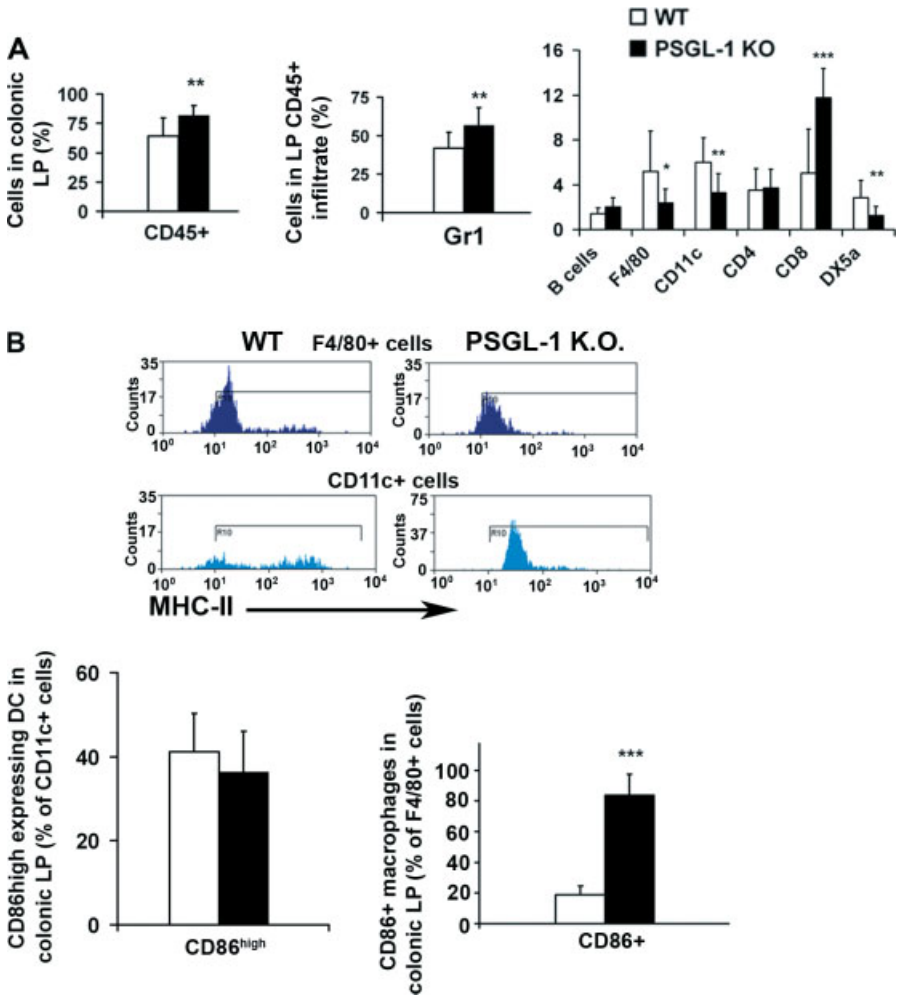


**Figure 2.** Resident leukocyte subpopulations in the colonic lamina propria (LP) of PSGL-$1^{-/-}$ mice. Cell suspensions were obtained from colons of WT and PSGL-1 KO mice, stained with a cocktail of antibodies and analysed by FACS. (A) Percentage of $CD45^+$ cells in the LP infiltrate and percentages of granulocytes ($Gr1^+$), B cells ($B220^+$), macrophages ($F4/80^+$), DCs ($CD11c^+$), $CD4^+$ T cells, $CD8^+$ T cells and NK cells ($DX5\alpha^+$) present in the $CD45^+$ cell population. Data are mean ± SD ($n = 10$ mice). (B) Histograms show a representative FACS analysis of the expression levels of MHC-II molecules in macrophages and DCs obtained from the colonic LP of WT and PSGL-1 KO mice. The bar charts show the percentage of $CD11c^+$ and $F4/80^+$ cells that express the co-stimulatory molecule CD86 (mean ± SD; $n = 5$ mice). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

most DCs and macrophages are in a higher activated state, likely acting as efficient antigen presenting cells.

## Effect of PSGL-1 treatment on the recruitment of leukocytes to the LP

In order to characterize the leukocyte subsets that require PSGL-1 for their recruitment to the colonic LP, we treated WT mice *in vivo* with the 4RA-10 anti-PSGL-1 antibody. Anti-PSGL-1 treatment reduced the total number of macrophages, DCs and B cells in the colonic LP, whereas other leukocyte subsets remained unchanged (Figure 3A). Anti-PSGL-1 treatment also resulted in a diminished expression of MHC-II by macrophages and DCs (Figure 3B), concurring with our previous results showing that signals through PSGL-1 result in induction of a tolerogenic phenotype with low class II expression in DCs [10].

## PSGL-1 deficiency generates a pro-inflammatory environment in colonic LP, with increased Th1 and Th2 cytokine levels and reduced numbers of Treg

We then assessed the profile of cytokine production by resident DCs and macrophages in the colonic LP of WT and PSGL-$1^{-/-}$ mice under steady-state conditions. As shown in Figure 4A, most DCs and macrophages from WT mice synthesized IL-10, with only a small percentage of cells producing IL-12, IL-4, IL-17 or IFNγ. In contrast, in PSGL-1 deficient mice, the number of $IL\text{-}10^+$ DCs and macrophages was markedly reduced, whereas the proportion of cells producing IL-12, IL-4 and IL-17 was increased, a cytokine profile that may favour the generation of inflammatory phenomena.

We also studied the characteristics of resident T lymphocytes in the colonic LP. As shown in Figure 4B, an enhanced number of $IFN\gamma^+$ or $IL\text{-}4^+$ CD4 T lymphocytes was detected in PSGL-$1^{-/-}$ mice. In addition, an increased proportion of $IL\text{-}4^+$ CD8 T cells

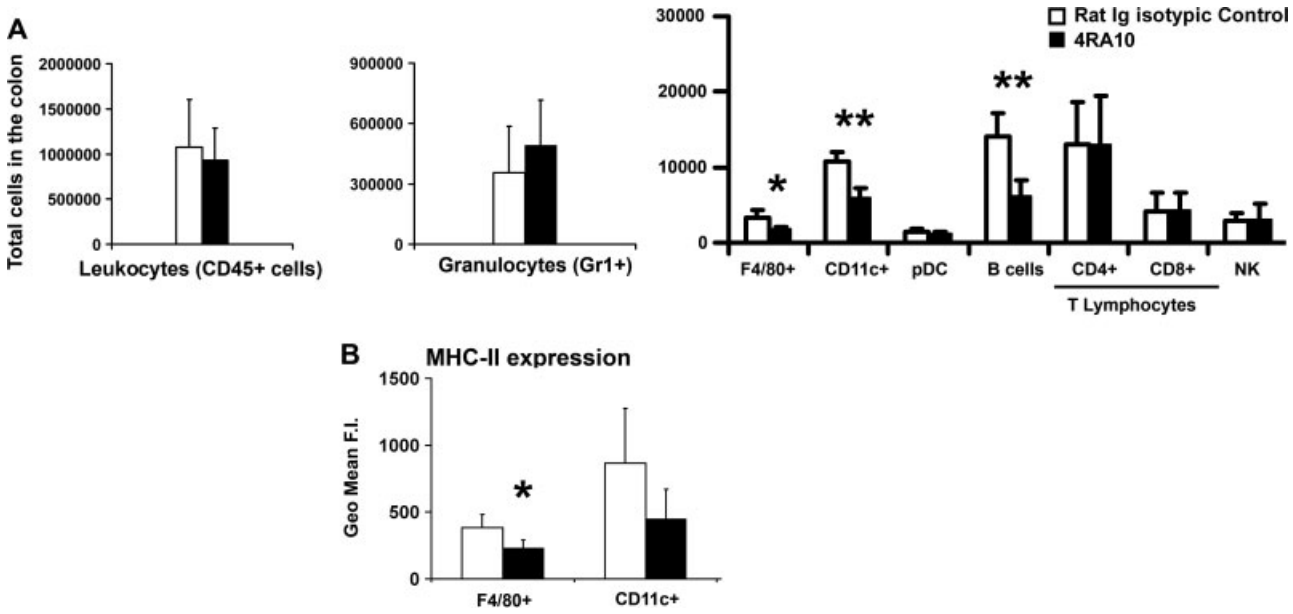


**Figure 3.** Effect of PSGL-1 blockade on the LP leukocytes. C57Bl/6 WT mice were injected i.v. three times with the blocking 4RA-10 anti-PSGL-1 antibody or rat IgG control, and the colonic LP leukocytes were analysed by flow cytometry. Total number of the indicated leukocytes and cell subsets are shown in (A) and the expression of MHC-II by DC and macrophages in (B) (mean ± SD; $n = 5$ mice). $^{*}p < 0.05$; $^{**}p < 0.01$. WBC, white blood cells; GRA, granulocytes; MID, myeloid cells; LYM, lymphocytes.

was observed in these animals (Figure 4B). Moreover, the percentage of T cells with regulatory phenotype ($CD25^{high}$ Foxp-$3^{+}$) was significantly reduced in the LP of PSGL-1 deficient mice (Figure 4C).

## PSGL-$1^{-/-}$ mice develop an exacerbated form of DSS-induced colitis

To assess the influence of the altered gut homeostasis of PSGL-1 deficient mice on the initiation and progression of inflammatory bowel disease, we used the dextran sulphate sodium salt (DSS)-induced colitis model. Addition of 1% DSS to drinking water causes barely detectable symptoms at day 7 of treatment in C57/Bl6 WT mice, while treatment with 4% DSS causes acute disease with severe clinical and histological symptoms [19]. At either dose, clinical symptoms started earlier in PSGL-$1^{-/-}$ mice (Figure 5A, B, E). Examination of disease induced by 4% DSS showed that the disease progressed faster and was more severe in PSGL-$1^{-/-}$ mice: at day 5 of treatment KO mice had lost on average 20% of their initial weight (Figure 5A), had a high disease activity index (DAI; Figure 5B) and showed more severe colon inflammation than WT (Figure 5C). Moreover, three of the 16 KO mice died on day 5 before the completion of analysis. Histological analysis showed that the colons of PSGL-$1^{-/-}$ mice were more distended and showed greater infiltration and a higher extent of crypt destruction than WT (Figure 5D). Treatment with 1% DSS induced clinical symptoms in PSGL-$1^{-/-}$ mice starting at 3 days, and maximum DAI was reached at day 8, with all animals presenting loose stools and bleeding (Figure 5E). These results indicate that PSGL-1 deficient mice initiate colitis earlier and develop a more severe disease than WT mice.

## PSGL-1 deficiency alters the profiles of inflammatory immune cell subpopulations in colonic LP affected by chemically induced colitis

We then analysed the colonic inflammatory cell infiltrate induced by DSS treatment. As shown in Figure 6A, B, a diminished number of DC and macrophages was observed in the inflammatory cell infiltrate of PSGL-1 mice. However, an enhanced expression of the activation marker CD69 was observed in the macrophage population of these animals (Figure 6C). Furthermore, an enhanced proportion of $CD8^{+}$ NKT cells was detected in PSGL-1 KO mice (Figure 6B). Analysis of cytokine production showed that the percentages of macrophages producing IL-12, IL-4 and IFN$\gamma$ were higher in the PSGL-$1^{-/-}$ mice (Figure 6D). In addition, the proportion of IL-$10^{+}$ DC was lower in PSGL-1-deficient mice, whereas the percentage of IL-4-producing cells was significantly enhanced (Figure 6E).

## T cell responses in colonic LP during DSS-induced colitis are exacerbated in PSGL-1 deficient mice

We next carried out a multi-cytokine analysis of colon homogenates. Interestingly, IL-1$\alpha$, IL-22 and IL-6 levels induced by DSS treatment were markedly higher in the colons of PSGL-$1^{-/-}$ mice (Figure 7A). In addition, an enhanced number of IFN$\gamma$ and IL-17 producing $CD4^{+}$ T cells (Th1 and Th17 cells, respectively) was detected in PSGL-1-deficient mice (Figure 7B). Analysis of $CD8^{+}$ T lymphocytes showed a similar increase in IFN$\gamma^{+}$ and IL-$17^{+}$ cells (Figure 7B). In contrast, similar levels of Treg cells were observed in WT and PSGL-$1^{-/-}$ mice (Figure 7C).

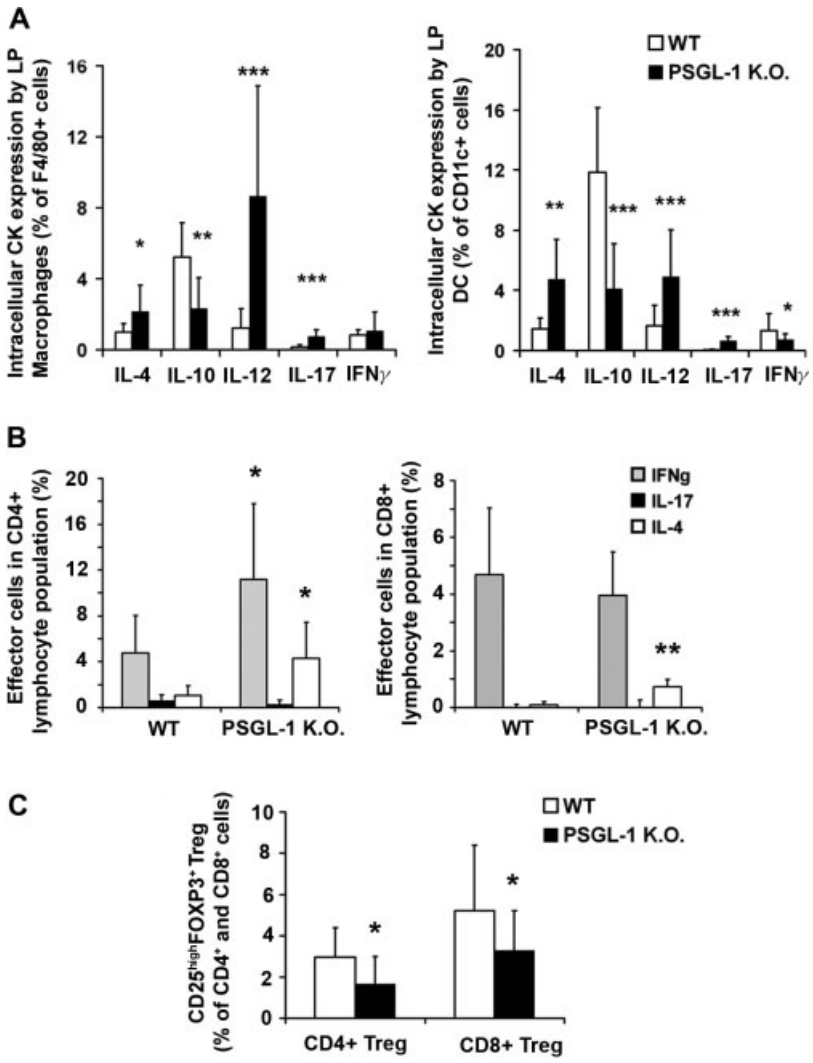


**Figure 4.** Innate and adaptive effector cells in PSGL-$1^{-/-}$ mice. (A) Percentage of different macrophage (F4/$80^{+}$) and DC ($CD11c^{+}$) effector subpopulations resident in the $CD45^{+}$ infiltrate of colonic LP from WT and PSGL-1 KO mice. Cells were analysed by FACS after intracellular staining for IL-4, IL-10, IL-12, IL-17 or IFNγ. Data are mean ± SD ($n = 6$ mice). (B) Percentage of $CD4^{+}$ and $CD8^{+}$ effector subpopulations in the $CD45^{+}$ infiltrate of colonic LP from WT and PSGL-1 KO mice. Cells were analysed by FACS after intracellular staining for IFNγ (Th1), IL-17 (Th17) and IL-4 (Th2). Data are mean ± SD ($n = 6$). (C) Percentage of Treg ($CD25^{high}$ Foxp-$3^{+}$) in the $CD4^{+}$ and $CD8^{+}$ subpopulations of colonic LP $CD45^{+}$ infiltrate. Data are mean ± SD ($n = 12$ mice); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

## Discussion

Commensal bacteria (microbiota) normally exist in a symbiotic relationship with the host intestine, but members of the microbiota can opportunistically invade mucosal tissues, causing serious problems in immunodeficient individuals. To ensure protection of the host against such opportunistic bacterial invasions, the balance between tolerance and immunity must be tightly regulated, and this intestinal homeostasis is maintained by the immune system [24,25]. When infectious bacteria traverse the epithelial barrier, macrophages and DC eliminate them by phagocytosis and produce proinflammatory cytokines to recruit neutrophils and other leukocytes and activate T cell responses. To maintain self-tolerance to microbiota and restore homeostasis after infection, inflammatory responses must be limited by the generation of Treg. In addition, another important role of intestinal macrophages after injury is to help restore the epithelial barrier, because epithelium damage can lead to bacterial infection, inflammation and sepsis [24]. However, it is evident that the molecules and cells that are involved in the regulation of the innate and adaptive responses that occur in mucosal tissues have not been fully characterized.

We have previously reported that the engagement of the adhesion receptor PSGL-1 on DCs enables them to generate Treg and that PSGL-1-deficient DCs are more immunogenic than their WT counterparts [10]. Accordingly, in this work we show that PSGL-1 seems to have a relevant role in the maintenance of the intestinal balance between tolerance and immunity, in addition to its role in controlling leukocyte recruitment to the LP. We have found that PSGL-1-deficient mice show reduced size of intestinal-associated lymphoid organs, and that their immune cell population profile is abnormal, suggesting an altered homeostasis. In this regard, it is very likely that the low proportion of DCs and macrophages

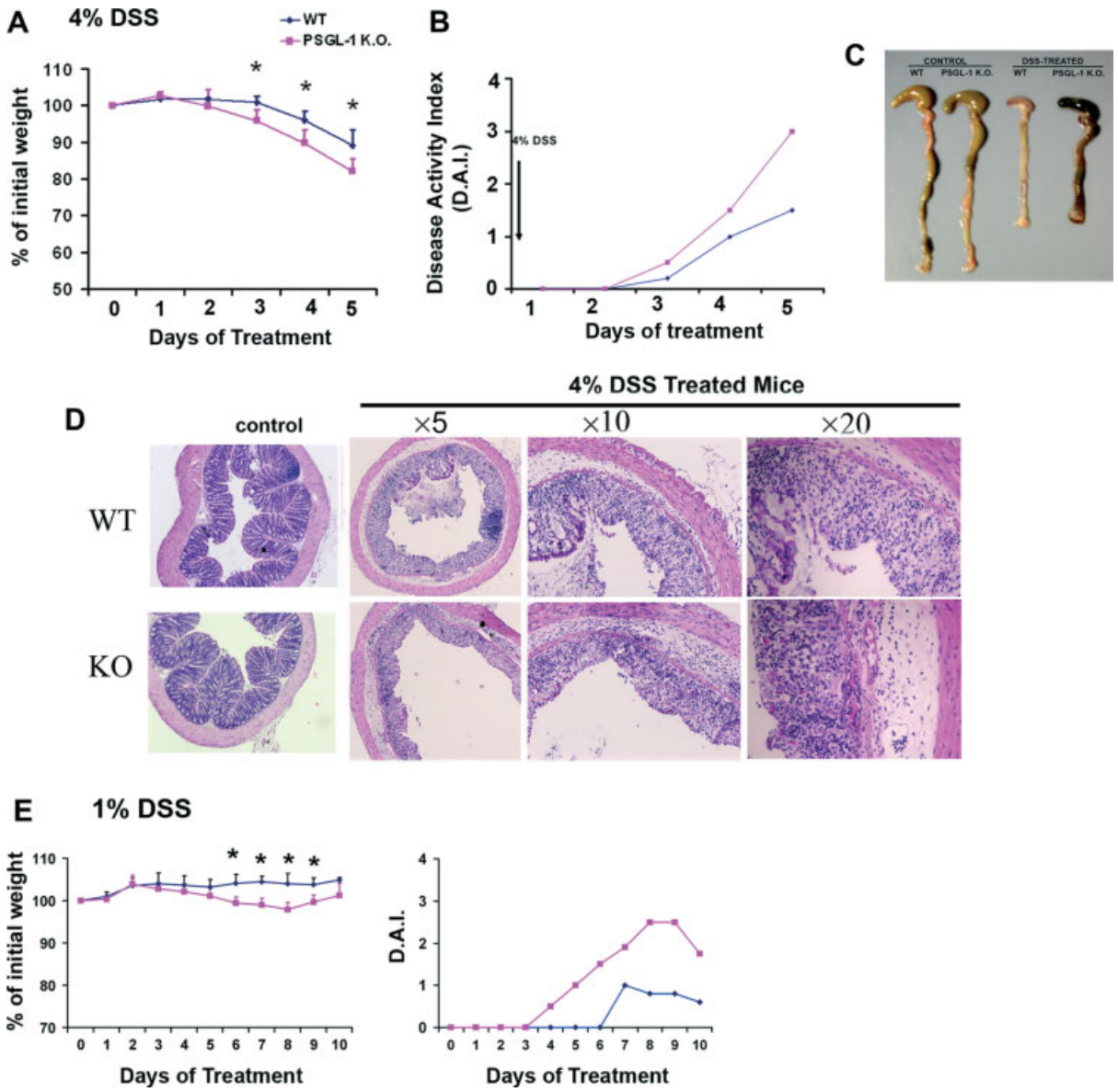


**Figure 5.** Progression of acute colitis induced by DSS in PSGL-$1^{-/-}$ mice. Acute colitis was induced in WT and PSGL-1 KO mice by including 4% DSS (A–D) or 1% DSS (E) in the drinking water. (A) Percentage of the initial weight from day 0 at day 5 of 4% DSS treatment. Data are mean ± SD ($n = 5$ mice) from a representative experiment of three; $^{*}p < 0.05$. (B) Time course of disease activity index (DAI), scored by clinical symptoms (see Methods). (C) Photographs of representative colons obtained after 5 days of treatment with 4% DSS. (D) H&E staining of sections of colon obtained after 5 days of treatment with 4% DSS. (E) Analysis of disease progression in WT and PSGL-1 KO mice treated with 1% DSS for 10 days. Data are mean ± SD ($n = 6$ mice) from a representative experiment of three; $^{*}p < 0.05$.

observed in these animals is due to a reduced recruitment of these cells, a possibility that is supported by our experiments of treatment with anti-PSGL-1, which blocks interaction with the natural ligand. This point is further supported by reports on the role of PSGL-1 in the recruitment of leukocytes to LN [21], ileum [13,14] and LP [15,16]. However, the cytokine profile and phenotype of these macrophages and DCs indicate that they are in an activated state (CD86 and MHC-II expression and enhanced synthesis of IL-4, IL-12 and IL-17, with a diminished production of IL-10), a phenomenon that could be causally associated with the increased numbers of granulocytes, B cells and $CD8^{+}$ T cells. It has been widely described that IL-10 is a potent suppressor of classical macrophage activation [26], and animals deficient in this cytokine show spontaneous intestinal inflammation [27] as well as enhanced synthesis of the pro-inflammatory cytokines IL-12 and TNF. Despite the pro-inflammatory environment detected into the colonic LP of PSGL-$1^{-/-}$ mice, these animals do not spontaneously develop intestinal inflammation or ulcerative colitis (UC). However, it has been reported that in the absence of PSGL-1, mice are highly sensitive to pathogens such as *Salmonella* [17]. It is therefore possible that the impaired leukocyte recruitment in PSGL-1 KO mice may prevent the surveillance of immune cells on overgrowth and inflammation induced by commensal microbiota. It is also feasible that the relative low proportion of Treg

observed in PSGL-1 KO mice is enough to limit the proliferation of Th1, Th2 and Th17 effector cells under steady-state conditions [28,29].

Concerning the role of PSGL-1 on T lymphocytes and the development of UC, apparently opposite results have been reported. Thus, it has been reported that PSGL-1 deficiency exacerbates the development of inflammation in a mouse model of ulcerative colitis (UC) induced by the adoptive transfer of naive T cells into RAG-deficient hosts [12]. However, using the

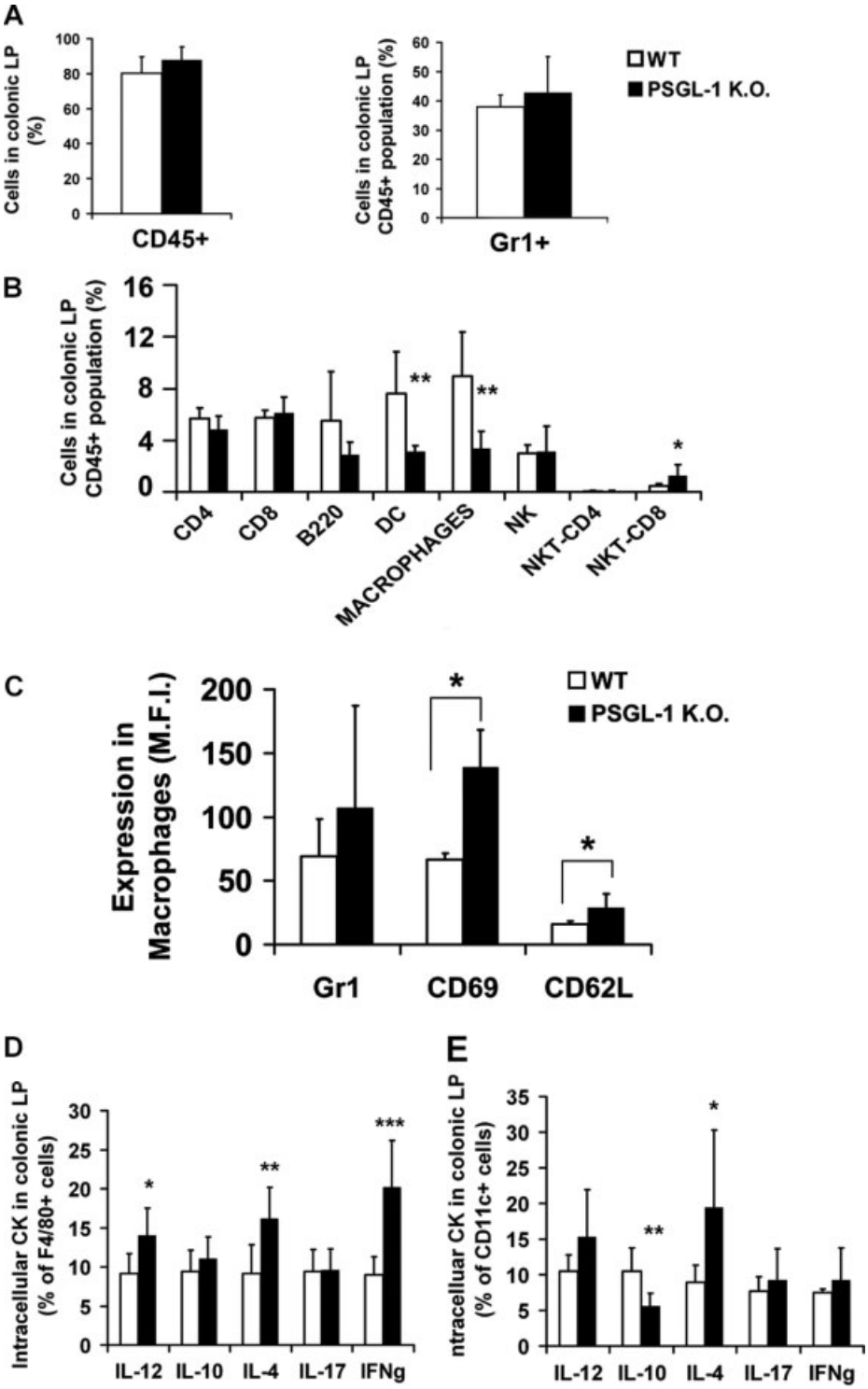


**Figure 6.** Leukocyte infiltrate in the colonic LP of PSGL-$1^{-/-}$ mice after acute colitis induction. Cell suspensions were obtained from colons of WT and PSGL-1 KO mice treated with 4% DSS (5 days). Cells were stained with a cocktail of antibodies and analysed by FACS. (A) Percentage of $CD45^+$ cells in the LP infiltrate and the percentage of $Gr1^+$ cells in the $CD45^+$ population. (B) Percentage of $CD4^+$ and $CD8^+$ T lymphocytes, B cells ($B220^+$), DC ($CD11c^+$), macrophages ($F4/80^+$) and NK cells ($DX5\alpha^+$) in the $CD45^+$ population. (C) Suspensions of $F4/80^+$ cells were stained with antibodies for surface markers and analysed by FACS ($n = 4$). (D, E) Macrophage (D) and dendritic cell (E) subpopulations analysed by intracellular cytokine staining and FACs. In all cases, data are mean ± SD from six mice; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

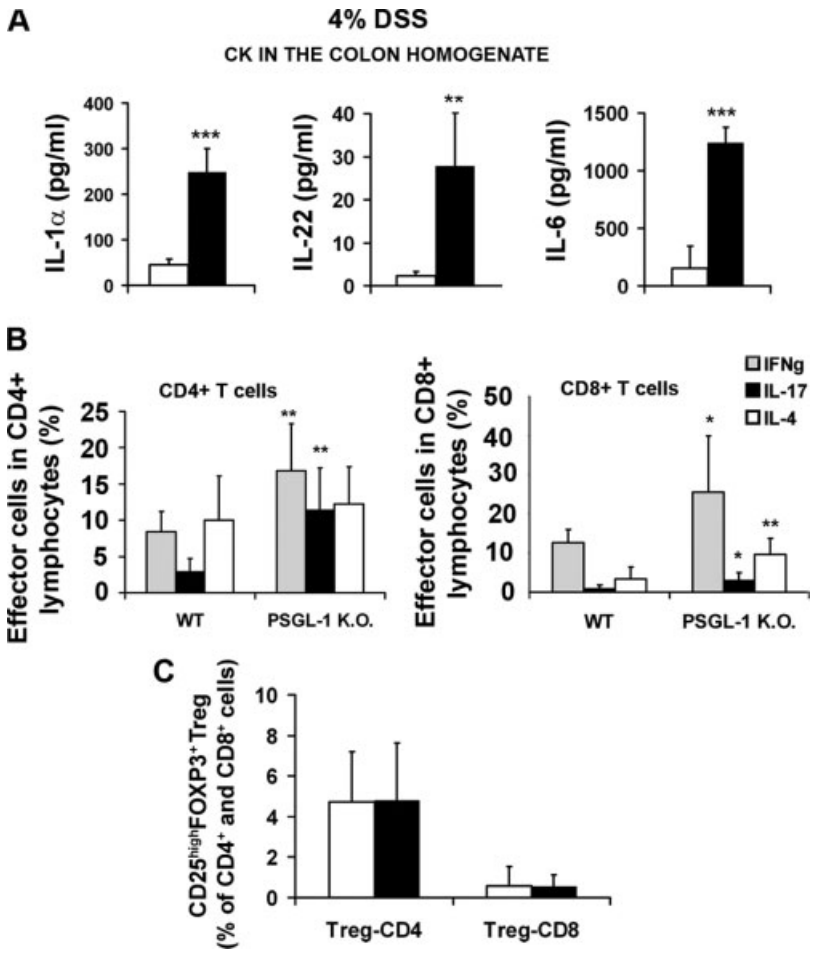


**Figure 7.** Increased inflammatory cytokine levels and Th responses in the colonic LP of PSGL-$1^{-/-}$ mice after acute colitis induction. Colons were obtained from WT and PSGL-1 KO mice treated with 4% DSS. (A) Total protein cell lysates were incubated with the antibody cocktail of the Th1/Th2/Th17 Flow Cytomix kit and analysed by FACS. The panel shows the amounts of IL-1α, IL-22 and IL-6 found in 300 ng total protein ($n = 12$ mice). (B) Percentages $CD4^+$ (left) and $CD8^+$ (right) T cells expressing IFNγ (Th1), IL-17 (TH17) and IL-4 (Th2) ($n = 6$). (C) Percentage of Treg ($CD25^{high}$ Foxp-$3^+$) in the $CD4^+$ and $CD8^+$ subpopulations ($n = 6$). In all cases, data are mean ± SD; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

same mouse model, another study reported that PSGL-1 is not required to initiate UC [30].

In spite of the relatively low number of macrophages and DCs in the LP of PSGL-1 KO mice under steady-state conditions, these animals rapidly develop an aggressive form of colitis upon treatment with DSS. This phenomenon suggests that these mice have an enhanced ability to generate innate immune responses [4], and is in agreement with the activated phenotype of LP macrophages. In this regard, it has been described that immunogenic DCs and activated macrophages are able to synthesize the pro-inflammatory cytokines IL-6 and IL-1, which induce the differentiation of Th17 cells [31]. Thus, it is very likely that in PSGL-1-deficient mice, the higher percentage of activated macrophages under steady-state conditions favours the differentiation of Th17 lymphocytes, in the presence of a triggering factor such as DSS. In turn, Th17 lymphocytes would be responsible for the observed over-production of IL-22, which along with IL-17 exerts an important pro-inflammatory effect. Interestingly, recent studies in humans with IBD have detected increased levels of *IL-17, IL-1* and *IL-6* mRNAs in intestinal mucosa and a reduced ratio of circulating Treg/Th17 [32].

We also detected an imbalanced Teff : Treg ratio in the colonic LP of PSGL-1 KO mice with DSS-induced acute colitis. These data further support that PSGL-1 deficient mice have a defective generation of adaptive Treg cells [10], a phenomenon that very likely contributes to the enhanced tissue damage observed upon induction of acute colitis. Therefore, our findings suggest that PSGL-1 contributes to the regulation of the innate and adaptive immune cell responses and hence participates in the initiation/resolution of inflammatory phenomena. We consider that this places PSGL-1 as a novel potential target for pharmacological intervention to ameliorate or resolve inflammatory diseases such as IBD.

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## Author contributions

NN-A, acquisition and analyses of data; AL, acquisition and analyses of data; DS, critical revision of the manuscript; JG, critical revision of the manuscript and statistical revision; RG-A, critical revision of the manuscript and drafting; FSM, conceptual and experimental design, discussion of data, funding and drafting; AU, conceptual and experimental design, acquisition, analyses and discussion of data, study supervision, funding and drafting.

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