CpG-matured Murine Plasmacytoid Dendritic Cells Are Capable of In Vivo Priming of Functional CD8 T Cell Responses to Endogenous but Not Exogenous Antigens

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Abstract

Plasmacytoid dendritic cells (PDCs) are a unique leukocyte population capable of secreting high levels of type I interferon (IFN) in response to viruses and bacterial stimuli. In vitro experiments have shown that upon maturation, human and murine PDCs develop into potent immunostimulatory cells; however, their ability to prime an immune response in vivo remains to be addressed. We report that CpG-matured murine PDCs are capable of eliciting in naive mice antigen-specific CTLs against endogenous antigens as well as exogenous peptides, but not against an exogenous antigen. Type I IFN is not required for priming, as injection of CpG-matured PDCs into type I IFN receptor–deficient mice elicits functional CTL responses. Mature PDCs prime CTLs that secrete IFN-γ and protect mice from a tumor challenge. In contrast, immature PDCs are unable to prime antigen-specific CTLs. However, mice injected with immature PDCs are fully responsive to secondary antigenic challenges, suggesting that PDCs have not induced long-lasting tolerance via anergic or regulatory T cells. Our results underline the heterogeneity and plasticity of different antigen-presenting cells, and reveal an important role of mature PDCs in priming CD8 responses to endogenous antigens, in addition to their previously reported ability to modulate antiviral responses via type I IFN.

Key words: PDC • T cell priming • type I IFN • H-Y • tetramers

Introduction

DCs play a pivotal role in the control of innate and adaptive immune responses (1). They consist of a heterogeneous cell population, classified into distinct subsets according to surface phenotype, functional properties, and localization (2). In humans, an immature DC subset with plasmacytoid morphology (plasmacytoid DC [PDC]) represents a unique leukocyte population capable of secreting high levels of type I IFN in response to viruses and bacterial stimuli (3, 4). It has been shown recently that human PDCs behave as bona fide DCs, as they efficiently prime naive antigen-specific CD8 T cells (5), and are capable of restimulating CD4 and CD8 responses upon influenza virus infection (6). In both experimental systems, CD4 and CD8 T cells expanded by PDCs were capable of IFN-γ secretion. However, other investigators have shown that PDCs can differentiate allo- geneic CD8 regulatory cells and Th2 responses (4, 7), suggesting that PDCs may have a certain degree of plasticity in their ability to prime T cell responses.

Murine PDCs have been identified recently on the basis of high type I IFN secretion and their unique surface phenotype (CD11chull, B220+, CD11b−, and Gr-1−; references 8–10). To date, all functional studies on murine PDCs have been performed in vitro using PDCs either isolated from spleen or differentiated from bone marrow precursors. It has been shown that freshly isolated murine PDCs express lower levels of MHC and costimulatory molecules than the myeloid CD11chigh CD11b+ subset (myeloid DC [MDC]), possibly accounting for their reported poor stimulatory capacity for allogeneic and naive T cells (8–11). In contrast, PDCs matured with viral or CpG stimulation are potent...
APCs, capable of stimulating proliferation of allogeneic T cells and naive transgenic CD4 and CD8 T cells (8, 10, 12–14). Depending on antigen dose and Toll-like receptor engagement, murine PDCs show flexibility in their T cell polarizing capacity, generally eliciting Th1 responses at high and Th2 responses at low antigen doses (15). In addition, immature PDCs have been shown to differentiate T regulatory cells, capable of suppressing antigen-specific T cell proliferation (13, 16, 17).

Although in vitro experiments indicate that mature PDCs are potent immunostimulatory cells, it remains unclear whether they can prime antigen-specific immune responses in vivo in naive nontransgenic animals. To address this question, we set up an in vivo priming model in which we monitored ex vivo by tetramer analysis the proliferation of antigen-specific T cells after injection of PDCs either freshly isolated from the spleen or from FLT3 ligand (FLT3-L)–supplemented murine bone marrow cultures (18). We report that CpG-matured PDCs prime CTLs specific for endogenous but not exogenous antigens. CTLs primed by PDCs acquire potent in vivo cytolytic activity, are capable of IFN-γ secretion upon peptide stimulation, and protect mice from a subsequent tumor challenge. Priming is dependent on direct presentation of the antigen by the injected DCs and does not require responsiveness to type I IFN. Conversely, immature PDCs do not induce proliferation of antigen-specific CTLs in vivo. However, in contrast to what was observed in vitro, administration of immature PDCs does not prevent responses to subsequent challenges with viruses or DCs expressing the relevant antigen.

Materials and Methods

Mice. C57BL/6, TAP-1−/− (on C57BL/6 background), 129A (lacking type I IFN receptor; reference 19), and 129 S1/SvEv mice were maintained at the John Radcliffe Hospital Biomedical Services and used at 7–12 wk of age according to institutional guidelines.

Peptides and Tetramers. UTY246–254 (WMHNNMDL), SMCY336–346 (KCSRNRRQYL), LCMV-gp34–41 (AVYNFATC), OVA257–264 (IQSAVHAAHAINEAGR), and OVA257–264 (SIINFELK) peptides were purchased from Sigma-Aldrich and were HPLC purified. UTY246–254-H2-Db, SMCY336–346-H2-Db, OVA257–264-H2-Kb, and LCMV gp34–41-H2-Kb fluorescent tetrameric complexes (tetramers) were synthesized as described previously (20). The LCMV gp34–41-H2-Kb tetramer was used instead of the LCMV gp33–41-H2-Db tetramer (21) because, upon priming by LCMV gp33–41 Peptide-pulsed DCs, responses to the octamer were dominant over those to the nonamer (unpublished data). Tetramers were validated by staining mice primed by vaccinia viruses encoding the relevant protein. Background levels of staining (<0.02% of total CTLs) were determined in naive mice.

Generation of Bone Marrow–derived DCs. Culture medium was RPMI 1640 supplemented with 2 mM l-glutamine, 1% nonessential amino acids, 1% pyruvate, 50 μg/ml kanamycin, 0.05 mM 2-mercaptoethanol (GIBCO BRL), and 10% FCS (Hyclone). Bone marrow cells were isolated by flushing femurs and tibia with complete medium. RBCs were lysed with RBC lysis solution (Puregene; Gentra Systems). The recovered cells were plated in culture medium containing 100 ng/ml FLT3-L (R&D Systems) at 10^6 cells/ml in six-well plates in a volume of 5 ml as described previously (18). Every 3–4 d, 2.5 ml of medium was replaced with fresh medium and FLT3-L. At day 10, half of the cultures were matured with 5 μg/ml phosphorothioate CpG DNA 1826 (Coley Pharmaceutical). In some experiments, bone marrow cultures were incubated with 500 μg/ml of soluble ovalbumin (fraction VII; Sigma-Aldrich) for 20 h before sorting (CpG was added to part of the cultures 4 h after ovalbumin). At day 11, the cultures were phenotyped by FACS® analysis with the following mAbs: CD11b-FITC, B220-PE, and CD19-APC (all obtained from BD Biosciences). PDCs (CD11b+B220+CD19+) and MDCs (CD11b+B220–CD19+) were isolated by a combination of magnetic (MACS; Miltenyi Biotec) and cell sorting (MoFlo; DakoCytometry). The purity was always >97%, and CD11b+ cells were not detected upon reanalysis of PDC preparations.

Isolation of Splenic DCs. C57BL/6 males were injected i.v. with 200 μg CpG DNA 1826. 16 h later, splenic DCs were enriched by magnetic sorting using CD11c beads (MACS; Miltenyi Biotec) after collagenase treatment of disrupted spleens. MDCs (CD11c+B220−Ly-6-G−) and PDCs (CD11c+B220−Ly-6-G−/C−) were subsequently purified by cell sorting (MoFlo; DakoCytometry) as described previously (11, 15).

Immobilization Protocols. Sorted cells were left unpulsed with 1 μg/ml of peptide in serum-free medium for 2 h at 37°C, extensively washed, and diluted in PBS. 200 μl of cells was injected into the lateral tail vein. Animals were boosted by i.v. injection of 3 × 10^5 BM-DCs or 10^5 PFCs of UV-inactivated recombinant vaccinia encoding the UTY246–254 or SMCY336–346 minigenes or the full length ovalbumin protein (22).

Generation of Recombinant Vaccinia Viruses. Recombinant vaccinia viruses (WR strains) encoding the UTY246–254 and SMCY336–346 minigenes were made by cloning each insert into the thymidine kinase gene using the vector pSC11 as described previously (23).

Isolation of PBLs and Tetramer Staining. Blood was taken from the tail vein, and PBLs were isolated after depletion of RBCs with RBC lysis solution (Puregene; Gentra Systems). Cells were resuspended in 25 μl of complete medium and incubated with 0.5 μg of tetramer for 25 min at 37°C. Cells were washed and incubated with rat anti–mouse CD8α (BD Biosciences) for 20 min at 4°C. Cells were washed twice and analyzed using a FACS Calibur™ with CELLQuest™ software.

In Vivo Killing Assay. To assess cytotoxicity, immunized and control mice were injected with a mixture of four differentially labeled syngeneic splenocyte populations, loaded or not with 10 μg/ml UTY246–254 Peptide; three populations were labeled with different concentrations of carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) and one with 10 μM of the dye 5-(and 6)-|{4-chloromethyl}benzoyl|amino| tetramethylrhodamine (Molecular Probes; references 24, 25). Labeled cells were pooled and injected at 10^7 cells/mouse into the tail vein. Cytotoxicity was assessed by FACS® analysis on blood taken from the lateral tail vein at different time points. The mean percentage lysis of peptide-loaded target cells was calculated relative to antigen-negative controls with the following formula: 100 − (100 × adjusted percent survival). Adjusted percent survival was calculated as follows: (percent survival Ag/percent survival Ag)/mean percent survival in control animals.

ELISPOT. Blood was taken from the tail vein, and PBLs were isolated after depletion of RBCs with RBC lysis solution (Puregene; Gentra Systems). In some experiments, RBC-depleted splenocytes were used as responders. Analysis of IFN-γ production in response to stimulation with 10 μM peptide for 16 h.
was performed on MultiScreen-IP high protein–binding 96-well plates (Millipore) using MabTech mouse IFN-γ ELISPOT kit according to the manufacturer’s instructions. In all experiments, stimulation with 1 μg/ml PHA served as positive control.

**Tumor Immunity Assay.** 10 d after priming, mice were challenged with subcutaneous injection of 10^6 B16-F10 tumor cells expressing the LCMV Gp33-41 minigene (26). Mice were monitored for tumor growth every 3–4 d, and the tumor size for each group was calculated as the mean of the products of bisecting diameters (± SEM). Measurements were terminated for each group when the first animal developed ascitis, when the tumor became ulcerated, or when it grew in excess of 200 mm^2.

**Intracellular Cytokine Staining.** Spleens were harvested 8–9 d after priming, and lymphocytes were isolated after depletion of RBCs with RBC lysis solution (Puregene; Gentra Systems). Cells were plated in complete medium and stimulated with 10 μ/ml ionomycin (Sigma-Aldrich), or left unstimulated. 5 μg/ml Brefeldin A (Sigma-Aldrich) was added after 1 h, and cells were collected after a total of 6 h. Cells were fixed in 2% paraformaldehyde, permeabilized in saponin buffer (27), and stained with antibodies to mouse IFN-γ-FITC, IL-2–PE, IL-10–PE, and IL-4–APC (BD Biosciences). Tetramer staining was performed on a sample of unstimulated splenocytes as described in previous paragraphs for blood PBLS.

**Online Supplemental Material.** Fig. S1 shows the phenotype of immature and CpG-matured PDCs and MDCs isolated from FLT3-L–supplemented bone marrow cultures. Fig. S2 supplements Fig. 1 and shows priming of SMCY738–746–specific CTLs by CpG-matured PDCs. Fig. S3 shows the phenotype of immature and CpG-matured PDCs and MDCs isolated from FLT3-L or GM-CSF + IL-4–supplemented cultures elicited qualitatively and quantitatively comparable CTL responses (unpublished data) and, therefore, restricted our subsequent analysis to FLT3-L–differentiated MDCs. The phenotype of bone marrow–derived PDCs and MDCs is shown in Fig. S1 (available at http://www.jem.org/cgi/content/full/jem.20031059/DC1).

In a first set of experiments, we investigated the response to an endogenous antigen, the male-specific transplantation antigen H-Y. When injected with male cells, female mice of the H-2^b haplotype develop an immunodominant H-2-D^b–restricted response against the UTY246–254 peptide (WMHHYNMDLI) that can be monitored by tetramer analysis (28, 29). UTY246–254–specific CTL responses were monitored ex vivo in the blood of female C57BL/6 mice injected with male PDCs or MDCs, either immature or CpG matured. Mature PDCs were as efficient as mature MDCs in eliciting UTY246–254–specific CTLs, as detected by tetramers (Fig. 1 A). In contrast, although immature MDCs always elicited a distinct CTL population (although lower in numbers as compared with mature MDCs), immature PDCs never induced proliferation of UTY246–254–specific CTLs above the detection limit of the tetramer staining (Fig. 1 A). Similar results were obtained when responses to the subdominant H-2-D^b–restricted SMCY738–746 epitope (28) were monitored (unpublished data; Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20031059/DC1).

To better evaluate quantitatively the CTL responses, we titrated the numbers of DCs. At low numbers (3,000 cells/mouse), MDCs were far more efficient than PDCs in eliciting UTY246–254–specific CTLs (Fig. 1 B), whereas at 10,000 cells/mouse, PDCs and MDCs elicited a similar

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**Results**

**Intravenous Injection of CpG-matured Male PDCs Induces Functional CTL Responses.** Thus far, the stimulatory capacity of murine PDCs has been investigated only in vitro against transgenic T cells (8, 10, 12–14). Therefore, we developed an in vivo model to study the ability of PDCs to prime and polarize antigen–specific T cells from naive non-transgenic precursors. We differentiated both PDCs and control MDCs from murine bone marrow cultures supplemented with FLT3-L (18) and isolated the two DC populations by a combination of magnetic and cell sorting. In preliminary experiments, we observed that MDCs isolated from FLT3-L or GM-CSF + IL-4–supplemented cultures elicited qualitatively and quantitatively comparable CTL responses (unpublished data) and, therefore, restricted our subsequent analysis to FLT3-L–differentiated MDCs. The phenotype of bone marrow–derived PDCs and MDCs is shown in Fig. S1 (available at http://www.jem.org/cgi/content/full/jem.20031059/DC1).

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**Figure 1.** Intravenous injection of CpG-matured male PDCs induces CTL responses. (A) C57BL/6 mice (n = 5) were injected i.v. with 10^5 male MDC of PDCs, immature or CpG matured. Control animals were injected with female MDC. CTL responses were assessed in the blood by ex vivo FAC® analysis using UTY246–254–H-2-D^b tetramers 7 d after priming. Mean proportions of tetramer− cells as a percentage of CD8 cells (± SEM) for each group are shown. (B) C57BL/6 mice (n = 5) were injected with graded numbers of male CpG-matured MDC (gray bars) or PDCs (black bars) and boosted after 1 wk with UV-inactivated vaccinia-UTY246–254 minigene. CTL responses were assessed in the blood by ex vivo tetramer staining 8 d after boosting. Tetramer stainings of control mice, primed by female MDC or by vaccinia-UTY246–254 minigene alone, are shown (white bars).

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569 Salio et al.
UTY246–254-specific CTL response. However, responses were weaker than in mice immunized with 10⁶ cells (Fig. 1 A) and were detected only upon in vivo restimulation with vaccinia virus encoding the UTY246–254 minigene.

The functional state of the induced UTY246–254-specific CTLs was investigated by assessing their cytotoxic capacity and cytokine secretion upon antigen exposure. Cytotoxicity was assayed in vivo against syngeneic splenocyte targets, either male or peptide-pulsed female that had been labeled with a fluorescent dye and injected into the lateral vein 10 d after priming. Specific lysis of the antigen-expressing cells was determined against control female splenocytes not pulsed with the UTY246–254 peptide. UTY246–254-specific CTLs primed by mature PDCs efficiently lysed pulsed with 10 μg/ml of peptide female cells, as well as unpulsed male splenocytes (Fig. 2, B and C). Lysis of unpulsed male splenocytes, expressing a much lower density of UTY246–254–Db complexes than peptide-pulsed cells, is indicative of expansion of high affinity CTLs. The majority of peptide-pulsed female targets were lysed within the first 17 h, whereas lysis of unpulsed male cells continued over the next 96 h, consistent with UTY246–254–Db complexes being presented at lower density but continuously over time (Fig. 2 B, not depicted). The cytolytic activity detected in mice primed by immature and mature MDCs correlated with the extent of CTL priming. No specific lysis above background was observed in mice primed by immature PDCs.

ELISPOT assays were performed to assess the capacity of UTY246–254-specific CTLs to secrete IFN-γ in response to peptide stimulation. As shown in Fig. 3, and in agreement with the tetramer data, mice primed by either mature PDCs or immature and mature MDCs were capable of recognizing the UTY246–254 peptide in an ex vivo assay, without further in vitro restimulation. No specific IFN-γ secretion was observed in mice primed by immature PDCs.

Intravenous Injection of CpG-matured Male PDCs Induces Type I Polarization of Splenic UTY246–254-specific CTLs. To further characterize the type of polarization induced by priming with mature PDCs, freshly isolated splenocytes
were stimulated in vitro with the UTY246–254 peptide or PMA + ionomycin 8 d after priming. Analysis of intracellular cytokines revealed that CD8 T cells secreted the type 1 cytokine IFN-γ in response to both the specific peptide and PMA + ionomycin. IL-2 was also detected in CD8 T cells stimulated with PMA + ionomycin. In contrast, CD8 cells did not secrete the type 2 cytokines IL-4 and IL-10 in response to PMA + ionomycin. A similar pattern of polarization was observed in mice primed by mature MDCs (unpublished data). Although the frequencies of tetramer+ cells in the spleen and in the blood were comparable, a higher proportion of splenic UTY246–254-specific CTLs secreted IFN-γ in response to the specific peptide (Fig. 4; not depicted), which may reflect homing of effector T cells to the spleen. We conclude that mature PDCs are able to efficiently prime antigen-specific CTLs, capable of cytolytic activity and IFN-γ secretion.

Priming of UTY246–254-specific CTLs by Freshly Isolated CpG-matured Splenic PDCs. It has been shown previously that freshly isolated splenic PDCs are less mature than their bone marrow–derived counterparts, hence they are also less immunostimulatory (15). Preliminary experiments showed that injection of immature splenic MDCs, in contrast to bone marrow–derived MDCs, did not elicit UTY246–254-specific CTLs detectable by ex vivo tetramer staining (unpublished data). Therefore, we isolated splenic PDCs and MDCs from male mice previously injected with CpG to induce in vivo DC maturation. Both MDCs and PDCs elicited UTY246–254-specific CTLs, although responses were much weaker than those elicited by equal numbers of bone marrow–derived DCs and were detectable only upon in vivo restimulation with vaccinia virus.
encoding the UTY_{246-254} minigene (Fig. 5 A). UTY_{246-254}-
specific CTLs primed by mature splenic PDCs and MDCs
were functional, as shown by IFN-\(\gamma\) secretion in response
to the cognate peptide in an ex vivo ELISPOT assay
(Fig. 5 B).

**Priming of UTY_{246-254}-specific CTLs Relies upon Direct Presentation of the Male Antigen by the Injected DCs.** Priming
of UTY_{246-254}-specific CTLs by CpG-matured PDCs and
MDCs could be due to direct presentation of the UTY_{246-254}
peptide by the male APCs. Alternatively, proliferation of
UTY_{246-254}-specific CTLs could be due to uptake and pre-
sentation of male APC debris by resident female DCs, a
mechanism known as cross-priming. Indeed, cross-prim-
ing has been shown to be effective for the generation of
cytotoxic T cells, and it may be the dominant route for
priming of some responses (30, 31). In vitro experiments
showed that presentation of the UTY_{246-254} epitope is en-
tirely TAP-dependent as UTY_{246-254}-specific T cells did
not recognize TAP-1−/− deficient male APCs (unpublished
data). Therefore, we used TAP-1−/− deficient male BM-DCs
as immunogens to distinguish between direct versus cross-
presentation. Injection of as many as 10^6 male TAP-1−/−
BM-DCs, either immature or CpG matured, failed to
prime UTY_{246-254}-specific CTLs in female C57BL/6 mice
(Fig. 6). In contrast, control mice developed good re-
sponses after injection with 10^5 wild-type BM-DCs. After
boosting with vaccinia-UTY_{246-254} minigene, only mice
that had been primed with 10^6 male APCs (i.e., 10 times more APCs than used in previous experiments)
showed enhanced CTL responses (unpublished data). In
agreement with the observation that UTY_{246-254}-specific
CTL responses cannot be efficiently generated upon prim-
ing by \(\beta\)-2m−deficient APCs (28), we conclude that the
role of cross-priming in generating UTY_{246-254}-specific
CTLs in this system is marginal, and can only be appreci-
at when animals are injected with large numbers of
APCs. Therefore, proliferation of UTY_{246-254}-specific
CTLs in our in vivo model can be accounted for by direct
presentation of the endogenous antigen by the injected
PDCs and MDCs.

**Lack of Priming by Intravenous Injection of Immature Male
PDCs Does Not Prevent Subsequent Induction of Functional
UTY_{246-254}-specific Responses.** The inability of immature
PDCs to prime antigen−specific CTLs could reflect the lack of
expression of costimulatory or adhesion molecules essen-
tial to trigger naive T cell proliferation. Alternatively, in-
mature PDCs could have induced the proliferation of aner-
gic or regulatory cells nonreactive to further antigenic
challenge or unable to bind UTY\textsubscript{246–254}-Db tetramers (32, 33). The possibility of anergy or negative regulation was ruled out by demonstrating that mice injected previously with immature PDCs developed cytolytic male-specific CD8 T cells upon boosting by splenocytes (Fig. 7, A and B). The high numbers of splenocytes used for the in vivo killing assay primes UTY\textsubscript{246–254}-specific CTL within 7 d (28), and this is reflected by the specific clearance of male splenocytes shown in B but absent at the earlier time point. The lack of clearance of peptide-pulsed female splenocytes reflects the short half-life of the peptide on the surface of these cells, which, therefore, represent an important internal control for the experiment. (C) Mean proportions of tetramer\textsuperscript{+} cells as a percentage of CD8 cells (± SEM) for each group 7 d after PDC priming (white bar) and 1 wk after the in vivo killing assay (black bars). (D) IFN-γ ELISPOT performed on blood PBLs to assess responsiveness to 10 µg/ml UTY\textsubscript{246–254} peptide 1 wk after the in vivo killing assay. All animals showed comparable responses to PHA stimulation (not depicted). The same groups of animals are shown in A–D. (E and F) Mean proportions of UTY\textsubscript{246–254}-H-2-D\textsuperscript{b} tetramer\textsuperscript{+} cells as a percentage of CD8 cells (± SEM) after in vivo boosting with male immature DCs (E, staining performed at day 7) or UV-inactivated vaccinia-UTY\textsubscript{246–254} minigene (F, staining performed at day 8).

Type I IFN Receptor–deficient CpG-matured Male PDCs Prime IFN-γ Secreting UTY\textsubscript{246–254}-specific CTLs. PDCs are a unique leukocyte population capable of secreting high levels of type I IFN in response to viruses and bacterial stimuli (8–10). Because type I IFN is important for the survival and proliferation of memory CD8 T cells (34, 35), we investigated the priming capacity of CpG-matured PDCs in mice lacking type I IFN receptor (129A mice) (19).

As compared with 129 wild-type mice, 129A mice had normal numbers of PDCs ex vivo in the spleen and in vitro after culturing bone marrow cells in the presence of FLT3-L (unpublished data). In addition, 129A PDCs underwent maturation after CpG treatment, although to a lower extent than their wild-type counterpart (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20031059/DC1). Female 129A mice injected with male CpG-matured 129A PDCs developed antigen-specific CTLs, which could also be boosted by UV-inactivated vaccinia UTY\textsubscript{246–254} minigene, as shown previously for C57BL/6 mice (Fig. 8 A). In addition, UTY\textsubscript{246–254}-specific CTLs primed by mature 129A PDCs secreted IFN-γ in response to the cognate peptide in an ex vivo ELISPOT assay (Fig. 8 B). These results suggest that in the absence of viral infection, type I IFN responsiveness is not essential for CTL priming by CpG-matured PDCs.
In Vivo Priming by Murine PDCs

Priming by CpG-matured PDCs Induces Protective CTL Responses. We have shown that PDCs can efficiently present an endogenous antigen and prime naive CTL precursor. We extended these results by analyzing the ability of peptide-pulsed PDCs to prime antigen-specific CTLs. We pulsed PDCs and MDCs with the LCMV gp34–41 peptide (Kb restricted) and monitored ex vivo LCMV gp34-CTLs by tetramer staining. In accordance with what was observed previously, CpG-matured PDCs and MDCs induced similar proliferations of LCMV gp34-CTL; immature MDCs were somewhat intermediate, and no priming was induced by immature PDCs (Fig. 9 A).

These animals were challenged at day 10 with B16-F10-gp33, a derivative of the aggressive melanoma B16 that grows rapidly when injected in C57BL/6 mice (26). As shown in Fig. 9 B, mice that had been primed by CpG-matured PDCs or MDCs were completely protected from tumor growth and remained tumor free for up to 2 mo (not depicted). Mice primed by immature MDCs succumbed to the tumors, although they did so 2 wk later than mice injected with immature PDCs (which did not develop LCMV gp34-CTL) or unimmunized control groups.

Lack of Presentation of Exogenous Antigens by Bone Marrow–derived PDCs. To further characterize the antigen-processing capacity of PDCs as compared with classical MDCs, we investigated the presentation of a model exogenous antigen, soluble ovalbumin. We sorted PDCs and MDCs from bone marrow cultures prepulsed with ovalbumin in the presence or absence of a maturation stimulus to induce cross-presentation (36). Ex vivo SIINFEKL–Kb tetramer staining detected primary CTL responses only upon injection of mature MDCs (unpublished data). After boosting with vaccinia virus encoding full-length ovalbumin, mice primed by ovalbumin-loaded PDCs did not expand SIINFEKL-specific CTLs above controls, regardless of the PDC maturation stimulus (Fig. 10 A). In contrast, CTL responses primed by MDCs were boosted two- to fourfold.

As a control, both CpG-matured PDCs and MDCs pulsed with the OVA257–264 peptide elicited functional SIINFEKL-specific CTLs detectable in the blood by tetramer staining (Fig. S4, available at http://www.jem.org/cgi/
Salio et al. demonstrated that PDCs present endogenous antigens for which the density of MHC–peptide complexes at the cell surface is much lower than after peptide pulsing. However, PDCs are not able to present exogenous antigens, in contrast to MDCs, thus underlining the heterogeneity within the DC populations.

Previous works showed that, upon in vitro maturation, PDCs develop into potent immunostimulatory cells, although not to the same extent as control MDCs (8, 10, 12–14). In our in vivo experiments, mature PDCs and MDCs injected at high numbers elicited comparable responses, qualitatively and quantitatively, whether presenting the endogenous antigen or peptide pulsed. Only when limiting numbers of DCs were injected, MDCs were more stimulatory than PDCs. In line with our results, Dalod et al. (37) showed recently that PDCs purified after in vivo challenge with murine cytomegalovirus (MCMV) became as potent as other DC subsets for activation of naive CD8 T cells. Dalod et al. did not address whether PDCs could also present endogenous viral antigens because MCMV did not infect PDCs, but only induced their maturation. Our results showing priming of UTY246–254–specific CTL responses by male PDCs strongly suggest that PDCs, infected by viruses inducing their maturation, would be capable of priming virus–specific T cell responses.

In contrast with the majority of the analyses, Krug et al. have shown that splenic PDCs failed to stimulate a strong proliferation of naive CD4 and CD8 T cells, even after in vivo viral stimulation, questioning whether PDCs belong to the DC system (11). Conversely, we have shown that, although less efficient than bone marrow–derived PDCs, freshly isolated in vivo–matured splenic PDCs are able to prime UTY246–254–specific CTLs. It is possible that maturation induced by either CpG (our model) and MCMV (37) or VSV (11) may not generate functionally equivalent cells as a consequence of different Toll-like receptor engagement and, therefore, account for the observed discrepancies. With respect to the model antigen used, we analyzed ex vivo CTL responses specific for the immunodominant H-Y UTY246–254–H-2-D^b epitope and the subdominant SMCY738–746–H-2-D^b epitope in nontransgenic animals. In contrast, Krug et al. have looked at the endogenous presentation of the SMCY738–746–H-2-D^b epitope using TCR transgenic mice (38). These mice fail to reject male skin grafts, and SMCY738–746 TCR transgenic CD8 T cells do not induce graft versus host disease when transferred in male nude mice (39, 40), suggesting that this receptor may have poor reactivity for its ligand. In addition, SMCY738–746 TCR transgenic CD8 T cells do not induce graft versus host disease when transferred in male nude mice (39, 40), suggesting that this receptor may have poor reactivity for its ligand. In addition, SMCY738–746 TCR transgenic CD8 T cells do not induce graft versus host disease when transferred in male nude mice (39, 40), suggesting that this receptor may have poor reactivity for its ligand.
ture PDCs might have primed CD4 as well as CD8 male-specific cells.

It has been shown that human immature MDCs induce differentiation of CD4 and CD8 regulatory T cells, both in vivo and in vitro (43, 44). In the mouse, targeting of antigen in vivo to immature DCs induces CD4+ CD25+ regulatory T cells and CD8 T cell tolerance (45, 46). Upon injection of immature MDCs, we elicited functional CTLs, although in lower numbers than with mature MDCs, and this can be explained by the fact that these cells may have undergone a limited degree of maturation during the culture/sorting period (unpublished data). Regulatory T cells have also been differentiated in vitro by CD40L-activated human PDCs (7) and murine immature PDCs (13, 16, 17), and it has been hypothesized that thymic PDCs might be involved in Treg differentiation (13). However, in our in vivo priming model, we did not elicit any antigen-specific CTLs when mice were injected with immature PDCs (male DCs or peptide pulsed), making it difficult to test for any regulatory activity. Nevertheless, exposure to immature PDCs did not prevent induction of immunity after subsequent challenge with DCs, recombinant virus, or splenocytes, ruling out establishment of long-term tolerance. Although these results might at first seem difficult to reconcile with published data, they may be explained by the different experimental systems used. Indeed, previous in vivo studies have been performed with adaptively transferred naive transgenic T cells, whereas our paper relies on endogenous CTL precursors present in much lower numbers. The development of antibodies to specifically target antigens to PDCs in vivo, as described for CD8α+ DCs via DEC-205 (45, 46), will help to address whether immature PDCs may also induce tolerance and T regulatory cells.

With respect to T cell polarization, we have shown that CTLs primed by CpG-matured PDCs secreted mainly IFN-γ in response to the cognate antigen both by ex vivo ELISPOT and intracellular cytokine assays. Other investigators have reported high IFN-γ by transgenic CTLs polarized in vitro by mature PDCs pulsed with OVA257–264 or LCMV gp33–41 peptides (12, 37). A flexibility of murine PDCs in directing Th1 and Th2 development of CD4 transgenic T cells has been described, depending on antigen dose and Toll-like receptor engagement (15). This is likely to reflect differences in polarization of CD4 and CD8 cells, consistent with the lack of secretion of IL-4 by PMA + ionomycin–stimulated CD8 splenocytes (Fig. 3). We have not been able to induce UTY246–254–specific CTL proliferation when mice were injected with immature PDCs (male PDCs or peptide pulsed); therefore, we cannot comment on the CTL polarizing capacity of PDCs at different stages of maturation.

We have shown that, in the absence of viral infection, type I IFN is not required for priming because injection of CpG-matured PDCs into type I IFN receptor–deficient mice (129A) elicits functional CTL responses. In contrast, during a viral infection, the importance of PDC-derived type I IFN in initiating innate immune responses and in the cross-talk with other DC subsets for induction of adaptive immunity has been very elegantly demonstrated (37, 47). However, we cannot exclude the possibility that other recently described members of the IFN family may play a key role in PDC stimulatory capacity (48, 49), and some redundancy in the IFN system may be envisaged. Such redundancy may be implied by the observation that 129A mice have PDCs (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20031059/DC1) in contrast to what was reported for IFN consensus sequence-binding protein (a transcription factor involved in the IFN signaling pathway)–deficient mice that lack PDCs and have a defect in the activation of CD8α+ DCs (50–52). In addition, 129A PDCs mature in response to CpG treatment, although to a lower extent than their wild-type counterpart, consistent which was with what was reported previously (53–55). This level of maturation is indeed sufficient for in vivo priming of functional CTL responses, although in other experimental models, amplification of DC maturation by IFN signaling may be required for optimal regulation of the immune response (37).

Although in vivo mature PDCs can induce proliferation of functional CTLs specific for an endogenous antigen as well as for exogenously loaded peptides, they are unable to present exogenous antigens, even in the presence of maturation signals known to activate cross-presentation in MDCs (36). This observation is consistent with a recent paper showing that PDCs were far less efficient compared with other DC subsets in presenting s.c. injected Leishmania major antigens (56). Lack of presentation of exogenous antigens could be due to poor endocytic activity compared with MDCs (unpublished data; references 57, 58), or to differences in the antigen-processing and -presenting machinery between MDCs and PDCs, consistent with the different expression pattern of cathepsins in human MDCs and PDCs (59). Our results underline the heterogeneity and plasticity within the APC family, highlighted previously between CD8α+ and CD8α– MDCs (60–63). We envision that in vivo PDCs may contribute to priming antigen–specific CD8 and CD4 T cell responses by efficiently presenting endogenous antigens, whereas their role in priming T cell responses to exogenous antigens would be negligible; in this respect, differing from both CD8α+ and CD8α– MDCs. In addition, PDCs could modulate the function of other DC subsets by inducing their functional maturation and promoting cross-presentation via type I IFN secretion (64). Finally, these data validate in vivo our previous results on the ability of human PDCs to prime antigen–specific naïve T cells (5), and provide a rationale for the combined use of mature MDCs and PDCs in vaccine trials to optimize the induction of innate and adaptive immune responses.

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