Stimulate Th1 and Th17 Memory Responses

Polarization of Naive T Cells but Do Dendritic Cells Fail to Induce Th17

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TLR-Stimulated CD34 Stem Cell-Derived Human Skin-Like and Monocyte-Derived Dendritic Cells Fail to Induce Th17 Polarization of Naive T Cells but Do Stimulate Th1 and Th17 Memory Responses

Sai Suda Duraisingham, Julia Hornig, Frances Gotch, and Steven Patterson

Dendritic cells (DCs) are important in linking innate and adaptive immune responses by priming and polarizing naive CD4+ Th cells, but little is known about the effect of different human DC subsets on Th cells, particularly Th17 cells. We have investigated the ability of TLR-stimulated human Langerhans cells (LC), dermal DCs (dDC), and monocyte-derived DCs (moDC) to affect naive and memory Th17 and Th1 responses. MoDCs stimulated greater memory T cell proliferation while LCs and dDCs more potently stimulated naive T cell proliferation, indicating functionally distinct subsets of DCs. TLR stimulation of all three DC types was unable to induce Th17 polarization from naive T cell precursors, despite inducing Th1 polarization. Dectin stimulation of DCs in IMDM was however able to produce Th17 cells. TLR-stimulated DCs were capable of inducing IL-17A and IFN-γ production from memory T cells, although the mechanism used by each DC subset differed. MoDCs partially mediated this effect on memory Th1 and Th17 cells by the production of soluble factors, which correlated with their ability to secrete IL-12p70 and IL-23. In contrast, LCs and dDCs were able to elicit a similar memory response to moDCs, but in a contact dependent manner. Additionally, the influence of microbial stimulation was demonstrated with TLR3 and TLR7/8 agonists inducing a Th1 response, whereas TLR2 or dectin stimulation of moDCs enhanced the IL-17 response. This study emphasizes the differences between human DC subsets and demonstrates that both the DC subset and the microbial stimulus influence the Th cell response.

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The CD4+ Th cell response comprises several subsets of cells characterized by the secretion of different cytokines, including the classical IFN-γ-producing Th1 cells and the IL-4-producing Th2 cells, which are involved in cellular or humoral immunity, respectively (1). Recently described IL-17-producing Th17 cells have been implicated in the immune response to extracellular bacteria and fungi as well as in several autoimmune diseases (2). Dendritic cells (DCs)3 play a key role in linking the innate and adaptive immune systems by priming naive T cells that can become polarized toward a particular Th cell phenotype. The triggering of TLRs by pathogenic molecules results in the up-regulation of MHC and costimulatory molecules and the secretion of cytokines by DCs, which can in turn skew effector Th cell differentiation (3).

In mice, the polarization of Th17 cells from naive T cells is initially dependent on the presence of TGF-β, IL-21, and IL-6, and at later stages on IL-23 (4). In humans, the polarizing cytokines that are necessary have not been definitively characterized, with studies variably citing the importance of IL-1, IL-6 (5, 6), and TGF-β (7–9). Additionally, IL-23 and IL-1β have been shown to induce the production of IL-17A from human memory T cells (10, 11). Alternatively, DC production of the IL-12p70 cytokine, which shares a common subunit with IL-23, is involved in the polarization of Th1 cells and the induction of IFN-γ from memory cells (12). Many of the studies regarding Th17 cell development have been conducted using APC-free cultures with anti-CD3/CD28 stimuli, exogenous cytokines, and IFN-γ/IL-4 neutralization. However, little is known about the physiological conditions needed for human Th17 development and maintenance, particularly the role of DCs. The variable production of key cytokines and other factors by DCs after TLR stimulation may therefore influence the differentiation and reactivation of Th17 and Th1 cells.

In humans, several distinct DC populations exist that can be broadly categorized into myeloid DC and plasmacytoid DC types (13). In human skin, there are two major myeloid DC subsets: epidermal Langerhans cells (LCs), which contain Birbeck granules and are langerin+, and the dermal DCs (dDCs), which express DC-specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and CD11b (14). DC subsets have been reported to express different arrays of TLR molecules, which may hint at a role for functional specialization in the response to pathogens (15, 16). Hence, the triggering of TLRs on each DC population may produce maturation signals that have differential effects on the resultant Th cell response. The study of human DC biology has been greatly assisted by the generation of DCs in vitro; the most commonly used model of human DCs is monocyte-derived DCs (moDCs) generated from blood monocytes (17). For LCs and dDCs, phenotypic equivalents can be generated in vitro from

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3 Abbreviations used in this paper: DC, dendritic cell; LC, Langerhans cell; dDC, dermal DC; moDC, monocyte-derived DC; DC-SIGN, DC-specific intracellular adhesion molecule 3-grabbing nonintegrin.
CD34+ hematopoietic stem cells (18). Understanding how human DCs respond to innate immune stimuli and the effect that this has on the adaptive immune response is important in generating vaccine adjuvants that can more efficiently induce an appropriate response. The study of human skin DCs may be particularly important due to the interest surrounding transcutaneous vaccination strategies (19).

In this study, we have examined the ability of TLR-stimulated LC, dDC, and moDC subsets to induce cytokine production from naive and memory Th cells. Human DCs stimulated through their TLRs were found to be unable to polarize Th1 cells from naive T cells but were able to induce Th1 and Th2 differentiation. Dectin-stimulated DCs were however able to polarize naive Th17 cells. All DC types were also capable of inducing IL-17A production from memory T cells. The production of the key cytokines IL-23/IL-12p70 and IL-23 by moDCs in response to TLR or dectin stimulation was associated with the corresponding Th1 or Th17 memory T cell response. In contrast, LCs and dDCs appeared to use an IL-12/IL-23-independent mechanism to elicit similar responses. This study highlights the differences between DC subsets and the importance of both the DC type and microbial stimuli in determining Th cell responses.

Materials and Methods

**Generation of DC subsets**

Umbilical cord blood samples were obtained from consenting full-term pregnant women in accordance with the local Ethical Approval Committee. CD34+ cells were isolated from cord blood by immunomagnetic beads (Miltenyi Biotec) and cultured in complete RPMI 1640 (100 IU/ml penicillin, 0.1 mg/ml streptomycin, 2 mM l-glutamine; Sigma-Aldrich) supplemented with 10% FCS (Invitrogen), 100 ng/ml GM-CSF (Immunex), 25 ng/ml stem cell factor, 25 ng/ml Flt3 ligand, and 5 ng/ml TNF-α (R&D Systems). After 6 days, the CD14+ cells were isolated with immunomagnetic beads and cultured for a further 7 days with 100 ng/ml GM-CSF and 1000 U/ml IL-4 (R&D Systems) to generate DC-SIGN+ CD11b+ dermal-type DCs. The remaining CD14+ cells were cultured for 7 days with 100 ng/ml GM-CSF and 5 ng/ml TGF-β (R&D Systems) to generate langerin+ Langerhans-type cells. Both DC types were finally isolated by MACS based on CD1a expression with a purity of >95%. For quantitative-PCR experiments, cells were passed over two MACS columns to yield a purity of 99%. Monocyte-derived DCs were generated from CD14+ peripheral blood monocytes isolated with immunomagnetic beads (Miltenyi Biotec) and cultured with 100 ng/ml GM-CSF and 1000 U/ml IL-4 (R&D Systems) to generate DC-SIGN+ CD11b+ dermal-type DCs. The remaining CD14+ cells were cultured for 7 days with 100 ng/ml GM-CSF and 5 ng/ml TGF-β (R&D Systems) to generate langerin+ Langerhans-type cells. Both DC types were finally isolated by MACS based on CD1a expression with a purity of >95%.

**RNA isolation and quantitative PCR**

RNA was isolated and cDNA synthesized from purified DC populations using the One-step cDNA kit according to manufacturer’s instructions (Miltenyi Biotec). Quantitative real-time PCR for TLR expression were prepared using SYBR Green I Mastermix (Roche Applied Systems) with the forward and reverse primers in supplemental Table I (Operon Biotechnologies) and additional 1 µM MgCl2 (Promega) for TLRs 1 and 5. The PCR were denatured for 8 min at 95°C and then amplified over 40 cycles of 95°C for 12 s, 56°C or 59°C for 8 s and 72°C for 10 s for data acquisition. Samples were run on a LightCycler 480 and analysis was conducted using Advanced Relative Quantitation software, where TLR relative expression was calculated by normalization to β-actin expression (Roche Applied Systems).

**T cell polarization induced by DCs**

The following TLR agonists from Invivogen were used: 1 µg/ml Pam3Csk4 (TLR1/2), 1 µg/ml P. gingivalis LPS (TLR2), 25 µg/ml poly(I:C) (TLR3), 1 µg/ml E. coli LPS (TLR4), 5 µg/ml CL097 (TLR7/8), 0.1 µg/ml zymosan (TLR2/12-1), and 1 µg/ml curdlan (dectin-1) (Wako). CD40L (5 µg/ml) was also added for some experiments (R&D Systems). DCs (107/ml) were stimulated for 24 h with the TLR agonists and the supernatants were assayed for IL-12p70, IL-23p70 and IL-23 by ELISA. IL-12p70 and IL-23 were assayed using available ELISA kits (eBiosciences). For IL-12p70 and IL-23, goat anti-mouse IgG precoated ELISA plates (Cayman Chemicals) were coated with capture Ab (1 µg/ml, clone 31052, R&D Systems) for 1 h. Supernatants and standards were added for 2 h at room temperature. The plates were washed and biotinylated-detection Ab were added (0.5 µg/ml, R&D Systems) for 1 h. The plates were developed with HRP avidin enzyme (1 µg/ml, Vector Laboratories), 3,3’,5,5’-tetramethylbenzidine substrate (Becton Dickinson), and stop solution (R&D Systems). Plates were read on an Anthos ELISA plate reader (ASYS Hitech) at 450 nm.

**Mixed lymphocyte reactions**

DCs (105/ml) in complete RPMI 1640 with 10% human AB serum (Sigma-Aldrich) were stimulated overnight with TLR or dectin agonists before culturing with different allogeneic T cell populations (106/well) for 5 days (proliferation assays) or 7 days (cytokine staining). In one experiment, complete IMDM supplemented with 10% AB or serum-free StemVivo Dendritic cell base medium (R&D Systems) was used for DC-T co-cultures. T cells alone were used as control wells. Naive CD4+ T cells were isolated from umbilical cord blood using the naive T cell isolation kit (Miltenyi Biotec) whereas memory CD4+ T cells were isolated from adult peripheral blood (Miltenyi Biotec), T cells were ≥98% CD45RO+ or CD45RA- , respectively. Where total lymphocytes were used, PBMCs were separated over a 50% Percoll gradient (Sigma-Aldrich) to yield enriched lymphocytes which were ≥75% CD3+.

For proliferation assays, T cells were cultured with graded numbers of DCs in triplicate. Cultures were pulsed on day 5 with 1µCi/well [3H]thymidine (PerkinElmer) for 16 h before harvesting and measurement of the cpm. For intracellular cytokine staining, cocultures were restimulated on day 7 with PMA (0.1 µg/ml) and ionomycin (1 µM) for 5 h, with Brefeldin A (10 µg/ml; Sigma-Aldrich) added for the last 4 h. Cells were surface stained with CD3-APC/Cy7 and CD4-PECy7 Abs (Becton Dickinson) before fixing with 2% paraformaldehyde. Cells were incubated in 0.1% saponin buffer with IFN-γFITC, IL-17-PE, and IL-4-allophycocyanin or IL-10-APC Abs (Becton Dickinson) and acquired on an LSRII.

**T cell cultures**

Cord blood naive or adult blood memory T cells (106/ml) in complete RPMI 1640 medium with 10% human AB serum were stimulated with anti-CD2/CD3/CD28 T cell activation beads (Miltenyi Biotec) with a bead to cell ratio of 1:2. The IL-17-inducing cytokines IL-1β, IL-6, IL-23 (all at 10 ng/ml; eBiosciences), and TGF-β (5 ng/ml; R&D Systems) were added to half of the wells. Control wells were cultured without these cytokines. On days 3 and 6, IL-2 (20 U/ml, R&D Systems) was added to all wells and IL-1β, IL-6, and IL-23 (10 ng/ml) were added to the cytokine wells. On day 7, cultures were restimulated and intracellular cytokine staining was conducted.

Cord blood naive or adult blood memory T cells (106/ml) with T cell activation beads were added to supernatants taken from DCs (107/ml) stimulated for 24 h with TLR or dectin agonists. On days 3 and 6 of culture, T cell cultures were gently pipetted, before being harvested and assessed for intracellular cytokines on day 7.

**Statistical analysis**

Data were analyzed using GraphPad Prism 5.0 software by ANOVA with Bonferroni’s post test for grouped comparisons and Mann-Whitney U test for comparison within groups. A p-value of <0.05 was considered significant.

**Results**

Human DC subsets express different TLRs and have differential abilities to induce T cell proliferation

Human Langerhans-type and dermal-type DCs were generated from CD34+ stem cells and their TLR expression levels were compared with conventional monocyte-derived DCs by quantitative real-time PCR. All three DC types expressed TLRs 1, 2, 4, 5, and...
6 at varying levels (Fig. 1A). LCs expressed higher levels of TLR1, TLR7, and TLR10 mRNA compared with dDCs and moDCs, but they did not express TLR3 or TLR8. dDCs expressed TLR8 but not TLR7, whereas moDCs expressed both TLR7 and TLR8. Stimulation of the DCs with agonists targeting each TLR resulted in the up-regulation of HLA-DR, CD80, CD83, and CD86 (Fig. 1C) in agreement with the TLR expression data for all DC types and agonists tested. For example, LCs that did not express TLR3 did not mature after treatment with the TLR3 ligand polyIC, but did have a greater maturational response to the TLR1/2 agonist Pam3Csk compared with dDCs or moDCs (data not shown).

To compare the intrinsic ability of each DC type to prime T cells, unstimulated DCs were cultured in MLRs with naive or memory CD4+ T cells. LCs and dDCs induced greater proliferation of naive T cells whereas moDCs were more efficient at inducing memory T cell expansion (Fig. 1B). There were, however, no apparent differences between the LCs and dDCs. Thus, each human DC type had a different TLR expression profile and differential ability to induce the proliferation of naive or memory T cells, suggesting that there may be functional specialization of the DC subsets.

**DC subsets stimulated through their TLRs induce different T cell cytokine production profiles**

DCs stimulated with TLR agonists were cultured with total lymphocytes enriched from peripheral blood (≥ 75% CD3+) and then CD4+ T cell cytokine production was assessed. LCs, and to a lesser extent dDCs, induced more IL-17A+ cells than moDCs regardless of the type of TLR stimulus (Fig. 2, A and D). LCs stimulated with Pam3Csk (TLR1/2), TLR4-LPS, and CL097 (TLR7/8) produced a significantly greater IL-17 response than moDCs. All three DCs induced strong Th1 IFN-γ+ responses, which were increased after stimulation with polyIC (TLR3), TLR4-LPS, or CL097 (TLR7/8) (Fig. 2B). Notably, the IFN-γ response induced by LCs was increased after polyIC stimulation despite the lack of TLR3 expression on LCs and may be due to signaling through cytoplasmic MDA5 that recognizes double-stranded RNA (21). Alternatively, there may be up-regulation of TLR3 on the LCs during the MLR culture or a role for other TLR3-expressing cell types in the total lymphocyte population. A minor population of IL-17A+ IFN-γ+ CD4+ T cells were consistently seen in all cultures (Fig. 2D) as reported previously (6, 22). LCs were also able to induce a greater IL-4+ Th2 response than dDCs and moDCs, which was statistically significant with TLR4-LPS stimulation (Fig. 2C). Hence, in the presence of a total lymphocyte population, LCs elicited a mixed Th cell cytokine profile but with significantly greater Th17 and Th2 responses, whereas dDCs and moDCs preferentially induced a Th1 response. The nature of the TLR stimulus also contributed to the type of response, with TLR3, TLR4, or TLR7/8 stimulation augmenting the IFN-γ response even in LCs.

Considering we had previously used total lymphocyte populations, we next investigated whether the DCs were exerting their...
effect on the differentiation of naive T cells or memory T cells. The ability of each DC type to polarize naive T cells purified from umbilical cord blood was compared. Cord blood was used to ensure a genuinely naive population, as IL-17A cells may be produced by the reactivation of contaminating memory T cells (23). Unexpectedly, none of the three DC types were able to induce the differentiation of Th17 cells, even after TLR1/2, TLR2, TLR3, TLR4, or TLR7/8 stimulation (Fig. 3A and data not shown). The DCs were however still able to efficiently polarize Th1 responses, particularly dDCs and moDCs stimulated with polyIC (TLR3) or CL097 (TLR7/8) (Fig. 3B). LCs induced lower levels of Th1 polarization, which could not be further augmented by TLR stimulation. All DC types also generated small percentages of Th2 and IL-10 T cells which were not significantly altered by TLR stimulation (Fig. 3C, D, and Fig. S1). Our inability to induce a Th17 response in naive T cells by stimulating with different DC types does not reflect an inherent incapacity of the naive T cells to produce IL-17 as demonstrated in later experiments (see Fig. 6).

Despite the poor ability of the DCs to polarize Th17 cells from naive precursors, all DC types could effect the secretion of IL-17A from memory CD4+ T cells, which was significantly increased upon stimulation with TLR2 or zymosan of the moDCs (Fig. 4A). All DCs also induced strong IFN-γ and moderate IL-4 memory responses that could not be further amplified by TLR stimulation (Fig. 4B and C). The moDCs induced a greater percentage of memory IL-10+, which did not coproduce IFN-γ or IL-17A (data not shown), than the LCs or dDCs (Fig. 4D, Fig. S1).

Polarization of Th17 cells from naive T cells

Because we were unable to induce Th17 polarization with any DCs tested, we wanted to confirm the ability of cord blood naive T cells to produce IL-17A. Naive T cells were stimulated with anti-CD2/CD3/CD28 beads and cultured in the presence of IL-1β, IL-6, IL-23, and TGF-β for 7 days. This combination of cytokines resulted in increased percentages of IL-17A+ cells from both naive and memory T cells with no observable effect on IFN-γ secretion (Fig. 6A), suggesting that the TLR-stimulated DCs may not be producing all of these factors or in sufficient quantities for naive Th17 polarization. These experiments were performed in RPMI.
1640 medium containing AB serum but similar results were obtained using StemX-vivo medium (not shown). Because DCs stimulated with TLR agonists were unable to induce Th17 cells, we tested whether the dectin-1 ligands zymosan or curdlan were able to polarise Th17 cells, as previously reported (24). In our culture conditions, dectin-stimulated moDCs were unable to induce Th17 cells (Fig. 6B). The presence of serum in culture medium has been reported to inhibit Th17 differentiation (7), however we were still unable to induce Th17 cells with TLR-treated DCs using serum-free X-Vivo medium (Fig. 6B). A recent report demonstrated that the use of IMDM enhanced Th17 differentiation compared with RPMI 1640 medium (25). Similar to the data with RPMI 1640 medium, TLR-treated DCs were unable to polarize Th17 cells even in the presence of IMDM. However, stimulation of the skin LCs and in vitro-derived LCs and dDCs with the dectin ligands zymosan or curdlan was able to induce 0.3–0.4% Th17 cells in IMDM but not RPMI 1640 medium (Fig. 6C). Interestingly, the moDCs induced the lowest percentage of Th17 cells in IMDM compared with the LCs and dDCs. The differences in responses induced by the skin LCs and the in vitro-derived LCs were more apparent in IMDM, compared with our previous experiments in RPMI 1640. Furthermore, the addition of exogenous IL-1β, IL-6, IL-23, and TGF-β cytokines to the unstimulated DC-T cell cultures was able to restore Th17 polarization of naive T cells (Fig. 6C), suggesting that these cytokines are not induced by TLR agonists. However, because the TLR-treated DCs were capable of inducing IL-17A secretion from memory but not naive T cells, the mechanisms by which DCs mediate their effect on memory T cells may differ to naive T cells.

**MoDCs but not LCs or dDCs partially mediate memory T cell cytokine production via soluble factors**

To ascertain whether DCs were inducing differential memory T cell cytokine production via soluble factors, memory T cells were cultured with anti-CD2/CD3/CD28 beads and supernatants taken from DCs stimulated with TLR-2, TLR-3, TLR7/8 or dectin ligands. Supernatants from LCs and dDCs had no significant amplifying effect on memory T cell cytokine production, above that induced by the activation beads alone (Fig. 7). In many cases the addition of LC or dDC supernatants led to a decrease in the percentage of cytokine-producing memory T cells. In contrast, supernatants from zymosan-stimulated moDCs increased the percentage of IL-17A memory T cells and supernatants from moDCs stimulated with polyIC (TLR3) and CL097 (TLR7/8) marginally increased IFN-γ (Fig. 7). DC supernatants were unable to induce any cytokine production from naive T cells, with the exception of moDCs stimulated with polyIC (TLR3), which could produce robust Th1 polarization (data not shown). Thus despite all three DC types having a similar ability to induce memory T cell IL-17A, IFN-γ, and IL-4 production, the mechanism by which this is mediated differs between the DCs. MoDCs but not LCs or dDCs appear to produce a

![FIGURE 3. DCs can polarise Th1, Th2, and IL-10+ cells but not Th17 cells from naive T cells. A–D, TLR-stimulated in vitro-derived DC types were cultured with allogeneic cord blood naive T cells for 7 days before re-stimulation and intracellular cytokine staining (N.D. indicates not determined). Data are the mean ± SEM of five to eight independent experiments. *, p < 0.05; ***, p < 0.001 (compared with unstimulated DC-T cells) by one-way ANOVA with Bonferroni's post test.](http://www.jimmunol.org/)}
soluble factor(s) which contribute to memory T cell cytokine production.

To explain this observation further, the TLR-stimulated DC supernatants were assayed for the IL-12/23p40 common subunit, Th17-inducing IL-23, and Th1-inducing IL-12p70 cytokines. CD40L costimulation was used for IL-12p70 and IL-23 to maximize DC cytokine secretion (26) and may be more representative of the conditions in DC-T cell cocultures. LCs and moDCs but not

FIGURE 4. DCs stimulated with TLRs are able to induce the production of IL-17A, IFN-γ, IL-4, and IL-10 from memory T cells. A–D, TLR- or dectin-stimulated in vitro-derived DC types were cultured with allogeneic memory T cells for 7 days before restimulation and intracellular cytokine staining (N.D. indicates not determined). Data are mean ± SEM of seven to eleven (LCs) or five to seven (dDCs and moDCs) independent experiments. *, p < 0.05 (compared with unstimulated DC-T cells) by one-way ANOVA with Bonferroni’s post test.

FIGURE 5. Human skin LCs are unable to polarize Th17 cells from naive T cells, but can efficiently induce IL-17A secretion from memory T cells. Human epidermal LCs (isolated from cells that have migrated out over 48 h) were stimulated with TLR agonists and cultured with allogeneic naive (A) or memory T cells (B) for 7 days before restimulation and intracellular cytokine staining. The percentage positive cells are shown in each quadrant (gated on the CD3⁺CD4⁺ cells). Results are representative of three independent experiments.
dDCs secreted large quantities of IL-12p70, even in the absence of CD40L costimulation, particularly after TLR2-LPS and CL097 (TLR7/8) stimulation (Fig. 8A). MoDCs produced IL-12p70 after stimulation with polyIC (TLR3) or CL097 (TLR7/8) and dDCs after stimulation with CL097 (TLR7/8) (Fig. 8B), although there was donor variation in IL-12p70 secretion. The LCs were unable to produce IL-12p70 even after TLR and CD40L stimulation. MoDCs also secreted significant quantities of IL-23 after stimulation with polyIC (TLR3) or CL097 (TLR7/8). LCs and dDCs produced lower amounts of IL-23 after TLR stimulation, however they were able to produce larger quantities when stimulated with zymosan (TLR2/dectin-1), which is known to induce IL-23 (24) (Fig. 8C). None of the three DC populations were able to secrete IL-1β after stimulation with the dectin-1 ligand curdlan (data not shown). Thus, moDCs produced both IL-12p70 and IL-23 after TLR stimulation, whereas dDCs did not secrete as much IL-12p70 or IL-23 and their supernatants could not induce memory T cell cytokine production, suggesting that their mechanism of action relies on cell-associated factors rather than soluble cytokines.

**Discussion**

DCs are important in driving T cell responses, but less is known about the influence of DC subsets on Th cell polarization, particularly Th17 cells. Distinct human DC subsets exist including LCs, dDCs, and moDCs, which were found to have different TLR expression patterns and thus have the potential to respond to different pathogen molecule agonists. CD34-derived LCs are widely used as models for skin LC. They express langerin and CD1a costimulatory molecules and have Birbeck granules but do not express markers associated with dDCs such as CD11b, DC-SIGN, and the mannose receptor. The TLR profile of our in vitro-generated dDC are similar to that described for dDC isolated from skin (28). There is lack of agreement between two independent studies of the TLR profile in LC isolated from skin. One reports expression of TLRs 1, 2, 3, 5, 6, and 10 (29) while a second observed expression of TLRs 1, 3, 6, and 7 (28). We find expression of TLRs 1, 2, 4, 5, 6, 7, and 10 in CD34-derived LCs. Studies using LCs derived from skin have shown an absence of TLR4 and TLR7/8 mRNA (28). Thus, there may be some differences between the CD34-derived in vitro model and ex vivo-isolated LCs. Interestingly, we show for the first time the three DC subsets tested had differing abilities to induce T cell proliferation. LCs and dDCs were more proficient at inducing naive T cell proliferation whereas moDCs were better at inducing the proliferation of memory T cells. Such differences may be partially due to the ability of moDCs to produce cytokines, including IL-12p70 and IL-23, which can induce memory T cell proliferation (30). These intrinsic differences between the DC subsets may indicate functional
specialization in primary and secondary responses and thus differing roles in guiding adaptive immunity.

Much work has been conducted on the differentiation of naive precursors into Th17 cells, however few studies have looked at the influence of DC subsets on naive T cell polarization. Studies have variably shown the importance of IL-1β, IL-6, IL-23, and TGF-β in human Th17 polarization (5–9), however these studies have often included a strong TCR stimulus, high concentrations of cytokines, and IFN-γ/IL-4/IL-12 neutralization, which may not represent the conditions present in vivo. We were unable to induce the polarization of Th17 cells from naive T cells using TLR-stimulated DCs even in IMDM, in agreement with the findings of other studies using moDCs (10) and monocytes (31). However, some studies have seemingly been able to induce Th17 polarization from naive T cells using DCs (5, 32). The differences between these data may be due to several technical factors including the addition of anti-CD3 Ab and IL-2, the type of medium (33), the presence of serum (7, 8), or the source and purity of naive T cells. In the study by Mathers and colleagues (32), CD45RA+ T cells were isolated from adult peripheral blood to a purity of >90%, however CD45RO+ memory T cells can revert to expression of CD45RA (23, 34), which means there may be contaminating memory Th17 cells that are expanded upon culture, thus confounding the data of apparent de novo Th17 differentiation. In our system, we have not used anti-CD3 Ab or IL-2, and have confirmed data with serum-free or IMDM and used naive CD4+ cells isolated from cord blood.

In contrast to TLR stimulation, DCs stimulated with the dectin ligands zymosan or curdlan were able to induce Th17 polarization.

**FIGURE 7.** MoDC supernatants induce IL-17A and IFN-γ production by memory T cells. Memory CD4+ T cells were cultured with anti-CD2/CD3/CD28 beads for 7 days with medium alone, supernatants from unstimulated DCs or TLR-stimulated or zymosan-stimulated DCs (in vitro-derived) before re-stimulation and intracellular cytokine staining. Data shown are the mean ± SEM of four to six independent experiments. *p < 0.05 analyzed by the Mann-Whitney U test compared with medium control.

**FIGURE 8.** Cytokine production by DCs stimulated with TLR agonists. In vitro-derived DCs were stimulated for 24 h with TLR or dectin agonists and CD40L, after which the supernatants were assayed for IL-12/23p40 (A), IL-12p70 (B), and IL-23 (C) by ELISAs. Data shown are the mean ± SEM of five to eight experiments. *p < 0.05 analyzed by the Mann-Whitney U test compared with unstimulated DCs.
of naive T cells in IMDM, which correlated with the ability of all three DC types to secrete IL-23 in response to zymosan. Thus potentially pathogenic Th17 cells may only be induced in response to a restricted range of pathogens that specifically stimulate dectin. The incapacity of TLR-stimulated DCs to induce Th17s appears to be due to the inability to produce key cytokines needed for Th17 development, because the DCs crucially did not secrete IL-1β and only moDCs produced significant amounts of IL-23 after TLR-stimulation. Additionally, the supplementation of MLR cultures with exogenous IL-1β, IL-6, IL-23, and TGF-β cytokines was able to re-establish Th17 development. This raises the question of whether in vitro T cell differentiation models using high concentrations of cytokines and strong TCR stimuli are comparable to the quality of signals offered by DCs in vivo. Nonetheless, it is also possible that in vivo, DCs are able to produce these cytokines in sufficient quantities, with the help of other cells and factors present in the lymph node environment. Other possible reasons for our inability to induce Th17 using TLR-stimulated DCs may include the kinetics of stimulation or the production of inhibitory factors by the DCs (including IL-12p70, IFN-γ, and IL-4) (4), although at least some of these factors would presumably be present in the in vivo situation. The presence of inhibitory factors such as IL-12p70 and IFN-γ may also partially explain why the moDCs induced lower levels of naive Th17 than the LCs and dDCs in IMDM.

Despite the inability of TLR-stimulated DCs to generate Th17 cells from naive precursors, DCs could efficiently polarize Th1 cells, particularly TLR3 or TLR7/8-stimulated dDCs and moDCs. MoDCs stimulated with these agonists induced high levels of IL-12p70, which is known to be a key Th1 polarising factor. Indeed, TLR3-stimulated moDC supernatants were also able to induce naive Th1 polarization, demonstrating the reliance on a soluble cytokine for this effect. Interestingly, dDCs treated with the TLR3 agonist induced equivalent levels of Th1 polarization as the moDCs, but in the absence of detectable IL-12p70, as reported previously for LCs and dDCs (20). Thus, even though dDCs and moDCs have a similar surface phenotype (35) and similar ability to induce Th1 responses after TLR3 or TLR7/8 stimulation, they have distinct modes of action.

All DC types analyzed were able to induce memory Th1 and Th17 responses with equal ability, although TLR stimulation of LCs and dDCs had no significant effect on the magnitude of the memory response, as reported previously for moDCs (10). The exception was dectin- or TLR2-stimulated moDCs which significantly increased the percentage of IL-17+ cells. At present it is unclear whether the DCs are causing the reactivation of precommitted effector memory Th17 cells (11) which may explain why TLR stimulation only has a minimal effect, because memory responses are less dependent on costimulatory signals induced by TLR stimulation (36). Alternatively, DC factors could be affecting un-polarised Ag-experienced cells, which may be why dectin and TLR2-stimulated moDCs augment the proportion of IL-17+ cells.

Even though all DC subsets induced a similar magnitude of Th1 and Th17 memory responses, the mechanism by which they mediated their effect differed. MoDCs supernatants, containing IL-12p70 and high levels of IL-23 were able to enhance the memory Th1 and Th17 responses, whereas LC and dDC supernatants could not. In accordance with this observation, LC and dDC supernatants did not contain IL-12p70 (except TLR7/8-stimulated dDCs) and contained lower amounts of IL-23, despite the production of the IL-12/23p40 common subunit as observed previously (20, 27, 37). Interestingly, unlike moDCs, supernatants from zymosan-stimulated LCs and dDCs which contained high levels of IL-23 were unable to enhance memory Th17 responses. Further studies that neutralize IL-12p70 and IL-23 should clarify to what extent these cytokines play a role. Several studies have highlighted the importance of IL-23 and IL-1 in memory IL-17 production (7, 9–11), however we have shown that in the presence of low amounts of IL-23, efficient memory IL-17 responses can still be induced by LCs and dDCs. Because the addition of LC or dDC supernatants had no enhancing effect on memory cell cytokine production, it may be that a cell contact mechanism is operating instead. Our findings are similar to that observed with TLR-stimulated monocytes, whose mode of action depended on cell contact and whose supernatants also had no amplifying effect on the IL-17+ cells (31).

In contrast to LCs and dDCs, moDCs induced memory IFN-γ and IL-17 by a mechanism partially dependent on soluble factors. MoDCs produced high levels of both IL-12p70 and IL-23 with TLR3 or TLR7/8 stimulation. IL-12p70 induces IFN-γ production from both naive and memory T cells (30) in accordance with the strong Th1 polarization seen with TLR3 and TLR7/8 stimulation. IL-23 induces memory IL-17 but also memory IFN-γ production (38), which may explain the particularly strong IFN-γ memory responses. Stimulation of moDCs through dectin or TLR2 as well as supernatants from dectin-stimulated moDCs significantly enhanced memory IL-17 production. In contrast to dectin stimulation which induced high levels of IL-23, TLR2-LPS only induced lower levels of IL-23 which may indicate that only low amounts of IL-23 are needed for IL-17 production. Additionally, IL-12 inhibits IL-17 induction and the IL-17-enhancing effect of IL-23 (38), suggesting that the outcome is dependent on the relative balance of IL-12p70 vs IL-23. Because dectin and TLR2-stimulated moDCs did not produce any IL-12p70 but still produced IL-23, this balance may have been sufficient to induce IL-17.

In experiments where total lymphocytes were used in the MLRs, LCs induced a significantly greater IL-17 and IL-4 response than dDCs and moDCs as previously observed (32, 39). In our cultures, this may have been due to the other non-T cells present in the lymphocyte-enriched population, which augmented the effect of the LCs. Although the composition of these other cells may not be representative of cells in the skin tissue or lymph node, this result highlights how the presence of other cells may dramatically alter the responses induced by different DCs, which should be taken into account when comparing in vitro and in vivo studies.

In conclusion, we have shown that functionally distinct populations of human DCs can be differentially stimulated by TLRs agonists, have distinct effects on T cell subsets and mediate these effects by different mechanisms. TLR-stimulated DCs were unable to induce Th17 polarization of naive T cells, despite strong Th1 polarization, but could still efficiently produce memory Th1 and Th17 responses. The effect of the type of TLR stimulus was also demonstrated, with TLR3 and TLR7/8 stimulation skewing toward a Th1 response, whereas TLR2 or dectin stimulation increased the IL-17 response in moDCs. MoDCs appear to partially mediate their effect via soluble factors, whereas LCs and dDCs can elicit a similar memory response to moDCs, but this appears to be dependent on cell contact.

Disclosures
The authors have no financial conflict of interest.

References


