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Severe Acute Respiratory Syndrome and the Innate Immune Responses: Modulation of Effector Cell Function without Productive Infection

Chien-Te K. Tseng, Lucy A. Perrone, Hongbing Zhu, Shinji Makino, and Clarence J. Peters

Severe acute respiratory syndrome (SARS), an acute and highly contagious pulmonary illness that spreads primarily via the respiratory route, has had a devastating social, economic, and medical impact worldwide (1). The causative agent of SARS was quickly identified as a novel coronavirus (CoV), now designated SARS-CoV, and genomes of a number of isolates were subsequently sequenced through an intense and unprecedented international effort (2–6). Additionally, angiotensin-converting enzyme 2 (ACE2) was recently identified as a functional receptor responsible for the entry of SARS-CoV into permissive cells, providing an important clue to the cellular and tissue tropism of this virus (7). Despite these remarkable achievements, the pathogenesis of SARS-CoV is still not understood.

The emergence of SARS-CoV infection results from the viral entry into permissive cells, providing an important clue to the cellular and tissue tropism of this virus. The skin, where most infections occur, makes them among the first sites of infection. The outcome of virus-host interaction depends on many factors of both viral and cellular origin. SARS is a highly contagious and life-threatening respiratory disease with the lower respiratory tract (e.g., the lungs) as its main target of the pathogenic process (3, 8, 9). Immunopathological examination of postmortem lung tissues from SARS patients revealed diffuse alveolar damage with prominent pneumocytic hyperplasia and accumulation of alveolar and interstitial macrophages (Mφ), as well as other pathological manifestations characteristic of acute respiratory disease syndrome resulting from massive inflammatory responses in the lungs. Intriguingly, these pulmonary manifestations usually occur after the clearance of viremia and in the absence of a consistent detection of other infectious agents, suggesting that an intense local inflammatory response, not directly induced by SARS-CoV or other opportunistic pathogens, could be responsible for the devastating outcome of SARS-CoV infection. The likelihood of SARS being an immune-mediated disease is also supported by the observation of a transient lymphopenia, especially in CD4+ and CD8+ T cells early in the SARS episode, and elevated serum levels of inflammatory cytokines in SARS patients (10–13).

Mφ and DC are two key cellular elements of the innate immune system with various effector functions, e.g., phagocytosis, Ag presentation, and cytokine production, facilitating their indispensable role in regulating the interplay between innate and adaptive immune responses. In addition, the fact that most resting Mφ and immature DC reside at sites of an interface with the environment, such as the mucosal epithelium, alveoli of the respiratory tract, and the skin, where most infections occur, makes them among the first and ideal targets for viral exploitation, contributing to viral pathogenesis (14, 15). Consequently, interactions between viruses and
Mφ or DC have been studied extensively for clarifying the pathogenesis of viral infections. Clearly, viruses can affect the biology of Mφ and DC in several ways. For instance, the persistent viruses, like human CMV and murine CMV, may be sequestered within these cells and impair their functions (16, 17). Cytolytic viruses can induce cytopathic effects on Mφ and DC, as shown with measles, vaccinia virus, and influenza virus (18–20). Importantly, viral infections can modulate host immune responses by either enhancing or dampening the effector functions of Mφ and DC, causing immunopathogenesis and/or immunoevasion (18, 21–25). Additionally, Mφ and DC can also harbor infectious viruses, serving as reservoirs for further dissemination (14, 26, 27).

Given the pivotal roles of Mφ and DC in host defense against viral infections, it is important to determine whether SARS-CoV can interfere with the function of these two critical innate immune cells, thereby influencing the outcome of the infection. This information is crucial not only for understanding SARS-CoV pathogenesis, but also for devising successful therapeutic strategies. Thus, in the present study we investigated the susceptibility of primary human Mφ and DC to SARS-CoV infection and also analyzed their responses by examination of their expression of costimulatory molecules, secretion of inflammatory cytokines, phagocytic capacity, and the potential for priming naive T cells.

Materials and Methods

Virus

The Urbani strain of SARS-CoV, kindly provided to us by Dr. T. G. Keisazer at the Centers for Disease Control (Atlanta, GA), was used throughout this study. The original stock of SARS-CoV was subjected to two additional passages in Vero E6 cells, generating a cell-free viral stock with a titer expressed as 50% tissue culture infectious dose (TCID₅₀/ml) sample (1 × 10⁴ TCID₅₀/ml), which was stored at −80°C. In addition, aliquots of this infectious stock were also irradiated (2 × 10⁵ rads) from a cobalt-60 source, according to a predetermined kill curve, and used as an inactivated SARS-CoV (gamma-inactivated) control in this study. All experiments involving infectious virus were conducted at the University of Texas Medical Branch (Galveston, TX) in an approved biosafety level 3 laboratory with medical monitoring of staff.

Virus titration

Supernatants collected at different time points postinfection were assayed for infectious viral titers in a TCID₅₀ assay on permissive Vero E6 cell monolayers in 24-well plates with 10-fold diluted samples. The titer of individual samples was expressed as TCID₅₀ per milliliter of sample.

Detection of SARS-CoV replication by quantitative real-time PCR

Total RNA was isolated from mock-infected and SARS-CoV-infected Vero E6 or U937 cells (10⁶ cells) at indicated time points after infection by a standard protocol using TRIzol reagent (Invitrogen Life Technologies). Contaminating genomic DNA was digested with DNase I during the extraction procedure. The primer pair and probe for detecting SARS-CoV-specific subgenomic mRNA 5 (M protein) were: forward 5'-AGGTTTCCTATTTCTCGAGCTTTAT, reverse 5'-GAGGCGCGGAGGAACACCCGCTTAT, and the sequence of ACCTGTTCCGATTAGAATAG as detection probe, all of which were derived by using Assays-by-Design (part #H11032/H9278). Specific subgenomic mRNA 5 for the M protein were: forward 5'-GGACGAGGAGAAACCA CGCTTAT, and the sequence of ACCTGTTCCGATTAGAATAG as detection probe, all of which were derived by using Assays-by-Design (part #H11032/H9278). Real-time PCR was performed on an ABI PRISM 7000 real-time thermocycler (Applied Biosystems) following a standard protocol using TRIzol reagent (Invitrogen Life Technologies). The original stock of SARS-CoV was subjected to two additional passages in Vero E6 cells, generating a cell-free viral stock with a titer expressed as 50% tissue culture infectious dose (TCID₅₀/ml) sample (1 × 10⁴ TCID₅₀/ml), which was stored at −80°C. In addition, aliquots of this infectious stock were also irradiated (2 × 10⁵ rads) from a cobalt-60 source, according to a predetermined kill curve, and used as an inactivated SARS-CoV (gamma-inactivated) control in this study. All experiments involving infectious virus were conducted at the University of Texas Medical Branch (Galveston, TX) in an approved biosafety level 3 laboratory with medical monitoring of staff.

Viral infections

Mφ and DC were infected with infectious SARS-CoV at a multiplicity of infection (MOI) of 1 unless indicated otherwise or incubated with gamma-inactivated virus at 37°C for 1 h. Mock-infected cells exposed to a similar volume of medium were included as controls. Cells were then washed twice with PBS to remove unbound virions and cultured at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (R-10) medium supplemented with GM-CSF (500 U/ml) alone or a mixture of GM-CSF (1000 U/ml) and IL-4 (500 U/ml) to generate Mφ and DC, respectively. Cytokines were replenished every 3–4 days. Cells were harvested at 6–7 days of culture for their morphologic and phenotypic characteristics. GM-CSF-seeded mononuclear cells consistently gave rise to highly adherent CD14⁺CD1a⁺CD40⁻CD86⁻HLA-DR⁺ cells; whereas the nonadherent or loosely adherent CD14⁺CD1a⁻CD40⁻CD86⁻/HLA-DR⁻/CD83⁻ cells, typical of immature DC, were derived from GM-CSF/IL-4-driven cultures.

Phenotyping of Mφ and DC

The cell surface phenotypes of Mφ and DC were determined with a panel of Ab to human CD40, CD86, CD1a, CD83, and HLA-DR molecules. Cells stained with a combination of Ab mix for enriching human CD4⁺ T cells were pulsed with 1 μCi/well [³H]thymidine (New England Nu- clear) for the last 12–16 h in the culture. The total incorporation of [³H]thymidine was determined by liquid scintillation counting, and expressed as cpm.

Allogeneic CD4⁺ T cell proliferation

Human PBMC were isolated from healthy individuals by Ficoll-Paque gradient centrifugation and CD4⁺ T cells were purified by negative selection with a combination of Ab mix for enriching human CD4⁻ T cells and a magnetic column (StemCell Technologies), as previously described (32). DC were preincubated with infectious virus or with gamma-inactivated virus or left untreated for 2 days, in 200 μl of R-10 medium in 96-well plates for a total of 6 days. A fixed number of allogeneic CD4⁺ T cells (1 × 10⁵/well) were cocultured with these DC at various ratios. The triplicate cultures were pulsed with 1 μCi/well [³H]thymidine (New England Nuc- lear) for the last 12–16 h in the culture. The total incorporation of [³H]thymidine was determined by liquid scintillation counting, and expressed as cpm.
Detection of IL-6 and IL-12

Cell-free supernatants, harvested at indicated time periods from different cultures of Mφ and DC, were subjected to gamma irradiation. The dosage of 2 × 10^3 rads was according to our kill curve studies against SARS-CoV, to ensure the absence of infectious virus, as evident by the absence of infectivity of irradiated samples in permissive Vero E6 cells, before assessing cytokine content in a biosafety level 2 laboratory. The amount of IL-6 and IL-12 in the supernatants was assessed by ELISA, as previously described (32, 33). Both capture mAb and biotinylated detection mAb for human IL-6 and IL-12p40 subunits were purchased from BD Pharmingen and used at the concentrations recommended by the manufacturer. Various concentrations of recombinant IL-6 and IL-12, ranging from 50,000 to 50 pg/ml, were included in the assay as the standard. Avidin-conjugated peroxidase and chromogenic substrate o-phenylenediamine dihydrochloride were obtained from Sigma-Aldrich. Spectrophotometer analysis was performed at a 405 nm wavelength on an Exmax spectrophotometer (Molecular Devices) using Softmax Pro software (Molecular Devices). The levels of IL-6 and IL-12 were expressed as the mean ± SD of duplicate samples.

Assessing phagocytosis by flow cytometry

Pellets of differentially treated Mφ (2 × 10^5 cells/pellet) were resuspended in 500 μl of R-10 medium in 1.5-ml Eppendorf tubes containing FITC-dextran (m.w. = 40,000, 40 μg/ml; Sigma-Aldrich) and were incubated at 37°C (or at 4°C for control) for 1 h. The cells were washed five times with cold R-10 medium and fixed with 2% paraformaldehyde, and the amount of the accumulated intracellular fluorescent probe was determined using a FACScan flow cytometer (BD Biosciences).

Results

SARS-CoV fails to productively infect human Mφ and DC

Mφ and DC are two of the most prominent cellular elements of innate inflammatory responses. To study the potential effect of SARS-CoV on the host defense system, we first determined whether this virus could productively infect Mφ and DC. For this study, we initially infected primary Mφ and DC, all of which were derived from the 6- to 7-day cytokine-driven CD14^+ monocytic cell cultures, as described in Materials and Methods. Permissive Vero E6 cell monolayers infected with SARS-CoV at a MOI of 1 were included as the positive control. Cell-free supernatants were harvested at indicated time points after infection and the titers of infectious viral particles were assessed by a standard TCID_{50} assay on Vero E6 cell monolayers. As expected, Vero E6 cells were highly permissive to SARS-CoV infection, in which a maximum viral yield (1–5 × 10^7 TCID_{50}/ml) could be consistently detected between 24 and 48 h after infection (Fig. 1). Additionally, foci of cytopathic effects in infected monolayers (cellular roundup and detachment) could be observed within the first 24 h after infection, which subsequently reached 70–80% within 36–48 h (data not shown). In contrast to the strikingly early burst of viral replication in Vero E6 cells, SARS-CoV did not seem to replicate in Mφ and DC. As shown in Fig. 1, representative of eight independent experiments using cells derived from six different individuals, the titers of infectious virus particles in the supernatants harvested over time after infection steadily dropped from 10^5 to 10^6 TCID_{50}/ml at day 0 (e.g., 1 h after infection) to become undetectable and 10^2 TCID_{50}/ml at day 5 for Mφ and DC, respectively. The nonproductive nature of SARS-CoV infection was also observed in repeated experiments by using Mφ-like U937 cells and DC-like KG-1 cells, showing very similar kinetics of decreasing infectivity in the supernatants (data not shown). To determine whether a small fraction of cells might be permissive for SARS-CoV, which may not be readily detected by the TCID_{50} assay, we used highly sensitive real-time PCR to quantify the expression of SARS-CoV-specific subgenomic sequences over time in infected cells. We chose monocytic U937 or DC-like KG-1 cells over primary Mφ and DC to ensure sufficient cell numbers for this study. We infected U937, KG-1, and Vero E6 cells with SARS-CoV (MOI = 1). Total RNA extracted from infected cells at indicated periods after infection were subjected to amplification and analysis of SARS-CoV-specific subgenomic mRNA 5 (M protein) by quantitative real-time PCR. As shown in Fig. 2, a dramatic increase in the copy number of mRNA 5 could be readily detected in permissive Vero E6 cells at 12 and 24 h after infection, paralleling the viral replication. Although mRNA 5 was readily detected in U937, KG-1, and Vero E6 cells at both 1 and 20 h after infection by qualitative PCR (data not shown), an increase in its copy number over time was not found in U937 cells or in KG-1 cells. Taken together, these results indicate that neither Mφ nor DC are permissive to SARS-CoV replication.

SARS-CoV does not add to the cell death of cultured Mφ and DC

Cell death is a likely outcome of viral infection, as exemplified by the apparently lytic infection of SARS-CoV in Vero E6 cells.
Thus, we investigated whether SARS-CoV could affect the viability of Mφ or DC. For these experiments, uninfected and SARS-CoV-infected cells (MOI = 1) were cultured in 24-well plates for 72 h in R-10 medium with or without the presence of exogenous cytokines used for in vitro differentiation (e.g., GM-CSF and GM-CSF plus IL-4 for Mφ and DC, respectively). The cell viability in the differential cultures was determined by a trypan blue exclusion technique. As shown in Fig. 3, total viable cells recovered from mock-infected cultures grown in R-10 medium alone (n = 4) were 20 and 50% of the original cellular input for DC and Mφ, respectively. To our surprise, these poor recoveries of viable cells were improved to 37% (DC, p ≤ 0.08) and 65% (Mφ) upon incubation with SARS-CoV. Because various cytokines are known to prevent cell death, the increased viability of Mφ and DC due to SARS-CoV exposure may suggest that this virus could be capable of inducing the release of cytokines from these cells. The requirement of soluble factors for better sustaining the viability of Mφ and DC was supported by the dramatically increasing viability of mock-infected cells cultured in the presence of exogenous cytokines (n = 2), in which the viability was increased from 20 to 50% for DC (p ≤ 0.05) and from 50 to 68% for Mφ. However, the difference in the viable cell recovery between mock-infected and infectious virus-exposed cells cultured in exogenous cytokine-containing R-10 medium was not significant (e.g., 50 vs 55% and 65 vs 68% for DC and Mφ, respectively). Although the molecular basis for the elevated viability in both Mφ and DC remains to be determined, our results clearly indicate that exposure of these cells to SARS-CoV does not add to the mortality of cultured Mφ and DC.

In addition to causing cell death, many viruses have been shown to modulate various functions of Mφ and DC, including activation, differentiation, maturation, and cytokine production (14, 21, 34). Importantly, the ability to alter the function of host cells by some viruses appears to be independent of viral replication (35, 36). Therefore, we designed a series of experiments, as described below, aimed to determine whether SARS-CoV could have any impact on the function of Mφ and DC.

**SARS-CoV induces the phenotypic maturation of DC, but not Mφ**

Activation and maturation of Mφ and DC are essential for initiating and/or promoting Ag-specific, T cell-mediated responses, largely due to their enhanced expression of costimulatory molecules, a process known as “phenotypic maturation” in DC (37, 38). We thus investigated whether SARS-CoV has the potential to influence this maturation process in Mφ and DC. For these experiments, mock-infected cells and cells that were infected with live virus (MOI = 1) or with gamma-inactivated virus for 72 h were analyzed by flow cytometry for their expression of various cell surface molecules, including costimulatory (CD40, CD86), MHC (HLA-DR), and a marker for mature DC (CD83). As shown in Fig. 4, representative of six independent experiments, the expression of CD40 and CD86 was not significantly altered in Mφ infected with either inactivated or live viruses, when compared with uninfected cells. The expression of HLA-DR on live virus or inactivated virus-treated Mφ was either unaffected (n = 4) or reduced (n = 2) in six independently conducted experiments. In contrast to Mφ, whose expression of costimulatory molecules, e.g., CD40 and CD86, appears to be rather refractory to manipulation, in seven of nine independent experiments using DC derived from six different individuals, an increased expression of at least one of the costimulatory molecules tested as a result of exposure to live SARS-CoV could be readily detected. As shown in Fig. 4B, representative of seven independent experiments demonstrating an enhanced expression of costimulatory molecules, the expression of CD40, CD86, HLA-DR, and CD83 was increased in DC incubated with live virus, when compared with mock-infected or inactivated virus-exposed DC. SARS-CoV most frequently enhances the expression of CD40 and CD83, among various markers tested, suggesting that such a virus-mediated enhancement in the expression of costimulatory molecules appears to be selective rather than global. Although inactivated virus did not exhibit any effect on the phenotypic maturation of DC in most of the experiments, a slight increase in the expression of selected molecules was occasionally observed. We also noted that no changes in the cell surface molecule expression were observed in two of nine experiments (data not shown), suggesting that DC that were derived from different individuals might not be universally responsive to SARS-CoV.

**SARS-CoV activates the production of IL-6 and IL-12 by Mφ and DC**

Both Mφ and DC have been identified as key producers of innate inflammatory cytokines in response to microorganisms and/or their microbial products, such as bacterial LPS (37–39). To investigate whether SARS-CoV could modulate their intrinsic ability to produce cytokines, Mφ and DC were incubated with live or inactivated SARS-CoV at an MOI of 1 for 48 h, followed by an additional 36-h incubation with or without a suboptimal dose of bacterial LPS (100 ng/ml). The contents of IL-12 (p40 subunit) and IL-6 in the resulting culture supernatants were assessed by ELISA.

As shown in Fig. 5, representative of six independent experiments using cells derived from five different individuals, mock-infected Mφ and DC spontaneously released low, but detectable, amounts of IL-12 and IL-6. Stimulation of Mφ and DC with a suboptimal dose of LPS resulted in a slight increase in secretion of both IL-12 and IL-6 in DC and of IL-6 but not IL-12 in Mφ. Additionally, neither spontaneous nor LPS-mediated IL-12 and IL-6 production was significantly altered in DC exposed to inactivated virus. However, there was a 2-fold increase in LPS-mediated IL-6 production in inactivated virus-exposed Mφ, compared with mock-infected cells. Incubation of either Mφ or DC with live virus consistently resulted in a slight increase in the production of both IL-12 and IL-6, when compared with those of mock or inactivated virus-exposed cultures. Remarkably, when live virus-exposed Mφ and DC were cocultured with a suboptimal dose of LPS (100 ng/ml), a dramatic increase in both IL-12 and IL-6 production.
FIGURE 5. SARS-CoV affects spontaneous and LPS-induced IL-12 and IL-6 production by MΦ and DC. Uninfected, inactivated SARS-CoV-infected, and live SARS-CoV-infected primary human MΦ (A and B) and DC (C and D) were stimulated with a suboptimal dose of LPS (100 ng/ml) at 48 h after infection or left untreated. The supernatants were harvested at 36 h after LPS stimulation and their contents of IL-12 and IL-6 measured by ELISA. Data shown are the mean ± SD of duplicate cultures in individual experiments. These results are representative of six independent experiments using cells derived from five different individuals. *, p < 0.05; **, p < 0.01 by Student’s t test, compared with mock-infected controls.
was observed, suggesting that infectious SARS-CoV can prime these two innate immune cells to a high cytokine production in response to secondary activation signals provided by LPS.

TLR4 is constitutively expressed on Mφ and DC and is a key LPS-responding molecule. We, thus, investigated whether the highly enhanced cytokine production in response to LPS by live virus-infected cells could be correlated with the extent of TLR4 expression. As shown in Fig. 6, representative of two independent experiments, ~17% of Mφ and DC constitutively expressed TLR4 on their surface. Incubation of DC with live, but not inactivated, virus for 2 days resulted in an increase of the total TLR4+ DC to 52%, with only minor changes in the MFI. However, as with the expression of costimulatory molecules, significant changes in the surface expression of TLR4 could not be observed in either live or inactivated virus-treated Mφ.

It was also noted that the ability of DC to secrete IL-12 and IL-6 in response to SARS-CoV does not always parallel with their phenotypic maturation because an enhanced production of both cytokines was also observed in live virus-exposed DC that did not show any phenotypic changes (data not shown). Taken together, these results strongly suggest that not only can SARS-CoV by itself induce the release of cytokines, it can also precondition Mφ and DC for a highly intense inflammatory response in response to bacterial LPS, resulting in massive inflammatory secretions.

SARS-CoV reduces the receptor-mediated endocytic activity of Mφ and enhances the T cell stimulatory capacity of DC

The ability to capture and process Ag and to subsequently present antigenic peptides to Ag-specific T cells are characteristic functions of Mφ and DC (40, 41). Therefore, we investigated whether SARS-CoV could modulate these two crucial functions of Mφ and DC. To study this, we exposed Mφ and DC to either inactivated or live SARS-CoV (MOI = 1) for a total of 3 days. Parallel cultures of mock-treated cells were also included as controls. Because receptor-mediated endocytosis is the most efficient mechanism for the uptake of Ags (42), we analyzed the effect of SARS-CoV on the mannose receptor-mediated uptake of FITC-conjugated dextran in Mφ. The endocytosis of various Mφ populations was assessed by quantifying the total uptake of FITC-dextran probe by flow cytometry. As shown in Fig. 7, an average of two independent experiments, mock-treated Mφ that were incubated at 4°C did not actively take up FITC-dextran and had a minimal MFI of 37.5 ± 7. However, at physiological temperatures they were quite active in endocytosis with an MFI of 291 ± 55. Interestingly, incubation with live virus reduced the MFI of captured FITC-dextran probes to 108 ± 15. Although the difference in the values of MFI between mock-infected and live virus-infected Mφ was not statistically significant (p ≤ 0.088), such obvious reduction in the phagocytic activity could be biologically relevant. Inactivated SARS-CoV-exposed Mφ had a lesser decrease in uptake (MFI = 210 ± 40). These results suggest that infectious SARS-CoV has the ability to compromise the phagocytic activity of Mφ, decreasing the potential of these cells to present Ags and increasing the threat of opportunistic infections.

The capacity to activate naive T cells is a characteristic feature of mature DC. To determine whether SARS-CoV could also affect the ability of DCs ability to prime naive T cells, DC were exposed to live virus (MOI = 1) or inactivated virus for a total of 3 days, and their ability to stimulate naive CD4+ T cells was analyzed by a standard one-way MLR. As shown in Fig. 8, representative of differentiation ratios, and cultured in 96-well, U-bottom microtiter plates for a total of 6 days. Individual wells were pulsed with [3H]thymidine at the concentration of 1 μCi/well for the last 12–16 h in the culture. The proliferation of allogeneic T cells was expressed as the average cpm ± SD of triplicate samples. Data shown are representative of four individual experiments. * p < 0.01 from Student’s t test, compared with mock-infected controls.

**FIGURE 6.** SARS-CoV modulates the expression of TLR4 expression on the surface of Mφ and DC. Primary Mφ (A) and DC (B) were mock infected, or infected with inactivated SARS-CoV or live SARS-CoV (MOI = 1) for 2 days before being harvested for analyzing their surface expression of TLR4 molecule by flow cytometry. Data presented are the histogram of FACS analysis showing the percentages of positive cells along with their MFI. Results are representative of two independent experiments using cells derived from different individuals.
four independent experiments using DC derived from different individuals, DC exposed to live virus stimulated a very active proliferation of the responder T cells, exceeding 300 times the efficiency of mock or inactivated virus-treated DC. These results are consistent with the expression level of costimulatory molecules, as shown in Fig. 4B, and suggest that live virus is also capable of inducing functional maturation of DC.

**Discussion**

In the present study we investigated, for the first time, the susceptibility of primary human Mφ and DC and their subsequent responses to SARS-CoV infection by focusing on their phenotypic expression, secretion of inflammatory cytokines, phagocytic activity, and T cell-priming capacity. The findings of our studies are: 1) SARS-CoV could not productively infect Mφ and DC (Figs. 1 and 2); 2) incubation with SARS-CoV did not add to the spontaneous cell death of primary Mφ and DC; on the contrary, it enhanced their viability (Fig. 3) independently of the presence of exogenous cytokines; 3) exposure to live SARS-CoV exerted a diverse effect on the biology of DC vs Mφ, in which it up-regulated the expression of MHC class II, CD40, CD83, and CD86, on the surface of DC but not Mφ. Consequently, it also greatly enhanced DC ability to stimulate the proliferation of allogeneic T cells (Figs. 4 and 8). In contrast, it impaired the phagocytic activity of Mφ (Fig. 7); and 4) not only can live SARS-CoV induce a low-to-moderate production of IL-6 and IL-12, but it also can prime for a much higher production of these proinflammatory cytokines by both Mφ and DC in response to a suboptimal dose of LPS from *Escherichia coli* (Fig. 5). Importantly, the ability of SARS-CoV to modulate Mφ and DC functions was independent of viral replication.

As mentioned, Mφ and DC are highly susceptible to many viruses. Thus, the prominent accumulation of interstitial and alveolar Mφ in the lungs of patients severely affected by SARS has raised the possibility that pulmonary Mφ could be one of the primary targets of SARS-CoV (8). However, the rarely detected viral genome and complete absence of viral Ags or viral particles in pulmonary Mφ, as revealed by subsequent studies, in which the cellular and tissue distribution of SARS-CoV was examined in postmortem lung tissues by in situ hybridization and immunohistochemistry, strongly suggest that they might function as scavengers rather than as the prime targets for this virus (9, 43). These results are in accordance with our in vitro results, in which no evidence of productive SARS-CoV replication in Mφ and DC was found. Our conclusion that neither Mφ nor DC were permissive to in vitro SARS-CoV infection is based on the comparison with the kinetics of infectious virus recovery and the expression level of subgenomic virus-specific mRNA 5 (M protein) in permissive Vero E6 cells. However, our conclusion is in contrast to that of a recent report, in which human monocytic cells in freshly isolated PBMC were shown to be capable of supporting a self-limited SARS-CoV replication with occasionally a maximal one-log increase in the recovery of infectious viral particles over a period of 4 days in infected PBMC cultures (44). Although the exact reason for the discrepancy between our data and those recently reported is not clear, the difference in the differentiation state of the target cells or in the strains of SARS-CoV used in these studies, e.g., the Urbani strain in one and the SIN 2774 isolate in the other, offer possible explanations.

Mφ and DC play an important role in the destruction of infectious agents and provide a link between innate and adaptive immunity. Ironically, they are also known to serve as vehicles for virus replication and further dissemination, as shown for HIV and measles virus (18, 45). Thus, the nonproductive nature of SARS-CoV infection, as reported in this study, would greatly reduce the likelihood of their role as viral reservoirs for disseminating the infection. However, from the pathological point of view, such a possibility may exist in that it took 5 days or longer for the infectivity of supernatants harvested from infected Mφ and DC cultures to become undetectable by the TCID<sub>50</sub> assay. This gradual loss of SARS-CoV infectivity associated with infected Mφ and DC cultures, along with an increased viability of these cells in response to SARS-CoV infection (Fig. 3), may thus give them the potential to play some role in further dissemination. Whether infectious viral particles remained bound to the surface at all times or were internalized later after initial contact with cells, as well as other aspects of this nonproductive interaction between SARS-CoV and Mφ or DC, are the subject of intense investigation in our laboratory. Our preliminary data revealed that although ACE2, the cellular receptor of SARS-CoV, could be detected in both cell types by Western blotting, we were unable to demonstrate its surface expression by flow cytometry (data not shown). CD209L (L-SIGN) has recently been identified as an additional receptor for SARS-CoV (46). Because CD209L is known to express on the surface of primary DC and DC-like KG-1 cells, the failure of SARS-CoV to productively infect these cells in our study indicates that this molecule may not be as critical as ACE2 in mediating SARS-CoV infection in Mφ and DC.

In addition to viruses and other pathogens, various inflammatory mediators, including LPS, dsRNA, and cytokines (e.g., TNF-α, IL-1, IL-6, IL-10, and others) can also induce maturation of DC (37). The stocks of SARS-CoV used in our study were derived from infected Vero E6 cells as crude cell-free supernatants, which might contain inflammatory cytokines that were released by infected Vero E6 cells, thus raising a possibility that the observed DC maturation in the current study could be a direct effect of contaminating cytokines in the virus stock rather than one of SARS-CoV infection. This is unlikely, because DC exposed to SARS-CoV from the same stock, which was irradiated by cobalt-60 at a dose (2 × 10<sup>6</sup> rad) shown to be sufficient to inactivate 10<sup>7</sup> TCID<sub>50</sub>/ml SARS-CoV without affecting cytokine activity (data not shown) (47), did not cause any significant phenotypic change. The ability of SARS-CoV to affect DC maturation is apparently dependent on live virus, as incubation with gamma-inactivated virus did not cause phenotypic and functional changes. This observation clearly suggests that a simple cellular receptor-mediated interaction with SARS-CoV is not by itself sufficient to activate DC. It has been reported that the ability to alter DC function by two large DNA viruses, HSV type 1 and human CMV, was mediated through soluble factors induced by immediate early and early gene products of viruses and was independent of a complete viral replication cycle (35, 36). Whether certain SARS-CoV-encoded signals were required for the manipulation of DC function is currently under investigation.

One of the characteristic features of the early stages of SARS is a decrease in the total counts of circulating CD4 and CD8 subsets of T lymphocytes (48). Significantly, the extent of this transient lymphopenia is associated with the severity of SARS. Although the mechanism underlying this hematological manifestation of SARS remains undefined, the sequestration of virus-specific T cells in the lungs or other infected peripheral tissues could result in such a transient loss of circulating T cells. In this context, the effectiveness of SARS-CoV to activate DC may be of significance. DC are known to be the only APCs capable of stimulating Ag-specific naive T cells in the draining lymph nodes, resulting in their expansion. These primed T cells subsequently transmigrate, via the bloodstream, to infected peripheral tissues in which they exert acquired immune functions by killing infected target cells, resulting
in tissue damage. Additionally, they can also exacerbate local inflammation by secreting inflammatory cytokines. Thus, the superb ability of live SARS-CoV-exposed DC to prime naïve T cells, as shown in this study, may suggest the existence of reasonably active virus-specific effector and/or memory T cell pool in patients recovered from SARS.

The ability of SARS-CoV to impair the phagocytic function of Mø is shared by many other viruses, some of which are porcine reproductive and respiratory syndrome virus, an animal coronavirus, respiratory syndrome virus, and HIV (49–52). Because the outcome of respiratory viral infections is often complicated by secondary bacterial infection (53), the reduced capacity of Mø to uptake the invading bacteria and other pathogens could pave the way for enhanced secondary infections, contributing to a situation of devastating pathology in the respiratory system.

It is clear that SARS-CoV induces activation of DC, resulting in phenotypic and functional maturation, as well as cytokine production, whereas its effect on Mø seemed to be restricted to selected functions, e.g., phagocytosis and cytokine production. The molecular basis for such a cell type-specific effect of this virus is unknown. SARS-CoV interaction with these cells may occur via the specific receptor, ACE2. Thus, a difference in the level of ACE2 expression between these two cells could thus be responsible for the discrete effect. Alternatively, virus may also interact with these cells via receptor-independent mechanisms, in which the kinetics and/or magnitude of such interactions would be largely based on the activation/differentiation stages of Mø vs DC. Thus, a detailed examination of Mø/DC-SARS-CoV interactions, especially in the early phase of interaction, is warranted for clarifying the differences in their responses to SARS-CoV infection.

Based on our findings, we propose the following model of SARS-CoV pathogenesis in which Mø and DC are of central importance. Alveolar Mø and DC residing in the airway epithelium interact with SARS-CoV, either inhaled via the aerosol route or released into airspaces by infected pneumocytes. Although there is not a productive infection, functional alterations occur in these cells. As a consequence of interaction with SARS-CoV, the phagocytic capacity of Mø decreases, posing an imminent threat of secondary bacterial and other pathogenic infections, whereas DC are activated, leading to a mature phenotype and subsequent migration from the lung to the lymph nodes where they efficiently stimulate T cell responses. Activated T cells efficiently transmigrate to the sites of pulmonary infection via the bloodstream, and firmly bind to and destroy infected pneumocytes and other permissive cells, causing extensive damage in the lung parenchyma. In addition, the inflammatory cytokines released by activated Mø and DC could further exacerbate unfavorable inflammatory responses in the lungs. The markedly enhanced secretion of proinflammatory cytokines by cells exposed to infectious SARS-CoV and low levels of LPS may also occur in the setting of Gram-negative pneumonia, stimulation of other TLR, or activities of transmigrated Ag-specific T cells, further contributing to an unfavorable outcome of SARS.

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Disclosures
The authors have no financial conflict of interest.

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