Dendritic Cell Responses to Early Murine Cytomegalovirus Infection: Subset Functional Specialization and Differential Regulation by Interferon α/β

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Abstract

Differentiation of dendritic cells (DCs) into particular subsets may act to shape innate and adaptive immune responses, but little is known about how this occurs during infections. Plasmacytoid dendritic cells (PDCs) are major producers of interferon (IFN)-α/β in response to many viruses. Here, the functions of these and other splenic DC subsets are further analyzed after in vivo infection with murine cytomegalovirus (MCMV). Viral challenge induced PDC maturation, their production of high levels of innate cytokines, and their ability to activate natural killer (NK) cells. The conditions also licensed PDCs to efficiently activate CD8 T cells in vitro. Non-plasmacytoid DCs induced T lymphocyte activation in vitro. As MCMV preferentially infected CD8α⁺ DCs, however, restricted access to antigens may limit plasmacytoid and CD11b⁺ DC contribution to CD8 T cell activation. IFN-α/β regulated multiple DC responses, limiting viral replication in all DC and IL-12 production especially in the CD11b⁺ subset but promoting PDC accumulation and CD8α⁺ DC maturation. Thus, during defense against a viral infection, PDCs appear specialized for initiation of innate, and as a result of their production of IFN-α/β, regulate other DCs for induction of adaptive immunity. Therefore, they may orchestrate the DC subsets to shape endogenous immune responses to viruses.

Key words: plasmacytoid dendritic cell • interferon α/β • murine cytomegalovirus • antigen presentation • CD8 T lymphocyte

Introduction

Dendritic cells (DCs),* initially identified by their ability to initiate adaptive immune responses through antigen-specific activation of naive T cells (1), are now known to be also involved in innate immune responses through the secretion of antimicrobial cytokines such as IFN-α/β (2) or activation of other cellular effectors such as NK cells (3).

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*Abbreviations used in this paper: DC, dendritic cell; GFP, green fluorescence protein; ie-1, immediate early gene-1; LCMV, lymphocytic choriomeningitis virus; MCMV, murine cytomegalovirus; MIP, macrophage inflammatory protein; PDC, plasmacytoid DC; PRR, pattern-recognition receptor.

They are heterogeneous populations. According to their frequency, DCs isolated from spleens of untreated, specific pathogen-free mice can be divided into three major subsets (4, 5): Ly6G/C^-CD8α^-CD11b^+ (later referred to as CD11b^+ DCs), Ly6G/C^-CD8α^-CD11b^- (CD8α^- DCs), and Ly6G/C^+CD8α^+/^-CD11b^- plasmacytoid DCs (PDCs). The subsets can all be derived from a common B220^+Ly6G/C^-CD8α^-CD11b^+ precursor (6, 7), depending on the cytokine environment (5, 8), but different patterns of cytokine production by DC subsets may occur in response to particular microbes (4, 9) and function for antigen presentation can be assigned to different DC subsets depending on conditions of challenge (10–13). For example, both CD8α^+ and CD11b^+ DCs can be potent activators of naive T lymphocytes, but CD8α^+ DCs have a unique ability to cross-present exogenous antigen in association with MHC class I molecules (11–13). By contrast, PDCs have been reported poor activators of CD4 T cells,
even after stimulation in vitro (14–16). Thus, a picture is emerging of specialization of DC subsets in recognition of, and responses to, specific molecules expressed during microbial infections. In turn, the activation of these subsets may lead to specific pathways for differential regulation of downstream immune responses. However, knowledge on the respective roles of DC subsets in the course of microbial infections in vivo is scarce (9, 17–19), and extensive simultaneous analyses of the different functions of the major DC subsets in a given system remain to be done.

We have reported that splenic DCs are maintained as plasmacytoid, CD8α+ and CD11b− subsets, and demonstrated the major contribution of PDCs to IFN-α/β and IL-12 production, during murine cytomegalovirus (MCMV) infection (9). However, the impact of in vivo viral infections on antigen presentation capability of PDCs and the relative consequences of particular DC functions on other DC subsets have not been evaluated previously. There is a high potential for PDCs to orchestrate both innate and acquired immune responses during certain viral infections, because the IFN-α/β cytokines produced by the subset can mediate multiple immunoregulatory functions (20). IFN-α/β regulate innate cytokine production during viral infections by promoting high levels of their own (9, 21) but limiting IL-12 production (9, 22). They also induce up-regulation of MHC class I molecules and regulate DC maturation in culture or in response to CFA or CpG in vivo (23–25).

The studies presented here were undertaken to examine the functional specialization of plasmacytoid, CD8α+ and CD11b− DC subsets, and their regulation by IFN-α/β functions, during MCMV infection in vivo. The following questions were addressed. (a) What is the relative contribution of DC subsets to production of a panel of innate cytokines involved in defense and to NK cell activation? (b) Can PDCs activate naïve T cells during viral infections? (c) Is MCMV replication within DCs part of the activation for the initiation of innate immune responses in vivo, and the relative consequences of particular DC functions and the relative consequences of particular DC functions on other DC subsets have not been evaluated previously.

**Materials and Methods**

**Mice, Virus, and Treatments.** IFN-α/β receptor-deficient (IFN-α/β/−/−) 129 mice were bred under pathogen-free conditions in the animal care facility at Brown University. Specific-pathogen-free wild-type 129 (129SvEvTacFBR) and C57BL/6 mice were purchased from Taconic Farms, as well as C57BL/6 RAG-2−/− mice transgenic for a TCR specific for the lymphocytic choriomeningitis virus (LCMV) epitope GP-33–41 (H2-Dk), strain 4113-TcR P14 (30). Mice were 5 to 12 wk of age. Handling of mice and experimental procedures were conducted in accordance with institutional guidelines for animal care and use. Infections were initiated on d0 by intraperitoneal injection of 10^6PFU of salivary gland–extracted MCMV Smith strain, WT, or clone RVG-102 (31) recombinant for the enhanced green fluorescence protein (GFP) under the promoter of the immediate early gene-1 (ie-1). In certain experiments, NK cells were depleted in vivo by intraperitoneal injection of anti-AGM1 antibody (Wako Pure Chemical Industries, Ltd.) 1 d before and 12 h after infection.

**Preparation of Cell Suspensions and Subset Enrichment.** Spleens were digested by collagenase and cell suspensions prepared as described (9). DCs and NK cells were enriched using anti-CD11c or anti-DX5 magnetic beads and positive selection columns MS+ according to manufacturer’s instructions (Miltenyi Biotec). Ly6G/C+ versus Ly6G/C−, CD11b+ versus CD11b−, or GFP+ versus GFP−, DC subsets were purified from enriched CD11c+ cells by fluorescence-activated cell sorting using a FACS Vantage™ with a 70-μm nozzle (9). Similarly NK cells were further purified as DX5+/CD3ε− cells. Purity and viability of sorted populations were analyzed immediately after sorting. CD8 T cells from P14 mice were prepared by negative selection using CD8 T cell enrichment kit, supplemented with CD11c-microbeads to ensure depletion of endogenous DC populations (Miltenyi Biotec).

**Quantification of Cytokines and Macrophage Inflammatory Protein 1α.** Supernatants from purified DC subsets or from cocultures of DCs with NK or T cells were harvested and tested in ELISA. Commercial kits were used for IL-12p40 and macrophage inflammatory protein (MIP)-1α (R&D Systems), as well as a custom anti-body pair for IL-12p40 as described previously (26). Limits of detection were 20 pg/ml for IL-12p40 and <1.5 pg/ml for MIP-1α. The IFN-α, IFN-γ, IL-18, and TNF-α ELISA have been described previously (9, 32, 33). Limits of detection were 1,500 pg/ml for IFN-α, 5 pg/ml for IFN-γ, 30 pg/ml for IL-18, and <5 pg/ml for TNF-α. Cytokine titers were expressed as pg/ml or ng/ml for 10^6 cells for analyses in overnight DC cultures and as pg/ml or ng/ml for analyses in DC/NK or T cells cocultures.

**Flow Cytometric and Immunofluorescence Analyses.** Cell surface stainings were performed as described (9), using the following antibodies: CD11c-FITC, CD11c-PE, CD8α-APC, CD11b-FITC, CD11b-APC, Ly6G/C-FITC, Ly6G/C-APC, Ly6G/C-PE/Cy5, I-A^d-PE, H-2K^d-PE, CD40-PE, CD80-PE, CD86-PE, CD3e-FITC, NK1.1.1-PE, and DX5-biotin with streptavidin-PE, purchased from BD Biosciences or eBioscience. Hyperimmune serum from MCMV-infected mice was used as a primary antibody and Cy5-conjugated affinity–purified F(ab’)2 fragment donkey anti–mouse IgG (Jackson ImmunoResearch Laboratories) as a secondary reagent for staining of viral membrane antigens. Freshly isolated cells were evaluated ex vivo for intracellular expression of IL-12 and IFN-γ as described (9), and of MCMV IE-1 protein using the Croma antibody (34) as a primary reagent and Cy5-conjugated donkey anti–mouse Ab as a secondary reagent. Samples were acquired using a FACS Calibur™ (Becton Dickinson), with the CELLQuest™ version 3.1 software package. Laser outputs were 15 mW at 488 and 635 nm.
wavelengths. For immunofluorescence studies of DC subset morphology and MHC class II expression, DCs were stained with CD11c-PE and Ly6G/C-FITC, purified by cell sorting in total CD11c+ cells or in Ly6G/C+ and Ly6G/C− subsets, retained in suspension with biotin-I-Ab followed by Texas Red-streptavidin and Ly6G/C-FITC. Specificity was assessed on total CD11c+ DCs using isotype control antibodies. Wright and Giemsa stainings were performed in parallel to ensure the lack of granulocyte contamination (unpublished data). Cytopsins were done at various cell inputs. Sorted GFP+ and GFP− DCs isolated from d1.5 RVG102-infected 129 mice were also examined by immunofluorescence for intracellular localization of GFP. Slides were dried, fixed with 1% paraformaldehyde, washed, and mounted with anti-fading medium Vectashield (Vector Laboratories). Microscopy was performed with an Axioskop microscope (Zeiss) equipped with a Spot, RT slider camera from Diagnostics, Inc. (MVI), and Vectashield anti-fading medium (Vector Laboratories). Images were collected digitally with a single filter cube with a Spot, RT slider camera from Diagnostics, Inc. (MVI), and processed for publication with Adobe Photoshop.

**RT-PCR for IL-15 mRNA.** Total RNA was extracted from ~5 × 10^6 cells and retro-transcribed as described previously (9). 5 μl cDNA was used as a template for PCR amplification using primers specific for IL-15 (35) synthesized by Operon. Amplifications were performed in a programmable thermal cycler (PTC-200; MJ Research) with 30 cycles and an annealing temperature of 54°C.

**Real-time RT-PCR for Quantification of MCMV mRNA.** Total RNA was isolated using RNeasy (Qiagen). To minimize contamination by viral genomic DNA, RNA was digested with DNase (RNase-Free DNase Set; Qiagen). Spin columns (Zymogen) were used to concentrate samples. 100 ng total RNA was used for reverse transcription of three RVG102 mRNAs - GFP, mouse interleukin-15 (IL-15), and a negative control. The sequences as well as cycling parameters used in the reactions are given in Table I.

### NK Cell Activation Assay.

**NK Cell Activation Assay.** DC subsets were sorted from uninfected or d1.5 MCMV-infected 129 mice. 5 × 10^4 DCs were plated in coculture with 5 × 10^4 NK cells purified from the spleen of uninfected 129 mice. After 24 h, 100 μl of supernatant was harvested from each well for IFN-γ titration and replaced by 5 × 10^4 YAC cells labeled with ^51Cr (PerkinElmer). In parallel, NK cells were sorted from uninfected or d1.5 MCMV-infected 129 mice, plated at 5 × 10^4/well, and used directly ex vivo for analysis of cytotoxic activity, or incubated for 24 h to measure spontaneous IFN-γ production. Wells with YAC cells alone were plated for evaluation of spontaneous and total releases. The chromium release assay was incubated for 6 h at 37°C, 5% CO2. Supernatant (50 μl) was transferred to a Lumaplate™, counted using the Topcount instrument (Packard Instrument Co./PerkinElmer), and the percentage specific release was calculated as described (37).

### IFN-γ and IFN-α Secretion Assays.

**CD8 T Cell Antigen-specific Activation Assays.** DC subsets were sorted from uninfected or d1.5 MCMV-infected 129 mice, pulsed for 3 h at 37°C, 5% CO2 in a solution of the LCMV peptide NP 396–404 (H-2Dd) or with the LCMV peptide GP33–41 (H-2Kd) as a negative control. Unless specified, peptides were used at 10 μM. DCs were washed three times and resuspended in fresh medium. 5 × 10^4 viable naive GP33–41-specific CD8 T cells purified from the spleens of P14 mice were plated in each well with serial dilutions of the various DC suspensions. After 2 d of culture, 100 μl of supernatant was harvested for IFN-γ titration, and cells were pulsed with 1 μCi of ^3H-thymidine (PerkinElmer) in 100 μl of fresh medium for 16 h. Supernatants were harvested and counted using a Topcount instrument (Packard Instrument Co./PerkinElmer) and the percentage specific release was calculated as described (37).

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### Table I. Oligonucleotides and Cycling Parameters Used for Real-time RT PCR on MCMV Genes

| Gene     | Primersa | Probes          | Cyclesb |
|----------|-----------|-----------------|---------|
| GFP      | 5’TGGCTCTGGATTTGTTG3’ | 5’LGRed640GCCGCAAGTCTCATATCATGGCGGCA3’ | 95°C 15 s |
|          | 5’AGGCAAGCTCTCTGAGTTG3’ | 5’GGGAGTCGAGCTCAACTACCAAGTTG3’ | 63°C 15 s |
|          | 5’CTTGATGCCGTTCTCTGG3’ | 5’TCCTAACGAGTTCGACAGTTG3’ | 72°C 15 s |
| ie-1     | 5’TATCATGAGGTTGCAATCT3’ | 5’LGRed640GCCGATCAAGTCTGCCAGGT3’ | 95°C 10 s |
|          | 5’ACACGTGGGAAATGATAACAGCGAACAT3’ | 5’GGGAGTCGAGCTCAACTACCAAGTTG3’ | 60°C 15 s |
|          | 5’CCCTGGACTCTGGAAAGAAA3’ | 5’GCGCCTGGGAGCTCAGCTGGCGT3’ | 72°C 10 s |
| gB       | 5’GTAGTTCGGATATATTGTAGG3’ | 5’LGRed640GGAACCTTTTGAGCGGAAACTGTGAC3’ | 95°C 10 s |
|          | 5’AGGCTGGGGAACAAACTTATGCTG3’ | 5’GCGCCTGGGAGCTCAGCTGGCGT3’ | 65°C 15 s |
|          | 5’AGGCAAGAGATCTCCTCTGG3’ | 5’GCGCCTGGGAGCTCAGCTGGCGT3’ | 72°C 10 s |

aThe first sequence corresponds to the primer used in the reverse transcription, the second sequence to the primers used in the real-time PCR.

bPolymerase was first activated by heating for 10 min at 95°C.

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the last 18 h. Cells were collected and counted using the Packard Filtermate Universal Harvester and Topcount instruments (Packard Instrument Co.).

**IFN-α ELISPOT Assays.** The IFN-α ELISPOT assays were adapted from IFN-γ ELISPOT described previously (38). In brief, Multiscreen-IP plates (Millipore) were coated overnight with 0.5 μg/well of the same primary antibody as used for the ELISA, washed, and blocked for 3 h with culture medium. Serial dilutions of enriched DCs from uninfected or d1.5 MCMV-infected 129 mice were then plated and incubated overnight at 37°C, 5% CO₂. Plates were washed and incubated with the same secondary antibody as in the ELISA, followed by biotinylated anti-rabbit antibody with minimal cross-reactivities between species (Jackson ImmunoResearch Laboratories) and extravidin-phosphatase alkaline (Sigma-Aldrich). Spots were developed and counted as described (38).

**Statistical Analyses.** Statistical analyses were performed in Microsoft Excel 5.0 (Microsoft Corporation, Redmond, WA) using student’s two-tailed t tests. Unless otherwise indicated, means ± SE are shown.

**Figure 1.** Morphology and MHC class II expression by DC subsets. DCs were purified from d1.5 MCMV-infected 129 mice and stained for FITC-Ly6G/C and Texas-Red MHC class II (I-A^k). Cytospins were analyzed by fluorescent microscopy using a double red/green filter. Representative photographs of individual cells are shown. Scale bar is 10 μm. (A–C) CD11c^+Ly6G/C^+ purified cells. Most cells show a round morphology (A and B) and do not express detectable levels of MHC class II. Only few cells have dendrites (C). (D–F) CD11c^+Ly6G/C^− purified cells. All cells express high levels of MHC class II and most of them have a clear web of long dendrites (E and F). (G) One example of the few cells coexpressing Ly6G/C and MHC class II. (H and I) Photographs taken from total CD11c^+ purified cells showing Ly6G/C^+ MHC class II^+ plasmacytoid cells and Ly6G/C^− MHC class II^+ cells with developed dendrites in a single magnification field.
Results

Innate Cytokine/Chemokine Production and NK Cell Activation by DC Subsets. The major producers of IFN-α/β and IL-12 during MCMV infections are CD11c<sup>hi</sup>Ly6G/C<sup>+</sup>CD8α<sup>+</sup>/CD11b<sup>−</sup> cells (9). To further characterize these cells, CD11c<sup>+</sup> populations were isolated from spleens of d1.5 MCMV-infected mice, stained for cell surface markers, and examined microscopically. The CD11c<sup>hi</sup>Ly6G/C<sup>+</sup>CD8α<sup>+</sup>/CD11b<sup>−</sup> cells had plasmacytoid morphology and low expression of MHC class II as compared with CD11c<sup>lo</sup>Ly6G/C<sup>−</sup> DCs (Fig. 1). Experiments were performed to determine if PDCs were induced to express other innate cytokines during MCMV infection. TNF-α, MIP-1α, IFN-γ, IL-15, and IL-18 were evaluated because these molecules also play critical roles early in the control of virus infection (32, 39, 40). In addition to IFN-α/β and IL-12, PDCs isolated from d1.5 MCMV-infected mice produced higher levels of TNF-α and MIP-1α than the other DC subsets (Fig. 2 A). They did not produce IFN-γ, which mostly originated from NK cells (Fig. 2 B) as reported previously (41). IL-15 mRNA expression was induced in total splenic leukocytes after MCMV infection. However, it segregated with non-DC populations upon sorting (Fig. 2 C). Similarly, IL-18 protein was induced in non-DC populations (unpublished data). Thus, PDCs are specialized in the production of a specific array of innate cytokines/chemokines in vivo during MCMV infection.
infection, and appear much more potent for this function than other DCs.

The ability of DC subsets to rapidly activate NK cells in vitro was assessed. Ly6G/C^+ and Ly6G/C^- DCs were isolated from uninfected or d1.5 MCMV-infected mice and cocultivated with NK cells freshly isolated from uninfected mice. After 24 h, parameters of NK cell activation were measured (Fig. 2 D). Low levels of cytotoxicity were detectable in cultures of DCs alone from infected mice, even after in vivo depletion of NK cells (unpublished data). However, a clear induction of cytotoxic activity and IFN-γ production was detectable after coculture of NK cells with PDCs from infected mice, but not with PDCs from uninfected mice or with Ly6G/C^- DCs. The levels of IFN-γ production and cytotoxic activity induced upon in vitro activation were within two- to fourfold of those of NK cells isolated at the peak time of in vivo activation, i.e., d1.5 after infection. No NK cell proliferation was detected in any of the conditions tested (unpublished data). Thus, in response to MCMV infection in vivo, PDCs specifically appear to acquire a potential for rapid activation of NK cell functions.

**Antigen Presentation Potential.** The maturation status and antigen presentation potential of PDCs were compared with those of other DCs (Fig. 3). Immature PDCs expressed far lower levels of MHC class II and costimulatory molecules than other DC subsets (Fig. 3 A). By contrast, their level of MHC class I expression was similar. After in vivo infection with MCMV, all DC subsets up-regulated MHC and costimulatory molecules. However, the expression of MHC class II and costimulatory molecules remained globally less on PDCs. Thus, PDCs mature in vivo in response to MCMV infection but may retain a lower potential for T cell priming than other DC subset.

The ability of DCs to activate CD8 T cells was evaluated next. As both CD8α^+ and CD11b^+ DCs are known to have a high potential for antigen-specific priming of T lymphocytes in vitro when pulsed with peptides, these two subsets were analyzed together (i.e., Ly6G/C^- DCs) for induction of CD8 T cell responses in vitro. Freshly isolated CD8 T cells expressing a transgenic TCR specific for the LCMV epitope GP-33–41 were cocultured with peptide-pulsed DC subsets (Fig. 3 B). Immature PDCs were far less efficient than their Ly6G/C^- counterparts at priming CD8 T cells, both in terms of proliferation and IFN-γ production. However, after in vivo challenge with MCMV, PDCs became as potent as other DCs for antigen-specific activation of naive CD8 T cells, and induced higher levels of IFN-γ. The responses occurred only in the presence of an antigen-specific activation of the CD8 T cells, as they were not observed when DCs were pulsed with a negative control peptide (data not depicted). Peptide concentrations required to induce efficient activation of CD8 T cells by nonplasmacytoid DCs and mature PDCs were similar, with responses reaching a plateau at 1 nM (Fig. 3 C). Thus, immature PDCs are not very efficient at priming T cells. However, PDCs become very potent antigen-presenting cells for CD8 T lymphocytes in vivo in response to MCMV infection.

To evaluate the potential role of DC subsets for in vivo activation of antiviral CD8 T cells, their accessibility to viral antigens for presentation with MHC class I was evaluated during MCMV infection. To accomplish this, a recombinant virus RVG-102 expressing GFP under the ie-1 promoter (31) was used to track the DCs replicating...
MCMV or taking up apoptotic bodies from infected cells in vivo. A small proportion of DCs was clearly positive for GFP at d1.5 after infection (Fig. 4 A, top left dot plot). Flow cytometric analyses, using serum of hyper-immune mice for detecting overall membrane antigens, or the Croma antibody for specifically detecting the IE-1 protein, showed that most of the GFP+ DCs had high intensity expression of the MCMV antigens, whereas no specific staining was observed on GFP– DCs (unpublished data). To test for cells that might have been GFP+ at the time of isolation because they were at very early stages of viral replication, sorted GFP– DCs (Fig. 4 A, bottom left dot plot) were reanalyzed for GFP expression after 24 h (bottom right dot plot) or 48 h (unpublished data) in culture. As compared with an initial fraction of 0.8–1% of GFP+ cells in total DCs, less than 0.03% of the negative DCs acquired GFP expression over a 48 h period of culture. Thus, at the time of ex vivo analysis, the populations at stages of productive virus replication preceding detectable GFP expression represented less than 4% of total infected DCs. The sensitivity of following GFP expression as an indication of infection was further demonstrated by ex vivo analysis of MCMV transcripts expression in total, GFP+ and GFP– DCs. These studies showed that most cells replicating MCMV score as GFP+ (Fig. 4 A, histograms) because GFP+ cells were highly enriched for mRNA expression of the ie-1 and gB MCMV genes as quantified by the sensitive real time RT-PCR analyses. Taken together, these results showed that analysis of GFP expression is a very sensitive assay for detecting infected cells in our system. Interestingly, most of the GFP+ cells appeared to be replicating the virus rather than taking up apoptotic bodies because 86% of the DCs expressed GFP throughout the cell as evaluated by fluorescent microscopy (Fig. 4 B, a–c). A punctuated pattern of expression was observed in only 14% of the cells (Fig. 4 B, d and e). Characterization of the DC subsets demonstrated that most of the GFP+ cells belonged to the CD8α+ population, whereas the number of GFP+ PDCs was very low (Fig. 4 C). Hence, although mature PDCs can efficiently prime naïve CD8+ T cells in vitro, their contribution for this function during MCMV infection in vivo may be low as compared with CD8α+ DCs, due to a restricted access to viral antigen for presentation with MHC class I molecules.

Figure 4. DC subset accessibility to MCMV antigens in vivo. (A) Analysis of the expression of MCMV transcripts in total, GFP+ and GFP– DCs. DCs were enriched from the spleen of 129 mice at 1.5 d after infection with the MCMV clone RVG-102, which express the GFP under the ie-1 promoter. DCs were then sorted in total, GFP+ and GFP– populations. The negative fraction was reanalyzed for GFP expression after 24 h in vitro culture, to allow expression of the marker in the eventual cells that were at very early stages of viral replication and scored negative at the time of isolation (bottom right dot plot). Number of events analyzed were 20,000 for total, 2,000 for GFP+, 770,000 for GFP–, and 650,000 for GFP– 24 h DCs. Percentages of GFP+ DCs are given above each dot plot. The data are from one representative experiment out of three. RNA was extracted from the GFP+, total and GFP– DC populations ex vivo, and transcripts for GFP, ie-1, and the glycoprotein-B (gB) were quantified by real-time RT-PCR. (bar graphs). For the gB transcripts, values were 1,258, 57 and undetectable for triplicates cell pellets of GFP+ DCs. The data are from one representative experiment of two. (B) Immunofluorescence analysis of GFP localization within positive DCs. Sorted GFP+ DCs were observed under a fluorescent microscope. No green signal was observed in the GFP– DCs under the same conditions (unpublished data). Scale bar is 10 μm. (C) Analysis of GFP expression in DC subsets. DCs were enriched from the spleen of 129 mice at 1.5 d after infection with RVG-102. The simultaneous expression of GFP (x-axis) and typical membrane markers (y-axis) was analyzed by flow cytometry within CD11c+ cells (left panel) or CD11c+Ly6C−/C− cells (middle and right panels). The percentages of cells in the quadrants are given above each dot-plot. The data are representative of at least three independent experiments.
components (42). However, it has been recently shown that IFN-α/β can also be induced in vitro in uninfected DCs through activation by glycosylated viral proteins (43) or double-stranded RNA (44). During ongoing viral infections in vivo, it is not known whether cytokine production is induced in single DCs as a result of viral replication inside the cell, of uptake of apoptotic bodies from dying infected cells, or of other signals delivered by neighboring infected cells. To address the relationship between expression of viral products and production of IFN-α/β, the frequencies of IFN-α spot-forming cells (SFCs) and of GFP+ cells were measured within DCs isolated from RVG-102–infected mice, by ELISPOT and flow cytometry, respectively (Fig. 5 A). After infection, the proportion of PDCs secreting IFN-α was consistently higher than those expressing GFP. These results were confirmed, and extended to IL-12 production, by comparing the levels of cytokines secreted by sorted GFP+ versus GFP− DCs (unpublished data).

Moreover, when GFP and IL-12 expression were analyzed simultaneously in DC subsets isolated from d1.5 MCMV-infected mice, by flow cytometry, it was demonstrated that the two occurred in largely distinct populations of cells (Fig. 5 B). This suggests that, at the single cell level, viral replication or uptake of apoptotic bodies from infected cells is neither required nor sufficient for induction of either IFN-α/β or IL-12 production in DCs.

Mice lacking IFN-α/β functions produce more IL-12, especially in CD11b+ DCs, and this is associated with enhanced viral replication in the spleen (9). To determine whether the increase in IL-12 production by CD11b+ DCs was a result of enhanced exposure to viral products in those cells, we analyzed the relationship between GFP expression and IL-12 production in DC from RVG-102–infected IFN-α/β−/− mice. In the absence of IFN-α/β functions, a significant increase in GFP expression was observed in all DC subsets (Fig. 5 C, Table II). An increase in the relative

**Figure 5.** Cytokine production and expression of MCMV products in DC subsets. (A) Comparison of the frequencies of cells secreting IFN-α or expressing viral products in PDCs. The left panel shows photographs of IFN-α ELISPOT assays performed with DC populations from splenic leukocytes of 129 mice uninfected (d0) or at 1.5 d after infection with the MCMV clone RVG-102 (d1.5). The frequencies of IFN-α spot forming cells (SFC) versus GFP+ cells within PDCs are given on the right (average and standard deviations for three mice). (B) Simultaneous analysis of GFP (x-axis) and IL-12 (y-axis) expression in DC populations from splenic leukocytes of 129 IFN-α/β−/− at 1.5 d after infection with the MCMV clone RVG-102. The frequencies of IFN-α spot forming cells (SFC) versus GFP+ cells within DCs are given on the right (average and standard deviations for three mice). (B) Simultaneous analysis of GFP (x-axis) and IL-12 (y-axis) expression in DC populations from splenic leukocytes of 129 IFN-α/β−/− at 1.5 d after infection with the MCMV clone RVG-102. The data are representative of at least three independent experiments. (C) Similar panel for IFN-α/β−/− mice. The data are representative of at least three independent experiments. (D) Impact of IFN-α/β functions on DC subset accumulation in the spleen. IFN-α/β−/− (○, △) versus IFN-α/β−/− (○, △) mice were infected with MCMV for various length of times and the frequency of subsets (Ly6G+/C−: ○, CD8α+: ●, CD11b+: △, ▲, CD11b+: △, ▲) in splenic DCs was analyzed by flow cytometry. The data shown are representative of at least three independent experiments for the 36 h time point.
The contribution of the CD11b+ DC subset to MCMV replication was consistently observed in the IFN-α/βR−/− mice at d1.5 and d2 after infection, where they accounted for 50% or more of the total GFP+ DCs versus less than 30% in the IFN-α/βR+/+ mice. However, in the absence of IFN-α/β functions, IL-12 production and GFP expression still occurred in largely distinct populations of cells (Fig. 5 C, Table II). Moreover, a significant increase in IL-12 production was observed in uninfected DCs, mostly in the CD11b+ subset but not in PDCs (Table II). These observations confirm that IFN-α/β inhibits IL-12 production preferentially in the CD11b+ DC subset, and show that this occurs in part independently of changes in expression of viral products within the cells.

We had previously reported that mice lacking IFN-α/β functions produced less of these cytokines (9). Here, the impact of IFN-α/β functions on the kinetics of DC subset accumulation in the spleen during MCMV infection was examined (Fig. 5 D). In contrast to other DC subsets, PDCs were necessary for the accumulation of PDCs, the major IFN-α/β producers, in the spleen during MCMV infection, and this likely contributes to the amplification of the cytokines.

Role of IFN-α/β on Maturation of DC Subsets. The expression of MHC and costimulatory molecules by DC subsets was next compared between IFN-α/βR−/− and IFN-α/βR+/+ mice.

### Table II. Impact of IFN-α/β Functions on MCMV Infection of, and IL-12 Production by, DC Subsets

| DC subset | Mouse strain | Population | IFN-α/βR+/+ (n = 3) | IFN-α/βR−/− (n = 4) | p* |
|-----------|--------------|------------|---------------------|---------------------|----|
| Ly6G/C−CD8α+/− | GFP IL-12− | 0.26 ± 0.04 | 3.38 ± 0.40 | <10−4 |
| Ly6G/C−CD8α+/− | GFP IL-12+ | 0.25 ± 0.05 | 5.16 ± 0.45 | <10−3 |
| Ly6G/C−CD8α+/− | GFP II-12+ | 7.15 ± 1.81 | 7.95 ± 0.61 | NS |
| Ly6G/C−CD8α+/− | GFP II-12+ | 2.63 ± 0.89 | 11.54 ± 0.83 | <10−4 |
| Ly6G/C−CD8α+/− | GFP II-12+ | 1.30 ± 0.26 | 8.17 ± 1.21 | <10−3 |
| Ly6G/C−CD8α+/− | GFP II-12+ | 2.63 ± 0.53 | 4.50 ± 0.09 | <10−2 |
| Ly6G/C−CD11b+ | GFP IL-12− | 0.29 ± 0.09 | 2.42 ± 0.23 | <10−3 |
| Ly6G/C−CD11b+ | GFP II-12+ | 0.08 ± 0.03 | 1.31 ± 0.23 | <10−3 |
| Ly6G/C−CD11b+ | GFP II-12+ | 0.79 ± 0.12 | 1.95 ± 0.21 | <10−3 |

*129 IFN-α/βR+/+ and IFN-α/βR−/− mice were infected for 1.5 d with MCMV RVG102 which encodes GFP under the promoter of the ic1 gene. DCs were enriched from spleens and stained for membrane markers and intracellular IL-12 as described in Materials and Methods. *Mean ± standard deviation.

Discussion

We have established the critical contribution of mouse PDCs to IFN-α/β production during MCMV infection (9). Here the functions and regulation of the three major splenic DC subsets (i.e., PDCs, CD8α+, and CD11b+) are further characterized at d1.5 after MCMV infection. PDCs from MCMV-infected mice secreeted high levels of innate cytokines and induced rapid activation of NK cells in vivo. PDCs from untreated mice had only weak antigen presentation capabilities. MCMV infection in vivo induced PDC maturation, licensing them to efficiently activate CD8 T cells in vitro. Non-plasmacytoid DCs did not produce IFN-α/β, secreted only low levels of the other innate cytokines, and did not induce detectable NK cell activation upon short coculture. However, they were very efficient at activating naive CD8 T cells in vitro. Expression of MCMV antigens seemed largely restricted to CD8α+ DCs, suggesting that the contribution of other DC subsets for in vivo activation of antiviral CD8 T cells may be low. Thus, PDCs were specialized in the activation of innate immune responses early during MCMV infection, whereas other DCs were likely to initiate acquired immune responses. However, PDC-derived IFN-α/β regulated multiple DC functions during MCMV infection in vivo, with different effects on DC subsets. In particular, the cytokines were necessary for maturation of CD8α+ DCs. Altogether, the results suggest that PDCs are a cornerstone for induction of both innate and adaptive immune responses to MCMV infection in vivo, through production of various cytokines, direct activation of NK cells, and indirect regu-
lation of T cell functions by IFN-α/β-mediated effects on other DCs.

Different mechanisms are in place to induce cytokine production by cells from the innate immune system in response to microbial challenges. Cells can produce cytokines as a consequence of recognition of molecular patterns shared within a class of microbes, through pattern-recognition receptors (PRRs; reference 45). Alternatively, responses can be triggered by host products generated during inflammation, such as heat shock proteins (hsp; reference 46). Both cytoplasmic and membrane-bound PRRs have been involved in IFN-α/β induction during in vitro viral challenges (42–44). All these mechanisms of danger recognition are relevant to DC physiology, because DCs are susceptible to infection by many viruses, can engulf apoptotic bodies from dying cells, and express a variety of membrane-bound PRRs or hsp ligands. Here, the relationship between expression of viral products and production of cytokines in DC subsets was examined during MCMV infection. A virus recombinant for the GFP under the ie-1 promoter was used to track DCs that were productively infected by MCMV in vivo or had engulfed whole apoptotic bodies from neighboring infected cells. GFP and cytokine expression occurred in largely distinct populations of DCs. As the results show that uptake of apoptotic bodies from infected cells or productive viral infection within DCs was neither required nor sufficient for induction of cytokine production, some of the other pathways must contribute to the induction of cytokine production by DCs during MCMV infection. These likely include recognition of viral components through Toll-like receptors (TLRs; reference 45) or other receptors. Although beyond the scope of this report, ongoing studies in our laboratory are aimed at defining these.

Several investigators have shown the occurrence of an auto-amplification pathway regulating the transcription of interferon genes during viral infections in vitro (47). Our group has demonstrated that intact IFN-α/β functions were required for high level productions of IFN-α/β during LCMV (21) and MCMV infections in vivo (9). As IFN-α/β have been shown to promote the survival of PDCs in vitro both in the mouse (16) and in the human (48), other mechanisms in addition to transcriptional regulation of the interferon genes could be involved in the pos-
itive feedback loop for production of the cytokines in vivo during viral infections. Indeed, IFN-α/β functions were required for accumulation of PDCs in the spleen at the time of local peak production of innate cytokines. Thus, one of the mechanisms by which IFN-α/β enhance their own production during MCMV infection is the promotion of PDC accumulation.

Both in mice and in humans, in vitro studies have demonstrated that DCs can activate NK cells through membrane contacts and that this may be at least partially independent of IFN-α/β and IL-12 production (for a review, see reference 3). DCs have also been shown to activate NK-dependent antitumoral responses in vivo (49). However, the roles of the different DC subsets for this function have not been reported. Here, only DCs isolated from d1.5 MCMV-infected mice were able to activate NK cytotoxicity and IFN-γ production in 24 h of coculture, and this function segregated with PDCs. Preliminary results suggested that IFN-α/β functions and IL-12 production were required for optimization of NK cell responses. Whether other cytokines or cell–cell contacts are involved still needs to be investigated. Whatever the case, these results demonstrate that PDCs are more efficient than other DC subsets at activating NK cells early during MCMV infection.

In consistency with the observations reported in vitro with resting human PDCs (48, 50), mouse PDCs isolated from uninfected mice were relatively poor at antigen presentation. Responses to MCMV infection induced PDCs to become very potent antigen-presenting cells for priming CD8 T cells in vitro, with induction of higher levels of IFN-γ production than other DCs. In vivo, expression of MCMV antigens seemed largely restricted to CD8α+ DCs. These cells have also been shown to have the unique ability to cross-present exogenous antigen in association with MHC class I (11–13). Therefore, CD8α+ but not CD11b+ or PDCs are likely to play a major role in the direct activation of MCMV-specific CD8 T cells in vivo. However, PDCs may be necessary for optimal induction of CD8 T cell responses during MCMV infection, through IL-12 or IFN-α/β–mediated effects on the T lymphocytes themselves or on other DC subsets. Indeed, it is shown here that IFN-α/β functions are required for CD8α+ DC maturation in vivo during MCMV infection, and we have previ-

![Figure 7](image-url)
ously reported that IFN-α/β promote CD8 T cell IFN-γ production in vivo during viral infections (33, 51, 52).

Altogether, the results presented here suggest a model of DC subset specialization and DC cross-talk during MCMV infection, with a central role for IFN-α/β functions in regulating both innate and acquired immune responses (Fig. 7). Early in response to MCMV challenge, PDCs are activated to produce IFN-α/β. The cytokines can elicit mechanisms directly mediating antiviral effects (arrow 1). They have also a number of important immunoregulatory functions. In particular, they induce NK cell cytotoxicity (arrow 2). Simultaneous PDC production of IL-12 is inducing NK cell IFN-γ responses. The PDC-derived IFN-α/β is also controlling the accumulation, maturation and cytokine production of DC subsets. IFN-α/β promote increased proportions of PDCs in the spleen, and this is likely to contribute to the amplification loop for secretion of the cytokines (arrow 3). PDC-produced IFN-α/β are also generally required for maturation of DCs, especially the CD8α+ subset (arrow 4). As this subset harbor viral products, it is likely to initiate acquired CD8 T cell responses by presentation of MCMV-derived epitopes in association with the MHC class I molecules. Thus, IFN-α/β induction of DC maturation may indirectly contribute to the activation of CD8 T cells. In addition, IFN-α/β are enhancing the maturation of, and inhibiting IL-12 production by, CD11b+ DCs (arrow 5). Because CD11b+ DCs have been reported particularly efficient for priming CD4 T cells in other systems (12), IFN-α/β-mediated regulation of this DC subset may be in place to shape CD4 T cell responses during viral challenges. Moreover, PDCs can participate in the activation of anti-MCMV CD8 T cell responses through mechanisms in addition to those downstream of IFN-α/β-induced CD8α+ DC maturation (arrow 6). In particular, if armed with antigen, they may directly support antigen-specific activation of the CD8 T cells. In addition, their production of cytokines may enhance IFN-γ production by CD8 T cells. Hence, during MCMV infection in vivo, PDCs are specialized in the activation of innate immune responses but, as a result of their maturation and production of IFN-α/β, they may also play a central role in the orchestration of global DC functions and downstream adaptive immune responses.

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