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Functional Genetics received a contract from DARPA, No. W911NF04-C-0039, from April 2004 to October 2006, to study 'TSG101 Based Antibody Therapeutic for Ebola and Related Viruses'. Our goal was to discover anti-TSG101 antibody therapeutics that would inhibit the budding of Ebola and related viruses which rely on TSG101, a member of the endosomal protein sorting machinery, to exit infected cells. After extensive screening of mouse monoclonal antibodies by traditional immunological assays, unlike the results from the polyclonal antibodies, we have not found monoclonal antibodies that directly inhibited viral budding and the consequent infection. Although we have not obtained antibody therapeutics for Ebola based on direct inhibition of budding through TSG101, we did make major progress in demonstrating that TSG101 was exposed extracellularly in virus infected cells but not in uninfected cells. A new antibody screening assay was developed based on the recognition of cell surface TSG101 and has resulted in the discovery of many monoclonal antibodies. We plan to engineer antibody-dependent cell killing function into these monoclonal antibodies to make them candidate therapeutics. Based on the progress made, this project has been transitioned to a new proposal funded by the Defense Treat Reduction Agency (DTRA) in August 2006.				
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Enclosure 1

Final Report

DARPA Contract No. W911NF04-C-0039

Title: TSG101 Based Antibody Therapeutic for Ebola and Related Viruses

Duration: 4/15/04 to 10/14/06

Contractor: Functional Genetics, Inc.

Report Date: 11_16_06

A. Summary

Functional Genetics received a contract from DARPA, No. W911NF04-C-0039, from April 2004 to October 2006, to study 'TSG101 Based Antibody Therapeutic for Ebola and Related Viruses'. We had set an overall goal for this contract to discover anti-TSG101 antibody therapeutics that would inhibit the budding of Ebola and related viruses which rely on TSG101 to exit infected cells. TSG101 is normally an intracellular protein and belongs to the endosomal ESCRT-I protein sorting complex. TSG101 is 'hijacked' by Ebola, HIV, and other viruses for 'budding' out of the infected cells to produce new viral particles. Because TSG101 binds directly to Ebola VP40 protein and HIV p6 through the same protein motif, PTAP, on these viral proteins, we have proposed to use HIV as a surrogate of Ebola due to the similarity in budding mechanisms and the difficulty in performing Ebola experiments. After extensive screening of mouse monoclonal antibodies by traditional immunological assays, unlike the results from the polyclonal antibodies, we have not found monoclonal antibodies that directly inhibited viral budding and the consequent infection. Although we have not obtained antibody therapeutics for Ebola based on direct inhibition of budding through TSG101, we did make major progress in demonstrating that TSG101 was exposed *extracellularly* in virus infected cells but not in uninfected cells. Previous literature has demonstrated the general movement of intracellular TSG101 to the cytoplasmic membrane location but has not demonstrated the *extracellular* exposure of TSG101. We have therefore devised a new approach to use TSG101 to specifically mark viral infected cells for antibody-mediated 'killing' or 'clearance', instead of finding antibodies that directly inhibit budding as a result of binding to TSG101. A new antibody screening assay was developed based on the recognition of cell surface TSG101 and has resulted in the discovery of many monoclonal antibodies that recognize cell surface TSG101. None of the monoclonal antibodies that were obtained from traditional immunological assays, which identifies antibodies that recognize intracellular TSG101, showed cell surface TSG101 recognition. We further demonstrated that cell surface TSG101 recognition by anti-TSG101 antibodies is clinically relevant because it was observed in the blood cells of HIV-infected patients. The next step in our new approach to developing an anti-TSG101 antibody therapeutic will be to demonstrate that cell surface TSG101 recognition by anti-TSG101 antibodies also exists in Ebola-infected cells and that it is feasible to

use anti-TSG101 antibodies to specifically clear infected cells through antibody-mediated cytotoxicity. Based on the progress made, this project has been transitioned to a new proposal funded by the Defense Treat Reduction Agency (DTRA) in August 2006.

B. Experimental Results

1. Mouse mAbs that interacted with TSG101 did not inhibit release of HIV.

In the beginning of the project, we tried to obtain mouse monoclonal antibodies (mAbs) that would directly inhibit viral budding and hence viral titer in the cell culture. In the absence of a high throughput assay relevant to viral budding, we initially screened mouse mAbs by immunological assays to demonstrate antibody interaction with TSG101. These assays include ELISA and immunoprecipitations using recombinant TSG101. We then proceeded to perform viral release inhibition assays, which involve huge amount of work in producing monoclonal antibody proteins, with the mAbs obtained from primary immunological screening. Although initial results showed that some polyclonal antibodies and one monoclonal antibody pool may have inhibitory activity against viral budding, with rigorous control experiments, we have now confirmed that these antibodies did not have inhibitory effects.

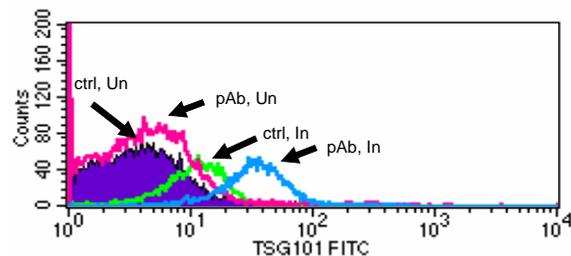
To date, we have developed a functionally more relevant high throughput assay (see section #2) compared to the antigen ELISA assay and the immunoprecipitation assays. The current assay can identify mAbs that recognize extracellularly (cell surface) exposed TSG101 on infected cells, in the context of its ESCRT I-viral budding complex, not just uncomplexed TSG101 as in an ELISA assay. We also learned that all of the mouse mAbs that we tested in viral release inhibition assays did not recognize extracellularly exposed TSG101. These results are consistent with their little or no inhibitory activity against budding. These mouse anti-TSG101 antibodies, although binding free TSG101 tightly, may not be able to access the extracellularly exposed TSG101 in its viral-ESCRT-I protein complex environment.

2. Both rabbit polyclonal and monoclonal antibodies can recognize extracellularly exposed TSG101

TSG101 is known to bind directly to the Ebola VP40 protein and HIV p6 protein through its UEV domain. Such binding results in the recruitment of TSG101 for Ebola or HIV to 'bud' from infected cells. In the current project, we have proposed the use of HIV as a surrogate for Ebola due to the similarity in molecular mechanisms of viral budding and the difficulty in performing Ebola experiments.

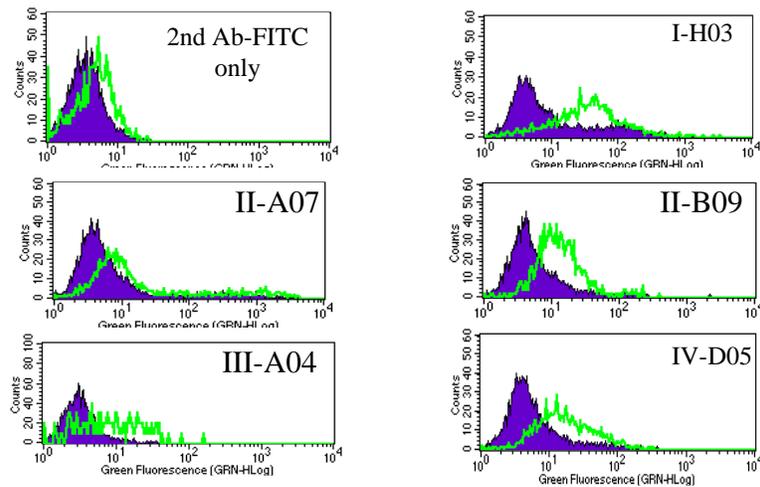
Previous literature has demonstrated the general movement of intracellular TSG101 to the cytoplasmic membrane location but has not demonstrated the *extracellular* exposure of TSG101. Because of the fact that we did not obtain any inhibitory antibodies for viral budding, we set out to investigate whether TSG101 is indeed exposed to the *extracellular* space at the moment HIV particles are released from the infected cells. Technically, the anti-TSG101 antibody staining of cell surface TSG101 must be done with live cells, before being 'fixed' by permeabilization reagents to avoid staining signals from antibodies that entered the cells after membrane permeabilization. A FACS assay was used to show binding by the right shift in fluorescence from the secondary antibody-FITC conjugate that was bound to the anti-TSG101 polyclonal antibody. Results from such a FACS assay demonstrate that in HIV-infected MT-T cell line, a polyclonal anti-TSG101 antibody could bind to TSG101 in infected cells (Figure 1, blue line) as indicated by the fluorescence shift from the control antibody (green line). There was no cell surface TSG101 staining in uninfected cells by either the anti-TSG101 polyclonal antibody (dark pink line) or the control antibody (solid purple histogram). The fact that only the infected, but not the uninfected, cells showed cell surface TSG101 staining supports our hypothesis that TSG101 can be a very specific 'marker' of virally infected cells. Such 'marker' could be utilized to clear infected cells through antibody-mediated killing of infected cells. The 'killing', or total clearance, of infected cells is of much value for curing infections by viruses, such as HIV, that permanently integrate their genomic material into the human genome in the infected cells.

Figure 1. Cell Surface TSG101 Staining by A Polyclonal Anti-TSG101 Antibody. MT-4 T cell line was infected with the NL4-3 HIV strain for 5 days. The cells were stained with a polyclonal anti-TSG101 antibody and a secondary goat-anti-rabbit antibody-FITC conjugate. Cells were then washed and fixed by glutaraldehyde for FACS analyses.



We then hypothesized that there would be monoclonal antibodies (mAb) that could also recognize cell surface TSG101, given sufficient screening effort. Such mAbs would be of much therapeutic potential when used as mediators for antibody-mediated killing of the infected cells. After screening 376 mAbs, we found 9 that recognized cell surface TSG101 in HIV infected MT4 cell line. Figure 2 shows a few of the representative mAbs from an initial screening assay. Currently, we are investigating the feasibility of using these antibodies to mediate antibody-dependent cell mediated cytotoxicity (ADCC) to clear HIV-infected blood cells.

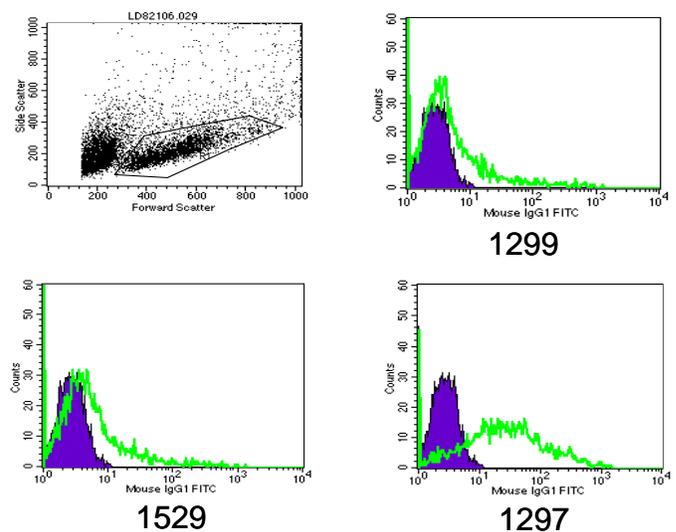
Figure 2. Cell Surface TSG101 Staining by Monoclonal Anti-TSG101 Antibodies. MT-4 T cell line was infected with the NL4-3 HIV strain for 5 days. The cells were stained with monoclonal anti-TSG101 antibody and a secondary goat-anti-rabbit antibody FITC conjugate. Cells were then washed and fixed by glutaldehyde for FACS analyses. Green line represents staining of infected cells while purple block represents staining of uninfected cells. The upper left panel shows control staining level by the secondary antibody-FITC coniuqate alone.



3. Extracellular TSG101 exposure exists in the blood cells of HIV patients

Having observed the cell surface TSG101 recognition by anti-TSG101 antibodies, we asked the question whether this phenomenon is clinically relevant or was it only a laboratory phenomenon. Thus, we tested blood cells from HIV patients for cell surface TSG101 recognition by anti-TSG101 antibodies. From four HIV patients tested, we found cell surface TSG101 recognition on the CD4+ cells of one patient (Figure 3). This recognition was shown by three polyclonal anti-TSG101 antibodies that recognize different epitopes at either the N or the Cterminus of TSG101. Although more experiments need to be performed to reveal the percentage of clinical HIV blood samples showing cell surfaceTSG101 exposure, this result clearly demonstrated that TSG101 cell surface exposure was a clinically relevant phenomenon. This result further supports the notion that an anti-TSG101 anti-viral therapeutic could be useful clinically, whether it is for HIV or for Ebola.

Figure 3. Cell Surface TSG101 Staining on CD4+ T Cells of an HIV Patient. Frozen blood cells of an HIV patient was thawed and cultured with PHA for three days before CD4+ cells were isolated. These cells were stained with monoclonal anti-TSG101 antibody and a secondary goat-anti-rabbit antibody FITC conjugate. Cells were then washed and fixed by glutaldehyde for FACS analyses. Green line represents staining of anti-TSG101 antibody while purple block represents staining of a control antibody. The upper left panel shows gating of live cells from the thawed frozen blood cells.



C. Outlook

We have originally proposed to discover antibody therapeutics for Ebola based on TSG101, but to use HIV as a surrogate due to the similarity in molecular mechanisms of viral release and the difficulty in performing Ebola experiments. Now we have obtained monoclonal antibodies that specifically recognize TSG101 on the surface of HIV infected cells, but not on that of uninfected cells. This cell surface TSG101 recognition is a clinically relevant phenomenon since it is observed also in HIV patient's CD4+ immune cells. We must now demonstrate that such phenomenon also exists for Ebola-infected cells. We expect this phenomenon to hold true since both Ebola and HIV used TSG101 in a similar manner for budding. The next key step to develop antiviral therapeutics from these monoclonal antibodies is to demonstrate that these monoclonal antibodies can mediate specific 'killing' or 'clearance' of infected cells through mechanisms such as ADCC (antibody-dependent cell mediated cytotoxicity). This work has recently become supported by the Defense Threat Reduction Agency. We hope to soon achieve the goal of finding an antibody therapeutic for Ebola and other viruses such as HIV through their specific recognition of cell surface TSG101 on, and only on, the infected cells and their ability to clear infected cells.