Immune responses to MERS coronavirus during the acute and convalescent phases of human infection

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Key points

Our study examined immune responses to MERS-CoV at the acute stage of human infection, and shows an association between the early CD8+ T cell response and the severity of the infection.

ABSTRACT

Background. An understanding of immune responses against the Middle East respiratory syndrome (MERS) is important for the development of treatments and preventive measures. Here, we investigated the spectrum of immune responses occurring in patients with MERS during the early period of infection.

Methods. We obtained peripheral blood samples from 27 hospitalized patients recruited during the epidemic that occurred in 2015 in South Korea. Plasma cytokines/chemokines and antibodies were quantified. Virus-specific T cells were examined by intracellular cytokine staining after stimulation of peripheral blood mononuclear cells (PBMCs) with overlapping peptides spanning whole virus structural proteins.

Results. At the acute phase of infection, elevated levels of plasma pro-inflammatory cytokines/chemokines were detected in proportion to the severity of the disease. Distinctively high frequencies of MERS-CoV-reactive CD8+ T cells were also observed in patients with severe/moderate illness, while antibody and CD4+ T cell responses were minimally detected at this stage. At the convalescent phase, disease severity-dependent antibody responses emerged and antigen-reactive cells were identified in both T cell subsets. These T cells belonged to the Th1 or Tc1 subtypes. While CD8+ T cells responded preferentially to the viral S protein than to E/M/N proteins especially at the acute stage, slightly more CD4+ T cells recognized E/M/N proteins versus S protein at the convalescent phase.

Conclusions. Our findings show an association between the early CD8+ T cell response and the severity of the infection, and also provide basic information that may help to prepare effective control strategies for MERS in humans.

Keywords: MERS coronavirus; Immune response; T lymphocytes; Acute phase of infection

INTRODUCTION

MERS-CoV mainly causes respiratory illness with a wide range of clinical severity varying from asymptomatic to severe pneumonia with respiratory failure [1]. While the clinical characteristics of MERS and the biology of the causative virus are well documented [2], the pathogenesis and host immune response during MERS-CoV infection have been poorly investigated. This has hampered the development of therapeutics and preventive measures. Recent studies have demonstrated that a strong antibody response develops in most patients after two to three weeks of illness and that this antibody response is not likely to be correlated with the elimination of the virus from the body [3, 4]. The elevated serum levels of proinflammatory cytokines and chemokines such as interleukin (IL)-6 and C-X-C motif chemokine (CXCL)-10 were also observed in patients during the early period of severe infection [5-8]. Furthermore, T cell responses to MERS-CoV have been recently measured in MERS survivors at late convalescent period and their association with disease course was analyzed [9]. However, information is lacking regarding the T cell responses in patients at the acute stage of infection. *In vitro* and animal studies showed that MERS-CoV preferentially infects respiratory epithelial cells and inhibits the type 1 interferon response [10, 11]. CD8+ T cells and antibodies were revealed to participate in clearing the invading MERS-CoV virions and to protect against subsequent infection, respectively [12, 13]. It was also reported that the local immune response likely plays a role in pulmonary pathology severity [14]. However, it remains to be seen whether the information obtained from animal studies also applies to humans.

In this study, we examined various immunological features, especially T cell responses, using blood samples obtained from patients during the acute and early convalescent stages of MERS-CoV infection. Our data provide basic information to understand the role of immune responses on the disease process of MERS.

MATERIALS AND METHODS

Patients and clinical samples

We recruited 27 patients with MERS who were hospitalized at the National Medical Center (NMC) in Seoul during the 2015 outbreak in South Korea. MERS-CoV infection was confirmed by real-time RT-PCR. Clinical information including laboratory data of individual patients is provided in Supplementary Table 1. Peripheral blood was collected from patients at the acute and convalescent phases of infection. In this study, the acute phase was defined as the period after the onset of symptoms but before the peak of illness, usually within two weeks after the onset of symptoms; the convalescent phase was defined as the period immediately after the negative conversion of real-time RT-PCR, usually between two and five weeks after symptom onset. The study was approved by the NMC Ethical Committee.

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by density gradient centrifugation using Ficoll-Paque solution (GE Healthcare, Sweden) and stored in liquid nitrogen. Paired plasma samples were collected to determine cytokine concentrations and MERS-CoV-specific antibody responses. Control PBMCs were obtained from three healthy persons at the NMC.

Peptide library

Although some viral peptides that trigger T cell responses in MERS-CoV infection were recently identified [9], the entire spectrum of viral antigens linked with diverse MHC molecules remains to be determined. The study of T cell responses to SARS-CoV, which is a member of the same coronavirus family and elicits a similar respiratory infection in humans, demonstrated that its structural proteins (spike, envelope, membrane, and nucleocapsid) were the most immunogenic to T cells, as compared with the nonstructural proteins [15]. We thus used overlapping synthetic peptides spanning all the four structural proteins of MERS-CoV (KOREA/Seoul/014-1-2015, Accession number KT374052) as viral antigen to analyze T cell responses to MERS-CoV. The peptide library comprised 507 peptides consisting of 15-mers overlapping at 11 amino acid residues; the peptides, with >80% purity as determined by mass spectrometry and HPLC, were manufactured by Mimotopes (Australia). We dissolved each peptide in DMSO at a concentration of 80 mg/ml and pooled those encompassing the viral S protein into two sets (S1: 168 N-terminal peptides; S2: 168 C-terminal peptides) and those of the E, M, and N proteins into one set (EMN: 171 peptides).

Cytokine assays

The concentration of plasma cytokines/chemokines (IL-1β, IL-1RA, IL-6, IL-8, IL-10, TNF-α, IP-10, MCP-1, MIP-1β, and RANTES) was quantified using Bio-Plex Multiplex Immunoassay Systems (Bio-Rad Laboratories, Hercules, CA, USA), according to the

manufacturer's instructions. Plasma IFN-α was measured by enzyme-linked immunosorbent assay (ELISA) using Verikine-HS ELISA kit (PBL Assay Science, Piscataway, NJ, USA).

Antibody assays

Anti-MERS-CoV immunoglobulin M (IgM) and IgG plasma titers were determined using an Indirect Immunofluorescence Test and ELISA kit (Euroimmun AG, Lubeck, Germany), respectively, following the manufacturers' instructions. Anti-MERS-CoV IgM titer was defined as the greatest dilution showing the identifiable specific fluorescence when two-fold serial dilutions (starting from 1:10) of plasma samples were analyzed. The amount of plasma IgG was evaluated semi-quantitatively by calculating the ratio of the extinction value of the patient sample over that of the calibrator, as suggested by the manufacturer. Neutralizing Ab against spike protein was measured by a pseudotype retrovirus-based neutralization assay as described previously [16, 17]. Neutralizing Ab titers were presented as the highest plasma dilution yielding more than 50% inhibition of luciferase activity.

Flow cytometric analysis

PBMCs were cultured in complete RPMI 1640 medium containing 10% (v/v) human serum (Biowest, Nuaille, France), and stimulated with 1 μg/ml of each peptide in the presence of 1 μg/ml each of anti-CD28 and anti-CD49d monoclonal Abs (mAbs) (BD Biosciences) for 1 h at 37°C. For negative and positive control cultures, PBMCs were incubated with DMSO alone and with anti-CD3 mAbs (BD Biosciences), respectively. After the addition of 1 μg/ml

Brefeldin A (eBiosciences) and 0.7 μg/ml Monensin (BD Biosciences), cells were further incubated for an additional 5 h. After staining dead cells using LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes), cells were subjected to surface staining with anti-CD3 BV421, anti-CD4 PerCP-Cy5.5, and anti-CD8 APC-H7 mAbs (BD Biosciences). For the detection of antigen-reactive T cells with degranulation activity, anti-CD107a FITC mAb (BioLegend) was added to the stimulation cultures, and also used for surface staining. After fixation and permeabilization using the Intracellular Fix and Perm Set (eBiosciences), cells were stained with either Set One (anti-IFN-γ PE-Cy7, anti-IL-2 PE, and anti-TNF-α APC) or Set Two (anti-IFN-γ PE-Cy7, anti-IL-4 PE, anti-IL-10 eflour660, and anti-IL-17 FITC) mAbs. At least 100,000 stained cells per sample were acquired using a FACSverse Flow Cytometer (BD Biosciences), and analyzed using FlowJo software (Tree Star, Ashland, OR). The data presented correspond to background-subtracted results using the negative control culture and the sum of responses against different peptide pools unless otherwise specified.

Statistical analysis

Statistical analysis was performed using Prism V5.04 software (GraphPad, San Diego, USA). The nonparametric Mann–Whitney *U* test and Wilcoxon signed rank test were used to compare the two groups with independent and paired samples, respectively. Correlation analysis was carried out using the Spearman test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Study patients

A total of 27 patients were enrolled in this study. As shown in Table 1, these patients were divided into three groups, depending on the severity of illness. Severe disease (n=12) included fatalities (n=5) and patients who required mechanical ventilation to relieve respiratory failure (n=7). Moderate disease (n=7) comprised patients with radiological evidence of pneumonia without respiratory failure. Mild disease (n=8) encompassed patients who were asymptomatic or who reported symptoms such as fever, headache, cough, and malaise, without distinctive pulmonary lesions. As reported previously [18, 19], older age was linked with severe disease (Table 1, $P < 0.05$). In addition, the decrease in lymphocytes was observed in patients with severe and moderate disease, especially at the acute phase of infection (Supplementary Figure 1).

Plasma cytokine profiles

To estimate the role of cytokines and chemokines in infection with MERS-CoV, we measured their plasma levels by cytokine bead array or ELISA. The plasma concentrations of IL-6, IL-1RA, IP-10, and MCP-1 were significantly elevated at the acute phase of infection (10.7-, 6.5-, 17.3-, and 4.0-fold increase in severe/moderate illness, respectively), the extent of which was correlated with disease severity, and declined to basal levels at the convalescent phase (Figure 1). However, IL-1 and TNF-α were not detected at either phase in most of the patients. The plasma IFN-α level increased in most patients at the acute stage, but this

increase was not proportional to the disease severity. The anti-inflammatory cytokine, IL-10, was rarely detected.

Antibody responses

Anti-MERS CoV Abs—including IgM, IgG and neutralizing Abs—were not detected in patients except for a case of severe disease within the first two weeks of illness. In the convalescent phase, patients with severe and moderate illness developed a neutralizing Ab response, which was also seen with IgM and IgG. Overall, the more severe the illness, the greater the antibody response detected in patients in the convalescent phase; mild or asymptomatic patients rarely develop antibody responses (mean±SD fold dilution of neutralizing Ab in severe vs. moderate vs. mild, 960.0±413.1 vs. 914±793.1 vs. 41.4±60.1, respectively) ($P < 0.05$) (Figure 2).

T cell immune responses to viral antigens

In order to assess T cell responses to MERS-CoV, PBMCs of patients were stimulated with three pooled viral peptides and analyzed for intracellular cytokines via flow cytometry (Supplementary Figure 2). As shown for one representative patient and healthy donor, virus-reactive cells were detected in T cells from patients, but not in those from healthy donors (Figure 3A). Details of IFN-γ-secreting T cell frequencies relative to the days after virus exposure and symptom onset in individual patients are provided in Supplementary Figure 3.

In summary, at the acute stage, virus-reactive CD4+ T cells—as determined by the secretion of IFN-γ, IL-2, or TNF-α upon antigenic stimulation—were detected in two of the nine patients in the severe group, but not in the moderate and mild groups (Figures 3B & 3C). At the convalescent phase, these CD4+ T cells appeared in most (18/21) of the patients regardless of the severity of infection. Unlike CD4+ T cells, high frequencies (> 0.3%) of CD8+ T cells secreting IFN-γ and/or TNF-α and expressing CD107a were seen in most (9/12) of the severe and moderate subjects (median [range] for IFN-γ secretion, 0.94% [0.32-5.44%]) but not in mild cases at the acute stage of infection. By the convalescent phase, the frequency of these antigen-reactive CD8+ T cells had diminished in a portion (4/7) of the patients with severe or moderate infection (Figures 3B & 3C). T cells secreting a cytokine triplet of IFN-γ, IL-2, and TNF-α are considered more potent than those producing one or two [20], and were mostly identified only in CD4+ T cells at the convalescent phase (Figure 3C). We also addressed whether MERS-CoV-reactive T cells produced cytokines other than Th1- or Tc1-types. As expected, IFN-γ-secreting T cells were predominant but IL-4, IL-10, or IL-17-secreting T cells were very rare in both CD4+ and CD8+ T cell populations (Figure 4).

Next, we analyzed the reactivity of T cells to different viral proteins. While more CD8+ T cells reacted to S protein than to E/M/N proteins especially at the acute phase of infection (0.26% [0.03-0.77%] vs. 0.07% [0.02-0.33%]; median [interquartile range]) ($P < 0.05$), slightly more CD4+ T cells recognized E/M/N than S proteins at the convalescent phase (0.10% [0.04-0.25%] vs. 0.09% [0.02-0.21%]) ($P = 0.057$) (Figure 5).

Finally, when we analyzed the relationship between serologic and T cell responses in individual patients at the convalescent phase, there was a significant correlation between IgG

titers and virus-reactive CD4+ or CD8+ T cell frequencies. A significant correlation was also found between the frequencies of virus-reactive CD4+ and CD8+ T cells (Figure 6).

DISCUSSION

In this study, we measured T cell responses together with antibody and pro-inflammatory cytokine responses in patients at the early period of MERS. Our data revealed that an initial severity-proportional, pro-inflammatory cytokine/chemokine secretion is followed by antibody and T cell responses in patients, which is a typical host response to many other acute viral infections. However, one peculiar finding was that extraordinarily high frequencies of MERS-CoV-reactive CD8+ Tc1-type T cells were observed in a large proportion of patients with severe and moderate illness at the acute stage prior to the detection of humoral and CD4+ T cell responses. At the convalescent phase, the magnitude of the CD8+ T cell response was not greatly augmented further, and actually contracted in some cases. Based on these data, we can infer that inefficient control of invading MERS-CoV brings about robust inflammatory and CTL responses, which clear the invading virions and also destroy lung tissue yielding to pneumonia.

The association of a strong inflammatory response with a severe form of MERS CoV infection has been revealed by histological and serologic findings in human and animal studies [7, 8, 21-23]. Our observation reinforces this association. Thus, the elevated serum levels of IL-6, IP-10, and MCP-1, which are associated with inflammatory process and recruitment of inflammatory cells, could reflect the ongoing inflammation at the infection site. In our study, two deceased patients who did not show any detectable T cell responses

had a very high level of plasma pro-inflammatory cytokines and chemokines at their acute phase of infection. Therefore, it is highly probable that the inflammatory reaction would damage diseased lung tissue in the absence of CTL responses in these two patients. Interestingly, the plasma level of RANTES was detected to be increased at the convalescent phase of infection irrespective of disease severity. We speculate this late increase of RANTES might be associated with the secretion of this chemokine by activated virus-reactive T lymphocytes [24].

A few reports explored the role of T cell responses in MERS-CoV infection using animal models. Examination of infected mice transiently expressing the human DPP-4 receptor revealed that T cell responses are required for viral clearance [12]. Interestingly, Coleman et al. reported that, using a mouse model in which hDPP4 is expressed under the endogenous mDPP4 promoter, CD8+ T cells may contribute to MERS-CoV-induced lung pathology [25]. The potential pathogenic contribution of strong CTL responses at the acute phase of infection has also been demonstrated in animal influenza virus infection [26]. Our data also suggest the pathogenic role of CD8+ T cells in human MERS-CoV infection. We posit two pathways for inducing a strong CD8+ T cell response at the acute phase of MERS-CoV infection. Antigenic stimulation caused by inefficient removal of invading virus and/or a large inoculum of virus could induce a rapid proliferation and differentiation of naïve CD8+ T cells in the absence of CD4+ T cells [27]. Otherwise, it could be due to heterologous immunity: memory CD8+ T cells—activated by prior infection with unrelated viruses—cross-reacting with MERS-CoV antigens [28]. Further studies will be needed to address these possibilities.

The spectrum of immune responses against MERS-CoV infection appears to be very similar to that against SARS-CoV infection. As reported in SARS [29], lymphodepletion occurs in

patients with severe MERS at the acute phase of infection. Moreover, the magnitude of the innate immune response—represented by the serum levels of inflammatory cytokines and chemokines—and humoral immune response was revealed to increase proportionally with the disease severity in both SARS and MERS-CoV infection [30]. Our study further demonstrates that, similar to B cell responses, T cell responses against MERS-CoV infection are also elevated in severe/moderate cases compared to mild infections. While there is a lack of data on the kinetics of T cell responses during SARS-CoV infection, numerous studies have investigated the memory T cell responses in recovered SARS patients [15, 31-36]. Among them, a comprehensive study of T cell responses against all the SARS-CoV proteins showed the dominance of CD8+ T cell responses over CD4+ T cells [15], which is in line with our findings in MERS, though the observations were made at different time points. However, the major protein recognized by CD4+ T cells seems to be different between SARS and MERS. While CD4+ T cell responses in SARS were clustered mainly in the spike protein [15], CD4+ T cells in patients with MERS responded slightly more to E/M/N rather than S protein. This difference is not likely due to the different time points at which CD4+ T cell responses were analyzed because the same recognition pattern of memory CD4+ T cells was observed in MERS patients at one-year post-infection (unpublished data).

According to a recent study conducted by Zhao et al., which examined T cell responses in patients at 6 or 24 months post-MERS infection [9], virus-specific neutralizing antibodies and CD4+ T cell responses correlated with severe disease. The same correlations were also observed at the early convalescent phase of the infection in our study. However, there is a different interpretation regarding CD8+ T cell responses. Based on the finding that patients with mild or subclinical illness develop prominent virus-specific CD8+ T cell responses,

Zhao et al. proposed that measurements of MERS-CoV-specific T cell responses may be useful for making prognoses [9]. Our study, which used acute stage samples, suggests a poor prognosis if a virus-specific CD8+ T cell response is detected at the acute phase of infection. Nevertheless, as the presence of virus-specific CD8+ T cells at the acute phase of infection was not shown in some patients with severe and moderate illness, it is not a reliable biomarker for poor outcomes.

Our patient cohort provided a unique opportunity to study immune responses in the early period of MERS-CoV infection. All of the patients were believed to have been exposed to MERS-CoV antigens for the first time in their lives. Thus, we assumed that the anti-MERS-CoV immune responses measured in this study would be a primary immune response. Moreover, the time course of antigen exposure and symptom onset was relatively well defined in these patients due to a sudden outbreak of MERS in South Korea. The limited number of patients recruited in this study, however, was an obstacle to reaching a conclusion. Another weakness of our study is the absence of viral load data. Consequently, we could not define the relationship between antigen dose and the magnitude of the cellular immune response. Nevertheless, our study provides valuable information about the cellular immune response against MERS-CoV infection in humans at the early period of infection.

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POTENTIAL CONFLICT OF INTEREST:

The authors have no potential conflicts to disclose.

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TABLE

Table 1. Characteristics of clinical samples

Group	No. of pts	Male/ Female (no.)	Age (mean ±SD)	Clinical findings			No. of samples		
				Fatal or mechanical ventilation	Develop-ment of pneumonia	Other mild symptoms	Acute	Convalescent	Paired
Severe	12	10/2	63±16	+	+	+	9	7	4
Moderate	7	4/3	54±20	-	+	+	3	7	3
Mild	8	3/5	44±25	-	-	±	5	7	4

Figure Legends

Figure 1. Plasma cytokine/chemokine levels of patients at the acute and convalescent phases of MERS infection. IFN-α was measured by ELISA, and the others by cytometric bead arrays. Each dot represents an individual patient and the lines indicate paired samples from the same patient.

Figure 2. MERS-CoV-specific antibody responses in patients at the acute and convalescent phases of infection. The magnitude of MERS-CoV-specific IgM, IgG, and neutralizing Ab responses was measured in patient plasma samples collected at the acute and convalescent phases of infection by IFA, ELISA, and pseudotype retrovirus-based neutralization assay, respectively. Fold dilutions ≥10 for IgM and neutralizing Ab, and ratio to calibrate ≥1.1 for IgG were considered to be positive. Each dot shown in the leftward figures represents an individual patient and the lines in the rightward figures indicate paired samples from the same patient. Mi, mild; Mo, moderate; S, severe.

Figure 3. T cell responses to MERS-CoV structural protein antigens in patients at the acute and convalescent phases of infection. PBMCs from patients were stimulated with MERS-CoV structural protein-peptide pools and virus-reactive T cells were examined by intracellular cytokine staining. A: Staining profiles of T cells from one representative patient (at the acute stage of infection) and a healthy donor. B: Frequency of IFN-γ-producing T cells in response to three pools of viral peptides. C: Summary of T cell responses producing Th1- or Tc1-type cytokines and molecules against all three peptide pools. Each dot represents an individual patient and the lines indicate paired samples from the same patient.

Figure 4. Frequency of various functional T cell subsets responding to MERS-CoV antigens in patients with MERS. PBMCs from patients were stimulated with MERS-CoV structural protein-peptide pools and the frequency of T cells secreting IFN-γ, IL-4, IL-10, or IL-17 was measured by intracellular cytokine staining. Each dot represents an individual patient.

Figure 5. Reactivity of T cells to different viral proteins in individual patients. Lines indicate the paired frequencies of IFN-γ-producing T cells responding to the S1 or S2 pool of peptides and the E/M/N peptides pool in individual patients. The two-tailed Wilcoxon signed rank test was used to compare the paired samples.

Figure 6. Correlation between T cells and Ab responses. Plasma IgG titers and the frequencies of IFN-γ-producing CD4+ and CD8+ T cells in the same patients were plotted. Spearman's rank correlation coefficient, the corresponding *P* value, and the line of best fit are shown.

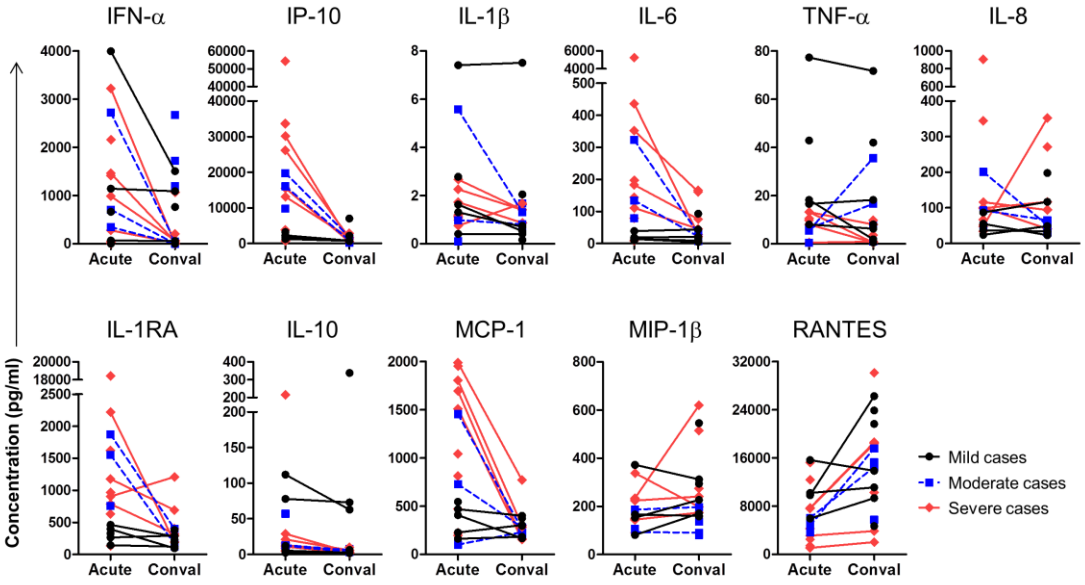

Fig. 1

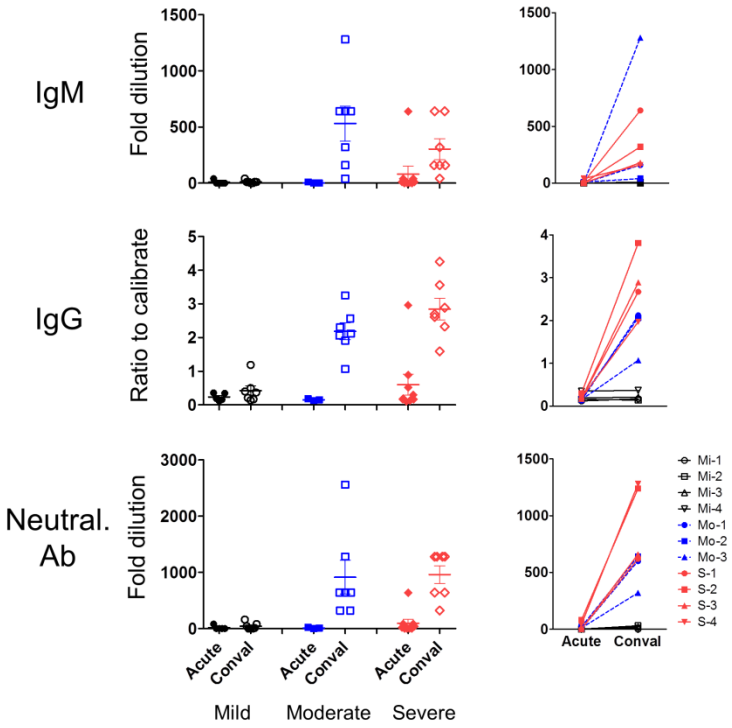

Fig. 2

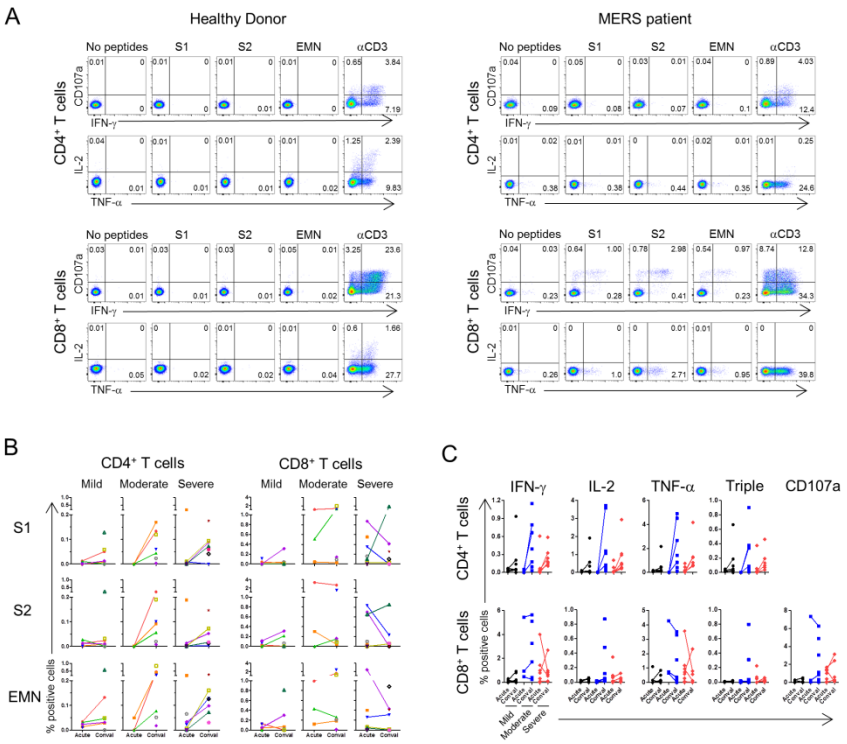

Fig. 3

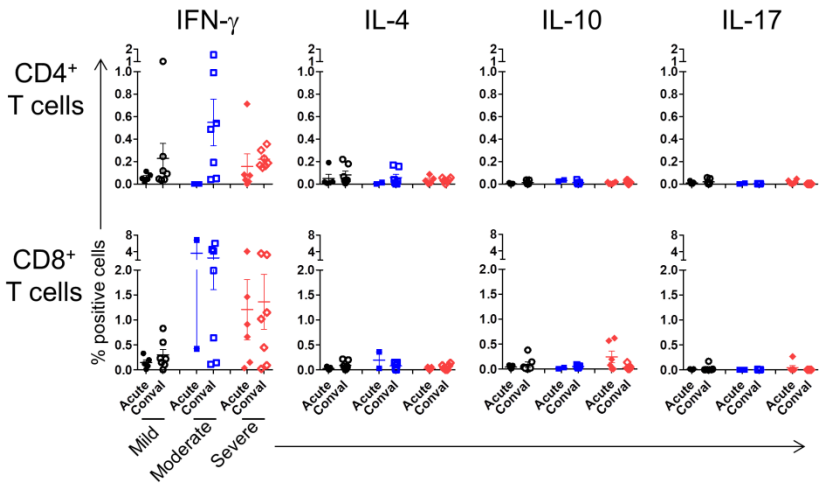

Fig. 4

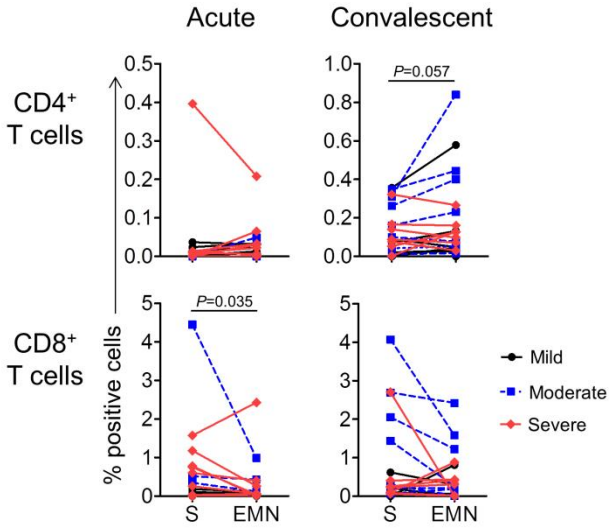

Fig. 5

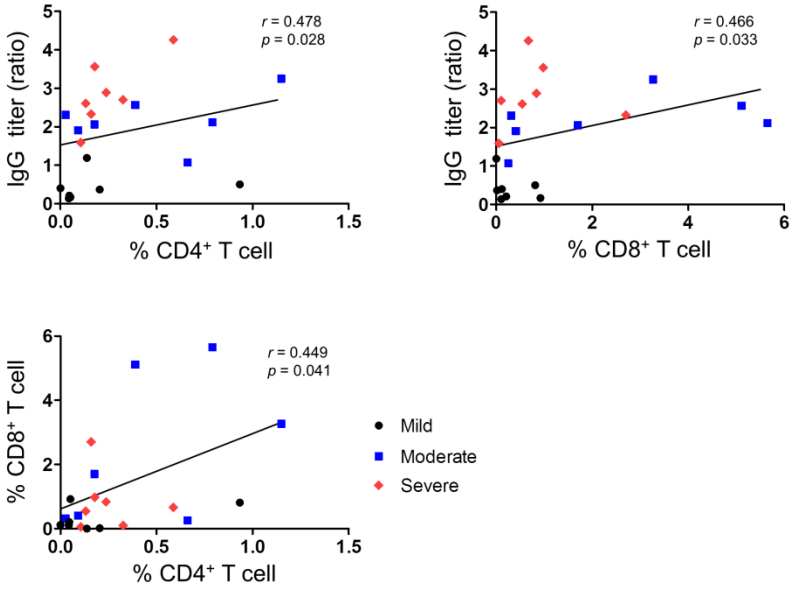

Fig. 6