

Activating Natural Killer Cell Receptors, Selectins, and Inhibitory Siglecs Recognize Ebolavirus Glycoprotein

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Keywords

Ebolavirus glycoprotein · Siglecs · Natural cytotoxicity receptors · Selectins · HPV

Abstract

Expression of the extensively glycosylated Ebolavirus glycoprotein (EBOV-GP) induces physical alterations of surface molecules and plays a crucial role in viral pathogenicity. Here we investigate the interactions of EBOV-GP with host surface molecules using purified EBOV-GP, EBOV-GP-transfected cell lines, and EBOV-GP-pseudotyped lentiviral particles. Subsequently, we wanted to examine which receptors are involved in this recognition by binding studies to cells transfected with the EBOV-GP as well as to recombinant soluble EBOV-GP. As the viral components can also bind to inhibitory receptors of immune cells (e.g., Siglecs, TIM-1), they can even suppress the activity of immune effector cells. Our data show that natural killer (NK) cell receptors NKp44 and NKp46, selectins (CD62E/P/L), the host factors DC-SIGNR/DC-SIGN, and inhibitory Siglecs function as receptors for EBOV-GP. Our re-

sults show also moderate to strong avidity of homing receptors (P-, L-, and E-selectin) and DC-SIGNR/DC-SIGN to purified EBOV-GP, to cells transfected with EBOV-GP, as well as to the envelope of a pseudotyped lentiviral vector carrying the EBOV-GP. The concomitant activation and inhibition of the immune system exemplifies the evolutionary antagonism between the immune system and pathogens. Altogether these interactions with activating and inhibitory receptors result in a reduced NK cell-mediated lysis of EBOV-GP-expressing cells. Modulation of these interactions may provide new strategies for treating infections caused by this virus.

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Published by S. Karger AG, Basel

Introduction

Ebolavirus (EBOV) and Marburg virus are two (2) genera of the Filoviridae family belonging to the most virulent viruses known, which in humans cause a rapidly fatal hemorrhagic fever [1, 2]. EBOV is able to infect al-

most every cell type with a rapid rate of viral replication [3]. Treatment is still largely symptomatic, but there are currently 2 licensed Ebola vaccines: The first licensed Ebola vaccine was the rVSV-ZEBOV vaccine made by Merck which was approved by the US FDA in 2019. The heterologous 2-dose Ad26.ZEBOV/MVA-BN-Filo vaccine by Janssen received marketing authorization approval under exceptional circumstances by the European Medicines Agency in 2020. Both of these vaccines have undergone in phase 3/2 trials, respectively (WHO; <https://www.who.int/groups/global-advisory-committee-on-vaccine-safety/topics/ebola-virus-vaccines>). In addition, a series of monoclonal antibodies have been used successfully alone or in combination [4].

The EBOV genome contains 7 different genes (3'-NP-VP35-VP40-GP-VP30-VP24-L-5'), out of which the GP gene forms at least 3 different glycoproteins (GPs) via alternative open reading frames (2–4). The other 6 genes code for structural proteins and the polymerase L of filovirus particles.

These GPs can be modified by various enzymes. After *N*- and *O*-glycosylation, GP0 is cleaved by the enzyme furin to yield the GP1 and GP2 subunits [5, 6]. GP is structured in a chalice-like shape with a trimer of GP1/GP2 heterodimers, out of which GP2 is forming the base and GP1 the cup [7]. This GP trimer can be cleaved from the viral surface via the tumor necrosis factor- α -converting enzyme [7]. GP1 is mucin-like and contains *N*-glycosylated as well as *O*-glycosylated areas, where mutations permanently take place, thus enabling immune escape [8, 9], whereas GP2 has only 2 *N*-glycosylated areas [10, 11]. GP1 is responsible for receptor binding (including α -dystroglycan, heparan sulfate, DC-SIGN, etc.) and GP2 mediates low pH-induced membrane fusion. Both proteins interact with each other to form a stable homotrimer complex on the viral envelope [12, 13].

Fuller and colleagues have shown that both Marburg virus-like particles and EBOV-like particles trigger a consistent upregulation of CD69 on the cell surface of polyclonal natural killer (NK) cells from different donors [14]. Upregulation of CD69 on NK cells is closely linked with activation of several signal transduction pathways including survival and induction of cytokine production and cytotoxicity of targets [15, 16]. In addition, EBOV infection induces massive NK cell apoptosis, thus avoiding NK function and impairing NK-mediated DC maturation [17–19].

The killer cell immunoglobulin-like receptors (KIRs) as well as Siglecs, and CD94-NKG2A, are involved in the inhibitory signal cascade of NK cells. Most inhibitory receptors recognize specific MHC class I isoforms and thereby

ensure tolerance of NK cells against self [20]. NK cell activation is mediated by receptors such as KIR2DS1-5, NK-G2D, CD16, or NCRs (natural cytotoxicity receptors NKp46, NKp44, and NKp30). In previous work, we were able to show that ligands for NKp30 and NKp44 can be detected on the surface and in intracellular compartments of different tumor cells [21]. These ligands often contain heparan sulfate linked to proteoglycans [22, 23]. Besides recognizing tumor cells, activating NK cell receptors have also been shown to be involved in detecting virus-infected cells by the interaction with various viral surface proteins. As an example, NKp30 and NKp46 present on NK cells play a key role in the immune response against vaccinia virus and mouse poxvirus (ECTV/ectromelia virus), as they bind to hemagglutinin (HA) – a component of the vaccinia virus envelope [24]. It should be noted that NKp30-triggered activation of NK cells is blocked by HA of vaccinia virus, whereas HA stimulates NK cells through NKp46 [24]. Moreover, the pp65 matrix protein of human cytomegalovirus binds NKp30 and inhibits its function [25]. The results indicate that NKp30 has a different role in NK-cell cytotoxicity [24, 26, 27]. NKp46 recognizes the sigma1 protein of reovirus [28]. In addition, the HA proteins of Sendai-, influenza-, and Newcastle disease viruses are able to bind NKp46 and NKp44, and induce NK cell activation [29–32]. NKp44 interacts with envelope glycoproteins from the West Nile and dengue virus E/M proteins [33].

DC-SIGN (CD209) and DC-SIGNR (DC-SIGN-related, CD299, CLEC4M) bind to soluble EBOV-GPs with similar avidity [34], as well as soluble human immunodeficiency virus type-1 (HIV-1) gp120. This interaction is inhibited in an environment with increased pH [34]. DC-SIGN and DC-SIGNR are calcium-dependent C-type lectins, which have high avidity for ICAM3 (CD50) [35]. DC-SIGN binds to weakly polysialylated NCAM-1 [36]. Recently, it was found that TIM-1 (T-cell immunoglobulin and mucin domain 1) is a filovirus receptor [37] and interacts by its phosphatidylserine (Ptd-L-Ser) binding pocket directly with Ptd-L-Ser located on the viral capsid [38–40]. Furthermore, TIM-1 binds the adhesion receptor P-selectin and mediates T-cell trafficking during inflammation and autoimmunity [41, 42].

L-selectin is a cell adhesion molecule expressed on most circulating cells, including neutrophils, dendritic cells, monocytes, B cells, NK cells, and T cells. L-selectin is a major regulator of transendothelial migration of leukocytes. E- and P-selectin are expressed on endothelial cells at sites of inflammation and interact with receptors on the surfaces of leukocytes. Also, selectins have been shown to interact with viral proteins. The glycoprotein

gp120 of HIV-1 binds L-selectin in solution and on the host cell membrane. Upon entry of HIV into CD4⁺ T cells, L-selectin is cleaved at the membrane proximal site by proteolysis, thus facilitating virus release from cells [43, 44]. Given that fact that many NK cell receptors can interact with viral proteins and that NK cells seem to be involved in EBOV infections, we wanted to investigate the interactions of EBOV-GP with different host cell surface proteins. Here we use purified EBOV-GP, EBOV-GP-transfected cells, and EBOV-GP-pseudotyped lentiviral particles to show an interaction with the NK cell receptors NKp44 and NKp46, Siglec-7, the host factors DC-SIGN and DC-SIGNR, P-selectin, and L-selectin. These interactions result in reduced NK cell-mediated killing of EBOV-GP expressing cells.

Materials and Methods

Cell Lines

Human embryonic kidney cells HEK-293 (ATCC CRL-3216) and CHO-K1 (ATCC CCL-61) were cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany) supplemented with 2 mM glutamine and 10% fetal calf serum (FCS). Human polyclonal NK cells were isolated by NK cell negative isolation kit (Miltenyi) from peripheral blood mononuclear cells or from healthy donor buffy coats. Between 95 and 99% of NK cells were CD3 negative and CD56 positive. Cells were grown in Iscove's modified Dulbecco's medium (Invitrogen) with 10% human serum, penicillin-streptomycin, and 100 IU/mL IL-2 (NIH Cytokine Repository, Bethesda, MD, USA).

Transfections

Prof. S. Becker (Institute of Virology, Marburg University, Germany) kindly provided the expression vector pCAGGS-ZEBOV-GP for transfection of HEK-293 cells together with empty pcDNA3.1(+) (Invitrogen) to provide for a neomycin resistance gene in trans. Furthermore, we used the expression vector pcDNA6/V5-His-A/ZEBOV-GP (plasmid 7616.5) for transfection of HEK-293 cells and CHO-K1 cells. Empty pcDNA6/V5-His-A was used for vector control transfectants. A total of 2.5×10^5 HEK-293 cells and CHO-K1 cells were cultured in 6-well plates and transfected with 4 μ g of the mentioned plasmids using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two days later, cells were selected with geneticin (1 mg/mL) or blasticidin (10 μ g/mL) and, after being sorted for high-level GP expression, maintained in 0.5 mg/mL geneticin or 5 μ g/mL blasticidin. GP expression was detected via mouse monoclonal anti-ZEBOV GP antibody 3B11 [45] (kindly provided by Prof. Stephan Becker, Institute of Virology, Marburg) and human monoclonal anti-ZEBOV GP antibody KZ52 [46] (dilution 1:200). KZ52 was purchased from IBT BioServices (Rockville, MD, USA).

Chromium Release Assay

EBOV-GP-transfected HEK-293 cells, which were used as target cells (0.5×10^6) in 100 μ L of assay medium (Iscove's modified Dulbecco's medium with 10% FCS and 1% penicillin-streptomycin),

were labeled with 100 μ Ci (3.7 MBq) of ⁵¹Cr (Hartmann & Braun, Frankfurt, Germany) for 1 h at 37°C. Cells were washed twice and resuspended in assay medium at 5×10^4 cells/mL. Isolated NK cells were seeded in 96-well round-bottom plates (Nunc) at a density of $1.5\text{--}2 \times 10^6$ /mL with irradiated feeder cells (K562-mbIL15-41BBL) in medium with 100 ng/mL IL-21 (Miltenyi Biotec) and 200 U/mL IL-2 (National Institutes of Health Cytokine Repository). After the first week, NK cells were restimulated with fresh feeder cells. In the next weeks, NK cells were split to a density of $1.5\text{--}2 \times 10^6$ /mL in the presence of 100 U/mL IL-2. On day 14, 2.5 ng/mL recombinant IL-15 (PAN Biotech) was added. After 3 weeks, NK cells were used for ⁵¹Chromium-release assay. These effector cells were resuspended in assay medium and mixed at different effector-to-target cell ratios with 5,000 labeled target cells/well in a 96-well F-bottom plate. Maximum release was determined by the incubation of target cells in 1% Triton X-100 solution. Spontaneous ⁵¹Cr release was measured by incubating target cells in the absence of effector cells. All samples were prepared in triplicate. Plates were incubated for 4 h at 37°C. Supernatant was harvested, and ⁵¹Cr release was measured in a γ -counter. The percentage of cytotoxicity was calculated according to the following formula: $([\text{chromium release for condition of interest} - \text{chromium release in spontaneous wells}] / [\text{max chromium release} - \text{chromium release in spontaneous wells}]) \times 100$. Representative examples from 3 similar experiments are shown.

Enzyme-Linked Immunosorbent Assay

For the direct detection of EBOV-GP via enzyme-linked immunosorbent assay (ELISA) plates, we obtained the human EBOV Zaire glycoprotein from Advanced Biomart (San Gabriel, CA, USA) and the recombinant human anti-EBOV GP antibody [KZ52] from Absolute Antibody (Oxford, GB). EBOV-GP was expressed with a polyhistidine-tag at the C-terminus and consists of 629 amino acids, predicting a molecular mass of 69 kDa. This antibody detects purified EBOV-GP coated on ELISA plates and HEK-293^{EBOV-GP} cells by IF. MicroTest III ELISA plates (BD Biosciences, Heidelberg, Germany) were coated overnight with EBOV-GP in 0.05 M NaHCO₃-Na₂CO₃ buffer (pH 9.6). They were also blocked using 3% skim milk powder (Merck, Darmstadt, Germany) in PBS-0.05% Tween 20 (PBS-T) (Sigma-Aldrich), as well as PierceTM Protein-Free (PBS) Blocking Buffer (Thermo Scientific), overnight at 4°C. The recombinant IgG-Fc fusion proteins NKp44-Fc (2249-NK-05), NKp46-Fc (1859-NK-025), NKp30-Fc (1849-NK-025), DC-SIGN-Fc/CD209-FC (161-DC-050), DC-SIGNR-Fc/CD299-FC (162-D2-050), Siglec-2-Fc (1968-SL-050), Siglec-3-Fc (1137-SL-050), Siglec-4-Fc (8940-MG-050), Siglec-5-Fc (1072-SL-050), Siglec-7-Fc (1138-SL-050), Siglec-10-Fc (2130-SL-050), PSGL-1-Fc (3345-PS-050), NKG2D-Fc (1299-NK-050), E-selectin-Fc (ADP1-050), P-selectin-Fc (137-LS-050), L-selectin-Fc (728-LS-100), TIM1-Fc (9319-TM-100), CD44-Fc (3360-CD-050), and CD24-Fc (5247-CD-050) were obtained from R&D (Planegg, Germany), and syndecan 1-Fc (PKSH033514) from Elabscience. All were analyzed regarding their binding ability to EBOV-GP-coated ELISA plates. All purified recombinant proteins (1 μ g/100 μ L in PBS-T with 1% bovine serum albumin) were added in triplicates to EBOV-GP-coated wells at 2 μ g/well for 1 h at room temperature. After washing 3 times with PBS-T, peroxidase-conjugated goat anti-hIgG-Fc or goat anti-mouse IgG-Fc (Dianova, Hamburg) in PBS-T (1:2,000 with 1% bovine serum albumin) was added for 1 h at room temperature. After washing 3 times with

PBS-T, a peroxidase substrate solution (*o*-phenylenediamine [Sigma-Aldrich] at 1 mg/mL in 0.1 M KH₂PO₄ buffer [pH 6.0]) was added for 20 min at room temperature in the dark. The substrate reaction was stopped with 50 μ L of 4 N H₂SO₄, and results were read out with a Titertek Multiscan plus MKII ELISA photometer (MP Biomedicals, Heidelberg, Germany) at OD450 nm and 570 nm for reference. The whole experiment was repeated at least 3 times.

Flow Cytometry

Flow cytometry was used to analyze binding of recombinant fusion proteins to EBOV-GP-transfected cell lines. For cell surface immunofluorescence staining, 0.5–1 \times 10⁶ cells were washed once in ice-cold fluorescence-activated cell sorter (FACS) buffer (D-PBS-2% FCS) and then incubated with a saturating amount of the primary mouse MAb for 45 min on ice. After 2 washes, cells were incubated with PE-labeled goat anti-mouse Ig for 30 min on ice. Complexes of the Fc fusion proteins listed above (1–2 μ g per staining) and PE-labeled goat anti-hIgG Fc (Dianova; 1:100 in FACS buffer) were allowed to form for 30 min before being added to cells for 60 min on ice. Cells were washed twice and resuspended in 200 μ L of FACS buffer with 0.05% propidium iodide (Sigma-Aldrich). Cytofluorometric analyses were done using a FACS Canto II or FACS LSR Fortessa flow cytometers and Diva software (Becton Dickinson, Heidelberg, Germany). For all FACS stainings, representative examples are shown from at least 3 repeats with similar results.

Heparanase and Neuraminidase Treatment of Cells

CHO-K1 transfected with pcDNA6/EBOV GP or empty pcDNA6 vector were treated with heparanase I and III (Sigma/Aldrich, 100 mU/100 μ L in PBS) or α 2-3,6,8,9-neuraminidase (Merck/Calbiochem, 5 mU/100 μ L PBS) for 1 h at 37°C, respectively. After enzymatic treatment and washing, cells were immediately stained on ice with Fc fusion proteins, complexed with goat anti-hIgG-PE secondary antibodies. The experiment was repeated 3 times.

P24 Antigen Capture Assay

Lentivirus particles based on the HIV-1 were produced in HEK-293 cells through transient transfection of 2 plasmids encoding components of the virus envelope as described below. Cell culture medium containing viral particles produced by packaging cells was harvested after 72 h. HEK-293 cells stably transfected with the plasmid pcDNA6/V5-HisA/EBOV-GP and control HEK-293 cells stably transfected with pcDNA6/V5-HisA were used to produce lentiviral particles displaying EBOV-GP (lenti-EBOV-GP) and control particles devoid of EBOV-GP. Briefly, 1 \times 10⁶ cells of each cell line were seeded in 60-mm cell culture dishes 1 night prior to transfection. The next day, 6 μ g of a 3:2 ratio of lentiviral transfer vector (pLOX-CWgfp) and packaging plasmid (psPAX2) were transfected into each cell culture dish using Turbofect Transfection Reagent (Thermo Fisher Scientific). Seventy-two hours posttransfection, viral supernatants were collected and centrifuged at 3,000 g for 15 min at 4°C to remove cell debris. To concentrate the viral particles, they were centrifuged at 48,000 g for 3 h at 4°C, and viral pellets were resuspended in cold PBS. Physical titration of the viral preparation was performed using p24 detection by sandwich ELISA. Microtiter plates precoated with anti-p24 Ab were incubated with increasing dilutions of the lentiviral suspension. After incubation and washing, p24 was quantified using a biotinylated anti-p24 Ab and detected using HRP-streptavidin. Color development was measured at 450 nm in a Bio-Rad spectrophotometer.

ELISA (Lenti-EBOV-GP)

Lentiviral vector titers are expressed in transducing units per mL. After physical titration of viral vectors, we immobilized HIV-lentiviral EBOV-GP particles (200 μ g/mL) on 96-well microtiter plates (Nunc, Maxisorp) in 0.1 M sodium bicarbonate buffer (pH 9.6) at 4°C for 18 h and blocked with protein-free blocking buffer (PBS) pH 7.4 (Thermo), which is recommended for viral particles with highly glycosylated proteins [47]. For the coating with EBOV-GP and control lentiviral particles, equal amounts of particles were added to each well. After blocking, the plates were washed 3 times with PBS containing 0.05% Tween-20. Dilutions of 10 μ g/mL of fusion proteins were prepared in PBS + 2% protein-free buffer, including NKp44-Fc, NKp46-Fc, NKp30-Fc, DC-SIGN-Fc, DC-SIGNR-Fc, Siglec2-Fc, Siglec3-Fc, Siglec4-Fc, Siglec5-Fc, Siglec7-Fc, Siglec10-Fc, PSGL-1-Fc, NKG2D-Fc, E-selectin-Fc, P-selectin-Fc, L-selectin-Fc, TIM1-Fc, CD44-Fc, CD24-Fc, syndecan 1-Fc (R&D), and human anti-Gp-Fc antibody as positive control. Each protein was added in triplicate to the respective wells and incubated for 1 h at room temperature. After incubation, the plates were gently washed 3 times with PBS containing 0.05% Tween-20. Then, the secondary antibody (goat anti-human IgG-Fc) was added to the wells and the plates were incubated for 1 h at room temperature. The plates were subsequently washed 3 times with PBS-T (PBS containing 0.05% Tween-20) and incubated with substrate (OPD) for 15–20 min. The reactions were stopped by adding 100 μ L of 1M sulfuric acid to each well. Finally, the absorbance of 450/570 nm was measured by a BioTek Synergy 4 Multi-Mode Microplate Reader. The whole experiment was repeated twice.

Statistical Analysis

Experimental values were obtained in triplicates and repeated at least twice. The corresponding mean values with SD were used for analysis of data. Significance was tested by unpaired *t* test with 2-sided *p* value. *p* values <0.05 were considered significant.

Results

EBOV-GP Binds NCRs, Homing Selectins, and Inhibitory Siglecs

We used ELISA plates coated with purified recombinant EBOV-GP produced in HEK-293 cells to assess the binding to different IgG1-Fc recombinant fusion proteins (Fig. 1). In accordance with previous results [34], DC-SIGN (CD209) and its related C-type lectin DC-SIGNR (CD299) bound strongly to EBOV-GP. Also, the activating NK cell receptors NKp44 and NKp46 but not NKp30 and NKG2D showed an interaction with EBOV-GP. Furthermore, P-selectin, L-selectin (CD62 P/L), and the inhibitory receptors Siglec-5 and Siglec-7 showed significant binding to EBOV-GP. However, we observed only weak or no binding of Siglec-3, E-selectin, PSGL-1, CD44, and CD24. These results were confirmed repeatedly under different conditions and with different batches of proteins, and the binding tendencies were reproducible throughout all experiments.

Fig. 1. Binding of EBOV-GP-His-tag to activating receptors, homing proteins (selectins), and inhibitory Siglecs. ELISA plates coated with recombinant Zaire Ebola-GP-His-tag protein were used to test the binding of the indicated recombinant proteins. Each recombinant protein was added in triplicates, noncoated plates and GahIg-GFc-Pox secondary antibody alone served as negative controls, and human anti-EBOV-GP antibody KZ52 as positive control. This whole test was repeated at least 5 times, with highly similar results. From these repeats, 1 representative ELISA experiment was selected for display. A significant difference between PBS controls and specific proteins was assumed when the 95% confidence limits did not overlap and the 2-sided *t* test for paired samples was equal or lower than $p = 0.05$ ($p < 0.05$; [*]: $p = 0.05$). EBOV-GP, Ebolavirus glycoprotein; ELISA, enzyme-linked immunosorbent assay.

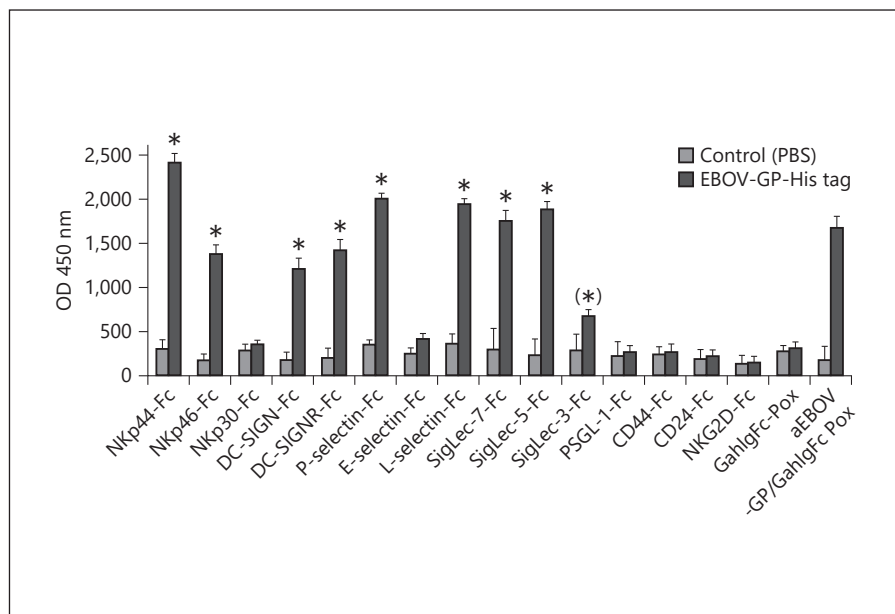
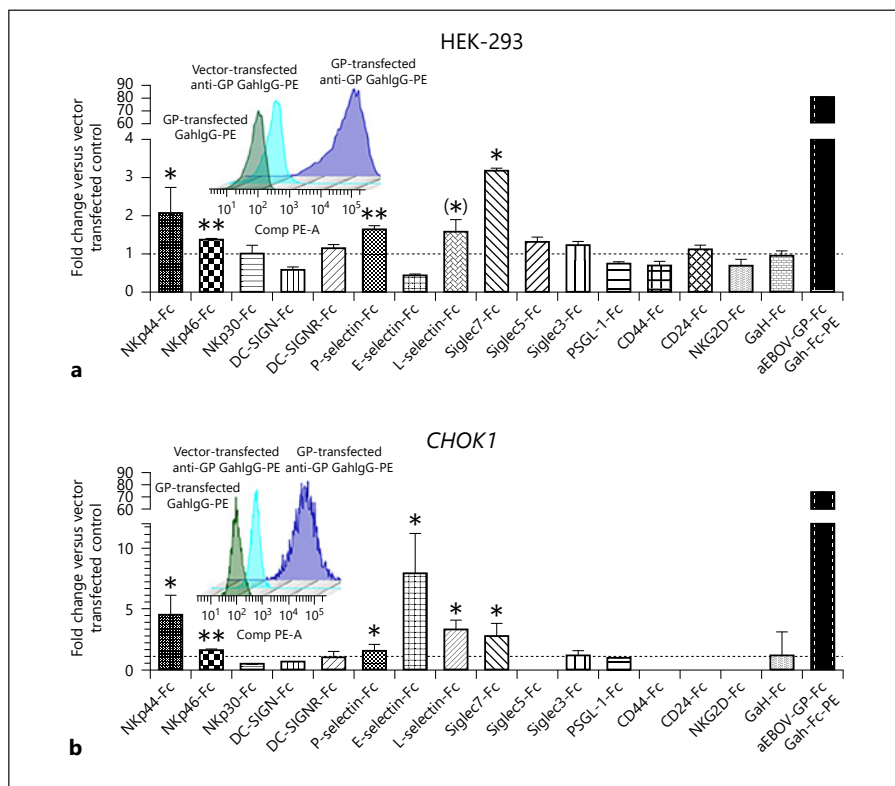


Fig. 2. NCRs, homing selectins, and inhibitory Siglecs bind to EBOV-GP-transfected HEK-293 and CHO-K1 cells: HEK-293 (a) or CHO-K1 (b) cells were stably transfected with EBOV-GP or with empty vector as control. GP expression efficiency was detected by anti-ZEBOV GP human antibody KZ52. The insets show peaks derived from the original flow cytometry analyses consisting of at least 3 repeats, which indicate the binding of the respective fusion proteins to EBOV-GP-transfected HEK-293 (a) or CHO-K1 (b) cells in relation to their binding to the vector control-transfected cells. Significance was calculated by 2-sided *t* test for paired samples, comparing the binding of recombinant proteins to vector- and GP-transfected cells. (*): $p < 0.1$; (*): $p < 0.05$; (**): $p < 0.001$. EBOV-GP, Ebolavirus glycoprotein; NCRs, natural cytotoxicity receptors.



Interactions with EBOV-GP Expressed in HEK-293 and CHO-K1 Cells

To confirm these interactions in a cellular context, we stably transfected HEK-293 cells with plasmids carrying the EBOV-GP gene. GP expression efficiency was con-

firmed using the human monoclonal anti-ZEBOV GP antibody KZ52 [46] (Fig. 2a). We then used the various Fc fusion proteins for immunofluorescence staining of the transfected HEK-293^{EBOV-GP} cells and analyzed the binding by flow cytometry (Fig. 2a; online suppl. Fig-

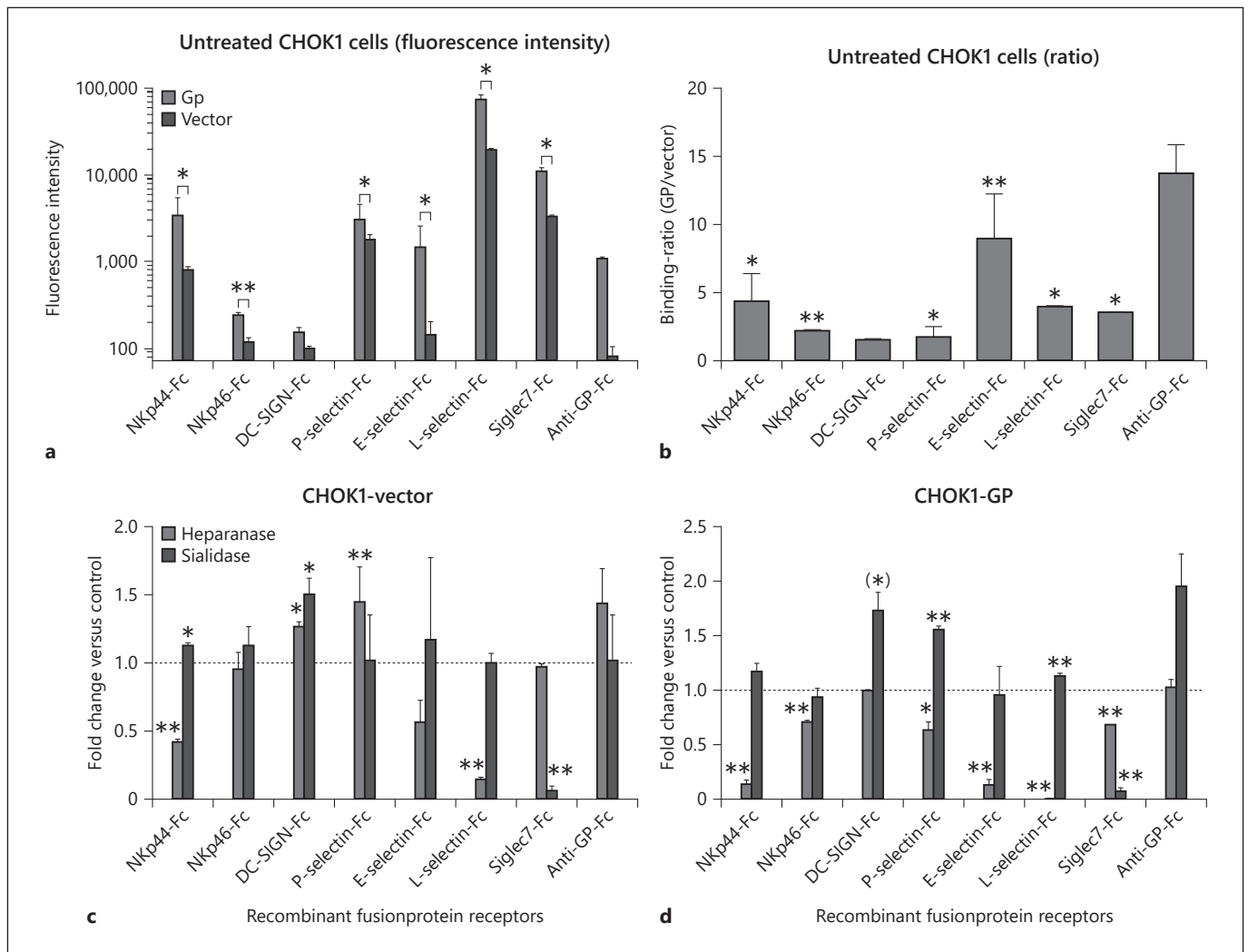


Fig. 3. Treatment of EBOV-GP-transfected CHO-K1 cells with heparinase I and III or sialidase. EBOV-GP- or vector control-transfected CHO-K1 cells were pretreated with heparinase I and III, or sialidase, or left untreated as a control. The binding of the indicated fusion proteins to the differential treated cells was analyzed by at least 2 experiments. Shown are the fluorescence intensities for binding (a), the corresponding ratios of GP- and vector-transfected CHO-K1 cells (b) in relation to the respective ratio in

binding to vector control- (c) and GP-transfected (d) cells, which had been pretreated with either heparinase or sialidase. Significance was calculated by 2-sided *t* test for paired samples, comparing the binding of recombinant proteins to vector- and GP-transfected cells. (*): $p < 0.1$; (*): $p < 0.05$; (**): $p < 0.001$. These results were also used for indicating significance of ratios. EBOV-GP, Ebolavirus glycoprotein.

ure 1; see www.karger.com/doi/10.1159/000517628 for all online suppl. material). Similar to our results with EBOV-GP-coated ELISA plates, we observed significantly enhanced binding of recombinant NKp44-Fc, NKp46-Fc, L-selectin-Fc, P-selectin-Fc, Siglec-7-Fc, and Siglec-5-Fc proteins to HEK-293^{EBOV-GP} cells in comparison to vector control-transfected cells. However, we could not confirm the binding of DC-SIGN-Fc and DC-SIGNR-Fc. Siglec-3, which only showed weak binding to the EBOV-GP-coat-

ed ELISA plates, exhibited similar binding in this cellular assay. For NKp30-Fc, NKG2D-Fc, E-selectin-Fc, PSGL-1-Fc, CD44-Fc, and CD24-Fc, we observed no increased binding to HEK-293^{EBOV-GP} cells exceeding the staining of endogenous ligands expressed by HEK-293 vector-transfected controls, confirming the ELISA results. Extending this analysis, we also did not observe enhanced binding of recombinant Siglec-2-Fc, Siglec-4-Fc, Siglec-10-Fc, L1-CAM-Fc, or TIM-1-Fc proteins to HEK-

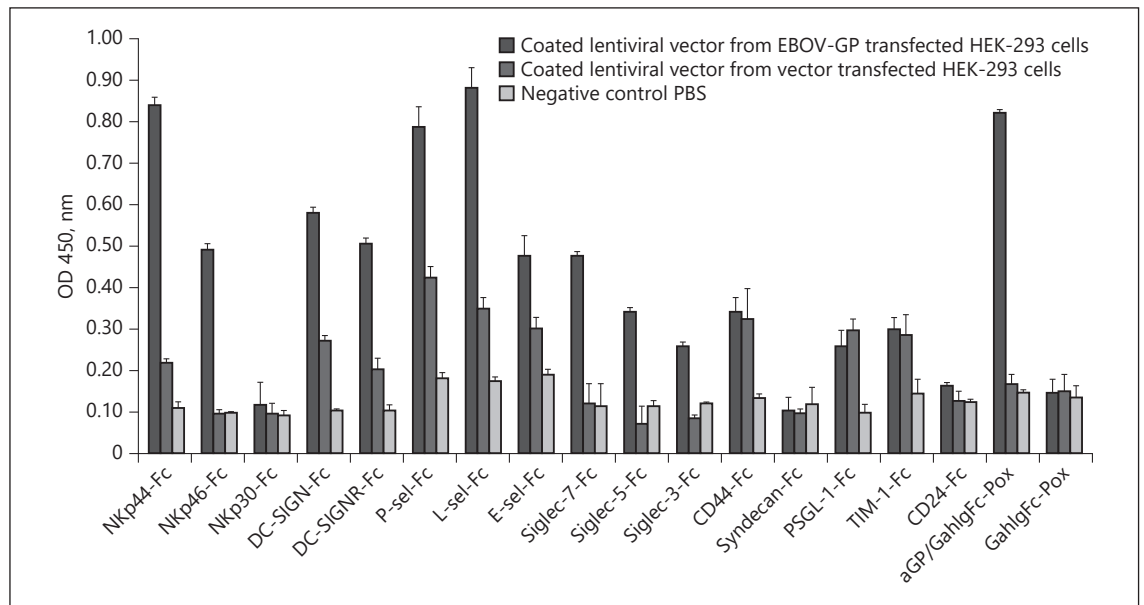


Fig. 4. Binding of fusion proteins to lentiviral particles displaying EBOV-GP. ELISA plates were coated with lentiviral particles originating from EBOV-GP- or vector control-transfected HEK-293 cells or with PBS as a control. The coating efficiency was determined by anti-ZEBOV GP monoclonal antibody KZ52. The binding of the indicated fusion proteins to the coated ELISA plates was analyzed. The experiment was performed in triplets and repeated twice. EBOV-GP, Ebolavirus glycoprotein; ELISA, enzyme-linked immunosorbent assay.

293^{EBOV-GP} cells (data not shown). Interestingly, we even observed reduced binding of some fusion proteins when comparing HEK-293^{EBOV-GP} to vector control-transfected cells. For example, binding of NKG2D to its cellular ligands was reduced in GP-transfected HEK-293 cells, which suggests a reduced availability of its ligands MIC-A/B, as it has been demonstrated before [48].

In addition to the HEK-293^{EBOV-GP} cells, we used EBOV-GP-transfected CHO-K1 cells to assess the binding of some of the recombinant fusion proteins to EBOV-GP in a nonhuman cell line (Fig. 2b and online suppl. Fig. 2). HEK-293 and CHO share similar posttranslational protein modifications in the endoplasmic reticulum and Golgi apparatus [49]. More importantly, CHO cells are able to produce complex types of recombinant proteins with human-compatible glycosylation. CHO cells, however, do not express Gal α -2,6-sialyltransferase, α -1,3/4-fucosyltransferase, or β -1,4-N-acetylglucosaminyltransferase III (GnT-III), which are enzymes expressed in human cells [50–52]. In agreement with the previous results, we found enhanced binding of NKp44-Fc, NKp46-Fc, L-selectin-Fc, P-selectin-Fc, and Siglec-7-Fc to EBOV-GP-transfected CHO-K1 cells. Again, DC-SIGN-Fc and DC-SIGNR-Fc did not show significant binding compared to control cells in this cellular assay. Also, we

found no enhanced binding of PSGL-1-Fc, NKp30-Fc, and Siglec-3-Fc, which is in agreement with the results of the ELISAs. However, in contrast to results mentioned above we observed strongly enhanced binding of E-selectin to EBOV-GP-transfected CHO-K1 cells. In summary, the cellular assays confirmed NKp44, NKp46, L-selectin, P-selectin, Siglec-7, and Siglec-5 as binding partners for EBOV-GP.

Binding of the Chimeric Soluble Receptors to Their Ligands Depends on Sialic Acid and Heparan Sulfate

P- and L-selectin recognize clustered sulfated *O*-sialoglycan epitopes, for example, on the proteoglycan CD44. L-selectin, like other selectins, recognizes sialylated Lewis^x and sialylated Lewis^a glycans (sLe^a; Neu5Aca2–3Gal β 1–3[Fuca1–4]GlcNAc) [53, 54]. All 3 selectins recognize sulfated and sialylated derivatives [54]. In addition, L-selectin binds to *O*-glycosylated proteins [55]. Many enveloped viruses, for instance, influenza virus and Newcastle disease virus (NDV), bind to sialic acid residues located on the surface of target cells [32]. In order to analyze the involvement of sialic acid and heparan sulfate moieties in the binding of the fusion proteins to EBOV-GP-transfected CHO-K1 cells, we treated the GP- and mock-transfected cells with heparanases I and III, and si-

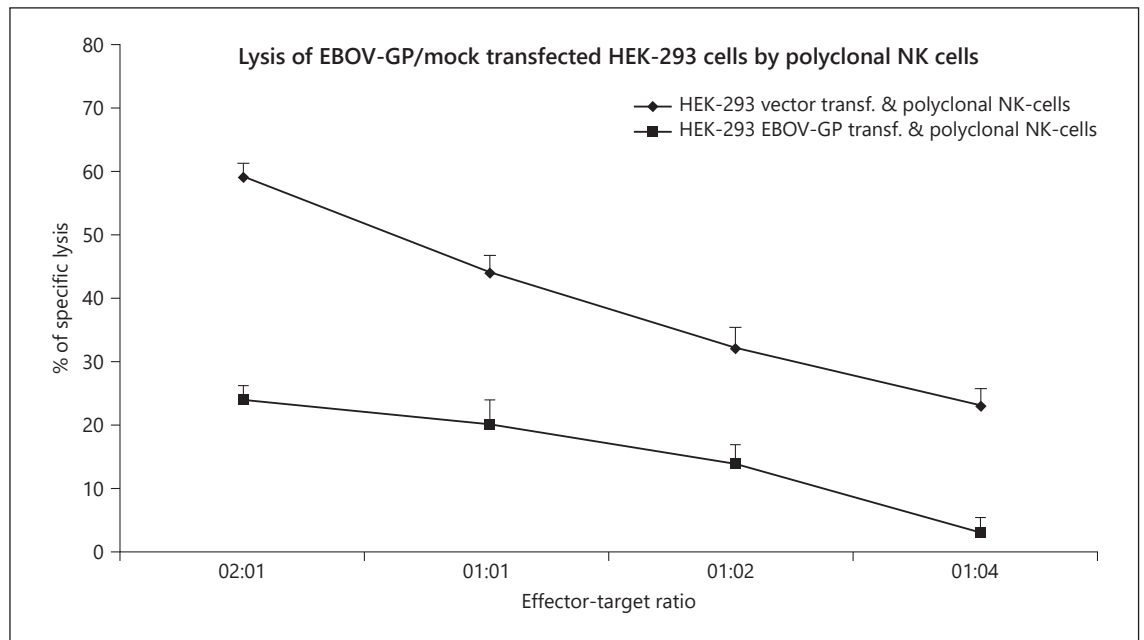


Fig. 5. Killing of HEK-293^{EBOV-GP} target cells by polyclonal NK cells. HEK-293 target cells transfected with empty vector as a control or with EBOV-GP were incubated with polyclonal human NK cells at the indicated effector to target ratios. Lysis of the HEK-293 cells was analyzed using a standard 4 h ⁵¹Cr release assay. The experiment was performed in triplets and repeated twice. EBOV-GP, Ebolavirus glycoprotein; NK, natural killer.

alidase. Interestingly, GP-transfected cells showed increased binding in terms of median fluorescence intensity of all fusion proteins shown compared to vector control cells (Fig. 3 top and online suppl. Fig. 3). Following treatment, we analyzed the impact of these enzymes on the binding of fusion proteins to EBOV-GP- and mock-transfected CHO-K1 cells (Fig. 3, middle and bottom; and online suppl. Fig. 3). Treatment with heparanase resulted in a significantly decreased binding of selectins to EBOV-GP-transfected CHO-K1 cells. Similarly, binding of NKp44 and NKp46 was reduced following heparanase treatment, as was expected since it is known that heparan sulfate is a ligand for these receptors [22, 23]. In contrast, binding of Siglec-7 was less (though still significantly) affected by heparanase, yet strongly sensitive to sialidase I/III treatment, which reduced its binding significantly by more than 90%, as can be explained by the fact that sialic acid is the main ligand of Siglecs.

Binding of Fusion Proteins to Lentiviral Particles Displaying EBOV-GP

The GP glycoprotein serves the EBOV to infect host cells by binding to various docking proteins. After transduction, viruses can incorporate or co-package many hosts cell-derived nonviral surface proteins into their

newly formed envelope [56–58]. Lately, this was demonstrated for infectious clones of HIV-1 propagated in HEK-293T cells [59]. We therefore produced recombinant lentiviral particles in HEK-293 cells stably transfected either with EBOV-GP or empty vector control. After concentrating the lentiviral particles, they were used for coating ELISA plates and coating efficiency was confirmed using an anti-GP antibody (Fig. 4). We then tested the binding of the different fusion proteins to the coated lentiviral particles (Fig. 4). We found significant binding to lenti-EBOV-GP by NKp44-Fc, NKp46-Fc, E-selectin-Fc, L-selectin-Fc, P-selectin-Fc, Siglec-7-Fc, Siglec-5-Fc, Siglec-3-Fc, DC-SIGN-Fc, and DC-SIGNR-Fc, essentially confirming our previous results. Consistent with experiments reported above, we found no binding of NKp30-Fc, CD24-Fc, or syndecan-1-Fc to the lenti-EBOV-GP. TIM-1-Fc bound to the lentiviral particles irrespective of their content of EBOV-GP. This may be due to the fact that TIM-1 binds to PtdSer, which originated from the cell surface of host HEK-293 cells and was taken up by the virus envelope [57]. Likewise, CD44-Fc and PSGL-1-Fc interacted with vector envelopes independent of GP expression, which can be explained by their binding to cellular ligands from the host plasma membrane, which were taken up by the lentiviral envelopes.

HPV-L1 Binds NCRs, Selectins, and Inhibitory Siglecs

To investigate if the proteins that we found to interact with EBOV-GP also bind to other viral surface proteins, we assessed the binding of the fusion proteins to purified human papillomavirus (HPV)-L1 virus-like particles (online suppl. Fig. S4). Interestingly, we observed specific binding of NKp44-Fc, NKp46-Fc, P-selectin-Fc, L-selectin-Fc, Siglec-7-Fc, and Siglec-5-Fc and no binding of NKp30-Fc, DC-SIGN-Fc, DC-SIGNR-Fc, E-selectin-Fc, Siglec-3-Fc, or PSGL-1-Fc, a pattern which was similar to the binding to EBOV-GP-pseudotyped lentiviral particles.

EBOV-GP Protects HEK-293 Cells from Lysis by Polyclonal NK Cells

Our data show that many NK cell receptors can interact with EBOV-GP. Therefore, we wanted to test how the expression of GP can influence the killing of target cells by NK cells. We used HEK-293^{EBOV-GP} or mock-transfected HEK-293 cells as targets for primary NK cells obtained from different donors in a ⁵¹Cr release assay. As shown in Figure 5, HEK-293^{EBOV-GP} cells were significantly less susceptible to NK cell-mediated lysis compared to mock-transfected cells, suggesting that the pleiotropic effects of EBOV-GP on the cell surface have an overall suppressive result on NK cells. Altogether, these results suggest a crucial role of EBOV-GP in mediating immune escape of transduced/infected cells.

Discussion/Conclusion

Our data show that EBOV-GP binds to the chimeric soluble proteins L- and P-selectin, Siglec-7 and Siglec-5, NKp44 and NKp46, and, to a lesser extent, to Siglec-3, DC-SIGN, and DC-SIGNR, which are known cellular receptors for EBOV [37, 39, 60–62]. We were able to demonstrate the binding of DC-SIGN-Fc and DC-SIGNR-Fc to purified recombinant EBOV-GP on ELISA plates, but not to HEK-293^{EBOV-GP} cells. A possible explanation is that EBOV-GP, which is highly glycosylated in human host cells, interacts with neighboring surface proteins. This, in turn, will block many epitopes, which are recognized by recombinant fusion proteins, including DC-SIGN-Fc and DC-SIGNR-Fc. In contrast, GP on the viral envelope remains free for interaction with both proteins. Like the HIV-1 gp120, the highly glycosylated EBOV-GP utilizes the C-type lectin receptor DC-SIGN (CD209) to infect dendritic cells, which are a major reservoir of EBOV [60–62]. We could not prove binding of TIM-1-Fc to HEK-293^{EBOV-GP} cells or to purified EBOV-

GP, yet we showed binding of TIM-1-Fc to lentiviral particles displaying EBOV-GP on their envelope (lenti-EBOV-GP), as well as to mock lentiviral particles. The binding of TIM-1-Fc to lentiviral particles irrespective of their content of EBOV-GP is due to the fact that TIM-1 binds to cohesive PtdSer, which was incorporated by the virus envelope. It originates from the inner plasma membrane of mock- or EBOV-GP-transfected HEK-293 cells [57]. TIM-3, TIM-4, and TIM-1 together inhibit HIV and EBOV release from infected cells [63]. TIM-1 also serves as a pattern recognition receptor on invariant NK cells, which mediate cell activation by TIMs binding to PtdSer on the surface of cells undergoing apoptosis [64]. It is likely that TIM-3, an inhibitory checkpoint receptor on effector cells, binds also to PtdSer of the EBOV envelope [41, 42]. We hypothesize that the integration of PtdSer into the virus envelope plays also a role in infected patients. Here, TIMs will elicit eventually a strong reaction (cytokine storm) when they are triggered by their ligand PtdSer. This will depend on the amount of PtdSer available and on the activation of stimulating receptors, which can cause that TIMs act as costimulatory receptors [65]. For NK cells, this can imply that the inhibitory activity caused by interaction of GP with Siglecs is diminished, and thus they contribute to the uncontrolled cytokine storm that has been observed after Ebola infection. This assumption is in line with reports showing a better survival of TIM1 double-knockout mice, which showed reduced cytokine release and better survival after infection with EBOV [66].

Our data show that L- and P-selectins and possibly also E-selectin bind to purified EBOV-GP coated on ELISA plates and to EBOV-GP-transfected cells (HEK-293 and CHO-K1). This is in line with recently published data that the viral particle gp120 of HIV-1 binds L-selectin (CD62L) [43, 44, 53, 67]. Similarly, human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71 [68]. Therefore, we believe that selectins play an important role in EBOV release from infected cells, as it has been shown for HIV, which binds L-selectin and CD34 to co-localize with ADAM17 [43, 44, 53, 67].

Siglecs often function as sensor for sialylated glycoproteins. Through their intracellular ITIM, they induce strong inhibitory signaling upon binding to different linkages of sialic acid [69]. Interestingly, this mechanism is used by tumor cells and pathogens to escape the immune system, by adding sialic acid residues to their glycan structures, thus highlighting that the sialic acid-Siglec interaction is key to the immune function against pathogens and cancer [70, 71]. NK and other effector cells express various Siglecs, for example, Siglec-3, 7, 8, and 9 [72,

73]. We observed binding of Siglec-7 and -5 to EBOV-GP in ELISA as well as to CHO-K1 EBOV-GP-transfected cells and lentiviral particles. Siglec-7 binding to EBOV-GP-transfected CHO-K1 is dependent on 2,6-sialic acid linkage, since treatment with sialidase reverts the binding. We have not tested Siglec-8 and Siglec-9, but assume that Siglec-8 and Siglec-9 might also bind to GP because of their similar avidity for 2,6- and 2,3-sialic acid linkages. In addition, they are MHC class I-independent inhibitory receptors on immune effector cells, which – when stimulated – prevent the activation of these cells.

The current study shows that Ebola-GP binds to several immune receptors. Notably, the binding differs between the principal assays: for example, EBOV-GP-overexpressing HEK-293 cells do not bind to E-selectin-Fc, while CHO-K1 cells bind strongly (Fig. 2). This might be explained by differences in the nature of these cells. EBOV-GP is highly glycosylated and therefore undergoes strong interactions *in cis*. As a consequence, there can be massive rounding and detachment of infected/transfected cells [74–76]. Concomitantly, the expression of receptors differs qualitatively and quantitatively between human HEK-293 and hamster CHO-K1 cells. Further, there are differences in glycosylation and accessibility of these receptors. Finally, human cells are more susceptible for EBOV infection than hamster cells. All these factors contribute to a (slightly to pronounced) different binding behavior of our recombinant proteins. For all these reasons, the binding behavior differs between cell lines from 2 different species.

NK cells are effector cells of the early innate immune response that play a critical role in the lysis of virus-infected cells and tumor cells without requiring prior antigen stimulation [77, 78]. We and others have previously shown the interaction of NCRs with HA-neuraminidase of NDV, poxviral HA, and influenza viruses [24, 29, 32]. Recently, it was reported that blocking NKp30 by a specific antibody reduced lysis of EBOV-infected dendritic cells by NK effector cells [14]. Our data show that NKp44 and NKp46 but not NKp30 directly bound with EBOV-GP if tested as soluble hIgG1 fusion proteins, while all 3 NCR Fc fusion proteins stained endogenous ligands on control cells. This interaction was likely dependent on heparan sulfate as it was reduced by heparinase treatment (see Fig. 4). Heparan sulfate binds many microorganisms and interacts with many viral envelope components, for example, from HIV-1 [79], hepatitis viruses [80, 81], flaviviruses [82, 83], vaccinia virus [84], HPV [85], human herpesvirus [86], HSV-1 [87], and EBOV [88]. The binding of NCRs to EBOV-GP as well as other viral envelope proteins led us to examine their binding also to HPV-L1.

NKp44 and P- and L-selectins showed strong binding to HPV-L1, while NKp46 and Siglec-5 and Siglec-7 showed low binding. Therefore, we hypothesized that NK cell receptors play a key role in the recognition of structural glycoproteins of virtually any virus by NK effector cells and thus facilitate the elimination of pathogens.

The mechanism by which EBOV-GP modulates the activity of NK cells may simultaneously involve (i) the binding of different inhibitory Sigeles, (ii) the interaction with the activating receptors NKp44 and NKp46, and (iii) a reduction of NKG2D ligands (MICA/B) as we observed a lower binding of NKG2D-Fc chimeric soluble receptors to EBOV-GP-transfected HEK-293 cells, as has been reported before [48]. The net result of the expression of EBOV-GP by HEK-293 cells was a reduction in NK cell-mediated lysis. This suggests that the reduced stimulation of NKG2D together with the engagement of inhibitory Sigeles outweighs the binding to the activating receptors NKp44 and NKp46.

For the observed cell growth inhibition of NK cells in response to EBOV-GP interaction, the binding to inhibitory Siglec receptors (e.g., Siglec-7 or Siglec-9) is decisive. In mock-transfected HEK cells, only heparan sulfate chains of proteoglycans, including CD44, will interact with NKp44 or NKp46 receptors of NK cells and thus activate them, which leads to an efficient cell kill. In GP-transfected cells, these groups are also available, but they are engaged by the *cis* interaction with GP. Therefore, these glycoproteins are less active in the interaction with activating receptors on NK cells, for example, NCRs and L-selectin. In addition, GP proteins bind with high affinity to inhibitory receptors, such as Sigeles. This causes an altered balance of activating and inhibitory receptors and in summary reduces the activation of NK cells. However, these thoughts are to be complemented with experiments involving the blockade of NK cell cytolytic activity to explore how the sublyng forces act together.

The reduced cell kill activity caused by the altered balance in NK cells based on activating and inhibitory receptors will be explored by blocking experiments in the future. The interaction of highly glycosylated GP *in cis* with other proteoglycans and their heparan sulfate chains will influence the interaction between target and effector cells (NK cells). Although it has been described that infection with EBOV is able to induce massive NK apoptosis, thus avoiding NK function and impairing NK-mediated effects [89], we have not observed massive apoptosis in our NK cells. We can only speculate that the GP load on our transfected HEK cells was lower than that of cells infected with the wild-type virus [24], which helped the NK cells to avoid induction of apoptosis.

To explain the reduced killing of GP-infected cells by NK cells (Fig. 5), we hypothesize that GP may interact in *cis* with certain adhesion molecules by binding to their heparan-sulfate chains. This is important, as in mock-transfected HEK-293 cells the heparan sulfate chains of proteoglycans, including CD44, will interact with NKp44 or NKp46 receptors of NK cells and thus activate them, which leads to an efficient cell kill. However, when engaged by the *cis* interaction with GP, these glycoproteins are less active in the interaction with activating receptors on NK cells, for example, NCRs and L-selectin. In addition, GP proteins bind with high affinity to inhibitory receptors, such as Siglec-3 or Siglec-7. This causes an altered balance of activating and inhibitory receptors and in summary reduces the activation of NK cells and thus prevents interaction and subsequent polarization of effector cells with their virus-infected targets. Alternatively, the blockade of receptors responsible for apoptosis induction may also be the reason for reduced killing of infected target cells, as described for filovirus particles [90]. More in-depth knowledge about the mechanisms by which EBOV-GP-expressing cells are able to directly switch off NK cell functions will certainly be helpful to develop an efficient therapy for EBOV infections [91].

Finally, we propose a possible therapeutic treatment for EBOV infections. We hypothesize that an anti-GP antibody retargeted to neuraminidase activity could reduce the virus's sialylation, thus reducing the binding to inhibitory Siglecs of immune effector cells and increasing the recognition of their activating receptor NCRs (see Fig. 4). This will bring about a higher sensitivity toward immune effector cells. Such a therapeutic approach is certainly interesting and must be followed up by future experiments.

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Acknowledgment

We are thankful to Professor Stephan Becker (Institute of Virology, Philipps-University, Marburg) for kindly providing pCAGGS/ZEVGV-GP plasmid.

Statement of Ethics

Not applicable because only laboratory experiments are described.

Conflict of Interest Statement

The authors declare no conflict of interest.

Funding Sources

This work received no funding.

Author Contributions

M.J. conceptualized, planned, and performed the experiments, reviewed the literature, and drafted the manuscript. F.M. designed the plasmid constructs. C.W. supervised the research and wrote the MS. L.K.B. performed the killing assay. K.A., R.A., and M.H.E. performed the antigen capture assay. A.K. edited the figures. M.R.B. supervised the research and statistical analysis. A.C.A., K.M., and N.B. all authors contributed equally in reading and editing the MS.

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