DC-SIGN activation mediates the differential effects of SAP and CRP on the innate immune system and inhibits fibrosis in mice

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Fibrosis is caused by scar tissue formation in internal organs and is associated with 45% of deaths in the United States. Two closely related human serum proteins, serum amyloid P (SAP) and C-reactive protein (CRP), strongly affect fibrosis. In multiple animal models, and in Phase 1 and Phase 2 clinical trials, SAP affects several aspects of the innate immune system to reduce fibrosis, whereas CRP appears to potentiate fibrosis. However, SAP and CRP bind the same Fcγ receptors (FcγR) with similar affinities, and why SAP and CRP have opposing effects is unknown. Here, we report that SAP but not CRP binds the receptor DC-SIGN (SIGN-R1) to affect the innate immune system, and that FcγR are not necessary for SAP function. A polycyclic aminothiazole DC-SIGN ligand and anti–DC-SIGN antibodies mimic SAP effects in vitro. In mice, the aminothiazole reduces neutrophil accumulation in a model of acute lung inflammation and, at 0.001 mg/kg, alleviates pulmonary fibrosis by increasing levels of the immunosuppressant IL-10. DC-SIGN (SIGN-R1) is present on mouse lung epithelial cells, and SAP and the aminothiazole potentiate IL-10 production from these cells. Our data suggest that SAP activates DC-SIGN to regulate the innate immune system differently from CRP, and that DC-SIGN is a target for antifibrotics.

fibrosis | pentraxin | DC-SIGN | serum amyloid P | IL-10

Fibrosing diseases such scleroderma, pulmonary fibrosis, and renal fibrosis are caused by aberrant scar tissue formation in internal organs and are associated with 45% of deaths in the United States (1). At a fibrotic lesion, monocytes leave the blood, enter the tissue, and differentiate into cells such as macrophages and fibrocytes (2). Fibrocytes and macrophages then secrete extracellular matrix (ECM) proteins, ECM modifying enzymes, and/or cytokines such as IL-4 to promote scar tissue formation and fibrosis (3, 4).

Pentraxins are a family of highly conserved secreted proteins that have a profound effect on the development of fibrosis and the regulation of the innate immune system (5–7). The pentraxin serum amyloid P (SAP) reduces neutrophil activation and recruitment (8, 9), inhibits the differentiation of monocytes into fibroblast-like cells called fibrocytes (8, 10), and promotes IL-10–secreting macrophages (11–13). In animal models and two human trials (6, 14, 15), injections of SAP decrease fibrosis, indicating that SAP has a dominant effect on a disease that is mediated in part by the innate immune system. Conversely, the closely related pentraxin C-reactive protein (CRP) is proinflammatory and promotes fibrosis (5, 16). However, under some conditions, CRP decreases inflammation, indicating that much remains to be understood about this molecule (5, 17). Despite the strong effects of pentraxins on the innate immune system and fibrosis (5, 6), little is known about their mechanism of action. For instance, pentraxins such as SAP and CRP appear to bind the same Fcγ receptors (FcγR) with similar affinities (7, 8, 18), but they generally have opposite effects. What causes this functional difference is not known.

Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN/CD209) is a C-type lectin found on dendritic cells, macrophages, and monocytes (19, 20). DC-SIGN mainly binds to mannosylated and fucosylated proteins (19, 20). Humans have DC-SIGN and L-SIGN, whereas mice have eight DC-SIGN orthologs called SIGN-R1–8 (21). SIGN-R1 most closely resembles DC-SIGN (21). DC-SIGN and SIGN-R1 also bind sialylated IgG (sIgG) (20). This interaction appears to be a protein:potein interaction and not a sialic acid:DC-SIGN interaction (22). Both sIgGs and SAP have α(2,6)-linked terminal sialic acids on the protein surface, and both sIgGs and SAP alleviate inflammation in mice (6, 20, 23).

In this report, we show that in absence of all of the FcγR, neutrophils, monocytes, and macrophages still respond to SAP, indicating that SAP uses other receptors. For SAP, we show that one of the other receptors is DC-SIGN. We also found that anti–DC-SIGN antibodies and a small-molecule DC-SIGN ligand mimic the effects of SAP. The synthetic DC-SIGN ligand shows efficacy in murine models of acute lung inflammation and pulmonary fibrosis. In contrast to SAP, we find that CRP requires the FcγR to regulate neutrophils and IL-10 secretion from macrophages, but not to increase ICAM-I$^+$ macrophages. This finding suggests that there are additional CRP receptors that regulate macrophage polarization. Our findings suggest the presence of a previously unidentified pentraxin target that accounts for the functional difference between SAP and CRP, and which might be useful as a therapeutic target to regulate the innate immune system and fibrosis.

Results

Fcγ Receptors Are Not Necessary for SAP Effects on the Innate Immune System. SAP and CRP both bind FcγR, but have different effects on the innate immune system (8, 18). To determine the role of FcγR in SAP and CRP function, we examined the effect of these proteins on mouse cells lacking all FcγR (FcγR

Significance

Pentraxins such as serum amyloid P (SAP) and C-reactive protein (CRP) have significant, and for SAP dominant, effects on the innate immune system. This report shows that contrary to the current model of how SAP and CRP are sensed by cells, Fcγ receptors are not necessary for SAP to regulate the innate immune system. This report considerably changes our understanding of the endogenous regulation of the innate immune system and connections between innate immune system signaling and epithelial cell signaling. The identification of DC-SIGN as a SAP receptor, the potential use of anti–DC-SIGN antibodies as a therapeutic, and the observation that remarkably low concentrations of a DC-SIGN ligand are therapeutic in a mouse model of fibrosis, create a new approach to treat fibrosis.

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Conflict of interest statement: Rice University has patents on the use of SAP to inhibit fibrosis. Texas A&M University has patent applications on the use of compound 1 to inhibit fibrosis. D.P. and R.H.G. are co-founders of and have equity in Promedior, a company that is developing SAP as a therapeutic. D.P. and R.H.G. receive a share of royalties paid by Promedior to Rice University.

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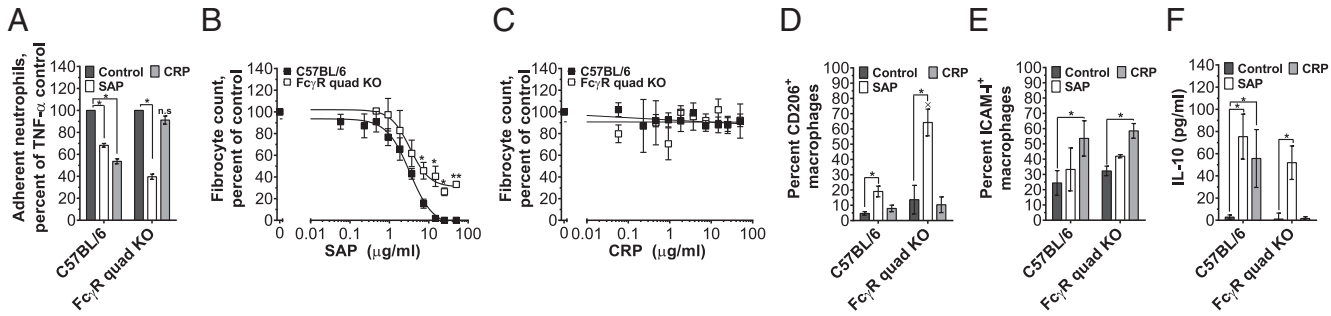

Fig. 1. Fcγ receptors are necessary for some but not all effects of SAP and CRP. (*A*) Mouse neutrophils were incubated with 10 μg/mL of the indicated pentraxin and TNF-α, and neutrophil adhesion to fibronectin was assayed, $n = 3$. (*B* and *C*) Mouse spleen cells were incubated with the indicated concentrations of pentraxin. After 5 d, the cells were fixed, stained, and fibrocytes were counted, $n = 3$–6. (*D* and *E*) Mouse bone marrow-derived macrophages were polarized in 0 (control) or 10 μg/mL of the indicated pentraxin. Cells were then fixed and stained, $n = 3$. (*F*) Macrophages were polarized as in *D*, and IL-10 levels in the supernatants were measured, $n = 3$–8. n.s. (not significant relative to the control), $*P < 0.05$ (*t* test), (*D*) ×, $P < 0.05$ (*t* test relative to SAP in C57BL/6). All values are mean ± SEM. Data were fit to sigmoidal dose–response curves with a variable Hill coefficient (*B*) or a line (*C*).

quad KO). As previously observed, SAP and CRP decreased the adhesion of C57BL/6 neutrophils to fibronectin (Fig. 1*A*) (8, 9). However, when added to FcγR quad KO neutrophils, SAP but not CRP significantly reduced neutrophil adhesion to fibronectin (Fig. 1*A*).

In addition to reducing neutrophil adhesion, SAP inhibits the differentiation of monocytes into fibroblast-like cells called fibrocytes (8, 10) (Fig. 1*B*). In the absence of FcγR, SAP reduced but could not completely inhibit fibrocyte differentiation (Fig. 1*B*). CRP had no effect on fibrocyte differentiation (Fig. 1*C*) (10). The absence of FcγR did not alter this response (Fig. 1*C*).

SAP potentiates $CD206^+$ antiinflammatory macrophages (13) (Fig. 1*D*). CRP, however, primarily promotes $ICAM\text{-}I^+$ M1-like inflammatory macrophages (16) (Fig. 1*E*). In our assays, both SAP and CRP were able to polarize FcγR quad KO macrophages as determined by CD206 and ICAM-I expression (Fig. 1 *D* and *E*). In agreement with its antiinflammatory function, SAP also increased IL-10 secretion from C57BL/6 and FcγR quad KO macrophages (Fig. 1*F*). We also observed that CRP increased IL-10 secretion from C57BL/6 macrophages but not from the FcγR quad KO cells, indicating that some but not all effects of CRP on macrophages are mediated by the FcγR (Fig. 1*F*). The increase in IL-10 secretion in response to CRP has been observed before (24, 25) and is most likely counteracted by CRP-induced TNF-α and IL-12 under inflammatory conditions (16). The related pentraxin PTX3 was also able to decrease neutrophil adhesion and alter macrophage phenotype in absence of the FcγR, although the PTX3-induced IL-10 secretion by macrophages was absent (Fig. S1). Together these results suggest that contrary to the current model of SAP signaling, the FcγR are not necessary for SAP effects. This observation suggests the presence of additional SAP receptors. Conversely, CRP requires the FcγR to reduce neutrophil adhesion and promote IL-10 secretion by macrophages, but not to increase $ICAM\text{-}I^+$ macrophages. This finding indicates that some aspects of the CRP effect on macrophages is mediated by an unknown receptor.

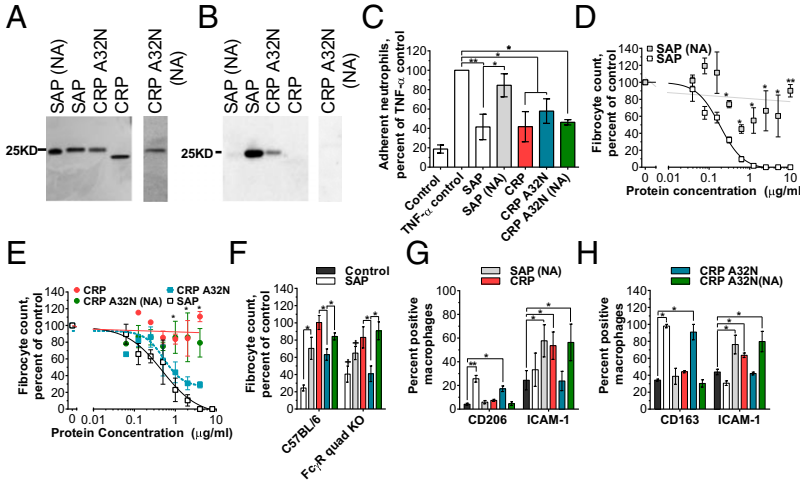

Fig. 2. Glycosylation mediates some effects of SAP. (*A*) Desialylated SAP [SAP (NA)], SAP, glycosylated CRP (CRP A32N), CRP, and desialylated CRP A32N [CRP A32N (NA)] were electrophoresed on SDS/PAGE gels and stained with silver stain. (*B*) Western blots of the samples in *A* were stained with *Sambucus Nigra* lectin to detect α(2,6)-linked terminal sialic acids. (*C*) Human neutrophils were incubated with 0 (control) or 10 μg/mL of the indicated pentraxin to assess neutrophil adhesion as in Fig. 1*A*, $n = 3$–5. (*D* and *E*) Human PBMC were incubated with the indicated concentrations of pentraxins. After 5 d, fibrocytes were counted, $n = 3$–5. (*F*) Mouse spleen cells were incubated in the presence or absence of 10 μg/mL of the indicated pentraxin. Fibrocyte counts were normalized to the no-pentraxin control, $n = 3$. (*G*) Mouse C57BL/6 bone marrow-derived macrophages were polarized in 0 (control) or 10 μg/mL of the indicated pentraxins. Cells were then stained for the indicated markers, $n = 3$. (*H*) Human macrophages were polarized by 3 μg/mL of the indicated protein and then stained, $n = 3$. $*P < 0.05$, $**P < 0.01$ (*t* test). (*F*) +, $P < 0.05$ (*t* test relative to the no-protein control). (*C*–*H*) Values are mean ± SEM. (*D* and *E*) Data were fit to sigmoidal dose–response curves with a variable Hill coefficient or a line where appropriate.

SAP Glycosylation Mediates SAP Effects on the Innate Immune System. CRP has sequence and structural similarity to SAP, and like SAP, binds FcγR (5, 6). However, SAP and CRP have different effects on monocyte and macrophage differentiation; for instance, whereas SAP inhibits fibrocyte differentiation (8) and promotes IL-10 secreting macrophages (3, 13), CRP has no effect on fibrocyte differentiation (10) and promotes proinflammatory macrophages (16). One possible reason for this functional difference is sequence divergence between SAP and CRP. However, we previously found that mutating SAP surface amino acid residues that are different between SAP and CRP has only a modest effect on SAP function (8). An alternative cause of this functional difference may be a difference in SAP and CRP glycosylation: SAP is glycosylated at N32 with α(2,6)-linked terminal sialic acids (23), which is exposed on a soluble surface, whereas CRP has no glycosylation (23). To determine whether the SAP glycosylation affects its function, we enzymatically removed the terminal sialic acids with neuraminidase. The desialylated SAP [SAP (NA)] could no longer be detected on Western blots stained with *Sambucus Nigra* lectin, which binds preferentially to α(2→6)-linked terminal sialic acids (Fig. 2 *A* and *B*).

SAP and CRP as observed previously reduced human neutrophil adhesion to fibronectin (9) (Fig. 2*C*). However as compared with SAP, SAP (NA) had a reduced effect on human neutrophil adhesion and was unable to inhibit fibrocyte differentiation when added to human PBMCs and mouse spleen cells (Fig. 2 *C*–*F*). These findings indicate a significant functional role for SAP glycosylation and provide a possible mechanism for the immune cells to differentiate SAP from CRP. Furthermore, these results suggest that a glycosylated CRP would be able to "trick" the innate immune cells and mimic SAP effects. To examine this possibility, we mutated CRP at position 32 from an alanine to an asparagine. The mutated CRP (CRP A32N) was glycosylated and had a lower mobility on SDS/PAGE gels compared with CRP (Fig. 2 *A* and *B*). However, on average only 40% of CRP A32N monomers were sialylated (Fig. 2*B*). This lack of complete sialylation is likely due to problems with recombinant protein expression and/or sequence differences between SAP and CRP (26). When tested on innate immune cells, CRP A32N similar to CRP and SAP reduced human neutrophil adhesion (Fig. 2*C*). However, unlike CRP, CRP A32N was able to inhibit fibrocyte differentiation when added to human PBMC or mouse spleen cells (Fig. 2 *E* and *F*). Furthermore, CRP A32N similar to SAP did not require the FcγR to inhibit fibrocyte differentiation (Fig. 2*F*).

In addition to regulating neutrophil adhesion and fibrocyte differentiation, SAP and CRP can polarize macrophages (3, 11, 13). To examine the role of SAP glycosylation on macrophage polarization, we added pentraxins to mouse and human macrophages. SAP and CRP A32N promoted CD206$^+$ M2 macrophages from mouse bone marrow-derived macrophages (Fig. 2*G*), whereas CRP and SAP (NA) increased ICAM-I$^+$ M1 macrophages (27) (Fig. 2*G*). We observed a similar trend in human monocyte-derived macrophages, where SAP and CRP A32N promoted CD163$^+$ M2 macrophages (28), whereas SAP (NA) and CRP potentiated ICAM-I$^+$ M1 macrophages (Fig. 2*H*). Neuraminidase-treated CRP A32N was essentially indistinguishable from CRP in our assays (Fig. 2), indicating a role of glycosylation in the effects of CRP A32N. Together, these results indicate that SAP glycosylation allows the innate immune cells to differentiate SAP from CRP and response appropriately to different pentraxins.

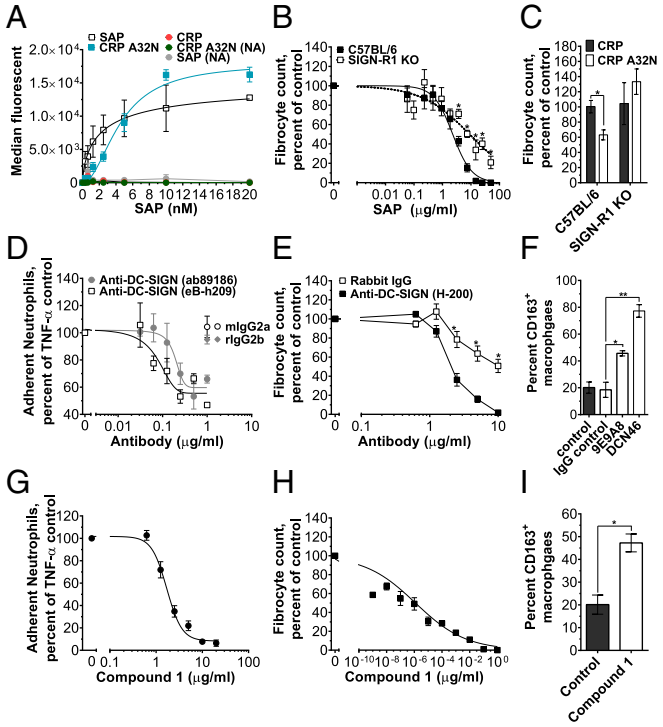

Fig. 3. DC-SIGN activation affects neutrophils and monocyte-derived cells. (*A*) DC-SIGN$^+$ HEK293 cells were incubated with fluorescently labeled SAP, CRP, CRP A32N, SAP (NA), or CRP A32N (NA), and binding was measured by flow cytometry. Mock transfected cells were used to estimate the nonspecific binding, $n = 3$. (*B*) The effect of SAP on fibrocyte differentiation in C57BL/6 and SIGN-R1–deficient cells was assessed, $n = 3$–5. (*C*) Mouse spleen cells were incubated with 0 or 10 μg/mL of the indicated pentraxin. Fibrocytes were counted as a percent of the no-pentraxin control, $n = 3$–5. (*D*) Human neutrophils were incubated with anti-DC-SIGN antibodies and then neutrophil adhesion to fibronectin was assessed, $n = 3$. (*E*) Human PBMCs were incubated with the indicated concentrations of a rabbit anti–DC-SIGN antibody or a rabbit isotype control. After 5 d, fibrocytes were counted, $n = 3$–5. (*F*) Human macrophages were polarized with 1 μg/mL of the indicated antibody. Macrophages were then fixed and stained, $n = 3$. (*G*) The effect of compound 1 on human neutrophil adhesion to fibronectin was assessed, $n = 3$. (*H*) The effect of compound 1 on human fibrocyte differentiation, $n = 3$. (*I*) Macrophage polarization by 10 pg/mL of compound 1 was examined in human macrophages, $n = 3$. *$P < 0.05$ (*t* test). All values are mean ± SEM. (*A*) Curves are fits to models of one-site binding with variable Hill coefficient where appropriate. (*B*, *D*, *G*, and *H*) Data were fit to sigmoidal dose–response curves with a variable Hill coefficient.

SAP but Not CRP Binds to DC-SIGN To Regulate Immune Cells. Sialylated IgG (sIgG) binds DC-SIGN to alter IgG responses (20). Because SAP shares the same type of glycosylation as sIgG (23), and SAP and the Fc domain of IgG bind to FcγR, we examined the possibility that SAP might bind to DC-SIGN. We expressed DC-SIGN on HEK293 cells and measured SAP binding to the transfected cells (Fig. 3*A* and Fig. S2). We used mock-transfected HEK293 cells to estimate the nonspecific binding. SAP bound to DC-SIGN with a K_D of 2.3 ± 1 μg/mL (19 ± 8 nM) and a Hill coefficient of 0.7 ± 0.3 (Fig. 3*A*). CRP A32N also bound to DC-SIGN (K_D of 3.4 ± 0.3 μg/mL, Hill coefficient = 1.9 ± 0.4) but with a lower affinity relative to SAP (Fig. 3*A*), suggesting that the SAP:DC-SIGN interaction may involve protein:protein interaction and, hence, not be limited to carbohydrate:lectin interaction. As expected, SAP (NA), CRP, and neuraminidase treated CRP A32N did not show detectable binding to DC-SIGN (Fig. 3*A*). This observation suggests that SAP and CRP A32N may bind to DC-SIGN to alter immune responses. To test this possibility, we examined the effect of SAP on spleen cells from mice lacking the mouse ortholog of human DC-SIGN, SIGN-R1. The absence of SIGN-R1 significantly decreased the inhibitory effect of SAP on fibrocyte differentiation (Fig. 3*B*). CRP A32N and PTX3 were also unable to alter fibrocyte differentiation from SIGN-R1–deficient spleen cells, suggesting that SIGN-R1 might mediate CRP A32N and PTX3 effects on fibrocyte differentiation (Fig. 3*C* and Fig. S3).

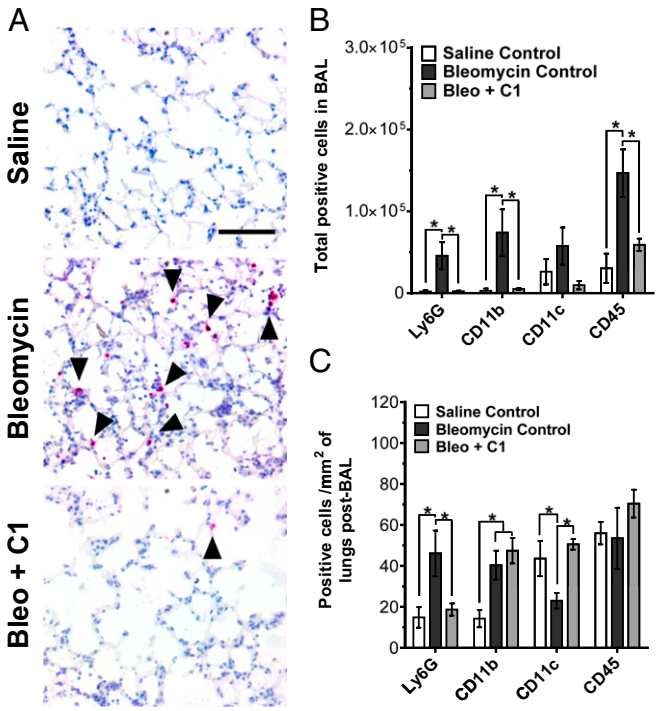

Fig. 4. Compound 1 decreases neutrophil accumulation in mouse lungs. (*A*) Mice received oropharyngeal bleomycin on day 0 to induce acute respiratory distress syndrome. The control mice received saline. Mice were then treated with i.p. injections of 0.1 mg/kg of compound 1 (C1) or an equal volume of vehicle control on days 1 and 2. On day 3, the mice were euthanized and lungs after BAL were stained for the neutrophil marker Ly6G. Images are representative of three independent experiments. (Scale bar: 100 μm.) (*B*) BAL cells were stained for the indicated markers, $n = 4$. (*C*) After collecting the BAL, lungs were stained for the indicated markers, $n = 4$. $^*P < 0.05$ (one-way ANOVA, Holm–Bonferroni post hoc test). (*B* and *C*) Values are mean ± SEM.

DC-SIGN is expressed on macrophages, dendritic cells, and monocytes (19, 20) (Fig. S4). Previously, DC-SIGN mRNA has been observed in human neutrophils (29, 30). We were also able to detect cell-surface DC-SIGN on human and mouse neutrophils (Figs. S4*A* and S5). Because the majority of cells expressing DC-SIGN appear to respond to SAP, we examined whether DC-SIGN activation by antibodies can mimic SAP effects on neutrophils, monocytes, and macrophages. Some, but not all, anti-human DC-SIGN antibodies decreased human neutrophil adhesion to fibronectin (Fig. 3*D*) and inhibited human fibrocyte differentiation ($IC_{50} = 2.4 \pm 0.4$ μg/mL) (Fig. 3*E* and Fig. S6 *A* and *B*). A different subset of anti-DC-SIGN antibodies also altered macrophage phenotype and increased $CD163^+$ macrophages similar to SAP and CRP A32N (Fig. 3*F* and Fig. S6*C*). We observed similar effects using F(ab2) fragments of anti-DC-SIGN antibodies, suggesting that our results are Fc-independent and most likely involve DC-SIGN activation by the antibodies (Fig. S6 *D–F*).

To further ascertain the role of DC-SIGN in SAP signaling, we examined whether DC-SIGN activation using synthetic ligands could mimic SAP effects. When added to immune cells, a polycyclic aminothiazole DC-SIGN ligand (compound 1; shown as compound 4 in figure 2 of ref. 31) decreased human neutrophil adhesion (IC_{50} of 1.7 ± 0.3 μg/mL and a Hill coefficient of 2.6 ± 0.2) (Fig. 3*G*) and inhibited human fibrocyte differentiation ($IC_{50} = 1.2 \pm 0.4$ pg/mL, Hill coefficient $= 0.30 \pm 0.01$) (Fig. 3*H*) without affecting cell viability below 0.1 μg/mL (Fig. S7 *A* and *B*). Other DC-SIGN ligands also inhibited human fibrocyte differentiation, although with lower potency (Fig. S8). In addition, compound 1 promoted $CD163^+$ M2 macrophages (Fig. 3*I*). Compound 1 similarly reduced neutrophil adhesion, inhibited fibrocyte differentiation, and promoted M2 macrophages in FcγR quad KO cells (Fig. S9). However, in SIGN-R1–deficient cells, compound 1 did not affect fibrocyte differentiation (Fig. S7*C*). These data indicate that DC-SIGN activation by antibodies or a synthetic ligand is sufficient to mimic SAP effects in vitro. In addition, these results suggest that SAP:DC-SIGN interaction may contribute to the functional differences in SAP and CRP effects on the innate immune system. However, it is not clear whether SAP glycosylation directly binds DC-SIGN (carbohydrate:lectin interaction) or alters the structure of SAP so that SAP can bind DC-SIGN similar to how sIgG binds this receptor (protein:protein interaction) (32).

Compound 1 Reduces Neutrophil Accumulation in the Lungs of Bleomycin-Treated Mice. SAP and PTX3 regulate neutrophil recruitment in mice (9, 33). To determine whether SIGN-R1 activation similarly affects neutrophils in mice, WT mice were given bleomycin to induce acute lung inflammation. We then injected the mice with compound 1 and examined neutrophil accumulation in the lungs. We did not use anti–SIGN-R1 antibodies because they would activate both SIGN-R1 and FcγR, therefore confounding the results. As observed before (9), oropharyngeal instillation of bleomycin significantly increased the number of $Ly6G^+$ neutrophils in the lungs (Fig. 4). When mice were injected with compound 1 at 0.1 mg/kg on days 1 and 2 after bleomycin, there was a significant decrease in $Ly6G^+$ cells in the lungs at day 3 compared with the bleomycin control (Fig. 4). Bleomycin treatment also resulted in a significant increase in $CD11b^+$ macrophages and $CD45^+$ immune cells in the bronchoalveolar lavage (BAL) fluid (Fig. 4*B*). This increase in infiltrating cells was absent when mice were given compound 1 (Fig. 4*B*). Additionally in the post-BAL lungs, compound 1 reversed a bleomycin-induced decrease in $CD11c^+$ cells (Fig. 4*C*). However, compound 1 did not alter the number of $CD11b^+$ macrophages in the lungs compared with the bleomycin control (Fig. 4*C*). Our results indicate that DC-SIGN ligands such as compound 1 can, similar to SAP and PTX3 (9, 33), reduce neutrophil accumulation in mouse lungs following an insult.

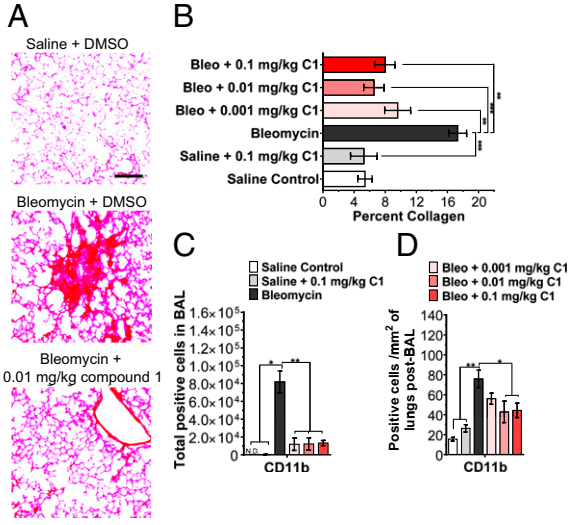

Fig. 5. Compound 1 alleviates pulmonary fibrosis in mice. (*A*) Mice received oropharyngeal bleomycin (Bleo) on day 0 to induce pulmonary fibrosis. Control mice received saline. Mice were then injected with compound 1 (C1) or vehicle control at the indicated dose daily starting on day 1 and ending on day 20. On day 21, mice were euthanized and after collecting BAL fluid, lungs were stained with PicroSirius red to estimate collagen deposition. Images are representative of three different experiments. (Scale bar: 200 μm.) (*B*) Quantification of PicroSirius red staining, $n = 3$. (*C*) BAL cell were stained for CD11b, $n = 3$. Rat IgG1 was used as the isotype control. (*D*) After collecting BAL, lungs were stained for CD11b, $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way ANOVA, Holm-Bonferroni post hoc test). (*B–D*) Values are mean ± SEM.

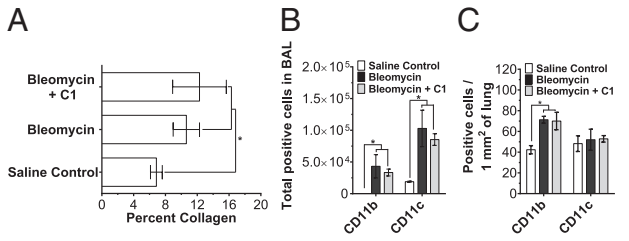

Fig. 6. IL-10 is necessary for the antifibrotic effect of compound 1. (*A*) Pulmonary fibrosis was induced by bleomycin instillation in IL-10–deficient mice. The bleomycin-treated mice were then injected with 0.1 mg/kg compound 1 (C1) or an equal volume of vehicle control daily. On day 21, mice were euthanized and lungs were stained with PicroSirius red to estimate collagen deposition, $n = 3$. (*B*) BAL cells were stained for the indicated markers, $n = 3$. Rat IgG1 was used as the isotype control. (*C*) After collecting BAL, lungs were stained for the indicated markers, $n = 3$. $^*P < 0.05$ (one-way ANOVA, Holm–Bonferroni post hoc test). All values are mean ± SEM.

Compound 1 Alleviates Pulmonary Fibrosis in Mice. Compound 1 alters macrophage phenotype and inhibits fibrocyte differentiation similar to SAP. Because macrophages and fibrocytes are implicated in fibrosing diseases (1, 2), we determined whether DC-SIGN activation by compound 1 in a murine model of pulmonary fibrosis was sufficient to mimic SAP and alleviate fibrosis. As observed before (34), oropharyngeal instillation of bleomycin resulted in increased collagen deposition and recruitment of $CD11b^+$ macrophages to the lungs (Fig. 5). Daily injections of compound 1 at doses as low as 0.001 mg/kg decreased collagen deposition in the lungs and improved overall health as indicated by weight change (Fig. 5 *A* and *B* and Fig. S10*A*). In addition, compound 1 reduced the number of $CD11b^+$ macrophages compared with the bleomycin control (Fig. 5 *C* and *D*). These data suggest that compound 1, similar to SAP, can alleviate pulmonary fibrosis and inflammation in mice.

IL-10 Is Necessary for the Antiinflammatory Effect of Compound 1. IL-10 is an antiinflammatory cytokine that is released in response to DC-SIGN activation (21). IL-10 is also necessary for the antifibrotic effect of SAP in a mouse model of renal fibrosis (12, 13). As compound 1 activates DC-SIGN to mimic SAP, we examined the efficacy of compound 1 on pulmonary fibrosis in IL-10–deficient mice. In IL-10–deficient mice, bleomycin instillation significantly increased collagen deposition and $CD11b^+$ and $CD11c^+$ macrophages in lungs (Fig. 6). Oropharyngeal instillation of bleomycin also resulted in significant decrease in body weight (Fig. S10*B*). Daily injections of 0.1 mg/kg of compound 1 had no significant effect on collagen deposition, $CD11b^+$ macrophage accumulation, $CD11c^+$ cell accumulation, or body weight in IL-10–deficient mice (Fig. 6 and Fig. S10*B*). These results suggest that IL-10 is necessary for the antifibrotic effects of compound 1 in a mouse bleomycin model of pulmonary fibrosis.

Lung Conducting Airway Epithelial Cells Express SIGNR-R1 and IL-10. Because compound 1 binds SIGN-R1 to regulate monocyte functions in mice, we examined the expression of this receptor in mouse lungs. We found that SIGN-R1 was expressed on $Epcam\text{-}1^+$ lung epithelial cells and on $CD45^+$ immune cells (Fig. 7*A*). To determine the source of IL-10, we stained mouse lungs for Epcam-1, CD45, and IL-10 by immunofluorescence. In saline-treated mice, $Epcam\text{-}1^+$ epithelial cells but not $CD45^+$ immune cells expressed detectable levels of IL-10 (Fig. S11). However, when mice were treated with bleomycin, the number of IL-10–expressing epithelial cells ($Epcam\text{-}1^+$) significantly decreased (Fig. 7*B* and Fig. S11*A*). This decrease in $IL\text{-}10^+$ $Epcam\text{-}1^+$ cells was reversed when mice were injected with compound 1 or SAP (Fig. 7*B* and Fig. S11*A*). These results suggest that compound 1 and SAP can bind to SIGN-R1 on $Epcam\text{-}1^+$ epithelial cells to induce IL-10 expression and reduce inflammation.

Discussion

SAP and CRP bind FcγR and regulate the innate immune system and fibrosis (8, 12, 18). In this report, we found that in the absence of all FcγR, SAP still reduces neutrophil adhesion, inhibits fibrocyte differentiation, and alters macrophage phenotype. Conversely, CRP requires FcγR to reduce neutrophil adhesion and promote IL-10 secretion by macrophages, but not to increase $ICAM\text{-}I^+$ M1-like macrophages. These observations suggest the presence of additional SAP and CRP receptors. We identified an additional SAP receptor as DC-SIGN, found that SAP binds to this receptor in a glycosylation-dependent manner, and observed that a DC-SIGN ligand mimics some SAP functions in vitro and in animal models of acute lung injury and pulmonary fibrosis. The DC-SIGN ligand alleviates pulmonary fibrosis in mice through an IL-10–dependent mechanism, with the IL-10 most likely originating from the epithelial cells in the lungs.

The FcγRs have been viewed as the main targets for SAP and CRP in the innate immune system (5, 18). Our data counter this view, because SAP is able to regulate the innate immune cells in absence of all FcγR. In fact, SAP is a more potent polarizer of FcγR-deficient macrophages than WT macrophages, suggesting that some of the FcγR may counteract the effect of SAP. In agreement with this observation, an anti-DC-SIGN antibody (which contains Fc regions and, thus, interacts with both FcγR and DC-SIGN) reduced, but did not abolish, neutrophil adhesion, whereas the F(ab2) fragment of the same antibody was significantly ($P < 0.05$ at 1 μg/mL) more potent and a DC-SIGN ligand completely abolished neutrophil adhesion. A similar trend was observed with SAP, which is a more potent inhibitor of FcγR-deficient neutrophil adhesion than WT neutrophils. This effect of FcγR appears to be cell-type dependent, because FcγR and DC-SIGN seem to act cooperatively to inhibit monocyte to fibrocyte differentiation. Both DC-SIGN and FcγR regulate the activity of Src kinases in innate immune cells (35, 36). The antagonism of DC-SIGN and FcγR signaling in some cells, and the cooperativity of

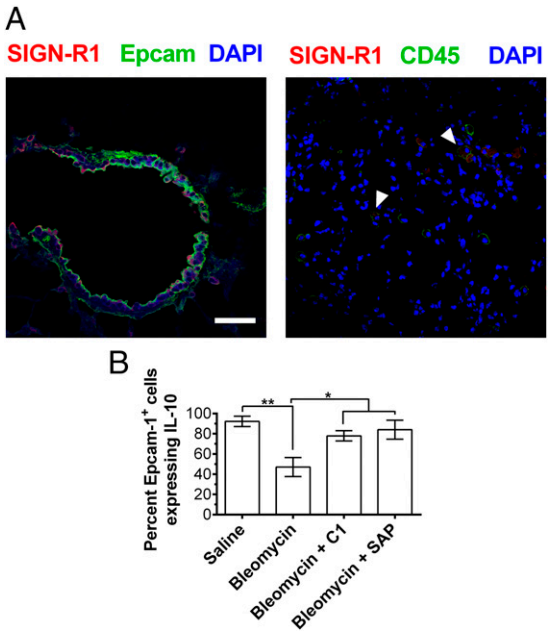

Fig. 7. Murine lung epithelial cells express SIGN-R1 and IL-10. (*A*) Mouse lungs following BAL were stained for the indicated markers. Images are representative of three different experiments. (Scale bar: 50 μm.) Arrows indicate $CD45^+$ cells expressing SIGN-R1. (*B*) The number of $Epcam\text{-}1^+$ cells expressing IL-10 in mouse lungs after BAL was quantified, $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, (one-way ANOVA, Holm–Bonferroni post hoc test). (*B*) Values are mean ± SEM.

DC-SIGN and FcγR signaling in other cells, may thus be due to their differential effects on Src kinases.

Although DC-SIGN/SIGN-R1 is considered to be primarily expressed by innate immune system cells, the majority of SIGN-R1 staining in mouse lungs was on Epcam-1$^+$ epithelial cells. These SIGN-R1$^+$ Epcam-1$^+$ expressed high levels of IL-10. Following a bleomycin insult, at day 21, although there was no appreciable reduction in the number of Epcam-1+ cells, there was a significant decrease in the number of IL-10$^+$ Epcam-1$^+$ cells. IL-10 inhibits apoptosis of epithelial cells (37) and increases the clearance of cell debris (38). As such, up-regulation of IL-10 by SAP or compound 1 may have a protective effect on lungs by limiting tissue damage and inflammatory responses. A similar role has been observed for epithelial cell-derived IL-10 in mouse models of inflammatory bowel disease (39). Alternatively, it is possible that IL-10 expression in SIGN-R1$^+$ epithelial cells is a function of their health. However, this possibility is unlikely because our studies in IL-10–deficient mice suggest a critical role for IL-10 in the antifibrotic role of compound 1.

Together, our data indicate that SAP binds DC-SIGN/SIGN-R1 to regulate innate immune cells and epithelial cells. Through its interaction with DC-SIGN, SAP distinguishes itself functionally from CRP. This observation suggests that DC-SIGN/SIGN-R1 is a key regulator of the innate immune system and is thus an interesting therapeutic target. Additionally, the functional interaction of SAP and PTX3 with DC-SIGN suggest that these pentraxins may regulate dendritic cells and, thus, the adaptive immune system.

Materials and Methods

All animals were used in accordance with National Institutes of Health guidelines and with a protocol approved by the Texas A&M University Institutional Animal Care and Use Committee. Human blood was obtained with written consent and with specific approval from the Texas A&M University human subjects Institutional Review Board. Human recombinant SAP and CRP were expressed in HEK293 cells and then purified y using affinity purification (8). Human PBMC, human neutrophils, mouse spleen cells, and mouse neutrophils were isolated and then incubated with antibodies and pentraxins, as described before (8, 9). Human monocytes were differentiated into macrophages in serum containing medium and then polarized for 3 d in serum-free medium. Pulmonary fibrosis and acute lung injury in mice were induced by bleomycin instillation (9, 40). Detailed information about mice, experimental procedures, and statistical analyses can be found in *SI Materials and Methods*.

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