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Antiviral-Activated Dendritic Cells: A Paracrine-Induced Response State

Antonio V. Bordería,2* Boris M. Hartmann,2† Ana Fernandez-Sesma,* Thomas M. Moran,* and Stuart C. Sealfon3†‡

Infection of immature dendritic cells (DCs) by virus stimulates their maturation into APC. Infected DCs can also expose uninfected DCs to a panoply of cytokines/chemokines via paracrine signaling. Mathematical modeling suggests that a high rate of paracrine signaling is likely to occur among DCs located in three-dimensional space. Relatively little is known about how secreted factors modify the early response to virus infection. We used a transwell experimental system that allows passage of secreted factors, but not direct contact, between virus-infected DCs and uninfected DCs to investigate paracrine signaling responses. Paracrine signaling from infected DCs induced an antiviral-primed DC state distinct from that of mature virus-infected DCs that we refer to as antiviral-activated DCs (AVDCs). AVDCs had increased surface MHC class II and CD86 levels, but in contrast to virus-infected DCs, their MHC class I levels were unchanged. Imaging flow cytometry showed that AVDCs had an increased rate of phagocytosis compared with naive DCs. Experiments with IFN-β cytokine indicated that it may be responsible for CD86, but not MHC class II regulation in AVDCs. Both IFN-inducible and IFN-independent genes are up-regulated in AVDCs. Notably, AVDCs are relatively resistant to virus infection in comparison to naive DCs and achieve accelerated and augmented levels of costimulatory molecule expression with virus infection. AVDCs show a distinct antiviral-primed state of DC maturation mediated by DC paracrine signaling. Although further in vivo study is needed, the characteristics of the AVDC suggest that it is well suited to play a role in the early innate-adaptive transition of the immune system. The Journal of Immunology, 2008, 181: 6872–6881.

Dendritic cells (DCs) are recognized as a key bridge between the innate and adaptive immune responses (1). A key event in the development of adaptive immunity upon exposure to infection is the maturation of DCs into APCs that instruct lymphocytes to generate responses to specific Ags. Activated DCs efficiently stimulate both innate immune cells, including NK cells (2) and NKT cells (3) as well as key components of adaptive immunity including naïve (4) and memory (5) B cells and T cells (1). Thus, DCs are important both for innate immunity as well as for various elements of adaptive immunity (6).

Our study focuses on DC activation by virus infection, using Newcastle disease virus (NDV), a RNA paramyxovirus that has been demonstrated to be a good model for immune activation (7). DC maturation is stimulated by detection of various pathogen-associated molecular patterns (1) that are characteristic of bacteria, fungi, protozoa, or viruses. DCs recognize virus infection either by TLRs or by IFN-inducible and IFN-independent genes are up-regulated in AVDCs. Notably, AVDCs are relatively resistant to virus infection in comparison to naive DCs and achieve accelerated and augmented levels of costimulatory molecule expression with virus infection. AVDCs show a distinct antiviral-primed state of DC maturation mediated by DC paracrine signaling. Although further in vivo study is needed, the characteristics of the AVDC suggest that it is well suited to play a role in the early innate-adaptive transition of the immune system.
is composed of two chambers separated by a membrane that allows soluble components such as cytokines and chemokines to diffuse between chambers, but prohibits direct contact between the cells placed in different chambers. DCs infected with NDV and naive noninfected DCs were placed in the upper and lower chamber, respectively. The culture was left for 18 h, allowing the infected DCs to initiate cytokine and chemokine secretion. We found that the naive DCs exposed to the specific cytokine/chemokine secretions released by infected DCs enter a partially activated state in which they are relatively resistant to virus infection and primed to generate a more rapid and enhanced response to virus infection.

Materials and Methods

**Differentiation of DCs**

All human research protocols for this work have been reviewed and approved by the Institutional Review Board of the Mount Sinai School of Medicine. Monocyte-derived DCs were obtained from healthy human blood donors following a standard protocol described elsewhere (7). All experiments were replicated using cells obtained from different donors. Briefly, human PBMC were isolated by buffy coats ficoll density gradient centrifugation (Histopaque; Sigma-Aldrich) at 1450 rpm and CD14+ monocytes were immunomagnetically purified by using a MACS CD14 isolation kit (Miltenyi Biotec). Monocytes were then differentiated into naive DCs by 5- to 6-day incubation at 37°C and 5% CO2 in DC growth medium, which contains RPMI 1640 medium (In-vitrogen/Life Technologies) supplemented with 10% FCS (HyClone), 2 mM l-glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin (In-vitrogen). Naive DCs were added in the upper compartment of the Transwell system with naive noninfected DCs in the lower compartment to mimic the condition during AVDC generation. We incubated cells for 18 h at 37°C. Naive DCs in the lower compartment were left at 37°C in the incubator. Fibroblasts were infected with NDV at a multiplicity of infection of 1 and left in the incubator for 40 min. Cells were then washed with PBS and fresh DC medium was added to the culture. Naive DCs were added in the upper compartment and left in the Transwell system for 18 h in the incubator.

**IFN-β treatment**

DCs were generated as described previously and IFN-β (PBL IFN Source) was added to the culture in a concentration of 2000 U/ml. The culture was left for 18 h in incubation as described before. After incubation, the cells were washed with PBS and harvested for flow cytometry analysis.

**Quantitative RT-PCR**

Viral and host RNA expression levels were quantified by real-time RT-PCR. RNA was isolated from cells using a Qiagen RNeasy kit according to the manufacturer’s protocol. cDNA was synthesized from total RNA with AffinityScript MultiTemp RT (Stratagene) with oligo(dT)1 as primer. For real-time PCR, PlatinumTaq DNA polymerase (Invitrogen) and a SYBR Green (Molecular Probes)-containing buffer were used. The real-time PCR was performed using a thermocycler (ABI7900HT; Applied Biosystems) as previously described (21). The RNA levels for the housekeeping genes ribosomal protein S11 (Rps11), tubulin (Tub), and β-actin (Actb) were also assayed in all samples to be used as an internal controls. mRNA measurements were normalized using a robust global normalization algorithm. All control crossing threshold (Ct) values were corrected by the median difference in all samples from Actb. All samples were then normalized by the difference from the median Ct of the three corrected control gene Ct levels in each sample, with the value converted to a nominal copy number per cell by assuming 2500 Actb mRNA molecules per cell and an amplification efficiency of 93% for all reactions. The primer sequences used for the assays were: NDV-NH sense, 5'-GACAATCGTGTGATTGCTGAAAC-3' and antisense, 5'-CAATGCTGAGACACTGCT-3'; NDV-IP-10 sense, 5'-GGGTTGA-3' and antisense, 5'-ACACCCTCACCTGTTTGGAG-3'; IFN-α1a sense, 5'-CTCAATGACCTGATGAGCCTGTCGTC-3' and antisense, 5'-ATTTTCCGCGCTGACACTC-3'; PKR sense, 5'-TTGTACGACAAGAGGAGGGT-3' and antisense, 5'-GTCGCTGTCCTTCAACCTGC-3'; OAS-1 sense, 5'-TTTTGATGCCTGATGAGCCTGTCGTC-3' and antisense, 5'-GTCGCTGTCCTGAGGAGAAGG-3'; RIG-I sense, 5'-AAAGCTCTGGATGATGAGG-3' and antisense, 5'-GGCTTGGGATGTCGTCGTC-3'; RANTES sense, 5'-AGAGCTCCTCTGAGGGTGTAG-3' and antisense, 5'-TGCCAGGAGGTGTTGACCA-3'; IL-6 sense, 5'-CTGGAGGCTCCCATCAGACATC-3' and antisense, 5'-AATGCGAGCTGGTGGTGAGGCA-3'; IP-10 sense, 5'-TCCCTATACCTCCTACATC-3' and antisense, 5'-TGAACAGGGCTACCTGAACAC-3'; IFN-β sense, 5'-CGTGGTGTATTTGGAAGAAG-3' and antisense, 5'-TGCAAGTGGAGGCTGATTC-3'; Rps11 sense, 5'-CCAGGCGACCTATAGACAGA-3' and antisense, 5'-GAGATGCTCCCCGGGAGA-3';
**FIGURE 2.** Expression of viral GFP protein and maturation markers in infected DCs, AVDCs, and noninfected DCs. Histograms show fluorescence intensity of cells for the viral GFP-tagged protein NDV-HN and the fluorochrome-conjugated Abs for CD86, HLA-ABC (MHC-I), and HLA-DR (MHC-II). Blue histograms indicate noninfected DC controls. Red histograms indicate infected DCs in the Transwell system (A), AVDCs (B), and infected DCs as positive control (C). The data shown are representative of three different experiments using three different donors that showed similar results.

**Actb** sense, 5'-GCCTCAACACCTCAAACCAC-3' and antisense, 5'-CCA CAGCTGAGAGGGAAATC-3'; and **Tuba** sense, 5'-AGCGCCCA ACCTACACTAAC-3' and antisense, 5'-GGGAAGTGGATGCGAGG GTA-3'.

**Flow cytometry analysis**

Cells were fixed with 1.5% paraformaldehyde (Sigma-Aldrich), washed with FACS staining buffer (Beckman Coulter), and stained with mAbs for

**FIGURE 3.** Quantitative RT-PCR of RNA expression for virus and type I IFN genes in infected control DCs (Inf-DCs), infected DCs from the Transwell cultures, AVDCs, and noninfected naive DCs (N-DCs) genes to viral infection. The results shown are averages of three independent experiments performed using different donors.
MHC-I, MHC-II, and CD86. NDV-GFP- or NDV-RFP-infected cells were stained with Abs for MHC-I, MHC-II, and CD86 (Beckman Coulter). Cells were assayed on an LSRII flow cytometer (Beckman Coulter) and data were analyzed with the FlowJo software (Tree Star). To provide higher throughput and reduce cell requirements per assay, we used a modified bar-coding method previously described by Nolan et al. (22). Briefly cells were analyzed with the FlowJo software (Tree Star). To provide higher throughput and reduce cell requirements per assay, we used a modified bar-coding method previously described by Nolan et al. (22). Briefly 

FIGURE 4. Multiplex ELISA for IL-6, IP-10, TNF-α, and IFNα levels in supernatants from the chambers containing infected DCs, AVDCs, and noninfected naive DCs (N-DCs) genes to viral infection, all in the Transwell system. The results shown are averages of three independent experiments performed using different donors. Inf-DCs, Infected DCs.

FIGURE 5. Quantitative RT-PCR of antiviral and inflammatory gene expression in infected control DCs (Inf-DCs), infected DCs from the Transwell cultures, AVDCs, and noninfected naive DCs (N-DCs) genes to viral infection. The results shown are averages of three independent experiments performed using different donors.
were fixed, then stained in DMSO containing different combinations of 0, 0.3, 1, 4, or 15 μg/ml Pacific Blue-NHS, 0, 1, 2.5, 5, or 20 μg/ml Alexa Fluor 350-NHS, and 0, 4, or 20 μg/ml Alexa Fluor 750-NHS for 15 min at 20–25°C (see Fig. 9A). Fluorescence minus 1 controls were obtained by staining naive DCs with all fluorochromes studied excluding the fluorochrome of interest, conjugated Ab against CD45 (23).

**Imaging flow cytometry analysis of phagocytosis and morphology**

Imaging flow cytometry was used to compare the morphology and phagocytosis levels of AVDCs and naive DCs. To detect phagocytosis, 1-μm 488-nm fluorescence-labeled latex microspheres (Polysciences) at a concentration of 50 beads/cell were cocultured for 2 h at 37°C with each cell type. Single-cell images were acquired using extended depth field imaging distortion to identify beads in different focal planes within a cell. The numbers of beads incorporated by cells were quantified in the images captured using image analysis software (IDEAS Software; Amnis). The distributions were compared using the Kolmogorov-Smirnov technique.

To measure the cellular morphology, AVDCs, naive DCs, and infected DCs were labeled with a live membrane dye, benzoxazolium, tributylphosphine oxide (DiO), following the manufacturer’s protocol (Invitrogen). Samples were then individually acquired by imaging flow cytometry. To analyze the morphology, an algorithm described by Haralick et al. (24), which describes the homogeneity of an given image, was used (IDEAS Software; Amnis).

**Multiplex ELISA**

Four different cytokines/chemokines (IL-6, IFN-γ-inducible protein 10 (IP-10), TNF-α, and IFNα) concentrations were assayed in the culture medium. To minimize the supernatant volume to assay, a Beadlyte Human Multiplex ELISA analysis (Millipore) was used per the manufacturer’s instructions. Briefly, 100 μl from each compartment per well was incubated in a 96-well filter polyvinylidene difluoride 1.2-μm plate specially designed to retain cytokines/chemokines, with a mixture of anti-cytokine IgG-conjugated beads for the different cytokines/chemokines assayed. After 2 h of incubation, the plate was filtered and washed three times with assay solution (PBS (pH 7.4) containing 1% BSA, 0.05% Tween 20, and 0.05% sodium azide). The washes were followed by a 1.5-h incubation with biotin-conjugated anti-cytokine IgG. After assay solution washing, streptavidin-PE was added followed by addition after 30 min of stop solution (0.2% (v/v) formaldehyde in PBS (pH 7.4)). The plate was then filtered and each well was resuspended in 125 μl of assay buffer and read in a Luminex 100 machine.

**Results**

**Effectiveness of Transwell chambers to study paracrine signaling**

To isolate the effects of direct infection by virus from that of paracrine signaling on naive DCs, we used a Transwell culture system. Naive, immature DCs were infected with NDV and immediately placed in the upper chamber. Noninfected naive DCs were placed in the lower chamber. The chambers were partitioned by a 0.4-μm pore membrane that allowed the diffusion of cytokines and chemokines (Fig. 1). After incubation, cells in each chamber were recovered and analyzed for their surface marker and gene expression patterns.

DCs do not support the productive infection of NDV (25). To ascertain whether viral particles could infect cells in the lower chamber during these experiments, we performed control experiments with a recombinant NDV containing a GFP-tagged HN protein (17). Infection of cells was assayed by flow cytometry. When NDV-GFP-infected cells were incubated in the upper chamber for 18 h, GFP fluorescence was detected only in cells from the upper, but not the lower chamber, indicating that the experimental system effectively separated infected and uninfected DCs (Fig. 2).

As a further test of the isolation of virus infection, the induction of the NDV-HN and NDV-NP viral genes and virus-induced type I IFN genes were compared in membrane-separated cocultured cells using real-time PCR assays. Both viral genes were present in virus-infected DCs from the upper chamber and were undetectable in noninfected control DCs and in DCs cocultivated in the lower chamber (Fig. 3). Viral components lead to activation of type I IFN via TLR activation. If uninfected cells were exposed to viral components that passed through the membrane, they would be expected to show induction of type I IFNs. Therefore, we also assayed the expression of IFN-β and IFN-α as a sensitive assay of exposure to very small amounts of virions, viral RNA, debris, or other small particles that might cross the membrane (26). Using a sensitive real-time PCR assay, we found that both IFNs were up-regulated in virus-infected DCs from the upper chamber, but were not induced in naive cells incubated in the lower chamber (Fig. 3). The results of flow cytometry and real-time PCR indicate that the culture system completely eliminates direct virus infection of uninfected, membrane-isolated cocultured cells. To test further the possibility that viral components such as RNA caused the surface marker up-regulation pattern observed (see below), we tested the effect of proteinase K digestion of supernatants from infected DCs on its capacity to affect naive DCs. Proteinase K-treated supernatant did not up-regulate the surface markers of DCs in the Transwell chamber. Supernatants from infected DCs that were not digested with proteinase K induced a similar surface marker pattern to that seen in AVDCs in the Transwell system (supplementary Fig. S1). Thus, any responses of the uninfected DCs in the lower chamber were the results solely of paracrine signaling initiated by infected DCs in the upper chamber.

**Effects of DC paracrine signaling on maturation markers**

Several cellular surface proteins have been described as maturation markers of DCs, including CD86, MHC-I, and MHC-II (6). There is an increase in the density of these markers in the cellular membrane of the DCs upon activation and maturation. In the coculture Transwell system, upper chamber-infected DCs and the positive control DCs showed a comparable up-regulation of MHC-I, MHC-II, and CD86 molecules (Fig. 2, A and C), indicating the maturation of those cells. Interestingly, lower chamber DCs in the coculture Transwell system also showed an up-regulation of MHC-II and CD86 molecules, while MHC-I remained at levels equivalent to the noninfected control DC levels (Fig. 2B).
To determine whether the changes in naive DCs induced by infected DCs were unique to infected DCs, we studied the effects of secreted factors from NDV-infected primary lung fibroblasts on naive DCs using the Transwell system. Our results showed that the presence of infected primary lung fibroblasts instead of infected DCs in the same Transwell system did not induce a comparable surface marker expression pattern in the naive DCs (supplementary Fig. S2). Furthermore, noninfected control DCs did not up-regulate any of the surface markers, even if cells were maintained in the Transwell system for periods up to 48 h (supplementary Fig. S2). These results indicate that the experimental manipulations alone were not affecting maturation marker up-regulation. Our results suggest that paracrine signaling alone between infected and uninfected DCs produces an increase of some DC maturation markers, leading to a unique DC state that differs both from naive DCs and from fully matured virus-infected DCs.

Levels of paracrine cytokines

We assayed cytokines that could contribute to paracrine activation of DCs. Factors secreted by DCs for which DCs expressed receptors include IL-6, TNF-α, IP-10, and type I IFN. Measurement of the levels of these cytokines in upper and lower chambers by ELISA during a coculture experiment indicated that high levels of all four cytokines were achieved in the lower chamber (Fig. 4).

Gene program response in AVDCs

To further understand the characteristics of the paracrine-generated AVDC state, the regulation of a variety of genes was assayed by real-time PCR in AVDCs, NDV-infected DCs, and naive DCs. Paracrine and autocrine IFN acting at the type I IFN receptor activates a number of genes through JAK-STAT signaling, including protein kinase R (PKR), 2’-5’-oligoadenylate synthetase (OAS),...
myxovirus resistance A (MxA), and IP-10. Consonant with the detection of high levels of IFN-γ in the medium by cytokine assays (Fig. 4), AVDCs showed robust induction of these IFN-activated genes (Fig. 5). OAS and MxA showed the same levels in the lower chamber as in infected DCs. Although PKR and IP-10 were lower than in infected DCs, they were still significantly induced in comparison with noninfected DCs. IFN is a key paracrine signaling factor during virus infection and has long been recognized to generate complex transcriptional responses (7, 26). However, the state of AVDCs appears to result from additional factors besides IFN, because genes such as TNF-α, which are not inducible by IFN are up-regulated in AVDCs (Fig. 5). The hypothesis that additional secreted factors contribute to the AVDC state was further supported by comparing the pattern of maturation markers in AVDCs and in IFN-β-treated naive DCs. The induction of the costimulatory molecule CD86 was similar in AVDCs and IFN-exposed DCs, suggesting that this regulatory event may result from IFN signaling. However, the increased expression of MHC-II was seen only in AVDCs (Fig. 6). Overall, the gene response patterns and maturation marker analysis indicate that type I IFN signaling represents only part of the paracrine environment necessary for the generation of AVDCs.

Quantification of AVDC morphology and rate of phagocytosis

The morphology of AVDCs and naive DCs was compared using imaging flow cytometry. Cells were stained with a live-cell membrane-localized dye, DiO, and morphology was measured using the texture analysis algorithm developed by Haralick et al. (24) as implemented in IDEAS software (see Materials and Methods). By this method, the spatial relationships between the texture features and the pixel values in an image were measured, and an H homogeneity mean and a SD value were obtained for each set of cells. The H homogeneity is a measure of the average shape of a cell. Representative raw images are shown in Fig. 7A. These analyses reveal that the average morphology of AVDCs is different from that of naive DCs. AVDCs showed an increase in textural homogeneity compared with naive cells that was comparable to the level seen in infected DCs (Fig. 7B).

The rates of phagocytosis of naive DCs and AVDCs were also compared using high-resolution imaging flow cytometry. Both

FIGURE 9. AVDCs resist viral infection. A, Bar coding for flow cytometry analysis. Cells were stained with different combinations of 0, 0.3, 1, 4, or 15 μg/ml Pacific Blue-NHS, 0, 1.25, 5 or 20 μg/ml Alexa Fluor 350-NHS, and 0, 4, or 20 μg/ml Alexa Fluor 750-NHS to enable 60 different conditions in one FACS run. B, Time course results for NDV-RFP expression following infection in naïve DCs and AVDCs. C, Time course for expression of MHC-I, MHC-II, and CD86 surface markers in the same cells studied in A and B. The results shown are representative of two independent experiments using cells from different donors.
cells types were incubated for 2 h with 1-μm 488-nm fluorescence-labeled latex microspheres. The number of particles taken up by each cell was quantified in approximately several thousand cells in each group using automated image analysis (Fig. 8A). In three independent experiments using cells from different donors, AVDCs showed significantly higher rates of phagocytosis.

**AVDCs resist viral infection and showed enhanced generation of maturation markers by infection**

We next investigated whether AVDCs differ from naive DCs in their response to direct virus infection by NDV. For this purpose, AVDCs generated by coculturing with NDV-GFP-infected DCs for 18 h using the Transwell system and naive DCs were exposed to a recombinant NDV virus expressing RFP (NDV-RFP) (19). The level of RFP signal generated over 12 h was assayed by flow cytometry, as a reflection of the generation of intracellular viral protein within the DCs following direct infection. To allow accurate comparison of the level of signal in different samples, a bar-coding flow cytometry approach was used (22). In this approach, each sample was first labeled with a characteristic pattern of three deconvolution dyes (Fig. 9A) and then the 60 mixed samples were labeled with fluorescently labeled surface marker Abs. Detection of virus protein expression and surface marker expression in each sample was then assayed in a single multispectral flow cytometry run. This analysis showed that AVDCs generated much lower levels of viral protein with infection than naive DCs (Fig. 9B). These results indicate that, in comparison to naive DCs, the AVDCs showed a relatively virus-resistant state.

Interestingly, AVDCs also expressed much higher levels of maturation markers than naive DCs following virus infection. Time course analysis by flow cytometry showed that the levels of CD86, MHC-I, and MHC-II were dramatically higher in infected AVDCs at all time points than in infected naive DCs (Fig. 9C). Notably, this enhanced activation state included both the CD86 and MHC-II markers that were increased in AVDCs without infection as well as the MHC-I signal that was unchanged in AVDCs before virus infection. These results indicate that the paracrine signaling environment cause the DCs to enter a state in which they are relatively resistant to infection by virus and their maturation response to infection is greatly enhanced.

**Discussion**

These experiments reveal that paracrine signals from virus-infected cultured human DCs act on naive uninfected DCs to induce an activated antiviral state. This AVDC state is characterized by basal up-regulation of CD86 and MHC-II cell surface markers, induction of a variety of antiviral response genes, altered morphology, and increased basal phagocytosis. The antiviral state of these AVDCs is evident from their relative resistance to virus infection and their enhanced rate and level of maturation marker expression following infection. These features of the AVDCs are well suited to facilitate the development of adaptive immunity to virus infection.

The Transwell experimental system with which we characterized the AVDC, while used in various studies of immune cell differentiation (27), has not previously been used to study paracrine signaling among immune cells. The pore size of the polyethylene terephalate membranes present in our experiments would not be expected to restrict virus particles or components. NDV does not cause productive viral infection in DCs (24). Nevertheless, we tested whether the Transwell culture system might effectively separate the paracrine-stimulated naive DCs from the effects occurring with direct encounter with virus. Using sensitive assays for expression of fluorescent viral protein and stimulation of virus infection-dependent gene expression (viral proteins and type I IFN induction), we demonstrated that the change in state seen in the AVDC results solely from the transmission of secreted factors and that AVDCs do not show any signs of infection by the virus or exposure to viral components.

Since the discovery of the effects of type I IFN more than 50 years ago, these cytokines have been well studied as inducers of a variety of antiviral responses that promote the development of DC maturation (28). We have shown previously that NDV infection of human DCs results in a strong activation pattern, including production of type I IFN, TNF-α, and other proinflammatory cytokines as well as IFN-inducible genes (7). We find that NDV-infected DCs, located in the upper chamber of the Transwell system, also secrete TNF-α, IL-6, and IP-10, which have receptors present on DCs and are therefore also potential paracrine factors (see Fig. 4). Several previous studies have considered the role of uninfected or “bystander” DCs in the response to virus infection (28–30). However, the effects of paracrine signaling on these bystander cells has either not been evaluated or attributed entirely to type I IFN. Therefore, we were interested in determining the role of type I IFN in generating the AVDC state that we have identified. We found that only some of the characteristics of AVDCs can be attributable to type I IFN signaling, indicating that the state of these cells reflects more complex, multifactor paracrine signals. The identification of which combinations and concentrations of factors are responsible for generating the AVDC state is an interesting subject for further investigation.

Pretreatment of immature DCs and/or DC precursors with single cytokines that are secreted either by DCs or other immune cells has been reported to lead to distinct cellular phenotypes. The effects of type I IFN (31–33), thymic stromal lymphopoietin (34, 35), TNF-α (36), IL-10 (37, 38), IFN-γ (39), and IL-15 (6, 40–42) have all been reported. During infection of a host, the extracellular environment of the DCs in the infected tissue in vivo contains multiple cytokines and chemokines and the response state of the uninfected DCs is likely to result from more than a single factor. Understanding the mechanisms underlying the effects of the integration of multiple signals has been the object of several experimental and computational systems biology studies (43–45). Recently, we have begun to develop models to describe paracrine and autocrine signaling among DCs located in three-dimensional spaces (15). Understanding the mechanisms by which AVDCs are generated is facilitated by studies integrating mathematical simulation with cytokine interaction studies.

Paracrine and autocrine signaling are recognized to contribute to the maturation of DCs following TLR stimulation (42, 46–49). Sporri and Reis e Sousa (49) found that paracrine signaling cannot substitute for contact for TLR-mediated contact with pathogen components in generating fully activated DCs. To our knowledge, the investigation of the role of paracrine signaling in modulating the DC response to subsequent encounter with virus, a sequence likely to occur during actual infection, has not been previously investigated.

During the early stages of infection, only a few cells are infected. In the case of respiratory virus, inhalation of only a few droplets containing virus particles can be sufficient to induce a successful infection, and virus transmission usually involves few viral particles (50). Thus, early in the infection, only a few DCs are likely to come into contact with virus or with virus-infected tissue. Therefore, we hypothesize that paracrine signaling events capable of inducing the generation of AVDCs are likely to play an important role in the early response stages of the immune system to virus infection (Fig. 10). Infection of primary lung fibroblasts was not able to generate AVDCs (supplementary Fig. S2), indicating that the AVDC is not generated by exposure to secreted factors from
any cell type. Pathogenic viruses express immune antagonists that prevent the activation of innate immune responses in infected cells (51–53). AVDCs could be able to respond even in the face of a viral antagonist due to their heightened activated state. Results obtained in cultured cells can be extrapolated only cautiously to the intact organism. However, characterizing the state of these cells and the mechanisms underlying their induction is important to set the stage for investigating the presence and role of AVDCs using in vivo models.

In conclusion, the characteristics of the AVDCs suggest that they are in an advanced state of readiness, but also capable of resisting the negative effects of the virus infection. In the case of human host viruses, the formation of AVDCs may provide an important process to generate APCs capable of overcoming the effect of viral protein inhibitors on the maturation of DCs. AVDCs were relatively resistant to virus infection and showed induction of several genes, including RIG-I, PKR, and OAS, that promote virus resistance. More importantly for their proposed role in the transition from innate to adaptive immunity, they showed an accelerated rate of maturation when infected and an increased level of expression of maturation markers needed for productive interaction with T cells. The increased phagocytosis rate and increased morphological texture of AVDCs also make them suitable for heightened antiviral surveillance and response.

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Disclosures
The authors have no financial conflict of interest.

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