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An epitope conserved in orthopoxvirus A13 envelope protein is the target of neutralizing and protective antibodies

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ABSTRACT

Primary immunization of humans with smallpox vaccine (live vaccinia virus (VACV)) consistently elicits antibody responses to six VACV virion membrane proteins, including A13. However, whether anti-A13 antibody contributes to immune protection against orthopoxviruses was unknown. Here, we isolated a murine monoclonal antibody (mAb) against A13 from a mouse that had been infected with VACV. The anti-A13 mAb bound to recombinant A13 protein with an affinity of 3.4 nM and neutralized VACV mature virions. Passive immunization of mice with the anti-A13 mAb protected against intranasal VACV infection. The epitope of the anti-A13 mAb was mapped to a 10-amino acid sequence conserved in all orthopoxviruses, including viriola virus and monkeypox virus, suggesting that anti-A13 antibodies elicited by smallpox vaccine might contribute to immune protection against orthopoxviruses. In addition, our data demonstrates that anti-A13 mAbs are effective for treating orthopoxvirus infection.

Introduction

Smallpox, once a deadly infectious disease afflicting millions of people, was officially eradicated more than 30 years ago through a global immunization campaign with live vaccinia virus (VACV). Routine smallpox vaccination has since stopped, as the vaccine carries risk for a significant portion of the population (Fulginiti et al., 2003), including pregnant women and immunocompromised individuals. The current population largely lacks protective immunity to smallpox, which is now considered to be a potential bioterrorism agent, and to monkeypox, which is still endemic in parts of Africa. Monkeypox virus causes a smallpox-like disease in humans with approximately 10% mortality rate (Parker et al., 2007). It was accidentally imported to the U.S. in 2003, causing a brief outbreak in the Midwest. Currently, the only licensed therapeutics to treat infection by an orthopoxvirus is Vaccinia Immune Globulin (VIG) (Hopkins and Lane, 2004), a blood product derived from people immunized with smallpox vaccine. VIG contains neutralizing antibodies against VACV and is used to treat complications of VACV vaccination. However, the exact composition of VIG is not well defined and its supply depends on the availability of people vaccinated with smallpox vaccine, so there has been considerable interest in developing well-defined immunotherapies for treating orthopoxvirius infection.

VACV, the prototypical orthopoxvirus, produces two types of infectious virions that are biologically and antigenically different (Condit et al., 2006; Moss, 2007; Smith et al., 2002). The majority of the virions produced is intracellular mature virions (MVs), which remain inside the cell until cell lysis. MVs contain an envelope with more than 20 envelope proteins. A fraction of MVs gain additional membranes inside the cells and eventually exit the cells as the extracellular enveloped viruses (EVs) (Smith et al., 2002). EV contains an additional envelope with at least six envelope proteins. Antibodies against both MV and EV are required for optimal immune protection against orthopoxvirus. Among the EV proteins, B5 is the major target of neutralization antibodies (Bell et al., 2004; Benhnia et al., 2009; Putz et al., 2006), while A33 is the target of protective antibody (Galmiche et al., 1999). Depletion of ant-B5 antibodies from sera of vaccinated individuals greatly reduced in vitro neutralization of EVs (Bell et al., 2004; Putz et al., 2006). Among the MV envelop proteins, A27 (Rodriguez et al., 1985), L1 (Ichihashi and Oie, 1996; Wolffe et al., 1995), D8 (Hsiao et al., 1999), H3 (Davies et al., 2005), A28 (Nelson et al., 2008) and A17 (Wallengren et al., 2001) are known to be the targets of neutralizing antibodies. However, no single protein has been found to be the dominant MV-neutralizing target, as depletion of individual or a combination of the major MV-neutralizing antibodies from sera of vaccinated individuals did not significantly reduce neutralization of MV (Aldaz-Carroll et al., 2005; Benhnia et al., 2008; He et al., 2007).

Primary VACV immunization in humans consistently elicits antibody response to at least 12 antigens (Davies et al., 2007), including membrane proteins on MV (A13, A17, D8 and H3) and EV (B5 and A33). However, it was unknown whether anti-A13 antibodies play any role in immune protection against orthopoxvirus. In the current

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study, we isolated an anti-A13 monoclonal antibody (mAb) from a mouse that had been infected with VACV. The characterization of the anti-A13 mAb shows that anti-A13 antibodies can contribute to immune protection against orthopoxviruses and that anti-A13 mAbs are effective for treating orthopoxvirus infection.

Results

Identification and characterization of an anti-A13 mAb

Similar to what we reported recently (Meng et al., 2011), we developed anti-VACV mAbs from a BALB/c mouse that had been infected with WR strain of VACV. Among the mAbs, one (clone name 11F7) immunoprecipitated a 12-kda protein from HeLa cells that had been infected with VACV (Fig. 1A). The 11F7 mAb also recognized the 12-kda protein in a Western blot of VACV-infected HeLa cells (Fig. 1B). Mass spectrometry finger-printing analysis of the 12-kDa protein matched two peptides with that of VACV A13 protein (30% sequence coverage, data not shown). This identification was confirmed by an enzyme-linked immunosorbent assay (ELISA) in which 11F7 specifically recognized recombinant A13 protein expressed in *E. coli.* (Fig. 1C). The isotype of 11F7 was determined to be IgG2a (data not shown). A13 has a theoretical molecular mass of 8-kda, but it was previously shown to migrate as a 12-kda protein on SDS-PAGE (Unger and Traktman, 2004). It is phosphorylated at a serine residue, thus explaining the faint band that migrated slightly above the 12-kda band in Fig. 1A. 11F7 stained viral factories and virion-size particles in immunofluorescence analysis of infected cells (Fig. 1C), consistent with A13 being a MV membrane protein.

Anti-A13 11F7 neutralizes VACV MV

Since A13 is a dominant antibody target in smallpox vaccine (Davies et al., 2007), we were interested in finding out whether anti-A13 antibody contributes to protection against orthopoxviruses. We thus tested the ability of 11F7 to neutralize VACV MV with a plaque reduction assay. Purified VACV MVs were incubated in the presence or absence of the antibody for one hour and then inoculated to a cell monolayer. The inoculum was removed after one hour, and the number of plaques that appeared after 2 days was enumerated. To facilitate plaque counting, the amount of viruses that were initially used for inoculation were just enough to yield on average 55 plaques per well in a 6-well plate (Fig. 2A). Under this condition, 11F7, at a concentration from 4 to 100 μg/ml, reduced the plaque number by approximately 30-40%, similar to a murine monoclonal antibody against H3 (#41, IgG2a) (McCausland et al., 2010). Complement has been previously reported to enhance the neutralization of MVs in vitro (Isaacs et al., 1992). Indeed, 11F7 together with 2% rabbit complement reduced the plaque number by 90%, while complement alone reduced the plaque number only by 20% (Fig. 2A).

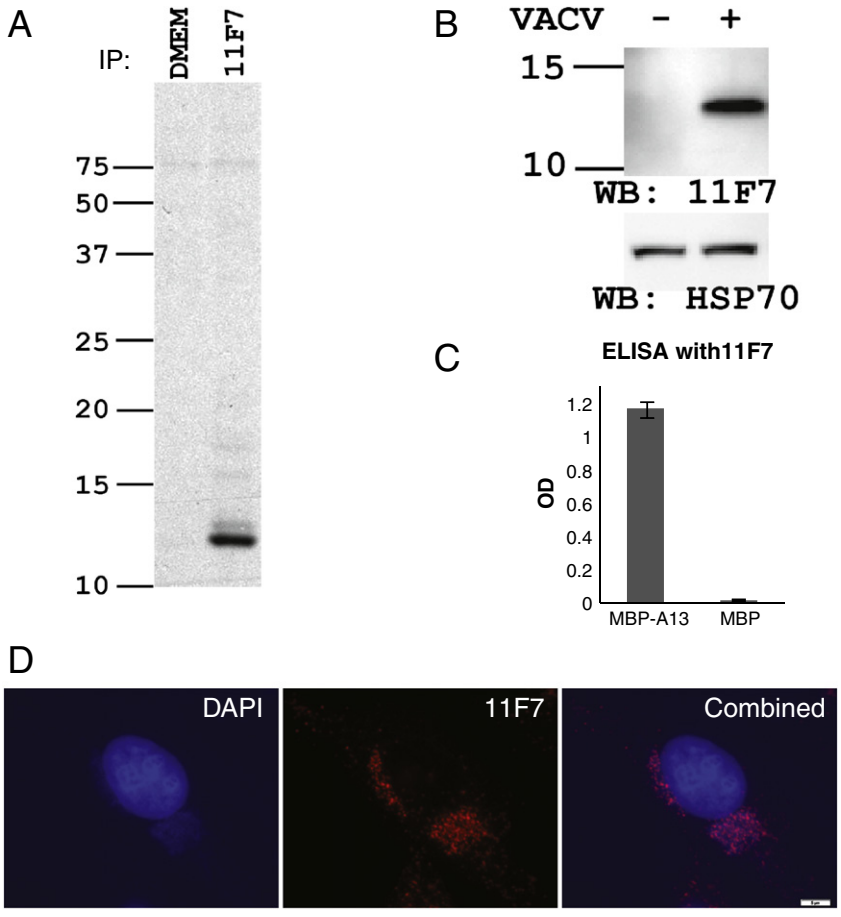

Fig. 1. Identification and characterization of an anti-A13 mAb 11F7. A). HeLa cells were infected with VACV WR at a MOI of 10 and metabolically labeled with ^{35}S-methionine and -cysteine from 8 to 16 hpi. The cells were lyzed and immunoprecipitated with either the culture supernatant from 11F7 hybridoma cells or DMEM medium only. The precipitated proteins were analyzed by SDS-PAGE, and the autoradiograph is shown. B). Proteins from uninfected (−) cells or cells infected with VACV WR (+) were analyzed by Western blot using the culture supernatant from 11F7. The same membranes were also blotted with anti-HSP70 antibody as a loading control. C). The same amount of purified recombinant MBP or MBP fused with VACV A13 were used to coat ELISA plates, and ELISA were performed with the culture supernatant from 11F7. Average OD from triplicate experiments is shown. D). BHK cells grown on cover-slips were infected with VACV WR at a MOI of 0.5 PFU/cell for 8 h and then analyzed by immunofluorescence with 11F7 supernatant. The primary antibody was stained with a Cy3-conjugated goat anti-mouse secondary antibody (red), and the DNA was stained with DAPI (blue).

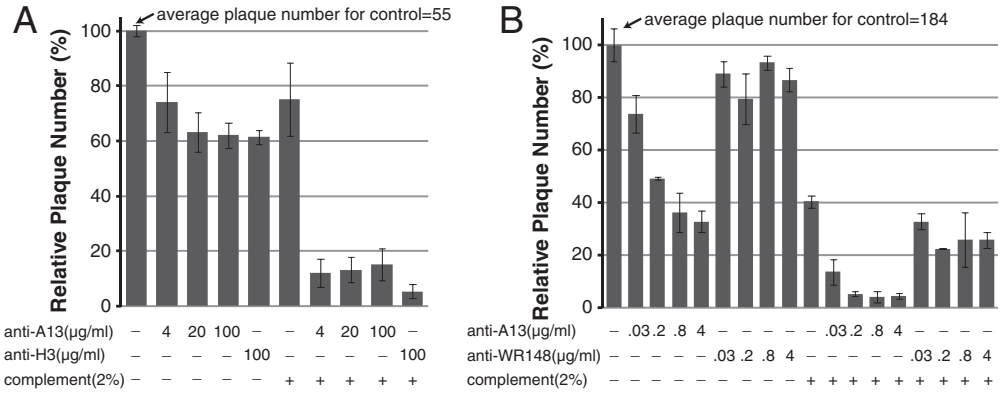

Fig. 2. Neutralization of VACV MV by 11F7. Sucrose-gradient purified VACV mature virions were incubated with the indicated amount of purified anti-A13 mAb 11F7, anti-H3 mAb #41, or anti-WR148 mAb HE7 in the presence (+) or absence (−) of rabbit complement for 1 h at 4 °C. The mixture was then added to monolayers of BS-C-1 cells, and the inoculum was removed after one hour. The number of plaques that appeared after 2 days was enumerated. The amount of viruses used for inoculation in (B) was three times of that in (A). The average number of plaques from untreated inoculum is 55 for (A) and 184 for (B). The number of plaques obtained under the indicated condition as the percentage to the number of plaques from untreated inoculums is shown. The average and standard deviation are from three independent inoculums.

To see whether a greater plaque reduction effect could be observed with a larger inoculum, we repeated the neutralization experiment by tripling the amount of input viruses (Fig. 2B). In the absence of any antibodies, the inoculum yielded on average 184 plaques per well in a 6-well plate. In the presence of an increasing concentration of 11F7 (from 0.03 to 4 μg/ml), there was a corresponding increase in % plaque reduction (from ~30% to ~70%). In contrast, a murine anti-WR148 mAb (HE7, IgG2a) (Meng et al., 2011) did not significantly reduce the plaque number. Complement (2%) alone or together with anti-WR148 reduced the plaque number by 60–70%, while complement and 11F7 reduced the plaque number by more than 90%. Comparing the two neutralization experiments, a greater plaque reduction by 11F7 was observed when a larger inoculum was used. This may due to that a small amount of input virus was somehow inaccessible for neutralization by antibodies and thus became a more significant background when the inoculum was smaller.

Anti-A13 11F7 protects mice against VACV infection

Next, the efficacy of 11F7 in protection against orthopoxvirus infection was tested with a mouse intranasal (i.n.) infection model using VACV WR strain. Groups of five BALB/c mice were given by the intraperitoneal (i.p.) route either purified antibodies or phosphate-buffered saline (PBS) and challenged subsequently with $1 \times LD_{50}$ of VACV WR by the i.n. route. The body weight and survival of the mice were monitored for at least 15 days after the challenge.

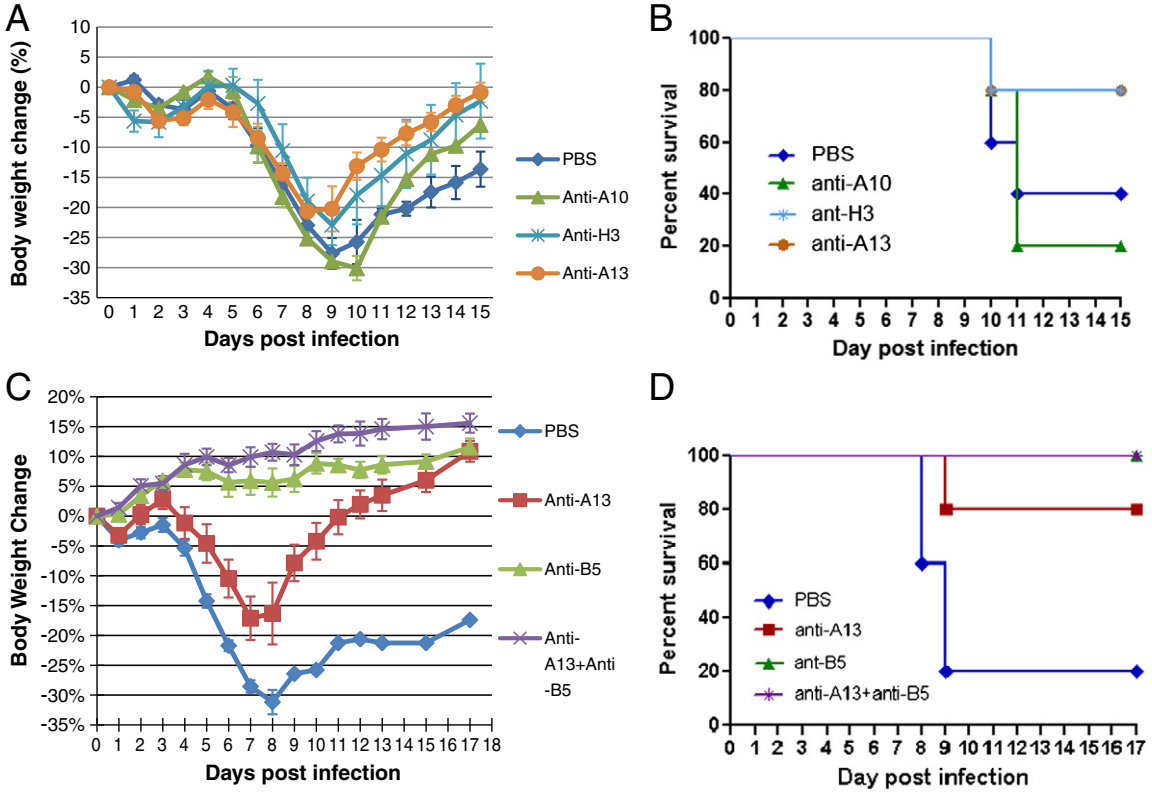

Fig. 3. Protection of mice from intranasal VACV infection by 11F7. (A and B). Groups of 5 BALB/c mice were given by the intraperitoneal route with either PBS or 2 mg of the indicated antibodies (anti-A10, anti-A13 11F7, and anti-H3 #41) on day −1 and challenged with 1×10^4 PFU of VACV WR by the intranasal route on day 0. The average body-weight changes with SEM (A) and survivals (B) of each group are shown. (C and D). The experiment was done as in (A and B) except with different antibodies.

In the first experiment (Figs. 3A and B), the efficacy of 11F7 was compared to anti-H3 antibody #41 and a murine anti-A10 antibody 9C2 (IgG2a). 9C2 does not neutralize VACV by plaque reduction assay (data not shown), so it was intended as a negative control. After challenged with VACV WR, all mice lost significant body weight, but mice that received either 11F7 or anti-H3 #41 lost less weight on average than mice that received either PBS or anti-A10 antibody (Fig. 3A). The difference in average body weight loss reached statistical significance (student t test, $p<0.05$) for those between 11F7-treated group and PBS-treated group after 10 days post infection. In addition, more mice that received 11F7 (80%) or anti-H3 (80%) survived the challenge than mice that received anti-A10 (20%) or PBS (40%).

In the second experiment (Figs. 3C and D), the efficacy of 11F7 was tested in comparison to and in combination with an EV-neutralizing murine anti-B5 antibody B126 (IgG2a) (Benhnia et al., 2009), which was previously shown to provide excellent in vivo protection against VACV WR challenge. Again, mice treated with 11F7 lost on average less body weight after WR challenge than mice mock-treated with PBS, and the difference in this experiment was statistically significant from day 5 post challenge. 80% of 11F7-treated mice survived the challenge in comparison to 20% survival in the mock-treated group. The survival rate of 11F7-treated mice was statistically better than that of mock-treated mice, when the survival data from both experiments are analyzed together to increase the group size to 10 (Log-rank test, $P=0.03$). Mice treated with anti-B5 antibody, either by itself or in combination with 11F7, did not lose significant body weight and all survived. Mice treated with the combination of ant-B5 and anti-A13 antibodies gained more weight over the time than mice treated with anti-B5 only, and the difference is statistically significant after 11 days post challenge.

Mapping the epitope of 11F7

To find out whether the epitope of 11F7 is conserved in orthopoxviruses, we mapped the epitope of 11F7 by using fragments of A13 proteins fused with maltose binding protein (MBP). The MBP-A13 fusion proteins were expressed in *E. coli*, and their bindings to 11F7 were evaluated by Western blot (Fig. 4A). Among the whole lysate of bacterial cells, 11F7 specially recognized A13 residues from 59 to 69 fused with MBP (MBP-A13aa-59–69) and all A13-MBP fusion proteins that contain this 10 amino acids. The identical 10 amino acids are present in A13 orthologues of variola virus and monkeypox virus (Fig. 4B).

To assess the affinity of 11F7 to A13, we purified recombinant A13 proteins from *E. coli* and studied the binding of the recombinant proteins to 11F7 with Surface Plasmon Resonance (SPR) (Fig. 5). 11F7 was immobilized on a BIAcore sensor chip and its binding to MBP, MBP fused with the ectodomain of A13 (residue 23–70), or MBP fused with A13 residue 59–69, was monitored in real time. As expected, 11F7 did not bind to MBP (data not shown). It binds to MBP-A13 (aa23-70) and MBP-A13(aa59–69) with an affinity of 3.4 nM and 0.2 μM, respectively. The reduced affinity to MBP-A13(aa59–69) is probably due to steric hindrance to antibody binding caused by the bulky MBP at the N-terminus.

Discussion

In this study, we identified VACV A13 as a neutralization target for VACV MVs. A13 was recently identified as one of the immunodominant targets of antibody response to smallpox vaccine (Davies et al., 2007), but it was previously unknown whether anti-A13 antibodies could contributes to immune protection against orthopoxviruses. In fact, the only study that tested neutralization potency of anti-A13 antibodies showed that a particular polyclonal anti-A13 antibody did not neutralize MV (Unger and Traktman, 2004). We showed here,

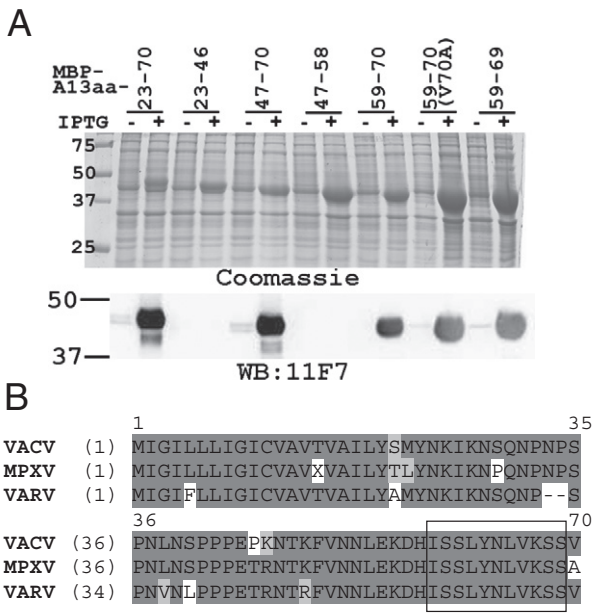

Fig. 4. Identification of epitope for 11F7. A). *E. coli* strains were either not induced (−) or induced with IPTG (+) to express fusion of MBP with the indicated A13 fragments. Proteins from the whole cell lysates were resolved by SDS-PAGE and analyzed by either Coomassie staining or by Western blot with 11F7. B). The multiple sequence alignment of A13 orthologues from vaccinia virus (VACV), monkeypox virus (MPXV) and variola virus (VARV). The box indicates the identified 11F7 epitope.

however, that an anti-A13 mAb 11F7 neutralized MV with similar potency as a proven neutralization antibody for VACV, an anti-H3 mAb. Furthermore, we revealed a neutralization epitope on A13 by mapping the epitope of 11F7. The minimal epitope that was found to bind 11F7 in Western blot is a 10-amino acid sequence located at the C-terminus of A13. A13 is a small membrane protein with only 70 amino acids. The transmembrane domain of A13 is located at the N-terminus with the rest of A13 pointing outside the virion (Salmons et al., 1997). The epitope at the C-terminal end of A13 probably extends furthest away from virion membrane and thus is most accessible for antibody binding. This may be one of the reasons why 11F7 neutralizes VACV while the previous polyclonal anti-A13 antibody does not. Most importantly, the neutralization epitope is conserved entirely in A13 orthologues of variola virus and monkeypox virus, suggesting that anti-A13 antibodies elicited by smallpox vaccination might contribute to immune protection against smallpox. The identification of A13 as the neutralizing target and mapping the neutralizing epitope provide knowledge for the development of next-generation subunit vaccine for smallpox.

Among all the known targets for MV neutralization (D8, H3, A27, L1, A28 and A17), A13 is one of the very few that are not involved in binding or entry/fusion of MV with the host cells. D8, H3 and A27 interact with cell surface molecules and mediate MV binding to the cells. A28 is part of the multi-protein entry/fusion complex (EFC) of VACV, while L1 associates with EFC. Both A28 and L1 are essential for VACV entry/fusion with the host cells. Antibodies to these proteins neutralize MV presumably by disrupting the functions of these proteins in MV binding or entry/fusion with the cells. In contrast, A13 is not known to interact with any cell surface molecule or the EFC but plays an essential role in virion morphogenesis (Unger and Traktman, 2004). A13 is an abundant virion membrane protein (Chung et al., 2006). One possible mechanism by which anti-A13 antibodies neutralizes VACV is that the binding of the antibodies with A13 on virion surface creates steric hindrance to prevent virions from binding and fusing with cell membrane. Another possible mechanism is that anti-A13 antibodies aggregate virions by crosslinking A13 on different virions. Similar to A13, A17 also plays a role in virion

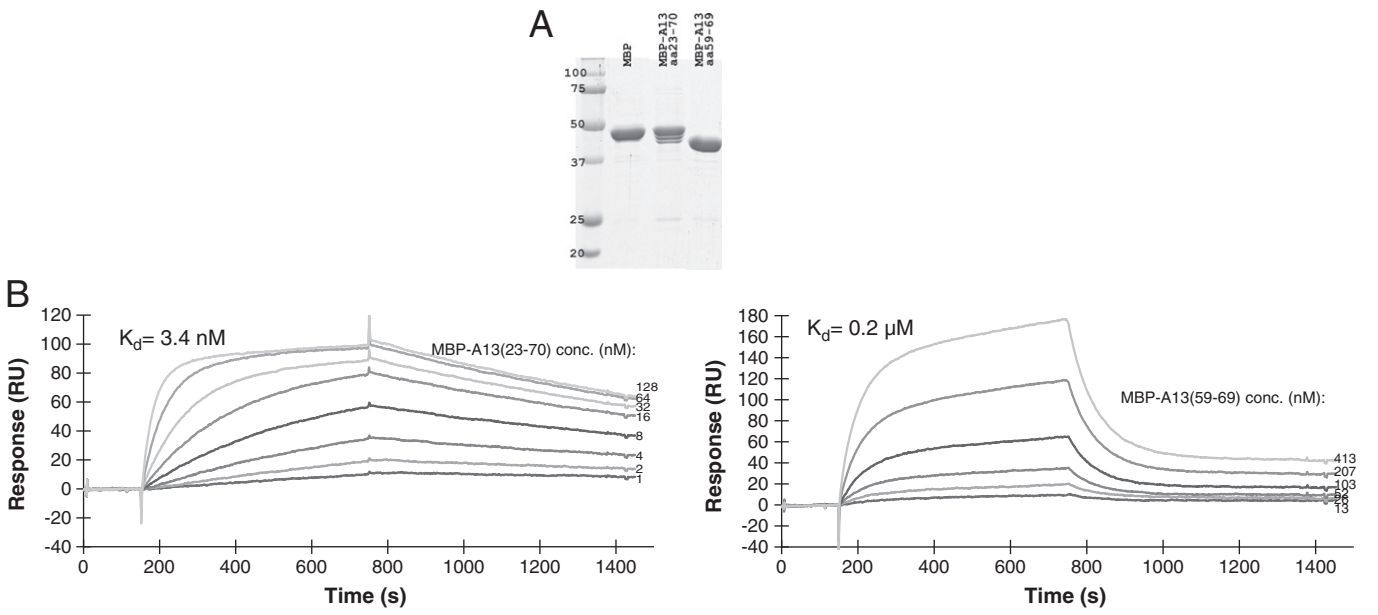

Fig. 5. Binding affinity of 11F7 to recombinant A13 proteins. A). Recombinant MBP, MBP fusion with A13(23–70) or MBP fusion with A13(59–69) was expressed in *E. coli* BL21 strains and purified with metal affinity chromatograph. Coomassie stain of the purified recombinant proteins is shown. B). 11F7 was immobilized on a BIAcore CM5 sensor chip, and its binding with MBP-A13(23–70) or A13(59–69) was then monitored with a BIAcore 3000 sensor. The lines are the responses obtained with the indicated concentrations of recombinant proteins. The response curves were globally fitted to a 1:1 binding model, and obtained affinity constants are shown.

morphogenesis (Rodriguez et al., 1995), although it was also suggested to be involved in fusion of VACV with the cells (Kochan et al., 2008). The finding of A13 as the neutralization target suggests that additional VACV virion membrane proteins that are not functional in VACV entry/fusion pathway may also serve as neutralization targets.

We showed that passive administration of the anti-A13 mAb 11F7 in mice significantly reduced the morbidity (measured by body weight loss) and mortality associated with i.n. infection by $1\times LD_{50}$ (10^4 PFU) of VACV WR. This level of protection is similar to what was reported for other MV-neutralizing antibodies. The i.n. challenge represented a more stringent test than the i.p. challenge for evaluating in vivo efficacy of VACV-neutralizing antibodies (Sakhatskyy et al., 2006). Significant protection from the i.n. challenge by MV-neutralizing antibodies is usually observed only when the challenge dose is rather modest, no greater than a few LD_{50}. For example, passive transfer of polyclonal MV-neutralizing antibodies protected mice against challenge of 3×10^3 PFU ($<1\times LD_{50}$) of VACV WR but failed to do the same when the challenge dose was 5×10^4 PFU ($>1\times LD_{50}$) (Law et al., 2005). Anti-L1 appears to be slightly more efficacious than other MV-neutralizing antibodies, providing significant protection against challenge with 2 to $3\times LD_{50}$ of VACV WR (Fogg et al., 2008; Lustig et al., 2004). In contrast, anti-A27 polyclonal antibodies failed to protect mice from challenge with $2\times LD_{50}$ of VACV WR, even though anti-A27 was more potent than anti-L1 at neutralizing VACV in vitro (Fogg et al., 2008). Anti-A28 polyclonal antibodies provided protection to mice that were challenged with 1×10^4 PFU ($\sim0.7\times LD_{50}$) of VACV WR (Nelson et al., 2008), similar to what we observed for 11F7. Anti-H3 polyclonal antibodies could protect mice from challenge with $1\times LD_{50}$ dose of VACV WR but could not reproducibly protect against challenge with $3\times LD_{50}$ dose of VACV WR (Davies et al., 2005). In our direct comparison between 11F7 and an anti-H3 mAb, we also found that these two mAbs are similar in their in vivo efficacy. In contrast, an EV-neutralizing anti-B5 mAb B126 was much more efficacious than 11F7 at protecting mice from VACV challenge. It was shown previously that, in addition to being able to neutralize EV, B126 could bind to VACV-infected cells and direct complement lysis of infected cells (Benhnia et al., 2009). These activities together may make B126 much more effective than other VACV-neutralizing antibodies at providing in vivo protection. The combination of 11F7 with the anti-B5 mAb provided a slightly better protection than the anti-B5 mAb alone, consistent with previous studies that showed EV- and MV-neutralization antibodies together provided the best protection against VACV challenge (Lustig et al., 2005).

VIG is currently the only licensed treatment for side effects associated with smallpox vaccination (Hopkins and Lane, 2004). Because the composition of VIG is ill-defined and its supply is uncertain, there is a need for highly specific monoclonal antibodies for replacing VIG. As illustrated by the fact that 11F7 neutralizes MV while polyclonal anti-A13 antibodies do not, a defined set of monoclonal neutralization antibodies should have a much higher efficacy than the polyclonal VIG for treating smallpox vaccine complication and possibly even for treating smallpox or human monkeypox. 11F7 binds to A13 with an affinity of 3.4 nM, recognizes an epitope that is conserved in all orthopoxviruses, and has similar in vivo efficacy as other MV-neutralizing mAbs, suggesting that an anti-A13 mAb could be a useful component of a mAb cocktail for replacing VIG.

Material and methods

Cells and viruses

BS-C-1 cells were maintained in minimum essential medium with Earle's salts supplemented with 10% fetal bovine serum (FBS). BHK and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. Wild-type (WT) WR viruses were propagated on BS-C-1 cells.

Hybridoma generation and characterization

The generation and characterization of the hybridomas were performed as described recently (Meng et al., 2011) except some changes in the immunization protocol described here. A six-week old BALB/c mouse was infected intranasally with 5×10^3 plaque-forming-unit (PFU) of WT VACV WR. Seven weeks after the infection, the mouse was injected intravenously with 7×10^7 PFU of UV-inactivated WR

virus. Three days afterwards, the spleen of the mouse was harvested for hybridoma generation. Anti-H3 mAb #41 (McCausland et al., 2010) and anti-B5 mAb 126 (Benhnia et al., 2009) were described before.

Antibody production and purification

Hybridoma cells were cultured in 175-mm flasks with hybridoma serum-free medium (Invitrogen) supplemented with OPI Media Supplement (Sigma-Aldrich, #O5003). Monoclonal antibodies were purified from the conditioned culture media with a HiTrap Protein G-Sepharose column (GE Healthcare Life Sciences). The antibodies are in PBS after going through a PD-10 desalting column (GE Healthcare Life Sciences). The concentration of the antibodies was calculated from OD_{280} using the formula (concentration in mg/ml = $1.43 \times OD_{280}$).

MV neutralization assay

WR viruses used in neutralization and in vivo protection assay were purified successively through a sucrose cushion and a sucrose gradient step, according to the standard protocol (Earl et al., 1998). The viruses were mixed with PBS or purified antibodies for 1 h at 4 °C (in a total volume of 1 ml). When complement is used in neutralization, baby rabbit complement (Cedarlane Laboratories) was added to the mixtures at a final concentration of 2%. For each neutralization condition, three independent mixtures were set up and inoculated onto monolayers of BS-C-1 cells in 6-well plate. After 1 h incubation at 37 °C, the inoculum was replaced with 2 ml DMEM supplemented with 1% FBS and 0.5% (w/v) methylcellulose (Sigma-Aldrich, #M0512). The plates were incubated for 2 days at 37 °C before being processed for plaque counting.

Expression and purification of recombinant proteins

The plasmids for expressing the fusion of MBP and A13 were constructed by PCR-amplifying the viral gene from WR DNA and cloning the PCR fragment into a modified pET28b vector that encodes maltose-binding protein (MBP) with an N-terminal 6-histidine tag (Krumm et al., 2008). The expression of the fusion protein in *E. coli* BL21 strain was induced with isopropyl-beta-D-thiogalactoside (IPTG; Invitrogen). For Western blot analysis, the bacteria were lyzed via sonication in SDS-PAGE sample buffer, and the clarified cell lysates were directly used in Western blot analysis. For recombinant protein purification, the bacteria were lysed via sonication in a buffer containing lysozyme. Recombinant proteins were purified from soluble fraction of the cell lysates through metal affinity chromatography with Ni-nitrilotriacetic acid resin (Qiagen). The protein concentrations were determined by Bradford protein assay (Biorad).

Surface Plasmon Resonance (SPR)

Purified 11F7 antibody was immobilized onto a BIAcore CM5 sensor chip through standard amino-coupling method as described (Xiang and Moss, 1999). The chip was injected with purified recombinant proteins at a flow rate of 20 μl/min and regenerated with a half-min pulse with 10 mM glycine (pH 1.5). Affinity constants were obtained by fitting the binding data to a 1:1 binding model with the BIAEVALUATION software (BIACORE).

In vivo protection

Groups of five female BALB/c 5-week-old mice were given intraperitoneal (i.p.) injection of 2 mg antibodies or PBS in equivalent volume. One day later, the mice were anesthetized and infected intranasally with 10^4 PFU of VACV WR in 20 μl PBS as described (Meng et al., 2008). Individual mice were weighed each day, and mice lost more than 30% original body weight were euthanized in accordance with an established IACUC protocol. Changes of body weight were analyzed with unpaired, two-tailed *T*-test with $P<0.05$ considered significant (Microsoft Excel). Survival curves significance was calculated using log-rank (Mantel-Cox) test of Kaplan-Meier curves (Graphpad Prism).

Acknowledgments

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