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Differential Capability of Human Cutaneous Dendritic Cell Subsets to Initiate Th17 Responses

Alicia R. Mathers,*§ Brian M. Janelins,** Joseph P. Rubin,† Olga A. Tkacheva,* William J. Shufesky,*† Simon C. Watkins,**§ Adrian E. Morelli,**§ and Adriana T. Larregina2*§

Human skin-migratory dendritic cells (DCs) have the ability to prime and bias Th1 and Th2 CD4+ T lymphocytes. However, whether human cutaneous DCs are capable of initiating proinflammatory Th17 responses remains undetermined. We report that skin-migratory DCs stimulate allogeneic naive CD4+ T cells that differentiate simultaneously into two distinct effector Th17 and Th1 populations capable of homing to the skin, where they induce severe cutaneous damage. Skin-migratory Langerhans cells (smiLCs) were the main cutaneous DC subset capable of inducing Th17 responses dependent on the combined effects of IL-15 and stabilized IL-6, which resulted in IL-6 trans-signaling of naive CD4+ T cells. Different from smiLCs, purified skin-migratory dermal DCs did not synthesize IL-15 and were unable to bias Th17 responses. Nevertheless, these dermal DCs were capable of differentiating Th17 cells in mixed leukocyte cultures supplemented with IL-15 and stabilized IL-6. Overall, our data demonstrate that human epidermal smiLCs induce Th17 responses by mechanisms different from those previously described and highlight the need to target clinical treatments based on these variations. The Journal of Immunology, 2009, 182: 921–933.

Efficient activation of naive CD4+ T cells requires the recognition of non-self Ag peptides in the context of MHC class II molecules (signal 1) followed by positive co-stimulation (signal 2). In addition, T cell polarization is determined by the presence of a specific cytokine profile secreted during Ag presentation (signal 3). These three signals are provided by professional APCs, such as dendritic cells (DCs) (1). Likewise, tissue resident DCs respond to inflammatory mediators and foreign Ags in their microenvironment by priming and biasing the differentiation of naive CD4+ T cells within the draining lymph nodes, while maintaining peripheral T cell tolerance to self-Ags (1, 2).

According to the pattern of cytokines secreted, CD4+ T lymphocytes are classified as Th1 or Th2 cells, which are effectors of cellular or humoral immunity, respectively. The recently described Th17 responses involve in host protection against infections and induction of chronic inflammation leading to autoimmunity (3–7). Th17 cells achieve these functions by secreting proinflammatory cytokines associated with chronic inflammation, the most relevant being IL-17A (IL-17), IL-17F, IL-22, and TNF-α, along with the proinflammatory chemokines CXCL1 and CCL20 (5, 8).

In mice, Th17 differentiation depends on the presence of IL-6 and TGF-β1 (9–13). However, in humans, the mechanisms regulating the initiation of Th17 differentiation are not completely elucidated. Accordingly, recent studies focused on the relevance of IL-1β and TGF-β1 to induce human Th17 responses demonstrated a dependence on IL-1β and either a suppressive or a beneficial effect of TGF-β1 (14–18). Additionally, Th17 responses are amplified by IL-23, IL-21, and IL-15 and suppressed by IL-4, IFN-γ, IL-12, and IL-27 (3, 19, 20). Finally, the role of APCs, particularly DCs, in biasing Th17 differentiation is not well clarified and it is not known whether tissue resident DCs are a source of Th17-driving cytokines. Therefore, in humans the role of different cytokines, and their cellular source, capable of initiating and sustaining proinflammatory Th17 immunity remains inconclusive.

The skin is a highly immunogenic organ capable of triggering inflammation and potent T cell responses by appropriately responding to antigenic stimuli. The immunogenicity of skin correlates with a substantial number of resident DCs including epidermal Langerhans cells (LCs) and dermal DCs (DDCs), which are both capable of activating naive T cells and biasing Th1 and Th2 immune responses (2, 21, 22). In addition, effector Th17 and Th1 cells infiltrate the skin during the development of chronic cutaneous inflammatory and autoimmune disorders, such as those observed in psoriatic plaques, indicating that skin resident DCs have a role in the initiation and maintenance of Th17 immunity (6, 23, 24).

Nevertheless, the capacity of different populations of human cutaneous DCs to initiate or inhibit Th17 differentiation has not been addressed mainly due to the lack of appropriate experimental models necessary to obtain a sufficient number of highly purified human LCs and DDCs. In this regard, the physiological model of human skin epidermal-dermal explants allows for the isolation of a high number of purified skin-migratory DCs (smiDCs) mobilizing from the skin to the skin draining lymph nodes via lymphatic vessels (22, 25). Therefore, the model of human skin explants represents a unique tool to address the T cell stimulatory and biasing functions of cutaneous DCs. The heterogeneity of smiDCs is identical to that obtained from human lymph after cannulation of lymphatic vessels, which based on their expression of CD1a and CD14 and according to their T cell stimulatory functions, can be classified as 1) CD1a+/CD14- skin-migratory
LCs (smiLCs) and CD14<sup>low</sup>/CD14<sup>−</sup> skin-migratory DDCs (smi-DDCs) (both potent T cell stimulators) or 2) CD1a<sup>+</sup>CD14<sup>−</sup> immature DCs (weak T cell stimulators) (22, 25, 26).

In the present study, using the ex vivo model of human skin epidermal-dermal explants, we addressed the ability of purified populations of human smiDCs to induce the physiologically relevant alloreactive Th17 immunity. We show that smiDCs induce Th17 responses, which readily coexist with Th1 cells. Furthermore, responder CD4<sup>+</sup> T cells have the phenotype of effector lymphocytes with skin-homing properties, as determined by the expression of skin homing receptors and their ability to infiltrate skin-explants and cause severe tissue damage. Under our experimental conditions, Th17 differentiation was induced by smiLCs rather than by smiDCs. A mechanistic analysis demonstrated that smiLCs and not smiDCs produced IL-15, and only smiLCs increased the secretion of IL-6 in ML. Thus, IL-6 trans-signaling synergized with the smiLC-secreted IL-15 to initiate Th17 differentiation. Purified smiDCs did not synthesize IL-15 and were unable to bias Th17 responses. However, smiDCs acquire the ability to initiate Th17 differentiation in MLC supplemented with IL-15 and stabilized IL-6.

**Materials and Methods**

### Isolation of smiDCs and naive CD4<sup>+</sup> T cells

Samples of normal human skin were obtained from healthy donors undergoing abdominal plastic surgery. Human peripheral blood samples (leukopacks) from healthy volunteers were obtained from the blood bank. Both skin and blood samples were obtained following Institutional Review Board approval and used according to the University of Pittsburgh guidelines.

The smiDCs, used as stimulators of CD4<sup>+</sup> T cells, were purified after migration from human skin explants as previously described (22, 27). Briefly, skin explants composed of epidermis and a thin layer of dermis (0.5 mm thick) were obtained from normal human skin samples using a cutaneous minikine (Integra-Padget). Following rinsing, skin explants were cultured epidermal-side up on top of sterile stainless steel mesh screens (0.1 mm pore) placed inside 100-mm<sup>2</sup> petri dishes. Culture plates were cultured epidermal-side up on top of sterile stainless steel mesh screens (0.1 mm pore) placed inside 100-mm<sup>2</sup> petri dishes. Culture plates were cultured skin-explants for 72 h. Following culture, the nonadherent cell fraction composed of smiDCs and lymphocytes was collected and quantified. Cell viability was determined using a beta scintillation counter. Assays were performed in triplicate and the results are expressed as mean of cpm ± 1 SD.

### MLC analysis

For T cell proliferation assays, 10<sup>5</sup> naive CD4<sup>+</sup> CD45RA<sup>+</sup> T cells were cocultured with 10<sup>5</sup> gamma-irradiated (2000 rad) total smiDCs or smi-DDCs. CD4<sup>+</sup> T cells at a 1:10 stimulator to responder ratio. Where indicated, the cytokine-specific Abs IFN-γ mAb (10 μg/ml; R&D Systems), IL-6 mAb (10 μg/ml; R&D Systems), IL-23 mAb or polyclonal Ab (10 μg/ml; R&D Systems), IL-17 mAb (10 μg/ml; R&D Systems), IL-6Rα mAb (10 μg/ml; R&D Systems), IL-15 mAb (10 μg/ml; R&D Systems), or mouse IgG2b isotype control (10 μg/ml; R&D Systems) were added alone or in combination with recombinant human IL-6 (20 ng/ml; R&D Systems), recombinant human IL-15 (20 ng/ml; R&D Systems), or gp130Fc chimeric protein (10 ng/ml; R&D Systems). Controls included naive CD4<sup>+</sup> T cells and smiDCs, smiLCs, or smiDCs cultured alone. Unless otherwise indicated culture supernatants were collected on day 5 of the MLC to perform cytokine-specific ELISA.

### smiDC cultures

For studies analyzing cytokine release and mRNA transcript expression by smiDCs, total smiDCs, smiLCs, or smiDCs were cultured at a concentration of 2.5 × 10<sup>5</sup>–1 × 10<sup>6</sup> cells/ml in 24-well plates in 500 μl of serum-free AIM V medium supplemented with 10 μg/ml gentamicin. Following 24 h of incubation at 37°C, the supernatants or cells were collected for cytokine protein analysis and mRNA detection, respectively. In experiments analyzing secretion of cytokines by activated DCs, smiDCs were plated in 24-well plates precoated with CD40 agonistic mAb (10 μg/ml; Caltag Laboratories). Where indicated, the culture medium was supplemented with recombinant human IFN-γ (1000 U/ml; R&D Systems), recombinant human IL-17 (50 ng/ml; eBioscience), or a combination of both.

### Skin-explant assay

Skin-explant assays were performed as previously described (28). Briefly, following 5-day MLC responder CD4<sup>+</sup> T cells were harvested. In some experiments, responder CD4<sup>+</sup> T cells were labeled with CFSE (Molecular Probes) and replated in 24-well tissue-culture plates at 1 × 10<sup>5</sup> cells/ml in 400 μl of serum-free AIM V medium with the addition of allogeneic skin explants composed of the epidermis and a thin layer of dermis. Skin-explant assays were incubated for 3 days at 37°C; explant sections were then collected for RNA isolation, processed for H&E staining, or snap-frozen and immunolabeled as described below.

### Staining and imaging of skin explants

To determine whether responder CD4<sup>+</sup> T cells, generated in 5-day MLC, have skin-homing capabilities, 1 × 10<sup>5</sup> responder CD4<sup>+</sup> T cells were labeled with CFSE (Molecular Probes) and replated in 24-well culture plates at 1 × 10<sup>5</sup> cells/ml in 400 μl of serum-free AIM V medium with the addition of allogeneic skin explants composed of the epidermis and a thin layer of dermis. Skin-explant assays were incubated for 3 days at 37°C; explant sections were then collected for RNA isolation, processed for H&E staining, or snap-frozen and immunolabeled as described below.

### Detection of secreted cytokines

The release of cytokines by smiDCs or responder CD4<sup>+</sup> T cells was analyzed in culture supernatants by ELISA or Searchlight Technologies. IFN-γ (OptEIA; BD Pharmingen), IL-17 (R&D DuoSet), soluble IL-6R (R&D DuoSet), IL-6 (OptEIA; BD Pharmingen), IL-1β (R&D DuoSet), IL-23 (eBioscience), and IL-13 (Quantikine; R&D Systems) production was quantified by ELISA, according to the manufacturer’s instructions. Plaques were analyzed using an E-max plate microplate reader (Molecular Devices). IL-6 and TGF-β1 were also analyzed by Endogen Searchlight Technologies (Thermo Fisher Scientific). Assays were performed in duplicate and the results are expressed as mean concentration ± 1 SD.
Detection of cytokine mRNA

For detection of cytokine mRNA transcripts total RNA was isolated from smiDC subsets following 24 h of culture or total RNA was isolated from skin explants using the Qiagen RNeasy mini kit, according to the manufacturer’s instructions, and eluted in 30 µl of water. RNA concentration and quality was determined by a 260/280 nm ratio using Biomate 3 spectrophotometer (Thermo Electron). One hundred to 500 ng of RNA were used to perform first-strand cDNA synthesis following removal of genomic DNA contamination using QuantiTect reverse transcription kit according to the manufacturer’s instructions (Qiagen). Finally, using the AmpliTaq Gold PCR master mix (Applied Biosystems) and 10X Quantitect primer assays (Qiagen) (IL-15 or the endogenous control SDHA (succinate dehydrogenase complex)), or IL-17 primer pairs (R&D systems), the PCR and amplification was setup according to the manufacturer’s instructions with the exception that only a 25-min hold at 72°C (Applied Biosystems). PCR product was run on a 2% agarose gel and visualized with ethidium bromide staining. Expected products at 131 bp for IL-15, 220 bp for IL-17, and 147 bp for SDHA.

Flow cytometric analysis

CD4+ T cells, collected on days indicated from MLC or following 5-day CD3ε stimulation (clone UCHT1, 20 µg/ml; BD Pharmingen), were blocked with 10% normal human serum. For surface staining, cells were incubated (30 min, 4°C) with combinations of 1) CyChrome-CD3 mAb; 2) FITC-CD45RO, FITC cutaneous lymphocyte Ag, FITC-CD25, or FITC-CD70 mAbs (BD Pharmingen); and 3) PE-IL-6Ra (BD Pharmingen), PE-CCR4, or PE-CCR10 mAbs (R&D Systems). To detect cytokine expression by responder T cells, we also performed intracellular staining procedures on day 3 of the MLC. Day 3 was chosen to compare the percentage of responder CD4+ T cells expressing IL-17 to IFN-γ while IL-17 is still actively expressed. For intracellular staining, cells were fixed in 4% paraformaldehyde following surface staining and then permeabilized in a 0.1% (w/v) saponin solution and stained with PE-IL-17 mAb (eBioscience) and FITC-IFN-γ, FITC-TNF-α, or FITC-IL-6 mAbs (BD Pharmingen). Appropriate fluorochrome-conjugated isotype-matched irrelevant mAbs were used as controls. After staining, cells were fixed with 2% paraformaldehyde and analyzed using a FACSCalibur flow cytometer (BD Immunocytometry Systems).

Statistical analysis

Using the GraphPad Prism 4 software, mean values from multiple groups were compared by ANOVA followed by Newman-Keuls post hoc test. Mean comparisons between two different groups were performed by two-tailed Student’s t test. A value of p < 0.05 was considered significant.

Results

smiDCs induce effector Th17 and Th1 responses

We and other researchers have demonstrated that generation and culture of human skin explants provides the proinflammatory signaling necessary to trigger the activation and migration of potent T cell stimulatory DCs via lymphatic vessels (21, 22, 27, 29-31). In a recent publication, we further demonstrated that alloreactive CD4+ T cells stimulated with either highly purified smiLC or smiDDCs proliferated and secreted significantly high levels of IFN-γ (21).

In the present study, we confirmed that smiDCs stimulate the proliferation of naïve allogeneic CD4+ T cells in 5-day MLC (Fig. 1A). Additionally, activated CD4+ T cells secreted IL-17 and IFN-γ, and in some cases low/intermediate levels of IL-5 and IL-13 (Fig. 1, B-D). IL-4 was not detected in any of the culture supernatants (data not shown). As expected, working with a model...
of human alloreactive cells, variations in the HLA mismatch accounted for variable levels of IL-17, IFN-γ, IL-5, and IL-13 observed in the MLC (Table I, data not shown and 21). However, secretion of IL-17 by responders CD4+ T cells increased significantly vs control naïve T cells (∼40-fold) in each independent experiment analyzed (Table I). The coexpression of IFN-γ and IL-17 by responder CD4+ T cells was further analyzed by flow cytometry in 3-day MLC, which demonstrated that IFN-γ and IL-17 were produced by two distinct CD4+ T cell subsets with a small percentage of CD4+ T cells synthesizing both cytokines (Fig. 1E). Approximately 80% of Th17 cells coexpressed TNF-α, a characteristic proinflammatory cytokine that is produced by both Th17 and Th1 cells (Fig. 1E). IL-17-secreting cells corresponded to 19.8–22.3% of cytokine-producing responder CD4+ T cells and represented 2.2–4.2% of the total CD4+ T cells present in the MLC (Fig. 1E). Responder CD4+ T cells expressed de novo memory (CD45RO) and activation (CD25 and CD70) markers (Fig. 2A). In addition smiDCs were capable of imprinting the responder CD4+ T cells for cutaneous homing by inducing the expression of the skin-homing molecules CCR4, CCR10, and cutaneous lymphocyte Ag and by down-regulating the lymph node homing molecule, CCR7 (Fig. 2A and data not shown). We further compared the activation and skin-homing phenotype of CD4+ T cells stimulated by smiDCs or a mAb directed to the CD3ε chain of the TCR. Stimulation of CD4+ T cells via TCR (Fig. 2B) lead to the increased expression of memory (CD45RO) and activation (CD25 and CD70) markers almost to the same extent as smiDCs (Fig. 2A). However, smiDCs were more efficient at inducing the up-regulation of skin-homing molecules in responder CD4+ T cells than CD3 stimulation (Fig. 2B). Together these results indicate that human smiDCs induce effector CD4+ T cells that have the potential to home to the skin and secrete IL-17 and IFN-γ.

The physiological relevance of effector CD4+ T cells stimulated with smiDCs was analyzed by means of an in situ skin explant assay (28). We addressed the ability of responder CD4+ T cells to 1) colonize the skin, 2) produce IL-17 in situ in the skin, and 3) cause skin damage. The capacity of effector CD4+ T cells to home into allogeneic skin samples was examined by confocal laser scanning microscopy. As shown in Fig. 3A, CFSE-positive CD4+ effector T cells from 3-day MLC infiltrated the upper dermis of the skin explants where the cells were arranged between dermal fibers. Naïve CD4+ T cells labeled with CFSE did not infiltrate the skin (data not shown). Importantly, cutaneous-infiltrating responder CD4+ T cells produced IL-17, as determined by the presence of IL-17 mRNA transcripts and protein in skin explants cocultured with effector CD4+ T cells, and not in explants cultured with naïve CD4+ T cells (Fig. 3, B and C, and data not shown). Furthermore, skin explants incubated with responder CD4+ T cells for 3 days exhibited significant damage characterized by cytoplasmic vacuolization and apoptosis of basal keratinocytes and focal detachment of the epidermal-dermal junction, resulting in cleft formation (Fig. 3D). Severe basal keratinocyte apoptosis was confirmed by positive TUNEL staining in skin explants incubated with responder CD4+ T cells (Fig. 3E). By contrast, this extensive skin damage was not observed in control explants cocultured with naïve CD4+ T cells (Fig. 3, D and E). To further determine whether the levels of IL-17 or IFN-γ secreted by effector CD4+ T cells were responsible for the severe skin damage observed, skin explants were incubated with 5-day MLC supernatants. As shown in Fig. 3F, skin samples cultured with MLC supernatants treated with IFN-γ- or IL-17-neutralizing mAbs or left untreated, presented similar pathological skin damage as samples observed when skin explants were cultured with effector CD4+ T cells. Conversely, the skin damage was completely abrogated by simultaneous neutralization of IFN-γ and IL-17 in MLC supernatants or in control skin samples cultured in serum-free medium alone (Fig. 3F). Together, these experiments indicate that smiDCs induce physiologically relevant effector Th17 and Th1 cells capable of homing to the skin and exerting a severe degree of tissue damage reminiscent of that previously described for graft-vs-host disease (28, 32, 33).

We next analyzed the kinetics of the secretion of IL-17 and IFN-γ from alloreactive CD4+ T cells, which demonstrated that IFN-γ secretion was maximal on day 5 and revealed a peak in IL-17 production on day 4 of the MLC (Fig. 4, A and B). With these results, we investigated the role that IL-17 might have in the induction and bias of Th1 responses by blocking IL-17 during MLC. Neutralization of IL-17 did not have a significant impact on the secretion IFN-γ by responder CD4+ T cells (Fig. 4A).
Because Th1 responses have been described as negative regulators of IL-17 production (12, 13, 20), we wanted to determine whether the secretion of IFN-γ that we observed suppressed IL-17 secreted by effector CD4+ T cells by the addition of an IFN-γ-neutralizing mAb in 5-day MLC. As shown in Fig. 4D, inhibition of IFN-γ during the MLC resulted in a significant decrease in IL-17 secreted by responder CD4+ T cells (from 630 ± 11 pg/ml to 410 ± 4 pg/ml), indicating that in our system IFN-γ enhances the production of IL-17.

**smiDCs induce allogeneic Th17 responses independent of IL-23**

The coexistence of responder Th17 and Th1 cells stimulated by smiDCs warranted further examination into the DC mechanisms that promote the simultaneous differentiation of Th17 and Th1 responses. It has previously been published that human cutaneous DCs do not produce IL-12p70 (21, 34). Moreover, our laboratory has demonstrated that the ability of cutaneous DCs to induce Th1 responses is dependent on IL-23 (21). Given that IL-23 is a cytokine known to promote the stabilization of Th17 responses (3, 8, 15, 17), we addressed whether the induction of Th17 immunity observed using our experimental model was mediated by IL-23.

We analyzed the secretion of IL-23 by smiDCs following 24 h of culture supplemented or not with agonistic CD40 mAb with or without IFN-γ or IL-17, conditions that mimic the microenvironment of the Th1 and Th17 immunological synapse. Under these conditions, IFN-γ increased the production of IL-23 by smiDCs, whereas IL-17 did not and the addition of IFN-γ plus IL-17 decreased the amount of IL-23 induced by IFN-γ alone (Fig. 5A). To further explore the role of IL-23 in the differentiation of Th17
responses, we analyzed the secretion of IL-17 by responder CD4+ T cells stimulated in MLC in the presence or absence of an IL-23-neutralizing Ab. As shown in Fig. 5B, inhibition of IL-23 did not reduce IL-17 production, whereas as expected according to our previous publication, the release of IFN-γ was significantly decreased (from 1340.0 ± 40.1 pg/ml to 759.0 ± 7.1 pg/ml) (Fig. 5C) (21). Furthermore, the neutralization of IL-23 did not abrogate CD4+ T cell proliferation induced by smiDCs (Fig. 5D). Taken together, these results confirm that the secretion of IL-23 by human smiDCs is necessary to bias a Th1 response but is dispensable for Th17 differentiation.

**smiDCs secrete IL-6 and IL-6 trans-signaling favors Th17 differentiation**

The differentiation of Th17 cells is initiated in mice by the combined effects of IL-6 and TGF-β1 (9–11). However, in humans, the mechanisms that initiate Th17 immunity are not fully elucidated. Moreover, recent publications exhibit controversial

**FIGURE 4.** IL-17 does not affect the secretion of IFN-γ, whereas IFN-γ enhances IL-17 production. IFN-γ (A) and IL-17 (B) secretion by responder CD4+ T cells stimulated with smiDCs. Data are representative of three independent experiments. Results are mean ± 1 SD from replicates.

**FIGURE 5.** smiDC secretion of IL-23 induces IFN-γ production but not IL-17 from responder CD4+ T cells stimulated in MLC in the presence or absence of an IL-23-neutralizing Ab. As shown in Fig. 5B, inhibition of IL-23 did not reduce IL-17 production, whereas as expected according to our previous publication, the release of IFN-γ was significantly decreased (from 1340.0 ± 40.1 pg/ml to 759.0 ± 7.1 pg/ml) (Fig. 5C) (21). Furthermore, the neutralization of IL-23 did not abrogate CD4+ T cell proliferation induced by smiDCs (Fig. 5D). Taken together, these results confirm that the secretion of IL-23 by human smiDCs is necessary to bias a Th1 response but is dispensable for Th17 differentiation.

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conclusions regarding the role of TGF-β1 in biasing Th17 cells and the effects of IL-6 during the initiation of Th17 responses has not been well defined (14–18, 35). In this context, we addressed the mechanisms by which human smiDCs promote the Th17 response observed in the present study.

First, we examined the potential of smiDCs to secrete IL-6 and TGF-β1 (Fig. 6, A and B) in the presence or absence of CD40 signaling with or without IFN-γ, IL-17, or a combination of both. As shown in Fig. 6A, smiDCs secreted moderate amounts of IL-6 (227 ± 8 pg/ml), which were slightly enhanced by stimulation with the CD40 mAb (297.8 ± 1.1 pg/ml). Secreted amounts were significantly increased following addition of IFN-γ (512 ± 21 pg/ml), IL-17 (478 ± 5 pg/ml), or a combination of the three stimuli (589 ± 33 pg/ml). In contrast, in the absence of CD40 stimulation, IFN-γ with IL-17 or IFN-γ alone did not induce a significant increase in IL-6 secretion (data not shown). The release of TGF-β1 by smiDCs remained unchanged irrespective of treatments (Fig. 6B). However, neutralization of TGF-β1 during MLC lead to an increase in IL-17 (Fig. 6C) and a decrease in IFN-γ secreted by responder CD4+ T cells (Fig. 6D), indicating that TGF-β1 negatively regulates the development of a Th17 bias. TGF-β1 might, however, be necessary to promote Th1 responses stimulated by smiDCs (Fig. 6, C and D). The role of IL-6 in the development of Th17 responses in our system was further analyzed by supplementing MLC with recombinant human IL-6 and stabilizing IL-6 mAbs. Addition of recombinant human IL-6 did not increase IL-17, whereas the presence of a stabilizing IL-6 mAb significantly augmented the production of IL-17 by responder CD4+ T cells by 11-fold (Fig. 7A). This increase was specific, as demonstrated by the lack of effect of an irrelevant isotype-matched mAb (negative control) (Fig. 7A). The enhanced secretion of IL-17 by the addition of the IL-6 mAb is explained by the fact that human IL-6 mAbs act as cytokine carriers to increase the half-life of IL-6 (36, 37). Together these data indicate that the IL-6 secreted during the MLC contributes to the initiation of Th17 differentiation.

To further analyze the functional role of IL-6 in our system, we treated the MLC with an IL-6Rα-neutralizing mAb. Specific inhibition of the IL-6Rα abrogated the IL-6-dependent increase in IL-17 (Fig. 7, A and B) and confirmed that the IL-6–IL-6 Rα complexes are necessary to stabilize and intensify the biological effects of IL-6. Additionally, equally robust T cell proliferation was observed regardless of treatments (data not shown), indicating that the IL-17 present in the MLC was not caused by an increase in T cell number. These results demonstrate that the differentiation of human Th17 cells by smiDCs is highly dependent on stable IL-6 signaling via the IL-6R.

The operational IL-6R has the following two subunits: 1) the α-chain (IL-6Rα) that binds IL-6 specifically and functions as either a membrane-bound IL-6Rα or a cleaved soluble IL-6Rα form and 2) the common gp130 subunit, which is the signal transducer (36). The gp130 signaling occurs following the interaction with either membrane-bound or soluble IL-6Rα. The process used by IL-6–IL-6Rα complexes to signal via gp130 is known as IL-6 trans-signaling. The availability of the common gp130 subunit on the surface of CD4+ T cells was increased by day 2 following smiDC stimulation and peaked by day 4 (data not shown). However, because T cells express variable levels of membrane-bound IL-6Rα, the effects of IL-6 on CD4+ T cell differentiation could have been induced by either binding to membrane-bound IL-6Rα or through the trans-signaling mechanism (38, 39). Therefore, to determine the signaling mechanisms involved in the IL-6-dependent Th17 differentiation stimulated by smiDCs, we measured the secretion of IL-17 in 5-day MLC supplemented with an IL-6Rα-neutralizing mAb that inhibits both membrane-bound and soluble IL-6Rα or with a soluble gp130/Fc chimera protein, which exclusively binds to the soluble IL-6Rα and specifically blocks the IL-6 trans-signaling mechanism (38). Simultaneous neutralization of membrane-bound and soluble IL-6Rα resulted in a similar decrease in IL-17 secretion compared with that observed after inhibition of soluble IL-6Rα alone (Fig. 7B). These data indicate that the Th17 differentiation observed in our experiments occurred through the IL-6 trans-signaling mechanism.

To further confirm that IL-6 can exert its effects by transsignaling naive CD4+ T cells, we analyzed the presence of soluble IL-6Rα in the supernatants of 5-day MLC. Naive CD4+
T cells released low amounts of soluble IL-6Rα, which was significantly increased following stimulation with smiDCs and was further augmented with the addition of the stabilizing IL-6 mAb (Fig. 7C). The high specificity of the neutralizing IL-6Rα mAb and gp130/Fc chimeric protein was demonstrated by the absence of soluble IL-6R detected in the supernatants of 5-day MLC by ELISA (Fig. 7C).

To determine the source of soluble IL-6Rα we compared the levels of expression of membrane-bound IL-6Rα by naive and responder T cells with smiDCs using flow cytometric analysis. Although few naive CD4+ T cells expressed membrane-bound IL-6Rα, the expression was significantly increased by effector T cells on day 4 following stimulation with smiDCs and significantly diminished following IL-6 stabilization (p < 0.05) (Fig. 7D and data not shown). The decrease in membrane expression of IL-6Rα upon further CD4+ T cell activation with IL-6 correlated with the increased soluble IL-6Rα detected in the MLR supernatants by ELISA (Fig. 7, C and D). Furthermore, smiDCs individualized by their expression of CD86, also expressed membrane-bound IL-6Rα that was decreased 24 h following agonistic CD40 mAb stimulation (Fig. 7E). Thus the early IL-6Rα cleaved from smiDCs likely contributes to the pool of soluble IL-6Rα detected in the MLC supernatants and that stimulates naive CD4+ T cells to differentiate into Th17 cells. The continued release of soluble IL-6Rα from responder CD4+ T cells is available to further augment the Th17 response. Based on these data, we can conclude that IL-6 trans-signaling is a dynamic mechanism that is supported by the secretion of soluble IL-6Rα, an active process enhanced by the stabilization of IL-6.

**smiDCs induce Th17 responses**

Given that human smiDCs consist of at least two subsets of immunostimulatory APCs, we sought to determine whether the Th17 differentiation observed in the MLC was preferentially induced by smiLCs or smiDDCs. To address this question, we used highly purified smiLCs or smiDDCs as stimulators of naive CD4+ T cells within the MLC and analyzed the release of IL-17. Under these conditions, the secretion of IL-6Rα by responder CD4+ T cells was induced preferentially by smiLCs (Fig. 8A). Although, we confirmed that smiDDCs stimulate naive CD4+ T cells to proliferate and differentiate into IFN-γ-secreting Th1 cells to the same extent as smiLCs (data not shown and 21). With these results, we asked whether smiDDCs...

**FIGURE 7.** Stabilizing IL-6 trans-signaling significantly increases IL-17 responses. A, Secreted IL-17 by responder CD4+ T cells in 5-day MLC with the addition or not (control) of recombinant human IL-6 (rhIL-6), IL-6 mAb, IgG control, IL-6Rα mAb, or a combination of recombinant human IL-6 and IL-6 mAbs. Data are representative of 10 independent experiments. Results are mean ± 1 SD from replicates. *, p < 0.001 compared with DCs plus T cells. B, Secretion of IL-17 by responder CD4+ T cells in 5-day MLC with the addition or not (control) of IL-6Rα mAb or gp130/Fc chimeric protein. Data are representative of three independent experiments. Results are mean ± 1 SD from replicates. *, p < 0.001 compared with DCs plus T cells. C, Soluble IL-6R detected in the supernatants of 5-day MLC with the addition or not (control) of IL-6 mAb, IL-6Rα mAb, or gp130/Fc chimeric protein. Data are representative of four independent experiments. Results are mean ± 1 SD from replicates. *, p < 0.05 or **, p < 0.01 compared with DCs plus T cells. D, Membrane IL-6Rα detection by responder CD4+ T cells following 5-day MLC in the presence or not (control) of IL-6 mAb. Data are representative of two independent experiments. E, Expression of membrane IL-6Rα by CD86+ smiDCs stimulated for 24 h or unstimulated with plate-bound agonist CD40 mAb. Data are representative of two independent experiments. N.D., Nondetected.
suppressed the Th17 response induced by smiLCs, by stimulating naive CD4+ T cells with a combination of smiLCs and smiDDCs at a 1:1 LC to DDC ratio. Similar levels of IL-17 production were initiated by smiLCs alone or in the presence of smiDDCs (Fig. 8A), demonstrating that smiDDCs do not affect the Th17 response initiated by smiLCs.

We next examined the differences between smiLCs and smiDDCs that might account for their differential capacity to promote Th17 responses. We first addressed the ability of highly purified smiLCs or smiDDCs to secrete IL-6 following 24 h of culture in medium alone or in 5-day MLC. As shown in Fig. 8B, low levels of IL-6 were detected in the supernatants of smiLCs and smiDDCs when cultured alone. However, the amount of IL-6 detected in MLC significantly increased (12.5-fold) in those cultures stimulated with purified smiLCs and not in cultures stimulated by smiDDCs (Fig. 8C). Furthermore, the addition of IL-6-IL-6 mAb complexes to MLC stimulated by smiDDCs was not sufficient to favor a Th17 bias (Fig. 9A). With these results, we further investigated the cellular source of secreted IL-6 in 3-day MLC stimulated with smiLCs by analyzing the intracellular expression of IL-6 by CD3+ IL-17+ responder CD4+ T cells. Flow cytometric analysis confirmed that the responder T cells did not synthesize IL-6 (Fig. 8D, left), as reported previously (3). However, we were able to detect the intracellular expression of IL-6 by smiDCs 24 h following migration (Fig. 8D, right). These results confirm that the high concentration of IL-6 detected in the MLC supernatants was produced by smiLCs during their interaction with CD4+ T cells. Overall, these data indicate that within our culture system smiLCs are the cutaneous DC population responsible for the high level of IL-6 that is required to initiate Th17 differentiation.

**IL-6 and IL-15 produced by smiLCs synergistically induce Th17 responses**

According to our results, inhibition of IL-6 trans-signaling was specific and significant and diminished the secretion of IL-17 by effector CD4+ T lymphocytes by ~50%, suggesting that smiLCs may have complementary mechanisms to promote the development of human Th17 responses. Therefore, we sought to determine whether there were other unique smiLC mechanisms coregulating the differentiation of Th17 cells. We examined whether IL-1β, a cytokine implicated in the generation of human monocyte-induced Th17 responses (14, 15), was preferentially secreted by smiLCs and thus favors the Th17 bias observed in this experiment. Under our experimental conditions, both smiLCs and smiDDCs secreted variable amounts of IL-1β (Fig. 9A). In addition, the levels of IL-1β secreted in the MLC did not increase regardless of the smiDC subset used as stimulators (data not shown), thus excluding IL-1β as a unique factor secreted by smiLCs.

We next examined the role of IL-15, a proinflammatory cytokine synthesized mainly by LCs, compared with DDCs, and which favors the production of IL-17 in patients with psoriasis and rheumatoid arthritis (20, 40–43). First, we compared the presence of IL-15 mRNA transcripts in highly purified smiLCs and smiDDCs and confirmed that IL-15 mRNA transcripts were present only in smiLCs and not in smiDDCs (Fig. 9B). Then, we addressed the ability of IL-15 to promote the secretion of IL-17 by neutralizing IL-15 in 5-day MLC. The inhibition of IL-15 significantly reduced the secretion of IL-17 during MLC (Fig. 9C). Importantly, simultaneous neutralization of IL-15 and IL-6R worked synergistically to abrogate the production of IL-17 by responder CD4+ T cells during MLC (Fig. 9D). Under these experimental conditions, the release of IL-5 by responder CD4+ T cells was not modified, demonstrating that the decrease in IL-17 was not due to overall T cell death (data not shown). Furthermore, there was a significant increase in IL-17 secretion by responder CD4+ T cells when cultures stimulated by smiDDCs were supplemented with IL-15 or a combination of IL-15 and IL-6-IL-6 mAb complexes (Fig. 9E). Together these results demonstrate that within our culture system, smiLCs favor Th17 differentiation by simultaneous secretion of IL-6 and IL-15 and although smiDDCs are unable to initiate Th17 responses, due to the lack of IL-15 expression, they can induce the differentiation of Th17 cells in the presence of IL-15 and IL-6.
Discussion

Th17 immunity has a beneficial role in host protection against extracellular bacteria and certain fungal infections, such as *Candida albicans* (3, 44). Conversely, pathogenic Th17 responses are linked with the onset of chronic inflammatory and autoimmune diseases including contact hypersensitivity and psoriasis vulgaris (5, 6, 24). Accordingly, human Th17 cells express CCR6 and CCR4, two chemokine receptors responsible for directional migration and skin homing of effector immune cells (44, 45). These findings indicate that cutaneous DCs likely play a key role in the initiation of Th17 differentiation. However, recent reports suggest that monocytes rather than DCs are responsible for the induction of human Th17 responses (14). In this study, we used an ex vivo model of human skin explants to harvest activated cutaneous DCs that exhibit potent T cell allostimulatory functions (21, 30, 46, 47). Moreover, in the absence of further proinflammatory or DC-biasing signals, cutaneous DCs induced the simultaneous differentiation of effector Th1 and Th17 cells, which have the capacity to home to the skin.

In the situ skin-explant assay was initially developed to predict the outcome of graft-vs-host disease in bone marrow transplantation patients. This unique model allows for the physiological assessment of the degree of incompatibility between donor and host by scoring cutaneous histological damage induced by effector T cell responses. Likewise in the present study, responder Th17 cells stimulated by smiDCs were able to infiltrate the skin explants and induce pathological damage similar to the cutaneous lesions characteristic of graft-vs-host disease (28, 32). Importantly, under our experimental conditions both IL-17 and IFN-γ were capable of inducing severe cutaneous pathology as confirmed by IL-17 and IFN-γ neutralization assays. Together these results demonstrate that cutaneous DCs initiate effector Th17 and Th1 responses with the ability to infiltrate the skin and cause severe cutaneous damage in humans. Moreover, the cutaneous pathologies described in this study are equivalent to those represented in chronic cutaneous inflammatory and autoimmune disorders (3–7, 33).

The simultaneous initiation of Th17 and Th1 cells stimulated by smiDCs in our model is in contrast to findings in both humans and mice, which demonstrate that IFN-γ suppresses the differentiation of Th17 cells (12, 15, 16). Conversely, we determined that IFN-γ has a positive effect on IL-17 secretion during MLC, suggesting that the proinflammatory properties of IFN-γ are beneficial for Th17 responses, whereas IL-17 did not have a significant impact on secretion of IFN-γ. Nevertheless, the latter observation does not rule out a positive role for IL-17 in certain Th1 functions. In fact, it has been described that Th17 cells homing to peripheral tissues secrete Th1 chemokines at sites of chronic inflammation (48). In agreement with these observations, our data strongly suggests that at least in the skin the coexistence of both Th17 and Th1 proinflammatory responses induced by cutaneous DCs collaborate in the induction of cutaneous immunity and autoimmunity. Furthermore, these results are consistent with the finding that both Th17 and Th1 cytokines present in human skin psoriatic lesions contribute to the immunopathogenesis of the disease (6, 15, 49–51).
The ability of smiDCs to initiate the concurrent differentiation of Th17 and Th1 responses might be explained in part by the fact that cutaneous DCs do not secrete IL-12p70 (21, 34), a cytokine known to inhibit Th17 differentiation. Likewise, studies have shown that the suppression of Th17 responses by IFN-γ can be overcome by neutralizing IL-12p70 (20). Moreover, smiDCs induce a Th1 bias by secreting IL-23 (21), a proinflammatory cytokine that promotes host protection in mice by sustaining the proliferation and stabilization of the development of Th17 responses (3, 8, 52). Likewise, DCs secreting IL-23 have been reported to be associated with the development of cutaneous autoimmune diseases, such as psoriasis vulgaris (35, 53–55). Regardless of the relevance of IL-23 in mice to sustain Th17 responses, it is still controversial whether IL-23 is necessary to initiate Th17 differentiation in humans (14, 15, 17, 44). In this study we have demonstrated that neither the proliferation nor secretion of IL-17 by effector alloreactive CD4+ T cells was dependent on the production of IL-23 by smiDCs. Nevertheless, we cannot rule out the possibility that IL-23 might contribute to the long-term maintenance of memory Th17 responses in humans. Based on our observations we conclude that following cutaneous stimulation, human smiDCs induce Th1 responses that are dependent on IL-23 whereas initiation of Th17 immunity is independent of IL-23.

In mice naive CD4+ T cells differentiate into Th17 cells following stimulation in the presence of IL-6 and TGF-β1 and are further amplified and stabilized by IL-21 and IL-23, (8–11, 19). Contrary to the murine model and consistent with recent findings using other human models, the secretion of TGF-β1 by smiDCs had a suppressive effect on the initiation of Th17 responses. Perhaps the secretion of TGF-β1 is a regulatory mechanism used by human DCs to prevent the overexpression of IL-17 and to regulate the inflammatory effects of IL-17 (14–16). Additionally, in humans the role of IL-6 in promoting Th17 responses has been largely under-appreciated. By stabilizing IL-6 with a mAb, we were able to demonstrate a specific and substantial increase in the IL-17 production induced by cutaneous DCs. This observation is supported in humans by previous publications demonstrating that IL-6 mAbs increased the half-life of serum IL-6 by 200-fold (36, 37). Therefore, controversial results regarding the effects of IL-6 on human Th17 differentiation are likely due to the fact that efficient IL-6 signaling requires stabilization of IL-6 by a carrier, a function that is performed in vivo by soluble IL-6Ra and that can be mimicked by IL-6 mAbs.

The membrane-bound IL-6Ra is expressed by a limited number of cells, which includes hepatocytes, neutrophils, monocytes, macrophages, and few lymphocytes (38). This response indicates that the effects of IL-6 might be restricted to these cells. Nevertheless, through a trans-signaling mechanism, IL-6-IL-6Ra complexes sensitize any cell expressing gp130, which is ubiquitous (36, 38). In this study we have demonstrated that naive CD4+ T cells do not express mIL-6Ra and Th1 responses are initiated by the IL-6 trans-signaling pathway, which has been linked to several chronic immunopathologies, perhaps due to the fact that IL-6 trans-signaling protects T cells from apoptosis (38).

According to their origin mouse and human DCs are classified as myeloid or plasmacytoid. Given that in mice IL-23 is important for the stabilization of Th17 immunity and myeloid DCs, but not plasmacytoid DCs, secrete IL-23 following TLR stimulation, it was suggested that myeloid DCs but not plasmacytoid DCs are relevant for the initiation of Th17 responses (56). However, there is no direct evidence linking a particular DC population with the induction of Th17 responses. In this study we have demonstrated that smiDCs, which belong to the myeloid lineage, can in fact initiate Th17 responses. Furthermore, recent literature indicates that in mice DDCs are proinflammatory DCs, whereas epidermal LCs are more tolerogenic or anti-inflammatory DCs (57–60), suggesting that DDCs would be more capable of inducing proinflammatory Th17 responses. Nevertheless, our results demonstrate that without further treatments added to the cultures, smiLCs rather than smiDDCs were capable of inducing Th17 differentiation, an effect that was dependent on the significant increase in IL-6 secretion by smiLCs during the MLC. The observation that only smiLCs initiated Th17 responses and that smiDDCs were not capable of differentiating Th17 cells even in the presence of IL-6-IL-6 mAb complexes indicates that other smiLC factors are involved in promoting Th17 differentiation.

In this study we have also demonstrated that IL-15, a proinflammatory cytokine synthesized exclusively by smiLCs, synergized with IL-6 to promote the secretion of IL-17 by responder CD4+ T cells. Accordingly, IL-15 has been described as a proinflammatory cytokine that enhances IL-17 production and has a strong genetic association with psoriasis (42, 43). In addition, other studies have also shown IL-15 to be preferentially secreted by LCs (40, 41). We confirmed IL-15 to be a unique factor expressed by smiLCs that is partially involved in the differentiation of Th17 cells. The capability of DDCs to initiate Th17 responses dependent on exogenous IL-15 further supports a major role for IL-15 in the biasing of Th17 cells. Thus, our data directly demonstrate that the Th17 differentiation induced by smiLCs is dependent on IL-6 trans-signaling in conjunction with smiLC secretion of IL-15. An explanation for why LCs express IL-15 and DDCs do not is likely due to differences in innate DC-biasing stimuli secreted into the epidermal and dermal microenvironments or differential expression of innate receptors by these cutaneous DC subsets (2, 61, 62).

The results presented in this study indicate that in humans, smiDCs induce effector Th responses by mechanisms unlike those used by monocytes and monocyte-derived DCs and support the hypothesis that human skin DCs have evolved to favor the coexistence of Th17 and Th1 immune responses observed in cutaneous immunity and autoimmunity diseases. Our data highlight not only the alternative routes of Th differentiation by cutaneous DCs but also the need to target clinical treatments based on these variations.

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drives a pathogenic T cell population that induces autoimmune inflammation.  


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