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A Novel Ebola Virus Antibody-Dependent Cell-Mediated Cytotoxicity (Ebola ADCC) Assay

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Abstract

Ebolaviruses are highly virulent pathogens that cause Ebola viral disease (EVD). Data from nonhuman primate (NHP) models and from human survivors of EVD suggest that anti-Ebola antibodies play an integral role in protection. Antibody-dependent cell-mediated cytotoxicity (ADCC) is a potential mechanism through which anti-Ebola antibodies may mediate protection. We developed a robust Ebola-specific ADCC assay for use in ongoing trials of Ebola vaccines. Stable cell lines for inducible Zaire ebolavirus glycoprotein (EBOV GP) expression were developed to provide a uniform source of target cells in the assay, and were combined with an existing human natural killer (NK) cell line as the effector cell. When applied to commercially available anti-EBOV GP monoclonal antibodies, the assay clearly differentiated antibody with high ADCC activity from those with low or no ADCC activity. The Ebola ADCC assay reported here will be a useful tool in studying the functionality of anti-EBOV GP antibodies elicited by Ebola vaccines in ongoing and future clinical trials.

Keywords

Ebola viral disease; Zaire; Ebolavirus; ADCC; Ebola glycoprotein

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Conflict of Interest

The Ebola ADCC assay procedure and the target cells described in this assay are the intellectual property of Emory University, Atlanta, GA. Paul Spearman, Karnail Singh, and Xuemin Chen are listed as inventors on this patent.

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Introduction

The 2013-2016 outbreak of Ebola virus disease (EVD) in West Africa resulted in over 28,000 cases and 11,310 deaths (World Health Organization, June 2016). The extent of the outbreak coupled with a high rate of mortality emphasized the importance of developing new therapeutics and preventive vaccines against Ebolaviruses. Assays to evaluate protective responses against Ebola are needed to evaluate vaccines as they move forward in human clinical trials.

Ebolaviruses are filamentous, negative-strand RNA viruses belonging to the *Filoviridae* family. There are five species of the Ebola viruses; Zaire ebolavirus (EBOV), Sudan ebolavirus (SUDV), Bundibugyo ebolavirus (BDBV), Tai-forest ebolavirus (TAFV) and Reston ebolavirus (RESTV). Ebolavirus structure consists of an inner nucleocapsid made up of nucleoprotein (NP) and containing the viral RNA, along with RNA polymerase L, transcription factor VP30 and cofactor VP35. The viral nucleocapsid is surrounded by a lipid bilayer that contains the envelope protein spikes of glycoprotein (GP). Between the viral envelope and nucleocapsid are the matrix proteins VP40 and VP24 (1). For the purpose of this report, GP is the relevant target of both neutralizing and non-neutralizing antibodies that can protect against EVD.

Data from infected humans and from the non human primate (NHP) model of EVD indicate that survivors of EVD mount effective humoral and cell mediated immune responses against Ebola virus antigens (2, 3). In support of antibody-mediated protection, administration of anti-Ebola GP antibodies has been shown to prevent the development of fatal disease in Ebola-infected NHP even when administered post-infection (4). The anti-Ebola GP monoclonal antibody cocktail, ZMapp, was administered to some EVD patients after the onset of the clinical symptoms of the disease. However it remains unclear how much, if any, the cocktail contributed to the survival of these patients (5). The recombinant vesicular stomatitis virus-based Ebolavirus vaccine rVSV-ZEBOV has been administered to workers post occupational exposure to Ebola virus, with the intent of raising protective humoral immune responses. These recipients developed strong Ebola-specific antibody responses, and none of them developed EVD, providing circumstantial evidence in favor of anti-Ebola antibodies in protection (6). Though anti-GP neutralizing antibodies are generally associated with protection (4), it has also been attributed in some studies to non-neutralizing anti-Ebola GP antibodies (7). The contribution of non-neutralizing antibodies towards protection against these viruses has not yet been fully defined. Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) is a potential mechanism of protection against EVD that requires further evaluation (8). In support of the importance of this mechanism, NHPs immunized with Ebola virus-like particles developed high-titer ADCC-mediating antibodies that protected the animals against subsequent Ebola virus challenge (2). Furthermore, clearance of Ebola-infected cells by three anti-EBOV GP monoclonal antibodies in a murine model correlated well with their *in vitro* ADCC activity (9).

ADCC activity in serum samples from Ebola-exposed or vaccinated individuals has previously been measured using target cells that have been transiently transfected to express EBOV GP on their surface, and employing peripheral blood mononuclear cells (PBMCs)

or Jurkat cells as effector cells (2, 8, 9). We sought to develop a robust and reliable Ebola ADCC assay that is not reliant on transient expression of EBOV GP and incorporates effector cells with uniform killing potential. Here we report the development of a robust non-radioactive Ebola ADCC assay that can accurately measure the ADCC activity of human anti-EBOV GP antibodies. This assay will be useful in evaluating the functionality of anti-EBOV GP antibodies in samples from EVD patients and from volunteers receiving candidate Ebola vaccines.

Materials and Methods

Antibodies:

Anti- EBOV GP monoclonal antibodies 4F3, KZ52, c6D8, c13C6FR1 and h13F6 were purchased from IBT Bioservices, Gaithersburg, MD.

Generation of Plasmids and Target cells:

An EGFP-IRES-Luciferase reporter cassette derived from plasmid pHAGE PGK-GFP-Luciferase-W (Addgene, Cambridge, MA) was cut with Not I and Cla I and then blunted by Klenow Fragment. The blunt fragment was introduced into EcoRV site of plasmid pcDNA4/TO (Invitrogen, Carlsbad, CA) to generate plasmid pcDNA4/TO GFP-IRES-Luc (Figure 1A). pcDNA5/TO-puro plasmid was created by replacing hygromycin resistance gene sequence of original pcDNA5/TO vector (Invitrogen, Carlsbad, CA) with a puromycin resistance gene. Briefly, the puromycin resistance gene was amplified by polymerase chain reaction (PCR) from pLPCX (Clontech) using cgcacgtgatgaccgagtacaagcc and cgcacgtgtcaggcaccgggcttg primers. The hygromycin resistance gene was deleted from the original pcDNA5/TO plasmid by digestion with enzyme Pml I, and the puromycin resistance gene inserted in its place. The sequence and orientation of the insert was verified. Codon-optimized EBOV GP Mayinga gene was synthesized by GenScript (Piscataway, NJ) and placed under a tetracycline-controlled cytomegalovirus (CMV) promoter into plasmid pcDNA5/TO-puro using BamHI and EcoRI sites to generate the plasmid pcDNA5/TO-puro EBOV GP (Figure 1A). Expression of GFP and luciferase reporters was evaluated by transfection of 293T cells with pcDNA4/TO GFP-IRES-Luc. 293T cells (ThermoFisher Scientific, Waltham, MA), cultured in DMEM media (Gibco, Grand Island, NY) supplemented with 10% FBS (Sigma-Aldrich, St Louis, MO), 1X Glutamax (Gibco), 100 IU/ml penicillin and 100 µg/ml streptomycin (both Mediatech, Manassas, VA), were transfected with pcDNA4/TO GFP-IRES-Luc using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 24 hours, cells were examined by fluorescence microscopy or lysed in Britelite Plus luciferase substrate (PerkinElmer, Waltham, MA) for examination of luciferase activity as determined using TopCount NXT Luminescence Counter (PerkinElmer). To examine GP protein expression, pcDNA5/TO-puro EBOV GP plasmid was transfected into 293T cells using Lipofectamine 2000, cells were harvested 24 hour later and cell lysates analyzed by western blotting using mAb 4F3.

To generate stably-transfected cells expressing EBOV GP and the dual GFP/luciferase reporter, T-REX 293 cells (ThermoFisher Scientific), cultured in DMEM media supplemented with 10% FBS, 1X Glutamax, 100 IU/ml penicillin and 100 µg/ml

streptomycin and 5 µg/ml blasticidin (Invivogen, San Diego, CA), were transfected with pcDNA4/TO GFP-IRES-Luc and pcDNA5/TO-puro EBOV GP together using Lipofectamine 2000. Forty-eight hours later, the cells were trypsinized and transferred into fresh complete media supplemented with 200 µg/ml Zeocin (Invivogen) and 0.3 µg/ml puromycin (Invivogen) and diluted by serial dilutions to achieve single foci on a culture plate. Single foci were selected and expanded over the coming weeks. Clonal populations growing in individual wells were duplicated and one part was induced with doxycycline (2 µg/ml) (Sigma-Aldrich) for 24 hours, cells harvested and tested for cellular cytotoxicity, luciferase, GFP and EBOV GP expression. Clones with high luciferase, GFP and EBOV GP expression were expanded further and used as target cells for the ADCC assay development.

Effector Cells:

CD16-176V-NK-92 cell line was obtained from Fox Chase Cancer Center (FCCC), Philadelphia, PA under a Material Transfer Agreement (MTA) between FCCC and Emory University. These are parental NK-92 cells that were retrovirally-transduced to express the 176V high affinity variant of CD16 in the pBMN vector. These cells express high amounts of CD16 on their surface and have been shown to be very suitable for ADCC assays (10).

Development of Ebola ADCC assay and evaluation of ADCC activity of Ebola monoclonal antibodies:

The ADCC assay developed in this study was modified from the one used by Binyamin et al (10). Target cells were initially cultured in DMEM media supplemented with 10% Tet-approved FBS (Clontech, Mountain View, CA), 1X Glutamax, 100 IU/ml penicillin and 100 µg/ml streptomycin, 5 µg/ml blasticidin, 200 µg/ml Zeocin and 0.3 µg/ml puromycin (complete selection media). Cells were grown in 75 cm² flasks and were induced with 2 µg/ml of doxycycline upon reaching 75% confluency. Twenty four hours later, cells were harvested by trypsinization, washed twice and re-suspended in Mylocult media (StemCell Technologies, Vancouver, BC, Canada) containing 4 µg/ml of doxycycline at a cell density of 5×10^5 cells/ml. Effector cells were cultured in 75 cm² cell culture flasks in Mylocult media supplemented with 200 IU/ml of recombinant human IL-2 (R&D Systems, Minneapolis, MN). On day 3, cells were washed twice and re-suspended in Mylocult media at a cell density of 5×10^6 cells/ml. The optimal target: effector cell ratio was determined by mixing 5×10^4 target cells/100 µl/well of 96-well round bottom culture plates (Corning, Corning, NY) with different target: effector cell ratios (1:1, 1:2, 1:5 and 1:10) in a final volume of 200 µl/well. Plates were centrifuged at 300xg for 2 minutes and incubated for 4 hours at 37°C in a 5% CO₂ incubator. Following this incubation, plates were centrifuged again at 300xg for 5 minutes, cells washed twice with phosphate buffered saline (PBS) and re-suspended in 100 µl of MyloCult media. 100 µl of Britelite Plus luciferase substrate was added to each well, and cells were lysed by repeated pipetting. 150 µl of the reaction mix from each well was then transferred to the corresponding well of the light protected black assay plates and plates read immediately on an a luminometer (TopCount NXT Luminescence Counter). A target: effector cell ratio of 1:2, giving minimum spontaneous target cell death, was selected as optimal ratio for further experiments.

Next, the assay was performed using four different commercially available anti-EBOV GP monoclonal antibodies (KZ52, c6D8, c13C6FR1 and h13F6). These antibodies were first tested by flow cytometry for their binding to EBOV GP expressed on the surface of the target cells following 24 hour induction with doxycycline. Each anti-EBOV GP monoclonal antibody and a control antibody was diluted in 50 µl of MyloCult media (final concentration 0.0016- 5 µg/ml). 5×10^4 target cells/100 µl/well were mixed with each antibody, and plates were incubated at 37°C in 5% CO₂ for 10 minutes. Effector cells were then added to each well at 1×10^5 cells/50 µl/well, and the assay performed as described above. The percent ADCC killing was calculated using the following formula:

$$\% \text{ ADCC killing} = \frac{((\text{Luciferase units})_{\text{no antibody control}}) - ((\text{Luciferase units})_{\text{antibody}})}{((\text{Luciferase units})_{\text{no antibody control}})} \times 100$$

Validation of Ebola ADCC assay using plasma samples from rhesus macaques immunized with a candidate Ebola vaccine:

Rhesus macaques (n=4), male, ~ 10 kg in body weight, were primed intramuscularly with a candidate Ebola VLP vaccine expressing EBOV GP (100 µg/dose based on GP content), developed by our laboratories (KS, PS). The VLPs contained an HIV Gag core and EBOV GP and were produced from a stably-transfected 293F cell line and purified by centrifugation. VLPs were combined with adjuvants CpG (500 µg/dose, Integrated DNA Technologies, Coralville, IA) and R848 (800 µg/dose, Invivogen) and administered to macaques intramuscularly, followed by a homologous booster dose four weeks later. Blood samples were collected before priming and two weeks after the booster dose, plasma isolated, heat-inactivated and tested for ADCC activity using Ebola ADCC assay as described above.

Ebola ADCC with PBMCs as effector cells:

In order to test the adaptability of the Ebola ADCC assay with PBMCs as effector cells, the assay was performed with 5×10^4 target cells/100 µl/well, 1.0 µg/ml of c13C6FR1 antibody and PBMC (as effector cells) at target: effector cell ratios of 1:2, 1:5 1:10 and 1:20. The plates were incubated either for 4 hours or overnight and ADCC activity was calculated as described above.

Results and Discussion:

Validation of reporter expression and EBOV GP expression:

The goal of this project was to develop an Ebola-specific ADCC assay employing reporter and effector cell lines that are standardized to facilitate reproducible evaluation of Ebola-specific functional activity. We chose to include both luciferase and GFP reporters in the target cell to enhance readout options available for the assay. We focused during development primarily on luciferase because of the dynamic range of this reporter. An important feature of the target cells is robust expression of the EBOV GP on the cell surface. We first examined luciferase, GFP and EBOV GP expression in transient expression assays. Transient transfection of 293T cells with pcDNA4/TO GFP-IRES-Luc resulted in abundant

expression of luciferase and GFP reporters (Figure 1B and 1C, respectively). EBOV GP expression was evaluated following transient transfection of 293T cells with pcDNA5/TO-puro EBOV GP. Western blotting revealed a characteristic EBOV GP band at approximately 130 Kd (Figure 1D). These data indicated that reporters and EBOV GP were expressed well in transient expression experiments and should be appropriate for the creation of stable, inducible cell lines.

Stable cell line selection:

To establish a clonal cell line, we selected T-Rex 293 cells as the cells of choice. These cells are adherent and express the Tet repressor protein, allowing regulated expression of reporters and EBOV GP. T-Rex 293 cells were transfected with pcDNA4/TO GFP-IRES-Luc and pcDNA5/TO-puro EBOV GP plasmids, followed by antibiotic selection and single-cell cloning. Clonal populations were examined for luciferase production and for cell surface expression of EBOV GP. Clone T-Rex 293 GFP-I-Luc EBOV GP15 demonstrated the highest luciferase activity and EBOV GP surface expression and was selected for further work. Figure 2 shows the results of luciferase assay and flow cytometry analysis before and after induction with doxycycline. Induced expression of EBOV GP did not result in significant cytotoxicity in the Trex cells compared to uninduced cells as indicated by low levels of 7-amino-actinomycin D (7-AAD) staining (Figure 2A). Both luciferase (Figure 2B) and surface EBOV GP (Figure 2C), as determined by staining with mAb 4F3, were abundantly expressed by this clonal population in an inducible manner.

Development and validation of Ebola ADCC assay:

The Ebola ADCC assay (Figure 3A) outlined here utilizes the CD16-176V-NK-92 cell line developed by the Campbell laboratory at Fox Chase Cancer Center (10). This line is a human NK cell line that has been well-characterized for its utility in the development of reproducible ADCC assays. This engineered cell line was chosen instead of random human donor NK cells to avoid donor-to-donor variation and enhance the reproducibility of the present assay. Initial mixing experiments were conducted to determine the optimal target:effector cell ratio. EBOV GP target cells were incubated together with effector NK cells for 4 hours. Figure 3B shows that target:effector ratios of 1:5 and 1:10 resulted in >30% spontaneous killing of targets, while 1:1 and 1:2 ratios produced <20% spontaneous target cell killing. We further evaluated the assay using a 1:2 ratio to minimize non-specific killing. Next, we evaluated specific ADCC killing using four different anti-EBOV monoclonal antibodies; c13C6FR1, KZ52, c6D8 and h13F6. All four anti-EBOV GP monoclonal antibodies bound to EBOV GP expressed on the surface of the target cells following induction with doxycycline (Figure 3C). The four antibodies were found to mediate ADCC to different degrees (Figure 3D). c13C6FR1, a human-mouse chimeric anti-EBOV GP monoclonal antibody that has neutralizing activity in the presence of complement, is a component of ZMapp and is protective in NHP model of EVD (4), demonstrated the highest level of ADCC. KZ52, another human anti-EBOV GP neutralizing monoclonal antibody demonstrated ADCC only at high concentrations. mAbs c6D8 (chimeric, neutralizing in the presence of complement) and h13F6 (human, non-neutralizing) did not mediate any significant ADCC killing of the target cells at the concentrations tested. It is not clear why c6D8 and h13F6 did not show ADCC activity.

These antibodies are directed to the mucin-like domain on GP, and all four monoclonals tested here bound well to GP on the surface of target cells (Figure 3D). The two monoclonals that mediated ADCC killing (c13C6FR1 and KZ52) showed slightly higher binding to EBOV GP than the other two that did not mediate any significant ADCC killing (Figure 3D), but this difference is not likely to explain the differences in ADCC. However, these results suggest the assay can differentiate antibodies that strongly mediate ADCC killing of EBOV GP expressing target cells from those that are weakly ADCC positive or are negative.

Next, we evaluated this assay for its ability to measure ADCC responses in the plasma samples from rhesus macaques that were vaccinated with a candidate Ebola VLP vaccine. The assay clearly detected ADCC mediating anti-EBOV GP antibodies in the plasma samples collected from all the four animals post vaccination. No ADCC was detected in pre bleed plasma samples from any of the animal (Figure 3E) indicating the specificity of the assay.

We believe the NK cells used in this assay are useful because they are a reproducible source of cell killing and can avoid donor-specific differences if random donor PBMCs are used as ADCC effectors. However, we also determined the performance of the reporter cell line when PBMCs were employed as effectors. In order to adapt Ebola ADCC with PBMCs as the effector cells, the assay was performed with target: effector cell ratios ranging from 1:2 to 1:20, 1.0 µg/ml of c13C6FR1 antibody and incubation period of either 4 hours or overnight. No detectable ADCC activity was observed at any of the target: effector ratios after 4 hours of incubation and at 1:2 and 1:5 ratios after overnight incubation (data not shown). ADCC activity comparable to that observed with NK cells as effector cells was detected only after overnight incubation and at target: effector ratios of 1:20 (Figure 3F). Thus the NK cell line works well at a higher target:effector ratio in this assay, but equivalent killing can be achieved with donor PBMCs when more cells are included along with longer incubation times.

In summary, we have developed and evaluated a novel Ebola ADCC assay that will provide a suitable tool for measurement of ADCC responses in plasma/serum samples from Ebola exposed individuals and for the evaluation of vaccines and antibody-based therapeutics against EVD.

Based on multiple experiments the following protocol was finalized:

Ebola ADCC Protocol:

A. Materials, Cells and Samples

1. T-Rex-293 GFP-I-Luc EBOV GP target cells (Spearman Lab, Cincinnati Children's Hospital Medical Center, Cincinnati, OH)
2. CD16-176V-NK-92 Effector cells (Campbell Lab, Fox Chase Cancer Center, Philadelphia, PA)
3. Plasma, serum samples or antibodies to be tested.

4. DMEM media (Catalog number: 11965-118, Gibco, Grand Island, NY)
5. Tet system approved Fetal Bovine Serum (Catalog number: 631106, Clontech, Mountain View, CA)
6. Pencillin/Streptomycin (Catalog number: 30-002-CI, Mediatech, Manassas, VA)
7. Glutamax (Catalog number: 35050-061, Gibco)
8. Puromycin (Catalog number: ant-pr), Zeocin (Catalog number: ant-zn-1), Blasticidin (Catalog number: ant-bl-1). All from Invivogen, San Diego, CA.
9. Doxycycline Hyclate (Catalog number: D9891, Sigma-Aldrich, St Louis, MO)
10. IX Phosphate buffered saline (PBS) (Catalog number: 10010-023, Gibco)
11. Trypsin/EDTA (Catalog number: 25300-054, Gibco)
12. MyloCult H5100 media (Catalog number: 5150, StemCell Technologies, Vancouver, BC)
13. Human IL-2 protein (Catalog number: 202-IL-050, R&D Systems, Minneapolis, MN)
14. Britelite Plus Luciferase substrate and development reagents (Catalog number: 6066769, PerkinElmer, Waltham, MA)
15. Anti-EBOV GP chimeric monoclonal antibody (c13C6FR1) (Catalog number: 0201-023, IBT Bioservices, Gaithersburg, MD)
16. 75 cm² Tissue culture flasks (Catalog number: 353136, Corning, Corning, NY)
17. U bottom 96-well plate (Catalog number: 351177, Corning)
18. 96 well assay plates (Catalog number: 3915, Costar, Corning, NY)
19. Adhesive Clear Polyester Seal Film.
20. Disposable reagent reservoir
21. 15 and 50 ml sterile tubes
22. 2.0 ml sterile aspirating pipettes
23. 5,10 and 25 ml sterile pipettes
24. 10, 20, 200 and 1000 µl sterile pipette tips

B. Equipment

1. Sterile Bio-safety Cabinet
2. Temperature controlled table top centrifuge
3. Hemocytometer or automated cell counter
4. 37°C, 5% CO₂ humidified incubator
5. Illuminometer with 96 well plate adapter

6. Timer
7. Pipet aid
8. Multichannel Pipettors

C. Preparation of culture Media:

Complete DMEM media:

1. DMEM media 440 ml
2. Tet system approved, heat inactivated fetal bovine serum 50 ml
3. Penicillin/Streptomycin 5 ml
4. Glutamax 5 ml
5. Puromycin Final Concentration (0.3 µg/ml)
6. Zeocin Final Concentration (200 µg/ml)
7. Blasticidin Final Concentration (5.0 µg/ml)

Complete MyloCult media

1. MyloCult H5100 media 500 ml
2. Recombinant human IL-2 Final Concentration (200 IU/ml)

D. Procedure

Day 1

1. Culture 4×10^6 T-Rex-293 GFP-I-Luc EBOV GP target cells in 25 ml of complete DMEM media in a 75 cm² tissue culture flask. Keep the flask(s) horizontally in a 37°C, 5% CO₂ humidified incubator.
2. Culture 6×10^6 CD16-176V-NK-92 effector cells in 25 ml of complete MyloCult media in a 75 cm² tissue culture flask. Keep the flask(s) vertical in a 37°C, 5% CO₂ humidified incubator.

Day 3

1. Induce the target cells with doxycycline (2 µg/ml) for 24 hours.

Day 4

Harvesting Target cells:

1. Using an aspirating pipet, remove all the medium from the flask.
2. Add 8.0 ml of room temperature 1X PBS. Gently wash the cells and then aspirate all the PBS.
3. Add 1.5 ml of trypsin/EDTA solution to the flask and make sure that it touches all surface area covered with the cells.
4. Let the flask sit horizontally for 2 minutes.

5. Detach the cells by gently shaking the flask. Add 18.5 ml of complete DMEM media and transfer the cells into a 50 ml tube.
6. Centrifuge the tube at $300 \times g$ for 10 minutes.
7. Discard the supernatant and re-suspend the cells in 20 ml of MyloCult media.
8. Centrifuge the tubes at $300 \times g$ for 10 minutes.
9. Repeat step 7 and 8.
10. Discard the supernatant and re-suspend the target cells in MyloCult media at a density of 0.5×10^6 cells/ml.

Harvesting Effector cells:

1. Transfer the CD16-176V-NK-92 effector cells from the flask into 50 ml tube.
2. Centrifuge the tube at $300 \times g$ for 10 minutes.
3. Discard the supernatant and re-suspend the cells in 20 ml of MyloCult media.
4. Centrifuge the tubes at $300 \times g$ for 10 minutes.
5. Repeat step 3 and 4.
6. Discard the supernatant and re-suspend the effector cells in MyloCult media at a density of 2.0×10^6 cells/ml.

Setting up of the plate and performance of the Ebola ADCC.

1. Add 50 μ l of appropriately diluted serum/plasma/antibody in MyloCult media, in triplicates, to the wells of a 96 well U-bottom plate. “No Antibody” control wells receive 50 μ l of media, “Target Cells Alone” wells receive 100 μ l media while “Effector Cells Alone” wells receive 150 μ l of media.
2. Add 100 μ l of target cells in each well (5.0×10^4 /well) except the “Effector Cells Alone” wells. Mix gently.
3. Incubate the plates for 10 minutes at 37°C in 5% CO₂ incubator.
4. Add 50 μ l of effector cells (1.0×10^5 /well) to each well except the “Target Cells Alone” wells.
5. Centrifuge the plates at $300 \times g$ for 2 minutes.
6. Incubate the plates for 4 hours at 37°C in a 5% CO₂ incubator.
7. Centrifuge the plates at $300 \times g$ for 5 minutes.
8. Discard the supernatant and wash the cells twice with 200 μ l/well of PBS.
9. Re-suspend the cells in 100 μ l/well of MyloCult media and add 100 μ l/well of Britelite Plus luciferase substrate.
10. Mix the reaction mix well by pipetting.

11. Transfer 150 µl of the reaction mix from each well to the corresponding wells of a 96 well light protected luciferase assay plate.
12. Read the assay plate in a luminometer within 5 minutes.
13. Calculate % ADCC by using the formula below.

$$\% \text{ ADCC} = \frac{(((\text{Luciferase units})_{\text{no antibody control}}) - ((\text{Luciferase units})_{\text{antibody}})) \times 100}{((\text{Luciferase units})_{\text{no antibody control}})}$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expression of luciferase (Luc), green fluorescent protein (GFP) and EBOV GP following transient transfection of 293T cells. (A) Schematic diagram of doxycycline-inducible reporter construct (top) and inducible EBOV GP expression construct (bottom), under separate antibiotic selection. (B) 293T cells were transfected with either pcDNA4/TO GFP-IRES-Luc, pcDNA5/TO-puro EBOV GP or empty vector using Lipofectamine 2000. 24 hour later cells were harvested and expression of (B) luciferase; (C) GFP expression by fluorescence microscopy; and (D) EBOV GP expression by Western blotting.

Figure 2.

Expression of Luc, GFP and EBOV GP in stably-transfected T-Rex 293 GFP-I-Luc EBOV GP cells after induction with doxycycline. Cells were induced with 2 $\mu\text{g/ml}$ of doxycycline (Dox). 24 hours later cells were analyzed for cytotoxicity and for the expression of luciferase, GFP and EBOV GP. (A) Percent frequencies of 7-AAD positive cells with and without Dox induction. (B) Luciferase activity vs target cell number per well with and without Dox induction. (C) Flow cytometric analysis of uninduced (left) and Dox induced (center and right) cells for GFP and EBOV GP expression (as determined by staining with mAb 4F3).

Figure 3.

Ebola ADCC assay and its evaluation with anti-EBOV GP monoclonal antibodies and plasma from rhesus macaques immunized with a candidate Ebola vaccine. (A) Schematic of Ebola ADCC assay. (B) Optimal target: effector cell ratio was determined by finding the cell ratio that gave minimal spontaneous cell killing after 4 hours of target and effector cells incubation together. Results are mean $\pm$ standard deviation (SD) of three independent experiments run in triplicates. (C) Binding of control and anti-EBOV GP monoclonal antibodies (c13C6FR1, KZ52, c6D8 and h13F6) to T-Rex 293 GFP-I-Luc EBOV GP cells 24 hours after induction with Dox as studied by flowcytometry. All four antibodies bound to EBOV GP expressed on the surface of the cells. (D) % ADCC observed with four anti-EBOV GP monoclonal antibodies (concentration range: 0.0016- 5 $\mu\text{g/ml}$). Target and effector cells were incubated together, in triplicates, in the presence of the five antibodies.

Four hours later luciferase activity was measured and % ADCC calculated. Results are mean $\pm$ SD of three independent experiments each done in triplicate. (E) Results of Ebola ADCC assay run on plasma samples from four rhesus macaques (RM1-4) before and after immunization with a candidate Ebola vaccine. (F) Ebola ADCC assay using PBMCs as effector cells. Target cells and PBMCs (T:E ratio:: 1:20) were incubated together, in triplicates, in the presence of 1.0 μ g/ml of c13C6FR1 antibody. After overnight incubation luciferase activity was measured and % ADCC calculated. Results from a representative experiment are shown as mean $\pm$ SD.

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