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Prophylactic and therapeutic efficacy of mAb treatment against MERS-CoV in common marmosets

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ABSTRACT

The high case-fatality rate of confirmed MERS-CoV infections underlines the urgent need for an effective treatment to reduce the disease severity and mortality. REGN3051 and REGN3048 are two fully human neutralizing monoclonal antibodies (mAb) against MERS-CoV that reduced virus replication in mice expressing human DPP4 upon prophylactic and therapeutic treatment. Here, we evaluated the prophylactic and therapeutic efficacy of REGN3048 and REGN3051 in the common marmoset model of MERS-CoV infection. Intravenous administration of mAb resulted in high levels of MERS-CoV-neutralizing activity in circulating blood. When animals were treated with mAbs one day before challenge, respiratory disease was less severe and, in animals treated with both REGN3048 and REGN3051, viral loads in the lungs were reduced. However, therapeutic treatment on day one after challenge was less efficacious as it did not prevent the development of severe respiratory disease and all treated animals developed bronchointerstitial pneumonia of similar severity as the control animals. Thus, mAb administration may be more effective in a prophylactic treatment regimen rather than treatment of MERS.

1. Introduction

Since the initial identification of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) as the causative agent of severe respiratory disease in 2012, 2121 cases have been detected with 740 fatalities (WHO, 2017). The high case-fatality rate of MERS-CoV infections underlines the urgent need for an effective treatment to reduce the burden of disease. Convalescent plasma therapy has been suggested as a promising option for improving disease outcome in MERS patients (Consortium, 2013). A meta-analysis of studies into the efficacy of convalescent plasma therapy in SARS patients showed a reduced case-fatality rate and reduced time to discharge in patients when treatment was administered early in the disease course; however, the quality of studies included in the meta-analysis was considered low and with a moderate to high risk of bias (Mair-Jenkins et al., 2015).

As a proof of concept for the therapeutic or prophylactic approach of convalescent plasma for MERS CoV, MERS-CoV-neutralizing serum from Egyptian dromedary camels naturally infected with MERS-CoV was transferred to IFNAR$^{-/-}$ mice transduced with an adenovirus expressing human DPP4 (Ad5-hDPP4) and inoculated with MERS-CoV, resulting in reduced weight loss and lung pathology in treated animals compared to controls (Zhao et al., 2015). Polyclonal immunoglobulin preparations from the plasma of transchromosomal cows or horses vaccinated against MERS-CoV were also effective in reducing lung virus titers when administered to Ad5-hDPP4 mice one day after inoculation with MERS-CoV (Luke et al., 2016; Zhao et al., 2017). However, despite promising results in mouse models, the high case-fatality rate in humans, low neutralizing antibody titers in survivors and the inability of many survivors to function as plasma donors due to underlying health issues are considerable hurdles in the implementation of convalescent plasma therapy for MERS patients (Arabi et al., 2016). Monoclonal antibody (mAb) treatment could provide an alternative for convalescent plasma, and several neutralizing monoclonal antibodies have been developed and tested in animal models (Agrawal et al., 2016;

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Corti et al., 2015; Houser et al., 2016; Johnson et al., 2016; Li et al., 2015; Pascal et al., 2015; Qiu et al., 2016).

REGN3051 and REGN3048 are two fully human, neutralizing monoclonal antibodies that bind to previously described distinct epitopes in the receptor-binding domain of the MERS-CoV spike protein (Pascal et al., 2015). Treatment of mice expressing human DPP4 under control of the mouse DPP4-regulatory elements with 200 μg of either REGN3048 or REGN3051 24hrs before inoculation with MERS-CoV significantly reduced virus titers in the lungs; similarly, treatment of mice with 500 μg REGN3051 24hrs after inoculation with MERS-CoV also reduced virus titers in the lungs. Inoculation of common marmosets with MERS-CoV results in coalescing bronchointerstitial pneumonia with clear signs of respiratory disease that may require euthanasia (Chan et al., 2015; Falzarano et al., 2014). Here, we evaluated the ability of the human, neutralizing mAbs REGN3048 and REGN3051 to reduce disease severity and virus replication in the lungs of common marmosets inoculated with MERS-CoV upon prophylactic and therapeutic treatment. In this model, there was a clear clinical benefit upon prophylactic treatment with mAb; however, the effect of therapeutic mAb treatment on the disease course was limited.

2. Materials and methods

2.1. Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of Rocky Mountain Laboratories, NIH and carried out by certified staff in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited facility, according to the institution's guidelines for animal use, and followed the guidelines and basic principles in the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals (available from http://grants.nih.gov/grants/olaw/references/PHSPolicyLabAnimals.pdf), and the Guide for the Care and Use of Laboratory Animals (available from https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-use-of-laboratory-animals.pdf).

The Institutional Biosafety Committee (IBC) approved work with infectious MERS-CoV strains under BSL3 conditions. Sample inactivation was performed according to IBC-approved standard operating procedures for removal of specimens from high containment.

2.2. Study design

To evaluate the effect of neutralizing monoclonal antibody treatment on MERS-CoV disease outcome, we used the common marmoset (Callithrix jacchus) MERS-CoV infection model that recapitulates the severe respiratory disease observed in hospitalized patients.

All animals were randomly assigned to groups and inoculated as described previously (Falzarano et al., 2014). Briefly, inoculation with MERS-CoV strain EMC/2012 was performed intranasally (100 μl per nare), orally (500 μl), intratracheally (500 μl) and ocular (50 μl per eye) with DMEM containing 4×10^6 TCID50/ml (total dose 5.2×10^6 TCID50). The animals were treated with mAb REGN684, REGN3048 or REGN3051. REGN684 is isotype control antibody and thus a non-specific treatment control in this experiment; REGN3048 and REGN3051 are both in vitro neutralizing mAbs against MERS-CoV that bind discrete epitopes in the MERS-CoV spike protein receptor binding domain (Pascal et al., 2015). The treatment groups, consisting of three female and three male common marmosets each, received a single treatment intravenously in a volume of ≤1 ml depending on bodyweight as indicated in Table 1.

The animals were observed twice daily for clinical signs of disease using a standardized scoring sheet as described previously (Falzarano et al., 2014). Based on the scoring sheet, euthanasia was indicated at a clinical score of 35 or more. The predetermined endpoint for this experiment was 6 days post inoculation (dpi). Clinical exams were performed on 0, 2, 4, and 6 dpi on anaesthetized animals. On exam days, clinical parameters such as bodyweight and respiration rate were collected, as well as dorsal-ventral and lateral radiographs and a blood sample. Terminal blood samples were obtained and samples of the following tissues were collected: conjunctiva, nasal mucosa, tonsil, trachea, all four lung lobes, mediastinal lymph node, liver, spleen, kidney, and bladder.

Table 1
Treatment schedule for determining the prophylactic and therapeutic efficacy of mAb treatment in common marmosets infected with MERS-CoV.

Group		N	Treatment	Dose	Time point
I	Control	3	REGN684	25 mg/kg	24 h before challenge
		3	REGN684	25 mg/kg	24 h after challenge
II	Prophylaxis	6	REGN3051	25 mg/kg	24 h before challenge
III	Prophylaxis	6	REGN3048 & REGN3051	12.5 mg/kg each	24 h before challenge
IV	Therapy	6	REGN3051	10 mg/kg	24 h after challenge
V	Therapy	6	REGN3051	25 mg/kg	24 h after challenge

2.3. Virus and cells

HCoV-EMC/2012 (Vero passage 6) was kindly provided by the Department of Viroscience, Erasmus Medical Center, Rotterdam, The Netherlands and propagated once in VeroE6 cells in DMEM (Sigma) supplemented with 2% fetal calf serum (Logan), 1 mM L-glutamine (Lonza), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco) (virus isolation medium). VeroE6 cells were maintained in DMEM supplemented with 10% fetal calf serum, 1 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

2.4. Virus neutralization assay

Two-fold serial dilutions of heat-inactivated (30 min, 56 °C) marmoset sera were prepared in DMEM containing 2% fetal calf serum, 1 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin, after which 100 TCID50 of HCoV-EMC/2012 virus was added. After 1hr incubation at 37 °C, this mix was added to VeroE6 cells. At 5 dpi, wells were scored for cytopathic effect. The virus neutralization titer was expressed as the reciprocal value of the highest dilution of the serum that still inhibited HCoV-EMC/2012 virus replication.

2.5. Quantitative PCR

Tissues (30 mg) were homogenized in RLT buffer and RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions. For detection of viral RNA, 5 μl RNA was used in a one-step real-time RT-PCR upE assay (Corman et al., 2012) using the Rotor-Gene probe kit (Qiagen) according to instructions of the manufacturer. In each run, standard dilutions of a titered virus stock were run in parallel, to calculate TCID50 equivalents in the samples.

2.6. Histopathology and immunohistochemistry

Histopathology and immunohistochemistry were performed on marmoset tissues. After fixation for 7 days in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were stained with hematoxylin and eosin (HE). To detect HCoV-EMC/2012 antigen, immunohistochemistry was performed using an in-house rabbit polyclonal antiserum against HCoV-EMC/2012 (1:1000) as a primary antibody.

Quantitation of antigen-positive lung tissues was done as described

previously (Baseler et al., 2016). Briefly, sections of lung from animals necropsied on 6 dpi were labeled for MERS-CoV antigen, digitized using an Aperio Digital Slide Scanner (Leica) and analyzed using the positive pixel count algorithm in ImageScope version 12.1.0.5029 (Leica).

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 7.01.

3. Results

3.1. High MERS-CoV neutralizing antibody titers in common marmosets after treatment

To test whether treatment with neutralizing mAbs can reduce disease severity caused by MERS-CoV in common marmosets, 5 groups of six marmosets were treated with an intravenous infusion of mAbs as indicated in Table 1. Control animals were treated with 25 mg/kg of REGN684, a non-MERS-CoV-S-binding isotype control. Therapeutic efficacy of two different doses of REGN3051 was tested in the first experiment; this experiment included one group of three REGN684 control-treated animals, and two groups treated with 10 and 25 mg/kg of REGN3051, respectively, treated 24 h after inoculation with MERS-CoV. When the results of this first experiment showed a limited efficacy of antibody treatment, a second experiment was performed. This experiment included one group of three REGN684 control-treated animals, and two groups treated with 25 mg/kg of REGN3051 or a combination of REGN3048 and REGN3051 at a concentration of 12.5 mg/kg each; animals were treated 24 h before inoculation with MERS-CoV.

Animals were randomly assigned to the following groups of 6 marmosets (Table 1): Three animals in the control group (group I) were treated prophylactically on day −1 and three animals were treated therapeutically on day 1 with 25 mg/kg REGN684. The marmosets in group II were treated prophylactically on day −1 with 25 mg/kg REGN3051; Group III was treated prophylactically on day −1 with a combination of REGN3048 and REGN3051 at 12.5 mg/kg each; group IV was treated therapeutically on day 1 with 10 mg/kg REGN3051 and group V was treated therapeutically on day 1 with 25 mg/kg. On 0, 2 and 6 days post inoculation (dpi), serum was collected from all animals to determine the level of MERS-CoV-neutralizing antibodies. As expected, no neutralizing antibodies against MERS-CoV were detected in the animals in group I that were infused with a control mAb (Fig. 1). The animals in groups II and III that were treated prophylactically had very high neutralizing antibody titers at the time of inoculation with MERS-CoV on day 0, ranging from 2560 to 7680 in individual animals. In five out of six animals in group II, the neutralizing titers had dropped between 0 and 6 dpi (Fig. 1). In group IV, neutralizing titers on 2 dpi ranged from 960 to 7680 and dropped to 960–1920 by 6 dpi. One animal in this group was euthanized due to severity of disease on 5 dpi; neutralizing titers in this animal were 2560 on 2 dpi and remained high at time of euthanasia (1280), so the severe disease observed in this animal was unlikely to be due to low neutralizing antibody titers. The animals in group V received a higher mAb dose than those in group IV, and this was reflected in the level of neutralizing antibodies detected in serum, that ranged from 1280 to 10,240 on 2 dpi. Titers dropped by 6 dpi in most animals to titers ranging from 2560 to 5120 (Fig. 1).

3.2. Clinical signs of disease after challenge with MERS-CoV

On day 0, all animals were challenged with 5.2×10^6 TCID50 of MERS-CoV isolate HCoV-EMC/2012 as described previously (Falzarano et al., 2014). After inoculation, animals were closely monitored for signs of disease and clinical scores were assigned according to a previously assigned scoring sheet (Falzarano et al., 2014). All the animals in group I treated with a control mAb showed signs of disease (Fig. 2A), such as decreased activity, starting on 1 or 2 dpi; all animals also had increased respiration starting on or after 1 dpi and lasting until the end of the experiment on 6 dpi. In contrast, four out of six marmosets in groups II and III that were treated prophylactically with different mAbs did not show obvious disease signs (Fig. 2A), other than loss of appetite in some of the animals that may have been the result of repeated anesthesia. The remaining two animals in group II and III showed decreased activity, but only one animal in each group showed increased respirations. All marmosets in groups IV and V, treated therapeutically with different doses of REGN3051, showed decreased activity starting on 1 dpi and lasting throughout the experiment and all but one animals showed increased respiration on several days after inoculation (Fig. 2A). One animal in group IV developed dyspnea, cyanosis and hypothermia (body temperature 33.9 °C) on 5 dpi and was euthanized.

All animals in group I lost bodyweight after inoculation with MERS-CoV, with animals losing between 6% and 17% of starting weight during the experiment (Fig. 2B). Weight loss in groups II and III was reduced, with a maximum observed weight loss of 6% and 7% respectively. Weight loss comparable to that in control group I was observed in all animals in group IV. In group V, five out of six animals showed weight loss ranging from 6% to 12% (Fig. 2B).

The respiration rate was established in all animals during clinical exams as an indicator of respiratory disease. Respiration rate clearly increased in all animals in control group I (Fig. 2C); no clear increases were observed in any of the animals in group II and in 4 out of 6 animals in group III. In group IV and V, there was a clear increase in respiration rate in 4 out of 6 animals (Fig. 2C).

Dorsal-ventral and lateral x-rays were collected to monitor signs of pneumonia on 0, 2, 4 and 6 dpi and analyzed by a clinical veterinarian according to a standard scoring system. The cumulative x-ray scores of all animals on 6 dpi, except for the one animal euthanized on 5 dpi for which x-ray score on 5 dpi is displayed, are shown in Fig. 2D. All animals treated with a control mAb in group I developed lung infiltrates visible on x-ray in two or more lung lobes. Pulmonary infiltrates were less prominent in animals in group II and most animals in group III. Pulmonary infiltrates in groups IV and V ranged from absent to severe (Fig. 2D).

3.3. Viral loads in lung tissue after treatment with mAb

All animals were euthanized on 6 dpi to determine the viral loads in tissues and perform histopathology. Since the lungs are the main site of MERS-CoV replication in common marmosets, we compared the viral loads, as determined by qRT-PCR (Corman et al., 2012), in all four lung lobes. In group I, viral loads in all lung lobes were consistently high; variation between animals was much larger in the groups treated with MERS-CoV-neutralizing antibodies (Fig. 3A). Further analysis of complete lung viral loads (Fig. 3B) showed that viral loads of animals in treatment groups III and IV were statistically significantly lower (One-way ANOVA with Dunnett's multiple comparisons test) than those in animals in group I; viral loads in the lungs of animals in treatment groups II and V did not differ significantly from those in group I. Of note, the animal in group IV that was euthanized on 5 dpi was excluded from this analysis; however, even when this animal is included there is still a statistically significant difference between lung viral loads in group I vs. group IV, but the difference is smaller. These differences do not correlate with the dose of mAb administered to the animals. Various other tissues were also tested for the presence of viral RNA. The viral loads in respiratory tissues and lymph nodes that drain those tissues were often significantly higher in the animals in group I than in the treated groups II-V. In extra-respiratory tissues there were few significant differences between the viral loads in animals in control group I versus any of the treatment groups (Fig. 4). Of note, the animal in group IV that was euthanized on 5 dpi due to severity of disease, was excluded from these analyses. Viral loads in tissues from this animal are displayed separately in Fig. S1.

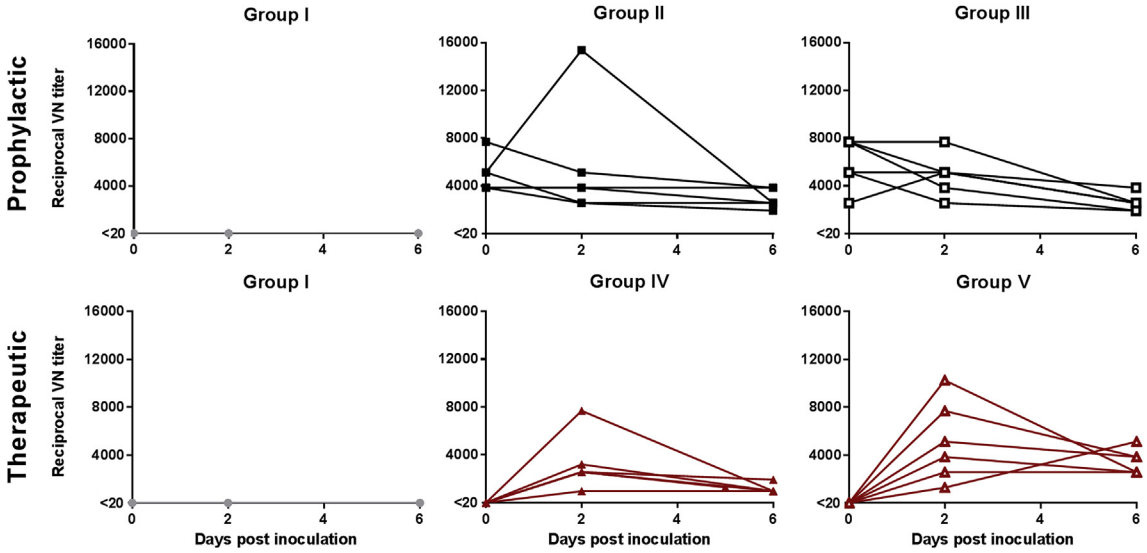

Fig. 1. MERS-CoV neutralization titers in the serum of common marmosets treated with monoclonal antibodies. Five groups of six marmosets were inoculated with MERS-CoV and treated with mAb as indicated in Table 1. On 0, 2 and 6 dpi, serum was collected and tested for the presence of neutralizing antibodies. Treatment groups: Group I: 25 mg/kg REGN684 administered prophylactically or therapeutically in three animals each; Group II: 25 mg/kg REGN3051 prophylactically; Group III: 12.5 mg/kg REGN3048 and 12.5 mg/kg REGN3051 prophylactically; Group IV: 10 mg/kg REGN 3051 therapeutically; Group V: 25 mg/kg REGN3051 therapeutically.

3.4. Lung histopathology in lungs after treatment with mAb

All animals euthanized on 6 dpi developed multifocal to coalescing, minimal to marked subacute bronchointerstitial pneumonia with type II pneumocyte hyperplasia as described previously (Baseler et al., 2016; Falzarano et al., 2014). Histopathologically, there was no difference in the severity or nature of the pneumonia between control animals in group I and prophylactically treated animals in group II; group III animals developed slightly less severe lesions as compared to controls (Fig. 5). There was no difference in the severity or nature of the pneumonia between animals in control group I and animals treated therapeutically with neutralizing mAbs in groups IV and V (Fig. 5).

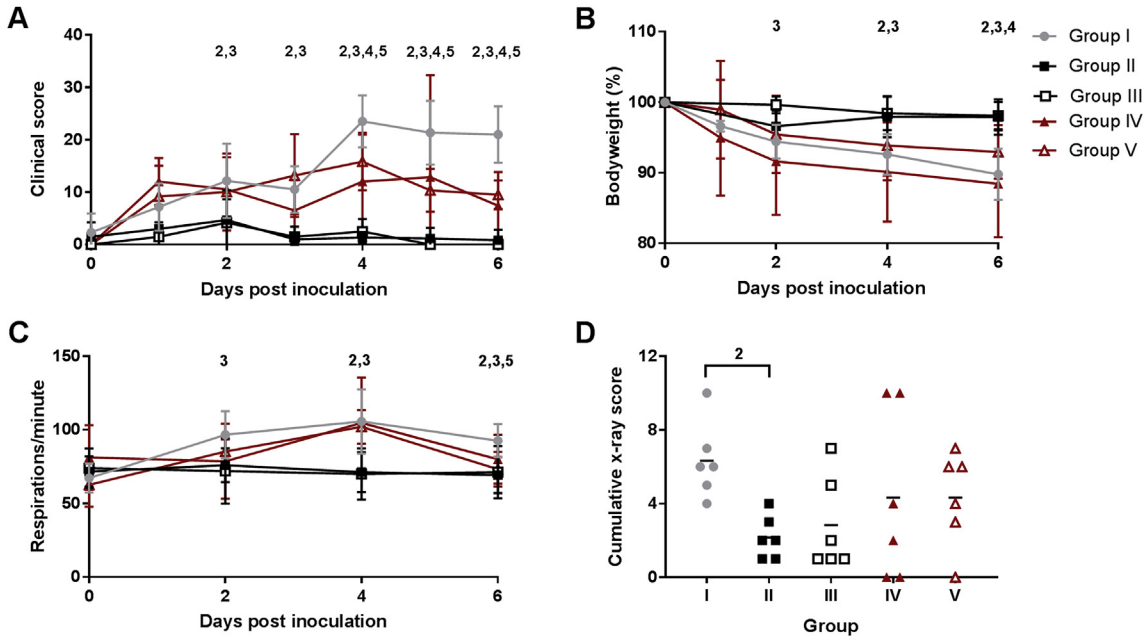

Fig. 2. Clinical findings in common marmosets inoculated with MERS-CoV and treated with neutralizing mAbs. Five groups of six marmosets were inoculated with MERS-CoV and treated with mAb as indicated in Table 1. After inoculation, the animals were observed twice daily for clinical signs of disease and scored using a clinical scoring system prepared for common marmosets (Falzarano et al., 2014) (A). On 0, 2, 4 and 6 dpi, clinical exams were performed during which bodyweight (B) and respiration rate (C) were determined and radiographs were taken. Radiographs were used to score individual lung lobes for severity of pulmonary infiltrates by a clinical veterinarian according to a standard scoring system (0: normal; 1: mild interstitial pulmonary infiltrates; 2: moderate pulmonary infiltrates perhaps with partial cardiac border effacement and small areas of pulmonary consolidation; 3: serious interstitial infiltrates, alveolar patterns and air bronchograms); the cumulative x-ray score is the sum of the scores of the four individual lung lobes per animal (D). Symbols indicate statistically significant difference in a 2-way ANOVA with Dunnett's multiple comparisons between 2: group I and II; 3: group I and III; 4: group I and group IV; 5: group I and V. Treatment groups: Group I: 25 mg/kg REGN684; Group II: 25 mg/kg REGN3051 prophylactically; Group III: 12.5 mg/kg REGN3048 and 12.5 mg/kg REGN3051 prophylactically; Group IV: 10 mg/kg REGN3051 therapeutically; Group V: 25 mg/kg REGN3051 therapeutically; when no symbols are present, differences were not statistically significant.

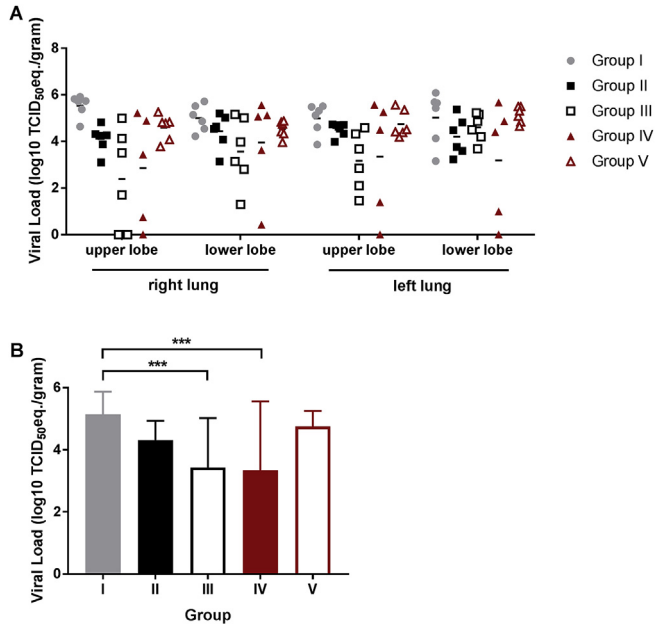

Fig. 3. Viral loads in the lungs of common marmosets inoculated with MERS-CoV and treated with neutralizing mAbs. Five groups of six marmosets were inoculated with MERS-CoV and treated with mAb as indicated in Table 1. On 6 dpi all animals were euthanized and tissue samples were collected from all 4 lung lobes, RNA was extracted and viral load determined as TCID50 equivalents per gram tissue. Individual animals and lung lobes are indicated (A) and averages and standard deviations per group (B). One-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significant differences between Group I and the other 4 groups. *** indicates $P < 0.0001$. Treatment groups: Group I: 25 mg/kg REGN684; Group II: 25 mg/kg REGN3051 prophylactically; Group III: 12.5 mg/kg REGN3048 and 12.5 mg/kg REGN3051 prophylactically; Group IV: 10 mg/kg REGN3051 therapeutically; Group V: 25 mg/kg REGN3051 therapeutically.

Immunohistochemistry was performed on lung tissues of all animals using an α-MERS-CoV antibody to look at the distribution of viral antigen. Small numbers of pneumocytes and rare macrophages were positive for viral antigen in all animals; these positive cells were predominantly associated with areas of pneumonia. Visually, there was no difference in the distribution of viral antigen between the five experimental groups (Fig. 4). An additional quantitative analysis of the number of antigen-positive pixels indicated that there was no difference in the number of MERS-CoV infected cells between animals in the control group and animals treated prophylactically or therapeutically, except a significantly higher number of antigen-positive cells in the left lung of animals in group IV as compared to group I (Fig. 6).

4. Discussion

Despite the urgent need for an effective treatment for MERS and the large number of monoclonal and polyclonal antibody preparations in development, very few of these have progressed beyond testing in small animal models. Three mAbs have been evaluated in nonhuman primate models of MERS-CoV infection. The prophylactic efficacy of mAb 3B11N was determined in rhesus macaques, where treatment resulted in a reduction in pathologic lung volume in computed tomography (Johnson et al., 2016). In the common marmoset model of MERS-CoV infection, some therapeutic efficacy of mAbs m336 and MCA1 was seen when animals were treated with m336 at 6 h and 2 days post inoculation, and with MCA1 at 2 or 12 h post inoculation (Chen et al., 2017; van Doremalen et al., 2017). Although some improvement in clinical disease was seen as compared to mock-treated control animals, neither treatment prevented MERS-CoV disease. Here, we tested the prophylactic and therapeutic efficacy of neutralizing mAbs against MERS-CoV in the common marmoset model of moderate to severe MERS. There was a clinical benefit of mAb prophylaxis in our study, with a reduction in clinical disease scores in all prophylactically treated animals. The effect of antibody administration on lung viral loads and lung histopathology was not consistent; although clinical scores were reduced in both prophylactic treatment groups, viral lung loads and histological

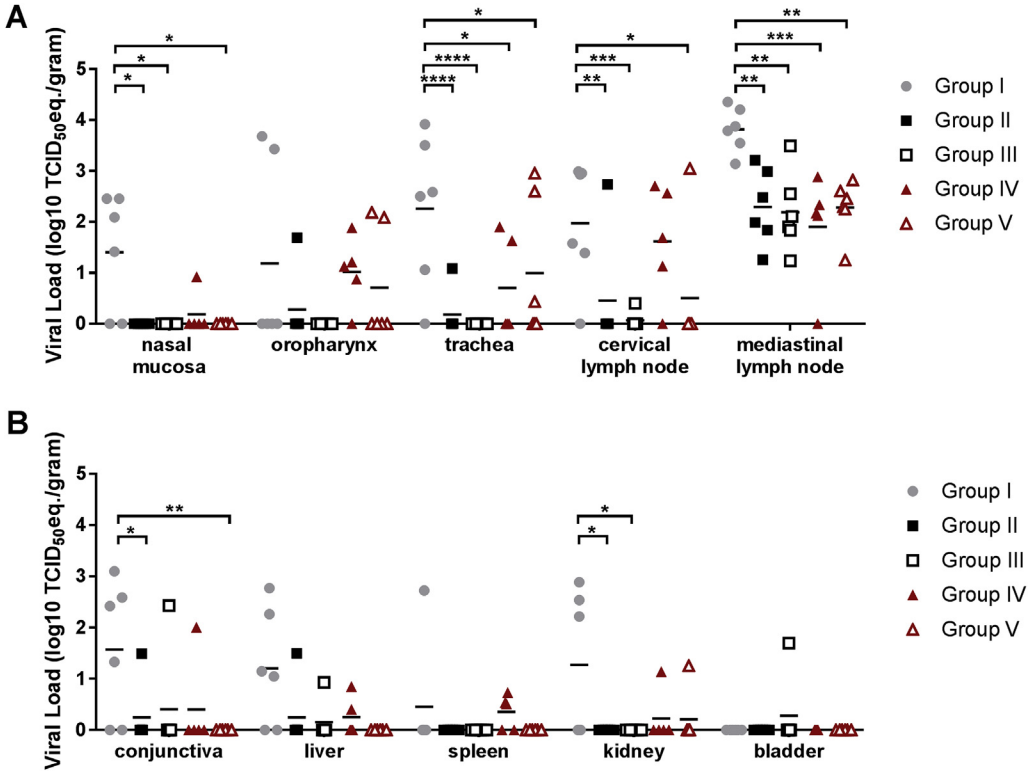

Fig. 4. Viral loads in tissues of common marmosets inoculated with MERS-CoV and treated with neutralizing mAbs. Five groups of six marmosets were inoculated with MERS-CoV and treated with mAb as indicated in Table 1. On 6 dpi all animals were euthanized and tissue samples were collected from all respiratory (A) and extra-respiratory (B) tissues, RNA was extracted and viral loads were determined as TCID50 equivalents per gram tissue. Two-way ANOVA with Tukey's multiple comparisons test was performed to determine statistical significant differences between Group I and the other 4 groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Treatment groups: Group I: 25 mg/kg REGN684; Group II: 25 mg/kg REGN3051 prophylactically; Group III: 12.5 mg/kg REGN3048 and 12.5 mg/kg REGN3051 prophylactically; Group IV: 10 mg/kg REGN3051 therapeutically; Group V: 25 mg/kg REGN3051 therapeutically.

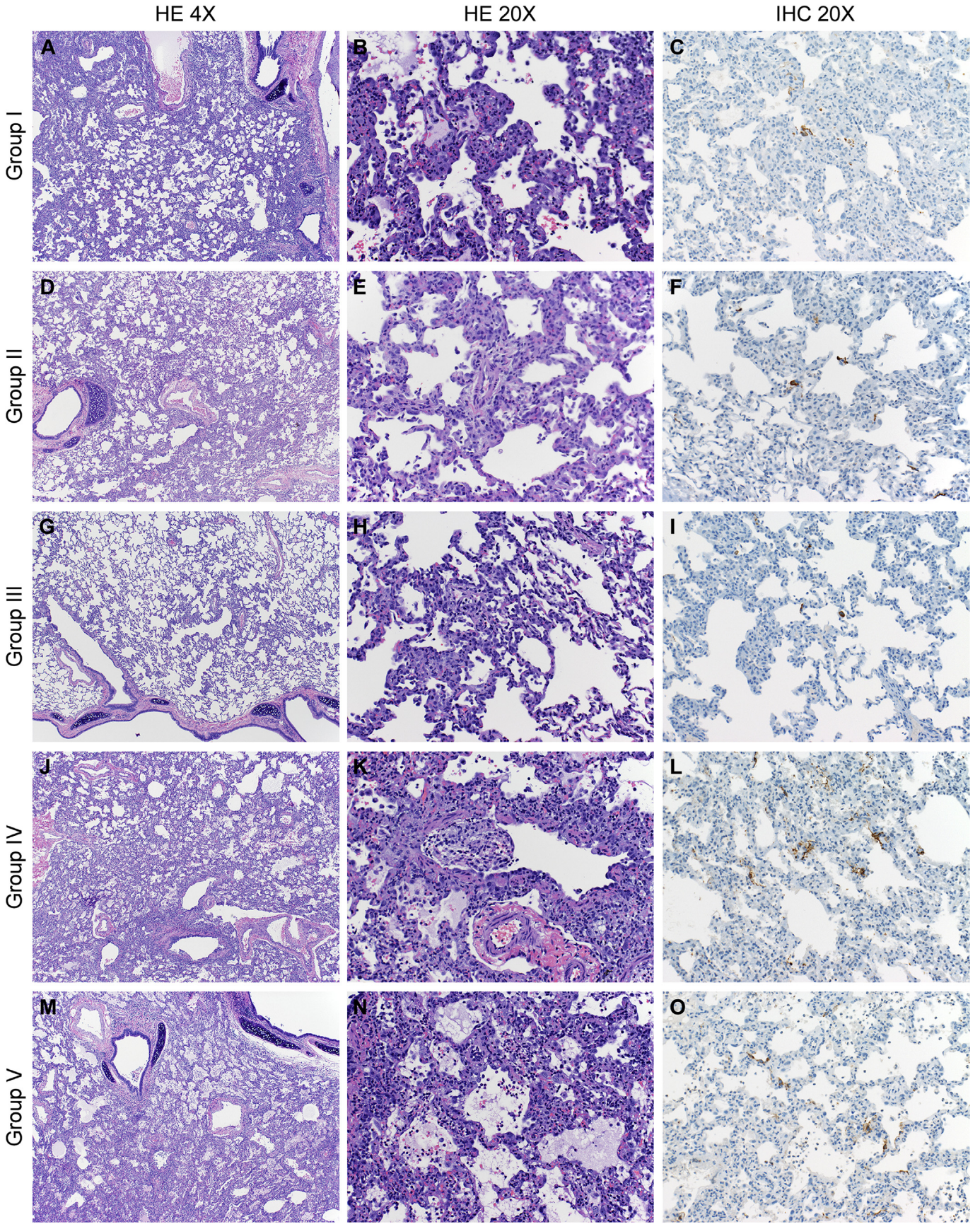

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Fig. 5. Histopathological changes in the lungs of common marmosets inoculated with MERS-CoV and treated with neutralizing mAbs. Five groups of six marmosets were inoculated with MERS-CoV and treated with mAb as indicated in Table 1. On 6 dpi all animals were euthanized and lung samples were collected and stained with hematoxylin and eosin (A, B, D, E, G, H, J, K, M, N) or a polyclonal α-MERS-CoV antibody (C, F, I, L, O). One representative image was chosen for group I (A, B, C), group II (D, E, F), group III (G, H, I), group IV (J, K, L) and group V (M, N, O). Treatment groups: Group I: 25 mg/kg REGN684; Group II: 25 mg/kg REGN3051 prophylactically; Group III: 12.5 mg/kg REGN3048 and 12.5 mg/kg REGN3051 prophylactically; Group IV: 10 mg/kg REGN3051 therapeutically; Group V: 25 mg/kg REGN3051 therapeutically. Panels were chosen to reflect the overall histopathology in the lung most accurately and were therefore not all collected from the same lung lobe; however, the 20x HE and IHC panels are from consecutive tissue sections. Magnification is indicated at the top of the figure.

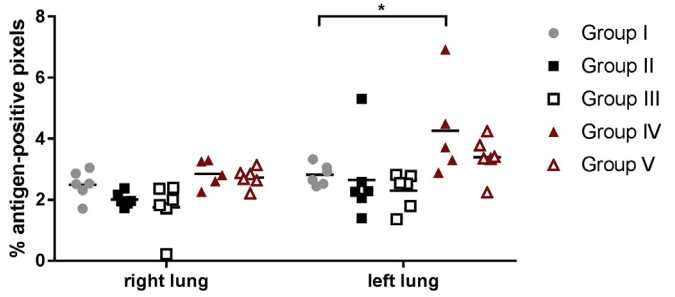

Fig. 6. Quantification of MERS-CoV-positive cells in the lungs of common marmosets inoculated with MERS-CoV and treated with neutralizing mAbs. Five groups of six marmosets were inoculated with MERS-CoV and treated with mAb as indicated in Table 1. On 6 dpi all animals were euthanized and lung samples were collected and stained with a polyclonal α-MERS-CoV antibody; slides of all four lung lobes of all animals were digitized and antigen-positive pixels were quantified using the ImageScope positive pixel algorithm. The percentage antigen-positive pixels was calculated as the number of pixels stained for MERS-CoV antigen divided by the total number of stained pixels (i.e. non-stained areas such as air spaces were excluded from the analysis). Two-way ANOVA with Dunnett's multiple comparisons test was performed to determine statistical significant differences between Group I and the other 4 groups. * indicates $P < 0.05$. Treatment groups: Group I: 25 mg/kg REGN684; Group II: 25 mg/kg REGN3051 prophylactically; Group III: 12.5 mg/kg REGN3048 and 12.5 mg/kg REGN3051 prophylactically; Group IV: 10 mg/kg REGN3051 therapeutically; Group V: 25 mg/kg REGN3051 therapeutically.

lesions were only significantly reduced in animals in group III treated with a combination of REGN3048 and REGN3051. When animals were treated therapeutically, we observed a smaller clinical benefit, with clinical disease scores returning to normal sooner in treated animals; Nevertheless, one of the therapeutically treated animals had to be euthanized due to signs of severe respiratory disease. However, there was no difference in the severity of lung pathology between control and therapeutically treated animals and thus no difference in the severity of the bronchointerstitial pneumonia in the marmoset model.

The poor correlation between lung viral loads, clinical disease and lung pathology we observed in mAb-treated animals may be explained by the fact that the immune response is an important contributor to the pathogenicity of MERS-CoV in nonhuman primates (Baseler et al., 2016). In a study in immunosuppressed rhesus macaques, viral loads in the lung were almost 100-fold higher than in controls, but lung pathology was reduced (Prescott et al., 2018).

Lung viral loads were statistically significantly lower in animals treated prophylactically with a combination of two neutralizing antibodies as compared to control animals, but this was not the case for animals treated prophylactically with a single neutralizing antibody at the same total dose. Moreover, lung lesions in animals treated with the combination of antibodies were slightly less severe than in the controls. Thus, a combination of two or more neutralizing antibodies targeting different epitopes on the spike protein may result in better treatment efficacy and could be considered for testing as prophylaxis in future studies.

In previously published common marmoset studies that showed clinical improvement with therapeutic mAb treatment after inoculation with MERS-CoV, mAb treatment was started sooner after inoculation (2, 6 and 12 h) than in the present study (Chen et al., 2017; van Doremalen et al., 2017), and in one study treatment was repeated 2 days post inoculation (van Doremalen et al., 2017). One factor that potentially affects treatment efficacy in all of these studies is the timing of peak virus replication in the lungs of inoculated common marmosets; it may only be possible to inhibit virus replication with neutralizing antibodies as long as the level of infectious virus particles in the lungs is still relatively low.

Another potential issue could be the intravenous administration route of the antibodies; upon intravenous administration the concentration of antibodies in the lungs is several hundred fold lower than in serum (Dall'Acqua et al., 2006; Hart et al., 2001), and penetration to the site of virus replication may be hampered even more once tissue damage occurs and alveolar septa thicken. One indication that penetration of mAb into the lung might at least partially explain the limited reduction in lung viral loads we observed, is the fact that viral loads in almost all other tissues were lower in all four treatment groups than in the control animals. It would thus be of interest to investigate alternative routes of administration such as nebulization to maximize the concentration of mAbs in the lungs (Respaud et al., 2015).

Although the MERS-CoV disease course in common marmosets is shorter than in human patients, thereby potentially shortening the window of opportunity for successful treatment in this model, the start of treatment on 1 dpi coincides with the onset of clinical signs. Should the impact on patient treatment be limited by the timing of health seeking behavior by patients, MERS-CoV neutralizing mAbs may still be useful for patient management during MERS-CoV outbreaks. Many MERS-CoV outbreaks have centered around health care settings, and the presence of comorbidities in MERS patients is linked to a poor prognosis. Thus, one could consider prophylactic treatment of specific target populations and their family members once several MERS cases have been identified in a health care facility, similar to the current practice of mAb prophylaxis of children at increased risk of serious lower respiratory tract infection caused by respiratory syncytial virus (American Academy of Pediatrics Committee on Infectious and American Academy of Pediatrics Bronchiolitis Guidelines, 2014). Moreover, it is possible that neutralizing mAbs will provide therapeutic benefit when combined with other antiviral treatment regimens that are currently under investigation for MERS.

Conflicts of interest

EdW, FF, AO, EvH, ElH, GS, DS, KJE and HF have no conflict of interest to declare. NS, LL and CAK are employees of Regeneron Pharmaceuticals, Inc.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2018.06.006.

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