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Research paper

Ebola virus secreted glycoprotein decreases the anti-viral immunity of macrophages in early inflammatory responses

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ABSTRACT

During Ebola virus (EBOV) infection, secreted glycoprotein (sGP) is found in large quantities in the serum of both patients and infected animal models. It is thought to serve as a decoy for anti-EBOV antibodies. Using an *in vitro* model incorporating treatment of non-infected human THP-1 macrophages with recombinant EBOV sGP, this study sought to examine the impact of sGP upon key macrophage functions. Macrophage polarization and phagocytic capacity of activated macrophages were found to be unaltered by sGP treatment. However, treatment with sGP inhibited macrophage production of the pro-inflammatory cytokines TNFα and IL-6 while the yield of anti-inflammatory cytokine, IL-10, remained intact. Interestingly, the migratory ability of macrophages was also diminished by sGP, potentially due to a decrease in expression of CD11b, a vital macrophage integrin. Thus, EBOV sGP may operate to diminish functional contributions of non-infected macrophages to increase the potential viral dissemination.

1. Introduction

The recent devastating outbreaks of *Zaire ebolavirus* (EBOV) in western Africa have shown the inadequacies of the world's healthcare system. Even though the first recorded EBOV outbreak was in 1976, much of the pathogenesis of the virion remains unclear [1]. While much effort has focused on the action of viral proteins produced within infected immune (macrophages and dendritic cells) and tissue cells, much is left to be discovered with regard to interactions of soluble Ebola virus proteins and infiltrating non-infected immune cells such as macrophages.

Within the order *Mononegavirales,* family *Filoviridae*, and genus *Ebolavirus*, there are currently five species of virus. The species include Zaire, Sudan, Reston, Bundibugyo, and Taï Forest. All species can infect and cause disease in humans, with the exception of the Reston virus. The Zaire strain is the most common during known outbreaks, and one of the most studied in laboratory settings [2,3].

The *Ebolavirus* virion is an enveloped virion with negative, single-stranded, RNA genome. The genome encodes for eight different proteins, including the envelope spike glycoprotein (GP). The glycoprotein consists of two subunits: G_1 and G_2 [4]. The G_1 subunit has a mucin-like domain, which is highly glycosylated and is usually implicated in attachment to host cells [5].

The glycoprotein has a transmembrane portion on the C-terminal end, which can be cleaved by tumor necrosis factor-alpha converting enzyme (TACE) [6]. This cleavage creates the shed glycoprotein and the delta peptide portion. The shed glycoprotein has been the subject of several studies; however, the biological function of the shed glycoprotein has not been completely defined [6–9]. There is some suggestion that it sequesters antibodies against the spike glycoprotein, and helps with virion immune evasion [6], or that it could cause the production of pro-inflammatory cytokines [7].

The glycoprotein can also be cleaved at the TACE cleavage site while it remains intracellular, and because this protein lacks a transmembrane domain and the delta peptide, it is secreted through the normal secretory pathway [10–13]. This protein is known as the secreted glycoprotein (sGP) [4,9,14,15]. The soluble forms of GP (both shed and secreted) have been measured at very high levels within patient blood serum [6,7,9].

Macrophages are critically important sentinel cells that detect and

Abbreviations: GP, glycoprotein; sGP, secreted glycoprotein; TACE, tumor necrosis factor-alpha converting enzyme; TLR, Toll-like receptor; VLPs, virus-like particles; LPS, lipopolysaccharide; PBS, phosphate buffered saline; CD, cluster of differentiation; IL, interleukin; TNF, tumor necrosis factor; OVA, ovalbumin; APC, allophycocyanin; FITC, fluorescein isothiocyanate; PMA, phorbol 12-myristate 13-acetate; EBOV, *Zaire ebolavirus*

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respond to threats within the body. Depending upon the type of challenge, they can acquire relative phenotypes that better equip them to more efficiently respond to threats. Macrophages that are facing a bacterial or viral infection can assume the classical or M1-like phenotype, associated with high pro-inflammatory (IL-6, TNFα, IL-12, etc.) cytokine production, and high phagocytic capabilities. Macrophages fighting a helminth infection can assume the M2-like phenotype, which is associated with higher production of anti-inflammatory cytokines (IL-4, IL-10, etc.), effector molecules (Arg1, FIZZ1, YM1, etc.), and cell markers (CD206, Egr2, etc.).

Macrophages and dendritic cells (DCs) are early targets in EBOV pathogenesis. Once infected, these cells gain access to the blood and disseminate the virus systemically into tissues and blood vasculature [10–12,16]. Viral invasion of endothelia and production of pro-inflammatory cytokines by the circulating infected macrophages and DCs lead to the development of viral hemorrhagic fever that is characteristic of EBOV infection [13].

Here we report that macrophages, and not monocytes, have reduced pro-inflammatory cytokine production when incubated with EBOV sGP. The inhibitory effects of sGP are insufficient to counter concomitant stimulation with lipopolysaccharide (LPS). Furthermore, sGP has no effect upon the capacity of activated macrophages to phagocytose antigen. However, chemotaxis of activated macrophages is significantly diminished by sGP and likely is associated with reduced levels of CD11b integrin. Overall, EBOV sGP may have a major role in the pathogenesis of EBOV through impairment of activated macrophages.

2. Materials and methods

2.1. Cell culture

Human THP-1 monocyte cells (ATCC) were cultured in complete media containing RPMI 1640 (GE Healthcare) supplemented with 10% FBS that had been heat-inactivated for 30 min at 57 °C, 0.05 mM 2-mercaptoethanol and gentamycin. Cells were maintained in an incubator at 37 °C in 5% CO_2-humidified atmosphere.

2.2. Macrophage differentiation and polarization

THP-1 monocyte cells were differentiated into M_0 macrophages over a 3-day incubation with 200 nM phorbol 12-myristate 13-acetate (PMA) stimulation followed by a 5-day rest [17]. The M0 macrophages were polarized into the M1 phenotype with 20 ng/mL IFNγ for 24 h, or the M2 phenotype with 20 ng/mL of IL-4 and 20 ng/mL of IL-13 for 24 h [18]. M1 and M2 phenotypes were confirmed using flow cytometry assessment of characteristic cellular markers (data not shown).

2.3. THP-1 cell activation and incubation with EBOV sGP

THP-1 monocytes or M0, M1, or M2 macrophages were treated with 1 μg/ml LPS (Sigma) or PBS for 24 h, washed and followed by incubation with low (4 μg/ml) or high (8 μg/ml) concentrations of sGP, recombinant EBOV sGP lacking the transmembrane domain (IBT Bioservices) [19], or PBS over 24 h. EBOV sGP used in this study is created in mammalian cells, is fully glycosylated, and has no inhibition on the formation of dimers or trimers that could occur.

Alternatively, to test sGP impact prior to stimulation of M1 and M2 macrophages, sGP (4 μg/ml) was added with cytokines during polarization over 24 h, cultures were washed, and then activated for an additional 24 h with LPS (1 μg/ml). Assessment of the effect of sGP upon M1 and M2 macrophages at the time of activation involved stimulation of both populations with LPS (1 μg/ml), sGP (4 μg/ml), LPS + sGP or PBS for 24 h. At the completion of the assay (48 h), cell supernatants were harvested and tested by ELISA.

2.4. Assessment of cytokine production

The ELISA assays were performed utilizing the Ready-Set-Go ELISA kits (ThermoFisher Scientific) for IL-6, TNFα and IL-10 following the manufacturer's instructions. All ELISA procedures were read on a BioTek Eon microplate spectrophotometer (BioTek Instruments Inc.) and analyzed using Gen 5 software version 2.01.14.

2.5. Flow cytometry

Following incubation of polarized macrophages with LPS, sGP, PBS or combinations of these as indicated, adherent cells were collected from culture plates via trypsinization with 0.25% trypsin-EDTA. Staining was performed using standard procedures and fixed with 10% formalin. Human-specific CD11b-APC (eBioscience) was utilized. Flow cytometry was performed using a Millipore Guava Cytometer (Millipore) and analyzed with GuavaSoft 2.6 software.

2.6. Phagocytosis assay

The ability to phagocytose foreign particles was measured utilizing a FITC labeled-chicken ovalbumin peptide (OVA-FITC, Fisher) [20]. Polarized macrophages were treated with LPS (1 μg/mL) or PBS for 24 h, washed, then treated with sGP (4 μg/mL) or PBS for an additional 24 h. The cells were washed, and OVA-FITC (0.25 mg/mL), complete unconjugated ovalbumin protein (1 mg/mL, Invivogen), or PBS was added. At 30 min, 1 h, and 3 h incubation time, the adherent cells were collected by treatment with 0.25% trypsin/EDTA. The cells were washed three times in cold PBS to remove any bound, but not phagocytosed molecules. Cells were fixed with 10% formalin and analyzed by flow cytometry. The cells were gated to exclude debris.

2.7. Chemotaxis assay

Chemotaxis was assayed as previously described [21]. In brief, Corning Transwell Plates (Sigma) with 8 μm pore size were used. M1 macrophages were treated with LPS, sGP, or PBS as indicated, trypsinized, and counted. M1 macrophages (3×10^5) were placed into the apical chamber in complete media. MCP-1, or CCL-2, (10 ng/mL, Peprotech) was added into the basal chamber of some wells, and PBS to the others. The cells were incubated for 24 h. Remaining macrophages in the wells were trypsinized, and counted using a hemocytometer (Becton, Dickinson, and Company), and measured via flow cytometry. The cells were gated to exclude debris, and cells that migrated across the membrane as events per 30 s. The flow data was represented as percent increase in chemotaxis and calculated as: 100% – [(number of cells that migrated to the basal chamber without chemokine/number of cells that migrated to the basal chamber in the presence of chemokine) × 100%].

2.8. Statistical analysis

Means and standard deviations were expressed in the data and assessed for statistical significance using the unpaired Student's *t*-test. When comparing two groups, a *p*-value of $\leq .05$ was considered significant, unless otherwise noted. Experiments were performed in duplicates and were performed a minimum of three times to observe consistent trends.

3. Results

3.1. EBOV sGP differentially inhibits production of IL-6 by macrophages but not monocytes

Certainly EBOV initiates infection by invading tissue macrophages and dendritic cells, ultimately resulting in systemic dissemination from

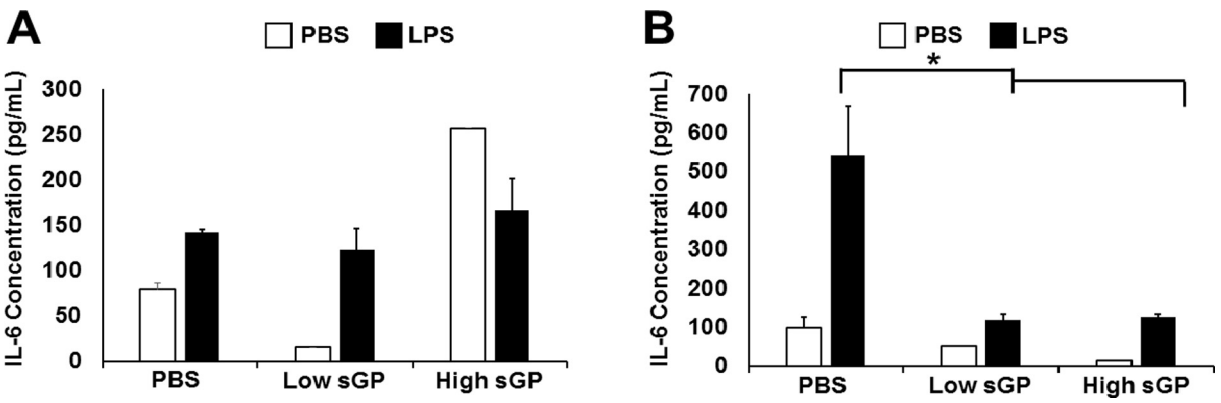

Fig. 1. Macrophage production of IL-6 in response to LPS is inhibited by the presence of EBOV sGP. (A) THP-1 monocytes were treated with LPS (1 μg/mL) or PBS for 24 h, then washed, and challenged with Low sGP (4 μg/mL), High sGP (8 μg/mL), or PBS for 24 h. (B) THP-1 monocytes were differentiated into macrophages with 200 nM PMA stimulation for 3 days followed by a 5-day rest. Then, the macrophages were treated with LPS (1 μg/mL) or PBS for 24 h, then washed and challenged with Low sGP (4 μg/mL), High sGP (8 μg/mL), or PBS for 24 h. The production of IL-6 was measured via ELISA. (n = 3, $^{*}P < .05$).

the entry point. Interestingly, the virus directs the synthesis and secretion of the modified glycoprotein (sGP) from these infected cells into the surrounding tissues and blood. The purpose of this maneuver may be to deter immune reactivity which could slow the replication and dissemination process rather than simply act as an anti-EBOV antibody sink. This is more likely the case during the early phases of disease prior to antibody synthesis. Given the importance of monocytes and macrophages in the early innate response to viral invaders, first we sought to examine the effect of EBOV sGP upon the activation of non-infected monocytes and macrophages. In order to test this *in vitro*, the human monocytic cell line, THP-1, which can be differentiated into macrophages by treatment with 200 nM PMA for three days, followed by a five-day rest, was used as a source of cells [17]. The THP-1 monocytes and macrophages were challenged with LPS for 24 h as a way to activate the cells and trigger cytokine production. Subsequently, the cells were washed with PBS and treated with either a low (4 μg/ml) or high (8 μg/ml) dose of sGP for 24 h. IL-6 production by monocytes was not statistically affected by either dosage of sGP (Fig. 1A). However, the production of IL-6 by macrophages was significantly inhibited in the presence of sGP (Fig. 1B). The low and high doses of sGP seemed to affect the activated macrophages equally; therefore, we chose to use the low dose throughout the remainder of the study. The results suggest that sGP may have a direct inhibitory effect on non-infected macrophages.

3.2. Polarization of macrophages is unaltered by EBOV sGP

THP-1 differentiated macrophages (M0) can be polarized into the M1 phenotype with the treatment of IFNγ. To test whether sGP will inhibit the polarization of M1 macrophages, M0 macrophages were incubated with sGP and IFNγ simultaneously for 24 h and then washed, and stimulated with LPS to induce cytokine production. When EBOV sGP was present during the polarization of M1 macrophages, there was no significant effect on the cytokine profile that is characteristic for M1 macrophages (Fig. 2A). The production of the pro-inflammatory cytokines, TNFα and IL-6, should be high for M1 macrophages as compared to resting M0 or M2 macrophages, and there was no significant change in these cytokine levels when macrophages were polarized with IFNγ in the presence of sGP. Typically, M1 macrophages produce low levels of the anti-inflammatory cytokine, IL-10, upon stimulation with LPS and the level of the cytokine was not affected by the presence of sGP during M1 polarization (Fig. 2A). Interestingly, the basal level of IL-10 production by the M1 macrophages was significantly greater than that of LPS-stimulated cells. This is likely due to the ability of LPS to promote M1 macrophage functions thereby resulting in a lower IL-10 production by these stimulated cells [22]. Of note, basal production of IL-10 by sGP-cultured M1 macrophages was decreased relative to LPS-stimulated cells. Possibly sGP ligation to an unknown receptor on the macrophages resulted in activation of an adaptor protein which operated to reduce basal release of IL-10. However, activation of sGP-treated M1 macrophages resulted in restoration of IL-10 levels to that of LPS-stimulated cells.

To test whether sGP had any effect upon the polarization of M2 macrophages, THP-1 macrophages were treated with IL-4, IL-13, and sGP for 24 h, washed, and subsequently activated by LPS an additional 24 h. M2 macrophages produce low levels of pro-inflammatory cytokines, and high levels of IL-10 as compared to M1 macrophages (Fig. 2B). When sGP was given at the same time as the polarizing cytokines, the production of TNFα, IL-6, and IL-10 by M2 macrophages was unchanged (Fig. 2B). In addition, the significant inhibition of basal IL-10 noted in sGP-treated M1 macrophages was not evident of M2 macrophages when given with M2-polarizing cytokines. Overall, the findings indicate sGP does not disturb the process of macrophage polarization but may have a role in the function of polarized macrophages.

3.3. Pro-inflammatory cytokine production by activated macrophages is selectively inhibited by EBOV sGP

When considering whether sGP can impact non-infected macrophage function, sGP, a soluble viral protein, would likely encounter macrophages within the body that were either previously activated or undergoing activation. To test the hypothesis that sGP inhibits functions of polarized non-infected macrophages that were previously activated by microbial stimuli (LPS), THP-1 macrophages were cultured with IFNγ for 24 h followed by LPS or PBS for 24 h. Subsequently, the cells were washed with PBS and treated with sGP or PBS for an additional 24 h. Therefore, total incubation time from polarization to activation and supernatant harvest is 72 h. Strikingly, sGP was able to inhibit the production of the pro-inflammatory cytokines, TNFα and IL-6, while not affecting the levels of the anti-inflammatory cytokine, IL-10 in both M1 and M2 macrophages (Fig. 3). While sGP alone did not provoke cytokine production by the macrophages, perhaps the treatment of polarized macrophages with LPS and then sGP is reducing cellular viability. In order to determine if the experimental approach is promoting cell death of the cells, M1 macrophages were cultured as above and then assessed for expression of 7-AAD (7-amino-actinomycin D), a cell viability stain, by flow cytometry. Viability greater than 90% was observed in all the groups (LPS, sGP and LPS → sGP) tested (Supplementary Fig. 1).

Therefore, sGP is able to inhibit the production of pro-inflammatory cytokines by previously activated polarized macrophages. Can sGP

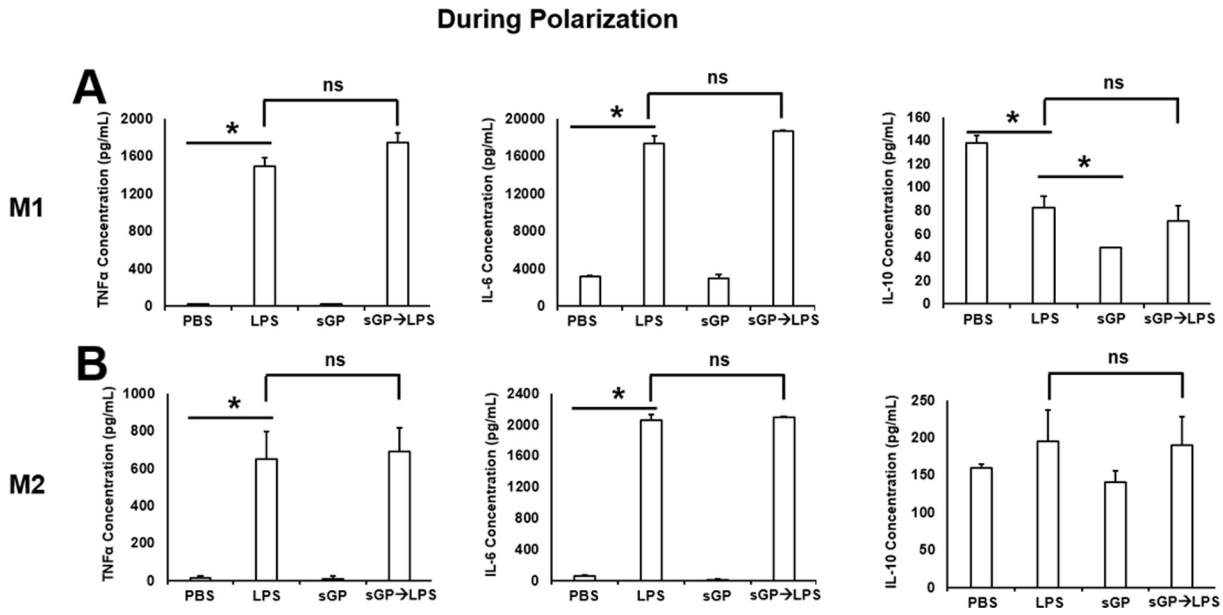

Fig. 2. The polarization of macrophages into the M1 or M2 phenotype is not affected by EBOV sGP. THP-1 monocytes were differentiated into macrophages. For the PBS, LPS, and sGP groups, macrophages were treated for 24 h with (A) IFNγ (20 ng/mL) or (B) IL-4 (20 ng/mL) and IL-13 (20 ng/mL). Subsequently, the macrophages were then stimulated with PBS, LPS (1 μg/mL) or sGP (4 μg/mL) for an additional 24 h. In the sGP → LPS group, IFNγ (20 ng/mL) or IL-4 (20 ng/mL) and IL-13 (20 ng/mL), and sGP (4 μg/mL) were given for 24 h, then the cells were washed and LPS (1 μg/mL) was added for 24 h. Supernatants from the cultures were harvested after a total of 48 incubation hours and the production of TNFα, IL-6, and IL-10 was measured via ELISA. (n = 3, $P < .05$, ns indicates not significant).

restrain polarized macrophages from producing cytokines when given simultaneously with the LPS stimuli? To test this possibility, M1 and M2 macrophages were cultured with both LPS and sGP for 24 h. Interestingly, there was no significant difference between the LPS + sGP and LPS only treated M1 or M2 macrophages in terms of IL-6 and IL-10 production (Fig. 3C and D).

These results suggest that EBOV sGP selectively inhibits the production of pro-inflammatory cytokines, but not anti-inflammatory cytokines from activated M1 and M2 macrophages. Furthermore, this inhibitory effect could be superseded by the stimulatory effect of LPS.

3.4. Phagocytosis is unaffected by the presence of EBOV sGP

One of the most important effector functions of macrophages is the capacity to phagocytose microbial pathogens [23]. Macrophages can recognize self- and non-self-particles and phagocytose them for destruction or antigen presentation. Given that EBOV sGP was able to interfere with pro-inflammatory cytokine production by previously activated macrophages (Fig. 3A and B); it may be acting to suppress the early immune response in general. As professional phagocytes, macrophages aid in the early response to control or clear the body of microbial invaders. Prior to assessment of sGP for the ability to impair phagocytic activity, M1 macrophages were treated with PBS, unconjugated, complete OVA protein (1 mg/mL), and OVA-FITC (0.25 mg/mL), and incubated for 30 min, 1 h, or 3 h to determine an optimum incubation time for the assay. After trypsinization, the cells were washed 3 times with cold PBS to stop antigen uptake and remove particles that only bound to the cellular surface, and assessed for antigen accumulation within the cells. As shown in Fig. 4A, over a 3-h period, the intensity of OVA accumulation increased and the 3-h culture time was determined as the optimal incubation time.

To test the capacity EBOV sGP to modulate the phagocytic capabilities of M1 macrophages, the cells were activated with either LPS or PBS for 24 h, washed, then treated with either sGP or PBS for an additional 24 h (Fig. 4B). The percentage of cells that phagocytosed the OVA-FITC particle were statistically unremarkable between the LPS only group and the group activated with LPS and treated with sGP (Fig. 4C), suggesting that the phagocytic ability of M1 macrophages, which is pivotal to macrophage functioning, is not significantly affected by the presence of EBOV sGP.

3.5. Chemotaxis to MCP-1 is inhibited by EBOV sGP

Interestingly, if phagocytic activity of macrophages is not altered by EBOV sGP but pro-inflammatory cytokine production is inhibited, the viral protein may be operating to promote EBOV survival and dissemination through the impairment of inflammation-related events associated with the migration and cytokine production by non-infected macrophages. Therefore, we tested the chemotactic ability of activated M1 macrophages after treatment with sGP. Previously activated M1 macrophages were incubated with sGP for 24 h, washed, and then placed in the apical chamber of a chemotaxis assay. The chemokine, monocyte chemoattractant protein-1 (MCP-1), or PBS was added to the basal chamber in complete media. The macrophages were allowed to migrate to the basal chamber for 24 h. Upon harvest, live cells from the basal chamber were counted by 30 s assessment of live cells from each well by flow cytometry. In Fig. 5, the activated M1 macrophages (LPS → PBS and LPS → sGP) showed a significant increase in the percent of chemotaxis compared to inactivated M1 macrophages (PBS → PBS and PBS → sGP). The percent of chemotaxis of the activated macrophages treated with sGP was decreased compared to activated M1 macrophages incubated with PBS. The results suggest that LPS provokes migratory functions of macrophages and sGP can operate to impair migration of the activated macrophages towards MCP-1.

3.6. EBOV sGP downregulates expression of macrophage integrin CD11b

Macrophage migration towards MCP-1 was compromised in the presence of EBOV sGP. Interestingly, the integrin, CD11b, has been shown to be critical for the migration of macrophages and not dendritic cells towards chemokines [24,25]. Therefore, EBOV sGP may be altering the expression of CD11b to impact the migratory capacity of activated macrophages. To test this possibility, activated M1 macrophages were cultured with sGP for 24 h and CD11b expression was assessed by flow cytometry. Indeed, levels of surface CD11b were decreased only in the activated macrophages incubated with sGP

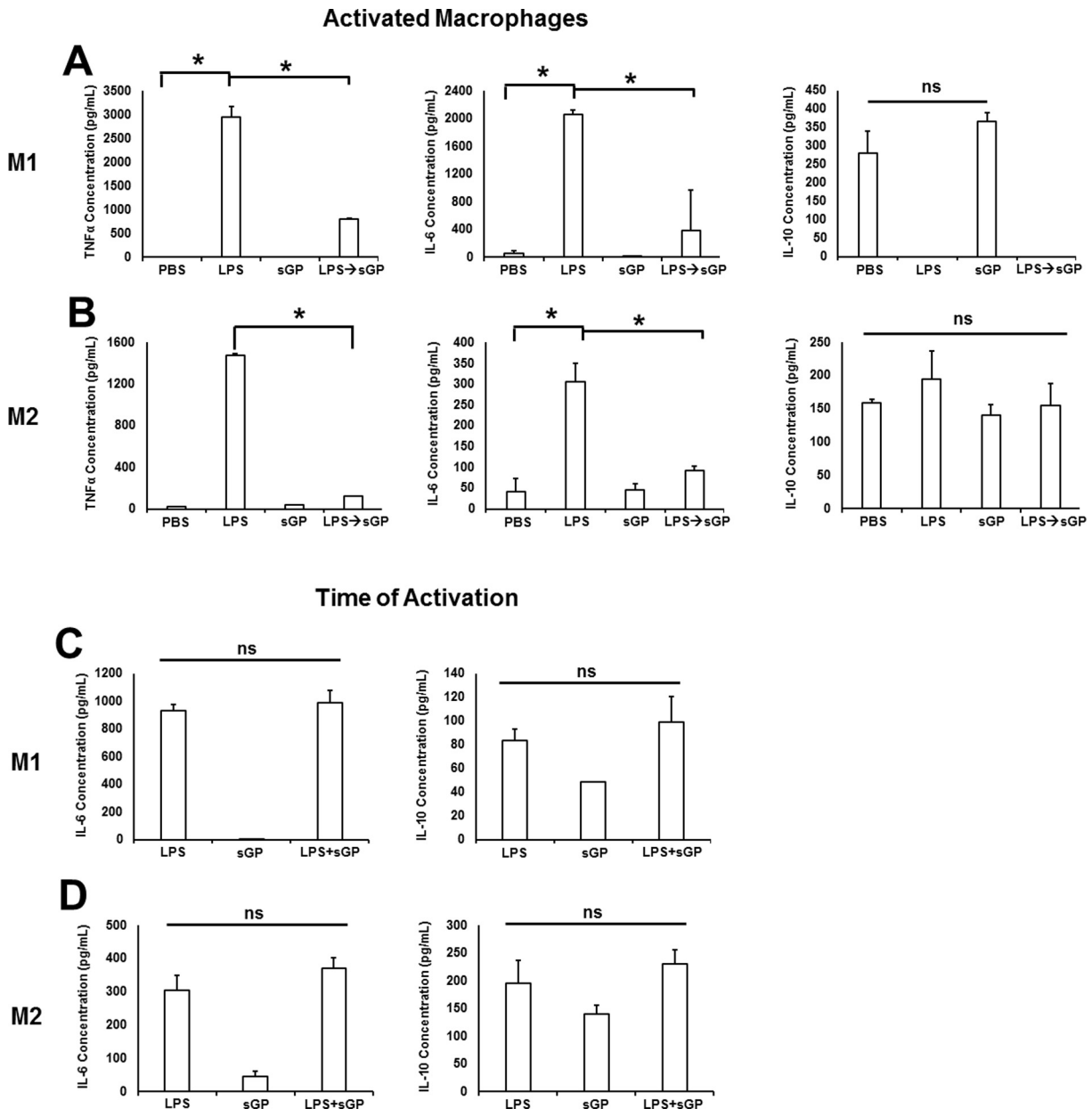

Fig. 3. M1 and M2 macrophage pro-inflammatory cytokine production is inhibited by EBOV sGP if given after activation, but not during activation. THP-1 monocytes were differentiated into macrophages. For the PBS, LPS, and sGP groups, macrophages were treated for 24 h with (A, C) IFNγ (20 ng/mL) or (B, D) IL-4 (20 ng/mL) and IL-13 (20 ng/mL). Subsequently, the macrophages were then stimulated with PBS, LPS (1 μg/mL) or sGP (4 μg/mL) for an additional 24 h. In the LPS → sGP group, (A) IFNγ (20 ng/mL) or (B) IL-4 (20 ng/mL) and IL-13 (20 ng/mL) were given for 24 h. Then the cells were washed and LPS (1 μg/mL) was given for 24 h. The cells were washed again and sGP (4 μg/mL) was added for 24 h. (C-D) In the LPS + sGP group, LPS (1 μg/mL) and sGP (4 μg/mL) were given simultaneously for 24 h after the polarization steps. Supernatants from the cultures were harvested and the production of TNFα, IL-6, and IL-10 was measured via ELISA. (n = 3, $P < .05$, ns indicates not significant).

(Fig. 5B). This suggests that EBOV sGP is decreasing the chemotactic ability of activated M1 macrophages by reducing CD11b expression.

4. Discussion

Currently, a major role of sGP in the EBOV pathogenesis has yet to be clearly demonstrated. Previously, it was thought that the primary role sGP served in pathogenesis was acting as a decoy molecule for antibody production [6]. In this study, we show for the first time that sGP has profound inhibitory effects upon activated macrophages that may condition the cells for susceptibility to infection by progeny virion. EBOV sGP inhibited the production of pro-inflammatory cytokines, impaired chemotaxis, and downregulated integrin expression. Phagocytosis of the activated macrophages was unaffected which is consistent with recent data supporting the notion that EBOV utilizes “apoptotic mimicry” and the phagocytosis mechanisms to invade the macrophages [16]. Thus, our findings suggest that sGP is produced to halt activated macrophage anti-viral activities and potentially preserving the entry site for the virion. In addition, once the activated macrophages are infected, EBOV glycoprotein and viral protein-40 may operate to override sGP-mediated inhibition to restore migration and cytokine production resulting in systemic dissemination of the virus and viral hemorrhagic fever.

EBOV titers have been shown to peak and plateau by day 6 post-infection at levels 10-fold greater than the titer levels of influenza virus infection [26]. This suggests an unusually high rate of viral replication that likely is accompanied by large amounts of pathogen-associated molecular patterns including the viral nucleic acids. In response,

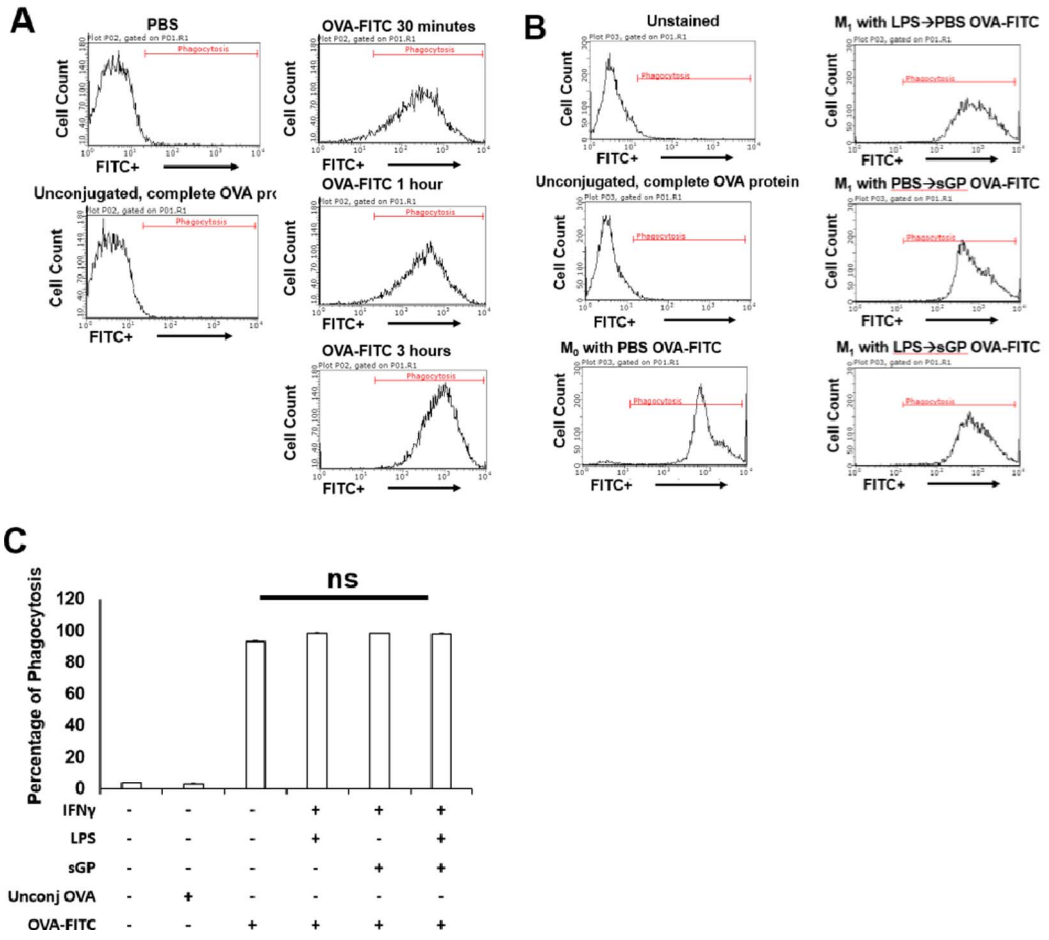

Fig. 4. Phagocytosis of M1 macrophages is unaffected by the presence of sGP. THP-1 monocytes were differentiated into macrophages. (A) Macrophages were treated for 24 h with IFNγ (20 ng/mL). Macrophages were then treated with sGP (4 μg/mL) or PBS for 24 h. The cells were washed, then treated with OVA-FITC (0.25 mg/mL) or unconjugated, complete OVA protein (1 mg/mL), or PBS for 30 min, 1 h or 3 h. The cells were washed, and analyzed on flow cytometry. (B) The macrophages were treated with either IFNγ (20 ng/mL), or PBS for 24 h. Washed and treated with LPS (1 μg/mL) or PBS for 24 h. Washed and treated with sGP (4 μg/mL) or PBS for 24 h. The cells were washed, and then treated with OVA-FITC (0.25 mg/mL), or unconjugated, complete OVA protein (1 mg/mL), or PBS for 3 h. The cells were washed, and analyzed on flow cytometry. (C) The percentage of the live population that was positive for OVA-FITC signaling was measured and compared. (n = 3, $P < .05$, ns indicates not significant).

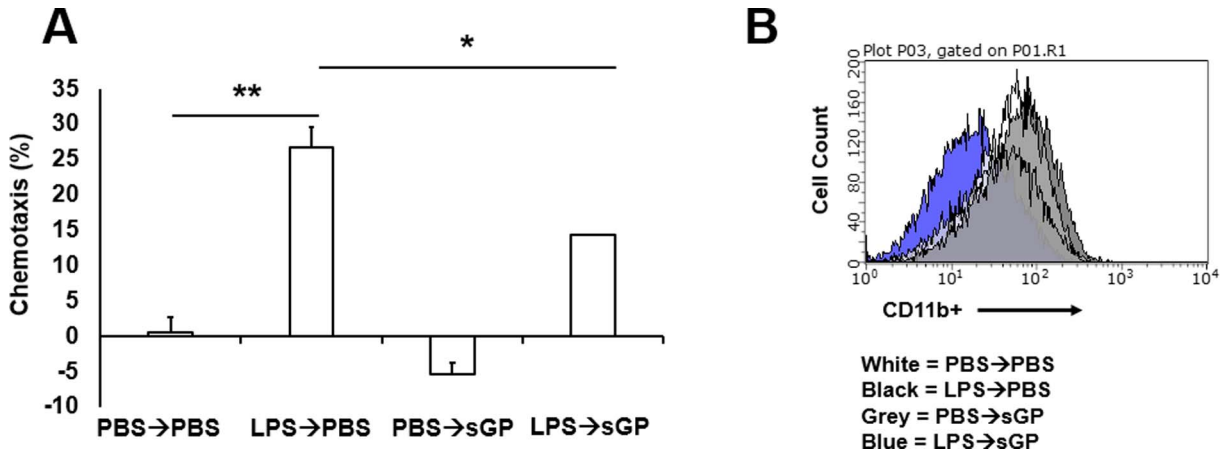

Fig. 5. Mobility of M1 macrophages is inhibited by the presence of sGP, which could be due to a downregulation of the macrophage integrin CD11b. THP-1 monocytes were differentiated into macrophages. Macrophages were treated for 24 h with IFNγ (20 ng/mL) or PBS. Macrophages were then treated with LPS (1 μg/mL) or PBS for 24 h. The cells were washed, and treated with either sGP (4 μg/mL) or PBS for 24 h. (A) The cells were collected and placed into the apical chamber of a chemotaxis plate. The chemokine, MCP-1 (10 ng/mL), was added to the basal chamber. The macrophages were incubated for 24 h. The chambers were then removed. The live cells in the basal chamber were counted via flow cytometry. The chemotaxis percent is calculated as spontaneous chemotaxis divided by the live cells in dorsal chamber with chemokine. (B) The cells were washed and stained for CD11b-APC, and analyzed on flow cytometry. (n = 3, $^{**} = P < .05$, $^{*} = P = .055$).

monocytes have been shown to emigrate from the bone marrow and into the bloodstream during the first 72 h of microbial infection [27]. However, systemic spread of the virus and high mortality suggests that despite the rapid mobilization of the innate response, EBOV is able to overwhelm the early host response in order to infect "carrier" cells (i.e. macrophages and DCs) for the purpose of disseminating the virus from the site of initial infection. The hypothesis put forward in this work was that EBOV sGP operates to inhibit effector activities of these immune cells prior to their infection. Efforts to assess the modulation of DCs by sGP are underway (unpublished data). Here, the focus was on monocytes and macrophages (resting and activated). Monocytes have little impact upon viral replication given their limited phagocytic ability and cytokine production and likely are not a target of EBOV sGP. This notion was supported by the inability of sGP to alter IL-6 production by THP-1 monocytes (Fig. 1A). By contrast, upon differentiation of monocytes into macrophages using PMA, and activation by LPS, THP-1 macrophages produced significantly less IL-6 (Fig. 1B). Therefore, sGP impacts macrophages only after differentiation from monocytes. This could suggest that the receptor on which sGP is binding is only expressed on differentiated macrophages and not monocytes. Work is proceeding to identify the sGP receptor as well as the signaling that may be initiated in macrophages upon incubation with sGP.

Differentiated macrophages undergo polarization into M1-like and M2-like effector cells in order to support microbial elimination; each genetic program configured for particular groups of pathogens, bacteria/viruses and helminths, respectively. Viruses such as severe acute respiratory syndrome coronavirus, human immunodeficiency virus and human cytomegalovirus have all been associated with promoting M2 polarization to support successful viral replication [28]. Thus, it was thought that EBOV may similarly promote M2 polarization through the secretion of a viral protein to modulate this process. Using the *in vitro* THP-1 model, EBOV sGP did not demonstrate the ability to affect the polarization of macrophages into M1 or M2 phenotypes (Fig. 2). This would seem to suggest that the spread of EBOV can benefit from correctly polarized macrophages. Again, because the macrophages were cultured with sGP prior to activation with LPS, it strongly suggests that the receptor for the viral protein is expressed or upregulated after the cells are stimulated.

Indeed, the production of IL-10 is not affected when sGP is given after macrophage activation, or during activation. The production is also not affected if sGP is given during the polarization of M2 macrophages as noted above. However, if sGP is given during the polarization of M1 macrophages then the production of IL-10 is significantly decreased. This could possibly be due to the activity of some sGP-mediated activation of a regulatory adaptor protein such as interferon regulatory factor-4 (IRF4), which has a profound inhibitory effect on pro-inflammatory cytokine production [29], but a milder inhibitory effect on IL-10 production [30,31]. The fact that sGP only interferes with the IL-10 production if given during the polarization step could be due to a role of a regulatory protein such as IRF-4 in the differentiation process of hematopoietic stem cells [31]. IRF-4 has been previously shown to be a target of viral processes to suppress the immune response and promote viral pathogenesis [32,33]. Therefore, it is possible that the presence of sGP promotes IRF-4 activity and suppresses pro-inflammatory cytokines, and only impacts the IL-10 production of M1 macrophages. This is a possibility that will be pursued in future work.

Previous studies have reported that shed EBOV glycoprotein can mitigate production of pro-inflammatory cytokines by innate immune cells, but the authors state that the secreted version was unable to stimulate the macrophages [7]. Other studies also show that soluble EBOV glycoprotein (shed, secreted, and spike) has no effect on macrophages [34]. Both of these studies were asking whether non-activated macrophages are stimulated to produce cytokines when treated with EBOV secreted glycoprotein; essentially asking whether sGP stimulates macrophage cytokine production. Thus, the finding that sGP can inhibit the production of pro-inflammatory cytokines by previously activated macrophages is novel (Fig. 3). The inhibitory effect of sGP upon IL-6 and TNFα production may occur at either the genetic or protein secretion level. When administered to mice, the half-life of LPS is 12 h and is likely much shorter *in vitro* [35]. Given that the macrophages are activated with LPS for 24 h and then washed to remove excess LPS before sGP is added, this suggests that sGP does not compete with LPS for TLR4 binding as is thought for shed GP [7]. So, EBOV sGP must affect LPS-induced signaling downstream, perhaps intervening to inhibit the activation of NF-κB by reinstating the action of IκB kinase (IKK) [36]. Another LPS-induced pathway that may be influenced by sGP are the mitogen-activated protein kinases, MAPK. LPS binding to Toll-like receptor 4 induces MyD88 signaling through recruitment and phosphorylation of IL-1 receptor-associated kinase (IRAK-4); which, in turn, activates IRAK-1. MAPK (and NF-κB) are subsequently activated leading to cytokine production [37]. Two proteins that block IRAK-4 may be triggered by sGP, Tollip and IRAK-M. Another protein, suppressor of cytokine signaling-1 (SOCS-1), could also be induced by sGP to interfere with cytokine production. Additionally, since IL-10 production by M2 macrophages was unchanged in the presence of sGP, it is highly unlikely that sGP impedes pro-inflammatory cytokine production by blocking protein secretory pathways. An understanding of how sGP affects NF-κB as well as the MAPK signaling pathways leading to cytokine production are underway.

While sGP appeared to have the capacity to interfere with LPS-mediated signals following activation, sGP was incapable of altering LPS signals when given at the same time as the stimulant (Fig. 3C and D). Possibly, sGP has a much shorter half-life than LPS; and thus, cytokines could be reduced for a short term and then restored by the time cytokine concentrations are determined at the 24-h endpoint of the experiment. An examination of the cytokine production at 12, 16, 20 and 24 h will address this issue. This rapid turnover may account for the projected high levels of sGP in the tissues and blood of infected animals [7]. EBOV GP and LPS have also been suggested to compete for the same receptor, TLR4; a possible attachment factor for the virus [7,13,38], which is expressed on macrophages. Whether the secreted, truncated version of GP may also bind to TLR4 is unknown. From these studies, sGP appeared not to have an impact upon cellular functions until the cells were activated suggesting that the receptor may not be constitutively expressed. Interestingly, related studies with natural killer (NK) cells lacking TLR4 and treatment with sGP have shown inhibition of NK effector functions indicating that sGP may at least have an alternate receptor which exists both on activated macrophages and NK cells (unpublished data).

Macrophages are widely known for their ability to phagocytose particles and clean up cellular debris in the tissues. Phagocytic action is increased when the macrophages assume the M1 phenotype, and is relatively lower for the M2 phenotype. The soluble antigen, ovalbumin, has been shown to be phagocytosed by DCs via the C-type lectin, mannose receptor [39]. C-type lectins have also been implicated as the possible attachment factor for EBOV entry into the host cells [11,40]. Interestingly, phagocytosis of ovalbumin by activated THP-1 M1 macrophages was not affected by treatment of sGP (Fig. 4). If the proposed hypothesis were correct, sGP would not interfere with the ability of the macrophages to uptake the virus through C-type lectins. Thus, C-type lectins may be important EBOV ligands for cell entry [16].

The migratory ability of macrophages is essential to their function as antigen-presenting cells. EBOV sGP exhibited the ability to diminish the capacity of THP-1 M1 macrophages to migrate in response to MCP-1 (Fig. 5A). Interestingly, the integrin CD11b is widely accepted as a marker of macrophage phenotypes; it has also been shown to be necessary for chemotactic ability of activated macrophages [41,42]. The assessment of CD11b in activated macrophages incubated with sGP demonstrated reduced expression of the cellular marker as compared to controls (Fig. 5B).

5. Conclusion

In conclusion, our results suggest a novel mechanism whereby EBOV sGP shuts down anti-viral immunity mediated by activated macrophages to create a pool of susceptible cells primed for infection. Indeed, EBOV sGP inhibited pro-inflammatory cytokine production and chemotaxis while preserving the cellular ability to take up antigens. Therefore, sGP may play a major role in EBOV pathogenesis in two ways. First, by impairing activated macrophages, the virus can rapidly produce progeny virion and spread to other parts of the body. Second, the paralyzed macrophages serve as a pool of susceptible host cells for infection. This information may be an important consideration when devising therapies given the unusually high replication rate of the virus and copious production of soluble viral proteins such as sGP, which may diminish efficacy of host immunity.

Conflict of interest

The authors have no financial conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2017.11.009.

References

[1] S. Flessa, M. Marx, Ebola fever epidemic 2014: a call for sustainable health and development policies, Eur. J. Health Econ. 17 (2015) 1–4, http://dx.doi.org/10.1007/s10198-015-0710-0.
[2] N. Wauquier, P. Becquart, C. Padilla, S. Baize, E.M. Leroy, Human fatal zaire ebola virus infection is associated with an aberrant innate immunity and with massive lymphocyte apoptosis, PLoS Negl. Trop. Dis. 4 (2010) e837, http://dx.doi.org/10.1371/journal.pntd.0000837.
[3] G. Simmons, R.J. Wool-Lewis, F. Baribaud, R.C. Netter, P. Bates, Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence, J. Virol. 76 (2002) 2518–2528, http://dx.doi.org/10.1128/JVI.76.5.2518-2528.2002.
[4] J.R. Francica, A Study of the Ebola Virus Glycoprotein: Disruption of Host Surface Protein Function and Evasion of Immune Responses, (n.d.). http://repository.upenn.edu/edissertations.
[5] N.J. Lennemann, B.A. Rhein, E. Ndungo, K. Chandran, X. Qiu, W. Maury, Comprehensive functional analysis of N-linked glycans on ebola virus GP1, MBio 5 (2014), http://dx.doi.org/10.1128/mBio. 00862-13.
[6] O. Dolnik, V. Volchkova, W. Garten, C. Carbonnelle, S. Becker, J. Kahnt, U. Stroher, H.-D.D. Klenk, V. Volchkov, U. Ströher, H.-D.D. Klenk, V. Volchkov, Ectodomain shedding of the glycoprotein GP of Ebola virus, Embo J. 23 (2004) 2175–2184, http://dx.doi.org/10.1038/sj.emboj.7600219/r7600219 [pii].
[7] B. Escudero-Pérez, V.A. Volchkova, O. Dolnik, P. Lawrence, V.E. Volchkov, Shed GP of ebola virus triggers immune activation and increased vascular permeability, PLoS Pathog. 10 (2014), http://dx.doi.org/10.1371/journal.ppat.1004509.
[8] D. Falzarano, O. Krokhin, V. Wahl-Jensen, J. Seebach, K. Wolf, H.J. Schnittler, H. Feldmann, Structure-function analysis of the soluble glycoprotein, sGP, of ebola virus, ChemBioChem 7 (2006) 1605–1611, http://dx.doi.org/10.1002/cbic.200600223.
[9] V.A. Volchkova, O. Dolnik, M.J. Martinez, O. Reynard, V.E. Volchkov, RNA editing of the GP gene of ebola virus is an important pathogenicity factor, J. Infect. Dis. 212 (2015) S226–S233, http://dx.doi.org/10.1093/infdis/jiv309.
[10] V. Wahl-Jensen, S. Kurz, F. Feldmann, L.K. Buehler, J. Kindrachuk, V. DeFilippis, J. da Silva Correia, K. Früh, J.H. Kuhn, D.R. Burton, H. Feldmann, Ebola virion attachment and entry into human Macrophages profoundly effects early cellular gene expression, PLoS Negl. Trop. Dis. 5 (2011) e1359, http://dx.doi.org/10.1371/journal.pntd.0001359.
[11] A. Takada, K. Fujioka, M. Tsuiji, A. Morikawa, N. Higashi, H. Ebihara, D. Kobasa, H. Feldmann, T. Irimura, Y. Kawaoka, Human macrophage C-type lectin specific for galactose and N-acetylgalactosamine promotes filovirus entry, J. Virol. 78 (2004) 2943–2947, http://dx.doi.org/10.1128/JVI.78.6.2943-2947.2004.
[12] O. Martinez, J.C. Johnson, A. Honko, B. Yen, R.S. Shabman, L.E. Hensley, G.G. Olinger, C.F. Basler, Ebola virus exploits a monocyte differentiation program to promote its entry, J. Virol. 87 (2013) 3801–3814, http://dx.doi.org/10.1128/JVI.02695-12.
[13] A. Okumura, P.M. Pitha, A. Yoshimura, R.N. Harty, Interaction between Ebola virus glycoprotein and host toll-like receptor 4 leads to induction of proinflammatory cytokines and SOCS1, J. Virol. 84 (2010) 27–33, http://dx.doi.org/10.1128/JVI.01462-09.
[14] J.E. Lee, T.P. Road, L. Jolla, M.-A. de La Vega, G. Wong, G.P. Kobinger, X. Qiu, The multiple roles of sGP in ebola pathogenesis, Viral Immunol. 28 (2015) 3–9, http://dx.doi.org/10.1089/vim.2014.0068.
[15] A. Sanchez, S.G. Trappier, B.W. Mahy, C.J. Peters, S.T. Nichol, The virion glycoproteins of ebola viruses are encoded in two reading frames and are expressed through transcriptional editing, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 3602–3607, http://dx.doi.org/10.1073/pnas.93.8.3602.
[16] F. Dahlmann, N. Biedenkopf, A. Babler, W. Jahnen-Dechent, C.B. Karsten, K. Gnirß, H. Schneider, F. Wrensch, C.A. O'Callaghan, S. Bertram, G. Herrler, S. Becker, S. Pöhlmann, H. Hofmann-Winkler, Analysis of ebola virus entry into macrophages, J. Infect. Dis. 212 (2015) S247–S257, http://dx.doi.org/10.1093/infdis/jiv140.
[17] M. Daigneault, J.A. Preston, H.M. Marriott, M.K.B. Whyte, D.H. Dockrell, The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages, PLoS ONE 5 (2010) e8668, http://dx.doi.org/10.1371/journal.pone.0008668.
[18] M. Genin, F. Clement, A. Fattaccioli, M. Raes, C. Michiels, M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide, BMC Cancer 15 (2015) 577, http://dx.doi.org/10.1186/s12885-015-1546-9.
[19] J.E. Lee, E.O. Saphire, Ebolavirus glycoprotein structure and mechanism of entry, Future Virol. 4 (2009) 621–635, http://dx.doi.org/10.2217/fvl.09.56.
[20] C.H. Lee, H.F. Nawar, L. Mandell, S. Liang, G. Hajishengallis, T.D. Connell, Enhanced antigen uptake by dendritic cells induced by the B pentamer of the type II heat-labile enterotoxin LT-IIa requires engagement of TLR2, Vaccine 28 (2010) 3696–3705, http://dx.doi.org/10.1016/j.vaccine.2010.03.016.
[21] P. Macanas-Pirard, T. Quezada, L. Navarrete, R. Broekhuizen, A. Leisewitz, B. Nervi, P.A. Ramírez, The CCL2/CCR2 axis affects transmigration and proliferation but not resistance to chemotherapy of acute myeloid leukemia cells, PLoS ONE 12 (2017) e0168888, http://dx.doi.org/10.1371/journal.pone.0168888.
[22] X.F. Zheng, Y.X. Hong, G.J. Feng, G.F. Zhang, H. Rogers, M.A.O. Lewis, D.W. Williams, Z.F. Xia, B. Song, X.Q. Wei, Lipopolysaccharide-Induced M2 to M1 macrophage transformation for IL-12p70 production is blocked by candida albicans mediated up-regulation of EBI3 expression, PLoS ONE 8 (2013), http://dx.doi.org/10.1371/journal.pone.0063967.
[23] Q. Zhang, Y. Wang, N. Zhai, H. Song, H. Li, Y. Yang, T. Li, X. Guo, B. Chi, J. Niu, I.N. Crispe, L. Su, Z. Tu, HCV core protein inhibits polarization and activity of both M1 and M2 macrophages through the TLR2 signaling pathway, Sci. Rep. 6 (2016) 36160, http://dx.doi.org/10.1038/srep36160.
[24] N.P. Podolnikova, J.A. Brothwell, T.P. Ugarova, The opioid peptide dynorphin a induces leukocyte responses via integrin Mac-1 ($\alpha_M \beta_2$, CD11b/CD18), Mol. Pain 11 (2015), http://dx.doi.org/10.1186/s12990-015-0027-0 s12990-015–0027.
[25] A.C. Kirby, J.G. Raynes, P.M. Kaye, CD11b regulates recruitment of alveolar macrophages but not pulmonary dendritic cells after pneumococcal challenge, J. Infect. Dis. 193 (2006) 205–213, http://dx.doi.org/10.1086/498874.
[26] V.K. Nguyen, S.C. Binder, A. Boianelli, M. Meyer-Hermann, E.A. Hernandez-Vargas, Ebola virus infection modeling and identifiability problems, Front. Microbiol. 6 (2015) 257, http://dx.doi.org/10.3389/fmicb.2015.00257.
[27] N.V. Serbina, W. Kuziel, R. Flavell, S. Akira, B. Rollins, E.G. Pamer, Sequential MyD88-independent and -dependent activation of innate immune responses to intracellular bacterial infection, Immunity 19 (2003) 891–901, http://dx.doi.org/10.1016/S1074-7613(03)00330-3.
[28] A.C. Labonte, A.-C. Tosello-Trampont, Y.S. Hahn, The role of macrophage polarization in infectious and inflammatory diseases, Mol. Cells 37 (2014) 275–285, http://dx.doi.org/10.14348/molcells.2014.2374.
[29] H. Negishi, Y. Ohba, H. Yanai, A. Takaoka, K. Honma, K. Yui, T. Matsuyama, T. Taniguchi, K. Honda, Negative regulation of toll-like-receptor signaling by IRF-4, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 15989–15994, http://dx.doi.org/10.1073/pnas.0508327102.
[30] K. Honma, H. Udono, T. Kohno, K. Yamamoto, A. Ogawa, T. Takemori, A. Kumatori, S. Suzuki, T. Matsuyama, K. Yui, Interferon regulatory factor 4 negatively regulates the production of proinflammatory cytokines by macrophages in response to LPS, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 16001–16006, http://dx.doi.org/10.1073/pnas.0504226102.
[31] S. Nam, K. Kang, J.S. Cha, J.W. Kim, H.G. Lee, Y. Kim, Y. Yang, M.-S. Lee, J.-S. Lim, Interferon regulatory factor 4 (IRF4) controls myeloid-derived suppressor cell (MDSC) differentiation and function, J. Leukoc. Biol. 100 (2016) 1273–1284, http://dx.doi.org/10.1189/jlb.1A0215-068RR.
[32] D. Xu, L. Zhao, L. Del Valle, J. Miklossy, L. Zhang, Interferon regulatory factor 4 is involved in epstein-barr virus-mediated transformation of human B lymphocytes, J. Virol. 82 (2008) 6251–6258, http://dx.doi.org/10.1128/JVI.00163-08.
[33] Q. Cai, S. Banerjee, A. Cervini, J. Lu, A.D. Hislop, R. Dzeng, E.S. Robertson, IRF-4-mediated CIITA transcription is blocked by KSHV encoded LANA to inhibit MHC II presentation, PLoS Pathog. 9 (2013), http://dx.doi.org/10.1371/journal.ppat.1003751.

[34] V. Wahl-Jensen, S.K. Kurz, P.R. Hazelton, H.-J. Schnittler, U. Ströher, D.R. Burton, H. Feldmann, Role of ebola virus secreted glycoproteins and virus-like particles in activation of human macrophages, J. Virol. 79 (2005) 2413–2419, http://dx.doi.org/10.1128/JVI.79.4.2413-2419.2005.
[35] H. Huang, T. Liu, J.L. Rose, R.L. Stevens, D.G. Hoyt, Sensitivity of mice to lipopolysaccharide is increased by a high saturated fat and cholesterol diet, J. Inflamm. (Lond.) 4 (2007) 22, http://dx.doi.org/10.1186/1476-9255-4-22.
[36] M. Karin, How NF-kB is activated: the role of the IkB kinase (IKK) complex, Oncogene 18 (1999) 6867–6874, http://dx.doi.org/10.1038/sj.onc.1203219.
[37] A.L. Blasius, B. Beutler, Intracellular toll-like receptors, Immunity 32 (2010) 305–315, http://dx.doi.org/10.1016/j.immuni.2010.03.012.
[38] J. Denner, Treatment of ebola virus infections with inhibitors of TLR4, Med. Hypotheses 85 (2015) 253–257, http://dx.doi.org/10.1016/j.mehy.2015.05.009.
[39] S. Burgdorf, V. Lukacs-Kornek, C. Kurts, The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation, J. Immunol. 176 (2006) 6770–6776, http://dx.doi.org/10.4049/jimmunol.176.11.6770.
[40] G. Simmons, J.D. Reeves, C.C. Grogan, L.H. Vandenberghe, F. Baribaud, J.C. Whitbeck, E. Burke, M.J. Buchmeier, E.J. Soilleux, J.L. Riley, R.W. Doms, P. Bates, S. Pöhlmann, DC-SIGN and DC-SIGNR bind ebola glycoproteins and enhance infection of macrophages and endothelial cells, Virology 305 (2003) 115–123, http://dx.doi.org/10.1006/viro.2002.1730.
[41] K. Simonin-Le Jeune, A. Le Jeune, S. Jouneau, C. Belleguic, P.F. Roux, M. Jaguin, M.T. Dimanche-Boitre, V. Lecureur, C. Leclercq, B. Desrues, G. Brinchault, J.P. Gangneux, C. Martin-Chouly, Impaired functions of macrophage from cystic fibrosis patients: CD11b, TLR-5 decrease and sCD14 inflammatory cytokines increase, PLoS ONE 8 (2013) e75667, http://dx.doi.org/10.1371/journal.pone.0075667.
[42] D. Maiguel, M.H. Faridi, C. Wei, Y. Kuwano, K.M. Balla, D. Hernandez, C.J. Barth, G. Lugo, M. Donnelly, A. Nayer, L.F. Moita, S. Schürer, D. Traver, P. Ruiz, R.I. Vazquez-Padron, K. Ley, J. Reiser, V. Gupta, Small molecule-mediated activation of the integrin CD11b/CD18 reduces inflammatory disease, Sci. Signal. 4 (2011) ra57, http://dx.doi.org/10.1126/scisignal.2001811.