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RAPID COMMUNICATION

Evidence against Ebola Virus sGP Binding to Human Neutrophils by a Specific Receptor

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The issue of whether Ebola secretory glycoprotein (sGP) binds to human neutrophils via the IgG Fc receptor IIIb (FcγRIIIb, CD16b) or other receptors has been controversial. To clarify this, FACS analysis, an sGP absorption assay, and direct binding of ^{125}I-sGP to neutrophils were performed. Results from FACS analysis demonstrated that limited washing conditions leads to the nonspecific formation of immune complexes on the neutrophil surface and this, but not a specific interaction between sGP and CD16b, is responsible for the previous observations. An sGP absorption assay also demonstrated that sGP is not specifically bound but is nonspecifically proteolysed by proteases released from neutrophils. Finally, there was no difference in ^{125}I-sGP binding to neutrophils compared to other control cell types. Taken together, these results demonstrate that neutrophils do not express a specific receptor for Ebola virus sGP. It is unlikely that sGP plays a role in the Ebola virus pathogenesis through interfering with the innate immunity by targeting neutrophils.

Introduction. Ebola virus, a member of family *Filoviridae*, causes acutely lethal hemorrhagic fever in humans and nonhuman primates. Two forms of glycoprotein, GP and sGP, are expressed from a single glycoprotein gene and have identical 295 aa at their NH_2-terminal ends but differ at the COOH termini. The GP is the structural membrane-anchored glycoprotein, which is expressed by two reading frames via transcriptional RNA editing. The sGP is a nonstructural, soluble glycoprotein which is directly synthesized from the unedited GP mRNA and secreted from infected cells as a disulfide-linked homodimer (*1–3*). Several reports have shown that GP, the only surface protein on the virions, is an important pathogenic determinant of Ebola virus. It is responsible for cell binding and entry of the virus and causes cytotoxicity (*4–7*). However, little is known regarding the pathogenic significance of sGP. It is speculated that the sGP may play an important role in the acute hemorrhagic fever as it can be detected in significant amounts in the sera of acutely infected patients (*8*).

Ebola sGP has been reported to bind to human neutrophils via the IgG Fc receptor IIIb (FcγRIIIb, CD16b) and to inhibit early neutrophil activation (*9*). This study received considerable attention because, for the first time, it provided evidence to suggest that sGP may be involved in disease pathogenesis by diminishing innate immunity to the virus through its specific binding to neutrophil which is not the target cell type for Ebola virus infection. It also provided a potential new approach to treat Ebola virus infection by blocking the binding of sGP to neutrophils (*10*). This study also raised the possibility that sGP interfered with neutrophil function by specific binding to CD16b and other possible coreceptors since CD16b was reported to be necessary but not alone sufficient for sGP binding. This report was later challenged by other investigators since sGP binding to neutrophils could not be detected with the use of anti-sGP Fab or $F(ab')_2$ fragments. These findings suggested that the binding signal generated with the rabbit antibody against sGP occurred through its Fc portion to FcγRIIIb as an immune complex with sGP (*11*). However, further data provided by the authors of the original report demonstrated that the binding of sGP to neutrophils as detected by the $F(ab')_2$ fragment could be restored by preincubating the neutrophils with purified nonspecific rabbit IgG (*12*). Therefore, the reports of sGP binding to neutrophils directly or indirectly has remained controversial as differences in experimental conditions, the source and purity of sGP, etc., may be responsible for the conflicting results of these studies. We have performed additional studies with purified sGP and human neutrophils to better understand the role of sGP in the pathogenesis of Ebola virus acute hemorrhagic fever.

Results and Discussion. As previously reported, the binding of sGP to neutrophils could be detected by FACS analysis using rabbit anti-sGP serum and anti-FcγRIIIb

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monoclonal antibody (Mab) 3G8 inhibited the binding of sGP, whereas several other antibodies against neutrophil surface antigens did not (9). FcγRIIIb is only expressed on neutrophils and is the dominant Fcγ receptor for neutrophil-mediated binding and clearance of immune complexes (IC), and the binding of IC to neutrophils can be totally inhibited by anti-CD16b antibody (3G8) (*13, 14*). This raises the question as to whether sGP/anti-sGP IC are involved in the binding of sGP to neutrophils, especially since the FACS assays were performed under conditions where IC may form (*9*). We performed FACS analysis of sGP binding to neutrophils using purified sGP under different incubation and washing conditions. Binding of the glutathione-*S*-transferase (GST) protein and human interleukin-8 (IL-8) was used as negative and positive controls, respectively. After sGP, GST, or IL-8 incubation, the cells were spun down and washed either once or twice followed by incubation with relevant primary rabbit antibody against sGP, GST, or IL-8 and FITC-conjugated secondary antibody. As shown in Fig. 1, we confirmed that purified sGP (A1), GST (A2), and IL-8 (A3) bound to neutrophils after a single washing step; however, after a second washing step, sGP (B1) and GST (B2) binding became barely detectable, while IL-8 (B3) binding remained unaltered. These results suggested that sGP binding to the neutrophil is not specific and could only be explained by ICs forming under single washing conditions but not under the condition of twice washing. To investigate this in more detail, we next examined whether Mab against CD16b clone 3G8 could inhibit binding of both sGP and GST to neutrophils under the same experimental conditions where the bindings occurred. GST and sGP were used as reciprocal controls for each other and anti-CD11b clone BEAR1 was used as the control of 3G8. The freshly isolated neutrophils were preincubated with 3G8 or BEAR1 and then followed by the immunostaining steps as described above. As seen in Fig. 1C, the anti-CD16b not only blocks the binding of sGP (C1), but also that of GST (C2). This confirmed that the "binding" of sGP and GST to neutrophils is due to the *in situ* formation of ICs on the neutrophil surface. In addition, we were not able to restore the binding of sGP to neutrophils as detected by the $F(ab')_2$ fragment of anti-sGP with the preincubation of neutrophils with either normal rabbit or human IgG (Fig. 1D), thus providing further evidence that mere occupancy of CD16b has no effect on sGP binding.

It was reported that the binding of sGP to neutrophils also occurred in the absence of antibody as shown by the depletion of sGP in an absorption assay (*9*). We also could obtain this similar result under certain assay conditions. It is well known that intracellular granules are a marked characteristic of neutrophils; the numerous proteases that are stored in the granules play an important role in the physiological and pathologic functions of neutrophils and that activation of neutrophils leads to the release of proteases and tissue injury. We asked whether the depletion of sGP by neutrophils could be due to degradation of sGP by cell-associated or proteases released from neutrophils that could be activated during the *in vitro* isolation and processing procedures. To address this, the absorption assay was performed by incubating neutrophils with sGP for different periods of time and either at 4 or 37°C. After incubation, cells were removed by centrifugation and the resulting supernatants were used for detection of sGP by Western blot. As shown in Fig. 2A, sGP appears to be depleted only when the incubations were performed at 37°C for 2 h in the absence of protease inhibitors, and the samples were resolved on 10% SDS–PAGE under reducing conditions. In contrast, sGP was not "depleted" when the incubations were performed in the presence of the protease inhibitors. Under other incubation conditions, such as at 37°C for 1 h or 4°C for 1–3 h in the absence of protease inhibitors, partial degradation of sGP was seen as evidenced by the appearance of two bands on SDS–PAGE under nonreducing conditions, an intact sGP with the expected molecular weight of 120–130 kDa, and a second smaller molecular weight form (around 100 kDa). The amount of the smaller band of sGP was temperature related and was not seen when the incubations were performed in the presence of protease inhibitors (Fig. 2B). This result indicated that the sGP was being degraded by neutrophil-associated proteases. To further examine whether the proteases were released from neutrophils and were specifically induced by sGP, we preincubated neutrophils and Jurkat cells with Dulbecco's 1× phosphate-buffered saline (PBS), bovine serum albumin (BSA), and sGP at 37°C for 1 h; then the cells were removed and the supernatants were harvested and incubated with fresh sGP for a further 1 h at 37°C. As shown in Fig. 2C, the later added sGP was not only proteolysed by the supernatant from neutrophils previously incubated by sGP, but also by supernatants from neutrophils previously incubated with PBS and BSA. In contrast, no proteolysis of sGP was seen with Jurkat cell supernatants obtained under identical incubation conditions. These data demonstrate that the previously reported depletion of sGP by neutrophils was not via specific binding, but through nonspecific proteolysis.

Finally, a direct binding assay was performed on neutrophils using ^{125}I-labeled sGP. The specific radioactivity of labeled sGP was estimated to be 7.5×10^6cpm/μg of protein. The ^{125}I-labeled sGP remained intact after labeling as shown in Fig. 3A. The binding assays were performed by using the indicated different concentrations of ^{125}I-sGP with neutrophils, Jurkat, and 293T cells. As shown in Fig. 3B, the total detectable binding of ^{125}I-sGP for the three types of cells were barely above background levels and were identical among neutrophils, Jurkat cells, and 293T cells.

In conclusion, we find no direct evidence that CD16b is

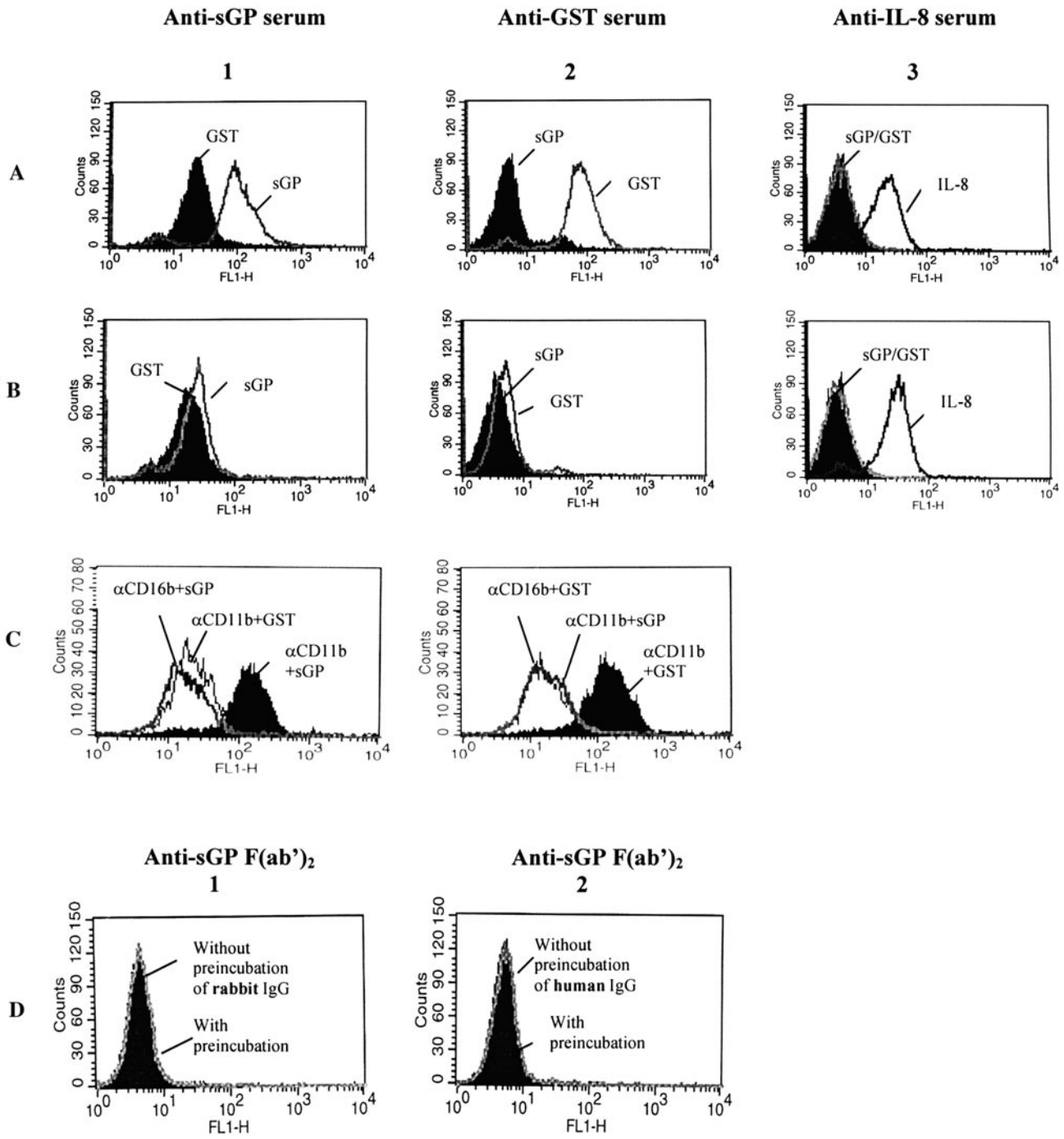

FIG. 1. (A) FACS analysis of purified sGP (A1), GST (A2), and IL-8 (A3) binding to human neutrophils under limited (1×) washing conditions as detected by specific rabbit antiserum. (B) FACS analysis of purified sGP (B1), GST (B2), and IL-8 (B3) binding to human neutrophils after more stringent (2×) washing conditions. (C) Binding of sGP (C1) and GST (C2) to neutrophils, under the conditions of A, are inhibited by CD16b Mab 3G8 but not by CD11b Mab. (D) No binding of sGP is detected by anti-sGP $F(ab')_2$ with the preincubation of neutrophils with nonspecific rabbit IgG (D1) or human IgG (D2). Secondary antibody is FITC-conjugated goat IgG $F(ab')_2$ fragments to rabbit IgG $F(ab')_2$.

a receptor or that other receptor(s) exists for sGP on neutrophils as compared to other protein and cell-type controls and as detected by FACS analysis, an absorption assay, and a direct-binding assay. We demonstrate that the nonspecific *in situ* immune complex formation and nonspecific proteolysis of sGP are responsible for the previously reported findings. In a more recent study sGP was reported to diminish the normal proximity of CD16b to CR3 on neutrophils, and as a consequence, the authors hypothesized that the inhibitory effects of sGP on neutrophil functions may be due to an sGP-mediated disturbance of the FcγRIIIB interactions with CR3 that

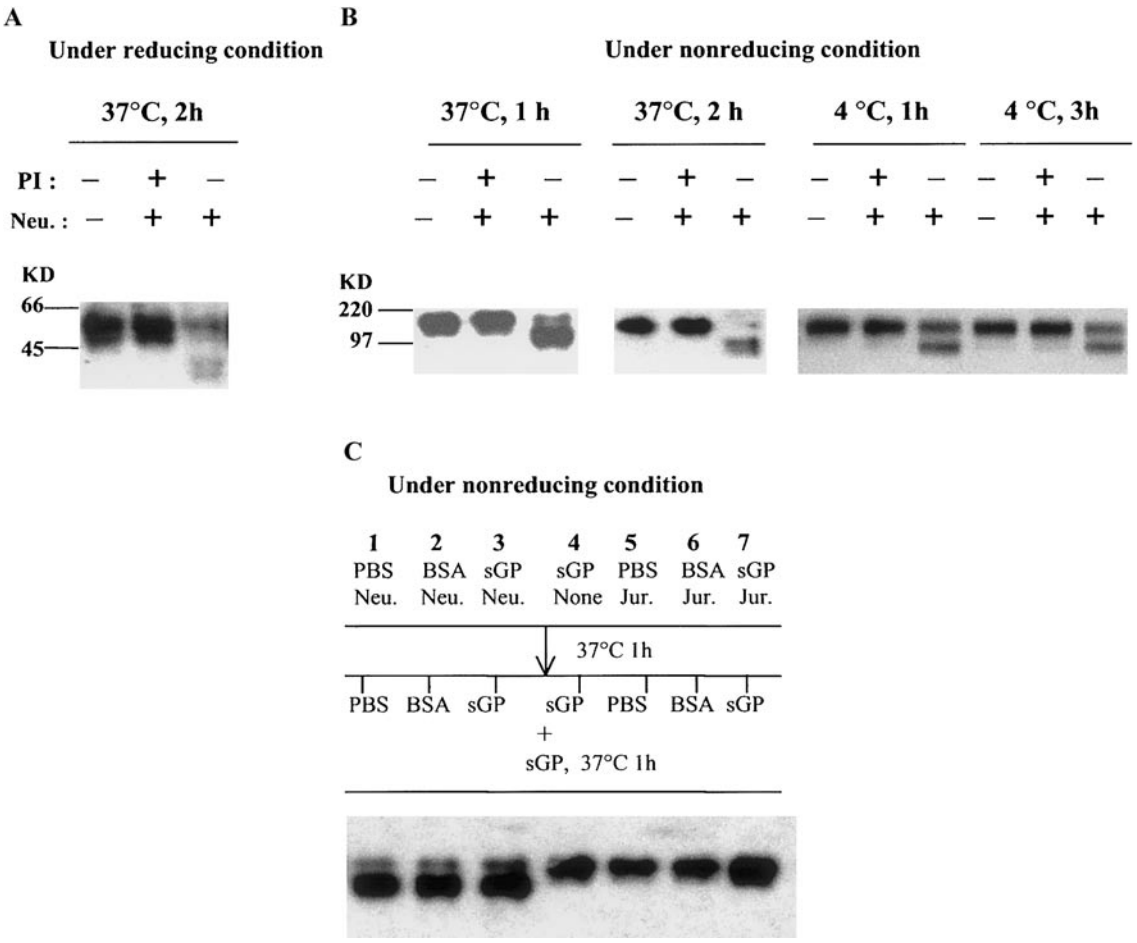

FIG. 2. The proteolysis of sGP results in the artificial absorption of sGP to neutrophils. (A) The "absorption" of sGP is detected under certain conditions, but is not detected in the presence of protease inhibitors. (B) Under some assay conditions and when nonreducing gels are used, the sGP does not appear "absorbed," but rather a smaller molecular weight band is seen. The intact form of sGP remains in the presence of protease inhibitors. (C) The proteases released from neutrophils result in the degradation of sGP and is not specifically induced by sGP. The same degradation occurs in the PBS and BSA incubated neutrophil group. (PI: protease inhibitors; Neu.: neutrophils; none: no cells; Jur.: Jurkat cells).

normally contribute to inflammatory signaling (*15*). However, sGP-containing or control-culture supernatants but not purified sGP were used in this study and unrelated protein controls as used in the present study were not reported. Thus, our findings together with Maruyama *et al.* (*11*) make it unlikely that sGP binds to neutrophils via any specific receptor or that it plays a significant role in Ebola virus pathogenesis through interfering with the innate immunity by targeting neutrophils.

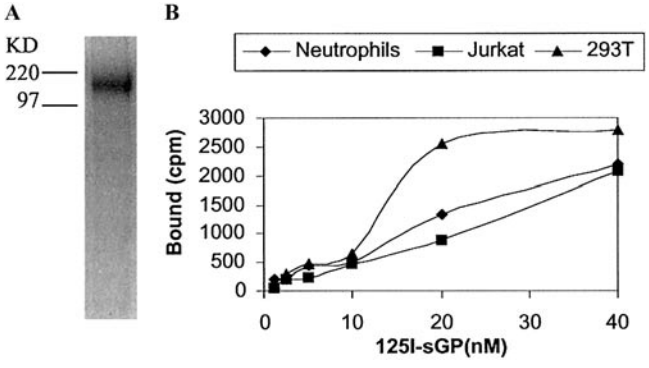

FIG. 3. Lack of binding to neutrophils as detected by ^{125}I-sGP binding assay. (A) ^{125}I-labeled sGP remains intact as shown on 10% SDS–PAGE under nonreducing conditions and visualized by autoradiography. (B) Total counts (cpm) of three cell types are plotted against ^{125}I-labeled sGP concentration (nM).

Abundant release of sGP occurs during Ebola virus infection and no homologues of Ebola sGP have been found to suggest another specific role of sGP in the pathogenesis of the acute hemorrhagic fever. However, a highly virulent Ebola virus variant only secretes minute amounts of sGP, and the apathogenic Reston subtype produces large quantities of sGP (*3*). In addition, Marburg virus, which causes comparable disease symptoms as Ebola virus, does not produce sGP by transcriptional RNA editing (*16*). A recent report demonstrated that sGP is not necessary for replication of Ebola virus *in vitro* cell culture, but may down-regulate GP-related cytotoxicity (*6*). It has also been suggested that sGP may act as a decoy to adsorb neutralizing antibodies because of its

ability to inhibit the neutralizing activity of anti-GP serum, although there is no direct *in vivo* evidence to support this possibility (*17*). Thus, further study of the biological activity of sGP, especially its interaction with other cell types, will be necessary to better understand the elusive role(s) of sGP in Ebola virus infections.

Materials and Methods. Expression and purification of Ebola sGP and GST. The sGP was expressed by transient transfection of 293T (ATCC) cells using expression plasmid pCMV-sGP (*9, 18*). The purified sGP was obtained by FPLC (Superdex-200 column, Amersham Pharmacia Biotech) from serum-free cell-culture supernatant. The GST was expressed from pGEX-2T expression vector in *Escherichia coli* and purified by affinity chromatography using Glutathione Sepharose 4B column (Amersham Pharmacia Biotech). The purity of sGP and GST were above 95% as analyzed by SDS–PAGE. The protein concentration was determined by protein assay reagent (Bio-Rad).

Neutrophil isolation. The neutrophils were isolated from heparinized peripheral blood of healthy adult donors using Ficoll–Paque (Amersham Pharmacia Biotech.) density gradient centrifugation. After separation, all further isolation steps were performed on ice. Erythrocytes were removed from the pellet by hypotonic lysis for 30 s and immediately balanced by adding 10× PBS. The remaining erythrocyte contamination was removed by isotonic lysis (ACK lysis buffer, Biosource International). Cells were washed twice and resuspended in 1× PBS. The purity of the neutrophils was ~95%; cell viability as assessed by trypan blue exclusion was at least 95%.

FACS assay. The immunostaining was performed by incubating 5×10^5 of fresh isolated neutrophils with roughly equal molar concentration of sGP (40 μg/ml), GST (20 μg/ml), and IL-8 (8 μg/ml, Cytoimmune Sciences Inc.), respectively, for 1 h, followed by incubating with relevant primary rabbit anti-sGP, anti-GST, or anti-IL-8 serum (1:200) and secondary antibody FITC-conjugated goat IgG $F(ab')_2$ fragments to rabbit IgG $F(ab')_2$ (1:75, Jackson ImmunoResearch Laboratory Inc.) for half an hour. All incubations were performed at 4°C in a 50 μl volume. All incubating and washing buffers were PBS containing 1% BSA and 0.1% NaN_3. After sGP, GST, or IL-8 incubation, the cells were spun down and washed either once or twice with 1 ml washing buffer. After antibody incubations, cells were spun down and washed twice with 1 ml washing buffer. After staining, cells were resuspended in 300 μl PBS for FACS analysis immediately. For the CD16b antibody blocking assay, the neutrophils were preincubated with 0.1 mg/ml mouse anti-CD16b clone 3G8 (IgG1) or anti-CD11b clone BEAR1 (IgG1) (Beckman Coulter Immunotech) in a 50 μl volume at 4°C for 30 min; cells were washed once and then incubated with the sGP or GST and washed once, and then followed by antibodies incubating and washing as described above. For the binding of sGP to neutrophils as detected by the $F(ab')_2$ fragment, the neutrophils were preincubated with 10 μg/ml nonspecific rabbit or human IgG (Sigma) in 100 μl at 4°C for 30 min; cells were washed once, followed by incubation with the sGP or GST and washed once, and then followed by anti-sGP $F(ab')_2$ fragment, secondary antibody incubation, and washed as described above.

Absorption assay. The absorption assay was performed by incubating 10^6 neutrophils with sGP (about 2–10 ng) in the absence or presence of 1× protease inhibitors (Roche) in a final volume of 50 μl at 37 or 4°C for 1–3 h. After incubation, cells were removed thoroughly by centrifuging and the resulting supernatants were resolved on 10% SDS–PAGE under reducing or nonreducing condition and blotted onto a NC membrane. Detection of the sGP was performed with rabbit anti-sGP serum and HPR-labeled goat anti-rabbit secondary antibody. The immune complexes were detected by chemiluminescence with SuperSignal chemiluminescent substrate reagents (Pierce). For further examining of whether the proteases released from neutrophils were specifically induced by sGP, the neutrophils and Jurkat cells (T cell leukemia) (ATCC, clone E6-1) were preincubated with PBS, BSA (Pierce, protease-free), and sGP, respectively, at 37°C for 1 h in 50 μl final volume; then the cells were removed completely and 40 μl supernatants were taken for a further 1 h incubation with 40 μl of fresh sGP at 37°C. Then they were analyzed by immunoblotting.

^{125}I-sGP-binding assay. To radiolabel sGP, 10 μg of pure sGP in 40 μl 0.1 M sodium borate buffer, pH 8.5, was added to 250 μCi [^{125}I]–Bolton-Hunter Reagent (New England Nuclear), incubated on ice for 2 h. Radiolabeled sGP preparations were purified by gel filtration on Pharmacia PD10 Sephadex G25 columns in 0.1 M phosphate-buffered saline. BSA was added to the purified ^{125}I-sGP to a final concentration of 1%. The ^{125}I-sGP were resolved on 10% SDS–PAGE under nonreducing conditions and visualized by autoradiography. The binding assays were carried out by incubating 2×10^6 neutrophils, Jurkat, or 293T cells with different concentrations of ^{125}I-sGP (1.25–40 nM) in 100 μl binding buffer (PBS containing 1% BSA) at 4°C for 2 h. Cells were washed twice with binding buffer; supernatants were removed, and cell sediments were counted by gamma counter.

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