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Inflammation Conditions Mature Dendritic Cells To Retain the Capacity To Present New Antigens but with Altered Cytokine Secretion Function

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Dendritic cells (DCs) are *directly* activated by pathogen-associated molecular patterns (PAMPs) and undergo maturation. Mature DCs express high levels of MHC class II molecules (“signal 1”), upregulate T cell costimulatory receptors (“signal 2”), and secrete “signal 3” cytokines (e.g., IL-12). Mature DCs efficiently present Ags linked to the activating PAMP and prime naive T cells. However, mature DCs downregulate MHC II synthesis, which prevents them from presenting newly encountered Ags. DCs can also be *indirectly* activated by inflammatory mediators released during infection (e.g., IFN). Indirectly activated DCs mature but do not present pathogen Ags (as they have not encountered the pathogen) and do not provide signal 3. Therefore, although they are probably generated in large numbers upon infection or vaccination, indirectly activated DCs are considered to play little or no role in T cell immunity. In this article, we show that indirectly activated DCs retain their capacity to present Ags encountered after maturation *in vivo*. They can also respond to PAMPs, but the previous encounter of inflammatory signals alters their cytokine (signal 3) secretion pattern. This implies that the immune response elicited by a PAMP is more complex than predicted by the examination of the immunogenic features of directly activated DCs, and that underlying inflammatory processes can skew the immune response against pathogens. Our observations have important implications for the design of vaccines and for the understanding of the interactions between simultaneous infections, or of infection in the context of ongoing sterile inflammation. *The Journal of Immunology*, 2014, 193: 3851–3859.

Dendritic cells (DCs) are found in peripheral tissues and secondary lymphoid organs displaying an *immature* phenotype characterized by high endocytic function and low expression of surface MHC class II (MHC II) and T cell costimulatory molecules, required for naive T cell priming. Immature DCs constitutively synthesize MHC II molecules, load them with peptides derived from proteins degraded in endosomes, and transiently display the resulting complexes on the plasma membrane (1). These complexes are short-lived because they are endocytosed and degraded in lysosomes, a process accelerated by

ubiquitination of the MHC II β -chain by the membrane-associated ubiquitin ligase, MARCH 1 (2, 3). The constant flow of newly generated MHC II–peptide complexes toward the cell surface via the endocytic route, and from the surface back to lysosomes for degradation, is responsible for the steady-state accumulation of MHC II molecules in endosomal compartments of immature DCs. Immature DCs also express receptors for pathogen-associated compounds (e.g., TLR ligands). Encounter of TLR ligands activates DCs and triggers a *maturation* program characterized by: 1) transient upregulation of macrocytosis/phagocytosis followed by its downregulation; 2) transient upregulation of MHC II synthesis followed by its near shutdown, a process controlled by changes in the expression of the transcription factor CIITA; 3) downregulation of MARCH 1 expression and MHC II ubiquitination; 4) upregulation of T cell costimulatory molecule expression; and 5) secretion of cytokines and chemokines that facilitate T cell priming (1–5). These features enable mature DCs to efficiently present Ags captured at the time of activation and to activate naive T cells. However, mature DCs are inefficient at presenting most forms of Ags encountered after maturation via MHC I and MHC II (6, 7), an exception being Ags captured with surface receptors (8, 9).

Immature DCs also express receptors for inflammatory molecules (e.g., type I IFN) released by DCs or other cells directly activated by pathogen-associated ligands. These molecules cause the DCs to undergo *indirect* (or *bystander*) activation (10). Indirectly activated DCs acquire a mature phenotype, increasing expression of MHC II and T cell costimulatory molecules. However, they do not secrete IL-12 in situations where directly activated DCs do (10); whether this is also true for other cytokines has not been determined. Because inflammatory compounds released at the point of infection can indirectly activate DCs at sites where the pathogen is not yet present, both directly (dir-mDCs) and indirectly

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Abbreviations used in this article: DC, dendritic cell; dir-mDC, directly activated mature DC; HEL, hen egg lysozyme; ind-mDC, indirectly activated mature DC; 2mDC, second wave of fully mature DCs; MHC II, MHC class II; OCS, OVA-coated splenocyte; PAMP, pathogen-associated molecular pattern; PIC, polyinosinic-polycytidylic acid; Tg, transgenic; WT, wild-type.

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activated mature DCs (ind-mDCs) are likely produced during infection (11, 12). The function played by the latter, if any, remains unknown. In principle, the ind-mDCs should not be able to present pathogen Ags because, by definition, these are not available at the site of indirect activation. This fact, and the observation that ind-mDCs do not secrete cytokines, has led to the logical conclusion that ind-mDCs do not participate in immunity against foreign Ags (11, 12).

In this article, we report that ind-mDCs retain their capacity to present subsequently encountered Ags and to prime naive T cells in vivo. They can also retain the capacity to secrete cytokines upon encountering TLR ligands, but their cytokine secretion profile is different from that displayed by dir-mDCs. Our results show that inflammation promotes the generation of *conditioned* DCs (ind-mDCs) still capable of presenting the same or a different pathogen to the one that triggered the inflammation, but with altered cytokine secretion profiles. This suggests that both directly and indirectly activated DCs contribute to the generation of immune responses against primary infection, and that the magnitude and/or type of immune response elicited by a pathogen can be modulated by pre-existing inflammation.

Materials and Methods

Mice

C57BL/6J, CBA (H-2^k), Ly5.1, TLR9^{-/-} (13), B6.CH-2^{bm1} (bm1), CIITA (H-2^k) (14), OT-I (15), OT-II (16), and MHCII^{-/-} (17) mice were bred under specific pathogen-free conditions at the Walter and Eliza Hall Institute and at the Bio21 Institute animal facilities. Sex- and age-matched mice (6–12 wk) were used and handled according to the guidelines of the National Health and Medical Research Council of Australia. Experimental procedures were approved by the Animal Ethics Committee, Melbourne Health Research Directorate.

Generation of mixed bone marrow chimeras and DC activation

For the generation of mouse chimeras, Ly5.1 recipient mice were gamma-irradiated (2 × 0.55 Gy) and reconstituted with 1 × 10⁶ T and B cell-depleted bone marrow cells of each relevant donor strain at a 1:1 ratio. Chimeras were then treated with 1.1 g/l neomycin (Sigma, St. Louis, MO) in the drinking water for the next 6 wk and were used for subsequent experiments after at least 8 wk from reconstitution. For activation of the DCs, mice were injected with 20 nmol CpG1668 (GeneWorks, Hindmarsh, Australia) 12 h before sacrifice. Chimerism and activation were always tested before or during experiments.

Purification of splenic DCs and generation of DCs from bone marrow precursors

Splenic DCs were isolated as described previously (18). Purity of the DC preparation was at least 75%. For further purification, the cells were fluorescently labeled and sorted using a FACSAria (Becton Dickinson) into the following subsets: CD8⁺ DCs (CD45RA⁻ CD11c⁺ CD8⁺ Sirpα⁻) and Sirpα⁺ DCs (CD45RA⁻ CD11c⁺ CD8⁻ Sirpα⁺), using the Ly5.1 or Ly5.2 markers to distinguish between the wild-type (WT; Ly5.1⁺) and TLR9^{-/-} (Ly5.2⁺) cells. Purity of the DC subsets was at least 95%. For *in vitro* experiments, DCs were incubated in DC media (RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10⁻⁴ M 2-ME) at 37°C and 10% CO₂. Where indicated, DC cultures included 25 mg/ml polyinosinic-polycytidylic acid (PIC; Sigma) or 1 μg/ml LPS. *In vitro*, DCs were generated from bone marrow precursors in culture medium supplemented with Flt3L as previously described (19).

Purification and labeling of OT-I and OT-II cells

OT-I (K^b-restricted anti-OVA_{257–264} CD8 T cells) and OT-II T cells (I-A^b-restricted anti-OVA_{323–339} CD4 T cells) were purified from pooled lymph nodes (s.c. and mesenteric) by magnetic depletion of non-CD8 or -CD4 T cells, respectively. T cells were then labeled by incubating them 10 min at 37°C in FCS-free PBS containing 0.1% BSA and 2.5 μM CFSE (Molecular Probes, Eugene, OR) or 5 μM Violet Cell Tracer (Life Technologies, Carlsbad, CA). T cell preparations were routinely >85% pure.

FACS analysis

DCs were stained with anti-CD11c (N418), anti-Sirpα (P84), anti-CD45RA (14.8), anti-CD8 (53-6.7), anti-CD86 (PO3.1), anti-MHC II (M5/114),

anti-Ly5.1 (A20), anti-Ly5.2 (104), or anti-I-A^k-hen egg lysozyme 48–62 (HEL_{48–62}; AW3.18) (7). For detection of transferred OT-I and OT-II cells, anti-CD4 (GK1.5) or anti-CD8 (53-6.7) and anti-TCR-Va2 (B20.1) were used. Propidium iodide (1 μg/ml) was added to the final cell suspension to stain dead cells. For intracellular staining, cells were incubated in the presence of GolgiPlug (Becton Dickinson) for 4–5 h, surface stained as indicated earlier, labeled with fixable viability dye (eBioscience) to stain dead cells, fixed and permeabilized with Fix/Perm kit (Becton Dickinson), and stained with anti-IFN-γ (XMG1.2) and anti-TNF (MP6-XT22; Becton Dickinson). Samples were acquired on an LSR-Fortessa or LSR-II (Becton Dickinson) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Formation of I-Ak-HEL_{48–62} complexes

DCs isolated from control or CpG-pretreated CBA mice, or generated *in vitro* from bone marrow precursors, were treated or not with 3 μg/ml CpG1668 or 1 μg/ml LPS and incubated in U-bottom 96-well plates (CoStar) with titrated amounts of HEL (Sigma) for 16 h. Cells were then stained with anti-I-A^k-HEL_{48–62} (7), washed, and analyzed by flow cytometry. Each determination was done in duplicate.

Metabolic radiolabeling

Metabolic labeling, immunoprecipitations, and SDS/PAGE analysis were performed as previously described (20). MHC II and MHC I were immunoprecipitated using a rabbit serum (JV1) (21) and monoclonal anti-MHC I (Y3), respectively.

Cytokine secretion assay

Cytokine quantification was performed as previously described (22). In brief, DCs were cultured in triplicate for 24 h, and cytokine contents in the supernatants were analyzed using a Bio-Plex cytokine kit according to manufacturer's instructions (Bio-Rad, Hercules, CA). Cytokines measured with this kit are mouse IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, Eotaxin, G-CSF, GM-CSF, IFN-γ, keratinocyte chemoattractant, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α. Only cytokines that reached detectable levels were analyzed.

Quantitative real-time PCR

Total RNA from purified cells was obtained using RNeasy Mini Kit (Qiagen, Limburg, the Netherlands). cDNA was then synthesized using SuperScript III Reverse Transcriptase (Life Technologies). SYBR green master mix (Roche) was used in quantitative PCRs on a LightCycler 480 (Roche). Expression of all molecules was normalized to housekeeping gene transferrin receptor 2.

Confocal microscopy

Protocol was adapted from Wilson et al. (23). In brief, DCs were placed on MHC II (N22 mAb)-coated coverslips. They were fixed and permeabilized, then blocked using 10% normal goat serum (Sigma) and labeled with anti-MHC II biotin (N22) and rat anti-Lamp1 (Abcam, Cambridge, U.K.). For detection, SAV-Alexa Fluor 488 (Life Technologies) and goat anti-Rat A594 (Life Technologies) were used. Samples were then stained with 5 μg/ml DAPI and mounted using Prolong Gold Antifade Reagent (Life Technologies). Images were acquired on an LSM700 confocal microscope (Zeiss, Jena, Germany) and analyzed using ImageJ software.

In vivo Ag presentation assays

Recipient mice were inoculated *i.v.* with 1 × 10⁶ Violet Cell Tracer-labeled OT-II cells and 0.1 mg OVA (Worthington, Lakewood, NJ) including or not 0.2 μg LPS (Sigma) as indicated. Sixty hours later, total splenocytes were stained with anti-CD4 and anti-TCRVα2, and resuspended in buffer containing 2.5 × 10⁴ blank calibration particles (Becton Dickinson). The total number of live dividing lymphocytes (propidium iodide⁻, Violet Cell Tracer⁴⁰) was calculated from the number of dividing cells per 5 × 10³ beads.

OVA-coated splenocytes

bm1 spleen cells were gamma-irradiated (1500 rad), washed, incubated with 10 mg/ml OVA in RPMI 1640 medium for 10 min at 37°C, and washed three times with RPMI 1640 medium supplemented with 3% FCS, to prepare cell-associated OVA.

In vitro Ag presentation assays

DCs were plated in DC media at 1 × 10⁴ cells/well in U-bottom 96-well plates (Costar, Cambridge, MA). For MHC II Ag presentation, DCs were

then pulsed for 1 h at the indicated concentration of soluble OVA (Worthington). Cells were then washed and resuspended with 5×10^4 CFSE or Violet Cell Tracer-labeled OT-II cells in DC media including 10 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ). For the cross-presentation assay, DCs were incubated in the presence of titrated amounts of OVA-coated splenocytes (OCSS) and 5×10^4 CFSE-labeled OT-I cells in DC media including 10 ng/ml GM-CSF (PeproTech). Proliferation of OT-II or OT-I T cells was determined after 60 h of culture as explained before for in vivo proliferation assays. For cytokine staining, cultures were left for 72 h and were incubated in the presence of GolgiPlug (Becton Dickinson) and 1 μ g/ml OVA_{323–356} peptide (Mimetopes, Melbourne, Australia) for 5 h before intracellular staining of the cells and analysis. Each determination was done in duplicate.

Statistical analysis

Data were plotted using GraphPad Prism (La Jolla, CA). Mann–Whitney unpaired test with two-tailed *p* values and 95% confidence intervals and two-way ANOVA test were used for all statistical analyses as indicated.

Results

Simultaneous generation of directly and indirectly activated DCs in vivo

To compare the functional properties of dir-mDCs and ind-mDCs generated in vivo within the same mice, we produced mixed bone marrow chimeras where lethally irradiated WT recipients were reconstituted with a 1:1 ratio of Ly5.1 WT and Ly5.2 TLR9^{-/-} bone marrow. The chimeric animals contained equivalent numbers of WT and TLR9^{-/-} DCs (Supplemental Fig. 1), which expressed similar levels of the classical maturation markers CD40, CD86, and MHC II (Fig. 1A). i.v. injection of the TLR9 ligand CpG induced direct activation of the WT DCs within 12 h, becoming dir-mDCs with high expression of the maturation markers (Fig. 1A). The TLR9^{-/-} DCs of these same mice also acquired a mature phenotype (ind-mDCs), in this case, because of release of inflammatory mediators produced by cells that responded to CpG (Fig. 1A). However, as previously shown (10), ind-mDCs did not secrete IL-12, whereas dir-mDCs did (Fig. 1B). This was also the case for IL-6, MIP-1 α , and RANTES (Fig. 1B). Secretion of 12 additional cytokines and chemokines also showed marked differences between ind-mDCs and dir-mDCs, although these differences did not achieve statistical significance (Fig. 1B).

Indirectly activated DCs can present Ags encountered after maturation

We have previously shown that normal mice cannot mount anti-OVA CD4 T cell responses if they are inoculated with CpG i.v. at least 9 h before immunization (7). This is because CpG causes direct activation of DCs systemically, and by the time of immunization they have already acquired a mature phenotype incapable of presenting the newly encountered OVA Ag (7). We tested whether this was also the case in mouse bone marrow chimeras reconstituted only with WT (WT:WT) or with both WT and TLR9^{-/-} (WT:TLR9^{-/-}) bone marrow (Fig. 2A). The mice were injected or not with CpG and 12 h later they were immunized with OVA plus LPS. MHC II Ag presentation was monitored by the adoptive transfer of Violet Cell Tracer-labeled anti-OVA, I-A^b-restricted OT-II cells. As expected, the OT-II cells proliferated in mice that had not received CpG before immunization, but they did not in WT:WT bone marrow chimeric mice pretreated with CpG (Fig. 2A) because of impaired Ag presentation by the systemically activated dir-mDCs. In contrast, CpG pretreatment did not prevent OT-II proliferation in WT:TLR9^{-/-} mixed bone marrow chimeras (Fig. 2A). This suggested that the TLR9^{-/-} ind-mDCs contained in these animals were still able to present OVA.

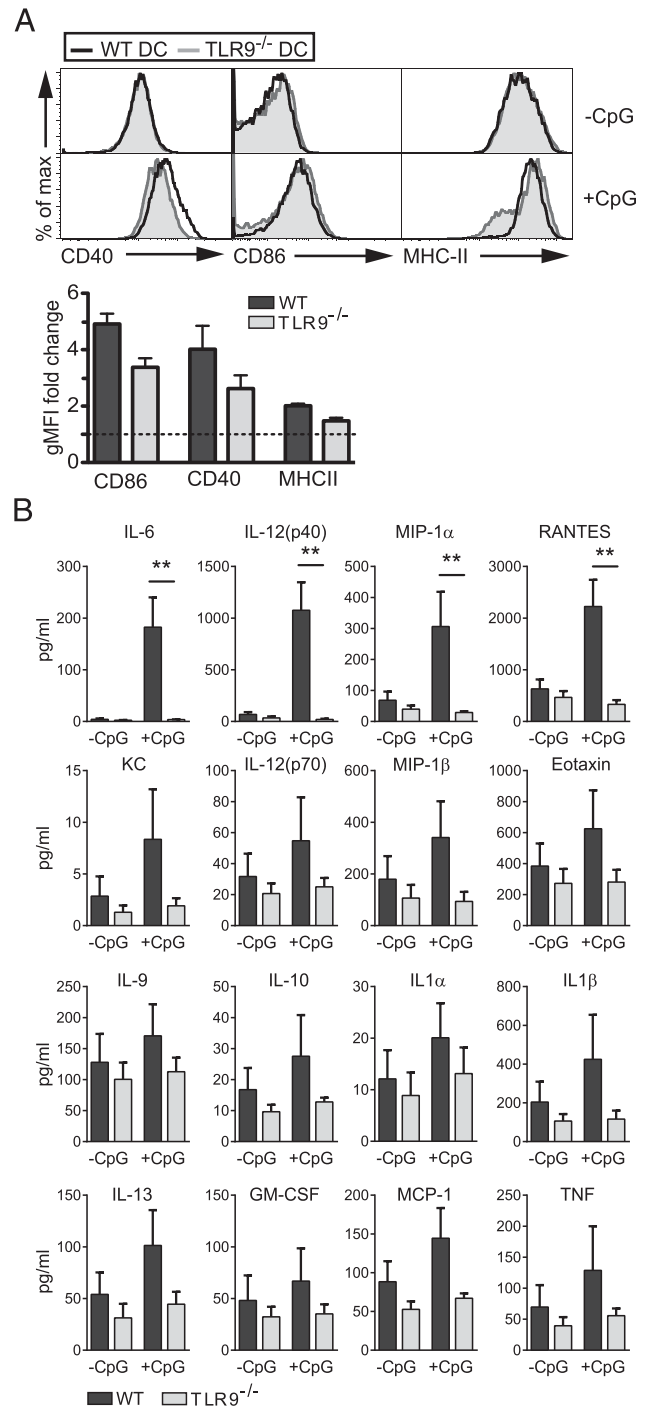


FIGURE 1. Indirect DC activation in vivo elicits upregulation of costimulatory molecules, but not cytokine production. WT:TLR9^{-/-} mixed bone marrow chimeras were injected or not with CpG, and 12 h later DCs were purified. (A, top panel) Representative histograms showing cell-surface expression of CD40, CD86, and MHC II of WT (Ly5.1⁺, solid black line) and TLR9^{-/-} (Ly5.2⁺, gray filled histogram) DCs from untreated (top) or CpG-treated (bottom) mice. (A, bottom panel) Geometric mean fluorescence intensity fold increase in CD86, CD40, and MHC II expression in WT or TLR9^{-/-} DCs as compared with their counterparts from untreated animals. Graph shows pooled data from nine experiments for CD86 and three experiments for CD40 and MHC II. (B) WT (Ly5.1⁺, dark bars) and TLR9^{-/-} (Ly5.2⁺, light bars) DCs were purified from untreated (-CpG) or CpG-treated (+CpG) mice as indicated and cultured. Cytokine secretion was quantitated 24 h later by a Bio-Plex cytokine assay. Data are shown as the mean of four independent experiments. Error bars indicate SE. ***p* < 0.01, Mann–Whitney unpaired test.

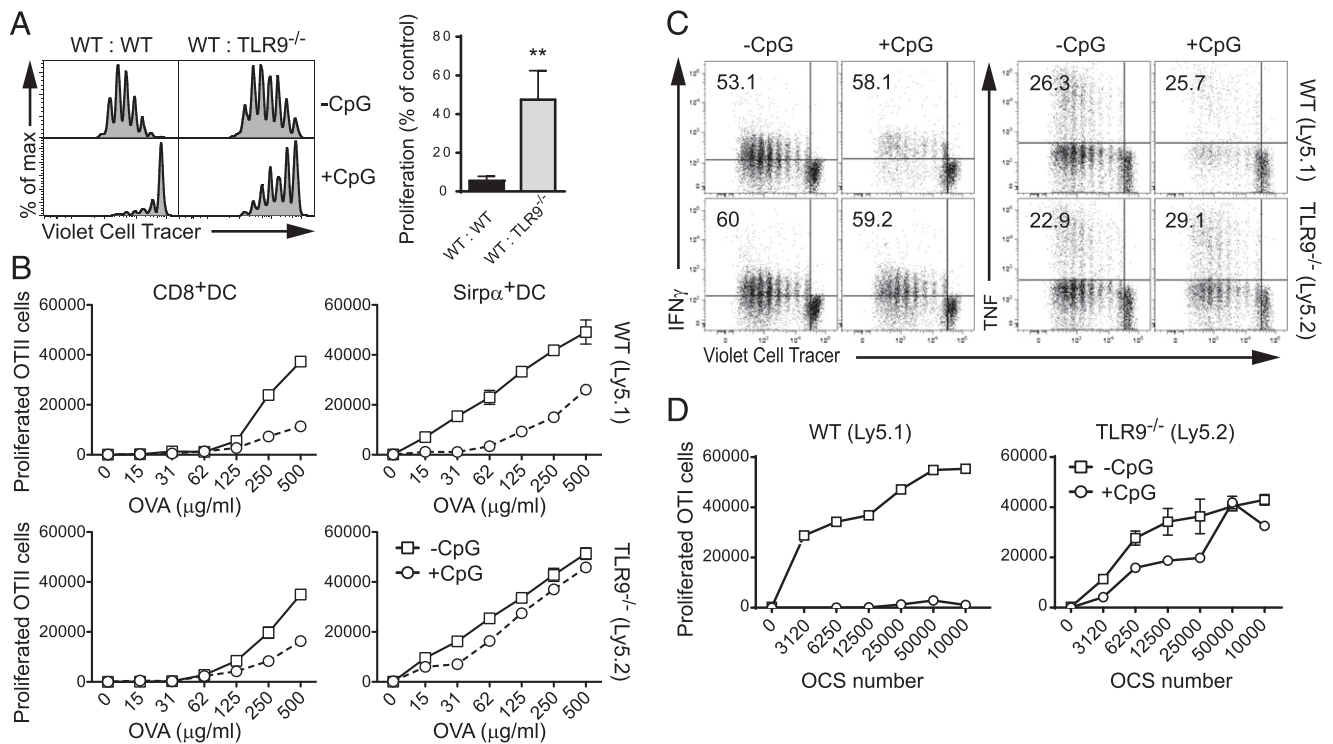


FIGURE 2. Ind-mDCs present newly found exogenous Ag in the context of MHC I and MHC II. **(A)** WT:WT or WT:TLR9^{-/-} mixed bone marrow chimeras were injected or not with CpG, and 12 h later mice were injected with soluble OVA (100 μg), LPS (0.2 μg), and Violet Cell Tracer–labeled OT-II cells (1 × 10⁶ cells). OT-II division in the spleen was assessed 60 h later by flow cytometry. *Left panel*, Representative histograms gated on Violet Cell tracer⁺ CD4⁺ TCRVa2⁺ cells. *Right panel*, Proliferation of OT-II cells is shown as percentage of the maximum proliferation for each chimera. Data shown are from a pool of different experiments. *n* = 7 for WT:WT chimeras; *n* = 12 for WT:TLR9^{-/-} chimeras. Data are shown as mean ± SE. ***p* < 0.01, Mann–Whitney unpaired test. **(B–D)** WT:TLR9^{-/-} mixed bone marrow chimeras were injected (circles) or not (squares) with CpG. Twelve hours later, WT (Ly5.1⁺) or TLR9^{-/-} (Ly5.2⁺), CD8⁺ or Sirpα⁺ DCs were purified. **(B)** DC were incubated with different concentrations of soluble OVA, washed, and incubated with CFSE-labeled OT-II cells for 60 h. Data represent the mean ± SE of the number of proliferated OT-II cells. *Left graphs*, CD8⁺ DCs. *Right graphs*, Sirpα⁺ DCs. *Upper graphs*, WT DCs. *Lower graphs*, TLR9^{-/-} DCs. Results are representative of three independent experiments. **(C)** Purified Sirpα⁺ DCs were incubated with soluble OVA, washed, and incubated with Violet Cell Tracer–labeled OT-II cells for 72 h. GolgiPlug was added for the last 4 h of incubation. OT-II cells were intracellularly stained for IFN-γ (*left*) and TNF-α (*right*). Numbers indicate the proportion of cytokine-producing cells after gating out nonproliferating cells. Results are representative of three independent experiments. **(D)** CD8⁺ DCs were incubated with different concentrations of OCSs and incubated with CFSE-labeled OT-I cells for 60 h. Data represent the mean ± SE of the number of proliferated OT-I cells. *Left graph*, WT DCs. *Right graph*, TLR9^{-/-} DCs. Results are representative of two independent experiments. Squares represent DCs purified from untreated mice; circles represent DCs from CpG-treated mice.

It should be appreciated that in the WT:TLR9^{-/-} mixed bone marrow chimeras treated with CpG, only half of the DCs (the ind-mDCs) were potentially capable of Ag presentation, so induction of OT-II proliferation in these animals could not be expected to be as efficient as in nontreated controls in which all of the DCs were fully functional. Nevertheless, it was possible that the systemic inflammation induced by CpG caused additional effects that limited the capacity of the DCs to capture Ag, or to interact with OT-II cells in vivo, so we also measured Ag presentation in vitro. This allowed us to compare the effect of CpG on the two major populations of conventional DCs, distinguished by their expression of CD8 (CD8⁺ DCs) and Sirpα (Sirpα⁺ DCs), respectively, because the latter play a more prominent role in MHC II presentation (24, 25).

We purified WT or TLR9^{-/-} CD8⁺ and Sirpα⁺ DCs from WT:TLR9^{-/-} mixed bone marrow chimeras that had been left untreated or injected i.v. with CpG 12 h previously. The four DC populations were incubated in vitro with soluble OVA and CFSE-labeled OT-II T cells, and OT-II proliferation was measured 60 h later (Fig. 2B). Both WT and TLR9^{-/-} DCs from untreated animals were immature at the time of purification. These DCs underwent spontaneous maturation in vitro during the Ag presentation assay (26) and induced OT-II proliferation similarly (Fig. 2B). As shown previously, the Sirpα⁺ DCs were more

efficient than the CD8⁺ DCs (24, 25). The DCs purified from CpG-treated animals were already mature (Fig. 1) and, as expected, the dir-mDCs (WT) had impaired capacity to present newly encountered OVA (Fig. 2B). However, Sirpα⁺ ind-mDCs (TLR9^{-/-}) maintained their Ag presentation capacity almost intact (Fig. 2B). Because the dominant DC subtype involved in MHC II presentation in vivo is the Sirpα⁺ DCs, the results of these in vitro experiments explain the incomplete but significant maintenance of MHC II presentation in CpG-treated WT:TLR9^{-/-} chimeric mice, where half the DCs were ind-mDCs.

We also assessed IFN-γ and TNF production by T cells that were activated by dir-mDC and ind-mDC Sirpα⁺ DCs in the Ag presentation assay in vitro. The percentage of proliferating OT-II cells that produced these cytokines was similar in all cases (Fig. 2C). Therefore, the main difference between dir-mDCs and ind-mDCs appeared to be at the level of Ag presentation, which determined how many naive T cells were primed and started to proliferate (Fig. 2B), but the rate of proliferation and differentiation of the primed T cells was similar regardless of which type of mDC caused the priming.

Indirect activation seemed to have a stronger effect on MHC II presentation by CD8⁺ than by Sirpα⁺ DCs (Fig. 2B). A possible

explanation for this difference was that inflammatory cytokines caused full activation of CD8⁺ DCs but not Sirpα⁺ DCs. Alternatively, it could be that because CD8⁺ DCs are inherently less capable of MHC II presentation, a small reduction in this capacity appeared to have a bigger overall impact on this population. We investigated this question further by assessing MHC I cross-presentation of cell-associated Ag, an activity restricted to the CD8⁺ DC population (27, 28). We purified WT or TLR9^{-/-} CD8⁺ DCs from WT:TLR9^{-/-} mixed bone marrow chimeras that had been left untreated or injected i.v. with CpG 12 h previously. The four DC populations were incubated in vitro with OCSs and CFSE-labeled OT-I cells, and proliferation was measured 60 h later (Fig. 2D). Immature WT and TLR9^{-/-} DCs showed a similar capacity to cross-present OCSs. As shown previously (6), dir-mDC (WT) obtained from mice pretreated with CpG failed to cross-present (Fig. 2D). However, the ind-mDCs (TLR9^{-/-}) retained most of their capacity to phagocytose and cross-present cell-associated Ag (Fig. 2D). These results support the conclusion that CD8⁺ DCs suffered only partial loss of their capacity to capture and (cross)present Ags via MHC I and II, although the relative effect on MHC II presentation was more pronounced.

MHC II Ag presentation is regulated in DCs chiefly by controlling MHC II synthesis

We have previously attributed the impairment in MHC II Ag presentation in mDCs to downregulation of MHC II synthesis (7), but formal demonstration that this is the major mechanism has been lacking. MHC II production is transcriptionally regulated by CIITA, whose expression is driven in DCs by a promoter that is silenced in the mature state (29). We examined MHC II Ag presentation by DCs from CBA (H-2^k) mice that, in addition to the endogenous CIITA, also express CIITA as a transgene controlled by an independent, constitutive promoter (14). These DCs upregulate surface MHC II expression during maturation like non-transgenic (non-Tg) DCs, but maintain MHC II synthesis in their mature state (Fig. 3A, 3B). We injected WT or CIITA Tg mice with CpG, and 12 h later purified their splenic DCs (which at this stage were dir-mDCs). Immature DCs were also purified from noninjected mice. The four groups of DCs were incubated for 12 h in vitro with HEL, and formation of I-A^k molecules loaded with the HEL₄₈₋₆₂ peptide was determined by FACS using the mAb AW3.18 (30). The magnitude of staining increased above background level in both WT and CIITA Tg DCs that were immature at the beginning of the assay, indicative of formation of I-A^k-HEL₄₈₋₆₂ complexes during the incubation in vitro (Fig. 3B, 3C). The WT dir-mDCs did not generate such complexes as expected, but CIITA Tg dir-mDCs did (Fig. 3B, 3C). Similar results were obtained with DCs generated in vitro from bone marrow precursors in the presence of Flt3L, activated with either CpG or LPS (Supplemental Fig. 2). Therefore, the impairment in MHC II presentation in dir-mDCs is primarily caused by shutdown of MHC II production (7, 20, 31) and can be rescued by sustaining MHC II synthesis.

The ind-mDCs maintain MHC II and MARCH1 expression

Next, we compared MHC II production in dir-mDCs and ind-mDCs purified from untreated or CpG pretreated mixed bone marrow (WT:TLR9^{-/-}) chimeras. MHC II protein synthesis by dir-mDC (WT) was nearly shut down, but ind-mDCs (TLR9^{-/-}) from these same mice synthesized similar amounts to their immature DC counterparts (Fig. 4A). As shown previously, MHC I synthesis was upregulated in mature DCs, and this was the case for both dir-mDCs and ind-mDCs (Fig. 4A).

The differences in MHC II expression between dir-mDCs and ind-mDCs were also evident at the transcription level, examined by quantitative real-time PCR (Fig. 4B), and correlated with changes in

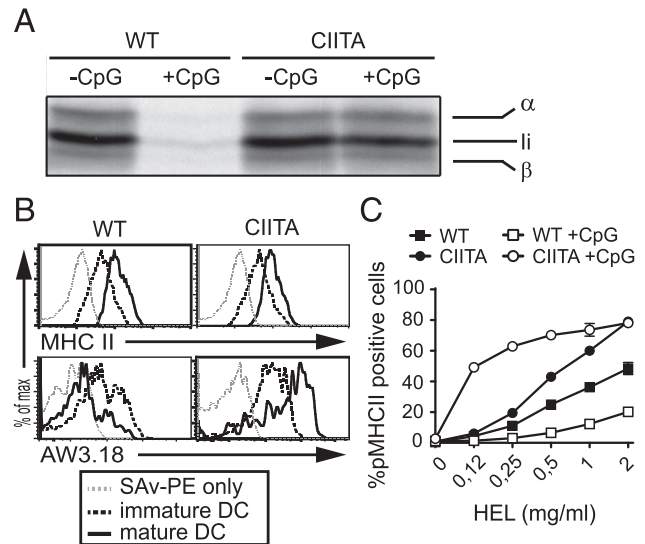


FIGURE 3. CIITA overexpression rescues the capacity of mature DCs to present Ag via MHC II. (A) DCs were isolated from WT or CIITA Tg mice and were immediately metabolically labeled after purification (-CpG) or after 18 h of culture in vitro in the presence of CpG (+CpG). MHC II molecules were then immunoprecipitated from normalized amounts of radiolysate and analyzed by SDS-PAGE. Results are representative of two independent experiments. (B and C) WT or CIITA Tg mice were injected with CpG or left untreated. Twelve hours later, DCs were isolated and cultured for 16 h in the presence of titrated amounts of HEL protein. Expression of MHC II and I-A^k-HEL₄₈₋₆₂ complexes were then measured by staining with AW3.18 mAb. (B) Representative histograms of MHC II (top) and AW3.18 (bottom) staining. Dotted gray lines represent background staining without primary Ab; black dashed lines represent staining of DCs from untreated mice; black solid lines represent staining of DCs from CpG-treated mice. (C) Graph showing the levels of AW3.18 staining after incubating with titrated amounts of HEL protein. Results are representative of three independent experiments. Error bars indicate SE.

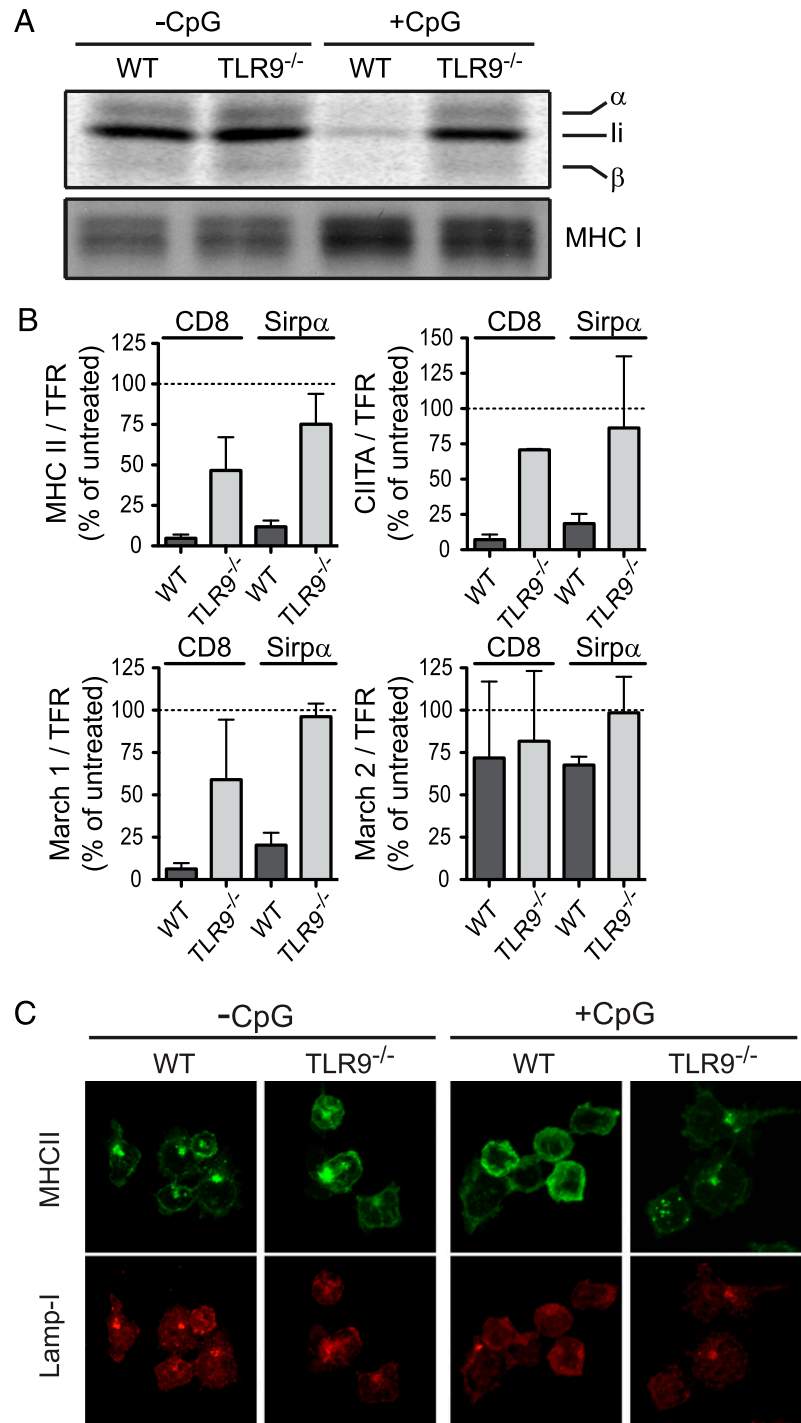
CIITA transcription (Fig. 4B). Directly activated mDCs also downregulate transcription of MARCH 1 (32, 33), a ubiquitin ligase that enhances turnover of MHC II-peptide complexes, but this was not the case in indirectly activated ind-mDCs (Fig. 4B). Expression of MARCH 2 was examined as a negative control, and indeed no differences were observed in MARCH 2 transcription between dir-mDCs and ind-mDCs (Fig. 4B). We observed that the transcription of MHC II, CIITA, and MARCH 1 was slightly more downregulated in CD8⁺ ind-mDCs than in their Sirpα⁺ counterparts, explaining the differential capacity of the two DC subsets to present Ag via MHC II encountered after indirect activation (Fig. 2B).

In immature DCs, continuous formation of new MHC II-peptide complexes, followed by transient cell-surface expression, ubiquitination, and delivery to lysosomes for degradation, causes a characteristic accumulation of MHC II in intracellular Lamp⁺ compartments (Fig. 4C). After direct activation of WT DCs by in vivo CpG treatment, MHC II was localized at the cell surface in dir-mDCs, but ind-mDCs displayed intracellular MHC II (Fig. 4C). We conclude that, despite the increased surface expression of MHC II and other characteristic markers of DC maturation, the MHC II Ag presentation pathway of ind-mDCs remains essentially as in immature DCs.

Pathogen-associated molecular pattern recognition by ind-mDCs induces a distinct cytokine secretion pattern

Next, we tested whether ind-mDCs are still able to recognize and respond to newly found pathogen-associated signals. WT:TLR9^{-/-} mixed bone marrow chimeras were left untreated or were injected

FIGURE 4. Indirect DC activation does not alter MHC II synthesis, transcription, and intracellular localization. WT:TLR9^{-/-} mixed bone marrow chimeras were injected or not with CpG. **(A)** WT (Ly5.1⁺) and TLR9^{-/-} (Ly5.2⁺) DCs were purified 12 h later, and MHC II synthesis was assessed by metabolic labeling. MHC II (*top*) or MHC I (*bottom*) were immunoprecipitated from normalized amounts of radiolysate and analyzed by SDS-PAGE. Results are representative of two independent experiments. **(B)** WT (Ly5.1⁺) or TLR9^{-/-} (Ly5.2⁺), CD8⁺ or Sirpα⁺ DCs were purified 12 h after CpG injection. Quantitative real-time PCR analysis of MHC II (*top left*), CIITA (*top right*), MARCH 1 (*bottom left*), and MARCH 2 (*bottom right*) are shown as percentage of the expression levels from identical subsets from untreated mice. All data were previously normalized to the expression of the housekeeping gene transferrin receptor (TFR). Bars represent the mean ± SE of two independent experiments. **(C)** Twelve hours after CpG inoculation, WT (Ly5.1⁺) and TLR9^{-/-} (Ly5.2⁺) DCs were purified, stained for MHC II (green) and LAMP-1 (red), and examined by confocal microscopy (original magnification ×100). Data are representative of two independent experiments.



with CpG to induce formation of dir-mDCs and ind-mDCs. After 12 h, these mice were immunized with OVA with or without LPS. The immunized mice also received CFSE-labeled OT-II cells to measure T cell proliferation *in vivo*. We observed little OT-II proliferation in mice that were immunized with OVA alone, but extensive proliferation in mice immunized with OVA plus LPS (Fig. 5A). This was the case both in untreated mice and in mice that had been pretreated with CpG. Because the only DCs capable of Ag presentation in CpG-pretreated mice were the ind-mDCs (Fig. 2), this result indicated that ind-mDCs responded to LPS, which increased their capacity to prime OT-II cells.

To further characterize how indirect activation affected the capacity of DCs to respond to a subsequent encounter of TLR

ligands, we purified immature WT and TLR9^{-/-} DCs from untreated WT:TLR9^{-/-} mixed bone marrow chimeras, and dir-mDCs and ind-mDCs from chimeras injected 12 h prior with CpG. The four groups of DCs were then incubated *in vitro* with LPS or PIC. After 24 h, we measured the secretion of cytokines and chemokines. The pattern of cytokine secretion induced by LPS on immature WT (Supplemental Fig. 4A) and TLR9^{-/-} (Fig. 5B) DCs was comparable. Secretion of cytokines by WT cells that first encountered CpG *in vivo* and subsequently LPS *in vitro* could be attributed to a direct effect of the first or the second TLR ligand (Supplemental Fig. 4A). The most striking result was observed with ind-mDCs (TLR9^{-/-}) stimulated *in vitro* with LPS, which secreted much higher levels of

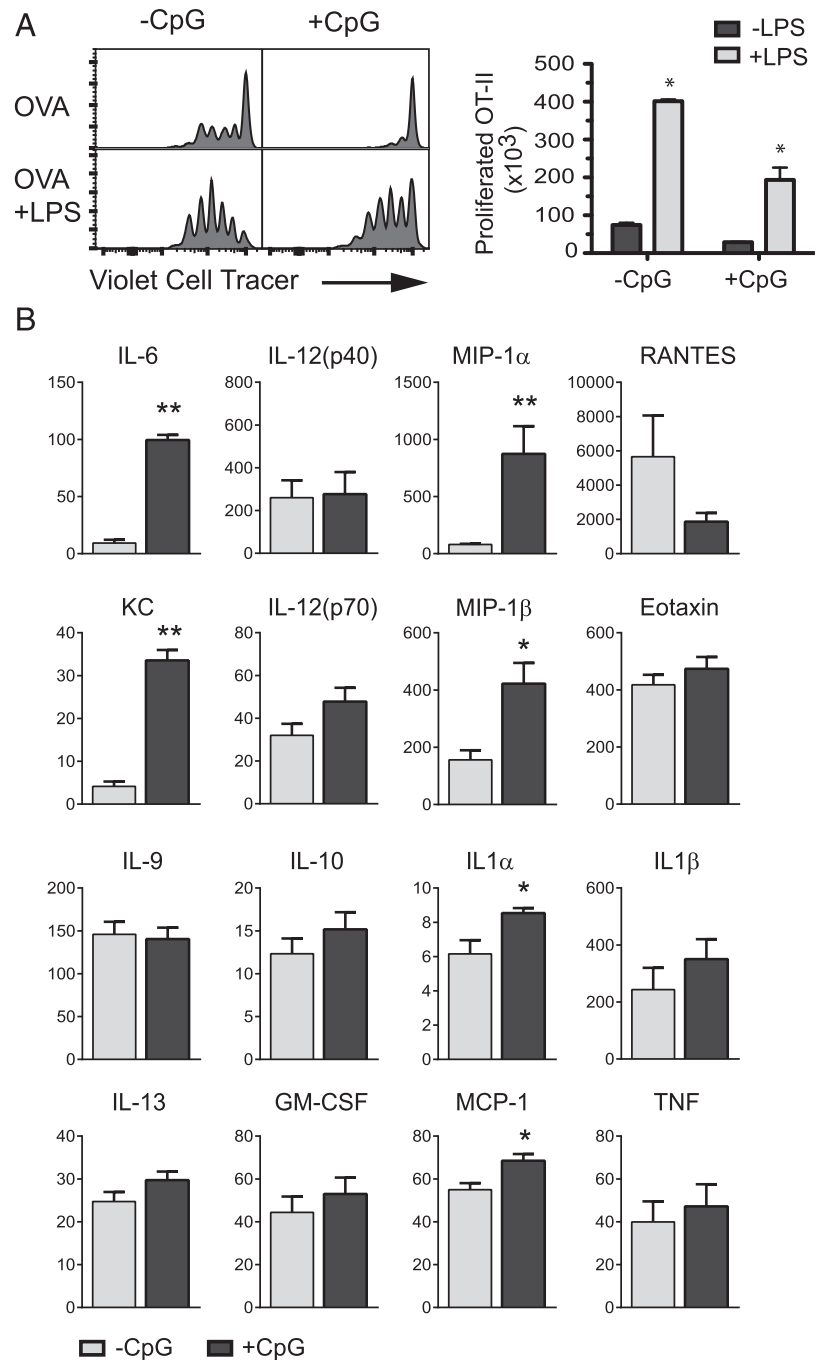


FIGURE 5. LPS recognition by ind-mDCs induces a differential cytokine secretion pattern. **(A)** WT: TLR9^{-/-} mixed bone marrow chimeras were injected or not with CpG. Twelve hours later, mice were injected with Violet Cell Tracer-labeled OT-II cells (1 × 10⁶ cells) and soluble OVA (100 μg) with or without LPS (0.2 μg). Proliferation of OT-II cells in the spleen was assessed 60 h later. *Left panel*, Representative histograms gated on Violet Cell tracer⁺ CD4⁺ TCRVα2⁺ cells. *Right panel*, Graph showing number of proliferated OT-II cells. *n* = 3 mice/group. Data are shown as the mean ± SE. **p* < 0.05 by two-way ANOVA. Experiment shown is representative of two independent experiments. **(B)** WT:TLR9^{-/-} mixed bone marrow chimeras were injected or not with CpG. Twelve hours later, TLR9^{-/-} DCs were purified, cultured for 24 h in the presence of LPS, and cytokines accumulated in the supernatant were measured by a Bio-Plex cytokine assay. Light bars represent DCs from untreated chimeras (-CpG); dark bars represent DCs from CpG-injected chimeras (+CpG). Data are shown as the mean of four independent experiments. Error bars indicate SE. **p* < 0.05, ***p* < 0.01, Mann-Whitney unpaired test.

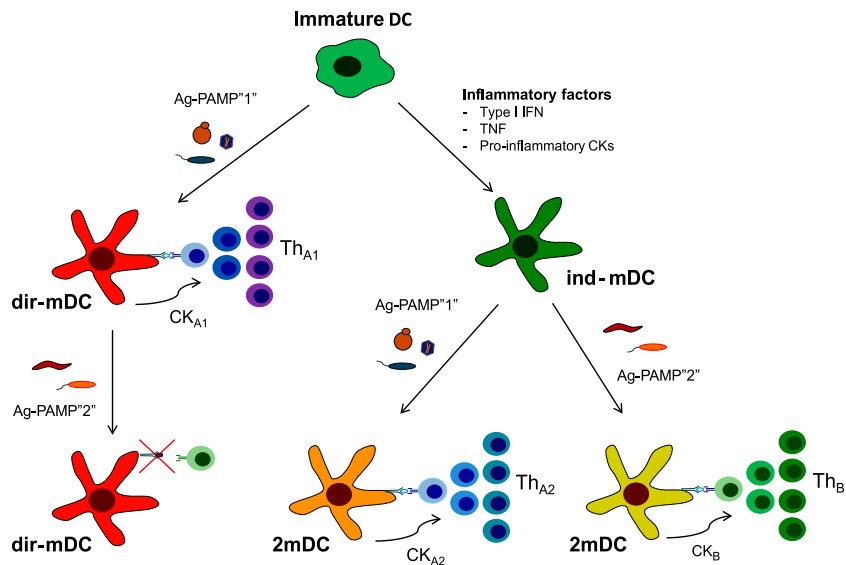
IL-6, MIP-1α, MIP-1β, and keratinocyte chemoattractant than their immature counterparts (Fig. 5B). IL-1α and MCP-1 were also secreted at statistically significant higher levels by the ind-mDCs, although the magnitude of the difference was not dramatic (Fig. 5B). In contrast, secretion of RANTES in response to LPS appeared inhibited in ind-mDC, although the difference did not reach statistical significance (Fig. 5B). These results show that although inflammatory stimuli were not sufficient to induce cytokine secretion by ind-mDCs (Fig. 1B), they conditioned these cells to change their pattern of cytokine secretion in response to subsequent encounter of a pathogen-associated ligand such as LPS. Conditioning did not affect the response to all TLR ligands. The pattern of cytokine secretion induced by PIC in WT and immature TLR9^{-/-} DCs was comparable and different from that induced by LPS (Supplemental

Figs. 3 and 4B), but this was little altered by pre-exposure of TLR9^{-/-} DCs to inflammatory cytokines in vivo (Supplemental Fig. 3).

Discussion

Pathogens can simultaneously induce two DC maturation pathways in vivo. DCs that directly encounter the pathogen become dir-mDCs; those responding to secondary inflammatory signals become ind-mDCs. It is well established that the function of dir-mDCs is to present the pathogen Ags and to prime naive T cells, but the function of ind-mDCs has been less clear (11, 12). In this article, we showed that ind-mDCs retain their capacity to present Ags encountered after the onset of maturation, both through MHC II and (for CD8⁺ DC) MHC I. We also showed that ind-mDCs can secrete T cell stimulatory cytokines in response to TLR ligands,

FIGURE 6. Functional properties of dir-mDCs, ind-mDCs, and 2mDC. See *Discussion* for details.



but their pattern of cytokine secretion was altered by the conditioning effect of the inflammatory signals.

Downregulation of MHC II synthesis and turnover enable dir-mDCs to present for long periods pathogen Ags captured in the mature state, but also prevents presentation of newly encountered Ags (7). Our analysis of DCs from CIITA Tg animals formally proved that downregulation of MHC II transcription and protein synthesis is the main mechanism responsible. Conversely, continued CIITA, MHC II, and MARCH 1 expression enable ind-mDCs to present newly encountered Ags. Similar conclusions were reached by Simmons and colleagues (34) in studies of the effect of inflammatory signals on DCs in vitro, and by Lutz (35) using cultured human DCs. Our results demonstrate for the first time, to our knowledge, that this is also the case in vivo and may explain why, in apparent contradiction to our own results, some reports concluded that mature DCs generated in vivo by TLR ligand inoculation retained their capacity to present new Ags (36). In these studies, the dose of TLR ligand used or the route of inoculation may have led to generation of both dir-mDCs and ind-mDCs, with the latter retaining their capacity for MHC II presentation.

CD8⁺ and Sirpa⁺ DCs are functionally distinct (37). CD8⁺ DCs have a high cross-presenting capacity and are the main producers of IL-12, whereas Sirpa⁺ DCs show better capacity to present Ags in the context of MHC II to CD4 T cells (24, 27). Our results show that this functional dichotomy is further exacerbated by indirect activation, because ind-mDCs CD8⁺ became less capable of Ag presentation via MHC II compared with their immature counterparts, but their capacity for MHC I cross-presentation remained largely intact.

By definition, ind-mDCs have not encountered, and therefore do not present, the pathogen Ag that triggered their differentiation (11, 12), but they may contribute to present that same pathogen if they encounter it subsequently. This implies that during any infection, the pathogen may be presented to naive CD4 T cells by two “waves” of mature DCs: first dir-mDCs and then ind-mDCs that later encounter the pathogen and undergo further differentiation. We term this second wave of fully mature DCs *2mDC* (Fig. 6). Importantly, the cytokines secreted by dir-mDCs and 2mDC may be different, and so may be the type of CD4 Th response they elicit. The result may be a more heterogeneous Th response than would be expected if only dir-mDCs participated in Ag presentation (Fig. 6). This conclusion may have implications for vaccine design. For example, a vaccine consisting of an

Ag coupled to CpG may induce secretion of a limited number of cytokines by DCs in vitro (e.g., IL-12), but in vivo that same vaccine may elicit other cytokines, which, in turn, may induce the formation of Th cells with unforeseen polarity. Another important implication of our results is that an ongoing immune response against a pathogen may influence the response against a coinfection (12). Likewise, underlying inflammatory processes may affect the type of immune response against an infection (12).

To evaluate the contribution of dir-mDCs, ind-mDCs, and 2mDC to immune responses in the scenarios outlined earlier, it will be important to identify differentially expressed molecules that may enable their discrimination and enumeration. At present, cytokine secretion and expression of Ag presentation genes are the only parameters that enable distinction of dir-mDCs and ind-mDCs, but they are not reliable for accurate quantitation. Definition of suitable surface markers for FACS analysis is now an important goal that will facilitate full characterization of the complex DC network in the steady-state and in different scenarios of infection and/or inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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