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Respiratory Syncytial Virus Infection of Monocyte-Derived Dendritic Cells Decreases Their Capacity to Activate CD4 T Cells

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Respiratory syncytial virus (RSV) is a major cause of severe lower respiratory tract infections in children, the elderly, and immune-compromised individuals. CD4 and CD8 T cells play a crucial role in the elimination of RSV from the infected lung, but T cell memory is not sufficient to completely prevent reinfections. The nature of the adaptive immune response depends on innate immune reactions initiated after interaction of invading pathogens with host APCs. For respiratory pathogens myeloid dendritic cell (DC) precursors that are located underneath the epithelial cell layer lining the airways may play a crucial role in primary activation of T cells and regulating their functional potential. In this study, we investigated the role of human monocyte-derived DC in RSV infection. We showed that monocyte-derived DC can be productively infected, which results in maturation of the DC judged by the up-regulation of CD80, CD83, CD86, and HLA class II molecules. However, RSV infection of DC caused impaired CD4 T cell activation characterized by a lower T cell proliferation and ablation of cytokine production in activated T cells. The suppressive effect was caused by an as yet unidentified soluble factor produced by RSV-infected DC.

Dendritic cells (DC)2 are APCs with the unique property of inducing primary T cell responses. In addition to the initiation of T cell proliferation DC also determine the development of T cell effector function (1, 2). Immature DC (iDC) localized in the tissues are among the first cells to interact with pathogens. Pathogen exposure triggers the maturation of DC that results in up-regulation of costimulatory molecules on their surface, the potency to produce cytokines, and their migration from peripheral tissues to the lymph nodes. In the lymph nodes, DC encounter and activate naive and central memory CD4 and CD8 T lymphocytes. Naive T cells proliferate and differentiate in response to TCR triggering by peptide-MHC complexes when appropriate costimulatory signals are provided on the surface of professional APC (3).

The efficacy of the initiation of the immune response as well as the type of immune response initiated are both components that are determined by the type and maturation status of DC (4). The T cell response will be programmed depending on the combined innate immune triggers caused by the pathogen via pattern recognition receptors on DC and the inflammatory milieu triggered by the pathogens in surrounding tissue (5). The type of immune response initiated after initial DC-T cell interaction in tissue draining lymph nodes, and the inflammatory environment in the affected tissue, both contribute to the character of the adaptive immune response in the periphery during the effector phase of the response (4–6).

Respiratory syncytial virus (RSV), a pneumovirus of the family Paramyxoviridae, is an important cause of lower respiratory tract infection in infants, the elderly, and immune-compromised people. The virus infects ~70% of infants in their first year of life, and all children are infected by age 3. Reinfection is common throughout life, and previous infection does not prevent subsequent re-infection (7, 8). CD4 as well as CD8 T cells play a crucial role in the elimination of RSV from the lung. However, they may also contribute to severity of disease via the orchestration of the inflammatory response in the lung. This was illustrated during a vaccine trial in the 1960s with a formalin-inactivated RSV virus, whereby enhanced disease occurred in vaccinees upon exposure with the natural virus (9). It has been hypothesized that this immune-mediated enhanced disease was caused by an unbalanced Th2 type recall response (10, 11), although this remains controversial (12). At present it is unclear what is causing severe lower respiratory tract disease during primary RSV infections, damage caused by the virus itself possibly as a consequence of an inadequate immune response, or immune pathology.

In the lung, iDC of myeloid origin are present underneath the epithelial cell layer lining bronchioli and alveoli (13, 14). During respiratory infections these DC have been postulated to be the primary cell involved in naive T cell stimulation (14). As a first step to determine whether there might be a possible role of innate effects of RSV via lung DC on the ensuing adaptive immune response during RSV infections in the lower respiratory tract, we have characterized the interaction of RSV with monocyte-derived DC in vitro. We have found that immature monocyte-derived DC can be infected with RSV, which results in DC maturation. When...
judged from the expression of costimulatory molecules CD80, CD86, CD40, CD54, CD83, as well as HLA class II, these RSV-infected DC (RSV-DC) are indistinguishable from DC matured by cytokines (IL-1β + TNF-α) or a control virus (influenza virus). However, RSV-DC were less effective in inducing proliferation of naïve CD4 T cells when compared with other types of mature DC. Furthermore, live T cells expanded after stimulation with RSV-DC for 10–12 days were poor inducers of both Th1 and Th2 cytokines. We found that a soluble factor produced by RSV-DC inhibited T naïve CD4 T cells when compared with other types of mature DC. However, RSV-DC were less effective in inducing proliferation of DC matured with cytokines IL-1β and TNF-α. Vice versa supernatants of DC matured with cytokines could reverse the inhibition of RSV-DC on T cell proliferation. The factors involved remain unidentified. However, we excluded a role for inhibitory cytokines IL-10, TGF-β, and IFN-α by Ab-blocking studies. Also inhibition by IL-1ra or a direct role of live virus produced by infected DC was unlikely. Moreover, a role for regulatory T cells that could have been either activated or de novo initiated upon DC-T cell interaction in the RSV-DC co-cultures was also excluded.

Materials and Methods

Generation of monocyte-derived DC

PBMC were isolated by ficoll-paque (Pharmacia Biotech) density gradient centrifugation of heparinized peripheral blood from healthy donors. DC were generated by culturing monocytes for 6 days in IMDM (Invitrogen Life Technologies) containing 1% l-glutamine (In vitrogen Life Technologies), 1% penicillin/streptomycin (Invitrogen Life Technologies) and 10% FCS (HyClone) supplemented with 500 U/ml GM-CSF (a gift of Schering-Plough) and 250 U/ml IL-4 (Strathmann). At day 6, maturation was induced by culturing the cells for 2 days using different maturation stimuli: 1) 50 ng/ml IL-1β (Strathmann) + 50 ng/ml TNF-α (Strathmann) referred to in the test as maturation factors (MF), 2) 20 µg/ml poly IC:LC (Sigma-Aldrich); 3) RSV (strain A2); 4) influenza virus (A/NT/60/68). For fluorescence-activated cell sorting (FACS) analysis, the cultures were exposed to UV light.

Preparation of RSV stocks

Virus stocks were prepared by infecting HEp-2 cells at a multiplicity of infection (moi) of 0.1. Infected cells were incubated at 37°C until extensive cytopathologic effect was induced at day 3 or 4. At this point, medium was removed, and fresh medium was added. After an additional 3–4 h at 37°C, the supernatant was collected and cell debris was pelleted by centrifugation. The supernatant was filtered sterilized (0.2 µM Filter-Sterilcop), Polyethylene glycol (PEG) (6000 Merck) was added to the supernatant at a final concentration of 10%, and the mixture was stirred at 4°C for 2 h. The virus was collected by centrifugation at 4000 rpm, for 30 min. The pellet was resuspended in 1/10 of the original volume in IMDM + 10% FCS. The titer of virus stocks was determined by inoculation of HEp2 cells with serial dilutions of the virus preparation. To inactivate RSV, aliquots of the virus were exposed to UV light.

Characterization of DC

The maturation status of DC was characterized by the expression of cell surface molecules on day 6 and day 8 of culture for every experiment by flow cytometry using three- or four-color direct immunofluorescence. The following anti-human mAbs were used: anti-CD3 PerCP, -CD4 PE, -CD11c PE, -CD14 FITC, -CD19 FITC, -CD80 PE, -CD86 PE, -HLA-DR PerCP, and isotype controls (mouse IgG1 and IgG2), all obtained from BD Pharmingen. Anti-CD83 APC was purchased from Caltag and anti-BDCA2 FITC was obtained from Miltenyi Biotec. The level of infection with RSV or influenza was measured by the cell surface expression of the fusion protein of RSV, anti-RSV-F FITC (Dako) and polyclonal anti-influenza A (Dako) respectively. Cells were washed twice with PBS containing 2% FCS, 2 mM EDTA and 0.02% NaN3. After saturation with FACS buffer containing 10% human serum for 20 min at 4°C, cells were incubated with the appropriate mAbs for 30 min at 4°C, washed twice with FACS buffer. Analyses were performed using FACScalibur and CellQuest software (BD Biosciences). Cell viability was routinely checked by staining dead cells with 7AAD (BD Pharmingen), TO-PRO-3 (Molecular Probes) or annexin V and phosphatidylinositol (Bender MedSystems) depending on staining combinations. Usually slightly more (1.5×) RSV-DC were apoptotic compared with DC matured by other stimuli.

Confocal microscopy

Monocyte-derived DC were infected with RSV moi 5 for 48 h, fixed with 2% paraformaldehyde, washed, and incubated with 0.1 M glycine for 10 min and permeabilized with 0.1% saponin/1% BSA in PBS. Immunostaining was performed with polyclonal goat-α-RSV (Biosidin; Campro Scientific) and mouse α-HLA-DR (clone B8.11.2) as primary Abs, and donkey anti-goat IgG(H+L)-FITC (Jackson Laboratory) and donkey F(ab’), anti-mouse IgG(H+L)-Cy3 (Jackson Laboratory) as secondary Abs. Cells were mounted in Vectashield, and fluorescence was imaged with a Bio-Rad 1024 confocal laser-scanning microscope equipped with an argon/krypton laser for double fluorescence at 488 nm and 568 nm, respectively, for FITC and Cy3 fluorescence. Confocal images were recorded with a ×60 Plan APO objective lens and processed with RASW software (Bio-Rad). FITC and Cy3 were pseudocolored in green and red, respectively.

Isolation and stimulation of naïve CD4 T cells

CD45RA-CD45RO+ naïve CD4 T cells were purified from PBL by negative selection using CD4+ MACS Multisort beads (Miltenyi Biotec), supplemented with PE-labeled CD45RO-Abs (DAKO). Naïve CD4 T cells were >96% pure and were further phenotyped for the expression of CD28 and CD72. Purified naïve CD4 T cells (2 × 106 cells) were cocultured with mDC (5 × 103 cells) in 200 µl of IMDM containing 10% FCS in the presence of the superantigen Staphylococcus aureus enterotoxin B (100 pg/ml SEB; Sigma-Aldrich), in 96-well flat-bottom culture plates (Nunc). At day 5, 10 U/ml recombinant human IL-2 (Roche) was added, and the cultures were expanded for 7 days.

Cytokine production by CD4 T cells

On day 12, the quiescent CD4 T cells were re-stimulated with 10 ng/ml PMA (Sigma-Aldrich) and 1 µg/ml ionomycin (Calbiochem) for 6 h. During the last 5 h, 10 µg/ml brefeldin A (Sigma-Aldrich) was added. Single cell IL-4 and IFN-γ production was measured intracellularly by flow cytometry. Cells were fixed in 2% paraformaldehyde (Fluka), permeabilized with 0.1% saponin (Sigma-Aldrich), and stained with anti-human IFN-γ FITC and anti-human IL-4 PE (both obtained from BD Pharmingen). In parallel, resting T cells were stimulated with 0.4 µg/ml anti-CD3 and 1 µg/ml anti-CD28 mAbs (both obtained from BD Pharmingen) to measure cytokine secretion in supernatants collected after 24 h by multiplex cyto- kine assay.

T cell proliferation assays

Naïve CD4 T cells were cocultured as described above with DC and SEB for 5 days. Cultures were performed in triplicate. For the final 16 h, cultures were pulsed with 1 µCi/well [3H]thyminidine. [3H]Thymidine uptake was measured using a liquid scintillation beta counter. To address the question whether a soluble factor might be responsible for the inhibitory effect, ultracentrifuged supernatants of DC cultured for 48 h with RSV were added to MF-DC/T cell cocultures in a 1/1 dilution. Vice versa supernatant of MF-DC was added to cocultures of RSV-DC with naïve T cells to test whether RSV-DC supernatants were lacking a cytokine/soluble factor necessary for effective T cell activation. In some experiments, blocking Ab specific for IL-10 (1 µg/ml) (R&D Systems) or clone JES5-9D7 (BD Pharmingen) or TGF-β (R&D Systems) or IFN-α receptor (1 and 5 µg/ml, clone MMHAR-2 anti-CD118, blocks the biological action of type I IFNs) were added to DC-T cell cocultures. Recombiant IL-1β (1 and 50 ng/ml) was added in some experiments to exclude a possible role of IL-1ra.

To obtain insight in the cell division kinetics, T cell stimulation assays were also performed using naïve CD4 T cells that had been labeled with CFSE (FACScalibur). For fluorescent cell labeling, naïve Th cells were washed twice with serum-free medium, labeled with 2.5 µM CFSE in serum-free medium for 5 min at room temperature. The cells were then washed three times with culture medium. The labeled cells were cocultured with DC and SEB or anti-CD3 and T cell proliferation was analyzed at different time points using FACScalibur. To obtain insight into the absolute numbers of expanding cells, a CFSE dilution index was calculated with TO-PRO-3-dye (which stains only dead cells), just before analyzing the cells with flow cytometry. Combining the total T cell numbers in the DC-T cell cocultures with the percentages of cells in each cell division obtained...
from FACS analysis, the exact live and dead T cell numbers per cell division could be calculated.

**T cell suppressor assay**

At day 12 of naive T cell cultures, resting T cells were harvested and washed three times with serum-free medium, before staining with PKH26 (Sigma-Aldrich), a red cell cycle tracking dye. Cells (0.5 × 10⁶) were stained with 1.2 × 10⁻⁴ M PKH26 for 5 min at room temperature according to the manufacturer’s instructions. After washing with IMDM + 10% FCS, 2.5 × 10⁴ cells were preactivated for 24 h with anti-CD3 and anti-CD28 in round-bottom 96-well plates. After overnight preactivation, these PKH-labeled cells were tested for their capacity to inhibit proliferation of CFSE-labeled CD4 T cells. Then 2.5 × 10⁴ CFSE-labeled responder T cells were added to each well together with the preactivated PKH-labeled cells. FACS-sorted CD4⁺CD25⁻ regulatory T cells were used as positive control.

**Statistical analysis**

Data were analyzed for statistical significance using ANOVA followed by Dunnett’s multiple comparisons test. Data are expressed as the mean ± SEM. A value of p < 0.05 was taken as the level of significance.

**Results**

**RSV infects monocyte-derived DC**

To study the effect of RSV on immature monocyte-derived DC, we first performed dose finding experiments to establish the moi that resulted in the highest possible infection rate. Infection efficiency was followed for several days by measuring intracellular and extracellular expression of RSV proteins using a fusion protein-specific Ab (Fig. 1) and a polyclonal anti-RSV goat serum (not shown). As a control (moi 0), DC were cultured with the combination of cytokines IL-1β and TNF-α. The percentage of RSV-infected DC was quantified by flow cytometry. As illustrated in Fig. 1A, DC can be infected with RSV. Forty eight hours after infection, >25% of the DC expressed RSV-F on the surface when a moi of 5 was used.

Confocal microscopy of RSV-infected DC that were costained with HLA-DR-specific mAb showed that the level of infection in individual DC varied widely and part of the cell population does not show infection. Viral proteins are located intracellularly and on the cell surface in heavily infected cells and mainly on the cell surface in less infected cells (Fig. 1B). We further confirmed findings earlier reported in the literature (15) that RSV replicates in iDC because low PFUs of RSV can be detected in the 48 h supernatants of DC that were pulsed with virus and then thoroughly washed (data not shown).

**RSV infection induces DC maturation**

We next evaluated the effect of RSV infection on the maturation status of the DC. Fig. 2A shows the phenotype and homogeneity of iDC at day 6 of culture, before they were exposed to different maturation stimuli. On day 8, the maturation by the different stimuli was evaluated. RSV infection significantly enhanced the expression of CD83, CD86, and HLA-DR (Fig. 2B), comparable to the levels expressed on DC that were exposed to MF (IL-1β and TNF-α) and influenza virus. Maturation induction by poly(I:C), a synthetic mimic for double-stranded viral RNA, was somewhat more efficient than RSV infection. Despite the fact that only a certain proportion of DC were infected, the DC population uniformly matured. UV-inactivated RSV-DC did not induce up-regulation of the surface markers mentioned, which indicates that virus replication is necessary (data not shown). The expression on RSV-DC of other surface markers (CD14, CD40, CD54, and CD80) was similar to the expression on the other groups of mature DC (data not shown).

**Naive T cell activation by RSV-DC is inefficient**

To determine the functional characteristics of RSV-infected mDC at day 8, cocultures were performed with naive CD45RO⁺RA⁺CD4 T cells in the presence of SEB or anti-CD3 as an antigenic stimulus.
Proliferation of the T cells was measured at different time points by \([^{3}H]\)thymidine incorporation (Fig. 3A) and by dilution of a CFSE label incorporated in T cells (Fig. 3B). Fig. 3C shows the results of two experiments in which the total number of live and dead T cells in the cultures was counted, and the absolute number of cells per division cycle was calculated (see Material and Methods). These data showed that RSV-DC are able to induce proliferation; however, lower numbers of T cells are found in higher cell divisions compared with mDC obtained with other maturation stimuli. In addition, when unsorted CD4 T cells were used, proliferation induced by RSV-DC was also suppressed (data not shown). Extra addition of IL-2 to these cultures did not enhance the proliferative response (Fig. 3B).

Cytokine production of T cells activated by RSV-DC is impaired

Pathogens can modulate the Th cell polarizing capacity of mature DC (4). Therefore, the cytokine production by T cells stimulated by RSV-DC was investigated. For this purpose RSV-infected DC were cocultured with naive CD4 T cells and SEB was added as antigenic stimulus. After 12 days, the production of IFN-γ and IL-4, respectively, representatives of Th1- and Th2-type cytokines, was measured intracellularly in the T cells after a short restimulation with PMA/ionomycin (Fig. 4A). The results were compared with T cells that had been cultured with several other groups of mDC: MF-DC, poly(I:C)-DC and Flu-DC. Compared with DC matured by MF only, there was a shift toward a Th1 profile in the T cell cultures that had been stimulated with poly(I:C)-DC and Flu-DC. In contrast, RSV-DC did not prime for an enhanced Th1 or Th2 polarization. With RSV-DC, the number of T cells that produced IFN-γ is similar to T cells stimulated with MF-DC but lower compared with Flu-DC or poly(I:C)-DC. The poor cytokine production of T cells activated by RSV-DC was also observed when the T cells that had been cultured with RSV-DC were restimulated with anti-CD3/CD28, and cytokine production was measured in 24 h supernatants. IFN-γ production was lower than in the other groups, and interestingly, the whole panel of Th2 cytokines measured was also suppressed (Fig. 4B).

Aptosis is not responsible for poor APC function of RSV-DC

Because there was more cell death observed in DC cultures when matured with RSV, we investigated whether DC cell death played a role in the poor activation of naive T cells. Therefore, RSV-DC were sorted in three groups: 1) live cells that don’t express the RSV-F protein on the cell surface; 2) live cells with an intermediate expression of the RSV-F protein; and 3) live cells that express high levels of the RSV-F protein (Fig. 5A). By selecting for the expression of the F protein, the role of F could also be investigated. This was done because a possible inhibitory role of the F protein on T cell activation has been described in the literature (16). T cell cocultures with these three groups of sorted DC, unsorted RSV-infected DC and control MF-DC were performed.
These experiments showed that it was unlikely that apoptotic cells in the cultures were the only reason for suppressed T cell activation. Moreover, high cell surface expression of the F protein was not required for the suppressive effect of RSV-DC (Fig. 5B).

**A suppressive soluble factor(s) is produced by RSV-DC**

The poor Ag presentation function of RSV-infected DC could not be ascribed to the expression of costimulatory/adhesion molecules CD86, CD80, CD83, CD40, CD54, or MHC class II because these molecules were expressed on RSV-DC to similar levels as induced after poly(I:C) or Flu-DC maturation (Fig. 2 and data not shown). Because apoptosis of DC and the F protein expression were not responsible for the impaired T cell activation, we next tested whether a soluble factor produced by RSV-DC could be involved in diminished T cell activation. Therefore, the ultracentrifuged supernatants of day 8 RSV-DC and MF-DC were exchanged in cocultures of DC and T cells (Fig. 6A). We observed that supernatant of RSV-DC added to cocultures of MF-DC + naive T cells inhibited T cell activation. Vice versa in four of five experiments MF-DC supernatant reversed the inhibition of T cell proliferation by RSV-DC. We performed CD40 ligation experiments to test the ability of the different groups of DC to produce cytokines. The results were highly donor dependent (data not shown). However, we found that RSV-DC produced IL-10 and IL-12 to a similar extent as other groups of DC. In direct ELISAs for TGF-β, this cytokine was not detectable in supernatants after CD40 ligation of RSV-DC (data not shown). To definitely exclude a possible role for the two inhibitory cytokines IL-10 and TGF-β in RSV-DC/T cell cocultures, we performed Ab-blocking experiments. However, using these antibodies, the impaired T cell activation could not be reversed (Fig. 6A). Because the supernatant of RSV-DC was ultracentrifuged, a possible role for the direct effect of live virus was excluded. This was also confirmed in experiments in which we added live virus to the coculture of MF-DC with T cells, which did not result in less proliferation (data not shown).

An inhibitory role for IFN-α on T cell proliferation was described by Preston et al. (17) in PBMC cultures stimulated with PHA in the presence of RSV. We excluded an inhibitory role for type I IFNs in our experiments because IFN-α was not measurable in RSV-DC supernatants by ELISA (data not shown). Furthermore, by using a blocking Ab to the IFN-α receptor chain 2 no increase in T cell proliferation was observed in RSV-DC/T cell cocultures. These results are in accordance with observations of Spann et al. (18) and Schlender et al. (19) that non-structural proteins NS1 and NS2 of RSV strain A2 strongly suppress type I IFN production by infected cells.

Another possible inhibitory factor might be IL-1ra, reported by Salkind et al. (20) to be involved in the inhibition of human lymphocyte proliferative responses in the presence of RSV. A role for IL-1ra would explain our observation that the supernatant of MF-DC counteracted the inhibitory effect of RSV-DC on T cell proliferation, because MF-DC were matured in the presence of the cytokines TNF-α and IL-1β. To test whether IL-1ra could indeed explain the poor T cell activation by RSV-DC we added rIL-1β to the cocultures of RSV-DC and naive T cells. A slight increase in T cell proliferation was observed in the cultures in which T cells were stimulated with iDC and poly(I:C) DC. However, Fig. 6C
shows that T cell proliferation in the cultures with RSV-DC was not increased.

In summary, these experiments suggested that an inhibitory soluble factor is produced by RSV-DC. A soluble component produced by MF-DC could counteract this inhibitory effect. We excluded some likely candidates, but the inhibitory factor in RSV-DC supernatants remains unidentified.

**No role for regulatory T cells (Tregs)**

It has been shown that some pathogen-derived factors are able to induce a suppressive type of DC that can activate naïve T cells to become regulatory cells. T cells stimulated by RSV-DC showed poor proliferation, a characteristic of Tregs. To investigate whether RSV-DC might induce Tregs, we tested the potential suppressive effect of the T cells primed by RSV-DC on the proliferation of peripheral blood-derived CD4 T cells (responder T cells) using a cell cycle tracking dye assay. In this assay, the two groups of cells can be analyzed separately: RSV-DC primed T cells were labeled with PKH26, a red cell cycle tracking dye, and cocultured for 5 days with responder T cells labeled with CFSE, a green cell cycle tracking dye (Fig. 7A). Suppression is evident when responder T cells proliferate slower than cultures to which no Tregs were added. As shown in Fig. 7B, the presence of T cells primed by RSV-DC did not inhibit the responder T cell proliferation, whereas sorted CD4⁻CD25⁺ Tregs did inhibit the proliferation of the responder T cells. These results suggested that regulatory T cells did not play a role in the decreased proliferation of naïve T cells primed by RSV-DC.

**Discussion**

DC of myeloid origin are present directly underneath the mucosal epithelium lining the airways and are a likely primary set of APC exposed to respiratory viruses (21, 22). Therefore, we set out to study the consequences of the interaction of RSV with in vitro cultured monocyte-derived DC. The aim of this study was to evaluate how the innate effects of the virus on this APC population might influence the efficacy of the initiation of a primary T cell response. We have previously found that during severe primary RSV infections in infants, T cell responses in the lung are low (unpublished manuscript). Furthermore, it is a well known fact that immune memory against RSV is incomplete in healthy adult individuals (7). Several reports exist showing that RSV has suppressive effects on T cell proliferation in vitro. In vitro studies with human PBMC RSV infection showed lower polyclonal T cell proliferation and cytokine production induced by lectins (16, 17, 20, 23). Moreover, Chang and Braciale have reported that the presence of infectious RSV in the mouse lung may result in functional impairment of T cell effector function and inefficient establishment of an effector memory T cell population (24). Nevertheless, T cell responses appear to be crucial to clear RSV from the infected lung, because patients with immune deficiencies in the T cell arm of the adaptive immune response are unable to clear the virus effectively (25).

Many viruses have developed ways to evade innate and/or adaptive immune responses. These include a wide variety of mechanisms such as interference with the immune-suppressive effects of IFNs, interference with Ag processing and presentation, suppression of the maturation of DC, interference with DC migration, or escape of immune surveillance by Abs and T cells caused by high mutation rate in viral proteins (26). In our study, we have found that RSV infection of monocyte-derived DC leads to impaired T cell activation. Both T cell proliferation as well as the functional capacity to produce cytokines were affected. We have found that in vitro cultured monocyte-derived immature DC could be infected with RSV, viral protein synthesis occurred and even some infectious virus was produced. RSV infection lead to the up-regulation of costimulatory molecules CD80 and CD86 and also the expression of CD40, CD54, and CD83 on RSV-infected DC was comparable to DC matured with several other stimuli that were effective as APC. Moreover, HLA class II was also expressed to similar levels on all groups of mature DC. Thus the impaired ability of RSV-DC to elicit a functional T cell response is not caused by an interference with DC generation or maturation as described for Vaccinia virus, human CMV, Lassa virus, and HSV (27–30), or Ag presentation function like HSV, EBV, and human CMV (31–33).

During the last decade it has become clear that DC might play a role in dictating the type of response they induce in the T cells they interact with. The maturation status of DC imprinted by the innate signals provided by pathogens may determine whether T cells are tolerized or activated, and whether T cells are polarized to become Th1-type, Th2-type, or regulatory Tr1- or Th3-type cells (34). Both the levels of expression of costimulatory/inhibitory molecules as well as cytokine produced by DC can contribute to the type of T cell response that is induced. It is still an ongoing debate whether skewed Th cell responses are a possible contributing factor during severe primary RSV infections. Some studies have suggested that Th2 responses and associated eosinophil recruitment to the lungs may be a critical component in severe lower respiratory tract infection caused by RSV (35). However, others have not found a Th2 skewing in T cell responses during severe primary RSV infections (36).

Using cord blood CD34⁺-derived DC, Bartz and colleagues (15) found that RSV infection of their DC population lowered the capacity of these cells to induce IFN-γ production in naïve T cells. They ascribed this effect of impaired APC function to induction of apoptosis by RSV and partial maturation of the DC, characterized by selective up-regulation of CD86 as maturation marker and low production of inflammatory cytokines. In contrast, our in vitro studies showed that CD14⁺ monocyte-derived DC can be fully matured by RSV and that expression of maturation markers is
indistinguishable from other DC matured by several other stimuli that induce maturation of effective APC. In addition, our experiments showed that indeed RSV induced a somewhat enhanced apoptosis in monocyte-derived DC when used at high moi. However, this did not appear to be the major reason why RSV infection induced impaired T cell activation. Differences with our results might be related to differences in the DC populations used, CD34+ cell-derived neonatal DC or CD14+ monocyte-derived adult DC. Bartz and colleagues have not looked at other cytokines produced by T cells upon stimulation by RSV-DC; thus, it is unclear whether in their experiments T cells were also generally affected in their potential to produce cytokines. Because we found that RSV-DC inhibited the production of type 2 and type 1 cytokines by T cells it appears unlikely that myeloid DC would play a role in Th2 cell skewing of RSV-specific T cell responses.

Recently it has been elegantly shown by Schlender et al. (16) that RSV-F expression on the surface of infected cells may have inhibitory effects on T cell proliferation. By transfecting human RSV-F and chimeric bovine/human RSV-F molecules into Vero cells, they showed that there was a species-specific effect of suppression on T cell proliferation by RSV-F. We have been able to reproduce these experiments using RSV-infected Vero cells added as third party cells to polyclonal stimulated PBL. Indeed when RSV-F is expressed at high levels on the third party cells, T cell proliferation was completely suppressed. However, it appeared that surface expression of F protein was not the major mechanism by which RSV infection of DC caused impaired T cell activation. DC derived from RSV-infected cultures that did not express the F protein on the cell surface were equally suppressive as the DC derived from the same cultures that did have a high surface expression of RSV-F (Fig. 5). Moreover, virus-free supernatant from RSV-DC suppressed T cell activation by MF-DC showing that unlike Vero-F cells, RSV-DC suppressed T cell activation without the requirement for cell contact.

It has been reported by Sporri et al. (37) that DC exposed to pathogens components produce soluble factors that can trigger the up-regulation of costimulatory molecules on bystander DC. These bystander DC are able to induce T cell proliferation but direct pathogen contact (Toll receptor triggering) is required to induce T cell effector function. In our experiments with RSV-infected DC cultures, we also found that DC populations uniformly matured whereas only a subpopulation of the DC was infected. However, unlike the experiments performed by Sporri et al. using CpG and LPS as pathogenic signals, we found that in RSV-infected DC cultures the inefficient T cell activation was present in both the directly infected and the uninfected DC (Fig. 5). Moreover, the effect was observed at the level of T cell proliferation as well as cytokine production.

Because we found that the suppressive effect of RSV was present in the supernatant of infected DC, we excluded a direct role by which RSV infection of DC caused impaired T cell activation. MF-DC did not cause suppression of T cell activation. A dose 100-fold higher than the amount of virus produced by RSV-infected DC did not inhibit T cell proliferation. A possible role for the well known suppressive cytokines IL-10 and TGF-β was ruled out because: 1) blocking Abs against these cytokines could not reverse the suppressive effect mediated by RSV-DC supernatants; 2) TGF-β production could not be detected in RSV-DC supernatants by ELISA; and 3) IL-10 production by RSV-DC was similar to levels of IL-10 produced by influenza DC that were not suppressive. Moreover, no Treg function was induced by RSV-DC, which could have been a consequence of TGF-β and/or IL-10 production by DC (38, 39). A role for IFN-α or IL-1ra was excluded as well.

In conclusion, RSV has developed several ways to evade immune responses. Suppression of the induction of type I and type II IFN by the NS1 and NS2 proteins in epithelial cells and macrophages has been described earlier (18). The expression of the viral F protein may inhibit T cell proliferation by direct cell to cell contact. Although the context of F expression, i.e., simultaneous expression of costimulatory molecules and cytokines, may be important because on RSV-infected DC, F expression did not seem to add much to the suppressive effect of a soluble factor. In the present study, we now show that a third way of immune suppression by RSV may be the induction of an as yet unidentified soluble suppressive factor by myeloid DC, which causes a form of functional anergy in T cells that cannot be overcome by the addition of IL-2.

Disclosures
The authors have no financial conflict of interest.

References


