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Combination therapy of vaccinia virus infection with human anti-H3 and anti-B5 monoclonal antibodies in a small animal model

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Abstract

Vaccinia virus possesses two immunologically distinct virion forms in vivo—mature virion (MV, IMV) and extracellular virion (EV, EEV). Here we show that combination therapy with two fully human mAbs against an immunodominant MV antigen, H3 (H3L), and an EV antigen, B5 (B5R), provides significantly better protection against vaccinia infection in a small animal model of progressive vaccinia (SCID mice infected with VACV_{NYCBOH} vaccine strain) than a single human monoclonal or human vaccinia immune globulin (VIG), the currently licensed therapeutic for side effects of smallpox vaccination.

Introduction

Smallpox is a highly lethal viral infection of humans (30% mortality) [1,2], which can spread rapidly through a population. Smallpox is a top bioterrorism concern, and is frequently considered the #1 bioterrorism danger [1,3–7]. The smallpox vaccine consists of live vaccinia virus (VACV) and, from a public health perspective, is the gold standard of vaccines since it has led to the complete eradication of wild smallpox (variola virus) from the human population [2]. Renewed fears that smallpox might be deliberately released in an act of bioterrorism have led to a resurgence in the study of treatment of smallpox infection. Individuals under the age of 35 (approximately 50% of the population) have not been vaccinated against smallpox, leaving them highly susceptible in the event of an outbreak. There is also substantial interest in better therapeutics for the treatment of the rare but severe side effects of the smallpox vaccine (vaccinia virus, VACV). There is an active smallpox vaccination campaign in the USA military, and VIG (Vaccinia Immune Globulin) is used to treat the rare side effects of vaccination. Finally, there is also interest in therapeutics for treatment of other poxviruses, such as monkeypox. A monkeypox outbreak occurred for the first time in the USA in 2003 [8–11], and monkeypox is transmitted among rodent populations.

The smallpox vaccine is administered as a series of 3–15 skin pricks using a bifurcated needle [12]. Four major smallpox vaccine strains were used during the massive vaccination program: Dryvax, Lister, Temple of Heaven and EM-63. In the USA, the VACV_{NYCBOH} (New York City Board of Health) strain has been used as the vaccine [12]. The vaccine was

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commercially produced as Dryvax (also known as the VACV Wyeth strain or substrain). A clonal isolate of VACV_{NYCBOH}, ACAM2000, has now been developed as a cell culture derived smallpox vaccine, with a comparable immunogenicity and safety profile to Dryvax, and ACAM2000 is now the currently licensed smallpox vaccine in the USA [13]. The vaccine “take” is observed as the formation of a pustule starting on approximately day 5 post-vaccination and lasting for 1–2 weeks thereafter [12,14,15]. The vaccine provides outstanding immunity, but causes a variety of side effects that have been reason for concern [16–18]. Common side effects include fever and satellite pocks (additional pustules near the primary pustule, also called mild “generalized vaccinia”). More severe side effects include progressive vaccinia, generalized vaccinia, encephalitis, vaccinia keratitis, and eczema vaccinatum [14,16,17,19].

Currently, VIG (Vaccinia Immune Globulin) is the only licensed therapeutic to treat the side effects of smallpox vaccination [16,18]. In addition, VIG has shown efficacy against smallpox itself, in clinical trials in the early 1960s. Meta-analysis of the four available controlled studies done with VIG indicates that VIG is protective and reduces smallpox cases by approximately 75% [20]. VIG reduced the spread of smallpox outbreaks when administered at the same time as smallpox vaccination to smallpox contacts [20–23]. A most impressive study demonstrating the utility of passive immunotherapy was published in 1941 [24]. In this study, a smallpox outbreak initially claimed the lives of 3 out of 10 patients. When patient care was expanded to include administration of high-titer smallpox-specific convalescent serum at the first signs of disease, the mortality rate dropped to 0% (0 deaths out of 250 subsequent smallpox infections reported) and the treated patients experienced fewer smallpox scars after recovery [24].

There is also compelling animal model data supporting the efficacy of VIG against pathogenic poxvirus infections. Licensed VIG has demonstrated efficacy by *in vitro* neutralization of VACV and *in vivo* treatment of SCID mice infected with VACV [18,25–29]. In rhesus macaque monkeypox studies, not only was it demonstrated that smallpox vaccine elicited neutralizing antibodies were necessary for protection, it was further shown that neutralizing antibodies were sufficient for protection against a lethal monkeypox challenge, as administration of VIG to unvaccinated macaques prior to monkeypox challenge provided protection [30]. Although animals developed skin lesions (i.e. pocks) in a dose-dependent manner with an inverse relationship to the amount of VIG administered, they were all fully protected from lethal infection [30].

Unfortunately, VIG is a poorly characterized, variable human product that is of limited potency [18,20]. Each of these issues is a major problem for biodefense preparedness against a smallpox bioterrorism event. These problems with VIG have led to great interest in the development of an alternative high potency anti-smallpox immunotherapy free of these issues.

Our goal is to develop a highly efficacious and standardized mAb anti-smallpox therapeutic that can be produced in large quantities and stored long term. Poxviruses (vaccinia, variola/ smallpox, monkeypox) have two virion forms, Intracellular Mature Virions (MV, IMV) and Extracellular Enveloped Virions (EV, EEV), each with distinct structure and biology. Importantly, the two virion forms do not share any surface proteins, and therefore the virion forms are immunologically distinct and are not neutralized by antibody of a single specificity [31]. VIG contains both anti-MV and anti-EV antibodies [29,32–37]. Therefore, an effective VIG replacement therapeutic product should contain one anti-MV mAb and one anti-EV mAb, each capable of neutralizing their respective virion form.

Here we report characterization of mAbs against H3 and B5, demonstrating their efficacy against VACV in an in vivo model of progressive vaccinia, using VIG as the benchmark for efficacy.

Materials and Methods

Viruses

VACV_{NYCBOH} (New York City Board of Health) vaccine strain was grown as a 2nd or 3rd passage stock from an aliquot of Dryvax®. VACV_{NYCBOH} and VACV_{WR} (Western Reserve) stocks were grown as described [36,38,39]. For experiments in Figure 7, a laboratory stock of the ACAM2000 clone of VACV_{NYCBOH} was used. The ACAM2000 stock was generated by single passage amplification in Hela cells of ACAM2000® vaccine virus (Acambis, UK).

Mice and infections

Female SCID mice on the BALB/c background (CBY.Smn.CB17^{PRKdc}SCID/J) between 7 to 12 weeks of age were purchased from Jackson Laboratories and preferably used in experiments at 8 weeks old. Animals were acclimatized for at least 1 week prior to infection. Animal health was assessed prior to infection. Five animals per cage were housed in micro-isolator cages in ventilated racks. All animal experiments were conducted using the Institutional Animal Care and Use Committee approved animal protocols.

Animals received food and acidified water (pH 2.5–3.5) *ad libitum*. The animals' diet was PicoLab® Autoclavable Rodent Breeder Diet (Cat #5013). Towards the end of these studies the vivarium diet was changed to irradiated PicoLab® Rodent Diet 20 (Cat #5053). After the change in feed it was noted that the mice exhibited slower disease kinetics. Therefore, the VACV_{NYCBOH} dose was changed in subsequent experiments from 1×10^4 PFU to 5×10^4 PFU. Doses used are detailed in the Figure Legends.

Mice were given intraperitoneal (i.p.) injection of antibodies in a volume of 200–500 μ L 18–24 hours prior to virus infection. Mice were infected with VACV_{NYCBOH}, ACAM2000 clone, or VACV_{WR} by tail vein intravenous injection in a volume of 200 μ L. Immediately prior to use, virus was diluted into plain DMEM from high titer stocks stored at -80°C . Of note, seizures were observed in mice given VACV_{WR} intravenously by the retro-orbital injection route. Therefore, all experiments shown in this manuscript were done using VACV injected via the tail vein route of intravenous injection.

Body weight—After infecting animals on day 0, weights were taken on day 0 for the initial body weight measurement. Beginning three days post-infection, body weights were taken every other day. Body weights were recorded until the animal reached 70% of initial body weight in all experiments (except experiment in Figure 7 when 75% of initial body weight was the endpoint), or if other external health variable became present for which euthanasia was the only humane course of action. Studies were ended on day 90.

Clinical score—Mice develop lesions (pox/pocks) on the tail between 6–8 days post-infection. The method of clinical scoring evaluates the number and severity of the lesions [19]. The tail is scored in three parts: the base, middle, and tip of the tail. The lesions are evaluated as follows: Score 0 = No pox. Score 1 = 1–3 pox. Score 2 = 3+ pox. Score 3 = 3+ pox and erupted. An additional evaluation of the paws is taken. A score of 1 is given if the animal only has pox on its hind paws, and a score of two is given when the animal has pox on the front and hind paws. 11 was the maximum score (e.g., Base = 3, Mid = 3, Tip = 3, Paws = 2). Clinical scores usually stabilized at 15–20 days post-infection.

Generation of recombinant H3

For cloning full length H3L, the DNA from cells infected with the NYBOH strain of vaccinia virus that was used as a PCR template. The sequence encoding the full-length open reading frame of vaccinia H3L was amplified by reverse-transcription polymerase chain reaction using primers H3L F NdeI pET-15b (5'-AGAGAGAGACATATGGCGGCGGCGAAAACT-3') H3L R Bam HI pET-15b (5'-AGAGAGAGACATATGGCGGCGGCGAAAACT-3'). As a cloning intermediate, this PCR product was cloned into the TA-topo 2.1 vector following manufacturers instructions (Invitrogen, Carlsbad CA). This H3L-TA-topo2.1 plasmid was then used as a template for PCR using primers H3L fwd NheI pET28 (5'-AGAGAGAGAGCTAGCGGCGGCGGCGAAAACT - 3') and H3L rev XhoI pET28 (5'-CTCTCTCTCTCTCGAGTTAGATAAATGCGGTAACGA. The amplified full-length PCR product was digested with NheI and XhoI and ligated into the NheI and XhoI sites of the bacterial expression vector pET28a to create pET28a-full length H3L-His, which encodes a H3L with a C-terminal 6xHis tag.

H3L production and protein purification

To produce H3L-His in bacteria, the expression vector pET28a-full length H3L-His was transformed into BL21 (DE3) pLysS competent cells and bacterial cultures were induced to express H3L-His by a 2 hr. incubation of diluted (1:20) overnight cultures in 1mM IPTG (isopropyl-beta-D-thiogalactopyranoside). Cells were harvested by centrifugation for protein purification. Recombinant full-length H3L with N-terminal HisTag® was purified by metal chelate affinity chromatography with Ni Sepharose 6 Fast Flow resin (GE Healthcare). Bacterial cells were lysed with microfluidizer (model M10L, Microfluidics, Inc.). Lysis buffer included 0.5% Triton X-100 (Calbiochem), and the chromatography was performed in the presence of appropriate non-ionic detergent, like 0.5% Triton X-100 or 0.58% Octyl Glucoside (Calbiochem). H3L was eluted from the column with 200 mM imidazole, and subsequently dialyzed against 20 mM Na phosphate buffer, pH 8.0, 0.25M NaCl, 10% glycerol, and appropriate detergent (see above). Protein concentration was determined by DC Lowry protein assay (Bio-Rad) using BSA standard (Pierce Biotechnology) in the same buffer.

Antibodies

Mouse antibody #41 was derived from a mouse infected twice with vaccinia virus WR. For the generation of human antibodies, KM mice were immunized subcutaneously with 25 to 50 µg of recombinant H3 or B5 proteins emulsified in complete Freund's adjuvant (CFA) (mixed 1:1). KM miceTM were generated by cross breeding double transchromosomal mice and transgenic mice [40]. KM mice possess the human chromosome fragments containing the entire human immunoglobulin heavy chain locus and the YAC transgene for 50% of the human immunoglobulin kappa light chain locus. KM mice are engineered to neither express endogenous immunoglobulin heavy chain (µMT) nor kappa light chain (κ-/-). KM mice are used to obtain human monoclonal antibodies. Generation of recombinant B5, immunizations of mice and generation of hybridomas were described previously [39,41]. Human anti-DNP IgG1 was derived from a CHO transfectant clone kindly provided by Dr. Hideaki Yoshida from Kyowa Hakko Kirin. For antibody purification, hybridomas or CHO stable transfectants were cultured in roller bottles or WaveTM bioreactors with hybridoma serum-free medium (Invitrogen, Corp.) supplemented with ultra low IgG fetal bovine serum (Invitrogen, Corp.) or CD CHO XP serum-free medium (Irvine Scientific), respectively. Monoclonal antibodies were purified from conditioned culture media using recombinant Protein A-Sepharose Fast Flow gel (GE Healthcare) as described in [41]. The purified antibodies were quantitated by the Lowry method using Bovine IgG (Pierce) as a standard.

and they were stored in aliquots at -80°C and diluted into PBS immediately prior to injection, as needed.

VIG (53 mg/ml) was the kind gift of Dorothy Scott and Robert Fisher (FDA). VIG was stored at 4°C and diluted into PBS immediately prior to injection, as needed. Human recommended dose was 50 mg/kg [25]. Maximum adult BALB/c SCID female mouse mass was 25g. Therefore the human equivalent VIG dose for a mouse based on mass was calculated to be 1.25 mg.

Virus neutralization assays

VeroE6 cells were seeded at 1.5×10^5 cells/well into 24-well Costar plates (Corning Inc, Corning, NY) and used the following day (75–90% confluence). Three different VACV MV neutralization assays were used: (i) overnight neutralization, (ii) 1 hour no complement, and (iii) 1 hour plus complement. (i) Overnight VACV MV neutralization as per Newman et al., [42]: diluted mAbs samples (10 $\mu\text{g/mL}$, final concentration) in D10 (Dulbecco's modified Eagle medium plus 10% fetal calf serum plus penicillin-streptomycin-glutamine) were incubated overnight at 37°C , 5% CO_2 , in an equal volume (50 μL) of sonicated VACV_{WR} MV (10^4 PFU/mL), as described previously [43]. Fetal calf serum was heat inactivated prior to use to eliminate complement function. In quantitative dose titration experiments, 2-fold dilution of mAb samples was used. VACV MV alone samples were used in each assay as negative control. (ii) Direct VACV MV neutralization: diluted mAbs samples (10 $\mu\text{g/mL}$, final concentration) were incubated for 1 hour at 37°C , 5% CO_2 , in an equal volume (50 μL) of sonicated VACV_{WR} MV. VACV MV alone samples were used as negative control. (iii) VACV MV neutralization in the presence of complement: diluted mAbs samples were incubated for 1 hour with the virus as described above in (ii) in the presence of 1% sterile baby rabbit complement (final concentration) (Cedarlane Laboratories, Ontario, Canada). VACV MV samples plus 1% baby rabbit complement were used as negative controls. Plaque assays were then done as described previously [36,38].

Statistics

Tests were performed using Prism 5.0 (GraphPad, San Diego, CA). Error bars are $\pm$ one SEM. Survival curves significance were calculated using Mantel-Cox statistical analysis of Kaplan-Meier curves. Statistical analysis of cumulative weight loss was calculated as the net area under the curve (AUC) (body weight vs. time) for each mouse and then statistical analysis was determined between experimental groups by two-tailed, unpaired T test with 95% confidence bounds without assuming a normal distribution (Welch's correction). Statistical analysis of time to 5% weight loss was done by tabulating the days until weight first dropped below 95.0% of starting weight for each mouse, and the statistical significance was calculated using two-tailed, unpaired T test with 95% confidence bounds without assuming a normal distribution (Welch's correction). Clinical score statistical analysis from a single timepoint was done using Mann-Whitney test. Cumulative clinical score data was calculated as the area under the curve (AUC) (clinical score vs. time) for each mouse and then statistical analysis was determined between experimental groups by two-tailed, unpaired T test with 95% confidence bounds without assuming a normal distribution (Welch's correction). If not otherwise indicated above, statistics were done using two-tailed, unpaired Student's T test with 95% confidence bounds.

Results

Characterization of murine anti-H3 mAb

Anti-H3 murine mAbs were generated using conventional techniques and then screened for neutralization of VACV in vitro. The neutralization potency of each mAb was determined

by quantitative dose titrations, measuring the lowest mAb concentration able to inhibit VACV infection 50%, as measured by plaque assay (PRNT₅₀). Clone #41 exhibited the most effective neutralization. Neutralization was observed in a standard overnight neutralization assay (Fig. 1A). Partial neutralization was observed in a one hour neutralization assay (Fig. 1B). This result is somewhat different than a previous report [44], where good neutralization was observed after short incubation of virus with antibody, perhaps due to residual complement present in previous experiments, or differences in the cell lines used for virus production. Enhanced neutralization was observed by mAb #41 in the presence of 1% complement in a one-hour neutralization assay (Fig. 1C).

In vivo protective efficacy of murine anti-H3 against progressive vaccinia

To examine in vivo efficacy of the anti-H3 mAb #41, we utilized SCID mice infected with VACV as a small animal model of human progressive vaccinia. In this system, SCID mice are infected with smallpox vaccine VACV_{NYCBOH} and then tracked for weight loss, pox formation, and survival. This model has been used by VIG vendors [28] and the FDA [29] to measure the efficacy of VIG. This model is valuable both because it uses the human vaccine strain of vaccinia and because it models immunodeficient or immunocompromised humans—a primary group of concern for failing to control VACV and develop progressive vaccinia or other life threatening side effects, clinical indications for which VIG is licensed. Treatment with a human mg/kg equivalent dose of VIG is able to extend life by > 1 week in this model [28,29,33]. Since SCID mice are fully immunodeficient, a complete cure is not expected from a single dose of therapeutic antibody. Nevertheless, this animal model readout of life extension correlates with the ability of VIG to ameliorate severe VACV side effects in humans [18], and provide apparently substantial (~75%) post-exposure prophylaxis against smallpox [20]. Since this assay uses the human smallpox vaccine strain, models vaccinia infection in an immunocompromised setting, and has been previously used with VIG, this animal model served as the primary benchmark by which we measured successful candidate mAbs. The mAbs were directly tested against VIG as the “standard of care”. Mabs were considered non-inferior to VIG if they extended survival time by an equivalent number of days, and mAbs were considered superior to VIG if they extended survival time longer than VIG.

VACV_{NYCBOH} was titrated in SCID mice, and disease progression observed was consistent with previous publications (Fig. 2A). Groups of SCID mice were then infected intravenously with 5×10^4 PFU VACV_{NYCBOH} after treatment with a single dose of anti-H3 mAb #41, VIG, or PBS one day prior to infection (day -1). Protection against weight loss was observed for treatment with anti-H3 #41 or VIG (Fig. 2B). Clinical scores were also measured, quantifying the number and severity of pox lesions on the tail and footpads (Fig. 2C). Anti-H3 #41 and VIG both provided partial protection against the development of pox lesions ($P < 0.0006$, anti-H3 #41 vs. PBS. $P < 0.0001$, VIG vs. PBS).

We also explored infection of SCID mice with a more virulent strain of vaccinia, VACV_{WR}. SCID mice succumb to weight loss more rapidly after infection with VACV_{WR} in comparison to VACV_{NYCBOH}. Nevertheless, anti-H3 mAb #41 was still effective at delaying weight loss ($P < 0.007$ vs. PBS) and extending survival time ($P < 0.008$ vs. PBS) of SCID mice infected with VACV_{WR} (Fig. 2D). VIG also exhibited efficacy at delaying weight loss ($P < 0.02$ vs. PBS) and extending survival time ($P < 0.005$ vs. PBS), but not as robustly as anti-H3 treatment (Fig. 2D).

Characterization of human anti-H3 mAb

Description of the development of murine anti-B5 mAbs has been reported [38], including efficacy of the mAb #B126 in vivo using the mouse intranasal VACV_{WR} infection model,

which is a small animal model for respiratory smallpox. The SCID mouse model was also used [38]. Having shown proof of principle with murine mAbs (Fig. 1–2, and ref. [38]), we then moved to develop fully human antibodies against H3 and B5 as our candidate therapeutics for use in humans. Kyowa Hakko Kirin California (KKC) used proprietary transchromosomal mice, which possess the human immunoglobulin loci and thereby produce fully human antibodies [40], to develop fully human mAbs against H3 and B5. The characterization of the fully human B5 mAbs has been described elsewhere [39]. Human anti-H3 mAbs were characterized *in vitro* and top candidate hV26 exhibited good neutralization of VACV (Fig. 3A–B), while clone hV25 exhibited no neutralization (Fig. 3B). When compared side by side, murine anti-H3 #41 and human anti-H3 hV26 exhibited comparable *in vitro* virus neutralization activity to each other (data not shown).

In vivo protective efficacy of human anti-H3 against progressive vaccinia

The fully human anti-H3 mAb hV26 was tested for efficacy in the *in vivo* progressive vaccinia model. Significant protection against weight loss was observed for treatment with human anti-H3 hV26 ($P < 0.003$ vs. PBS) (Fig. 3C). A dramatic increase in survival was also observed ($P < 0.004$ vs. PBS) (Fig. 3D). Anti-H3 mAb hV26 provided equivalent protection to that of VIG, both by weight loss (hV26 vs. VIG, $P = \text{ns}$) and survival (hV26 vs. VIG, $P = \text{ns}$). Clinical scores were measured (Fig. 3E), quantifying the number and severity of pox lesions on the tail and footpads. Human anti-H3 hV26 and VIG both provided significant protection against the development of pox lesions, both in terms of magnitude and kinetics ($P < 0.003$, hV26 vs. PBS. $P < 0.0005$, VIG vs. PBS. Cumulative severity measured as area under the curve.).

Dose titrations of human anti-H3 mAb hV26 were performed to determine potency. Anti-H3 hV26 exhibited a clear dose titration, with 100 μg or 200 μg /mouse providing substantially better protection than 10 μg /mouse (Fig. 4A). Even 10 μg /mouse provided protection similar to that of VIG. Anti-H3 hV26 doses of 50 μg , 100 μg , and 200 μg provided significant protection against weight loss ($P < 0.05$, $P < 0.0002$, and $P < 0.0002$ vs. PBS). Furthermore, anti-H3 hV26 doses of 100 μg and 200 μg provided significantly better protection than VIG against weight loss ($P < 0.02$ and $P < 0.04$). This was also reflected in improved survival of mice treated with 100 or 200 μg hV26 ($P < 0.002$, 100 μg anti-H3 hV26 vs. VIG. $P < 0.007$, 200 μg anti-H3 hV26 vs. VIG) (data not shown). Pox lesion development was also reduced in the hV26 treated mice (clinical scores for 10 μg , 20 μg , 50 μg , 100 μg , 200 μg ; $P < 0.05$, $P < 0.006$, $P < 0.0001$, $P < 0.0002$, $P < 0.0001$ vs. PBS, respectively) (Fig. 4B). Pox lesion reduction was similar between hV26 and VIG.

In vivo protective efficacy of human anti-B5 against progressive vaccinia

The fully human anti-B5 mAb h101 was tested for efficacy in the *in vivo* progressive vaccinia model. The fully human mAb h101 performed comparably to the murine anti-B5 mAb *in vivo*, showing efficacy comparable to or better than VIG treatment (Fig. 5A–B). Cumulative weight loss was significantly ameliorated by single treatment with h101 (AUC analysis, $P < 0.007$, h101 vs. PBS). As a measure of early disease, the number of days until 5% weight loss was quantitated. Time to 5% weight loss was significantly better in h101 treated mice ($P < 0.002$) (Fig. 5B), and comparable to, or better than, VIG. Pox lesions were also significantly inhibited after treatment with human anti-B5 h101 ($P < 0.0001$ vs. PBS) (Fig. 5C), and comparable to VIG treatment. Not only did h101 treatment delay onset of disease (Fig. 5B–C), it also extended overall lifespan, as animal survival was significantly improved after treatment with h101 ($P < 0.0001$ vs. PBS) (Fig. 5D).

Dose titrations of human anti-B5 h101 were performed to determine potency (Fig. 6). A control irrelevant human IgG1 mAb (DNP hapten specific) was also included as a more

specific negative control. Results with PBS or anti-DNP IgG1 treated mice were comparable (Fig. 6). Compared to control human IgG1 mAb (DNP), weight loss was substantially improved in mice receiving 100 μ g, 50 μ g ($P < 0.02$), or 25 μ g anti-B5 mAb h101 ($P < 0.02$) (Fig. 6A), though the 100 μ g h101 group did not reach significance due to an early death of one mouse. Delay of early stage disease, measured as time to 5% weight loss, was significantly better in h101 treated mice than in control mAb treated mice (h101 100 μ g, 50 μ g, 25 μ g. $P < 0.03$, $P < 0.006$, $P < 0.005$ respectively) (Fig. 6B). All doses of h101 treatment were equal to or better than VIG, as measured by time to 5% weight loss (Fig. 6B), or overall weight loss kinetics (Fig. 6A). Pox lesions were also significantly inhibited at all doses of h101 treatment compared to control IgG1 (DNP) (100 μ g $P < 0.02$. 50 μ g $P < 0.02$. 25 μ g $P < 0.03$) (Fig. 6C). Survival was extended in all h101 treated mouse groups, but did not reach statistical significance due to the small group sizes ($N = 5$) (Fig. 6D). Larger group sizes were used in subsequent studies.

In vivo protective efficacy of combination human anti-H3/anti-B5 against progressive vaccinia

Again, our proposed therapeutic is a combination of an anti-MV and anti-EV antibody, and therefore we next tested whether combination therapy with anti-H3 (hV26) plus anti-B5 (h101) fully human mAbs exhibited increased protective efficacy. Groups of SCID mice were infected with VACV_{NYCBOH} after treatment with a single dose of human anti-H3 mAb hV26, human anti-B5 mAb h101, human anti-H3/B5 together, VIG, or control human IgG1 at day -1. As before, significant protection against weight loss and pox formation was observed after treatment with anti-H3 or anti-B5 mAb alone compared to treatment with an irrelevant mAb (weight loss $P < 0.005$ and $P < 0.004$, respectively. Pox formation $P < 0.0001$ and $P < 0.0001$, respectively)(Fig. 7).

50 μ g combination treatment was significantly better than control IgG1 as measured by the early disease parameter of days to 5% weight loss ($P = 0.0009$), the end stage disease parameter of survival ($P < 0.0001$), total weight loss disease burden ($P = 0.0006$), or pox lesion development ($P < 0.0001$, day 17). A lower dose of combination treatment of anti-H3/B5 was also tested (25 μ g each), with results more similar to single treatment with either mAb alone (Fig. 7), and still statistically significantly better than control IgG1 by time to 5% weight loss ($P < 0.03$), cumulative weight loss disease burden ($P < 0.004$), clinical disease ($P < 0.003$, day 17), and survival ($P < 0.0001$).

Impressively, 50 μ g (each) anti-H3/B5 combination therapy provided significantly enhanced efficacy compared to VIG (Fig. 7A). This was quantifiable as measured by delay of early disease (time to 5% weight loss)($P = 0.07$)(Fig. 7B), total weight loss disease burden (AUC) ($P < 0.04$) (Fig. 7A), severity of pox lesions (clinical score) ($P = 0.06$)(Fig. 7C), or survival ($P < 0.02$)(Fig. 7D). Combination therapy with 50 μ g anti-H3/B5 also provided substantially enhanced efficacy compared to single therapy with either mAb alone (Fig. 7A–D), though these differences did not quite reach statistical significance, except survival of 50 μ g anti-H3/B5 combination vs. 50 μ g hV26 alone ($P < 0.04$). The borderline significance of combination therapy in comparison to single therapy with either mAb alone was due to underpowered experiment group sizes, as combined statistical analysis of the two largest experiments showed that combination mAb therapy robustly outperformed VIG (Table 1, meta-analysis), and provided a statistically significant improvement over single mAb therapy in survival ($P = 0.006$), overall weight loss ($P = 0.049$), and time to 5% weight loss ($P = 0.046$) (Table 1).

Discussion

Antibodies against vaccinia and smallpox are critical components of the protective immunity elicited by the smallpox vaccine [31,45]. Monoclonal antibodies to VACV MV and EV have been previously demonstrated to be protective in a variety of small animal experimental models [35,37,38,46–50]. Our primary goals here were to develop human anti-MV and anti-EV mAbs that could be used in combination as a clinical therapeutic that would be a replacement for VIG. The rationale for this approach is that human mAbs can be manufactured in virtually unlimited quantities, under well defined conditions. Furthermore, mAbs were expected to have a higher specific activity than polyclonal VIG, resulting in the twin benefits of lower doses and higher efficacy.

We demonstrate here that fully human mAbs against either H3 or B5 were efficacious at providing protection equal to VIG. Furthermore, a combination of the two mAbs provided impressively increased protective efficacy beyond that observed for VIG. Therefore, the pre-clinical studies validate that this combination of human antibodies is a promising approach as a poxvirus therapeutic for use in humans. In future studies, post-exposure prophylaxis against vaccinia virus will be tested. Combination therapy with human antibodies may also be a useful treatment for smallpox infections.

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References

1. Henderson DA, Inglesby TV, Bartlett JG, Ascher MS, Eitzen E, Jahrling PB, Hauer J, Layton M, McDade J, Osterholm MT, O'Toole T, Parker G, Perl T, Russell PK, Tonat K. Smallpox as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. *Jama* 1999;281:2127–2137. [PubMed: 10367824]
2. Fenner, F.; Henderson, DA.; Arita, I.; Jezek, Z.; Ladnyi, ID. Smallpox and its eradication. World Health Organization; Geneva: 1988.
3. NIAID. NIAID Category A, B & C Priority Pathogens. Edited by Editor|. Year|; p.^pp. Pages|. City|: Publisher|
4. NIAID. NIAID High Priority Biodefense Products. 2007.
5. Meltzer MI, Damon I, LeDuc JW, Millar JD. Modeling potential responses to smallpox as a bioterrorist weapon. *Emerg Infect Dis* 2001;7:959–969. [PubMed: 11747722]
6. LeDuc JW, Jahrling PB. Strengthening national preparedness for smallpox: an update. *Emerg Infect Dis* 2001;7:155–157. [PubMed: 11266310]
7. O'Toole T, Mair M, Inglesby TV. Shining light on “Dark Winter”. *Clin Infect Dis* 2002;34:972–983. [PubMed: 11880964]
8. Huhn GD, Bauer AM, Yorita K, Graham MB, Sejvar J, Likos A, Damon IK, Reynolds MG, Kuehnert MJ. Clinical characteristics of human monkeypox, and risk factors for severe disease. *Clin Infect Dis* 2005;41:1742–1751. [PubMed: 16288398]
9. Hammarlund E, Lewis MW, Carter SV, Amanna I, Hansen SG, Strelow LI, Wong SW, Yoshihara P, Hanifin JM, Slifka MK. Multiple diagnostic techniques identify previously vaccinated individuals with protective immunity against monkeypox. *Nat Med* 2005;11:1005–1011. [PubMed: 16086024]
10. Karem KL, Reynolds M, Olson V, Li Y, Damon IK. Monkeypox outbreak diagnostics and implications for vaccine protective effect. *Nat Med* 2006;12:495–496. author reply 496–497. [PubMed: 16675987]

11. Reynolds MG, Yorita KL, Kuehnert MJ, Davidson WB, Huhn GD, Holman RC, Damon IK. Clinical manifestations of human monkeypox influenced by route of infection. *J Infect Dis* 2006;194:773–780. [PubMed: 16941343]
12. Fulginiti VA, Papier A, Lane JM, Neff JM, Henderson DA. Smallpox vaccination: a review, part I. Background, vaccination technique, normal vaccination and revaccination, and expected normal reactions. *Clin Infect Dis* 2003;37:241–250. [PubMed: 12856217]
13. Marriott KA, Parkinson CV, Morefield SI, Davenport R, Nichols R, Monath TP. Clonal vaccinia virus grown in cell culture fully protects monkeys from lethal monkeypox challenge. *Vaccine* 2008;26:581–588. [PubMed: 18077063]
14. Fulginiti VA. Risks of smallpox vaccination. *Jama* 2003;290:1452. author reply 1452. [PubMed: 13129976]
15. Neff JM, Lane JM, Fulginiti VA. Smallpox and smallpox vaccination. *N Engl J Med* 2003;348:1920–1925. author reply 1920–1925. [PubMed: 12740965]
16. Cono J, Casey C, Bell D. Smallpox Vaccination and Adverse Reactions. Morbidity and Mortality Weekly Report (MMWR) 2003;52:1–28.
17. Fulginiti VA, Papier A, Lane JM, Neff JM, Henderson DA. Smallpox vaccination: a review, part II. Adverse events. *Clin Infect Dis* 2003;37:251–271. [PubMed: 12856218]
18. Wittek R. Vaccinia immune globulin: current policies, preparedness, and product safety and efficacy. *Int J Infect Dis* 2006;10:193–201. [PubMed: 16564720]
19. Kawakami Y, Tomimori Y, Yumoto K, Hasegawa S, Ando T, Tagaya Y, Crotty S, Kawakami T. Inhibition of NK cell activity by IL-17 allows vaccinia virus to induce severe skin lesions in a mouse model of eczema vaccinatum. *J Exp Med*. 2009
20. Hopkins RJ, Lane JM. Clinical efficacy of intramuscular vaccinia immune globulin: a literature review. *Clin Infect Dis* 2004;39:819–826. [PubMed: 15472814]
21. Kempe CH. Studies on smallpox and complications of smallpox vaccination. *Pediatrics* 1960;25:176–189. [PubMed: 14408455]
22. Kempe CH, Berge TO, England B. Hyperimmune vaccinia gamma globulin. *Pediatrics* 1956;18:177–188. [PubMed: 13349330]
23. Marennikova SS. The use of hyperimmune antivaccinia gamma-globulin for the prevention and treatment of smallpox. *Bull World Health Organ* 1962;27:325–330. [PubMed: 13932966]
24. Couzi G, Kircher JP. Immunotherapie de la Variole. *Bulletin de l'Institut d'hygiene du Maroc* 1941;1:59–68.
25. Corporation, BH. Vaccinia Immune Globulin (Human) package insert. Edited by Editor|. Year|; p.^pp. Pages|. City|: Publisher|
26. Dynport Vaccine Company. Vaccinia Immune Globulin Intravenous (Human) (VIGIV) package insert. Edited by Editor|. Year|; p.^pp. Pages|. City|: Publisher|
27. Cangene Corporation. Vaccinia Immune Globulin Intravenous (Human) (VIGIV) package insert. Edited by Editor|. Year|; p.^pp. Pages|. City|: Publisher|
28. Shearer JD, Siemann L, Gerkovich M, House RV. Biological activity of an intravenous preparation of human vaccinia immune globulin in mouse models of vaccinia virus infection. *Antimicrob Agents Chemother* 2005;49:2634–2641. [PubMed: 15980330]
29. Goldsmith JC, Eller N, Mikolajczyk M, Manischewitz J, Golding H, Scott DE. Intravenous immunoglobulin products contain neutralizing antibodies to vaccinia. *Vox Sang* 2004;86:125–129. [PubMed: 15023182]
30. Edghill-Smith Y, Golding H, Manischewitz J, King LR, Scott D, Bray M, Nalca A, Hooper JW, Whitehouse CA, Schmitz JE, Reimann KA, Franchini G. Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. *Nat Med* 2005;11:740–747. [PubMed: 15951823]
31. Amanna IJ, Slifka MK, Crotty S. Immunity and immunological memory following smallpox vaccination. *Immunol Rev* 2006;211:320–337. [PubMed: 16824139]
32. Bell E, Shamim M, Whitbeck JC, Sfyroera G, Lambris JD, Isaacs SN. Antibodies against the extracellular enveloped virus B5R protein are mainly responsible for the EEV neutralizing capacity of vaccinia immune globulin. *Virology* 2004;325:425–431. [PubMed: 15246280]

33. He Y, Manischewitz J, Meseda CA, Merchlinsky M, Vassell RA, Sirota L, Berkower I, Golding H, Weiss CD. Antibodies to the A27 protein of vaccinia virus neutralize and protect against infection but represent a minor component of Dryvax vaccine--induced immunity. *J Infect Dis* 2007;196:1026–1032. [PubMed: 17763325]
34. Davies DH, Molina DM, Wrammert J, Miller J, Hirst S, Mu Y, Pablo J, Unal B, Nakajima-Sasaki R, Liang X, Crotty S, Karem KL, Damon IK, Ahmed R, Villarreal L, Felgner PL. Proteome-wide analysis of the serological response to vaccinia and smallpox. *Proteomics* 2007;7:1678–1686. [PubMed: 17443847]
35. Davies DH, McCausland MM, Valdez C, Huynh D, Hernandez JE, Mu Y, Hirst S, Villarreal L, Felgner PL, Crotty S. Vaccinia virus H3L envelope protein is a major target of neutralizing antibodies in humans and elicits protection against lethal challenge in mice. *J Virol* 2005;79:11724–11733. [PubMed: 16140750]
36. Benhnia MR-E-I, McCausland MM, Su H-P, Singh K, Hoffmann J, Davies DH, Felgner PL, Head S, Sette A, Garboczi DN, Crotty S. Redundancy and plasticity of neutralizing antibody responses are cornerstone attributes of the human immune response to the smallpox vaccine. *Journal of Virology* 2008;82:3751–3768. [PubMed: 18234801]
37. Lustig S, Fogg C, Whitbeck JC, Eisenberg RJ, Cohen GH, Moss B. Combinations of polyclonal or monoclonal antibodies to proteins of the outer membranes of the two infectious forms of vaccinia virus protect mice against a lethal respiratory challenge. *J Virol* 2005;79:13454–13462. [PubMed: 16227266]
38. Benhnia MR-E-I, McCausland MM, Moyron J, Laudenslager J, Granger S, Rickert S, Koriazova L, Kubo R, Kato S, Crotty S. Vaccinia virus extracellular enveloped virion neutralization in vitro and protection in vivo depend on complement. *Journal of Virology* 2009;83:1201–1215. [PubMed: 19019965]
39. Benhnia MR, McCausland MM, Laudenslager J, Granger SW, Rickert S, Koriazova L, Tahara T, Kubo RT, Kato S, Crotty S. Heavily isotype-dependent protective activities of human antibodies against vaccinia virus extracellular virion antigen B5. *J Virol* 2009;83:12355–12367. [PubMed: 19793826]
40. Tomizuka K, Shinohara T, Yoshida H, Uejima H, Ohguma A, Tanaka S, Sato K, Oshimura M, Ishida I. Double trans-chromosomal mice: maintenance of two individual human chromosome fragments containing Ig heavy and kappa loci and expression of fully human antibodies. *Proc Natl Acad Sci U S A* 2000;97:722–727. [PubMed: 10639146]
41. Benhnia MR, McCausland MM, Moyron J, Laudenslager J, Granger S, Rickert S, Koriazova L, Kubo R, Kato S, Crotty S. Vaccinia virus extracellular enveloped virion neutralization in vitro and protection in vivo depend on complement. *J Virol* 2009;83:1201–1215. [PubMed: 19019965]
42. Newman FK, Frey SE, Blevins TP, Mandava M, Bonifacio A Jr, Yan L, Belshe RB. Improved assay to detect neutralizing antibody following vaccination with diluted or undiluted vaccinia (Dryvax) vaccine. *J Clin Microbiol* 2003;41:3154–3157. [PubMed: 12843056]
43. Benhnia MR, McCausland MM, Su HP, Singh K, Hoffmann J, Davies DH, Felgner PL, Head S, Sette A, Garboczi DN, Crotty S. Redundancy and plasticity of neutralizing antibody responses are cornerstone attributes of the human immune response to the smallpox vaccine. *J Virol* 2008;82:3751–3768. [PubMed: 18234801]
44. Lin CL, Chung CS, Heine HG, Chang W. Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. *J Virol* 2000;74:3353–3365. [PubMed: 10708453]
45. Moyron-Quiroz JE, McCausland MM, Kageyama R, Sette A, Crotty S. The smallpox vaccine induces an early neutralizing IgM response. *Vaccine* 2009;28:140–147. [PubMed: 19822230]
46. Sakhatsky P, Wang S, Chou TH, Lu S. Immunogenicity and protection efficacy of monovalent and polyvalent poxvirus vaccines that include the D8 antigen. *Virology*. 2006
47. Chen Z, Earl P, Americo J, Damon I, Smith SK, Zhou YH, Yu F, Sebrell A, Emerson S, Cohen G, Eisenberg RJ, Svitel J, Schuck P, Satterfield W, Moss B, Purcell R. Chimpanzee/human mAbs to vaccinia virus B5 protein neutralize vaccinia and smallpox viruses and protect mice against vaccinia virus. *Proc Natl Acad Sci U S A* 2006;103:1882–1887. [PubMed: 16436502]
48. Chen Z, Earl P, Americo J, Damon I, Smith SK, Yu F, Sebrell A, Emerson S, Cohen G, Eisenberg RJ, Gorshkova I, Schuck P, Satterfield W, Moss B, Purcell R. Characterization of chimpanzee/

human monoclonal antibodies to vaccinia virus A33 glycoprotein and its variola virus homolog in vitro and in a vaccinia virus mouse protection model. *Journal of Virology* 2007;81:8989–8995. [PubMed: 17581986]

49. Fogg CN, Americo JL, Earl PL, Resch W, Aldaz-Carroll L, Eisenberg RJ, Cohen GH, Moss B. Disparity between levels of in vitro neutralization of vaccinia virus by antibody to the A27 protein and protection of mice against intranasal challenge. *Journal of Virology* 2008;82:8022–8029. [PubMed: 18524827]
50. Nelson G, Sisler J, Chandran D, Moss B. Vaccinia virus entry/fusion complex subunit A28 is a target of neutralizing and protective antibodies. *Virology*. 2008

Figure 1. In vitro neutralization of murine anti-H3 mAb #41

A) Neutralization of VACV_{WR} by murine anti-H3 mAb #41 in a standard overnight neutralization assay. “VACV” = no mAb added. **B)** Neutralization of VACV_{WR} by murine anti-H3 mAb #41 in a 60 minute neutralization assay. **C)** Neutralization of VACV_{WR} by murine anti-H3 mAb #41 in a 60 minute neutralization assay in the presence of 1% complement. Anti-H3 mAb #41 was used at 10 µg/mL in all conditions. Data are representative of three or more experiments.

Figure 2. In vivo protection of murine anti-H3 mAb vs. VIG

A) SCID model VACV_{NYCBOH} titration. Adult SCID mice were infected intravenously (i.v.) with 1×10^3 PFU, 1×10^4 PFU, or 1×10^5 PFU VACV_{NYCBOH}. Body weight was tracked. Dotted lines indicate starting body weight (upper) and terminal body weight (bottom). **(B–C)** SCID mice were treated with a single dose of 1.25 mg VIG, 200 μ g anti-H3 murine mAb #41, or PBS at day -1 and then infected with 1×10^4 PFU VACV_{NYCBOH} i.v. Body weight, clinical disease (pox), and survival were tracked. N = 5/group. Data are representative of four independent experiments. **B)** Body weight kinetics. **C)** Clinical scores. **D)** Efficacy of murine anti-H3 mAb against virulent VACV_{WR} in SCID mice. SCID mice were treated with 200 μ g anti-H3 murine mAb #41, 600 μ g VIG, or PBS at day -1 and then infected with 1×10^3 PFU VACV_{WR} i.v. Body weight was tracked. N = 6/group. Data are representative of two experiments. Error bars are $\pm$ SEM.

Figure 3. In vivo protection of human anti-H3 mAb vs. VIG

(A–B) In vitro neutralization of fully human anti-H3 mAbs. **A)** Neutralization of VACV_{WR} by human anti-H3 mAb hV26 in a standard overnight neutralization assay. Dose titration showed a 50% neutralization (PRNT₅₀) at ~100 ng/mL. Dashed lines indicate VACV plaque number in the absence of mAb (top line) and the 50% plaque number (middle). **B)** Neutralization of VACV_{WR} by hV26. Three neutralization assays were used. (left) Overnight neutralization assay. **, $P = 0.006$. (middle) one hour neutralization in absence of complement. **, $P = 0.0012$. (right) one hour neutralization in the presence of 1% complement (1hr + C'). **, $P = 0.005$. “VACV” = no mAb added. hV25 = an anti-H3 mAb exhibiting no neutralization. Data are representative of three or more independent experiments. (C–E) SCID mice were treated with a single dose of 100 μg human anti-H3 mAb hV26, 1.25 mg VIG, or PBS at day -1 and then infected with 1×10^4 PFU VACV_{WR} i.v. Body weight, clinical disease (pox), and survival were tracked. $N = 6$ /group respectively. All data are representative of three or more experiments. **C)** Body weights, averages per group. Anti-H3 hV26 vs. PBS, $P < 0.003$. VIG vs. PBS, $P < 0.0002$. **D)** Survival curves, per treatment group. Anti-H3 hV26 vs. PBS, $P < 0.0006$. VIG vs. PBS, $P < 0.004$. **E)** Clinical scores, per treatment group. Anti-H3 hV26 vs. PBS, $P < 0.0003$. VIG vs. PBS, $P < 0.0005$. Error bars are $\pm$ SEM.

Figure 4. Titration of human anti-H3 mAb protection

SCID mice were treated with a single dose of human anti-H3 mAb hV26 (10 µg, 20 µg, 50 µg, 100 µg, or 200 µg), 1.25 mg VIG, or PBS at day -1 and then infected with 5×10^4 PFU VACV ACAM 2000 strain i.v. N = 5/group, except PBS group N = 4. **A**) Body weights. **B**) Clinical score. Data are representative of two independent experiments. Errors bars are $\pm$ SEM.

Figure 5. In vivo protection of human anti-B5 mAb vs. VIG

SCID mice were treated with a single dose of human anti-B5 mAb h101 (50 μ g), 1.25 mg VIG, or PBS at day -1 and then infected with 1×10^4 PFU VACV_{NYCBOH} i.v. N = 6 mice/group for VIG and PBS, 10/group for h101. **A)** Body weights. **B)** Time (days) to 5% weight loss. ** P < 0.01. **C)** Clinical scores. P < 0.0001, h101 vs. PBS. **D)** Survival. P < 0.0001, h101 vs. PBS. Data are representative of three or more experiments. Error bars are $\pm$ SEM.

Figure 6. Titration of human anti-B5 mAb protection

SCID mice were treated with a single dose of human anti-B5 mAb h101 (25 µg, 50 µg, or 100 µg), 1.25 mg VIG, control human mAb (IgG1 anti-DNP hapten), or PBS at day -1 and then infected with 5×10^4 PFU VACV (ACAM 2000 strain) i.v. $N = 5/\text{group}$. **A)** Body weights. **B)** Time to 5% weight loss. **C)** Clinical score. Error bars are $\pm$ SEM. **D)** Survival curves.

Figure 7. In vivo protection of anti-H3 plus anti-B5 vs VIG

SCID mice were treated with a single dose of human anti-H3 mAb hV26 (50 μ g), human anti-B5 mAb h101 (50 μ g), 25 μ g combination of human anti-H3 mAb hV26 and anti-B5 mAb h101 (25 μ g or each), 50 μ g combination of human anti-H3 mAb hV26 and anti-B5 mAb h101 (50 μ g or each) 1.25 mg VIG, or control human anti-DNP mAb (50 μ g) at day -1 and then infected with 5×10^4 PFU ACAM2000 clone of VACV_{NYCBOH} i.v. N = 9–10/group. **A**) Body weights. **B**) Time to 5% weight loss. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **C**) Clinical score. **D**) Survival. Data are representative of two to four independent experiments. Experiment was ended at day 81, with 50% of h101 + hV26 50 μ g combination group still healthy ($> 100\%$ initial body weight). Error bars are $\pm$ SEM.

Table 1

Results from all combination treatment studies.

	Time to >5% Weight Loss	Overall Weight Loss	Clinical Score	Survival
Study 1 (n=5 or 6)				
Combo vs. PBS	P=0.049	P=0.003	P=0.003	P=0.0005
Combo vs. VIG	P=0.82	P=0.06	P=0.006	P=0.005
Study 2 (n=5)				
Combo vs. control mAb	P=0.02	P=0.017	P<0.0001	P=0.003
Combo vs. VIG	P=0.15	P=0.98	P=0.01	P=0.49
Study 3 (n=10)				
Combo vs. control mAb	P=0.0009	P=0.0006	P<0.0001	P<0.0001
Combo vs. VIG	P=0.07	P=0.04	P=0.06	P=0.03
Combo vs. C12	P=0.34	P=0.16	P=0.42	P=0.22
Combo vs. D67	P=0.19	P=0.051	P=0.16	P=0.036
Study 4 (n=10)				
Combo vs. control mAb	P<0.0001	P<0.0001	P<0.0001	P=0.0015
Combo vs. VIG	P=0.0009	P=0.039	P=0.0036	P=0.14
Combo vs. C12	P=0.084	P=0.40	P=0.03	P=0.37
Meta-Analysis				
Combo vs. control mAb	P<0.0001	P<0.0001	P<0.0001	P<0.0001
Combo vs. VIG	P=0.0002	P=0.0004	P=0.0006	P=0.0003
Combo vs. C12	P=0.046	P=0.049	P=0.096	P=0.006