Ebola Virus, Neutrophils, and Antibody Specificity

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Zhi-yong Yang *et al*. (*1*) report that the secreted form of the Ebola virus glycoprotein (sGP) specifically interacts with neutrophils through the immunoglobulin G (IgG) Fc receptor IIIb (FcγRIIIb, CD16), which is exclusively expressed on neutrophils. Yang *et al*. conclude that sGP "inhibits early neutrophil activation, which likely affects the host response to [Ebola] infection." The study raised considerable interest because it provided at least a partial explanation for the high pathogenicity of Ebola virus (*2, 3*). The study, however, did not exclude the possibility that the rabbit antibody used to detect the binding of Ebola sGP mediated the binding of rabbit IgG-sGP immune complexes to neutrophil FcγRIIIb through its Fc portion. To clarify this issue, we performed flow cytometry to study the putative interaction of sGP with neutrophils with the use of three human monoclonal antibody Fab fragments specific for sGP (Fab KS14, K518, and LS4) (*4, 5*) and rabbit IgG $F(ab')_2$ fragments to sGP (*6*).

First, we confirmed that we could detect the binding of sGP-rabbit antiserum immune complexes with neutrophils in flow cytometry with the use of a fluorescein isothiocyanate (FITC)–labeled goat antibody to rabbit IgG, as previously shown by Yang *et al*. (*7*). In contrast with this result, however, we could not detect binding of sGP to neutrophils with the use of three human monoclonal Fab fragments under similar conditions (Fab LS4 shown in Fig. 1A; Fab KS14 and K518 yielded similar results). It is unlikely that this result is due to overlapping binding sites of the Fab fragments and the putative receptor binding site on sGP, because strong binding of all three human Fabs to neutrophil-bound sGP was found in the presence of the rabbit antibody to sGP (Fig. 1B). These data suggest that the binding of Ebola sGP to neutrophils may be mediated by the rabbit IgG used to detect the sGP, rather than by a specific sGP-neutrophil interaction.

To investigate whether the rabbit antibody–dependent binding of Ebola sGP was mediated by the Fc moiety of the rabbit IgG, we prepared $F(ab')_2$ fragments. Rabbit $F(ab')_2$ retained the same reactivity against sGP as did the whole rabbit IgG to sGP, as measured by enzyme-linked immunosorbent assay (ELISA) (Fig. 2). The rabbit IgG tested alone gave a weak signal in flow cytometry, which was increased significantly in the presence of sGP (Fig. 1C). In contrast, rabbit IgG $F(ab')_2$ fragments to sGP did not bind to neutrophils either in the absence or presence of sGP (Fig. 1D).

Finally, in contrast, to Yang *et al*., we did not detect any absorption of sGP by purified neutrophils (Fig. 3), which makes it difficult to predict the presence of any low affinity receptor for sGP on neutrophils.

Thus, we conclude that Ebola sGP does not bind FcγRIIIb (CD16) or any other receptor on neutrophils and that the rabbit IgG against sGP used for detection (*1*) bound to FcγRIIIb through its Fc moiety as an immune complex with sGP.

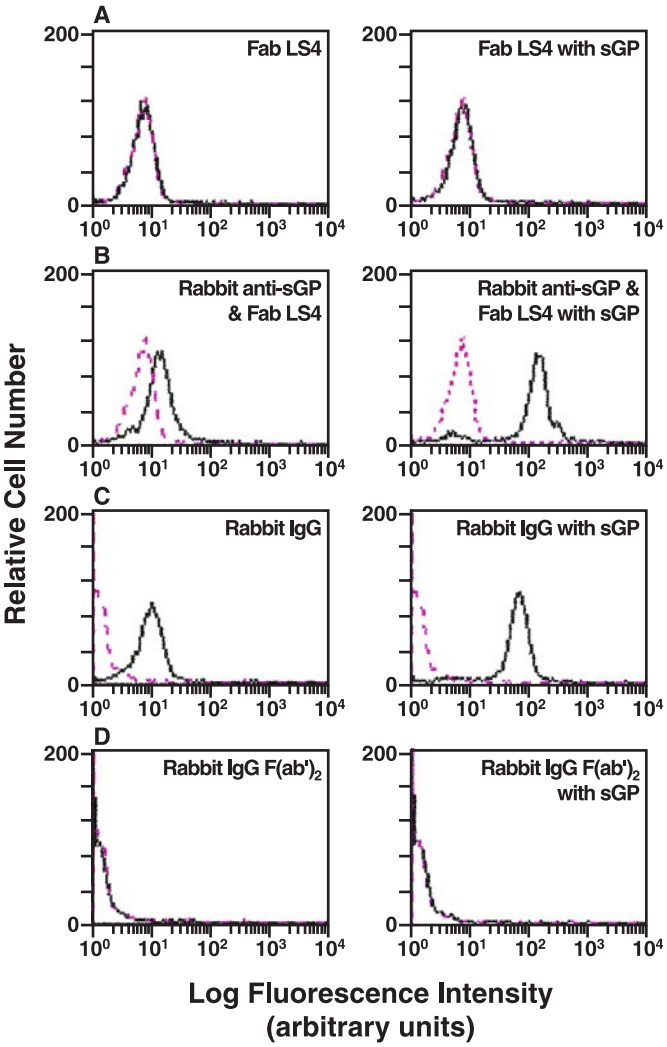
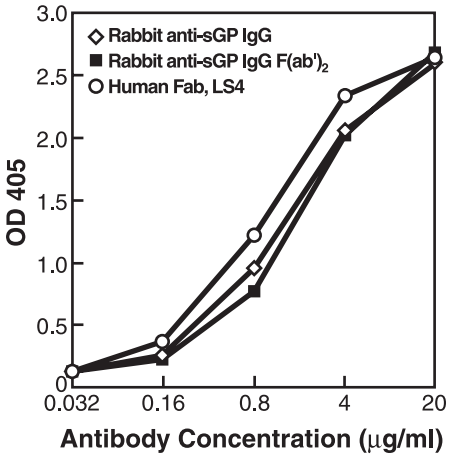


Fig. 1. Flow cytometry assays. Incubation with primary antibody, black solid lines; control FITC-conjugated antibodies alone, red dotted lines. (**A**) Binding of sGP and Fab fragment (LS4) to neutrophils. No binding was detected when Fab fragments (LS4, KS14, and K518) (20 μg/mL) were premixed with sGP and incubated with neutrophils (left panel: without sGP, right panel: with sGP). Detection was performed with fluorescein isothiocyanate (FITC)–conjugated goat IgG $F(ab')_2$ fragments to human IgG $F(ab')_2$ (1:100) (Pierce, Rockford, Illinois) (data of KS14 and K518, not shown). (**B**) Binding of Fab fragment LS4 and sGP to neutrophils in the presence of rabbit anti-sGP serum. Fab LS4 (20 μg/ml) and rabbit anti-sGP serum (1:250) were mixed in the absence (left panel) or the presence (right panel) of sGP and incubated with neutrophils (1.25×10^5 cells). Binding of Fab LS4 to neutrophils was detected only in the presence of both sGP and rabbit anti-sGP serum. Detection was performed with FITC-conjugated goat IgG $F(ab')_2$ fragments to human IgG $F(ab')_2$. (**C**) Binding of sGP (1:10) and purified rabbit IgG against sGP (5 μg/ml) to neutrophils without (left panel) and with (right panel) sGP. Detection was done by FITC-conjugated goat IgG $F(ab')_2$ fragments to rabbit IgG $F(ab')_2$ (1:100) (Jackson, West Grove, Pennsylvania). (**D**) Binding of sGP and rabbit IgG $F(ab')_2$ fragments against sGP (5 μg/mL) to neutrophils. No binding of rabbit IgG $F(ab')_2$ fragments against sGP to neutrophils was observed in the absence (left panel) or presence (right panel) of sGP. Detection was done with the use of FITC-conjugated goat IgG $F(ab')_2$ fragments to rabbit IgG $F(ab')_2$. Incubations were performed in a 50-μL volume at 4°C for 60 min with sGP and the primary antibody, followed by a 20 min incubation with the FITC-conjugated antibodies. Dilution of sGP, antibodies, serum, and neutrophils and the washing of cells were done with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) and 0.01% NaN_3.

Fig. 2. Binding of antibodies and antibody fragments to sGP by ELISA. Wells were coated with sGP (1:10 in PBS) at 4°C overnight. After washing 5× with PBS/0.05% Tween 20, the wells were blocked with 4% (w/v) nonfat dry milk (Bio-Rad, Hercules, California)/PBS. Rabbit IgG and IgG $F(ab')_2$ fragments to sGP were serially diluted and added to the wells. Detection was done with alkaline phosphatase-conjugated goat IgG to rabbit IgG $F(ab')_2$ (1:500) (Pierce, Rockford, Illinois). Human Fab LS4 was included as a control and detected by alkaline phosphatase-conjugated goat IgG to human IgG $F(ab')_2$ (1:500) (Pierce). Incubations were performed at 37°C for 60 min. Rabbit IgG and IgG $F(ab')_2$ fragments showed similar binding reactivity to sGP.



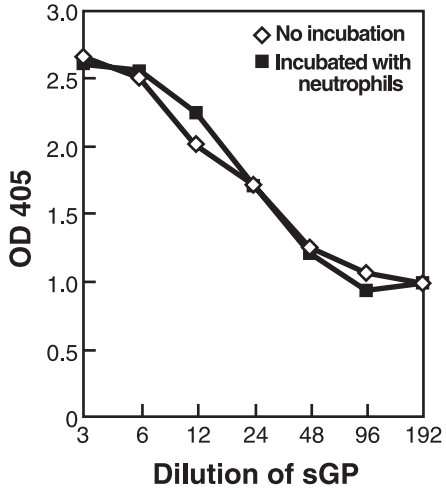


Fig. 3. Absorption test of sGP with purified neutrophils. sGP (1:10) was incubated with purified neutrophils (1×10^7) at 4°C for 60 min in a 50-μl volume, after which the cells were removed by centrifugation. Supernatant containing unabsorbed sGP was serially diluted in 1% FCS/PBS/0.01% NaN3 and transferred to ELISA wells coated with rabbit IgG against sGP (4 μg/ml). Plate was washed and incubated with Fab LS4 (4 μg/ml). Bound Fab was detected with alkaline phosphatase-conjugated goat IgG to human IgG $F(ab')_2$ (1:500) (Pierce, Rockford, Illinois). sGP not incubated with neutrophils was also serially diluted and detected in the same ELISA. ELISA incubations were performed at 37°C for 60 min, and wells were washed 5× with 0.05% Tween 20–PBS at each step. No absorption of sGP by neutrophils was as observed compared with the control wells (no incubation with neutrophils).

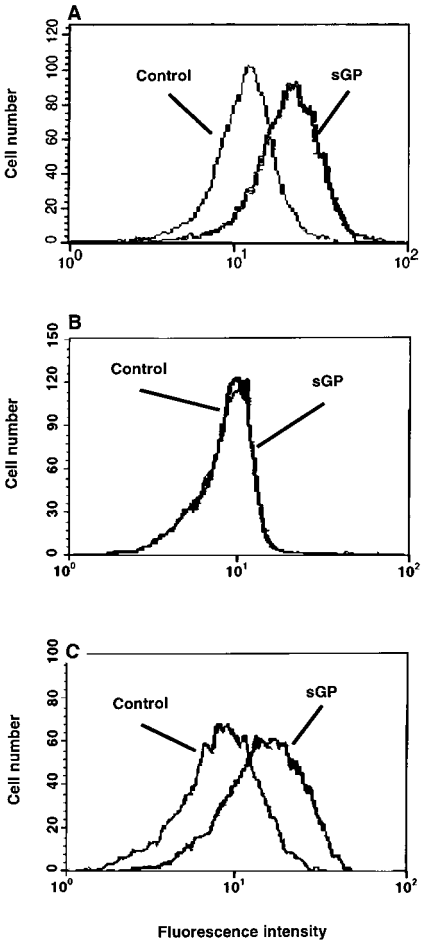


Fig. 1. Potentiation by IgG of sGP binding to human neutrophils detected by $F(ab')_2$ reagent. (**A**) Freshly isolated human neutrophils from healthy donors (2×10^6) were incubated with sGP or control supernatants for 30 min on ice. Cells were spun and washed once with 1 ml of ice-cold PBS. One hundred microliters of diluted antiserum to GP/sGP (1:1000) was added and incubated on ice for 30 min. Cells were spun again and washed with 1 ml of PBS. Finally, 100 μl of 1:75 diluted FITC-conjugated affiniPure $(Fab')_2$ fragment goat anti-rabbit IgG-$(Fab')_2$ (Jackson, West Grove, Pennsylvania) was added and incubated on ice for 30 min. Cells were washed once with PBS and resuspended in PBS + 1% formaldehyde for fluorescence-activated cell sorter (FACS) analysis. (**B**) Cells were incubated with sGP and control supernatants as in (A), and followed by an incubation of 1:100 diluted purified IgG $(Fab')_2$ fragment rabbit antibody to GP/sGP (0.4 mg/ml). Finally, the cells were incubated with FITC $(Fab')_2$ fragment goat antibody to rabbit IgG$(Fab')_2$ for 30 min on ice. Cells were washed once with 1 ml of ice-cold PBS after each step and resuspended in PBS + 1% formaldehyde for FACS analysis. (**C**) Cells were pre-incubated with 1 μg of rabbit IgG (Sigma, St. Louis, Missouri) in 100 μl on ice for 30 min, followed by the incubations described in (B). Cells were washed once with 1 ml of ice-cold PBS after each step.

References and Notes

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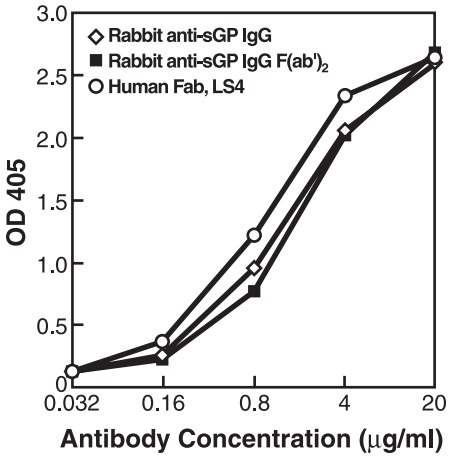

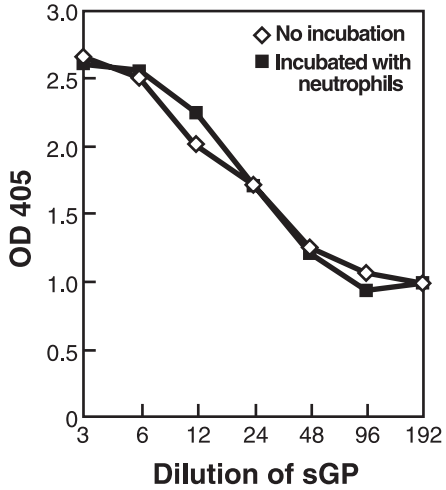

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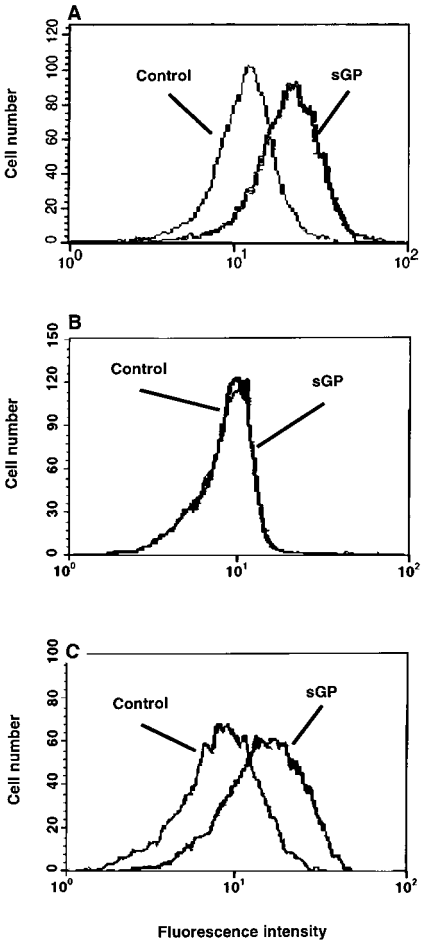

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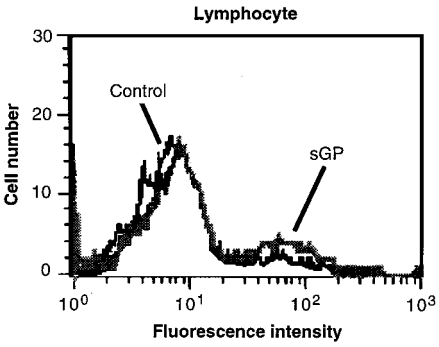

Fig. 2. Correct image, showing a FACS profile from lymphocytes, that should have appeared in our report (*1*, p. 1034) as the far-left graph in figure 1A.

trophil. In this case, FcR occupancy would be necessary for sGP binding to be detected by the antibody to sGP $F(ab')_2$ fragment and is consistent with the result of Maruyama *et al*. A third potential explanation, suggested by a reviewer, is that engagement of FcγIII by immunoglobulin may trigger neutrophils to express another activation antigen that binds sGP. This model is also consistent with the available data. Because neutrophils undergo variable activation during isolation in vitro, this alternative could also account for differences observed in sGP depletion by cellular absorption.

In summary, the observation of Maruyama *et al*. provides further insight and suggests alternative mechanisms of sGP interaction with neutrophils. We had previously reported that CD16 alone was not sufficient for binding to sGP. Together, the data suggest that sGP binding to neutrophils is dependent on CD16, although additional studies will be required to determine whether CD16 is directly or indirectly responsible for the interaction. Regardless of the details of the interaction of sGP with CD16 and binding to neutrophils, our observation (*1*) that the secreted and transmembrane forms of the Ebola glycoproteins have distinct cellular specificities remains unchanged. It also remains important to determine whether sGP can be detected on neutrophils from infected patients before its relevance to disease progression and pathogenesis can be fully understood.

We would like to take this opportunity to correct an error in our report. A FACS profile from the monocyte cell population was inadvertently scanned into the far-left graph in figure 1A in our report (*1*, p. 1034). The correct figure part is shown here (as Fig. 2). This change does not alter the conclusion of the report in any way. We would like to thank Brett Lindenbach and the Virology Journal Club at the Washington University School of Medicine for bringing this error to our attention and also apologize for any confusion this error may have caused.

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