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Development and characterization of two GP-specific monoclonal antibodies, which synergistically protect non-human primates against Ebola lethal infection

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ABSTRACT

Ebola fever is an acute highly contagious viral disease characterized by severe course, high mortality and development of hemorrhagic syndrome (tendency to skin hemorrhage and bleeding of mucous membranes). The mortality rate of the disease 60–90%. Nowadays, there are no licensed specific therapeutic agents for Ebola in the world. Monoclonal antibodies (MAbs) having viral neutralizing activity with high specificity to the GP protein of the Ebola virus are considered as candidate highly effective antiviral drugs.

In our study, for the first time a panel of mouse monoclonal antibodies specifically binding to EBOV GP protein was obtained using recombinant human adenovirus 5 serotype, expressing GP protein (Ad5-GP). The virus-neutralizing capacities of antibodies were evaluated on the Ebola virus cell infection model, as well as recombinant vesicular stomatitis virus pseudotyped by GP Ebola virus protein (rVSV-GP) cell infection model. Based on the results of virus neutralization, two most promising clones were selected, the specific and protective capacities of which were determined. The study of the protection of selected individual antibody clones, as well as their combinations on the model of lethal infection of rhesus macaques with Ebola virus showed that intravenous administration of a mixture of antibodies in the amount of 50 mg/kg 24 h after infection leads to the survival of 100% of the animals, while individual clones of antibodies possess partial protection (0–30%). The results of the study suggest the important role of antibodies in controlling replication of the Ebola virus in vivo and show the possibility of using a mixture of antibodies specific to the GP to protect against lethal infection with the Ebola virus in the post-infected mode of administration.

1. Introduction

The virus of Ebola hemorrhagic fever belongs to the family Filoviridae, genus Ebolavirus. The disease caused by EBOV (Zaire ebolavirus) viruses is accompanied by 60–90% mortality (Qiu et al., 2012a,b). Near the end of 2013, an outbreak of EBOV began in Guinea, subsequently spreading to neighboring Liberia and Sierra Leone (Kaner and Schaack, 2016). The total number of registered Ebola virus disease (EVD) cases were more than 28,000 of which more than 11,000 (40%) were fatal (Kaner and Schaack, 2016; CDC, 2019, https://www.cdc.gov). New outbreaks of EVD started in August 2018 in Congo and in June 2019 in Uganda (https://ecdc.europa.eu), thus EVD problem is currently particularly acute. However, there is no FDA approved anti-EBOV treatment available, but many drug candidates are currently being developed.

The key target of anti EBOV immunotherapy is a viral glycoprotein (GP), which is the most important factor of pathogenicity (Audet et al., 2014). Monoclonal antibodies (MAbs) with high specificity to the GP

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protein of the Ebola virus with viral neutralizing activity are considered as candidate highly effective antiviral drugs (Moekotte et al., 2016). Currently, about 20 MAbs to EBOV GP have been developed and characterized, which have demonstrated promising results in animal models studying (González-González et al., 2015). For example, MAb KZ52, isolated from the surviving human, successfully protected mice and Guinea pigs from lethal infection, but failing to protect nonhuman primates (NHPs) when administered as single antibody (Murin et al., 2014). The most promising drugs are cocktails from several MAbs: MB-003, ZMAb, MIL77E, MAb114/MAb100, which targeting to the EBOV GP and shown 100% protection on NHPs lethal Ebola virus infection model (Moekotte et al., 2016; Corti et al., 2016). The first drug to treat patients with Ebola fever was ZMapp, consisting of a cocktail of three chimeric mouse MAbs, developed at 2014. ZMapp antibodies were tested in human trials during the outbreak in West Africa and possible to achieve a full recovery of a number of patients (Moekotte et al., 2016). However, further studies have shown that ZMapp specifically neutralizes the Zaire strains, and has no effect on the Sudan strains (Moekotte et al., 2016).

Given that the GP is a critical and highly abundant viral protein, we sought to produce a set of mouse MAbs specific for multiple epitopes of EBOV GP and investigate its immunological capacities, as well as to study its neutralizing and protective properties on the model of lethal Ebola virus infection of non-human primates. For the first time to obtain immune library against EBOV GP, we used recombinant human adenovirus 5 carrying the gene of the protein GP of the Ebola virus (Ad5-GP), which showed high protection in experiments in vivo and was approved for clinical use in Russia according to the results of clinical studies (Dolzhikova et al., 2017). The use of Ad5-GP for mice immunization leads to expression of GP protein with its native conformation and glycan profile. Using this approach we selected two clones of MAb (2c8, 6g3) which in mixture showed high protective capacity against lethal infection non-human primates with the Ebola virus in the post-infected mode of administration.

2. Materials and methods

2.1. Viruses and antigens

Ad5-GP – Recombinant replication-defective adenovirus type-5 expressing GP protein (H.sapiens-wt/SLE/2014/Makona-G3735.1, GenBank accession no. KM233056) $2.5*10^{11}$ viral particles per dose has been generated as described previously (Dolzhikova et al., 2017; Shcherbinin et al., 2014);

VSV-GP – Live-attenuated recombinant vesicular stomatitis virus expressing Ebola virus glycoprotein (H.sapiens-wt/SLE/2014/Makona-G3735.1, GenBank accession no. KM233056) $2.5*10^{7}$ plaque forming units per dose has been generated as described previously (Dolzhikova et al., 2017);

Mucin-like domain of Ebola virus glycoprotein was expressed in E. coli and purified with HisTrap column (GE healthcare, Waukesha, USA);

The EBOV GP1 glycoprotein was generated by transient transfection of CHO–S cell line with recombinant plasmid pShuttle-CMV-GP1, containing gene of GP1 strain Ebola virus/H. sapiens-wt/SLE/2014/Makona-G3735.1, GenBank accession no. KM233056 (Makona) and purification with HisTrap column (GE healthcare, Waukesha, USA);

The EBOV GP glycoproteins strains H. sapiens-wt/GIN/2014/Kissidougou-C15(Kissidougou) and strain Mayinga 1976 (Mayinga) were obtained from Sino Biological (Beijing, China), cat. no. 40442-V02H and 40304-V08B1 respectively; The EBOV secreted glycoprotein (sGP) was obtained from IBT Bioservices (Rockville, USA), cat. no. 0565-001;

Ebola virus strain Zaire was obtained from 48 Central Research Institute, Ministry of Defense of Russian Federation, Sergiev Posad-6, Russia.

2.2. Cell lines

CHO–S cell line was obtained from Thermo Fisher Scientific (Waltham, USA) cat. no. R80007;

Vero E6 (ATCC CRL 1586), A549 (ATCC CCL 185), Sp2/0-Ag-14 (ATCC CRL 1581) and GMK-AH-1(D) (CVCL_L878) cell lines were obtained from Russian collection of vertebrate cell lines (Saint Petersburg, Russia).

2.3. MAbs production and purification

8 week-old BALB/c female mice 18–20 g obtained from the Pushchino Branch of the Institute of Bioorganic Chemistry, RAS (Pushchino, Russia). The Ad5-GP with 10^{8} pfu/ml (100 µl/mouse) was intramuscularly injected 3 times with 2 weeks intervals, without any adjuvants. Recombinant EBOV protein GP (H. sapiens-wt/GIN/2014/Kissidougou-C15) was used for booster immunization. A booster dose (50 µg/mouse) was administered intraperitoneal without any adjuvants, 4 days prior to spleen isolation. Suspension of mouse splenocytes was isolated by a standard method in a cooled growth medium without serum. Sp2/0-Ag-14 cells were used to generate hybridoma cell lines, according to the Kohler G. and Milstain C. method (Kohler and Milstain, 1975). After 12 days of growth in 96-well plates, the clones were tested for the presence of antibodies to the EBOV GP1 (H.sapiens-wt/SLE/2014/Makona-G3735.1) protein by ELISA. Clones with a specific activity were subcloned by limiting dilutions method in the presence of mice peritoneal macrophages and then again tested for the presence of specific antibodies. Antibodies isotypes determination was performed using the Mouse-Hybridoma-Subtyping Kit (Sigma), according to manufacturer's instruction. MAbs production was performed by standard cultivation methods with ADCF-MAb media (GE healthcare, Waukesha, USA). Purification of MAbs was performed using Mabselect Sure resin (GE healthcare, Waukesha, USA) and Sartobind Q (Sartorius stedim biotech, Goettingen, Germany). Concentrations of MAbs were evaluated with Nanodrop 2000c (Thermo Fisher Scientific, Waltham, USA).

2.4. Indirect ELISA

High binding polysterene microtitre plates (Greiner bio one, Frickenhausen, Germany) were coated with 100 ng of antigen per well in carbonate-bicarbonate buffer at 4°C overnight. Next day, MAbs diluted in blocking solution (100 µl) or cultural media were added and incubated at 37°C for 1 h. After washing the plates 5x, the peroxidase-conjugated affinity purified goat anti-mouse IgG detection antibody (Sigma) diluted 1:5000 in blocking solution was added for 1 h at 37°C. After washing 5x the substrate TMB was added and the plates were incubated at room temperature for 15 min and then, reaction was stopped with 1N H2SO4. The OD signals were determined with Varioskan Lux (Thermo Fisher Scientific, Waltham, USA) at 450 nm.

2.5. Cross-inhibition assay

Conjugation of MAbs with horseradish peroxidase was performed according to the Tjissen P. method (Tjissen, 1985).

The activity of conjugates was evaluated with cross-inhibition assay using the immobilized EBOV GP1 (Makona). High binding polysterene microtitre plates (Greiner bio one) were coated with 100 ng of antigen per well in carbonate-bicarbonate buffer at 4°C overnight. Next day, MAbs diluted in blocking solution (100 µl) were added at 0.1 mg/ml concentration and incubated for 1 h at 37°C. Then conjugated with horseradish peroxidase MAbs were added at dilutions to obtain an optical density within 1–1.5 in the interaction with the antigen in the absence of competing antibodies. Determination of MAbs activity was provided by spectrophotometer Varioskan Lux (Thermo Fisher Scientific, Waltham, USA) at 450 nm. Cross-inhibition between MAbs

(CIM) is calculated by the formula:

CIM = 100 – (A 450 samples with competing MAb/A 450 samples without competing MAb) x 100

2.6. MAbs kinetic binding analysis by Biolayer Interferometry (BLI)

The OctetRed system (Pall Biotech, New-York, USA) was used to measure binding affinity of 2c8 and 6g3 with different EBOV GP strains. Anti-Mouse IgG Fc Capture (AMC) Dip and read biosensors (Pall Biotech, New-York, USA) were used to capture 2c8 or 6g3 respectively at 1x kinetics buffer (PBS supplemented with 0.002% Tween-20 and 1 mg/ml BSA). Binding of test EBOV GPs to 2c8 and 6g3 were also performed at 1x kinetics buffer with MAbs concentration 100 μg/ml and EBOV GPs concentrations 50, 25, 12.5, 6.25, 3.125, 1.525, 0.78 mg/ml, respectively. The base lines and dissociations were carried out in 1x kinetics buffer according to the manufacturer's recommendations. Data Analysis 10.0 program software was used for KD evaluations.

2.7. Virus plaque reduction assay (PRNT50)

Dilutions of the virus (VSV-GP or Ebola) were prepared with buffer (10 mM Tris-HCl pH = 7.5, 1 mM EDTA, 10% sucrose). A mixture of equal volumes of MAbs and virus stocks was incubated for 60 min at 37°C and then applied to a one-day monolayer of Vero E6 (for VSV-GP) or GMK-AH-1(D) (for Ebola) cell plates. After adsorption of the MAbs + virus complex on the cells for 120 min at 37°C, an agar coating was applied. Plates were incubated at 37°C, 5% CO2 for 48 h (Vero E6) or 7 days (GMK-AH-1(D)). Results were evaluated by counting the plaque number under a microscope. The assay was performed in triplicate. The following formula was used to determine plaque forming units

(PFU): PFU/ml = (mean PFU count/0.2 ml) × dilution factor

2.8. Immunofluorescence assay (IFA)

The A549 cells were seeded at density 10^6 cells per well to 6-well cultural plates with sterile cover glasses in DMEM (10% FBS and 4 mM Glutamine) and cultivated overnight at 37°C and 5% CO2. Next day, cells were transduced with Ad5-GP (1 pfu/cell) and cultivated 5 days at 37°C and 5% CO2. After 5 days of cells cultivation IFA was performed using 2c8 and 6g3 clones and secondary antibodies conjugated with fluorescent dye (Dylight® 488) as described previously (Tutykhina et al., 2018).

2.9. Passive immunization and protection in non-human primates

30 healthy male Rhesus macaques (*Macaca mulatta*; 2.0–2.9 kg) were obtained from Scientific Research institute of Medical Primatology (Veseloe, Russia) and challenged with 15LD50 (1000 pfu according to Dolzhikova et al., 2017) of EBOV (1 ml each intramuscularly). 24 h after challenge animals were treated intravenously with purified MAbs (50 mg/kg). Intravenous infusion of MAbs were repeated 5 times on days 1,3,5,7 and 9 respectively. For the immobilization of animals before treatment Zoletil 100 (1,5 mg/kg) was used. The infected and control animals were monitored for 30 days. Specificity of animal death was determined by the presence of the virus in the liver suspension. Negative colonies of specific morphology formed by the Ebola virus in cell culture testified to the specificity of animal death.

2.10. Statistical analysis

Data were analyzed in EXCEL 2010 and STATISTICA 7.0. For antibody titers study The Mann ± Whitney *U* test was used to evaluate differences in antibody titers in ELISA. Changes in survival were measured by calculating percentage compared with baseline in each group. Comparison of survival used Mann-Whitney test and Gehan-Wilcoxon test. $p < 0.05$ indicates statistical significance.

2.11. Ethics statement

The experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication #85–23, revised 1996) and National Standard of the Russian Federation GOST R 53434–2009, approved by the Institutional Animal Care and Use Committee (IACUC) of the Federal Research Centre of Epidemiology and Microbiology named after Honorary Academician N.F. Gamaleya. All persons using or caring for animals in research underwent yearly training as required by the IACUC.

3. Results

3.1. Characterization of MAbs

The panel of MAbs specific for the EBOV GP was generated. The 8 weeks old BALB/c female mice were immunized with Ad5-GP intramuscularly 3 times with 2 weeks interval. For boost immunization EBOV GP (H.sapiens-wt/GIN/2014/Kissidougou-C15) was used at concentration 50 μg/mice (intraperetaneously without any adjuvants 2 weeks after the last Ad5 administration). 4 days after the last immunization spleen and splenocytes were isolated. The scheme of mice immunization is shown on Fig. 1. Sp2/0-Ag-14 myeloma cell line and isolated splenocytes were used for hybridization according Kohler and Milstain, 1975. The limited dilution method was performed for obtaining the panel of hybridoma clones.

During the hybridization 600 hybridoma cell lines were obtained. After the initial screening with indirect ELISA, 49 hybridoma clones producing specific antibodies to GP1 (with OD450 ≥ 2.5) were selected. At the next step clones were evaluated in a light microscope by cell size and condition, as well as by OD450(≥3.0) in indirect ELISA

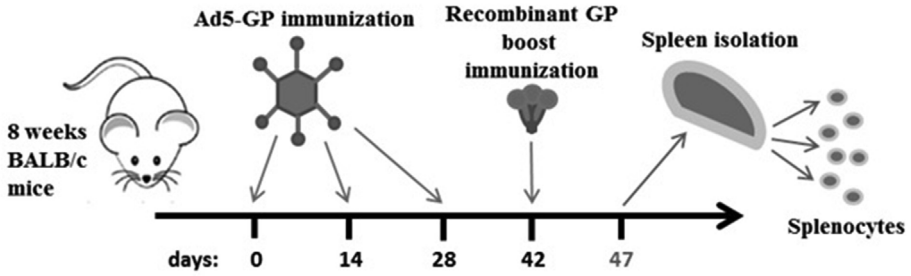

Fig. 1. Scheme of mice immunization for generation of hybridoma cell lines. BALB/c female mice (8 weeks) were immunized intramuscularly with Ad5-GP 3 times with 2 weeks interval and then boosted with EBOV GP (H.sapiens-wt/GIN/2014/Kissidougou-C15) 50 μg/mice.

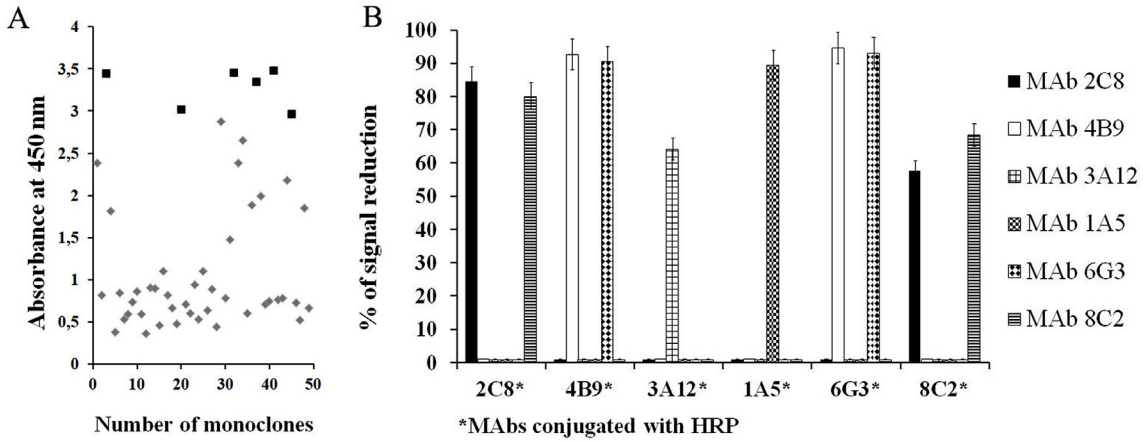

Fig. 2. Results of screening of 49 hybridoma cell lines (A) and cross inhibition assay of 6 selected MAbs (B).
A. High binding polysterene microtitre plates were coated with 100 µl of EBOV GP1 (H.sapiens-wt/SLE/2014/Makona-G3735.1). Next day, cultural media from hybridoma clones were added and incubated at 37 °C for 1 h. After washing, the peroxidase-conjugated affinity purified goat anti-mouse IgG detection antibody was added for 1 h at 37 °C. After washing 5x the substrate TMB was added and results were evaluated. B. High binding polysterene microtitre plates were coated with 100 µl of EBOV GP1 (H.sapiens-wt/SLE/2014/Makona-G3735.1). Next day wells were washed 5x with 0,1% TPBS and then blocked with 5% non-fat drying milk on TPBS. MAbs diluted in blocking solution were added at 0.1 mg/ml concentration and incubated for 1 h at 37 °C. Then MAbs conjugated with horseradish peroxidase were added at dilutions to obtain an optical density within 1–1.5 in the interaction with the antigen in the absence of competing antibodies.

Table 1
Primary screening of selected MAbs clones with virus-neutralization and isotyping assays.

MAbs clones	Ebola virus plaque assay	VSV-GP plaque assay	Heavy chain isotype	Light chain isotype
2c8	neutralization	lack of neutralization	IgG2a	kappa
4b9	neutralization	neutralization	IgG1	kappa
3a12	lack of neutralization	lack of neutralization	IgG1	kappa
1a5	lack of neutralization	lack of neutralization	IgG2b	kappa
6g3	neutralization	neutralization	IgG1	kappa
8c2	neutralization	lack of neutralization	IgG1	kappa

(Fig. 2A). Finally 6 clones (2c8, 3a12, 6g3, 8c2, 1a5, 4b9) were selected, antibodies produced and purified at approximately 5–10 mg quantity. Then EBOV virus-neutralizing activity of purified clones was evaluated using VSV-GP and Ebola virus plaque assays (Table 1). For that purpose about 110 pfu of virus (EBOV or VSV-GP) and about 100 µg of MAbs were mixed and added to GMK-AH-1(D) or Vero E6 cells, respectively. Isotypes of heavy and light chains of 6 clones were also identified (Table 1). The results indicated four MAbs with pronounced EBOV virus-neutralizing activity and two MAbs with pronounced VSV-GP virus-neutralizing activity.

Therefore 1a5 and 3a12 clones were excluded from further studies and epitopes binding specificity of the others 2c8, 8c2, 6g3 and 4b9 clones was evaluated using cross inhibition assay. The results suggest that only two of four clones have the ability to bind different EBOV GP epitopes (Fig. 2B). Apparently 6g3 and 4b9 bind the same EBOV GP epitope as well as 2c8 and 8c2, so clones 6g3 and 2c8 were chosen for the next studies.

3.2. Characterization of MAbs with different EBOV GPs

First, specificity of 2c8 and 6g3 clones to different EBOV GPs: Kissidougou, Mayinga, Makona, mucin-like domain of GP and sGP were characterized using indirect ELISA method. Commercially available anti-EBOV GP MAb (clone c13c6 FR1) (0201-023, IBT Bioservices) was used as a control to evaluate antigenic properties of different EBOV GPs (Fig. 3C). The results indicated that clone 2c8 authentically bound to Mayinga, Makona, mucin-like domain of GP and Kissidougou at titers 1 ng/ml, 1 ng/ml, 3.9 ng/ml and 1 mkg/ml, respectively, and didn't bind to sGP as well as BSA (negative control). In turn clone 6g3 bound to Mayinga, Kissidougou, Makona and sGP at titers 250 ng/ml, 1 ng/ml, 1 ng/ml and 3,9 ng/ml respectively and didn't bind to mucin-like domain of GP. The indirect ELISA results are shown on Fig. 3.

To investigate the capacity of 2c8 and 6g3 binding to EBOV GP expressed on cell surface, IFA was used. A549 cells were transduced with Ad5-GP at 1 pfu/cell. After 5 days IFA with 6g3 and 2c8 was performed. The results indicated that cells transduced with Ad5-GP and treated with 2c8 and secondary antibodies or treated with 6g3 and secondary antibodies had strong green fluorescence unlike negative control cells (Fig. 4).

Then affinity constants of 2c8 or 6g3 and different EBOV GPs were measured by Biolayer Interferometry with OctetRed96. The results suggest that clone 2c8 demonstrates high affinity to EBOV GPs Mayinga, Makona and low affinity to Kissidougou and 6g3 demonstrates high affinity to EBOV GPs Kissidougou, Makona and lower affinity to GP Mayinga respectively (Table 2).

Before animal experiments PRNT50 of 2c8 and 6g3 was determined with EBOV. For that purpose the following dilutions of MAbs: 40, 8, 4, 2.5, 1.25 and 0 µg/ml, were mixed with 110 pfu of EBOV and added to GMK-AH-1 cells. After 7 days of cultivation the results were evaluated (Fig. 5). MAbs dilutions that reduced > / = 50% of plaques compare to negative control were taken for PRNT50. PRNT50 was evaluated to be at least 8 µg/ml for clone 2c8, at least 4 µg/ml for clone 6g3 and, the most important, at least 1,25 µg/ml for mix of both clones.

All data suggest that 6g3 and 2c8 demonstrate significant affinity to different EBOV GP and potentially protect against the Ebola virus.

3.3. Passive therapy with characterized MAbs

Finally, protective capacity of 2c8 and 6g3 clones was evaluated with passive intravenous immunization of male Rhesus macaques, challenged intramuscularly with 15LD50 of EBOV 24 h before therapy. The experiment included groups threated with 2c8 or 6g3 separately

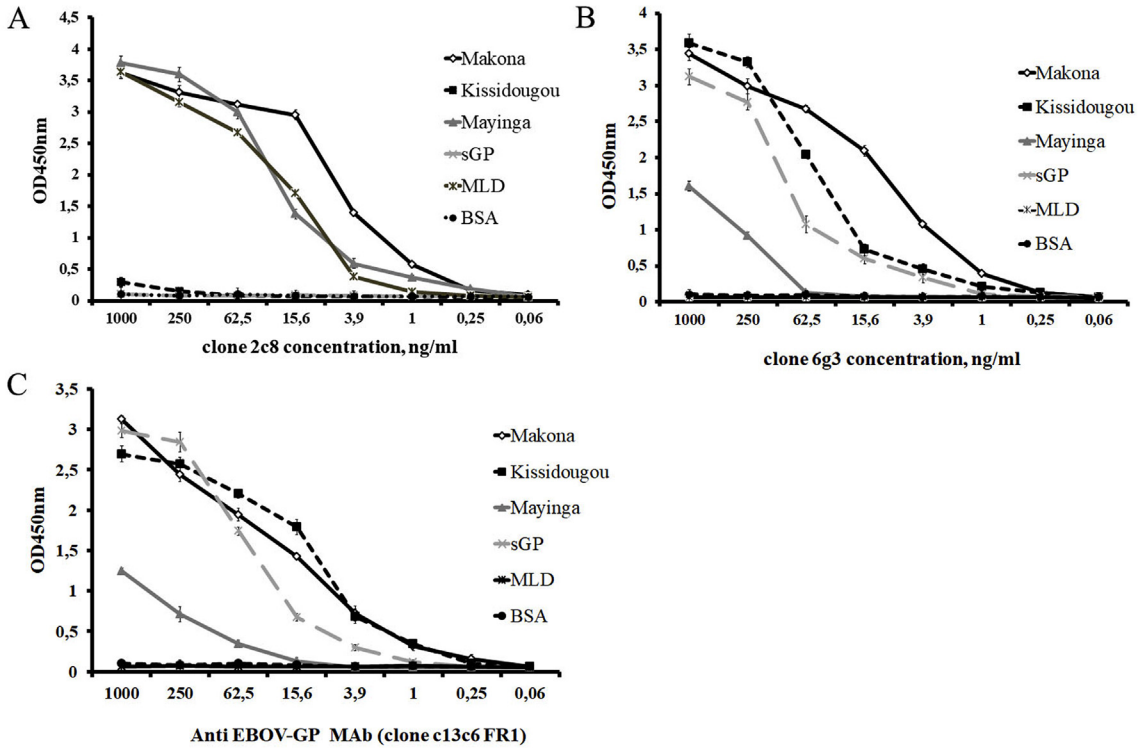

Fig. 3. Determination of 2c8 (A), 6g3 (B) and anti-EBOV GP (clone c13c6 FR1) (C) MAbs titers on different EBOV GPs.
Makona – GP of strain Makona, Kissidougou – GP of strain Kissidougou, Mayinga – GP of strain Mayinga, sGP – secreted form of GP, MLD – mucin-like domain of GP, BSA – bovine serum albumin.
High binding polysterene microtitre plates were coated with 100 µl of different EBOV GPs. Next day wells were washed 5x with 0,1% TPBS and then blocked with 5% non-fat drying milk on TPBS. Different dilutions of MAbs (2c8, 6g3 or c13c6 FR1) in blocking solution (100 µl) were added and incubated at 37 °C for 1 h. After washing the plates 5x the goat anti-mouse IgG antibodies (A9044-2 ML, Sigma) (for 6g3 and 2c8 detection) or the goat anti-human IgG antibodies (A0170, Sigma) (for c13c6 FR1) in blocking solution were added for 1 h at 37 °C. After washing 5x the substrate TMB was added and results were evaluated.

and group threated with both 2c8 and 6g3 to evaluate potential synergistic capacity of two MAbs. Intact animals were used as positive control and animals which received placebo (PBS) were used as negative controls. The animals from 6g3, 2c8 and 6g3 + 2c8 groups were treated with respective MAbs (50 mg/kg) 5 times on days 1,3,5,7 and 9 after challenge respectively (Fig. 6). The results show that the animals from the positive control group survived till the end of the experiment. Animals from the viral dose control and the placebo groups all died on days 8 and 10 respectively. Animals treated with 2c8 all died on day 12, whereas only 2 of 3 animals treated with 6g3 died and one animal survived till the end of the experiment. The best results were obtained

Table 2
Affinity KD of 6g3 and 2c8 with different EBOV GPs.

Clone	EBOV GPs			BSA
	Kissidougou	Mayinga	Makona	
6G3	$3.4*10^{-9}$ M	$2,2*10^{-8}$ M	$2.0*10^{-9}$ M	no binding
2C8	$1.02*10^{-7}$ M	$1,6*10^{-9}$ M	$5,4*10^{-9}$ M	no binding

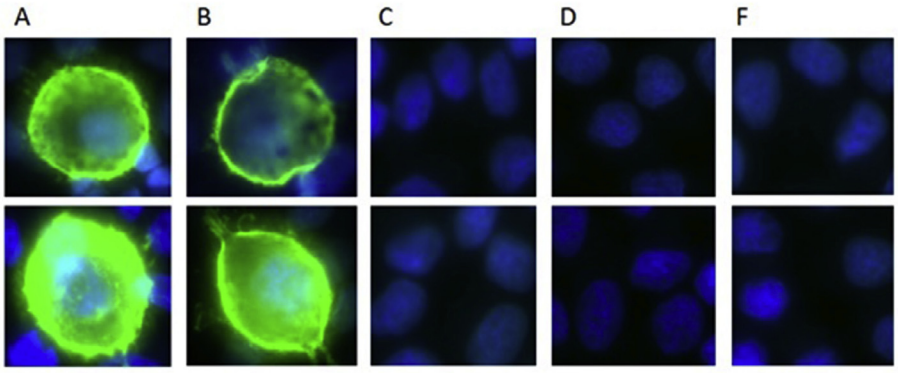

Fig. 4. Results of IFA with A549 cells, transduced with Ad5-GP and treated with 2c8 or 6g3.
Experiment was performed in duplicate – top and bottom row. A. – Cells, transduced with Ad5-GP and treated with 6g3 and conjugate; B. – Cells, transduced with Ad5-GP and treated with 2c8 and conjugate; C. – Cells, transduced with Ad5-GP and treated with 2c8 or 6g3 without conjugate; D. – non transduced cells treated with 2c8 or 6g3 with conjugate; F. – Cells, transduced with Ad5-GP and treated with conjugate only.
The A549 cells were seeded at density 10^6 cells per well on a 6-well plate with sterile cover glasses. Next day cells were transduced with Ad5-GP and cultivated 5 days and then were fixed with 0,1% glutaraldehyde follow by washing 5 times with TPBS. 2c8 and 6g3 clones were added to wells at 10 µg/ml concentration in blocking buffer and incubated for 1 h at 37°C and follow washed 5 times with TPBS. Secondary antibodies conjugated with fluorescent dye (Dylight® 488) were added 1:500 in blocking buffer. After 5 times washing DAPI stain was added to wells and incubated 30 min at 20–25°C. Finally, cover glasses with cells were up taken from plates and visualized under UV using CARL ZEISS Axio Imager Z1 (Oberkochen, Germany). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

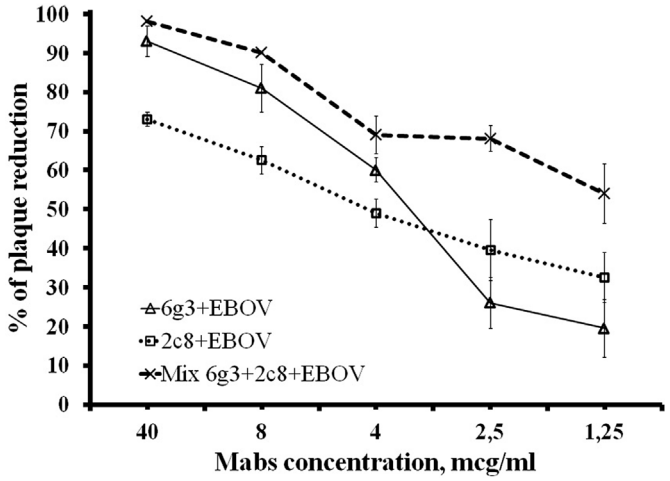

Fig. 5. Results of virus-neutralization assay with EBOV and 2c8 or 6g3 clones or its mix respectively.
Ebola virus strain Zaire dilutions were prepared with a concentration of 110 pfu/ml. A mixtures of equal amounts of MAbs (40, 8, 4, 2.5, 1.25, 0 μg/ml, respectively) and Ebola virus were incubated for 60 min at 37 °C, then 0.5 ml was applied to a 1-day monolayer of GMK-AH-1(D) cells, pre-draining the growth medium and after adsorption of the complexes on the cells for 60 min at 37 °C, decanted then applied to the primary agar coating. After 7 day the infected monolayer of cells was stained with 0.1% neutral red and was incubated for 48 h at 37 °C, the results were evaluated under microscope.
EBOV –Ebola virus strain Zaire; 6g3 – clone 6g3; 2c8 – clone 2c8.

from the group treated with 6g3 and 2c8 together, where no animals died till the end of the experiment, suggesting a synergistic effect of MAbs protective capacity. The results of the experiment are shown on Fig. 8.

Thus, as a result of the experiment 100% survival rate of infected animals was demonstrated by treatment with a cocktail of 2 monoclonal antibodies one of which is specific to the mucin domain of the GP protein.

4. Discussions

Drug development for Ebola treatment is still an urgent task. Studies have been conducted showing the successful use of monoclonal antibodies for Ebola therapy (Qiu et al., 2012a,b; Wec et al., 2019). In this case the most suitable target is the surface glycoprotein GP of Ebola virus which is the most impotent protein for viral pathogenesis. It was shown that antibodies specific to GP protein have protective activity against lethal infections of mice, Guinea pigs and non-human primates (Qiu et al., 2012a,b; Wec et al., 2019).

In our study we generated a panel of monoclonal antibodies specifically binding to the GP protein of the Ebola virus using for the first time a recombinant Ad5-GP to express the GP protein with native conformation and glycan profile. Further in accordance with ELISA neutralizing activity and cross-inhibition assay two most perspective clones (2c8 and 6g3) were selected. In addition in the process of studying the clone's isotypes, it was found that 2c8 belongs to the IgG2a isotype, and 6g3 to IgG1 isotype respectively which can presumably activate various effector functions of antibodies which are implemented through the Fc-fragment with the elimination of the virus. Thus, 2 of the most promising clones 6g3 and 2c8 with specificity to various epitopes of the protein and GP belonging to different isotypes of immunoglobulins were selected for further study.

As a result of further studies it was found that the 6g3 clone specifically binds to the GP proteins of the Makona, Mayinga and Kissidougou strains as well as to the secreted form of the GP protein and does not bind to the mucin-like domain. Thus, it can be assumed that clone 6g3 interacts with the epitope of the GP1 protein and the amino acid residues formed in the region with 33–295 amino acids (Fig. 8). Unlike 6g3, clone 2c8 does not interact with the secreted form of the GP protein but interacts well with its mucin-like domain. As 6g3, clone 2c8 interact well with the protein GP of Makona, Mayinga and weaker with GP Kissidougou. Consequently the 2c8 clone interacts presumably with an epitope in the mucin domain of the GP1 protein (Fig. 8). In addition the results show the presence cross-reactivity to GP protein of the different strains of EBOV, by how 2c8 and 6g3 antibodies interact not only with strains Kissidougou and Makona but also with GP protein of the Mayinga strain which is important in the case of prevention against different EBOV strains.

As a result IFA demonstrated the ability of the selected antibodies to interact with the GP protein on the surface of the infected cell which serves as an indirect confirmation of the ability of 2c8 and 6g3 clones to bind GP protein during natural infection (Fig. 5 and Table 3).

Data of Biolayer Interferometry (BLI) for clone 6g3 is quite correlated with the data of ELISA where the titers on Mayinga GP were significantly lower than on GP of Makona and Kissidougou strains (Table 3). Similarly, the clone 2c8 affinity to GP of Makona and Mayinga strains was approximately the same as titers in ELISA and very low affinity and signals to GP of strain Kissidougou (Table 3). Thus, a sufficiently high specificity and affinity of the selected clones to various EBOV GP proteins with BLI was demonstrated.

After determining the concentration of antibodies capable of suppressing viral activity in the plaque formation assay on the EBOV, it was found that clone 2c8 has a 50% virus-neutralizing activity at a dose of at least 8 μg/ml and 6g3 at least 4 μg/ml respectively (Fig. 5 and Table 1). It is interesting that the mix of two clones showed the highest virus-neutralizing activity at a dose of at least 1,25 μg/ml, which quite correlates with the data of the animals experiment, discussing bellow.

The protective capacities of antibodies were demonstrated from 0 to 100%, with passive immunization of rhesus macaques at a dose of 50 mg/kg, with a 5-fold scheme of administration on 1,3,5,7 and 9 days after infection with a lethal dose of 15LD50 Ebola virus (Fig. 7). A dose of 50 mg/kg was chosen according studying of different EBOV neutralizing MAbs (Moekotte et al., 2016). In the group of animals that

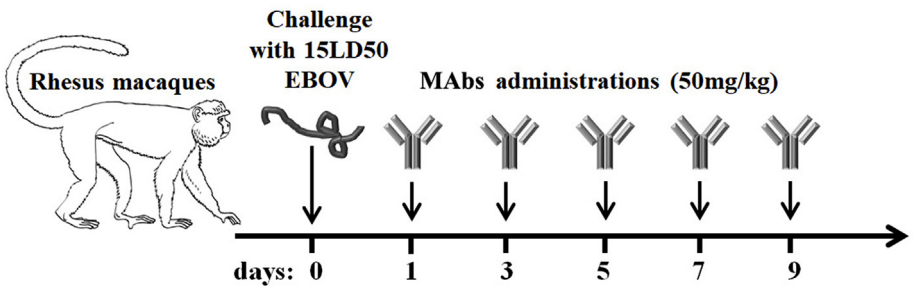

Fig. 6. Scheme of animal challenge with EBOV lethal dose and passive immunization with MAbs.
Rhesus macaques received 15LD50 of EBOV (1 ml each intramuscularly) and 24 h after challenge, animals were treated intravenously with MAbs (50 mg/kg) on days 1,3,5,7 and 9 respectively.

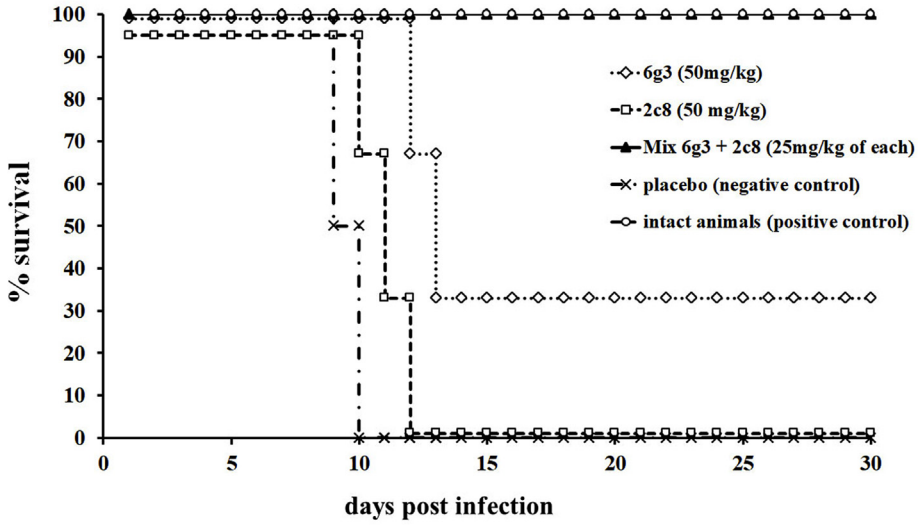

Fig. 7. Protective activity of MAbs.
6g3 – rhesus macaques administered with clone 6g3 after challenge; 2c8 – rhesus macaques administered with clone 2c8 after challenge; 6g3 + 2c8 – rhesus macaques administered with both 2c8 and 6g3 clones after challenge; placebo – animals received PBS as negative control; intact animals – control of animals keeping (positive control).
Animals from the 1st group were treated intravenously with 6g3- (50 mg/kg), animals from the 2nd group were treated intravenously with 2c8- (50 mg/kg) and animals from the 3rd group were treated intravenously with 6g3- & 2c8-(50 mg/kg) 24 h after challenge with 15LD50 of EBOV (1 ml each intramuscularly). The 4th group received PBS as placebo and the 5th group included intact animals as positive control. Specificity of animal death was determined by the presence of the virus in the liver suspension. Negative colonies of specific morphology formed by the Ebola virus in cell culture testified to the specificity of animal death.

Fig. 8. Presumptive scheme of 2c8 and 6g3 interaction with different parts of EBOV GP.
2c8 and 6g3 – Mabs clones; sp – signal peptide; GP1, GP2 – GP protein subunits; sGP – secreted form of GP; mucin – mucin part of GP1; 1, 33, 295, 312, 501 – amino acid residues numbers.

received 2c8, death was observed on 10,11 and 12 days respectively which indicates a very weak protective activity of this clone or even its absence, since the delay in death was insignificant (2–3 days) if compare with control group. In the group of animals treated with 6g3 alone, 33% survival rate of primates and delayed death of the remaining individuals for 4–5 days was observed, which indicates the presence of protective properties in clone 6g3 but not sufficient to provide full protection against 15LD50 at a dose of 50 mg/kg and 5-fold administration scheme. Important data were obtained in the group that received both 2c8 and 6g3 where 100% survival rate with no clinical manifestation of EBOV disease was observed. These data demonstrate a pronounced additive effect on protection against the lethal dose of Ebola virus when two clones are used in cocktail. Importantly the additive protective activity of antibody, specifically binding mucin domain of GP, in the mix with antibody to another epitope of GP, was shown for the first time.

Perhaps this phenomenon is due to the fact that 6g3 interacts with the GP protein epitope which plays a key role in the cellular pathogenesis of the virus. However, apparently, this antibody is not able to completely block the interaction of the GP protein with the cell receptor and thus has only partial protection. Clone 2c8, on the contrary, apparently interacts with the GP protein epitope, binding to which does not interfere with the implementation of the cellular pathogenesis of the virus. In the case of using a cocktail of two clones, it is likely that the overlap of the GP protein epitopes involved in the pathogenesis of the virus occurs most fully in consequence of which, there is a pronounced additive effect and 100% protection of primates. In addition the 2c8 clone has an IgG2a isotype, which should cause high affinity to Fc receptors, and therefore a high ADCC (antibody-dependent cell cytotoxicity), ADCP (antibody-dependent cellular phagocytosis) and CDC (complement-dependent cytotoxicity) activity (Beers et al., 2016). Thus, despite the fact that individually clone 2c8 is not protective, it may provide an additive effect in a cocktail with 6g3 by activation of Fc-mediated functions of the immune system and, thus, together they provide 100% protection of primates.

Thus, in our work, the possibility of generating protective mouse monoclonal antibodies against Ebola virus was demonstrated for the first time by primary immunization of mice with recombinant human adenovirus 5 serotype expressing EBOV GP and subsequent booster immunization with recombinant EBOV GP. We selected two monoclonal antibodies with virus-neutralizing activity against the lethal strain of Ebola virus. Finally, the protective capacities of two antibodies were determined on the model of lethal Ebola infection of nonhuman primates. We have shown that the cocktail of the two MAbs binding different GP epitopes with one of the epitopes acting as a mucin domain has the greatest protective activity in post-infected mode of administration. On the basis of 2c8 and 6g3 clones, the drug GamEMab was developed and the first phase of clinical trials was completed (ClinicalTrials.gov: NCT03428347).

Table 3
Summary of immunogenic capacities of MAbs.

Assay	Protein	Clone 2c8	Clone 6g3
Elisa (titers)	GP Makona	positive signal at conc. 1 ng/ml	positive signal at conc. 1 ng/ml
	GP Maiynga	positive signal at conc. 1 ng/ml	positive signal at conc. 250 ng/ml
	GP Kissidougou	positive signal at conc. 1 mkg/ml	positive signal at conc. 1 ng/ml
	sGP	negative	positive signal at conc. 1 ng/ml
	MLD	positive signal at conc. 3,9 ng/ml	negative
Immunofluorescence assay (IFA)	GP on cells surface	bind GP on cell surface	bind GP on cell surface
Biolayer Interferometry (BLI)	GP Kissidougou	affinity constatnt, KD = 1.02e-7	affinity constatnt, KD = 3.4e-9
	GP Makona	affinity constatnt, KD = 5.4e-9	affinity constatnt, KD = 2.0e-9
	GP Maiynga	affinity constatnt, KD = 1.6e-9	affinity constatnt, KD = 2.2e-8

Declaration of competing interestCOI

The authors report no potential conflicts of interest.

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References

Audet, J., Wong, G., Wang, H., Lu, G., Gao, G.F., Kobinger, G., Qiu, X., 2014. Molecular characterization of the monoclonal antibodies composing ZMAb: a protective cocktail against Ebola virus. Sci. Rep. 4, e6881. https://doi.org/10.1038/srep06881.

Beers, S.A., Glennie, M.J., White, A.L., 2016. Influence of immunoglobulin isotype on therapeutic antibody function. Blood 127 (9), 1097–1101. https://doi.org/10.1182/blood-2015-09-625343.

CDC, 2019. 2014-2016 Ebola Outbreak in West Africa. URL: https://www.cdc.gov/vhf/ebola/history/2014-2016-outbreak/index.html, Accessed date: 9 August 2019.

Clinicaltrials, 2019. An open, dose escalation safety and pharmacokinetics study of the medicine for ebola fever emergency prevention based on monoclonal recombinant antibodies with a single application in 3 groups of healthy volunteers. URL: https://clinicaltrials.gov/ct2/show/NCT03428347?term=GamEmab&rank=1, Accessed date: 22 August 2019.

Corti, D., Misasi, J., Mulangu, S., Stanley, D.A., Kanekiyo, M., Wollen, S., Ploquin, A., Doria-Rose, N.A., Staupe, R.P., Bailey, M., Shi, W., Choe, M., Marcus, H., Thompson, E.A., Cagigi, A., Silacci, C., Fernandez-Rodriguez, B., Perez, L., Sallusto, F., Vanzetta, F., Agatic, G., Cameroni, E., Kisalu, N., Gordon, I., Ledgerwood, J.E., Mascola, J.R., Graham, B.S., Muyembe-Tamfun, J.J., Trefry, J.C., Lanzavecchia, A., Sullivan, N.J., 2016. Protective monotherapy against lethal Ebola virus infection by a potently neutralizing antibody. Science 351, 1339–1342. https://doi.org/10.1126/science.aad5224.

Dolzhikova, I.V., Zubkova, O.V., Tukhvatulin, A.I., Dzharullaeva, A.S., Tukhvatulina, N.M., Shcheblyakov, D.V., Shmarov, M.M., Tokarskaya, E.A., Simakova, Y.V., Egorova, D.A., Scherbinin, D.N., Tutykhina, I.L., Lysenko, A.A., Kostarnoy, A.V., Gancheva, P.G., Ozharovskaya, T.A., Belugin, B.V., Kolobukhina, L.V., Pantyukhov, V.B., Syromyatnikova, S.I., Shatokhina, I.V., Sizikova, T.V., Rumyantseva, I.G., Andrus, A.F., Boyarskaya, N.V., Voytyuk, A.N., Babira, V.F., Volchikhina, S.V., Kutaev, D.A., Bel'skih, A.N., Zhdanov, K.V., Zakharenko, S.M., Borisevich, S.V., Logunov, D.Y., Naroditsky, B.S., Gintsburg, A.L., 2017. Safety and immunogenicity of GamEvac-Combi, a heterologous VSV- and Ad5-vectored Ebola vaccine: an open phase I/II trial in healthy adults in Russia. Hum. Vaccines Immunother. 13 (3), 613–620. https://doi.org/10.1080/21645515.2016.1238535.

ECDC, 2019. Ebola outbreak in the democratic Republic of the Congo – ongoing. URL: https://ecdc.europa.eu/en/ebola-virus-disease-outbreak-democratic-republic-congo-ongoing, Accessed date: 9 August 2019.

González-González, E., Alvarez, M.M., Márquez-Ipiña, A.R., Trujillo-de Santiago, G., Rodríguez-Martínez, L.M., Annabi, N., Khademhosseini, A., 2015. Anti-Ebola therapies based on monoclonal antibodies: current state and challenges ahead. Crit. Rev. Biotechnol. 26, 1–16. https://doi.org/10.3109/07388551.2015.1114465.

Kaner, J., Schaack, S., 2016. Understanding ebola: the 2014 epidemic. Glob. Health 12 (1), 53. https://doi:10.1186/s12992-016-0194-4.

Kohler, G., Milstain, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497. https://doi:10.1038/256495a0.

Moekotte, A.L., Huson, M.A., van der Ende, A.J., Agnandji, S.T., Huizenga, E., Goorhuis, A., Grobusch, M.P., 2016. Monoclonal antibodies for the treatment of Ebola virus disease. Expert Opin. Investig. Drugs 25 (11), 1325–1335. https://doi.org/10.1080/13543784.2016.1240785.

Murin, C.D., Fusco, M.L., Bornholdt, Z.A., Qui, X., Olinger, G.G., Zeitlin, L., Kobinger, G.P., Ward, A.B., Saphire, E.O., 2014. Structures of protective antibodies reveal sites of vulnerability on Ebola virus. Proc. Natl. Acad. Sci. U.S.A. 111, 17182–17187. https://doi.org/10.1073/pnas.1414164111.

Qiu, X., Fernando, L., Melito, P.L., Audet, J., Feldmann, H., Kobinger, G., Alimonti, J.B., Jones, S.M., 2012a. Ebola GP-specific monoclonal antibodies protect mice and Guinea pigs from lethal Ebola virus infection. PLoS Neglected Trop. Dis. 6 (3) e1575 (a). https://doi:10.1371/journal.pntd.0001575.

Qiu, X., Audet, J., Wong, G., Pillet, S., Bello, A., Cabral, T., Strong, J.E., Plummer, F., Corbett, C.R., Alimonti, J.B., Kobinger, G.P., 2012b. Successful treatment of ebola virus-infected cynomolgus macaques with monoclonal antibodies. Sci. Transl. Med. 4 (138) 138ra81 (b). https://doi:10.1126/scitranslmed.3003876.

Shcherbinin, D.N., Esmagambetov, I.B., Noskov, A.N., Selyaninov, Y.O., Tutykhina, I.L., Shmarov, M.M., Logunov, D.Y., Naroditskiy, B.S., Gintsburg, A.L., 2014. Protective immune response against Bacillus anthracis induced by intranasal introduction of a recombinant adenovirus expressing the protective antigen fused to the Fc-fragment of IgG2a. Acta Naturae 6 (1), 76–84.

Tjissen, P., 1985. Practical and theory of enzyme immunoassay. Lab. Tech. Biochem. Mol. Biol. 280–294.

Tutykhina, I., Esmagambetov, I., Bagaev, A., Pichugin, A., Lysenko, A., Shcherbinin, D., Sedova, E., Logunov, D., Shmarov, M., Ataullakhanov, R., Naroditsky, B., Gintsburg, A., 2018. Vaccination potential of B and T epitope-enriched NP and M2 against Influenza A viruses from different clades and hosts. PLoS One 13 (1), e0191574. https://doi.org/10.1371/journal.pone.0191574.

Wec, A.Z., Bornholdt, Z.A., He, S., Herbert, A.S., Goodwin, E., Wirchnianski, A.S., Gunn, B.M., Zhang, Z., Zhu, W., Liu, G., Abelson, D.M., Moyer, C.L., Jangra, R.K., James, R.M., Bakken, R.R., Bohorova, N., Bohorov, O., Kim, D.H., Pauly, M.H., Velasco, J., Bortz, R.H., Whaley, K.J., Goldstein, T., Anthony, S.J., Alter, G., Walker, L.M., Dye, J.M., Zeitlin, L., Qiu, X., Chandran, K., 2019. Development of a human antibody cocktail that deploys multiple functions to confer Pan-ebolavirus protection. Cell Host Microbe 25 (1), 39–48e5. https://doi:10.1016/j.chom.2018.12.004.