Distinct Fcγ Receptors Mediate the Effect of Serum Amyloid P on Neutrophil Adhesion and Fibrocyte Differentiation

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Distinct Fcγ Receptors Mediate the Effect of Serum Amyloid P on Neutrophil Adhesion and Fibrocyte Differentiation

Nehemiah Cox, Darrell Pilling, and Richard H. Gomer

The plasma protein serum amyloid P (SAP) reduces neutrophil adhesion, inhibits the differentiation of monocytes into fibroblast-like cells called fibrocytes, and promotes phagocytosis of cell debris by macrophages. Together, these effects of SAP reduce key aspects of inflammation and fibrosis, and SAP injections improve lung function in pulmonary fibrosis patients. SAP functions are mediated, in part, by FcγRs, but the contribution of each FcγR is not fully understood. We found that aα Q55 and E126 in human SAP affect human fibrocyte differentiation and SAP binding to FcγRI. E126, K130, and Q128 affect neutrophil adhesion and SAP affinity for FcγRIIa. Q128 also affects phagocytosis by macrophages and SAP affinity for FcγRI. All the identified functionally significant amino acids in SAP form a binding site that is distinct from the previously described SAP-FcγRIIa binding site. Blocking FcγRI with an IgG-blocking Ab reduces the SAP effect on fibrocyte differentiation, and ligating FcγRIIa with Abs reduces neutrophil adhesion. Together, these results suggest that SAP binds to FcγRI on monocytes to inhibit fibrocyte differentiation, and binds to FcγRIIa on neutrophils to reduce neutrophil adhesion. The Journal of Immunology, 2014, 193: 1701–1708.

A berrant scar tissue formation is the hallmark of fibrosing diseases such as end-stage kidney disease, liver cirrhosis, pulmonary fibrosis, and congestive heart disease (1–3). The inappropriate scar tissue in fibrosis ultimately leads to organ failure and/or death. Fibrosing diseases are associated with 45% of deaths in the United States, but despite their high prevalence, there are no Food and Drug Administration–approved therapies (1, 4).

Serum amyloid P (SAP) component is a pentameric protein that belongs to the pentraxin family of evolutionarily conserved proteins. Pentraxins also include C-reactive protein (CRP) and the long pentraxin PTX-3 (5). SAP, CRP, and PTX-3 all have regulatory roles in the immune system (6–8). Injections of SAP inhibit inflammation and fibrosis in mouse models of pulmonary fibrosis, ischemic cardiac fibrosis, and renal fibrosis (9–12), and in a phase 1b clinical trial, SAP injections appear to improve lung function in pulmonary fibrosis patients (13).

At the onset of tissue damage and inflammation, neutrophils are recruited to the tissue in response to chemokines such as CXCL2 and CXCL8 to remove pathogens and/or cell debris via phagocytosis (14). This migration and activation of neutrophils is tightly regulated by factors expressed and secreted by endothelial cells, macrophages, and other cell types (14). When this regulation is compromised, the elevated influx of neutrophils and recruitment of other immune cells by activated neutrophils can cause severe organ damage and fibrosis (14–16). SAP binds neutrophils to inhibit their spreading and adhesion to components of extracellular matrix and endothelial cells (12, 17). Injections of SAP decrease the infiltration of neutrophils into the lungs after bleomycin insult in mice (12). However, the mechanism for this function is not well understood.

After neutrophil migration into the inflammation site, CD14+ monocytes enter and differentiate into macrophages and fibrocytes (3). Fibrocytes are CD45− collagen I+ fibroblast-like cells that share characteristics of both hematopoietic and stromal cells (18). Fibrocytes are found in healing dermal wounds and some fibrotic lesions, and secrete collagen and enzymes that modify the extracellular matrix (3, 9, 10, 19, 20). SAP inhibits fibrocyte differentiation partly through a group of receptors called FcγR (11, 21–24). These receptors bind IgG and consist of FcγRIIb, FcγRIIa, FcγRIIb, FcγRIIa, and FcγRIIIa (25). We have previously shown that FcγRI is one of the receptors responsible for the effect of SAP on fibrocyte differentiation in both humans and mice (21). SAP also binds the IgA receptor FcαRI (26).

In addition to modifying neutrophil adhesion and monocyte differentiation, SAP can also enhance phagocytosis of cell debris by professional phagocytes such as macrophages (24, 27). The SAP pentamer forms a flat disk, and binds to bacteria and cell debris on one surface and to FcγRs on the other surface, to promote phagocytosis by cells (24). Previous studies have implicated FcγRI as the key receptor for SAP-induced phagocytosis, but the precise role of each FcγR in this process is unclear (24, 27).

In this report, we examined how SAP interacts with FcγRs to regulate different aspects of the immune system. We found that SAP inhibits fibrocyte differentiation and promotes phagocytosis by macrophages through FcγRI, whereas it reduces neutrophil adhesion via FcγRIIa. Using site-directed mutagenesis, we determined that although the same site on SAP affects monocytes, macrophages, and neutrophils, it is possible to affect specific SAP functions without altering the other functions in an appreciable way. In addition, we identified a novel FcγR binding site that is distinct from the site previously identified in a cocrystal structure of SAP and FcγRIIa (23).

Materials and Methods

PBMC and neutrophil isolation, cell culture, and fibrocyte and macrophage differentiation

Human peripheral blood was collected into heparin tubes (BD Bioscience, San Jose, CA) from healthy adult volunteers who gave written consent and with specific approval from the Texas A&M University human subjects
SAP USES DIFFERENT RECEPTORS FOR DIFFERENT FUNCTIONS

We previously made site-directed mutations of human SAP at amino acids that interact with human FcγRIIα in a SAP-FcγRIIα cocystal structure (23), and observed that changes to these amino acids had no significant effect on the ability of SAP to inhibit fibrocyte differentiation (21). To better understand the interaction of SAP with FcγRs, we compared the amino acid sequence of human SAP with the related pentraxin CRP. SAP and CRP have 51% sequence identity and similar crystal structures, but have different affinities for FcγRs and different roles in the immune system (6, 11, 23, 27). Thus, the sequence differences can be used to identify structurally and functionally significant amino acids. Of the amino acids that were different between SAP and CRP, we mutated only the ones that were exposed on the surface of SAP (23). E153, which is at the interface between SAP monomers, was also mutated in an attempt to destabilize the pentameric protein and introduce functional defects. We then expressed all the generated SAP variants in HEK293 cells. All the SAP variants eluted at 10–12 ml from a Superose 12 size exclusion chromatography column, indicating the absence of aggregates larger than pentamers, and the absence of free monomers (Fig. 1A and Supplemental Table I). We subsequently tested the ability of these variants to decrease neutrophil adhesion, inhibit fibrocyte differentiation, and promote phagocytosis. In addition, we examined the binding of these variants to FcγRs.

We first examined the ability of our SAP variants to reduce neutrophil adhesion to human fibroblasts. We screened 29 SAP variants for their ability to reduce neutrophil adhesion and then, based on our preliminary data, focused on 13 of the examined variants (data not shown). These 13 SAP variants were screened at 80 nM (10 μg/ml) WT SAP or mutant SAP in 20 mM Tris, 140 mM NaCl, 2 mM CaCl2 for 1 h. Using a biochip reader, we then measured the binding of WT SAP or mutant SAP to cells that were then treated with 10% FBS (Caisson). Phagocytosis of FITC-conjugated zymosan A particles (Life Technologies) by macrophages was assayed as described previously (24). To measure the binding of SAP to zymosan A particles, we added bioparticles with 240 nM (30 μg/ml) zymosan A particles (Life Technologies) to HEK293 cells and analyzed the number of bioparticles bound to the cells using a flow cytometer (24). When measuring SAP binding to HEK293 cells expressing FcγRI or FcγRIIb, mock-transfected HEK293 cells were used to estimate the nonspecific binding. K562, HEK293, FcγRII (clone 10.1; Biolegend), and FcγRII (clone 3G8; Biolegend) to determine the expression of the indicated receptor by flow cytometry (29). Leukocytes were assayed for CD3 (Biolegend), CD14 (Biolegend), CD15 (Biolegend), CD19 (Biolegend), CD45 (Biolegend), FcγRI (clone 10.1; Biolegend), FcγRII (clone FUN-2; Biolegend), and FcγRII (clone 3G8; Biolegend) were assayed by flow cytometry to determine the presence of different immune cell populations as previously described (29, 30, 35).

Statistical analysis

Data were analyzed by ANOVA (with Dunnett’s posttest) or t test when appropriate using Prism software (GraphPad, San Diego, CA). Data were fit to the appropriate model of binding as determined by F-tests. Normality was tested using Shapiro–Wilk and D’Agostino–Pearson omnibus tests when applicable.

Results

Identification of SAP amino acids that affect neutrophil adhesion

We previously made site-directed mutations of human SAP at amino acids that interact with human FcγRIIα in a SAP-FcγRIIα cocystal structure (23), and observed that changes to these amino acids had no significant effect on the ability of SAP to inhibit fibrocyte differentiation (21). To better understand the interaction of SAP with FcγRs, we compared the amino acid sequence of human SAP with the related pentraxin CRP. SAP and CRP have 51% sequence identity and similar crystal structures, but have different affinities for FcγRs and different roles in the immune system (6, 11, 23, 27). Thus, the sequence differences can be used to identify structurally and functionally significant amino acids. Of the amino acids that were different between SAP and CRP, we mutated only the ones that were exposed on the surface of SAP (23). E153, which is at the interface between SAP monomers, was also mutated in an attempt to destabilize the pentameric protein and introduce functional defects. We then expressed all the generated SAP variants in HEK293 cells. All the SAP variants eluted at 10–12 ml from a Superose 12 size exclusion chromatography column, indicating the absence of aggregates larger than pentamers, and the absence of free monomers (Fig. 1A and Supplemental Table I). We subsequently tested the ability of these variants to decrease neutrophil adhesion, inhibit fibrocyte differentiation, and promote phagocytosis. In addition, we examined the binding of these variants to FcγRs.

We first examined the ability of our SAP variants to reduce neutrophil adhesion to human fibroblasts. We screened 29 SAP variants for their ability to reduce neutrophil adhesion and then, based on our preliminary data, focused on 13 of the examined variants (data not shown). These 13 SAP variants were screened at 80 nM (10 μg/ml; Fig. 1B). We chose this concentration because it was close to the IC_{50} (67 ± 7 nM) of SAP for reducing neutrophil adhesion (Fig. 1C), and hence allowed us to detect both increases and decreases in the SAP effect on neutrophils. Following our screen, we observed that SAP variants E126A and Q128A had significantly reduced inhibitory effect on neutrophil adhesion compared with WT SAP (Fig. 1B). SAP variant K130V conversely had a significantly reduced inhibitory effect on neutrophil adhesion (Fig. 1B).

Identification of SAP amino acids that affect fibrocyte differentiation

Because SAP inhibits fibrocyte differentiation, we also screened the 29 SAP variants for their ability to inhibit the differentiation of...
monocytes into fibrocytes (Supplemental Table I) (21, 28, 29, 36). Using PBMCs from a variety of donors, we observed 1200–3100 fibrocytes per 10^6 PBMCs. Because of this variability, fibrocyte counts were normalized to the no-SAP control, as described previously (21, 28, 37). WT SAP inhibited fibrocyte differentiation with an IC_{50} of 2.9 ± 0.3 nM, similar to previously published data (21, 36). Twenty-five of the 29 variants tested did not significantly alter the ability of SAP to inhibit fibrocyte differentiation (Supplemental Table I). Compared with WT SAP, variants Q55A and K130V were more effective at inhibiting fibrocyte differentiation, whereas variants E153A and E126A had reduced activity (Fig. 2 and Supplemental Table I). In addition, we observed significant changes in the Hill coefficient of SAP variants V68A and Q128A compared with WT SAP (Supplemental Table I). This change could be because of alterations in SAP variant binding to FcγRs and/or how these variants activate the receptors.

Identification of SAP amino acids that affect phagocytosis

SAP enhances phagocytosis of pathogens and cell debris through FcγRs (24, 27). However, the exact receptor and amino acids involved are unknown. We first screened the 29 SAP variants for their ability to enhance phagocytosis and then, based on our preliminary data, focused on 13 variants (data not shown). The 13 SAP variants were screened at 240 nM (30 µg/ml, physiological concentration in the human plasma) for their ability to promote phagocytosis of zymosan A bioparticles by macrophages (Fig. 3A). Eleven of the SAP variants examined did not significantly alter the ability of SAP to enhance phagocytosis (Fig. 3A). Compared with WT SAP, SAP variants Q128A and E153A had significantly reduced ability to promote phagocytosis by macrophages (Fig. 3A).

We then measured the binding of WT, Q128A, and E153A SAP to zymosan A bioparticles to determine whether these SAP variants had deficiencies in binding the bioparticles. Compared with WT SAP, we found no statistically significant differences in the binding of Q128A or E153A to zymosan A (Fig. 3B). Together, this indicates that SAP variants Q128A and E153A have defects in binding and/or activating FcγRs to promote phagocytosis of zymosan A.

SAP binds to endogenous FcγRI and FcγRIIa on immune cells

Much of the work done on SAP binding to FcγRs has focused on SAP binding to rFcγRs or to receptors expressed on non-human cells such as COS-7 and NIH-3T3 (11, 23, 24). This is problematic because the affinity of FcγRs for their ligands is sensitive to the receptor glycosylation state and the presence of intracellular signaling proteins (38–41). Therefore, to identify functionally significant receptors in the SAP response, we examined the binding of SAP to endogenous FcγRs on human immune cells and to receptors expressed on the human-derived cell line HEK293. We SAP-f and then measured the binding to different peripheral blood cell populations as identified by their flow characteristics and receptor expression (Supplemental Fig. 1). We tested the activity of SAP-f on neutrophils, monocytes, and macrophages, and observed no functional defects compared with unlabeled SAP (Supplemental Fig. 2). When SAP-f was incubated with leukocytes, we observed no binding to the lymphocyte population (Fig. 4A). Because B cells (~5% of lymphocytes) express FcγRIIb (25), and NK cells (~5–10% of lymphocytes) express FcγRIIIa (25), this suggests that SAP does not bind to these receptors under our experimental conditions. However, SAP-f did bind to monocytes and neutrophils (Fig. 4A). Monocytes express FcγRI, FcγRIIa, and some FcγRIIIa (Supplemental Fig. 1) (12, 30). This indicates that SAP could be binding to any or all of the FcγRs on monocytes. Because NK cells express FcγRIIIa, and we
we incubated SAP-f with Fcγ.

SAP-f binds to endogenous Fcγ on neutrophils, but not to Fcγ on monocytes, and we hypothesize that this is likely due to the presence on neutrophils. We observed more SAP binding to monocytes than to neutrophils. This then suggests that SAP binds to FcγRIIa on neutrophils. We observed more SAP binding to monocytes than to neutrophils, and we hypothesize that this is likely due to the presence of FcγRIIa on monocytes. Following our initial binding assays using human immune cells, we investigated the binding of our SAP variants to FcγRI and FcγRIIa. Of the 29 SAP variants, we chose 6 that had altered functions as determined by human IgG binding (Fig. 4B, 4C, and Supplemental Fig. 3). This then suggests that SAP binds to FcγRIIa on neutrophils. We observed more SAP binding to monocytes than to neutrophils, and we hypothesize that this is likely due to the presence of FcγRIIa on monocytes (Fig. 4A). Together, our data indicate that SAP-f binds to endogenous FcγRI and FcγRIIa on monocytes and neutrophils, but not to FcγRIIb, FcγRIIIa, and FcγRIIIb (Supplemental Fig. 4).

**SAP binds to FcγRI and FcγRIIa on HEK293 cells**

Following our initial binding assays using human immune cells, we investigated the binding of our SAP variants to FcγRI and FcγRIIa. Of the 29 SAP variants, we chose 6 that had altered functions as measured by neutrophil adhesion assays, fibrocyte differentiation assays, and macrophage phagocytosis assays. The six SAP variants were fluorescently labeled and then incubated with K562 cells to measure the binding to FcγRIIa. The only known receptor that binds SAP on the surface of K562 cells is FcγRIIa (34) (Supplemental Fig. 3). WT SAP bound to FcγRIIa with a $K_d$ of 19.7 ± 3.4 nM (Fig. 5A and Table I). Previous measurements of the $K_d$ for SAP binding to FcγRIIa range from 0.29 nM to 1.4 μM (11, 23, 24). As previously described, these inconsistencies are most likely caused by the method of receptor expression and how the $K_d$ was estimated (38). Of the 6 SAP variants tested, compared with WT SAP, E126A, Q128A, and K130V had significant differences in their binding to FcγRIIa (Fig. 5A and Table I). These changes in affinity correlate with the ability of these SAP variants to reduce neutrophil adhesion. Variants E126A and Q128A have a higher affinity for FcγRIIa and have an increased inhibitory effect on neutrophil adhesion (Fig. 1 and Table I). Conversely, variant K130V has a decreased affinity for FcγRIIa and has a reduced inhibitory effect on neutrophil adhesion to fibronectin (Fig. 1 and Table I). In addition, variant E126A has a Hill coefficient of 3.3 ± 0.5, indicating cooperativity in SAP E126A-FcγRIIa binding. This cooperativity is absent from the other SAP variants because their Hill coefficient is not significantly different from the 1.2 ± 0.2 we measured for WT SAP. One possible explanation for this increased Hill coefficient is self-aggregation of SAP E126A after binding to cells. This would then manifest as an increase in the maximal binding ($B_{\text{max}}$) of SAP E126A to cells. However, the SAP E126A $B_{\text{max}}$ was 69.7 ± 10.8% of WT SAP (mean ± SEM, $p$ not...
significant by t test), indicating that SAP E126A was not aggregating on the surface of cells.

To measure the binding of our six SAP variants to FcγRI, we used FcγRI HEK293 cells (Supplemental Fig. 3). The FcγRI HEK293 cells were cotransfected with FcγRy, because this intracellular protein is necessary for FcγRI localization to the cell membrane (42). The mock-transfected cells were used to estimate the nonspecific binding (Fig. 6). WT SAP bound to FcγRI with a $K_d$ of 4.6 ± 0.8 nM and a Hill coefficient of 2.1 ± 0.6 (Table I). This affinity matches the previously published 

<table>
<thead>
<tr>
<th>SAP Variants</th>
<th>FcγRI $K_d$ (nM ± SEM)</th>
<th>Hill Coefficient</th>
<th>FcγRIIa $K_d$ (nM ± SEM)</th>
<th>Hill Coefficient</th>
</tr>
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<tbody>
<tr>
<td>WT SAP</td>
<td>4.6 ± 0.8</td>
<td>2.1 ± 0.6</td>
<td>19.7 ± 3.4</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>I23G</td>
<td>9.8 ± 5.8</td>
<td>1.1 ± 0.4</td>
<td>25.2 ± 9.8</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>Q55A</td>
<td>2.6 ± 0.1*</td>
<td>6.7 ± 0.2*</td>
<td>22.3 ± 3.3</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>E126A</td>
<td>36.9 ± 2.8*</td>
<td>0.9 ± 0.1</td>
<td>11.9 ± 0.6**</td>
<td>3.3 ± 0.5**</td>
</tr>
<tr>
<td>Q128A</td>
<td>24.7 ± 6.0**</td>
<td>0.9 ± 0.1**</td>
<td>11.9 ± 2.1**</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>K130V</td>
<td>4.5 ± 0.7</td>
<td>3.7 ± 1.9</td>
<td>43.7 ± 8.7**</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>E153A</td>
<td>5.3 ± 0.6</td>
<td>3.2 ± 0.9</td>
<td>27.8 ± 1.4</td>
<td>4.7 ± 0.9</td>
</tr>
</tbody>
</table>

HEK293 cells expressing FcγRI were incubated with Alexa Fluor 647–labeled SAP variants. The cells were then washed and the binding of the labeled SAP to the cells was measured by flow cytometry. Mock-transfected cells were used to estimate the nonspecific binding. K562 cells were used to measure the binding of SAP variants to FcγRIIa. Values are mean ± SEM, $n$ = 3–6.

$p < 0.05$, **$p < 0.001$, t test when compared with the corresponding WT control.

FIGURE 5. SAP variant binding to FcγRIIa and FcγRI. (A) K562 cells, which express FcγRIIa, were incubated with fluorescently labeled SAP variants. The cells were then washed, and the binding of the labeled SAP to the cells was measured by flow cytometry. (B) HEK293 cells expressing FcγRI were incubated with fluorescently labeled SAP variants and then binding was measured by flow cytometry. Mock-transfected cells were used to estimate the nonspecific binding. Median fluorescence intensity values were normalized to the intensity value of the highest SAP concentration. Values are normalized mean ± SEM, $n$ = 3–5. Curves are fits to models of one-site binding with variable Hill coefficient. The absence of error bars indicates that the error was smaller than the plot symbol.
FcγRIII Abs in the presence or absence of WT SAP. The anti-FcγRII and anti-FcγRIII Abs used in this experiment do not discriminate between the different FcγRII or FcγRIII isoforms (43). However, they do block IgG binding to all FcγRII or FcγRIII receptors (43). Mouse IgG1, anti-FcγRII, and anti-FcγRIII Abs had no effect on the inhibitory effect of SAP on fibrocyte differentiation (Fig. 8). However, the F(ab’)2 fragment of anti-FcγRI Ab clone 10.1 reduced the ability of SAP to inhibit fibrocyte differentiation (Fig. 8). This indicates that SAP binds to FcγRI to inhibit fibrocyte differentiation.

Identification of a novel FcγR binding site on SAP

Following our functional assays, we mapped all the mutated amino acids onto the SAP structure (Fig. 9A). Excluding aa E153, all the functionally significant amino acids form a distinct binding site on the surface of SAP (Fig. 9B, 9C). This novel binding site is different from the previously identified FcγRIIa binding site (Fig. 9B) (24). The position of this novel binding site on SAP may allow for the binding of multiple FcγRs (Fig. 10).

Discussion

The pentraxin SAP is an antifibrotic agent that inhibits aberrant scar tissue formation by regulating neutrophils, monocytes, and macrophages (11, 12, 24, 36, 44). All SAP functions appear to be mediated partly through FcγRs (11, 21, 45). Because there are multiple FcγRs on neutrophils, monocytes, and macrophages, we determined how each receptor contributed to different SAP functions. We found through site-directed mutagenesis that SAP binds to FcγRI on monocytes to inhibit fibrocyte differentiation and to FcγRIIa on neutrophils to reduce adhesion to fibronectin. In addition, we identified a novel FcγR binding site on SAP.

Mutations in SAP that affect binding to FcγRIIa significantly change the ability of SAP to reduce neutrophil adhesion to fibronectin. Similar to SAP, ligating FcγRIIa by anti-FcγRII Abs decreases neutrophil adhesion. This suggests that SAP binds to FcγRIIa in a manner analogous to that of FcγRIIa.
The activation of FcγRIIIa results in the phosphorylation of the ITAM in the cytosolic region of this receptor (46). ITAM phosphorylation is implicated in inside-out signaling and regulation of adhesion molecules (46). This then suggests that FcγRIIIa activation can reduce adhesion of neutrophils to fibronectin by regulating adhesion molecules on neutrophils. In addition, ligating FcγRIIIb by an anti-FcγRIII Ab reduces neutrophil adhesion to fibronectin, suggesting that ligands of FcγRIIIb such as Ig could also regulate neutrophil adhesion to fibronectin.

Mutations in SAP that affect binding to FcγRI significantly alter the ability of SAP to inhibit fibrocyte differentiation. In addition, blocking FcγRI with an IgG blocking Ab reduces the SAP effect on fibrocyte differentiation. Together, this suggests that although there are multiple FcγRs on monocytes, SAP activates FcγRI to inhibit fibrocyte differentiation. This is in agreement with our previous results where we observed that small interfering RNA knockdown of FcγRI in humans results in decreased inhibitory effect of SAP on fibrocyte differentiation (21), and that cross-linking FcγRI with Abs can mimic the inhibitory effect of SAP on fibrocyte differentiation (45). In this study, we have identified an FcγRI binding site on each SAP monomer, suggesting that SAP can cross-link multiple FcγRs (Fig. 9). Together, this suggests a role for FcγRI cross-linking in SAP inhibition of fibrocyte differentiation.

SAP appears to promote phagocytosis of bioparticles such as zymosan A through FcγRI (Table I) (27). However, not all SAP variants with alteration in FcγRI binding have defects in phagocytosis. For instance, variant E126A has an ~10-fold reduction in affinity for FcγRI and defects in inhibiting fibrocyte differentiation, but has no deficiencies in promoting phagocytosis. This suggests that a SAP oposoned bioparticle activates FcγRI to promote phagocytosis in a manner that is distinct from how SAP activates FcγRI to inhibit fibrocyte formation. This is supported by the fact that FcγRI-mediated phagocytosis is Syk-kinase dependent, but inhibition of fibrocyte differentiation by SAP is Syk-kinase independent (45, 47–49). It is also possible that SAP binding to FcγRI is sufficient to promote phagocytosis irrespective of changes in SAP-FcγRI affinity. Alternatively, it is feasible that Q128A and E153A modulate zymosan A phagocytosis by altering macrophage activation. However, SAP oposoned zymosan A particles were incubated with macrophages for a short time (60 min), which would not allow for significant alteration in macrophage activation and phenotype.

In surface plasmon resonance experiments, SAP binds to all of the FcγRs (11, 23). However, we observed that SAP only binds to endogenous FcγRI and FcγRIIa on immune cells. This inconsistency can be explained by the differences in the glycosylation state of the receptors and/or the lack of some intracellular signaling components (38). FcγRIIa is a highly glycosylated receptor in humans. Modifying FcγRIIa glycosylation changes its affinity for IgG and perhaps SAP (38). FcγRI and FcγRIIa in humans interact with an intracellular protein called FcγR (38, 42). The absence of FcγR alters the affinity of FcγRI and FcγRIIa for IgG in humans (38, 42). This can potentially alter SAP binding to FcγRI and FcγRIIa. Together, this suggests that the SAP affinity for FcγRs is dependent on the modification of these FcRs and the interactions they make before binding SAP.

Our findings indicate that it is possible to mimic specific SAP functions by targeting particular FcγRs (Fig. 10). For instance, activation of FcγRIIa by Abs or small molecules could be used to decrease neutrophil adhesion and hence reduce neutrophil accumulation in lungs of patients suffering from acute respiratory distress syndrome or cystic fibrosis. Similarly, blocking SAP binding to FcγRI might promote fibrocyte differentiation and wound healing. In addition, our results suggest that altering the SAP sequence could improve its ability to inhibit fibrocyte differentiation and/or reduce neutrophil adhesion. This could lead to the development of a more potent SAP antifibrotic.

Acknowledgments
We thank Jeffrey R. Crawford for guidance and constructive discussion. We also thank the staff at Beutel student health center for drawing blood from volunteers. In addition, we thank the PSLBiology-materials repository for providing FcγRI, FcγRIIa, and FcγRplasmin.

Disclosures
D.P. and R.H.G. are inventors on patents for the use of SAP as a therapeutic for fibrosing diseases and patents for the use of SAP-depleting materials to enhance wound healing. D.P. and R.H.G. are members of the Science Advisory Board of, and have stock options from Promedior, a start-up company that is developing SAP as a therapeutic for fibrosing diseases, and they receive a share of milestone payments made by Promedior to Rice University.

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