Middle East Respiratory Syndrome Coronavirus Efficiently Infects Human Primary T Lymphocytes and Activates the Extrinsic and Intrinsic Apoptosis Pathways

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(See the editorial commentary by Ying, Li, and Dimitrov on pages 877–9.)

Middle East respiratory syndrome (MERS) is associated with a mortality rate of >35%. We previously showed that MERS coronavirus (MERS-CoV) could infect human macrophages and dendritic cells and induce cytokine dysregulation. Here, we further investigated the interplay between human primary T cells and MERS-CoV in disease pathogenesis. Importantly, our results suggested that MERS-CoV efficiently infected T cells from the peripheral blood and from human lymphoid organs, including the spleen and the tonsil. We further demonstrated that MERS-CoV infection induced apoptosis in T cells, which involved the activation of both the extrinsic and intrinsic apoptosis pathways. Remarkably, immunostaining of spleen sections from MERS-CoV–infected common marmosets demonstrated the presence of viral nucleoprotein in their CD3+ T cells. Overall, our results suggested that the unusual capacity of MERS-CoV to infect T cells and induce apoptosis might partly contribute to the high pathogenicity of the virus.

Keywords. MERS-CoV; T lymphocytes; apoptosis; caspase; tonsil; spleen; marmosets.

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Preparation of Peripheral Blood Mononuclear Cells (PBMCs), T Cells, and Spleen- and Tonsil-Disassociated Cells

PBMCs and T cells were prepared as previously described [28]. Tonsil- and spleen-disassociated cells were obtained...
from individuals undergoing splenectomy and tonsillectomy; all individuals provided written consent before cells were collected. Details are presented in the Supplementary Materials.

Infection of Common Marmosets
Approval by the institutional animal care and use committee was obtained for animal experiments [29]. Common marmosets were inoculated with $5 \times 10^6$ 50% tissue culture infective doses (TCID$_{50}$) MERS-CoV intratracheally in 500 µL of Dulbecco’s modified Eagle’s medium. Organ samples were collected on necropsy as described previously [29–31].

Antibodies
Cell surface markers were obtained from BD Pharmingen, BioLegend, and Abcam. DPP4 antibody was obtained from R&D Systems. Immunostaining of MERS-CoV [14, 22] and SARS-CoV nucleoprotein (NP) [32] were performed as previously described.

Virus and Infections
MERS-CoV strain EMC/2012 (passage 8) was provided by Dr Ron Fouchier (Erasmus Medical Center) [1, 33]. MERS-CoV and SARS-CoV were cultured from Vero E6 cells. For infections, cells were inoculated with MERS-CoV or SARS-CoV at 2 TCID$_{50}$ per cell for 1 hour at 37°C.

Reverse Transcription–Quantitative Polymerase Chain Reaction (RT-qPCR)
RNA extraction and RT-qPCR were performed as previously described [14, 22, 34].

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assays
The TUNEL assay kit was purchased from Life Technologies. Labeling procedures were performed following protocols recommended by the manufacturer.

Active Caspase Assays
Active caspase 3 antibody was obtained from BD Pharmingen. CaspGLOW fluorescein active caspase 8 and 9 staining kits were obtained from eBioscience. Labeling was performed according to the manufacturer’s protocol and is outlined in the Supplementary Materials.

Flow Cytometry
Immunostaining for flow cytometry was performed following standard procedures. Flow cytometry was performed using BD FACSCanto II (BD Biosciences), and data were analyzed using FlowJo vX (Tree Star).

Confocal Imaging of Marmoset Spleen Sections
MERS-CoV NP was labeled with guinea pig anti-MERS-CoV NP serum as mentioned above. CD3$^+$ T cells were identified with a mouse anti-human antibody (clone SP34-2) from BD
Figure 2. Middle East respiratory syndrome coronavirus (MERS-CoV) efficiently infects CD4+ and CD8+ T cells and downregulates surface dipeptidyl peptidase 4 (DPP4) in the infected cells. A, T cells were infected with MERS-CoV and severe acute respiratory syndrome CoV (SARS-CoV) at 2.50% tissue culture infective doses (TCID₅₀) per cell. Cells were fixed at 24 hours after infection and immunolabeled for detection of expression of MERS-CoV NP or SARS-CoV NP. The shaded curve and the solid line represent virus NP expression from mock-infected and MERS/SARS-CoV–infected cells, respectively. B, T cells were infected with MERS-CoV at 2 TCID₅₀ per cell. Cells were fixed at 24 hours after infection and immunolabeled for detection of CD4 or CD8 and MERS-CoV NP expression. The shaded curve and the solid line represent NP expression from mock-infected and MERS-CoV–infected cells, respectively. C, Uninfected T cells were fixed and immunolabeled for detection of CD4 or CD8 and surface DPP4 expression. The shaded curve and the solid line represent isotype and DPP4-specific staining, respectively. D, T cells were infected with MERS-CoV at 2 TCID₅₀ per cell. Cells were fixed at 24 hours after infection and immunolabeled for detection of CD4 or CD8, DPP4, and MERS-CoV NP expression. Surface or total DPP4 was detected by labeling the cells with the DPP4
Pharmingen. Images were acquired with a Carl Zeiss LSM 780 system.

Statistical Analysis
Statistical comparison was performed by the Student t test, using GraphPad Prism 6.

RESULTS

MERS-CoV Differentially Infects Subsets of Human PBMCs, and the Infectivity Correlates With Surface DPP4 Expressions

We hypothesized that T cells were potential targets of MERS-CoV infection. To test our hypothesis, we inoculated PBMCs with MERS-CoV and evaluated the proportion of MERS-CoV–infected T cells, B cells, natural killer (NK) cells, and monocytes with flow cytometry. Notably, a substantial proportion of T cells were infected by MERS-CoV. In contrast, MERS-CoV only modestly infected monocytes and NK cells, whereas it marginally infected B cells (Figure 1A). Quantitatively, the average percentages (± standard deviation [SD]) of MERS-CoV–positive T cells, B cells, NK cells, and monocytes were 65.6% ± 5.4%, 5.4% ± 2.4%, 18.5% ± 4.8%, and 13.5% ± 6.6%, respectively (Figure 1A). As expected, the percentage of MERS-CoV infection was in direct accordance with the level of surface DPP4 expression on these PBMC subsets (Figure 1B). Overall, we showed that MERS-CoV was capable of infecting T cells, NK cells, monocytes, and, to a less extent, B cells. The efficiency of infection highly correlated with the level of surface DPP4 expression.

MERS-CoV Efficiently Infects CD4+ and CD8+ T Cells and Downregulates Surface DPP4 in the Infected Cells

To further characterize MERS-CoV infection in T cells, we first challenged purified T cells with MERS-CoV and SARS-CoV and checked for NP expression. Strikingly, whereas a substantial number of T cells were infected by MERS-CoV, they were largely refractory to SARS-CoV infection (Figure 2A). This differential susceptibility might be in part attributed to the discrepancy in the expression levels of DPP4 and angiotensin converting-enzyme 2 in T cells (Supplementary Figure 1). Next, we asked whether the 2 major populations of T cells, CD4+ and CD8+ T cells, were equally susceptible to MERS-CoV infection. As shown in Figure 2B, substantial numbers of both populations were infected by MERS-CoV. However, a significantly higher proportion of CD4+ T cells (mean ± SD, 72.1% ± 9.1%) were infected, compared with CD8+ cells (50.9% ± 7.7%). The degree of infection again correlated with surface DPP4 expression (Figure 2C). Next, because DPP4 plays essential roles in a broad array of T-cell functions [24–26], we evaluated the expression level of surface and total DPP4 on CD4+ or CD8+ T cells after MERS-CoV infection. Remarkably, MERS-CoV significantly downregulated surface DPP4 in infected CD4+ T cells by 80% and CD8+ T cells by 70% (Figure 2D). Changes in the total DPP4 level were less dramatic, with values of 50% and 30% for CD4+ and CD8+ T cells, respectively (Figure 2D). In contrast to DPP4, surface or total levels of CD4 and CD8 were unaltered in infected T cells (Supplementary Figure 2). Together, our data demonstrated that MERS-CoV but not SARS-CoV efficiently infected T cells and significantly downregulated surface DPP4 in the infected cells.

MERS-CoV Infection of Human T Cells Is Abortive

To grasp a more comprehensive picture of the replication kinetic of MERS-CoV in T cells, we measured the genome copy number of MERS-CoV from samples harvested at different time points. In stark contrast to MDMs [14] and ModDCs [22], in which MERS-CoV infections are productive, T cells failed to support MERS-CoV replication (Supplementary Figure 3). The number of MERS-CoV genome copies in the cell lysates (Supplementary Figure 3A) and supernatants (Supplementary Figure 3B) of infected T cells remained largely steady over time. As expected, SARS-CoV similarly failed to replicate in T cells. Intriguingly, the number of intracellular MERS-CoV genome copies was 2–3 logs higher than that of SARS-CoV (Supplementary Figure 3A). Therefore, our findings supported the notion that MERS-CoV efficiently entered T cells, although the infection was not productive, whereas SARS-CoV failed to enter T cells.

MERS-CoV but Not SARS-CoV Induces Apoptosis in T Cells

MERS-CoV is known to cause cytopathology in a number of cell lines [35, 36]. To examine whether MERS-CoV infection induced cell death in T cells, we assessed TUNEL activity in MERS-CoV–infected T cells and compared the findings to those in SARS-CoV– or mock-infected T cells. TUNEL assays detect DNA fragmentation that results from apoptotic cascades and identifies cells in the last phase of apoptosis [37]. We detected an increased number of TUNEL-positive cells in MERS-CoV–infected T cells at as early as 6 hours after infection, compared with SARS-CoV– or mock-infected T cells. At 24 and 48 hours after infection, significantly more TUNEL-positive cells were detected in MERS-CoV–infected T cells comparing with findings for the 2 other groups (Figure 3A). Quantitatively, the mean percentages (±SD) of TUNEL-positive T cells among MERS-CoV infection were 8.2% ± 2.7%, 20.0% ± 7.8%, and 27.8% ± 9.7% at 6, 24, and 48 hours after infection, respectively (Figure 3B). Thus, we concluded that MERS-CoV but not SARS-CoV induced apoptosis in T cells.
Apoptosis is a coordinated and energy-dependent process that can be triggered through the extrinsic (death receptor) or the intrinsic (mitochondrial) caspase-dependent pathways [38]. The 2 pathways end and converge at the execution phase, which involves the activation of execution caspases, including caspases 3, 6, and 7 [39]. Among them, caspase 3 is considered to be the most important executor caspase and is activated by any of the initiator caspases (caspases 2 and 8–10) [38]. Therefore, we evaluated the extent of caspase 3 activation in infected T cells. Remarkably, substantial active caspase 3 expression was evident in MERS-CoV–infected T cells as early as 6 hours after infection, and expression remained high over time (Figure 3C). Specifically, active caspase 3 was detected at a mean frequency (±SD) of 74.9% ± 6.8%, 75.3% ± 10.2%, and 63.2% ± 7.6% among MERS-CoV-NP–positive T cells at 6, 24, and 48 hours after infection, respectively (Figure 3D). Caspase 3 activation was absent in mock-infected cells or in T cells that remained uninfected in the MERS-CoV–treated samples (Figure 3C). In summary, we demonstrated that caspase 3 was substantially activated in MERS-CoV–infected T cells early upon infection and remained highly expressed.
Extrinsic and Intrinsic Caspase-Dependent Apoptosis Pathways Are Triggered in MERS-CoV–Infected T Cells

We then examined the degree of caspase 8 and caspase 9 activation, which represented the involvement of the extrinsic and intrinsic apoptosis pathway, respectively. Intriguingly, our results suggested that both caspase 8 and caspase 9 were activated starting as early as 1 hour after infection with MERS-CoV. The degree of caspase 8 activation in MERS-CoV–infected T cells was rather consistent over time (Figure 4A), with mean levels (±SD) of 14.3% ± 2.7%, 11.4% ± 4.8%, and 15.4% ± 3.0% at 1, 6, and 24 hours after infection, respectively (Figure 4B). An increasing level of caspase 9 activation was detected in MERS-CoV–infected T cells over time (Figure 4C). Specifically, the mean frequencies (±SD) of MERS-CoV–infected T cells expressing active caspase 9, at 1, 6, and 24 hours after infection were 13.5% ± 4.1%, 17.6% ± 1.2%, and 31.3% ± 7.3%, respectively (Figure 4D). Together, our results suggested that the extrinsic and intrinsic apoptosis pathways were triggered in MERS-CoV–infected T cells.

MERS-CoV–Induced Caspase 3 Activation in T Cells Occurs Rapidly Upon Infection and Is Not Inhibited by UV-Inactivated MERS-CoV

To compare the extent of MERS-CoV–induced cytopathology between cell lines and primary T cells, we infected Vero E6
cells and compared the level of caspase 3 activation with that in infected T cells. Our data suggested that caspase 3 was also activated in Vero E6 cells, although the dynamic of activation was drastically different from that in T cells. Remarkably, at 6 hours after infection, only a subtle population of Vero E6 cells was infected by MERS-CoV, with no caspase 3 activation (Figure 5A). Intriguingly, at 24 hours after infection, essentially all Vero E6 cells were infected, and the vast majority of the infected cells were expressing activated caspase 3 (Figure 5A). On the other hand, a substantial number of T cells were infected at 6 hours after infection, with accompanying excessive caspase 3 activation. The level of infection and caspase 3 activation in T cells remained largely constant between 6 and 24 hours after infection (Figure 5A). Since Vero E6 cells are known to support robust MERS-CoV replication, we speculated that caspase 3 activation in Vero E6 cells was a result of virus replication. To test this hypothesis, we inoculated Vero E6 cells and T cells with UV-inactivated MERS-CoV. The replication capacity of MERS-CoV was completely abolished with 10 minutes of UV inactivation (Supplementary Figure 4). Strikingly, UV inactivation completely obliterated MERS-CoV infection and caspase 3 activation in Vero E6 cells (Figure 5B). In stark contrast, MERS-CoV entry and MERS-CoV–induced caspase 3 activation in T cells remained largely unaltered by UV inactivation (Figure 5B). NP staining with no cell permeabilization (Supplementary Figure 5A) or on ice before cell permeabilization (Supplementary Figure 5B) both picked up little to no signal, suggesting the detected MERS-CoV NP signal was indeed intracellular but not

Figure 5. Middle East respiratory syndrome coronavirus (MERS-CoV)–induced caspase 3 activation in T cells occurs rapidly upon infection and is not inhibited by UV inactivation of the virus. A, T cells and Vero E6 cells were infected with MERS-CoV at 2 50% tissue culture infective doses (TCID₅₀) per cell. Cells were fixed at the indicated time points and immunolabelled for detection of MERS-CoV nucleoprotein (NP) and active caspase 3. B, T cells and Vero E6 cells were infected with MERS-CoV at 2 TCID₅₀ per cell. In parallel, equal volumes of UV-inactivated MERS-CoV were applied to Vero E6 cells and T cells. At 24 hours after infection, cells were harvested, fixed, and immunolabeled for detection of MERS-CoV NP and active caspase 3. The illustrated result was a representative of 2 independent experiments that showed similar results.
from surface-attached viruses. Together, these data illustrated intrinsic differences in the dynamics of MERS-CoV infection and MERS-CoV–induced caspase 3 activation in Vero E6 cells and T cells.

**T Cells in Human Lymphatic Organs, Including the Tonsil and the Spleen, Are Highly Susceptible to MERS-CoV and MERS-CoV–Induced Apoptosis**

Recent pathological studies detected the presence of viral RNA in the tonsil [30, 40, 41], lymph node [30, 40, 41], and spleen [30] of MERS-CoV–inoculated animals. To investigate whether T cells in these lymphoid organs are as susceptible to MERS-CoV infection as those in the peripheral blood, we inoculated disassociated cells from human spleen and tonsil with MERS-CoV and estimated the percentage of infected T cells by using flow cytometry. Remarkably, CD3⁺ T cells from the spleen and tonsil were infected to a similar extent as T cells in the peripheral blood (Figure 6A). We further assessed the level of MERS-CoV–induced apoptosis in these cells. Our data suggested that the percentage of TUNEL-positive (Figure 6B) and active caspase 3–positive (Figure 6C) cells among MERS-CoV–infected CD3⁺ T cells from the spleen and tonsil highly resembled that among the peripheral blood T cells. In this regard, we demonstrated that T cells in the human spleen and tonsil were highly susceptible to MERS-CoV infection and MERS-CoV–induced apoptosis.

**MERS-CoV Infects CD3⁺ T Cells in the Spleen of Common Marmosets**

Our in vitro and ex vivo data have, so far, consistently demonstrated the high susceptibility of human T cells to MERS-CoV infection. We next used the common marmoset animal model [30, 31] to evaluate the involvement of T cells in MERS-CoV infection in vivo. Two male common marmosets (Callithrix jacchus; CM1 and CM2) were inoculated with 5 × 10⁶ TCID₅₀ of MERS-CoV intratracheally. Upon necropsy of these infected animals, tissue samples were collected and analyzed for the presence of viral RNA. Notably, viral RNA was detected from the spleen tissues of both animals, with a viral gene to GAPDH ratio of 7.3 × 10⁴ and 7.6 × 10⁵ in CM1 and CM2, respectively (Supplementary Figure 6). Next, immunostaining was performed on paraformaldehyde-fixed and paraffin-embedded sections of spleen from the infected animals. Remarkably, MERS-CoV NP was readily detected in the spleen (Figure 7D and 7G). More importantly, MERS-CoV NP was detected in T cells, which was suggested by the colocalization between MERS-CoV NP and CD3 (Figure 7D–I). As a control, no MERS-CoV NP signal was detected when preimmune serum was used in place of MERS-CoV NP antibody (Figure 7A–C). Overall, we demonstrated the capacity of MERS-CoV to disseminate to the spleens of common marmosets and infect CD3⁺ T cells therein, despite respiratory transmission of the pathogen.

**DISCUSSION**

T cells and T-cell responses play central roles in the pathogenesis of SARS [42, 43]. Currently, little is known about the virus-host interaction of MERS-CoV infection in T cells. In this study, we demonstrated that human T cells were highly susceptible to MERS-CoV infection and MERS-CoV–induced apoptosis while remaining impervious to SARS-CoV. Most importantly, we showed in a common marmoset model that MERS-CoV efficiently targeted the CD3⁺ T cells in the spleen of infected animals. Together, our results suggested that its distinct capacity to invade T cells might contribute to the high pathogenicity of MERS-CoV.
Viremia has been frequently reported in patients with SARS and those with MERS. Viral RNA was detected in the sera of SARS patients during the acute phase of infection [44, 45]. MERS-CoV RNA could be detected in the blood of infected patients for a prolonged period [46–48]. The viremia can be attributed to circulating free viral RNA that should be transient, free virion in blood or virus in infected cells. Remarkably, our current study demonstrated that MERS-CoV but not SARS-CoV efficiently infected T cells and induced substantial apoptosis in the infected cells. This discovery indicates that the viremia in patients with SARS and those with MERS may have different clinical significance. In particular, given the capacity of MERS-CoV to invade T cells, patients with MERS who develop viremia may have a more severe outcome. Of note, viremia is not the only route for MERS-CoV to infect T cells. T cells could be attracted to the infected lung under the action of chemoattractants and therefore be infected at the primary organ of MERS-CoV infection. Alternatively, dendritic cells, which we have previously shown to support robust MERS-CoV replication [22], can be infected, migrate to the draining lymph nodes, and transmit the virus to naive T cells during antigen presentation. Importantly, lymphopenia is a common hematological abnormality that has been frequently reported in SARS and MERS cases [7, 49]. It has been speculated that lymphopenia detected in SARS cases might be due to direct infection, tissue sequestration of lymphocytes, cytokine-mediated cell death, as well as suppression of bone marrow or thymus for T-cell generation. In this study, we demonstrated that MERS-CoV but not

Figure 7. Middle East respiratory syndrome coronavirus (MERS-CoV) infects CD3+ T cells in the spleen of infected common marmosets. Immunostaining was performed on parafomaldehyde-fixed and paraffin-embedded sections of spleen obtained on day 1 after virus challenge of animals. Colocalization between MERS-CoV nucleoprotein (NP; D and G) and CD3 (E and H) were detected in the spleen of the infected animals (arrows; D–F and G–I). CM1 and CM2 represent 2 individual common marmosets. MERS-CoV NP was not detected in the preimmune serum control (A–C). Bars represent 25 µm.
SARS-CoV could directly infect T cells and induce apoptosis in the infected cells. Therefore, the underlying mechanism for SARS-CoV- and MERS-CoV–induced lymphopenia could be distinct. In particular, lymphopenia in MERS but not SARS cases could be a result of direct infection of T cells and infection-induced apoptosis. In addition to apoptosis, the dramatic depletion of surface DPP4 in T cells upon MERS-CoV infection may substantially disrupt the proliferation and cellular function of T cells. The mechanism and implication of MERS-CoV–directed DPP4 depletion in T cells deserve further investigation.

Multiple genes of SARS-CoV have been implicated in caspase induction [4]. We showed in the current study that MERS-CoV–induced apoptosis in T cells was accompanied by the activation of caspases 3, 8, and 9. The maturation of caspases 8 and 9 suggested that the extrinsic and intrinsic apoptosis pathways were triggered by MERS-CoV infection. Currently, the exact mechanism of MERS-CoV–induced caspase upregulation is unknown. However, since UV-inactivated MERS-CoV induced caspase 3 upregulation to an extent similar to that of untreated MERS-CoV, we postulate that the detected caspase upregulation is likely attributed to the structural components of MERS-CoV, including the E, M, S, and N proteins. Nevertheless, our data demonstrated that, while approximately 70% of MERS-CoV–infected T cells were expressing active caspase 3, a considerably lower percentage of infected T cells showed signs of caspase 8 or caspase 9 activation. Although the relationship of the quantity of initiator caspases (caspases 8 and 9) and execution caspase (caspase 3) is not linear, because they are catalyzed in a cascade, it is possible that other, yet-to-be-identified pathways are also involved in caspase 3 activation and in contributing to MERS-CoV–induced apoptosis in infected T cells. Remarkably, the dynamics of MERS-CoV infection and MERS-CoV–induced apoptosis in T cells were drastically different from findings in cell lines. We demonstrated that, whereas caspase 3 induction in cell lines was gradual and replication dependent, it was abrupt and replication-independent in T cells. This discrepancy is likely due to the difference in the efficiency of MERS-CoV entry of cell lines versus primary T cells, which may in turn be a result of differential surface DPP4 expression.

In this regard, if virus-induced T-cell apoptosis is a pathogenic determinant of MERS, a novel treatment regimen such as one involving anti-apoptotic agents in addition to antivirals inhibiting virus replication may be considered to improve the outcomes of patients with MERS, as in the case of VX-765 for human immunodeficiency virus infection [50].

Recent animal studies have identified MERS-CoV RNA in the lymphoid organs of infected animals, including common marmosets [30], rhesus macaques [40], and dromedary camels [41]. However, owing to the lack of reports describing samples obtained from patients with MERS during autopsy, whether the virus could invade human lymphoid organs remained unknown. To this end, we inoculated disassociated human spleen and tonsil cells with MERS-CoV. Remarkably, CD3+ T cells in the spleen and tonsil were highly susceptible to MERS-CoV infection and MERS-CoV–induced apoptosis. Intriguingly, T cells from these organs appeared to demonstrate a higher degree of infection and apoptosis upon MERS-CoV challenge. This suggested that T cells at different developmental stages might be differentially affected by MERS-CoV. Finally, we examined the spleen of MERS-CoV–infected common marmosets. Importantly, our data demonstrated that MERS-CoV could disseminate to the spleens of infected animals and infect their CD3+ T cells. Collectively, our ex vivo and in vivo findings suggest that MERS-CoV can invade not only T cells in the peripheral blood, but also the large repertoire of T cells in lymphoid organs, which may result in a more severe immunopathology.

In summary, our study provided in vitro, ex vivo, and in vivo evidence for MERS-CoV infection of human T cells. We demonstrated that the infection is associated with apoptosis induction, which may provide a new target for effective therapeutic interventions.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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