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Pregnancy specific glycoprotein 1 (PSG1) activates TGF-β and prevents dextran sodium sulfate (DSS)-induced colitis in mice

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Abstract

Transforming growth factor beta (TGF-βs) are secreted from cells as latent complexes and the activity of TGF-βs is controlled predominantly through activation of these complexes. Tolerance to the fetal allograft is essential for pregnancy success; TGF-β1 and -β2 play important roles in regulating these processes. Pregnancy-specific β-glycoproteins (PSGs) are present in the maternal circulation at high concentration throughout pregnancy and have been proposed to have anti-inflammatory functions. We found that recombinant and native PSG1 activate TGF-β1 and TGF-β2 *in vitro*. Consistent with these findings, administration of PSG1 protected mice from DSS-induced colitis, reduced the secretion of pro-inflammatory cytokines and increased the number of T regulatory cells. The PSG1-mediated protection was greatly inhibited by the co-administration of neutralizing anti-TGF-β Ab. Our results indicate that proteins secreted by the placenta directly contribute to the generation of active TGF-β and identify PSG1 as one of the few known biological activators of TGF-β2.

INTRODUCTION

Transforming growth factor beta (TGF-β) are involved in the regulation of several processes including cell growth, differentiation and angiogenesis [1]. In addition, TGF-β regulates diverse innate and adaptive immune functions ranging from immune tolerance, tumor rejection and suppression of autoimmune disorders [2]. TGF-β exists in three isoforms in

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DISCLOSURE

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mammals: TGF-β1, -β2 and -β3, which are secreted from cells in biologically inactive forms, either as part of a large latent complex (LLC) or the small latent complex (SLC). The LLC is composed of the latent TGF-β binding protein (LTBP), the latency associated protein (LAP) and the mature 25 kDa TGF-β while the SLC lacks LTBP and is comprised only of LAP and mature TGF-β [3]. The association of LAP with the mature TGF-β homodimer is non-covalent and essential for latency, as it is only when this interaction is disrupted that the 25 kDa growth factor can bind to its receptors [4]. Most cells secrete at least one TGF-β isoform and express TGF-β receptors; which have known biological activators. For instance, activators release TGF-β either by degrading LAP and/or LTBPs or by modifying the conformation of the latent complex. Integrins αvβ6, αvβ8 and reactive oxygen species are specific activators for particular TGF-β isoforms, due to differences in the sequence of LAP of each isoform. In addition, other activators (e.g. platelet thrombospondin 1 (TSP-1), certain proteases, low pH conditions, and high temperatures) activate all three isoforms [5–7].

Pregnancy-specific β-glycoprotein 1 (PSG1) is synthesized by the human placenta and is secreted into the maternal circulation in increasing concentration from the time of trophoblast differentiation until term [8]. PSG1 is the most abundantly expressed of the 11 different PSG genes, which reach a median concentration of 168μg/ml at week 36 of pregnancy, when a plateau is reached [9]. PSGs belong to carcinoembryonic antigen (CEA) family but differ from other CEA family members in that they are secreted rather than membrane-bound. Human PSGs are highly glycosylated proteins composed of an immunoglobulin (Ig) variable-like domain followed by constant-like domains [8]. Genes coding for PSGs have only been described in species with haemochorial placentation in which fetal cells are in direct contact with the maternal circulation posing a risk of immune rejection by the mother.

There are 17 murine PSG genes and the few that have been studied so far appear to have a similar function as demonstrated by *in vitro* studies [10–11]. The murine PSG genes are interspersed within chromosome 7 with other members of the CEA family making it technically difficult to evaluate whether PSGs are essential for pregnancy success [12]. At this time, the importance of these proteins in pregnancy is supported by clinical studies, which indicate that concentrations of PSGs below the normal range are associated with adverse pregnancy outcome [13–14]. During pregnancy, TGF-β regulates trophoblast invasion, angiogenesis and extracellular matrix production [15]. Additionally, TGF-β suppresses $CD8^+$ T- and NK-cell cytotoxic functions and is essential for differentiation of extrathymic regulatory T cells, which are necessary for the development of tolerance of the maternal immune system to paternal antigens expressed by the fetus [16–17]. Studies of isoform-specific TGF-β-null mice demonstrated non-redundant roles of the different TGF-β isoforms in development. While the three isoforms have been shown to be expressed in mucosal tissues and signal through a common receptor subunit, their expression varies in different cell types. In addition, the different TGF-β isoforms have recently been reported to vary in their ability to induce the pathogenic function of effector TH-17 cells [18–19].

Treatment of different cells with PSG1 increased the secretion of total TGF-β1 in the supernatant as determined by ELISA [20–21]. In addition, we observed that PSG1 induced VEGF-A in a trophoblast cell line in a TGF-β-dependent manner [22]. This observation as

well as other observations described below prompted us to investigate whether PSG1 bound TGF-β and whether PSG1 also could play a role in the process of TGF-β activation.

RESULTS

Recombinant and native PSG1 bind TGF-β

First, we determined by ELISA that purified recombinant PSG1-Fc generated in CHO-K1 cells was associated with total (latent + active) TGF-β1. Next, we explored whether besides total TGF-β1, PSG1-Fc contained the active form of the cytokine and if the presence of latent and active TGF-β1 also could be detected in recombinant PSG1 preparations generated in other cell lines. We found that Protein A-purified PSG1-Fc harvested from the supernatant of transfected HeLa and HEK-293T cells also was associated with TGF-β1. At concentrations of PSG1-Fc higher than 15μg/ml, some of the TGF-β1 was in the active form, as detection by ELISA did not require prior acidification. Table S1 shows results obtained with individual PSG1-Fc preparations. Active and latent TGF-β1 was also detected in recombinant PSG1-His-FLAG secreted from stably transfected CHO-K1 cells after elution from a His-Trap and an anti-FLAG agarose column (Table S1). Besides mature TGF-β1, PSG1 purified from HeLa and HEK-293T cells contained LAP-β1 (Figure 1a). We did not test for the presence of LAP in PSG1 made in CHO-K1 cells due to the lack of available reagents to detect hamster LAP. The PSG1-LAP interaction was confirmed using HeLa cells expressing a recombinant PSG1 that contains the transmembrane-anchorage domain of CEACAM1 (HeLa-PSG1) [8]. HeLa-PSG1 cells had significantly higher levels of LAP bound to their membrane when compared to untransfected HeLa cells (Figure 1b). PSG1-Fc secreted from transfected MEFs derived from TGF-β1-null mice and PSG1 generated in insect cells, which we had used for our initial studies in monocytes, had undetectable levels of associated TGF-β1. This is expected as these cells do not express this cytokine and were grown in serum-free conditions [20]. Interestingly, PSG1-Fc generated in the TGF-β1-null fibroblasts contained latent TGF-β2, which could only be detected at 30μg/ml or higher concentrations of PSG1, with some variations in the concentration of TGF-β2 observed between preparations (Table S1). These results indicate that recombinant PSG1 generated in different cell lines can bind to TGF-β1 and TGF-β2. CEACAM9, like PSG1, is a member of the CEA family expressed in the placenta and FLAG-Fc is a recombinant protein containing the same tags as the recombinant PSG1 used for most of our studies. CEACAM9-Fc and FLAG-Fc were generated and purified under identical conditions as PSG1-Fc. These proteins were used as controls and were evaluated in parallel at equimolar concentrations as the different preparations of PSG1 in each experiment. We did not detect TGF-β1, -β2 or LAP-β1 in the control proteins up to the highest concentration tested, which was 100μg/ml. All recombinant proteins utilized for these studies are shown in Figure 1c.

To determine whether native PSG1 bound LAP-β1 and mature TGF-β1, we affinity purified PSG1 from sera of pregnant women (Figure 1d). We found that all four native PSG1 preparations, each purified from different batches of pooled sera from pregnant women, carried TGF-β1 and LAP-β1. As observed with recombinant PSG1 preparations, there was some variation in the concentration of PSG1 at which LAP-β1 and mature TGF-β1 were detected and in the amounts of these moieties carried by the different preparations. In

addition, we tested for the presence of TGF-β2 in different preparations of native PSG1 up to a PSG1 concentration of 80μg/ml. We found that latent TGF-β2 was detected in two out of four pregnant sera PSG1 preparations. Table 1 shows the ELISA results obtained for one of the native PSG1 preparations that carried TGF-β2.

PSG1 activates latent TGF-β1 and TGF-β2

We next examined whether PSG1 is a passive TGF-β carrier, like apolipoprotein E3 and surfactant protein A, or if unlike these molecules, PSG1 functions as an activator of the latent form of this cytokine [23–24]. To address this issue, we added PSG1 generated in the MEF TGF-β1-null cells, to TGF-β reporter cell lines, which are useful for detecting the presence of active TGF-β but that themselves only secrete the latent form of this cytokine. Importantly, in these experiments, PSG1 was used at concentrations at which we could not detect any isoform of TGF-β associated with the protein. To test whether PSG1 could activate latent TGF-β1 secreted from cells, we generated a stable TGF-β reporter cell line by transducing HEK-293T cells with lentivirus particles containing the firefly luciferase gene under the control of a minimal CMV promoter and tandem repeats of the SMAD transcriptional response element (HEK-SMAD reporter). Specific ELISAs for all three isoforms demonstrated that this cell line expresses only latent TGF-β1 with an average of 182pg/ml detected after acid activation of the supernatant harvested after 16h. Treatment of the HEK-SMAD reporter cell line with PSG1-Fc generated in TGF-β1-null cells resulted in a dose-dependent increase in luciferase expression compared to the CEACAM9-Fc treated cells (Figure 2a).

The second reporter system tested relies on cells (TMLECs) transfected with a truncated PAI-1 promoter fused to the firefly luciferase reporter gene [25]. The third bioassay consists of embryonic fibroblasts derived from TGF-β1-null mice, which were stably transfected with a reported plasmid consisting of TGF-β responsive SMAD-binding elements coupled to a secreted alkaline phosphatase (SEAP) reporter gene (MFB-F11) [26]. We found that incubation of the reporter TMLEC and MFB-F11 cell lines with PSG1-Fc resulted in a dose-dependent increase of light units (Figure 2b) and of SEAP activity (Figure 2c), respectively. The TGF-β2 secreted by TMLECs and MFB-F11 cells was all in the latent form, as active TGF-β2 could be measured only after acid activation of the conditioned media (average of 280pg/ml and 440pg/ml of TGF-β2 after 16h incubation, respectively). We could not detect TGF-β1 or TGF-β3, with or without acid activation, in the conditioned media of these cells. These results strongly suggest that PSG1 converts latent TGF-β2 secreted by these cells into its active form.

Importantly, we found that the native PSG1 preparations, which had undetectable levels of all three TGF-β isoforms at the concentration utilized for these experiments, had the ability to activate TGF-β1 secreted from the HEK-SMAD reporter cells (Figure 2d). Furthermore, native PSG1 activated latent TGF-β2 secreted from the reporter TMLEC and MFB-F11 cells (Figure 2e–f). As controls, we treated the reporter cells with the control proteins CEACAM9-Fc, Flag-Fc or with media alone. We did not observe a significant difference in the response of any of these cells to CEACAM9-Fc and Flag-Fc treatments; which were not significantly different to media alone (not shown). As positive control, we treated the cells

with different concentrations of mature TGF-β1 or TGF-β2 (Figure 2g–i). The increase in luciferase and SEAP activity mediated by recombinant and native PSG1 in the three reporter bioassays employed was specific, as it was inhibited by the addition of the ALK5 inhibitor SB 431542 and the pan neutralizing anti-TGF-β Ab 1D11 (Figure 3a–d).

Because some metalloproteinases and serine proteases activate TGF-β [27], we tested whether the activation of TGF-β1 and -β2 mediated by PSG1 that was observed in the TMLEC, MFB-F11, and HEK-SMAD reporter assays was blocked by the addition of inhibitors of serine and cysteine proteases (leupeptin), aspartate proteases (pepstatin), amino peptidases (bestatin), metallo endopeptidases (phosphoramidon), matrix metalloproteinases (galardin), and anti-papain. None of these protease inhibitors blocked the PSG1-mediated increase in luciferase activity and SEAP expression, which are the indicators of the presence of the active cytokine (Figure S1).

PSG1 activates SLC in cell-free conditions

To determine whether PSG1 activated the small latent complex (SLC) of TGF-β1 under chemically defined, cell-free conditions, we incubated different concentrations of recombinant and native PSG1 or control protein with SLC of TGF-β1 and measured the amount of active TGF-β1 as indicated in the Methods section. In addition, as the SLC may contain a small amount of active TGF-β1, we incubated SLC in PBS to determine the concentration of active TGF-β1 in each SLC preparation (insert in Figure 4a). We employed two ELISAs to measure active TGF-β1, one in which the wells were coated with TGF-β RII-Fc and the other, in which the wells were coated with an Ab that binds only to active TGF-β. In both assays, we observed that PSG1 induced an increase in the concentration of active TGF-β1 in a dose dependent manner (Figure 4a–b and **S2**). Recombinant and native PSG1 without SLC were added to the wells as controls to show that these preparations did not carry any active TGF-β1 at the concentrations utilized in these experiments (data not shown). Recombinant PSG1 generated in insect cells, GST-PSG1d, was also able to activate SLC in the cell-free assay (Figure S2). While PSG1 could activate SLC, the amount of active TGF-β1 generated was always lower than what was observed upon acid treatment of the SLC, which determines the total available substrate in this assay (Figure 4c and **S2**).

Prevention of DSS-induced colitis by administration of PSG1

The DSS model of colitis has demonstrated several correlations with inflammatory bowel disease. In this model, an alteration of the mucosal barrier results in a macrophage-induced inflammatory response superimposed to T-cell mediated inflammation [28]. Several studies strongly suggest that activated TGF-β1 plays an important role in maintaining intestinal tolerance [29–31]. Therefore we hypothesized that administration of PSG1 would ameliorate the intestinal pathology observed in acute colitis induced by DSS administration as TGF-β1 and -β2 have been suggested to play a non-redundant role in the control of intestinal inflammation [32]. Mice were given DSS in the drinking water and were injected i.p. with 100 μg of recombinant PSG1-Fc or an equimolar concentration of the control protein Flag-Fc, as described in the Methods section (Figure 5a). As shown in Figure 5b, animals receiving PSG1 were protected from weight loss and colon shortening, the standard clinical features observed in mice receiving DSS [28]. In addition, histologic analysis revealed that the colons

obtained from PSG1-treated mice had a marked reduction in thickening of the colonic wall, as well as transmural leukocyte infiltration and lost significantly less goblet cells than protein control-treated mice (Figure 5c). Furthermore, there was no evidence of bloody stools in mice receiving PSG1 in contrast to the protein control-treated mice, in which diarrhoea and bloody stools were evident at day 6.

PSG1 effect on cytokine production in mice undergoing DSS-colitis

Next, we determined the effect of i.p. administration of recombinant PSG1 on cytokine production in mice subjected to induction of DSS colitis. For these studies, after the mice were killed, MLN cells were obtained from PSG1-Fc or control protein-treated mice. After culture with anti-CD3/anti-CD28 or IFN-γ and *Staphylococcus aureus* Cowans (SAC) (described in Methods), different cytokines were measured in the culture supernatant. As shown in Figure 5d, MLN cells obtained from mice given control protein produced increased amounts of IL-6, TNF-α, IFN-γ upon stimulation with anti-CD3/anti-CD28. On the other hand, secretion of these cytokines was not increased upon stimulation, when MLN cells were obtained from PSG1-treated mice. Similarly, increased secretion of IL-6 and TNF-α was only observed in MLN cells obtained from control protein but not from PSG1-treated mice upon stimulation with IFN-γ and SAC (Figure 5e). Finally, we observed that low amounts of IL-10 could be detected in stimulated MLN cells obtained from both PSG1 as well as control-protein treated mice (Figure 5d–e). Analysis of cytokine gene expression in the colon showed that mice receiving PSG1 expressed lower levels of IFN-γ, TNF-α and IL-17 mRNA than control protein-treated mice (Figure 5f). In addition, we observed an increase in IL-10 mRNA expression in the colon upon PSG1 treatment and no significant changes in the expression of TGF-β1 mRNA (Figure 5f). Taken together these results show that PSG1 can significantly reduce the generation of two Th1-cytokines implicated in the development of mucosal inflammation, TNF-α and IFN-γ, which are negatively regulated by TGF-β and of the pro-inflammatory cytokine IL-17.

To evaluate whether the *in vivo* protective effect of PSG1 in the DSS-colitis model could be correlated with the activation of TGF-β by PSG1 observed *in vitro*, we administered anti-TGF-β Ab or a control antibody together with PSG1 or the control protein at days 0 and 3 (Figure 6a). We observed that co-administration of neutralizing anti-TGF-β Ab precluded the protective effect of administration of PSG1 as shown in Figure 6b–c. In accordance with these results, we observed that administration of anti-TGF-β Ab to the PSG1-treated mice revealed increased histological signs of inflammation with clear signs of immune cell infiltration and epithelial erosion when compared to mice receiving PSG1 alone or PSG1 with control Ab (Figure 6d). On the other hand, co-treatment with anti-TGF-β antibody did not have a significant effect in the protein control-treated mice, which again experienced significant weight loss, colon shortening and pathology (Figure 6b–d). In response to anti-CD3/anti-CD28 stimulation, MLN cells obtained from PSG1-treated mice co-injected with anti-TGF-β Ab secreted significantly more IL-6, TNF-α, and IFN-γ than MLN cells obtained from PSG1-treated mice that received the control Ab (Figure 6e). On the other hand, no significant change in IL-17 was observed when comparing the stimulated cells obtained from PSG1-treated mice upon administration of the neutralizing anti-TGF-β Ab and the control Ab (Figure 6f). An increase in IL-17 secretion was observed in the protein

control-treated mice, which received the neutralizing anti-TGF-β Ab (Figure 6f). When MLN cells were stimulated with IFN-γ and SAC, there was a significant increase of IL-6 and TNF-α in the PSG1-treated mice receiving the anti-TGF-β Ab compared to the mice that received PSG1 and control Ab (Figure 6g).

PSG1 increases the frequency of lamina propria CD4$^+$ T cells expressing LAP and FoxP3 in a TGF-β dependent manner

TGF-β converts Foxp3$^-$ CD4 T cells into FoxP3$^+$ regulatory cells [2]. In addition, stimulation of mouse CD4$^+$CD25$^-$ T cells with recombinant TGF-β increases the cell surface expression of LAP, which reflects the protective regulatory function of these cells in intestinal inflammation [33]. Therefore, we hypothesized that administration of PSG1 might increase the number of FoxP3$^+$ and LAP$^+$ cells in the colonic lamina propria, a normal site of accumulation of Treg cells specific for commensal bacteria. Lamina propria mononuclear cells were isolated and stained for surface expression of CD4 and LAP and for intracellular expression of FoxP3 as indicated in Methods. We observed a significant increase in the percentage and absolute number of cells expressing both LAP and FoxP3 in mice that received PSG1 when compared to protein-control treated mice (Figure 7a–b). This increase was markedly inhibited in mice receiving PSG1 in conjunction with the neutralizing anti-TGF-β Ab, strongly suggesting that the effects of PSG1 resulting in the increase of Tregs was at least partially mediated by active TGF-β (Figure 7a–c). In addition, administration of anti-TGF-β Ab had an effect in lowering the number of FoxP3$^+$/LAP$^+$ cells in the protein control-treated mice (Figure 7a–c).

DISCUSSION

Recombinant PSG1 induces the secretion of TGF-β1 by human monocytes, bone-marrow derived dendritic cells and the RAW 264.7 macrophage cell line [20–21]. Here we report that PSG1 has associated LAP-β1 and mature TGF-β1. This interaction was detected by ELISA and was observed in different forms of recombinant PSG1, regardless of the strategy employed to purify the protein and the nature of the tag present in the recombinant PSG1. In addition, we demonstrated the interaction between PSG1 and LAP-β1 by expressing a membrane-bound form of PSG1 in HeLa cells. Therefore, our results indicate that LAP and TGF-β, which are non-covalently bound to each other, associate with PSG1 rather than co-purify with it. As expected, recombinant PSG1 generated in insect cells, which do not produce TGF-β, had no detectable associated TGF-β, while PSG1 generated in mouse embryonic fibroblasts obtained from TGF-β1-null cells only had associated TGF-β2. Importantly, PSG1 purified from pooled serum of pregnant women also contained LAP-β1 and mature TGF-β1 and some preparations contained TGF-β2. We could not assay these samples for the presence of LAP-β2 as there are no available reagents for its detection. At this time, we cannot explain why all preparations of PSG1 did not contain both TGF-β isoforms, but this may be related to the fact that the serum was pooled irrespective of gestational age or that the concentration of TGF-β2 may vary between individuals.

Using three different TGF-β reporter cell lines, we observed that addition of recombinant and PSG1 purified from pregnant serum resulted in an increase of active TGF-β that could

signal in these cells. Addition of different proteases inhibitors did not preclude the increase in active TGF-β mediated by PSG1 observed in the TGF-β reporter assays, suggesting that PSG1's mechanism of action is protease-independent and may be similar to that of TSP-1 [7]. To understand the mechanism by which PSG1 addition resulted in an increase of TGF-β signalling in the reporter cell lines, we incubated PSG1 with latent small latent complex in a cell-free system. As shown in figures 4 and S2, PSG1 converted some of the latent small latent complex into its active form. Further studies are required to determine whether, like TSP-1, PSG1 induces a conformational change in the TGF-β latent complex of the TGF-β1 and -2 isoforms and also activates TGF-β3. In addition, we observed that recombinant and native PSG1 activated the small latent complex of TGF-β1 in a cell-free system in a dose-dependent manner. This result indicates that neither PSG1 nor the latent complex have to be anchored to the cell membrane, which is a requirement for some TGF-β activators [34].

TGF-β1 and TGF-β2 are considered potent anti-inflammatory cytokines and have been shown to mediate suppression of the Th1-response in models of induced colitis and to regulate intestinal macrophage cytokine production. We explored the therapeutic potential of PSG1 administration in a murine model of acute-DSS induced colitis and evaluated the role of TGF-β in this system. We observed that administration of PSG1 was highly effective in preventing induction of colitis. The mechanism by which PSG1 exerts this protective effect is in great part mediated by one or more TGF-β isoforms, as the PSG1 protective effect was not observed in the presence of the pan neutralizing anti-TGF-β Ab. The inhibition of TNF-α and IFN-γ by PSG1 in the colitis model is likely relevant in the context of pregnancy, as these cytokines have been reported to have potent abortogenic properties [35]. In addition to TGF-β, IL-10 has been previously linked to the prevention of colitis. The relationship of TGF-β and IL-10 is complex; TGF-β induces IL-10 secretion and IL-10 secreted by a subset of macrophages in the lamina propria maintains Foxp3 expression and suppressive function in mice with colitis [36–37]. While PSG1 treatment increased the mRNA expression of IL-10 in the colon, we did not detect a further increase in IL-10 protein upon stimulation of MLN cells isolated from PSG1-treated mice over the increase observed upon stimulation of MLN cells isolated from the protein control-treated mice. This may be explained by a difference in the production of IL-10 by myeloid cells in the colon and in the mesenteric lymph nodes, and a difference in the level of TGF-β between these tissues. Previously, we reported an increase of TGF-β1 secretion by PSG1 in monocytes but we could not detect an increase of TGF-β1 mRNA. This result is in agreement with the lack of increase of TGF-β1 mRNA in the colon upon PSG1-treatment. The release of intracellular existing TGF-β1 rather than new synthesis has been previously shown to contribute to the increase in TGF-β1 in several experimental conditions[38].

PSG1 binds to heparan sulfate (HS) proteoglycans, including the HS chains in all four syndecans. This interaction is required for the ability of PSG1 to induce endothelial tube formation but it is not known whether cell surface HS can modulate the ability of PSG1 to induce the secretion of cytokines [39]. Interestingly, mature TGF-β1 trapped on the T-cell surface by syndecan 4, has been proposed to potentiate inhibition of T-cell activation [40]. The domain(s) of PSG1 involved in PSG1 binding to HS and to LAP and/or mature TGF-β have yet to be identified. It is therefore possible that while bound to HS moieties in syndecan-4 expressed on activated T cells, PSG1 could activate TGF-β and that some of the active TGF-

β remains bound to HS on syndecan. This would increase the availability of mature TGF-β that could be presented to another cell as well as its half-life. A similar mechanism could be envisioned in the surface of the placenta. Active TGF-β has been described associated with the syncytiotrophoblast plasma membrane and microvilli. The syncytiotrophoblast exhibits apical expression of syndecan-1, which also binds active TGF-β [41]. Therefore TGF-β may bind to TGF-β receptors on the trophoblast or it may stay associated with syndecan-1 and PSG1 and be presented to maternal cells. In this context, decidual NK cells are susceptible to TGF-β regulation and have a distinct phenotype from those from the peripheral blood [42]. It is therefore possible that PSG1 increases the availability of active TGF-β and in this way influences NK-phenotype during pregnancy.

While pregnancy has a clear beneficial effect in diseases such as rheumatoid arthritis, multiple sclerosis and systemic sclerosis; there is no consensus regarding the beneficial effects of pregnancy in diseases affecting mucosal surfaces such as inflammatory bowel disease. The rate of disease flare during pregnancy is similar to non-pregnant flare rates although there have been some suggestions that Crohn's disease symptoms might improve during gestation [43–44]. While PSG1 may have some therapeutic potential for inflammatory bowel diseases, the increase in regulatory T cells could potentially increase the susceptibility to infections. Therefore further studies are required before the use of PSG1 as a therapeutic agent is considered. Recently, Martinez and co-workers showed that PSG1 affects the maturation of bone-marrow derived dendritic cells and that intra-peritoneal infection of mice with a vaccinia virus expressing PSG1 promoted the expansion of Ag specific $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells in response to *Listeria monocytogenes* infection [21]. In our experiments, using a different model, we observed an increase in the frequency of $CD4^+$ cells expressing FoxP3 and cell surface LAP. Further studies are required to understand the contribution of PSGs in the expansion of Tregs, which occurs physiologically during pregnancy [17]. In addition, the possibility that PSG1 may increase the suppressive activity of Tregs and/or the conversion of naïve cells to Tregs by activating the membrane-bound form of latent TGF-β1 expressed on activated Tregs remains to be explored.

Phylogenetic analysis of the CEA family strongly suggests that one of the primordial genes in this family is CEACAM1 (CEA-related cell adhesion molecule), which has been shown to have immune regulatory functions, the nature of which is dependent on the splice variant expressed [45]. Our observations and those of others indicate that some members of the CEA family regulate the innate and adaptive immune response by using different mechanisms, which may be related to their sites of expression and may have evolved in parallel to the type of placentation. This newly described activity of PSG1 reinforces previous clinical observations on the importance of PSGs in the establishment and maintenance of a successful pregnancy in humans and provides a mechanism for their proposed role as immune-modulators.

METHODS

Protein production and purification

PSG1-Fc, PSG1-His-FLAG, and the control proteins were harvested from the supernatant of transiently or stably transfected cells and purified as previously reported [22]. GST-PSG1

(isoform d) was generated as previously described [20]. Detailed description is available as Supplementary information.

Induction of colitis and PSG treatment

Dextran Sodium Sulfate (DSS, 36–50 kDa, MP Biomedical) was used to supplement the drinking water of 6–8 week old C57BL/6 mice at 2.5–3% (w/v) solution for 7 days. Fresh solution was replaced after 3 days and given *ad libitum*. On day 7, mice were returned to normal drinking water and monitored for an additional 3 days. Body weight and diarrhoea were recorded daily. At time of sacrifice, mice were evaluated for colon length and a portion of the colon then removed, fixed in 10% buffered formalin and embedded in paraffin. Colon samples were cut in 4-μm sections, stained with H&E and examined by light microscopy. PSG1-Fc (100 μg in 200 μl of PBS) or an equimolar concentration of the control protein (Flag-Fc) were injected i.p. the day before the mice were given DSS (experimental day (ED) 0), on the first day of DSS administration (ED1) and every other day until DSS-containing water was replaced for normal drinking water (ED7). When indicated, 1 mg of anti-TGF-β or isotype control antibodies were administered together with the first and third protein injections. Procedures that involved mice were approved by state authority and the Medicine University of Berlin committee on Animal Use in Research and Education.

Isolation of lamina propria (LP) and mesenteric lymph nodes (MLN) cells

Lamina propria mononuclear cells (LPMCs) were isolated from LP by gradient centrifugation with the Lympholyte-M density separation medium. For a detailed description please see the Supplementary information.

Cytokine determination by cytometric bead arrays (CBA)

Cytokines were analysed in cell culture supernatants using CBAs from BD Biosciences. Detailed description is available as Supplementary information.

RNA isolation and quantitative PCR analysis

Total RNA was isolated and converted into cDNA with Superscript II (Invitrogen) and random primers. Real-time PCR was performed with the Power SYBR Green PCR master mix and the specific primers. Detailed description is available as Supplementary information.

Luciferase and SEAP Assays

Reporter bioassays were performed as previously described [25–26]. Detailed descriptions of these protocols are available as Supplementary information. Results are expressed as Luciferase activity fold induction (FI) or SEAP activity fold induction (FI) after normalization with values obtained upon treatment of the cells with DMEM-0.1% ITS or DMEM for MFB-F11 cells.

Statistics

Treatments presented in Fig 1 to 4 were performed in triplicate and values are given as mean ± standard error of the mean (S.E.M.) of the triplicate wells for each treatment. Statistical

analysis was performed using Student's *t*-test and $p<0.05$ was considered statistically significant. Differences between treatments with the same protein at different concentrations were calculated by one-way ANOVA. Statistical analysis for *in vivo* experiments was calculated by one-way ANOVA and Bonferroni's multiple comparison tests. Statistically significant differences between groups are indicated (*$p<0.05$; †$p<0.01$; ‡$p<0.001$).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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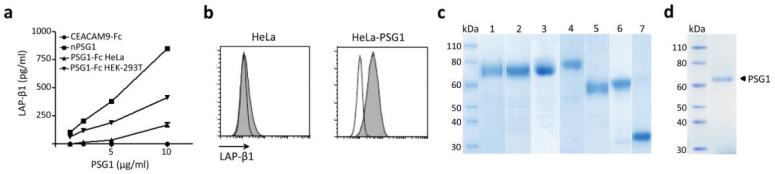

Figure 1.

LAP-β1 is present in recombinant and native PSG1-TGF-β complexes. (**a**) Different concentrations of recombinant PSG1 or control protein CEACAM9-Fc generated in HEK-293T and HeLa cells and PSG1 purified from pooled sera of pregnant women (nPSG1) were tested by ELISA for the presence of LAP-β1 as indicated in Methods. (**b**) HeLa-PSG1 and the parental HeLa cells transfected with empty plasmid were incubated with PE-labeled anti-human LAP-β1 mAb (full grey) or PE-labeled isotype control (empty white). (**c**) PSG1-Fc was generated in CHO-K1 (lane 1), HEK-293T (lane 2), HeLa (lane 3), Mouse embryonic fibroblast cells derived from TGF-β1 null-mice (lane 4). The supernatant of the transfected cells was purified on a protein A column. PSG1-His-FLAG was generated in CHO-K1 cells (lane 5) and was purified with a HisTrap column, followed by an anti-FLAG column. The control proteins Ceacam9-Fc (lane 6) and FLAG-Fc (lane 7) were purified from the supernatant of transfected CHO-K1 cells on a protein A column. After purification, proteins were concentrated, buffered exchange into PBS and run on SDS-PAGE. The GelCode Blue stained-gel shown here is a composite of lanes run in different gels. (**d**) PSG1 was purified from pooled serum of pregnant women by affinity chromatography with anti-PSG1 mAb #4. The eluted material was concentrated, buffered exchange into PBS run on SDS-PAGE and stained with GelCode Blue.

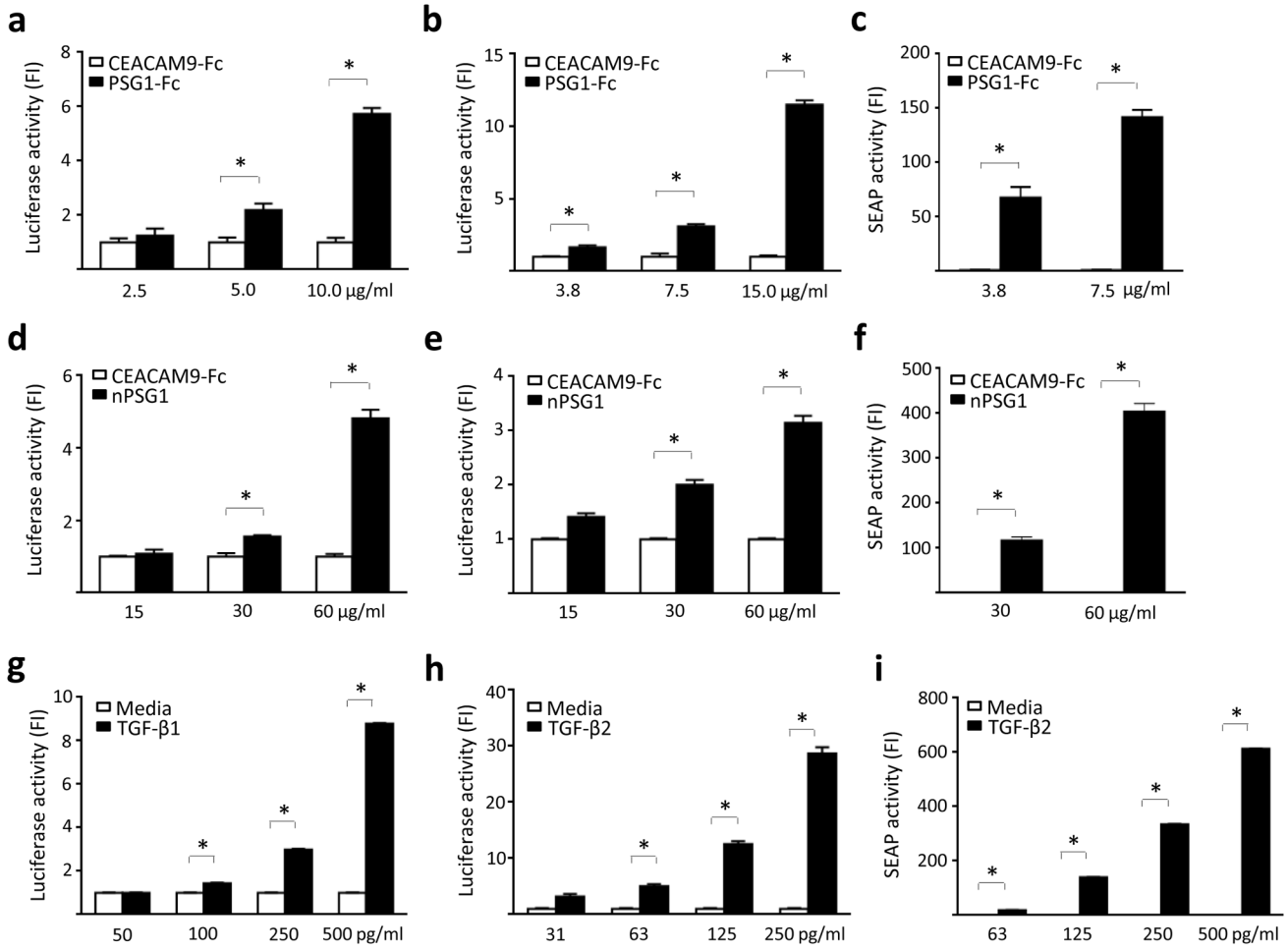

Figure 2.

Recombinant and native PSG1 activate TGF-β secreted in latent form by TGF-β reporter cell lines. HEK-SMAD cells were treated with different concentrations of PSG1-Fc made in TGF-β1-null MEFs (**a**); native PSG1 (**d**) or mature TGF-β1 (**g**) in DMEM-0.1% ITS and light units were measured in the lysates after 16h. TMLECs were incubated with different concentrations of PSG1-Fc made in TGF-β1-null MEFs (**b**); native PSG1 (**e**) or mature TGF-β2 (**h**) in DMEM-0.1% ITS. Light units were measured in the cell lysates 16h post-treatment MFB-F11 cells were incubated with different concentrations of PSG1-Fc made in TGF-β1-null MEFs (**c**); native PSG1 (**f**) or mature TGF-β2 (**i**) in DMEM. The reporter (SEAP) was measured in the cell supernatant 24h post-treatment. The three reporter cell lines were also incubated with the control protein CEACAM9-Fc at the same concentration or with DMEM with 0.1% ITS (HEK-SMAD and TMLECs) or DMEM only (MFB-F11 cells). Results are expressed as Luciferase activity fold induction (FI) or SEAP activity fold induction (FI) after normalization with values obtained upon treatment of the cells with DMEM-0.1% ITS or DMEM for MFB-F11 cells. Treatments with PSG1 and CEACAM-Fc were performed in triplicate wells. Treatments with recombinant mature TGF-βs were performed in duplicate wells. All experiments were performed three independent times with

similar results. In all figures, significant differences are noted as *$p<0.05$ as analysed by Student's t –test.

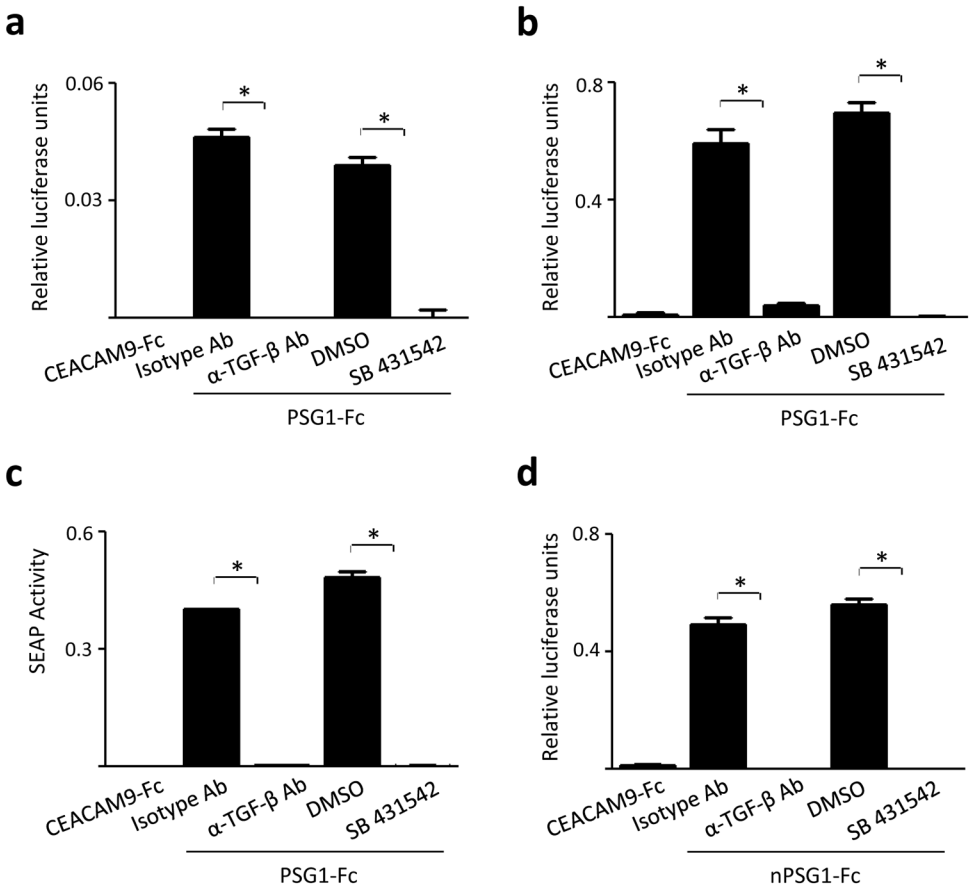

Figure 3.

PSG1-mediated activation of TGF-β is inhibited by the pan TGF-β-neutralizing 1D11 mAb and the TGF-β receptor I kinase inhibitor SB 431542. (**a**) TMLECs, (**b**) HEK-SMAD, and (**c**) MFB-F11 cells were treated with 7.5μg/ml recombinant PSG1 in the presence of 60μg/ml anti-TGF-β 1D11 Ab, isotype control Ab at the same concentration, 5 μM SB 431542 or DMSO. (**d**) HEK-SMAD were treated with 20 μg/ml native PSG1 (nPSG1) in the presence of 60 μg/ml anti-TGF-β 1D11 Ab, 5 μM SB431542 and the respective controls. The cells were also incubated with the control protein CEACAM9-Fc at 7.5 μg/ml (**a** – **c**) and 20 μg/ml in (**d**). Results are expressed as Luciferase activity or SEAP activity after subtraction of the values obtained upon treatment of the cells with DMEM-0.1% ITS or DMEM for MFB-F11 cells. All treatments were performed in triplicate in three independent experiments. In all figures, significant differences are noted as *$p<0.05$ as analysed by Student's t-test.

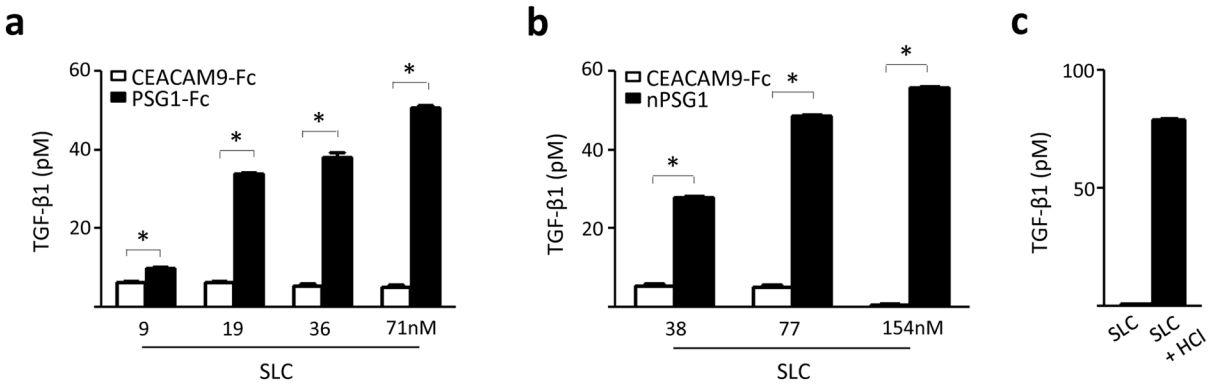

Figure 4.

Recombinant and native PSG1 activate the small latent complex (SLC) of TGF-β1 in a cell-free system. PSG1-Fc generated in MEFs derived from TGF-β1-null mice (**a**); native PSG1 (**b**) or the control protein CEACAM9-Fc were incubated in triplicate at increasing concentrations with PBS or with SLC (0.5nM) for 1h at 37 °C, after which they were added to wells coated with TGF-β RII-Fc. The presence of active TGF-β1 was quantified after incubation with biotin-labelled anti-mature TGF-β1 Ab and streptavidin-HRP. (**c**) The amount of TGF-β1 already in the active form in the recombinant SLC and the concentration of active TGF-β1 that was obtained upon acid activation of the SLC were determined in parallel. All treatments were performed in triplicate in three independent experiments. In all figures, significant differences are noted as *$p<0.05$ as analysed by Student's t-test.

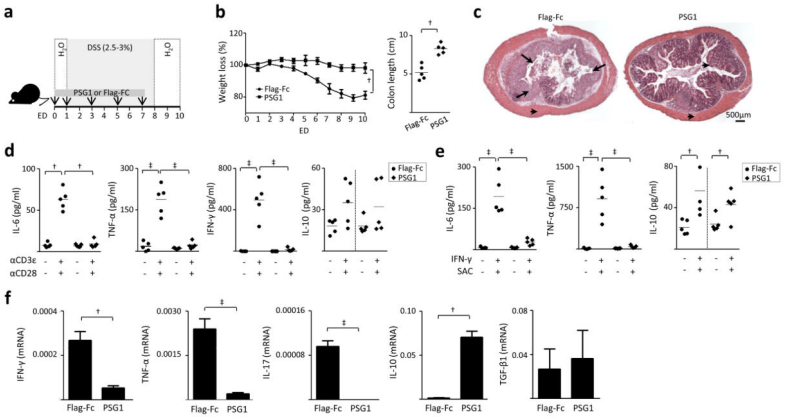

Figure 5.

PSG1 prevents DSS-induced colitis in mice. (**a**) Acute colitis was induced by administration of 2.5–3% DSS to the drinking water starting from day one to day 7 of the experiment. In addition mice were injected (i.p.) either with Flag-Fc (control protein, 30µg/day) or PSG1 (100µg/day) on day 0, 1, 3, 5 and 7 of the experimental day (ED) as described in Methods. (**b**) Weight loss (%) in control (Flag-Fc) and PSG1 treated mice (n=5/ group) was monitored every day. Colon length was recorded on ED 10. (**c**) Haematoxylin- and eosin-(H&E) stained colon sections on ED10 (original magnification ×50; scale bar= 500µm). Short arrows, epithelium (top arrow, right panel) and smooth muscle (bottom arrow, right panel); large arrows, cellular infiltrates (left panel).(**d**) IL-6, TNF-α, IFN-γ and IL-10 production by lymphocytes isolated from mesenteric lymph nodes (MLN) and stimulated with CD3ε and CD28 for 48h as described in Methods. (**e**) IL-6, TNF-α and IL-10 production by lymphocytes isolated from MLN and stimulated with IFN-γ and Pansorbin (SAC) for 48h as described in Methods. In Figures (**d**) and (**e**) cytokine concentrations were determined in culture supernatants by Cytometric Beads Array (CBA). (**f**) IFN-γ, TFN-α, IL-17, IL-10 and TGF-β1 expression in colon tissues on ED10 as analysed by qPCR. In all figures, significant differences are noted as †p<0.01 and ‡p<0.001 as analysed by one-way ANOVA followed by Bonferroni test. Data shown are mean values ± SD derived from five mice per group each analysed in duplicate.

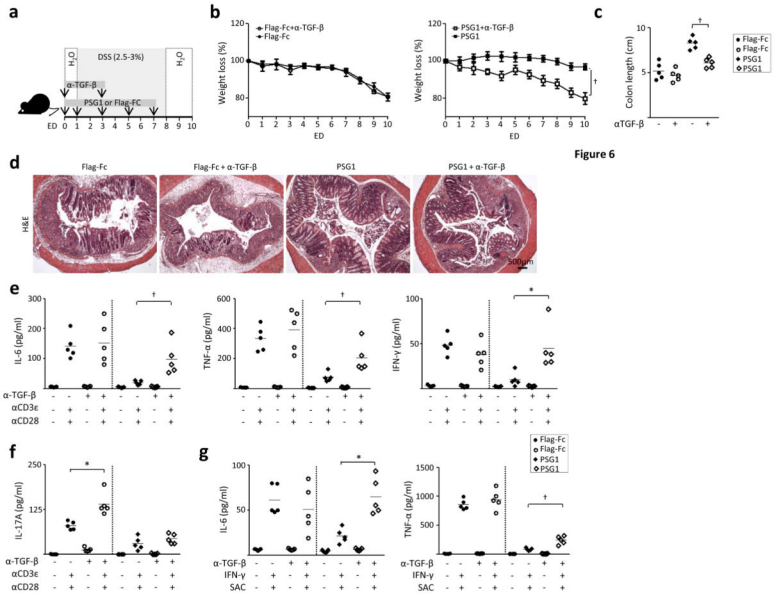

Figure 6.

Administration of anti-TGF-β abrogates the protective effect of PSG1 in DSS-induced colitis. (**a**) Experimental design. DSS-treated mice were injected (i.p.) either with Flag-Fc (control protein, 30μg/day) or PSG1 (100μg/day) on ED0, 1, 3, 5 and 7. In addition the mice received anti-TGF-β (1mg/day) or isotype control antibody on ED0 and ED3 as described in Methods. (**b**) Weight was monitored every day. Data are presented as weight loss (%). (**c**) Colon Length recorded on ED10. (**d**) H&E stained colon sections on ED10. (**e**) IL-6, TNF-α and IFN-γ and (**f**) IL-17 production by lymphocytes isolated from mesenteric lymph nodes (MLN) and stimulated with CD3ε and CD28 for 48h as described in Methods. (**g**) IL-6 and TNF-α production by lymphocytes isolated from MLN and stimulated with IFN-γ and Pansorbin (SAC) for 48h as described in Methods. In figures (**d**) and (**e**) cytokine concentrations were determined in culture supernatants by Cytometric Beads Array (CBA). In all panels, significant differences are noted as *$p<0.05$ and †$p<0.01$ as analysed by one-way ANOVA followed by Bonferroni test. Data shown are mean ± SD derived from five mice per group each analysed in duplicate.

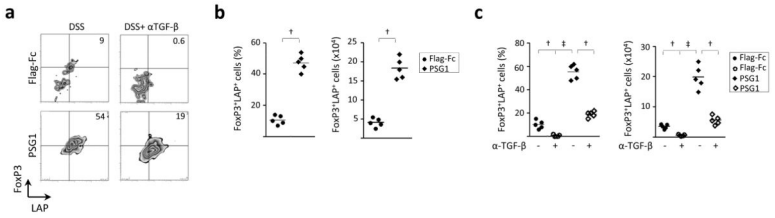

Figure 7.

Administration of PSG1 increases the frequency of $CD4^+FoxP3^+LAP^+$ lymphocytes in the lamina propria. (**a**) Representative plots of FoxP3 and LAP stained $CD4^+$ lymphocytes isolated from lamina propria (LP) were analysed by flow cytometry (FACS). (**b**) Percentages and absolute numbers of $FoxP3^+LAP^+$ LP lymphocytes isolated from Flag-Fc or PSG1 DSS-treated mice are depicted. (**c**) Percentages and absolute numbers of $FoxP3^+LAP^+$ LP lymphocytes isolated from Flag-Fc or PSG1 DSS-α-TGF-β treated mice are shown. All treatments were performed in triplicate in two independent experiments using 5 mice per group. In all figures, significant differences are noted as †$p<0.01$ and ‡$p<0.001$ as analysed by one-way ANOVA followed by Bonferroni test. Data shown are mean ± SD derived from five mice per group each analysed in duplicate.