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Monocyte-Derived Dendritic Cells Promote Th Polarization, whereas Conventional Dendritic Cells Promote Th Proliferation

Kevin V. Chow,*‡ Andrew M. Lew,*‡§ Robyn M. Sutherland,*‡§ and Yifan Zhan*‡§

Monocyte-derived dendritic cells (moDCs) dramatically increase in numbers upon infection and inflammation; accordingly, we found that this also occurs during allogeneic responses. Despite their prominence, how emergent moDCs and resident conventional DCs (cDCs) divide their labor as APCs remain undefined. Hence, we compared both direct and indirect presentation by murine moDCs versus cDCs. We found that, despite having equivalent MHC class II expression and in vitro survival, moDCs were 20-fold less efficient than cDCs at inducing CD4+ T cell proliferation through both direct and indirect Ag presentation. Despite this, moDCs were more potent at inducing Th1 and Th17 differentiation (e.g., 8-fold higher IFN-γ and 2-fold higher IL-17A in T cell cocultures), whereas cDCs induced 10-fold higher IL-2 production. Intriguingly, moDCs potently reduced the ability of cDCs to stimulate T cell proliferation in vitro and in vivo, partially through NO production. We surmise that such division of labor between moDCs and cDCs has implications for their respective roles in the immune response. The Journal of Immunology, 2016, 196: 624–636.

Dendritic cells (DCs) are heterogeneous and can be divided into subsets based on selective characteristics including appearance, location, phenotype, transcription factors, function, and ontogeny (1, 2). For example, plasmacytoid DCs have a smooth surface, are located ubiquitously, express E2-2 transcription factor, siglec-H, B220, BDCA-2, and TLR-7 and abundantly secrete type 1 IFN (3, 4), whereas CD8+ cDCs have a dendritic appearance, are located in lymphoid tissue, express ID2 and BATF3 transcription factors Clec-9A, XCR1, and TLR-3, and produce IL-12 and IFN-γ. CD8+ cDCs are highly efficient at presenting exogenous Ag on MHC class I (6), whereas CD8– cDCs are specialized at priming CD4+ T cells (7).

Unlike other DC populations, moDCs are inconspicuous in the steady state and only become prominent during inflammation (8, 9). They are recruited from Ly6C+ inflammatory monocytes via a CCR2-dependent process (8, 10, 11) and are prominent in the immune response to infections such as Listeria (8, 12), Leishmania (9), Brucella (13), Trypanosoma (14), and Salmonella (15). They have been implicated in experimental autoimmune encephalomyelitis (EAE) (16, 17) and in the regulation of gastrointestinal production of IgA through interactions with microflora (18). Of note, an additional, unique subset of moDCs expressing DC-specific intercellular adhesion molecule–3–grabbing nonintegrin (SIGN) has been reported to accumulate in skin-draining lymph nodes (but not in other lymph nodes or the spleen) after LPS stimulation (but not other inflammatory stimuli). These DC-SIGN–positive moDCs were found to be efficient at presenting Ags to both CD4+ and CD8+ T cells (19). Its relationship to the conventional, well-described Ly6C+ moDCs that arise in systemic bacterial infection (8) remains unclear.

Reports on how moDCs interact with and regulate T cell responses are conflicting. As previously mentioned, moDCs in some studies have been reported to be effective at priming T cells to proliferate (19), whereas moDCs in other studies can be redundant (8) or even unable to drive T cell proliferation (15). Differences in the type of Th polarization by moDCs have also been found to vary depending on the inflammatory stimuli. For example, during Leishmania and influenza infection, moDCs induced Th1 (9, 11), whereas during dust mite allergy, they induce Th2 (20) and in EAE, rheumatoid arthritis, and cancer they induce Th17 (17, 21, 22). In addition to influencing adaptive immune responses, a role for moDCs in the innate response against invading pathogens has also been shown. For example, TNF-α and inducible NO synthase (iNOS) produced by moDCs have been shown to be bactericidal in Listeria infection (8). Whether the variations in these findings reflect the subphenotypes, origin, location, activation state and inciting condition remains moot.
The role of moDCs in the setting of organ transplantation is poorly defined. The possibility that transplant-induced inflammation could promote the differentiation of monocytes into moDCs has led some to hypothesize that they may be important in the rejection of transplanted allografts. For example, one report suggests that monocytes could innately recognize and mediate a local inflammatory response against allogeneic Ag deposited in the skin (23). In a subsequent study, the majority of tissue-infiltrating DCs in heart allografts were found to be C<sub>X</sub>C<sub>R</sub>R<sub>C</sub>1 positive, suggesting a monocytic origin (24). Interestingly, these moDCs were found to arise specifically in response to the allograft and not to transplanted related inflammation, as was presumed previously. Nevertheless, how moDCs respond to allogeneic Ags remains unclear.

DCs can capture exogenous Ag and present antigenic peptides on self-MHC (25–27). This is termed the indirect pathway of Ag presentation. In the transplantation setting or when pathogens infect DCs, an additional pathway for DC-T cell interaction exists, where DCs capable of directly stimulating host T cells present endogenously generated peptides (28). This is termed the direct pathway. How moDCs and cDCs fulfill their roles as APCs through these two pathways have not been carefully examined.

Whereas cDCs are located in lymphoid organs (where primary immune responses occur), moDCs are found in both lymphoid and parenchymal tissues (where secondary immune responses can occur). As such, we wondered whether their Ag presentation function might be different. In this study, we aimed to better define the role of moDCs in direct and indirect Ag presentation and compared moDCs and cDCs for their capacity to stimulate CD4<sup>+</sup> T cells both numerically and qualitatively in their capacity to drive Th differentiation. Furthermore, we also interrogated how these two subsets coordinate to induce T cell responses. Overall, the two DC subsets exhibit great functional diversity in the induction of the T cell response: cDCs as initiators predominantly of proliferation and moDCs as initiators predominantly in differentiation and as regulators.

Materials and Methods

Mice and bone marrow chimeras

C57BL/6 (wild-type [WT]), GM-CSF transgenic (29), OT-II (30), CCR2.DTR (31), B6.C-H<sup>2<sub>bm1</sub></sup> (C57BL/6 with the bm1 mutation of H-2<sup>K<sub>bm1</sub></sup>), and BALB/c mice were bred under specific pathogen-free conditions compared moDCs and cDCs for their capacity to stimulate CD4<sup>+</sup> T cells. Whereas cDCs are located in lymphoid organs (where primary immune responses occur), moDCs are found in both lymphoid and parenchymal tissues (where secondary immune responses can occur). As such, we wondered whether their Ag presentation function might be different. In this study, we aimed to better define the role of moDCs in direct and indirect Ag presentation and compared moDCs and cDCs for their capacity to stimulate CD4<sup>+</sup> T cells both numerically and qualitatively in their capacity to drive Th differentiation. Furthermore, we also interrogated how these two subsets coordinate to induce T cell responses. Overall, the two DC subsets exhibit great functional diversity in the induction of the T cell response: cDCs as initiators predominantly of proliferation and moDCs as initiators predominantly in differentiation and as regulators.

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DC preparation and enrichment

Spleens were digested with collagenase-DNase, and light density cells were selected after centrifugation in Nycodenz medium (1.077 g/cm<sup>3</sup>; Nyegaard, Oslo, Norway) (32).

Flow cytometry

After Fcγ blockade (2,4G2; produced in-house) single-cell suspensions were stained with CD11b (M1/70), CD11c (HL-3), Ly6C (AL-21), MHC class II (MHCI-F-A/E [M5/I14/15.2]), CD4 (GK1.5), TCRβ (H57-597), TCRα (B20.1), CD25 (PC61), CD40 (3/23), CD69 (H1.2F3), CD86 (GL1), programmed death ligand 1 (PD-L1), CD45.1 (A20), or CD45.2 (B6.2F1) from BD Biosciences and propidium iodide to determine the numbers of viable cells.

Cell culture

Single-cell suspensions from spleens of GM-CSF transgenic, BALB/c, and OT-II mice were enriched using FACS sorting. Enriched CD4<sup>+</sup> T cells (purity > 95%) were labeled with CellTrace Violet (CTV; Life Technologies, Grand Island, NY) or CFSE (Life Technologies, Grand Island, NY) and cultured with enriched DCs (purity > 95%) at various ratios (as described in the figure legends) with or without soluble OVA Ag (OVA protein 1 mg/ml or OVA 323–339 peptide 10 μg/ml as indicated) for 96 h (indirect cultures) or 120 h (direct cultures). IL-2 (100 U/ml) or L-NMMA (0.5 mM) was added as indicated.

Assessment of CD4<sup>+</sup> T cell proliferation

Proliferation of naive CD4<sup>+</sup> T cells in response to priming by DCs was assessed by FACS, according to reduction in dye intensity of CD4<sup>+</sup> T cells after culture with moDCs or cDCs, as described above. To assess proliferation of activated CD4<sup>+</sup> T cells, OT-II T cells were cultured with cDCs and OVA protein 1 mg/ml for 96 h, after which proliferated CD4<sup>+</sup> T cells were enriched using FACS sorting and recultured with moDCs or cDCs with 1 mg/ml OVA protein. Further proliferation of CD4<sup>+</sup> T cells was assessed by reduction in dye intensity of recultured CD4<sup>+</sup> T cells.

Assessment of CD4<sup>+</sup> T cell activation

Activation of CD4<sup>+</sup> T cells by DCs was assessed by FACS; according to expression of CD69, CD25 and pS6 by OT-II T cells after culture with moDCs or cDCs with OVA Ag (as described in the figure legends) for 4–72 h.

Measurement of cytokine protein

To examine CD4<sup>+</sup> T cell differentiation in response to DC stimulation, cytokines in supernatants from DC-T cell cultures were analyzed using BioPlex Assay kits on the BioPlex 2200 system, according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Cytokine production was also assessed by intracellular staining of CD4<sup>+</sup> T cells following culture with DCs.

To confirm that polarizing cytokines were actually being produced by CD4<sup>+</sup> T cells, OT-II T cells were cultured with moDCs or cDCs for 96 h with 1 mg/ml OVA protein, after which they were enriched by FACS sorting and reprinted in equal numbers with anti-CD3 Ab and anti-CD28 Ab for 12–36 h. Cytokines in the supernatants from these cultures were again analyzed using BioPlex Assay kits on the BioPlex 2200 system.

Infection with Listeria monocytogenes

Listeria monocytogenes was diluted in normal saline from concentrated stock. Six- to 8-wk-old mice were infected i.v. with 2 x 10<sup>7</sup> Listeria. The infection dose was checked retrospectively by plating serial 10-fold dilutions of the inoculation suspension on a horse blood agar plate and incubating at 37°C for 24 h. Spleens from 3-d infected and uninfected mice were then harvested for analysis.

In vivo T cell proliferation

CCR2.DTR BM chimera mice received 10<sup>5</sup> enriched CTV labeled OT-II CD4<sup>+</sup> T cells i.v. One day later they received 2 x 10<sup>7</sup> OVA coated (10 mg/ml) bm1 splenocytes with 1 μg LPS i.p. (33). Spleens were harvested 3 d later. Proliferation of CD4<sup>+</sup> T cells was assessed as above.

In vivo T cell differentiation

CCR2.DTR BM chimera mice received 10<sup>5</sup> enriched CTV-labeled OT-II CD4<sup>+</sup> T cells i.v. One day later, they received 100 μg OVA protein emulsified in 100 μl CFA s.c. Spleens were harvested 6 d later. Cytokine production by adoptively transferred T cells was assessed by flow cytometry following intracellular staining.

Diphtheria toxin treatment

To deplete CCR2<sup>+</sup> cells, CCR2.DTR chimera mice were given 20 ng/g diphtheria toxin (DT) (CSL, Parkville, VIC, Australia) i.p. every 2 d.

DC survival assays

Enriched DCs from spleens of GM-CSF transgenic mice were cultured with or without OT-II CD4<sup>+</sup> T cells and 1 mg/ml OVA protein in FACS tubes to minimize transfer losses (5 x 10<sup>5</sup> DCs in 200 μl for up to 120 h). Cells were resuspended in the presence of EDTA, and survival was assessed by flow cytometry with FITC-conjugated FACS calibration beads (BD Biosciences) and propidium iodide to determine the numbers of viable cells.
Nitrite was detected by Griess reaction (34). Fifty microliters of supernatant were read at 540 nm. Nitrite concentrations were calculated using a sodium nitrate standard curve.

**Statistical analysis**

Mean and SD values were calculated, and two-tailed Student t tests (for experiments with two groups) or one-way ANOVA with post hoc Tukey’s honestly significant difference tests (for experiments with more than two groups) were performed, with GraphPad Prism Software.

**Results**

**moDCs are increased in response to allogeneic Ag**

We defined splenic DC populations according to surface phenotype as we and others have previously described: moDCs as CD11c<sup>int</sup> CD11b<sup>high</sup>MHC-II<sup>+</sup>Ly6C<sup>+</sup> and cDCs as CD11b<sup>high</sup>CD11b<sup>low</sup>high MHC-II<sup>+</sup>Ly6C<sup>-</sup> (9, 12, 35). Two additional markers CD64 and MAR1 were used to further delineate moDCs from CD11b<sup>+</sup> cDCs (Fig. 1A) (20, 36). We confirmed our previously reported findings that moDCs are rare in the steady state but become abundant in inflammation such as infection with *Listeria* (12, 35) or where systemic levels of GM-CSF are high, such as in GM-CSF transgenic mice (35). In contrast, cDCs are abundant at steady state as well as during inflammation (Fig. 1B, Supplemental Fig. 1A).

moDCs under *Listeria* infection or in GM-CSF transgenesis had a similar phenotype, with equivalent expression of MHC-II and Ly6C as well as the costimulatory molecules CD40 and CD86 (Fig. 1C).

To test whether moDCs may increase in response to allogeneic Ag, we administered 5 × 10<sup>7</sup> C57BL/6 (syngeneic) or BALB/c (allogeneic) splenocytes to C57BL/6 hosts i.v. One day later, recipients of allogeneic splenocytes had significantly more splenic moDCs than untreated hosts or recipients of syngeneic splenocytes. In contrast, cDC numbers were similar in all conditions (Fig. 1D, 1E, Supplemental Fig. 1B). Thus, we demonstrate that moDCs can be induced by allogeneic Ag.

To examine the relationship between these Ly6C<sup>+</sup> splenic moDCs and the DC-SIGN expressing moDCs that accumulate exclusively in skin-draining lymph nodes after LPS stimulation (19), we examined the spleen and skin-draining lymph nodes of mice 24 h after they had been challenged with LPS. As reported (19), we confirmed a significant expansion of a CD206A<sup>+</sup>DC-SIGN<sup>+</sup>MHC-II<sup>+</sup>CD11b<sup>+</sup> population in skin-draining lymph nodes (Supplemental Fig. 2A, 2C). This population did not significantly accumulate in the spleen of LPS-treated mice (Supplemental Fig. 2B, 2C). In contrast, Ly6C<sup>-</sup> monocytes and cDCs increased significantly after LPS administration (data not shown). As such, conventionally defined Ly6C<sup>+</sup> splenic moDCs appear to be distinct from DC-SIGN<sup>+</sup> moDCs that arise in skin-draining lymph nodes.

moDCs are inefficient at inducing T cell proliferation by either direct or indirect Ag presentation

Given that moDCs increase in allogeneic responses, we investigated their role in priming T cells through direct and indirect presentation. Initial analyses were performed in vitro. Although moDCs are rare in unmanipulated mice, by using GM-CSF transgenic mice, we were able to isolate sufficient moDCs for analysis while avoiding prior antigenic exposure (12).

To examine their ability to present Ag directly, we cultured sorted ex vivo moDCs with allogeneic CD4<sup>+</sup> T cells from BALB/c mice in an MLR. CD4<sup>+</sup> T cells were labeled with dye (CTV) prior to culture. After 5 d, proliferated CD4<sup>+</sup> T cells were detected by dye dilution and quantified by flow cytometry. moDCs were able to prime allogeneic CD4<sup>+</sup> T cells to proliferate in comparison with cultures of CD4<sup>+</sup> T cells alone (Fig. 2A). However, moDCs were significantly less efficient at directing T cell proliferation than cDCs isolated from the same GM-CSF transgenic mice, generating 20-fold less numbers of proliferating T cells (Fig. 2B). We also confirmed that T cell priming through direct Ag presentation by both DC subsets is alloantigen specific by comparing the response to syngeneic DCs (Supplemental Fig. 3A).

Because direct Ag presentation in vivo occurs only in the setting of a transplanted allograft or an infected DC, we next sought to examine the ability of moDCs to act by indirect Ag presentation. We cultured sorted moDCs from GM-CSF transgenic mice with syngeneic CD4<sup>+</sup> T cells from OT-II mice (transgenic for a TCR that recognizes OVA peptide on I-A<sup>B</sup>). As before, T cells were labeled with CTV prior to culture. OVA protein was added to the culture as cognate Ag. After 4 d of culture, we found that moDCs are capable of priming CD4<sup>+</sup> T cells through indirect Ag presentation (Fig. 2D). We then sought to compare indirect T cell priming by moDCs and cDCs. In accordance with our results with direct presentation, we found that moDCs were 10-fold less efficient than cDCs at stimulating T cell proliferation through indirect presentation (Fig. 2E). We confirmed that T cell priming through indirect Ag presentation by moDCs and cDCs required the presence of cognate Ag (Supplemental Fig. 3B).

We confirmed that these findings are not restricted to GM-CSF transgenesis by observing similar results in experiments conducted using DCs isolated from WT C57BL/6 mice (Supplemental Fig. 3C) and *Listeria*-infected C57BL/6 mice (Supplemental Fig. 3D).

We then sought to investigate whether the relative inefficiency of moDCs to stimulate T cells extends also to activated T cells. To this end, proliferated OTII CD4<sup>+</sup> T cells from cDC-stimulated T cell cultures were restimulated by cDCs or moDCs in the presence of OVA protein. In this setting, moDCs remained less efficient than cDCs at driving T cell proliferation (Supplemental Fig. 3E).

The differential effect on T cell proliferation between moDCs and cDCs occurs early but is not due to differential DC survival, Ag processing, or IL-2

We then investigated potential mechanisms underlying the inefficiency at inducing T cell proliferation by moDCs. We sought to determine whether differential expression of MHC-II and other costimulatory molecules contributed to the Ag presentation potential of the two DC subsets. Comparison of moDC and cDC surface marker expression revealed no significant differences in expression of MHC-II, the costimulatory molecules CD40 or CD86, or the inhibitory molecule programmed death ligand 1, suggesting that there are no underlying differences between the surface interactions required to provide activating signals to T cells (Fig. 3A).

Different DC subsets may vary in their survival. To determine whether differential survival occurred in our culture conditions and thus affect Ag presentation capacity, we conducted DC cultures over a 4- to 5-d period in similar conditions to those present in our previous experiments. We enumerated moDCs and cDCs at the beginning of the culture and then at various time points. We found no significant differences in moDC and cDC numbers at any time point, whether DCs were cultured alone or with CD4<sup>+</sup> T cells (Fig. 3B), indicating that survival differences did not contribute to our findings.

To examine whether the efficiency of moDC mediated presentation was affected by the ability to process Ag, we used OVA peptide to bypass the need for Ag processing. We found that OVA peptide did not alter T cell proliferation when cultured with moDCs (Fig. 3C), that is, proliferation induced by moDCs was dramati-
FIGURE 1. moDCs are rare at steady state and become abundant during inflammation or following allogeneic exposure. (A) Gating strategy to identify moDCs and cDCs. (B) Representative FACS plots showing increased numbers of CD11c<sup>hi</sup>CD11b<sup>int</sup> cells in *Listeria* infection and GM-CSF transgenesis. (C) Cell surface marker expression on moDCs arising in *Listeria* infection (dashed line) and in GM-CSF transgenesis (solid lines). (D) Representative FACS plots showing numbers of CD11c<sup>hi</sup>CD11b<sup>int</sup> cells following adoptive transfer of syngeneic and allogeneic splenocytes. (E) Enumeration of splenic moDCs and cDCs following adoptive transfer of syngeneic and allogeneic splenocytes. FACS plots in (A), (B), and (D) are gated on total viable mononuclear splenocytes. Data in (C) and (E) refer to moDC and cDC populations as indicated. Error bars indicate SD. Data are representative of at least three independent experiments with three to six mice per group. **p < 0.01 one-way ANOVA (E).
cally lower than that by cDCs whether OVA protein or peptide was used. This finding indicated that Ag processing ability does not explain the inefficiency of moDCs to induce T cell proliferation.

To investigate whether a deficiency of IL-2 production by T cells that receive their activating signals from moDCs contributes to inefficiency of T cell proliferation, we cultured CD4+ T cells with moDCs or cDCs with or without the addition of exogenous IL-2. Although T cell priming by cDCs increased with exogenous IL-2, the addition of IL-2 had no effect on moDC-induced T cell priming (Fig. 3D), suggesting that IL-2 deficiency is not responsible for the lack of T cell priming.

moDCs are highly efficient at the production of NO by upregulation of iNOS (8). NO can act as a suppressive mediator. To investigate whether the lack of T cell proliferation induced by moDCs is due to NO-mediated suppression, we conducted cultures with moDCs and CD4+ T cells with or without the addition of the iNOS inhibitor L-NMMA. Supplemental Fig. 3F shows that L-NMMA indeed strongly inhibits the production of NO. Nevertheless, we found that this inhibition had no effect on T cell proliferation in either the direct pathway or indirect pathway of Ag presentation, suggesting that the inefficiency of moDCs at inducing T cell proliferation is not due to active suppression by NO (Fig. 3E).

When APCs interact with and prime T cells, one of the earliest detectable T cell responses is upregulation of activation markers preceding T cell proliferation. CD69 is an early T cell activation marker. To investigate the relative efficiency of moDCs and cDCs to induce early activation of CD4+ T cells, we cultured OT-II CD4+ T cells alone or with sorted moDCs or cDCs in the presence of OVA peptide. After 4 h, we examined the T cells for expression of CD69. The numbers of T cells induced to upregulate CD69 expression by moDCs were 3-fold less than that by cDCs (Fig. 3F, 3G). This low level of early T cell activation suggests that the inefficiency of moDC-induced T cell proliferation is associated with very early events in the DC–T cell interaction. However, although deficient CD69 upregulation by moDC-primed T cells was observed for up to 72 h, at higher peptide doses (100 μg/ml), this deficiency was much smaller, and CD69 expression was more similar to that of cDC-primed T cells (Supplemental Fig. 4A, 4B).

Aside from CD69, we also probed two additional molecules that are critical for T cell activation: CD25 and pS6, a key component of PI3K/Akt/mTOR pathway. Similar to what we observed for CD69, T cell activation by moDCs, based on the expression of surface CD25 and intracellular pS6, was impaired compared with cDCs (Fig. 3H). Again, this deficiency was seen with different doses of Ag peptides and over various times points (from 4 h to 3 d) after antigenic exposure; although again, these differences were smaller when very high doses of OVA peptide (100 μg/ml) were used (Fig. 3I).

moDCs suppress CD4+ T cell proliferation induced by cDC mediated direct or indirect presentation

So far, we have investigated the way that moDCs or cDCs interact with CD4+ T cells in isolation. However, in most pathophysiological immune responses, both subsets of DCs are present, therefore, allowing for more complex DC–T cell interactions. To determine how moDCs, cDCs, and CD4+ T cells could interact with each other and influence T cell responses, we next conducted experiments with all three cell types together.

We first sought to determine whether moDCs could affect T cell proliferation induced by cDCs. To do this, we cultured cDCs with allogeneic CD4+ T cells alone or with varying numbers of moDCs

FIGURE 2. moDCs are inefficient at inducing T-cell proliferation by either direct or indirect Ag presentation. (A–C) Proliferation of allogeneic BALB/c CD4+ T cells after 5 d of culture alone or with GM-CSF transgenic moDCs or cDCs as indicated. (D–F) Proliferation of syngeneic OT-II CD4+ T cells after 4 d of culture with OVA alone or with GM-CSF transgenic moDCs or cDCs as indicated. FACS plots in (C) and (F) are gated on viable CD4+ T cells. Error bars indicate SD. Data are representative of four to five independent experiments with three to four replicates per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 two-tailed Student t test.
We found that moDCs were able to significantly inhibit direct cDC induced T cell proliferation; indeed, the number of proliferated T cells was inversely proportional to the number of moDCs added (Pearson $R = 0.99$; Fig. 4A, 4B). At a 1:1 ratio, T cell proliferation was $\sim 3$-fold less than in cultures without moDCs.

We then investigated whether this observation could be extended to the indirect pathway of Ag presentation. To determine this, we conducted cultures of cDCs with syngeneic OT-II cells in the presence of OVA protein, with or without moDCs. Consistent with our findings from the direct pathway, we found that moDCs were capable of suppressing T cell proliferation induced by cDCs acting through indirect Ag presentation (Fig. 4C, 4D); the number of proliferated T cells was again inversely proportional to the numbers of moDCs present (Pearson $R = -0.97$). At a 1:1 ratio, T cell proliferation was $\sim 3$-fold less than in cultures without moDCs.

**Suppression of cDC function by moDCs is Ag independent and partially dependent on NO**

To better understand the mechanisms by which moDCs suppress cDC induced CD4$^+$ T cell proliferation, we next sought to determine whether the ability for moDCs to suppress cDC induced T cell proliferation was Ag dependent or not. The allogeneic MLR system is well suited for this because we could use syngeneic DCs as non–Ag-bearing DCs. We cultured T cells with allogeneic cDCs. As expected, the addition of equal numbers of syngeneic cDCs, which do not present Ag to the T cells in this system, did not alter the numbers of proliferating T cells (Fig. 4E). In contrast, the addition of syngeneic moDCs, which also do not present Ag to T cells, significantly inhibited T cell proliferation (Fig. 4F).

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FIGURE 4. moDCs suppress direct and indirect cDC-induced CD4+ T cell proliferation through an Ag-independent NO-dependent process. (A and B) Proliferation of allogeneic BALB/c CD4+ T cells after 5 d of culture with 50,000 cDCs and variable numbers of moDCs as indicated. (C and D) Proliferation of syngeneic OT-II CD4+ T cells after 4 d of culture in OVA with 20,000 cDCs and variable numbers of moDCs as indicated. (E) Proliferation of CD4+ T cells after 4 d of culture with allogeneic cDCs and equal numbers of syngeneic cDCs or moDCs as indicated. (F) Proliferation of allogeneic BALB/c CD4+ T cells after 5 d of culture with equal numbers of moDCs and cDCs with (black) or without (gray) L-NMMA. (G) NO production in cultures of moDCs and cDCs with allogeneic CD4+ T cells with or without L-NMMA. (H) Proliferation of syngeneic OT-II CD4+ T cells after 4 d of culture in OVA with equal numbers of moDCs and cDCs with (black) or without (gray) L-NMMA. (I) NO production in cultures of moDCs and cDCs with syngeneic OT-II CD4+ T cells in OVA. (J) Recovery of cDCs after 4 d of culture in OVA of 20,000 cDCs and 50,000 OT-II CD4+ T cells, or 20,000 cDCs, 20,000 moDCs, and 50,000 OT-II CD4+ T cells with or without L-NMMA. FACS plots in (B) and (D) are gated on viable CD4+ T cells. Error bars indicate SD. Data (Figure legend continues)
the T cells, profoundly suppressed T cell proliferation, demonstrating that the observed suppressive effect is independent of cognate recognition between T cells and moDCs.

We next sought to determine whether NO production by moDCs was responsible for this observation. Although we had shown above that NO production does not suppress moDC-induced T cell proliferation, it remains possible that NO could act to suppress cDC induced T cell proliferation. To examine this question, we conducted direct pathway moDC–cDC–T cell cocultures with or without L-NMMA. Inhibition of iNOS by L-NMMA had no effect on T cell proliferation when only cDCs were present; however, it significantly reversed the suppressive effect of moDCs (Fig. 4F). Consistent with this observation, the concentration of NO in moDC–cDC–T cell cocultures was significantly lower when L-NMMA was added (Fig. 4G).

To determine whether this was also true for indirect presentation, we repeated this experiment with indirect moDC–cDC–T cell cocultures with or without L-NMMA. Partial reversal of moDC induced suppression confirmed that this effect was also at least partially because of iNOS-induced production of NO (Fig. 4H, 4I). To examine whether NO produced by moDC could adversely affect cDC survival and, therefore, partially explain the discrepancy between the effect of NO on T cell proliferation in moDC cultures and moDC–cDC cocultures, we conducted further experiments to examine cDC survival when cultured with T cells alone, with T cells and moDCs, or with T cells, moDCs and L-NMMA. cDC survival was reduced in cultures with moDCs, an effect that was partially reversed by the addition of L-NMMA, suggesting that NO produced by moDCs may indeed impair cDC survival (Fig. 4J).

**CCR2**^+^ cells suppress CD4^+^ T cell proliferation in vivo

The above experiments were performed in vitro. Next we sought to determine whether moDCs influence T cell activation and differentiation in vivo. To do this, we used CCR2.DTR mice, which have been genetically modified to express the DT receptor (DTR) under the CCR2 promoter. Because monocytes and moDCs, but not other types of DCs, express high levels of CCR2 (37), the administration of DT to these mice allows for conditional depletion of CCR2 expressing cells including monocytes and moDCs in the spleen (Fig. 5A, 5B). In contrast, WT mice are not susceptible to DT treatment.

We adoptively transferred OT-II cells into syngeneic CCR2.DTR mice, followed by OVA-coated bm1 splenocytes as cognate Ag. DT-treated hosts, which had effective ablation of CCR2^+^ cells, had significantly greater proliferation of the adoptively transferred OT-II cells (Fig. 5C). Presumably, the OT-II cells were being primed by CCR2^+^ APCs, most likely cDCs. To confirm that this observation was not due to a direct DT effect, we repeated this experiment using C57BL/6 hosts. Administration of DT to these hosts, in which CCR2^+^ cells were unaffected by DT, had no effect on the proliferation of transferred OT-II cells (Fig. 5C). This suggests that CCR2^+^ cells are also capable of suppressing T cell proliferation in vivo. We surmise that the effect is due to moDCs and not monocytes, even though both express high levels of CCR2. However, monocytes are generally poor APCs (38, 39). Moreover, we found that under inflammatory conditions, monocytes readily differentiate into MHC-II^−^CD11c^−^ moDCs so that the >90% of CCR2^+^ cells in spleens are MHC-II^−^CD11c^+^ moDCs, that is, at the physiological sites of T cell priming most CCR2^+^ cells are moDCs not monocytes.

moDCs are highly efficient at inducing Th1 and Th17 and inhibiting Th2 CD4^+^ T cell polarization

In addition to stimulating T cell proliferation, DCs are capable of influencing T cell polarization into a number of different subtypes by producing instructing cytokines. To determine the efficacy of T cell polarization by the two different DC subsets under investigation, we analyzed cytokine levels in the supernatants from mixed cultures of moDCs versus cDCs with CD4^+^ T cells (Fig. 6A).

We found that cDC–T cell cultures produced ~10-fold greater amounts of IL-2 compared with moDC–T cell cultures. The higher production of IL-2 induced by cDCs is commensurate with the higher T cell proliferation observed in these cultures, although it is clear from the exogenous IL-2 experiments that IL-2 alone is not sufficient for optimal proliferation.

Despite mounting a lower CD4^+^ T cell proliferative response, moDC–T cell cultures produced significantly greater amounts of Th1- and Th17-associated cytokines compared with cDC–T cell cultures. For example, moDC–T cell cultures produced ~8-fold greater amounts of IFN-γ. Similarly, there was a 3- and 2-fold greater production of IL-6 and IL-17A, respectively. Although very pleiotropic, IL-6 is essential in the establishment of the Th17 response, through at least in part the activation of RORγt. These observations are even more striking considering the significantly lower levels T cell proliferation seen in the moDC cultures. On a per-cell basis, the amount of IFN-γ, IL-6, and IL-17A produced was ~100-, 35-, and 25-fold higher than that produced in cDC–T cell cultures.

To demonstrate that moDCs are indeed superior to cDCs at inducing Th1 and Th17 differentiation, intracellular cytokine staining was performed to detect IFN-γ and IL-17A. Despite overall lower proliferation of T cells cultured with moDCs, T cells stimulated by moDCs produced more IFN-γ and IL-17A in each cell division (Fig. 6B, 6C).

To conclusively show the greater efficiency of Th1 and Th17 differentiation induced by moDCs compared with cDCs and to remove the potential confounding effect of different numbers of proliferating T cells induced by the two DC subsets, we next purified OT-II T cells that had been previously induced to proliferate by either moDCs or cDCs (in the presence of OVA Ag) and recultured them in equal numbers in the presence of anti-CD3 Ab and anti-CD28 Ab for 2 d. T cells that had been previously primed by moDCs continued to produce significantly higher amounts of IFN-γ and IL-17A than T cells that had been previously primed by cDCs (Supplemental Fig. 4C).

We then aimed to determine the ability for moDCs to influence T cell polarization in vivo. To investigate this we again used CCR2.DTR mice. We adoptively transferred OT-II CD4^+^ T cells into syngeneic CCR2.DTR mice, followed by OVA protein emulsified in CFA. CFA has been shown to influence activated T cells to differentiate into Th1 (40, 41) or Th2 (42) phenotypes. The administration of CFA emulsified OVA effectively primed the adoptively transferred T cells. In this setting, even with effective ablation of CCR2^+^ cells (Fig. 5A), we did not observe a significant difference in T cell proliferation (data not shown). One explanation may be that the strong adjuvanticity of CFA masked much of the inhibition by moDCs. Nevertheless, we detected increased Th2 polarization with significantly greater expression of IL-13 and GATA-3 when CCR2^+^ moDCs were deleted (Fig. 7A, 7B), whereas we could not detect a significant difference in the expression of IFN-γ or IL-17A in this system.

are representative of two to four independent experiments with three to four replicates per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 one-way ANOVA (A, C, E, F, H, and J), two-tailed Student t test (G and I).
Taken together, these observations suggest that, although moDCs are not efficient at inducing T cell proliferation, they are able to effectively influence T cell differentiation, particularly toward Th1 and Th17 and away from Th2 differentiation.

**Discussion**

The fact that DC subsets are located differently anatomically and secrete different cytokines would suggest some division of labor. Indeed, moDCs but not cDCs seem important in the pathogenesis of EAE (17, 43), whereas plasmacytoid DCs are pivotal in systemic lupus erythematosus pathogenesis (44, 45). Still, little is known about how moDCs, particularly relative to cDCs, affect T cell responses. We found that moDCs are inefficient at initiating CD4+ T cell proliferation through either direct or indirect Ag presentation, compared with cDCs. In contrast, despite their poor ability to induce T cell proliferation, moDCs potently induce T cell differentiation. T cell cultures stimulated with moDCs contained higher levels of IFN-γ and IL-17A, indicating that moDCs have a propensity toward inducing Th1 and Th17 polarization while suppressing Th2 polarization. This is further evident from in vivo experiments showing that removal of CCR2+ DCs increase Th2 cytokines.

Like many other types of DCs, moDCs are also likely to be heterogeneous and consist of different subsets. Serbina et al. (8) coined the term TNF/iNOS-producing DCs to describe a type of moDC that becomes abundant upon systemic bacterial infection. Most of these DCs express Ly6C and the chemokine receptor CCR2. We observed that these Ly6C+CCR2+ moDCs also become abundant in mice immunized with CFA (17) and in mice with high levels of GM-CSF (35). Because Ly6C can be downregulated, additional markers like CD64 and MAR1 have been applied to distinguish moDCs in peripheral tissues (skin and lung) from lymph-resident cDCs (20, 36). In this study, splenic moDCs also express CD64 and MAR1. Interestingly, LPS induces a type of DC-SIGN–expressing moDCs only in skin-draining lymph nodes but not in the spleen (19). Because we believe that the splenic Ly6C+ moDCs that we have studied here are distinct from the LPS induced DC-SIGN–expressing moDCs in lymph nodes, the precise relationship of two subsets remains to be established. It is tentatively speculated that the two moDCs may have different precursors or maturation sites, notwithstanding that both types can be derived from BM monocytes in the presence of GM-CSF (19, 46).

We observed that moDCs are vastly inferior to cDCs at stimulating T cell proliferation but superior at driving Th1 and Th17 differentiation. The division of labor for lymphoid-resident cDCs to stimulate proliferation and moDCs to stimulate differentiation is conceptually attractive. T cell priming occurs in lymphoid organs, and therefore, it would make functional sense for these cDCs to stimulate proliferation of T cells. moDCs are abundant at inflammatory sites, including at sites of infection, and differentiation of T cells into effectors at these sites would seem desirable (9). Of note, moDCs remain less efficient than cDCs at stimulating activated T cells to proliferate.

We have searched for the basis of the inability for moDCs to induce T cell proliferation. Several parameters influencing T cell proliferation are surprisingly not different between moDCs and cDCs. These include the expression of costimulatory molecules and MHC-II as well as NO production. Bypassing antigenic processing, inhibiting production of NO and supplementation of the T cell growth factor IL-2 all failed to enhance T cell proliferation by moDCs. This differential effect of moDCs appears to be an early event in T cell activation as indicated by the finding that moDCs, in comparison with cDCs, were poor inducers of CD69 expression on T cells. At the signaling level, we also noted that T cell activation induced by moDCs was suboptimal in the PI3K/Akt/mTOR
Expression of pS6 was lower in T cells activated by moDCs than those activated by cDCs. This defect persisted over time and was still evident after 72 h. Nevertheless, increasing the intensity of TCR stimulation by exposure to higher concentration of Ag peptide can rectify the defect to some degree. This is also true for the induction of CD25 expression on activated T cells.

Given the important role that moDCs play in the innate immune responses against certain infections (8, 47, 48), it is possible that they are more functionally suited to degrading and removing potentially pathogenic Ag rather than retaining internalized peptides for presentation to T cells. This property is reminiscent of several populations of macrophages, which are professional phagocytes and share monocytic origins with moDCs (49). Although once considered to be prototypical APCs, it has now been established that these macrophages contain high levels of lysosomal proteases that allow them to rapidly degrade and remove internalized proteins (50). It is thought that this phenomenon prevents them from being able to efficiently prime T cells, in contrast with DCs, which have a more favorable internal environment, which allow for persistence of MHC–peptide complexes (51). Nevertheless, our peptide presentation studies show that differences in processing alone cannot explain the low efficacy of moDCs at initiating T cell proliferation. Although moDCs have similar levels of MHC-II and costimulatory molecule expression to cDCs, further studies are required to determine whether differences in their internal compositions may prevent moDCs from effectively initiating T cell proliferation.

Apart from being poor inducers of T cell proliferation, we found that moDCs also effectively suppress cDC-induced T cell proliferation through both pathways of presentation. We also found that the in vivo removal of CCR2 expressing cells enhanced the proliferation of transferred TCR transgenic T cells induced by Ag coated cells. The inhibition was, at least partially, mediated by an iNOS-dependent mechanism and independent of cognate Ag.

FIGURE 6. moDCs induce Th1 and Th17 CD4+ T cell polarization in vitro. (A) Data show cytokine concentrations (picograms per milliliter) in culture supernatants after 4 d of culture of syngeneic OT-II CD4+ T cells with OVA and moDCs or cDCs as indicated. (B) Intracellular staining for IFN-γ and IL-17A of OT-II CD4+ T cells after 4 d of culture with syngeneic moDCs or cDCs with OVA. (C) Analysis of IFN-γ- and IL-17A–positive OT-II CD4+ T cells by division number as assessed by intracellular staining. Error bars indicate SD. Data are representative of two to four independent experiments with three to four replicates per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 two-tailed Student t test.
recognition by moDCs. This finding confirms iNOS-mediated NO production as a critical mediator of moDC function. First discovered in activated macrophages (52), the complex role played by NO in various biological systems is becoming increasingly appreciated (53). Aside from direct toxic effects exerted against infectious microorganisms (8, 54, 55), it appears that NO can also act in an inhibitory manner to limit the proliferation of pathogens such as Leishmania, thereby favoring clearance of the infection by the immune system (56). This inhibitory capacity has been previously noted in respect to T cells exposed to NO produced by activated macrophages (57) or monocytes (58). NO appears to inhibit T cell proliferation through direct cytostatic effects on T cells and APCs or through depletion of L-arginine during the iNOS reaction (59). In support of a role for NO inhibition of APCs, we found that recovery of cDCs was lower when cocultured with moDCs and that blocking of NO synthesis partially reversed this effect. These negative regulatory roles of moDCs on T cells and other APCs may restrict any excessive activation of T cells educating immunopathology or autoimmunity.

Although moDCs and cDCs may sometimes function in different locations (peripheral sites of inflammation and secondary lymphoid organs, respectively), under certain circumstances, such as infection, there is both inflammation (so moDCs become prominent) and Ag delivery to lymphoid organs. Thus, lymphoid-resident cDCs and inflammation-induced moDCs would coexist in the same location, whereupon moDCs can directly inhibit T cell proliferation induced by cDCs. Furthermore, activated T cells traffic to peripheral inflamed tissues, where moDCs can potentially exhibit regulatory effects to control the size of the local T cell response and its polarization.

These negative regulatory roles of moDCs are similar to those of myeloid-derived suppressor cells (MDSCs) that have been extensively studied in cancer immunology. Cotransplantation of islet allografts with MDSCs led to improved survival of islet grafts (24). Our work adds to the body of knowledge about how moDCs respond to allogeneic Ag, such as that found in transplantation, and about how they orchestrate the CD4+ T cell response through the direct and indirect pathways of Ag presentation. We show in this study that moDCs in the spleen are expanded in response to exposure to allogeneic Ag. Currently, the basis of innate recognition of allogeneic cells by monocytes or moDCs remains unidentified. Further molecular dissection of the innate recognition of allogeneic cells may provide new avenues for the modification of allogeneic responses.

moDCs belong to an extended family of mononuclear phagocytes which consist of moDCs, precursor monocytes, site-unique nonlymphoid tissue DC subsets and different subsets of macrophages. Mouse monocytes that give rise to different DC subsets can be divided into two main subsets in mice and three subsets in humans (66). Inflammatory moDCs, in which we are mostly interested, are derived from Ly6C+ monocytes. In contrast, Cx3CR1-expressing DCs in heart and kidney allografts (24) could be derived from other (Ly6C−) monocyte subsets. At mucosal surfaces,


moDCs are rare at steady state and become abundant during inflammation or following allogeneic exposure – supplemental data. (A) Representative FACS plots (according to gating strategy in Fig.1A) showing increased numbers of moDCs in Listeria infection and GM-CSF transgenesis. (B) Representative FACS plots showing numbers of moDCs following adoptive transfer of syngeneic and allogeneic splenocytes. FACS plots are gated on total viable mononuclear splenocytes.
LPS induced DC-SIGN+ moDCs in skin draining lymph nodes are distinct from splenic Ly6C+ moDCs. (A) & (B) Representative FACS plots showing accumulation of CD206A+DC-SIGN+ moDCs in skin-draining lymph nodes and lack of accumulation in spleen of mice 24 h after treatment with 5 µg LPS i.v. (C) Enumeration of CD206+DC-SIGN+ moDCs in skin-draining lymph nodes and spleen of mice 24 h after treatment with 5 µg LPS or vehicle i.v.
(A) Proliferation of CD4⁺ T cells after 5 d of culture with syngeneic or allogeneic moDCs or cDCs as indicated. (B) Proliferation of CD4⁺ T cells after 4 d of culture with moDCs or cDCs with or without OVA as indicated. (C) Proliferation of syngeneic OT-II CD4⁺ T cells after 4 d of culture with OVA alone or with wild-type C57BL/6 moDCs or cDCs as indicated. (D) Proliferation of allogeneic BALB/c CD4⁺ T cells after 5 d of culture alone or with moDCs or cDCs from listeria-infected C57BL/6 mice as indicated. (E) Proliferation of previously activated syngeneic OT-II CD4⁺ T cells after 4 d of culture with OVA alone or with GM-CSF transgenic moDCs or cDCs as indicated. (F) NO production after 2 d of cultures with peritoneal macrophages from GM-CSF transgenic mice with or without LPS or L-NMMA. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, two-tailed Student t test (A) – (E), one-way ANOVA (F). Error bars indicate SD.
A) Representative histograms and quantification of expression of CD69 on OT-II CD4+ T cells after 4 h of culture with syngeneic GM-CSF transgenic moDCs or cDCs with various doses of OVA peptide and OVA protein as indicated. (B) Expression of CD69 on OT-II CD4+ T cells after culture with syngeneic GM-CSF transgenic moDCs or cDCs with various doses of OVA peptide and OVA protein and for 1-3 d as indicated. (C) Cytokine concentrations (pg/mL) in culture supernatants after 2 d of culture of 50,000 syngeneic OT-II CD4+ T cells with 5 µg/mL anti-CD3 Ab and 2 µg/mL anti-CD28 Ab. T cells had been previously cultured for 4 d with syngeneic GM-CSF transgenic moDCs or cDCs as indicated with OVA. Histograms in (A) and (C) are gated on viable CD4+ T cells. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, two-tailed Student t test. Error bars indicate SD.