TLR2-activated human langerhans cells promote Th17 polarization *via* IL-1β, TGF-β and IL-23

Ehsan Aliahmadi[*1], *Robert Gramlich*[*1], *Andreas Grützkau*[2],
Manuel Hitzler[3], *Melanie Krüger*[2], *Ria Baumgrass*[2],
Maximilian Schreiner[1], *Burghardt Wittig*[1], *Reinhard Wanner*[1]
and Matthias Peiser[1,3]

[1] Charité – University Medicine Berlin, Institute of Molecular Biology and Bioinformatics, Berlin, Germany
[2] German Arthritis Research Center (DRFZ), Berlin, Germany
[3] Federal Institute for Risk Assessment, Berlin, Germany

The cytokines IL-6, IL-1β, TGF-β, and IL-23 are considered to promote Th17 commitment. Langerhans cells (LC) represent DC in the outer skin layers of the epidermis, an environment extensively exposed to pathogenic attack. The question whether organ-resident DC like LC can evoke Th17 immune response is still open. Our results show that upon stimulation by bacterial agonists, epidermal LC and LC-like cells TLR2-dependently acquire the capacity to polarize Th17 cells. In Th17 cells, expression of retinoid orphan receptor γβ was detected. To clarify if IL-17^{+}cells could arise *per se* by stimulated LC we did not repress Th1/Th2 driving pathways by antibodies inhibiting differentiation. In CD1c^{+}/langerin^{+} monocyte-derived LC-like cells (MoLC), macrophage-activating lipopeptide 2, and peptidoglycan (PGN) induced the release of the cytokines IL-6, IL-1β, and IL-23. TGF-β, a cytokine required for LC differentiation and survival, was found to be secreted constitutively. Anti-TLR2 inhibited secretion of IL-6, IL-1β, and IL-23 by MoLC, while TGF-β was unaffected. The amount of IL-17 and the ratio of IL-17 to IFN-γ expression was higher in MoLC- than in monocyte-derived DC-cocultured Th cells. Anti-IL-1β, -TGF-β and -IL-23 decreased the induction of Th17 cells. Interestingly, blockage of TLR2 on PGN-stimulated MoLC prevented polarization of Th cells into Th17 cells. Thus, our findings indicate a role of TLR2 in eliciting Th17 immune responses in inflamed skin.

Key words: Cytokines · DC · Human · Skin · T cells

Introduction

Recently, Th17 cells are described as a further specialized subset of CD4^{+} effector cells in addition to the well-known Th1 and Th2 cells [1–3]. Upon stimulation Th17 cells acquire the capacity to produce the inflammatory cytokine IL-17 [4]. Recent reports demonstrate that Th17 cells are involved in processes of defence against extracellular bacteria and fungi, cancer and autoimmunity [5–7]. Furthermore, it was observed that the Th2-derived IL-17E, which is a member of the IL-17 cytokine family (A–F), plays a role in boosting allergic reactions by induction of IL-4, IL-5, and IL-13. IL-17E expression was found in human keratinocytes and atopic dermatitis skin lesions [8, 9]. In addition, there are other diseases affecting the skin with participation of IL-17. IL-17 mRNA was detected in skin cells of sensitized mice and in T cells of psoriatic human skin [10, 11]. However, naive T cells from patients

Correspondence: Dr. Matthias Peiser
e-mail: matthias.peiser@bfr.bund.de

*These authors contributed equally to this work.

suffering from hyper-IgE syndrome lost the potential to differentiate into Th17 cells [12]. In human donors suffering from Langerhans cell (LC) histiocytosis, IL-17A was found in skin LC, CD3/CD28-activated T cells, and monocyte-derived DC (MoDC), but not in MoDC from healthy donors [13]. Murine OVA-pulsed CD4^{+} T cells secreted IL-17A after stimulation with skin-derived DC [14]. Considering a central role for IL-17 in the molecular processes of infectious diseases and autoimmunity, so far the Th17 driving factors remained obscure and species specific. A panel of cytokines have been proposed for induction of Th17 development. In mice, TGF-β together with IL-6 [15], with IL-23 [16], or with IL-6 and TNF-α [17] were reported to drive the Th17 cell fate. In humans, IL-1β and IL-6 [18] or IL-23 and IL-1β [19] were observed to be crucial for Th17 commitment. The question, which cells are producing these Th17 differentiation factors, is under discussion as well. Although IL-6, IL-1β, and IL-23 are known to be produced by activated DC, the source of TGF-β remains unclear. In general, Treg are able to secrete TGF-β in cocultures with LPS-stimulated DC [17]. However, the involvement and presence of suppressive cells in the early acute inflammation phase is still controversially discussed. TGF-β is required for differentiation and survival of LC and is produced in an autocrine or paracrine way [20, 21]. We have recently shown that epidermal LC express TLR2 protein for TLR2 and reported that agonists could activate the NFκB pathway, induce cell migration and IL-6 expression in monocyte-derived LC-like cells (MoLC [22, 23]).

It was reported that TLR2 together with Dectin-1 is capable of sensing zymosan in murine bone marrow-derived DC and that hyphae of *Candida albicans* prime monocyte-mediated Th17 responses [24, 25]. In case of injury and breakdown of the skin barrier or otherwise microbial invasion, LC gain access to

TLR-agonist-bearing pathogens. Therefore, we hypothesized a role of TLR2-expressing LC for polarizing Th17 immune responses and investigated their potential to induce IL-17. In this study we show that peptidoglycan (PGN)-stimulated MoLC secreted IL-6, IL-1β, IL-23, and TGF-β Blocking of TLR2 by specific antibody inhibited IL-6, IL-1β, and IL-23. Th cells in coculture with epidermal LC, MoLC, or MoDC released IL-17; however, the relative IL-17A amount was higher in coculture with MoLC. In addition, agonists for TLR2 on MoLC provoke IL-17 in CD4^{+} CD25^{−} T cells. Flow cytometry detected IFN-γ^{+}IL-17^{+} T cells and, after treatment with anti-IL-1β and anti-IL-6/-IL-23, diminished release of IL-17A. Because TLR2-blocked and PGN-stimulated MoLC are not able to induce IL-17 in cocultered Th cells, we propose that TLR2 plays a role in Th17 differentiation. This receptor seems to be important even for adaptive skin immune reactions against bacterial insult.

Results

TLR2-activated LC secrete IL-6, IL-1β, TGF-β, and IL-23

To date, IL-6 and TGF-β are prominent cytokine candidates for polarization of naive Th cells into Th17 cells, whereas IL-23 is discussed for Th17 outgrowth of already differentiated cells [5–7, 26]. In human skin, among other cytokines IL-6 is produced by LC upon microbial stimulation by PGN and other TLR2 agonists [23]. After stimulation with different agonists for PRR like LPS, DC acquire the capacity to release IL-6, IL-1β, and IL-23 [18]. Based on this background, we hypothesized that LC might induce Th17 differentiation. In detail, we studied whether TLR2-
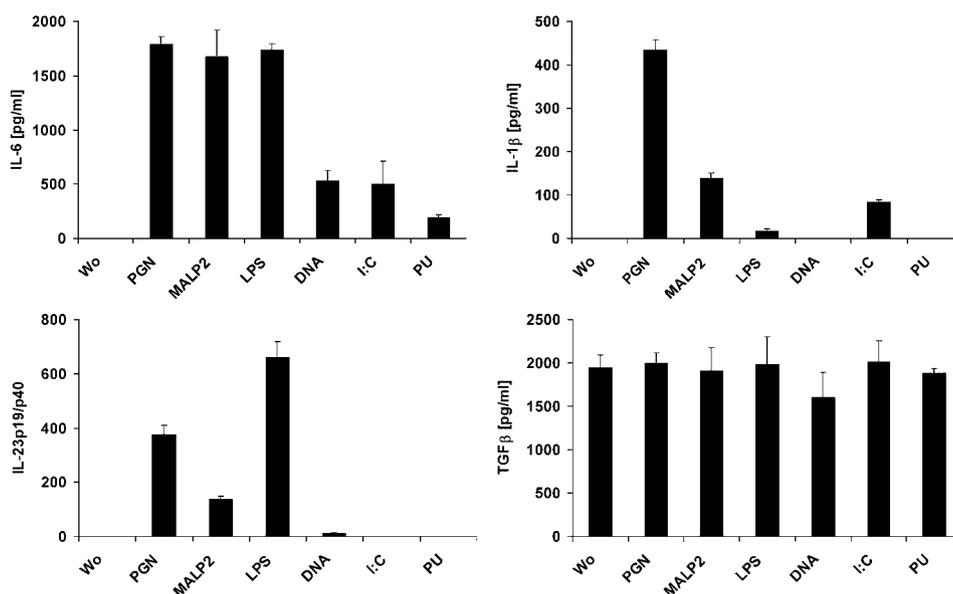
Figure 1. TLR-stimulated CD1c^{+} MoLC release Th17-driving cytokines. CD1c^{+} MoLC were enriched from human blood by depletion of non-CD14^{+} cells and cell culture with GM-CSF, IL-4, and TGF-β, followed by CD1c-sorting. Cells were stimulated for 48 h by PGN, MALP2, LPS, bacterial DNA, polyI:C (I:C), and polyU (PU) to activate cells *via* TLR2, TLR2/6, TLR4, TLR9, TLR3, TLR8, respectively, or remained without stimulus (Wo). Supernatants were analysed for IL-6, IL-1β, IL-23p19, and TGF-β by ELISA. Data are mean+SEM of four independent experiments with different donors.

stimulated DC with a pronounced LC phenotype could drive Th17 differentiation by providing TGF-β, a cytokine crucial for LC differentiation. Phenotypical homogeneous, langerin-expressing MoLC [22] were enriched by CD1c-sorting (CD1c^{+} MoLC [27]). Cells were stimulated by PGN, *Mycoplasma*-derived macrophage-activating lipopeptide 2 (MALP2), LPS, bacterial DNA, polyI:C and polyU for triggering TLR2/6, TLR2/6, 4, 9, 3, and 7/8, respectively. In supernatants of stimulated cells we found that TLR2 agonists PGN and MALP2 induced IL-6, IL-1β, and IL-23 (Fig. 1). Spontaneous release of TGF-β was not affected by any agonist. However, bacterial DNA, polyI:C, and polyU induced secretion of only low amounts of IL-6 and, in case of polyI:C, IL-1β.

Stimulation of TLR2 on CD1c^{+} MoLC induces IL-17 and ROR-γt in Th cells

Although TLR agonists induced Th17-promoting cytokines it remained unclear whether the presence of DC like CD1c^{+} MoLC is essential for the induction of the Th17 phenotype. To address this question, CD4^{+} T cells highly enriched by depletion were directly stimulated with PGN or beads coated with anti-CD3/CD28 (Fig. 2A). PGN-stimulated CD4^{+} T cells did not elicit Th17 cells, whereas CD3/CD28 stimulation promoted intracellular IL-17 in CD4^{+} T cells. Unstimulated CD1c^{+} MoLC and LPS stimulated CD1c^{+} MoLC induced the same frequency of IL-17^{+} cells in the cocultures with Th cells. LPS, however, elevated the frequency of IFN-γ-producing cells. Compared with LPS, PGN (agonists of TLR2/6) and Pam$_3$CSK$_4$, (agonists of TLR1/2 strongly enhanced the occurrence of IL-17^{+} cells and moderately enhanced the frequency of IFN-γ^{+} cells (Fig. 2A).

To confirm that LC can drive Th17, the mRNA expression of Th17-specific transcription factor retinoid orphan receptor γβ (ROR-γβ) was analyzed. ROR-γβ was induced in Th cells cocultured with PGN (*Staphylococcus aureus*)-activated MoLC, less transcripts were detected when stimulated with PGN from or *Bacillus subtilis* or with Pam$_3$CSK$_4$. Lower expression levels of ROR-γt were also detected in Th cells from cultures with unstimulated MoLC (Fig. 2B).

## IL-6, IL-1β, and IL-23 but not TGF-β are TLR2-dependently released

As along with TLR4 agonist LPS, agonists for TLR2 evoke significant amounts of IL-6, IL-1β, and IL-23 in CD1c^{+} MoLC, we analyzed if cytokine release could be inhibited by an antibody blocking TLR2. TLR2 was previously found to be expressed by epidermal LC, CD1c^{+} MoDC and CD1c^{+} MoLC [23, 28]. In cultures of CD1c^{+} MoLC, TLR2 was treated by saturating concentrations of an antagonistic monoclonal antibody, T2.5, before stimulation by PGN. The blockage completely abrogated secretion of IL-6, IL-1β, and IL-23, although it showed no effect on secretion of TGF-β (Fig. 3).
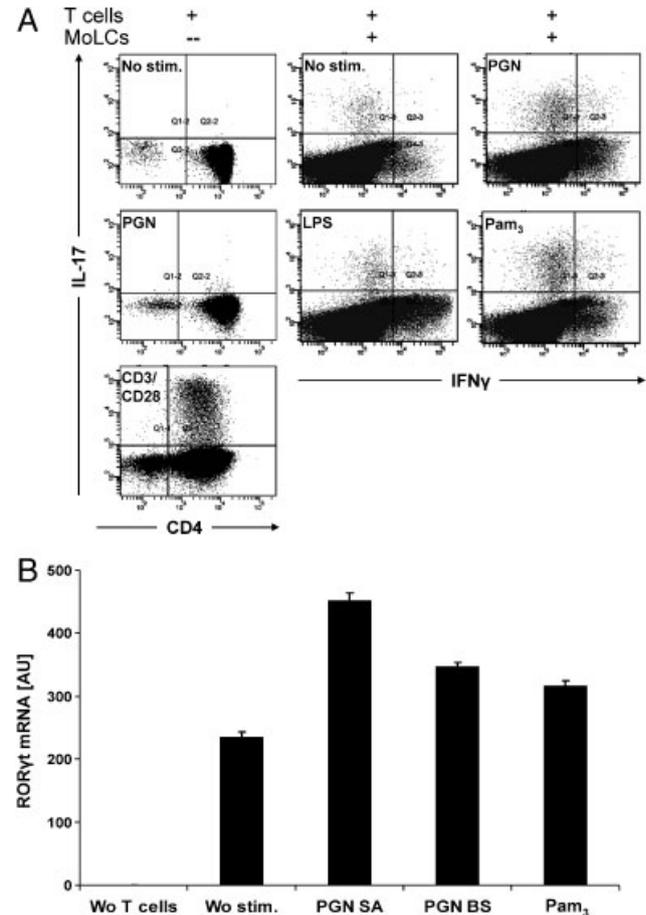
Figure 2. TLR2 ligands promote induction of Th17 cells in the presence of CD1c^{+} MoLC. (A) Intracellular IL-17 and IFN-γ were analysed by flow cytometry. Untouched CD4^{+} T cells were enriched from PBMC by depletion. In the absence of MoLC, CD4^{+} T cells were stimulated with PGN or beads coated with anti-CD3/anti-CD28 antibodies. After 5 days, CD4 and IL-17 expression were detected by flow cytometry (left panel). CD1c^{+} MoLC were stimulated by 20 μg/mL PGN, 1 μg/mL LPS, or Pam$_3$CSK$_4$ for 48 h and extensively washed. CD1c^{+} MoLC were cocultured with allogeneic CD4^{+} T cells for 5 days, stained for IL-17 and IFN-γ and analysed by flow cytometry (right panel). Dot plots show intracellular cytokine expression and represent results from three independent experiments with different donors. (B) TLR2-activated MoLC induce expression of ROR-γt in Th cells. CD1c^{+} MoLC were stimulated with 20 μg/mL PGN from *S. aureus* (PGN SA) or *B. subtilis* (PGN BS) or with 1 μg/mL Pam$_3$CSK$_4$ for 48 h and cocultured with allogeneic CD4^{+} T cells at a ratio of 1:10 for 5 days. Expression of ROR-γt was quantified by quantitative RT-PCR analyses of total MLR cell extractions. Data shown are fold differences+SEM relative to LC from a culture without T cells. Data were generated from the mean of three different MoLC with three different T-cell donors; expression was determined as triplicates; AU, arbitrary units.

TLR2-activated MoLC induce IL-17 and IFN-γ but not IL-4 in CD4^{+} T cells

To investigate if CD1c^{+} MoLC that secrete polarizing cytokines after activation *via* TLR2 could elicit a Th17 response, we measured IL-17, IFN-γ, and IL-4 in cocultures of CD1c^{+} MoLC and allogeneic T cells. CD1c^{+} MoLC and CD1c^{+} MoDC were generated from monocytes of the same donors and stimulated by
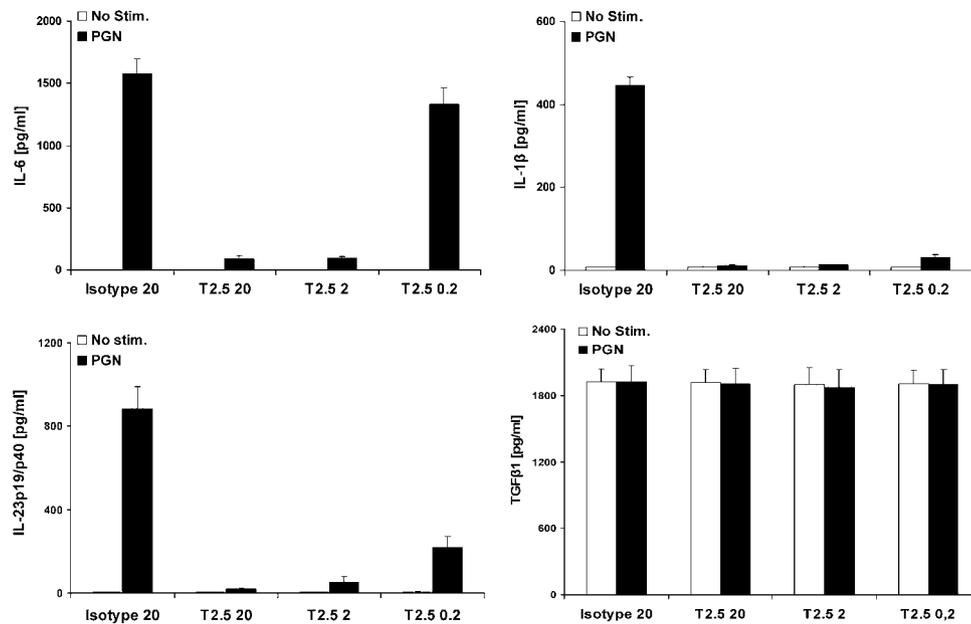
Figure 3. Anti-TLR2 blocks expression of IL-6, IL-1β, and IL-23p19 but not TGF-β. CD1c-isolated CD1c^{+} MoLC preincubated for 0.5 h with 20, 2, and 0.2 µg/mL monoclonal anti-TLR2 (T2.5) or with isotype control IgG1 (MOPC-21) were stimulated with (shaded bars) or without (open bars) 20 µg/mL PGN for 48 h. Data show mean+SEM of cytokine in supernatants (triplicate) from one donor detected by ELISA. Results are representative of four independent experiments with different donors.

PGN. After extensive washing, these cells were exposed to CD4^{+} T cells and cytokines typical for Th1, Th2, and Th17 cells were measured. We used a stimulation protocol representing pure cell interactions including no external T-cell signals, blocking antibodies and IL-2 or other T-cell-expanding cytokines. Not only the amount of IL-17 production was found to be significantly increased in CD1c^{+} MoLC-stimulated T cells but also the ratio of IL-17 to IFN-γ production was higher in CD1c^{+} MoLC than in CD1c^{+} MoDC cultivated Th cells (Fig. 4A). Unstimulated CD1c^{+} MoLC/MoDC released IFN-γ but not IL-17. Th2 cytokine IL-4 was not detected under any condition. To prove whether CD1c^{+} MoLC could represent true LC for the capacity of Th17 polarization we used LC from human epidermis. Because LC show a reduced viability after digestion of adhesion molecules and detachment from neighbouring keratinocytes, a short-term stimulation and coculture was applied. However, epidermal LC released IL-6 and IL-1β after stimulation with PGN and MALP2 (Fig. 4B). Both TLR2 ligands also enhanced the potential of bona fide LC to induce Th17. We next evaluated, which Th cell subpopulation could elicit the highest amounts of IL-17 after stimulation with TLR agonists. In all of the ten different stimuli, T-cell separation approaches revealed highest amounts of IL-17 in CD4^{+}CD25^{−} T cells, Th cells not expressing IL-2 receptor α subunit (Fig. 4C). Based on investigations showing Th17 polarization by adding differentiating cytokines in the absence of antigen-presenting cells [16–19], we examined whether IL-6 and TGF-β could enhance the secretion of IL-17 in CD4^{+} T cells. Interestingly, supplement of external IL-6 and TGF-β to the cocultures reduced the potential to secrete IL-17 at stimulation by

PGN, synthetic lipopeptides, and LPS in CD1c^{+} MoLC and CD1c^{+} MoDC (Fig. 4D). In contrast, in DC-free cultures containing anti-CD3/CD28 coated beads, IL-6 and TGF-β enhanced the release of IL-17.

Anti -IL-1β, -TGF-β, and -IL-23 decreased Th17 in cocultures of PGN-stimulated MoLC and Th cells

To verify if IL-6, IL-1β, IL-23, and TGF-β derived from TLR2-stimulated CD1c^{+} MoLC were involved in Th17 differentiation, we neutralized the cytokines by antibodies. CD4^{+} T cells were preincubated with anti-IL-6, -IL-1β, -IL-23, and -TGF-β before coculturing with PGN-stimulated CD1c^{+} MoLC. Single anti-IL-1β, anti-TGF-β and combinations of anti-IL-6 and anti-IL-23 abrogated secretion of IL-17 (Fig. 5A). IL-1β and IL-23 also diminished IFN-γ, whereas anti-TGF-β/-IL-6 did not inhibit the release of IFN-γ. Flow cytometric dot plot analyses revealed that TLR2-activated CD1c^{+} MoLC induced a population of IL-17/IFN-γ double positive T cells (Fig. 5B). Compared with isotype, anti-IL-6/-IL-23, and anti-TGF-β/-IL-1β reduced differentiation of Th17 cells to 89% and most of the blocking effect was mediated by anti-TGF-β and anti-IL-1β. Interestingly, the reduced frequency of IL-17^{+} cells in anti-IL-23- or anti-TGF-β-treated cultures did not coincide with a marked reduction of IL-17 protein in the supernatant of these cultures. In contrast, data from analyses of IL-1β- or/and IL-6-blocked cultures showed similar results by both methods, FACS and ELISA.
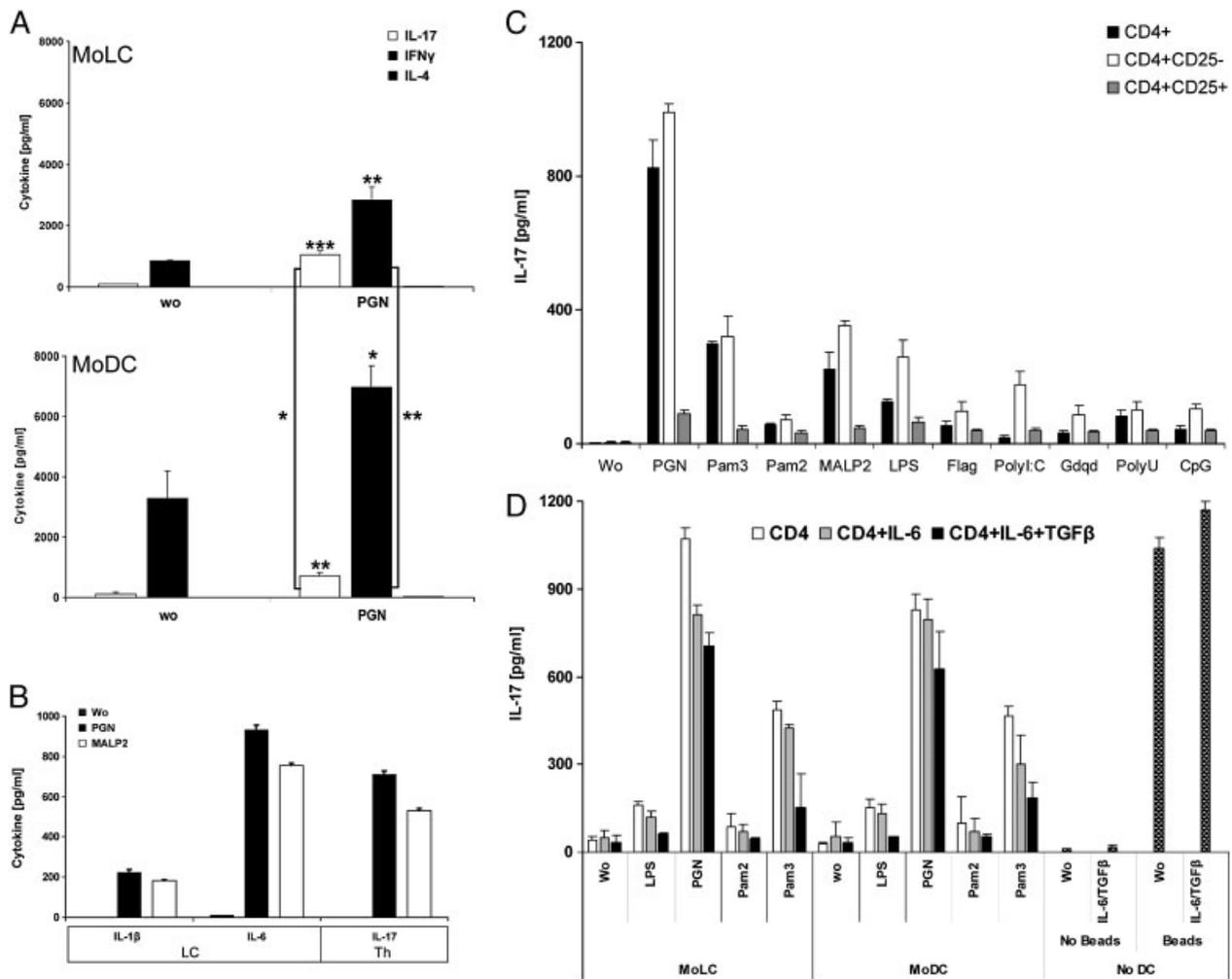
Figure 4. Th17 differentiation by PGN-stimulated MoLC. (A) Cytokine secretion from T cells differentiated in the presence of TLR2-activated CD1c$^+$ MoLC and CD1c$^+$ MoDC. CD1c$^+$ MoLC (upper panel) and CD1c$^+$ MoDC (lower panel) were generated from monocytes of the same donors and stimulated with PGN for 48 h. Cells were cocultured with allogeneic CD4$^+$ T cells. Bars show IL-17 (white), IFN-γ (black), and IL-4 (grey) in the supernatant after 5 days and represent the mean+SEM of five independent donors (***$p \leq 0.0005$, **$p \leq 0.005$, *$p \leq 0.05$). (B) Epidermal LC release IL-6 and IL-1β and induce Th17 after TLR2 stimulation. LC were isolated from human skin explants by CD1c-MACS. Cells were stimulated with PGN (black bars) or MALP2 (white bars) and then cocultured with allogeneic CD4$^+$ T cells. For IL-6 and IL-1β, secretion was detected by ELISA after 24 h, for IL-17 after additional 3 days. Data shown mean+SEM of triplicate cultures from one donor and are representative of three donors. (C) Secretion of IL-17 by CD1c$^+$ MoLC-cocultured subsets of Th cells in response to various TLR ligands. CD1c$^+$ MoLC were stimulated with TLR agonists for 48 h; flagellin (Flag), Gardiquimod (Gdqd). After extensive washing, cells were cocultured with CD4$^+$ T cells (black bars), CD4$^+$CD25$^+$ (grey bars) or CD4$^+$CD25$^-$ (white bars). Data are the mean+SEM and represent one out of four independent experiments. (D) External IL-6 and TGF-β decrease the release of IL-17 by differentiated Th17 cells. CD1c$^+$ MoLC/MoDC of one donor were activated by TLR agonists for 48 h and cocultured with CD4$^+$ T cells without external cytokines (white bars), with IL-6 (grey bars), and with IL-6 and TGF-β (black bars). As controls T cells without DC were stimulated by anti-CD3/anti-CD28-coated beads and cytokines (dashed bars). Data show mean+SEM from triplicate cultures of one donor and are representative of results from three different donors. To determine p values, Student's t test for unpaired data was used.

Blocking TLR2 on PGN-stimulated MoLC prevents Th17 differentiation

As reported recently, TGF-β-releasing Treg might promote Th17 differentiation. Human Treg although expressing weak TLR2 respond to agonist heat shock protein HSP60 [29, 30]. To discriminate whether CD1c$^+$ MoLC or induced Treg are the crucial source for TGF-β and thereby for Th17 induction in our cultures, we blocked TLR2 on CD1c$^+$ MoLC before coculturing

them with CD4$^+$ T cells. CD1c$^+$ MoLC and CD1c$^+$ MoDC were preincubated with anti-TLR2, stimulated by PGN, and after extensive washing, seeded in culture together with CD4$^+$ T cells. TLR2-blocked CD1c$^+$ MoLC/MoDC strongly decreased the IL-17 release of the cocultured T cells (Fig. 6). Th17 induction might be induced by superantigens in PGN preparations from gram-positive bacteria [31]. In blocking experiments, however, we could rule out superantigens as the main source for Th17 induction. Anti-TLR2 significantly ($p \leq 0.005$) minimized IL-17

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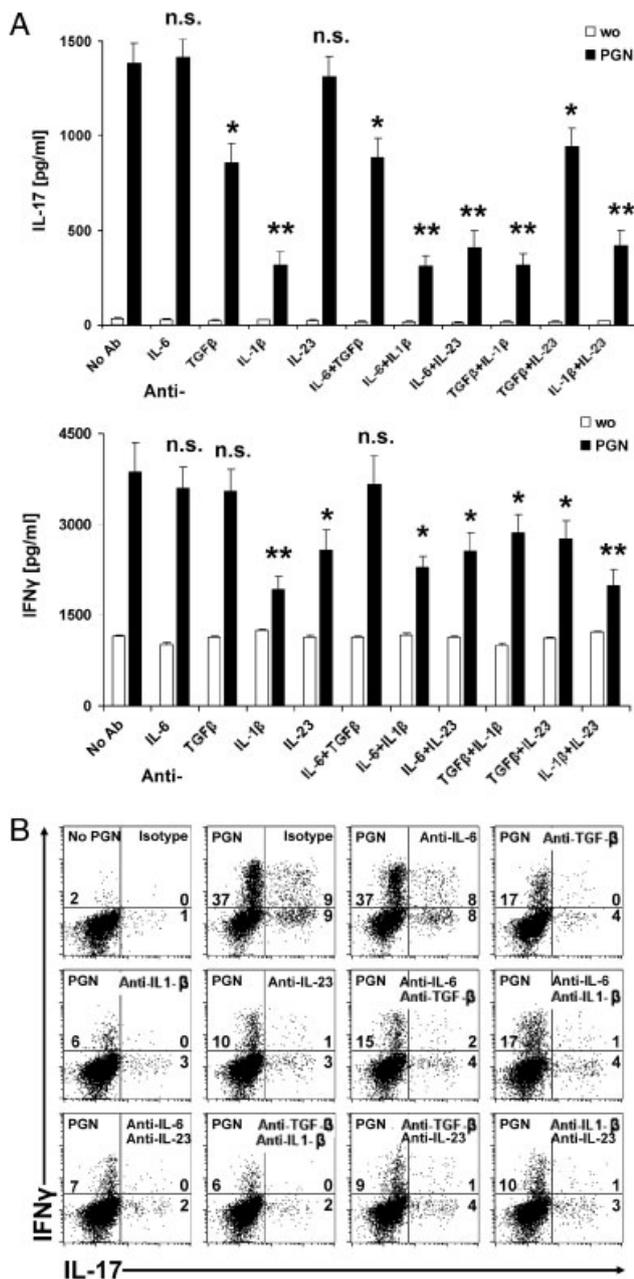
Figure 5. Cytokine-neutralizing antibodies prevent Th17 differentiation. PGN-activated CD1c⁺ MoLC and CD4⁺ T cells were treated with antibodies blocking IL-6, IL-1β, IL-23p19, and TGF-β at the onset of coculture. (A) Amounts of IL-17 and IFN-γ protein after 5 days in the supernatant were detected by ELISA. Bars (open, without stimulation; shaded, 20 μg/mL PGN) represent results of experiments with five different donors (mean+SEM; **p≤0.001, *p≤0.01, n.s. = not significant). (B) Intracellular costaining of IL-17 and IFN-γ. Cocultured cells were analyzed after 5 days by flow cytometry. Dot plots show results from the same donor as in (A). One experiment out of three with different donors and similar results is shown. Numbers represent the percentage of IL-17⁺ and/or IFN-γ⁺ cells among CD4⁺ T cells. Statistical significance was determined by the Student's t test for unpaired data.

secretion to a very low level. Finally, in our observations blocking of TLR2 more prominently in CD1c⁺ MoLC than in CD1c⁺ MoDC reduced the level of IL-17 in cocultured Th cells.
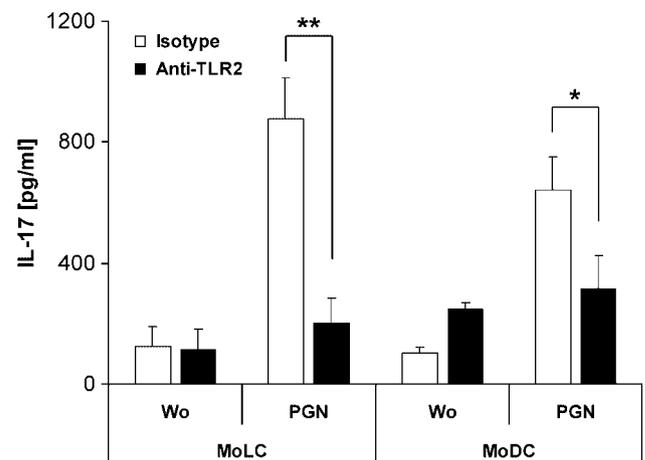
Figure 6. TLR2 blocking on MoLC inhibits Th17 differentiation of CD4⁺ T cells. CD1c⁺ MoLC and CD1c⁺ MoDC derived from one donor were incubated for 30 min with 10 μg/mL anti-TLR2 (T2.5, closed bars) or isotype control (open bars). After 48 h stimulation with PGN, cells were extensively washed and cocultured with allogeneic CD4⁺ T cells. After 5 days, supernatants were analysed for IL-17 by ELISA. Data are mean of experiments with five different donors (mean+SEM; **p≤0.005, *p≤0.05), unpaired t test.

Discussion

In this study, we could give evidence for generation of Th17 cells by TLR-stimulated LC. Previously, we have shown that human LC and surrogates for LC, MoLC express protein for TLR2 and stimulation by TLR2 agonists induced phophorylation of IL-1R-associated kinase-1 and NF-κB, increase of CD86, CD83, CCR7, and release of TNF-α and IL-6 [23]. In addition to IL-6, we now found IL-1β and IL-23 in the supernatant of PGN- and MALP2-stimulated cells. These results are supported by observations in MoDC [32] that mature after stimulation with microbial lipopeptides via TLR2 [33]. In accordance with expression of TLR4 observed in human MoLC but not in epidermal LC [23], we could stimulate TLR4 on MoLC by LPS and detected secretion of IL-6 and IL-23. In our study TGF-β was found to be spontaneously released, independent of any agonist. By detection of IL-1β, IL-6, IL-23, and TGF-β we found cytokines that recently have been proposed to be crucial for Th17 commitment [15–19]. However, even in human T cells, IL-23 seems to be responsible rather for enhancement than commitment of IL-17 [34], but in human CD8⁺ T cells also IL-17 induction was observed [35]. The cytokine profile found in our experiments may be specific for the CD1c⁺ phenotype, which is consistent with detection of IL-6 and IL-1β in CD1c⁺ circulating DC, stimulated by TLR agonists [18]. Microbial compounds, in consequence, demonstrate the capacity to provoke Th17-driving cytokines in MoLC.

Consistently, the cytokine levels for IL-6, IL-1β, and IL-23 detected in MoLC in our studies were significantly lower than the levels found in MoDC. This difference in cytokine secretion level was reported for IL-6 and is suspected to depend on TGF-β added to the cultures in the differentiation process of the MoLC [36, 23]. Unsorted MoDC are reported strongly and weakly to produce

IL-6 after activation with PGN [36, 18], whereas Pam$_3$CSK$_4$ induced equal amounts of IL-6 in these studies. However, it seems to be likely that LC as cells that control a defined immunological area in the suprabasal keratinocyte environment can perform this task rather by paracrine cytokine communication and even lower cytokine amounts than MoDC representing DC localized in human blood. If epidermal LC *in vivo* really release lower protein level for IL-6 and IL-1β than LC *in vitro* upon TLR2 stimulation, this could not be identified by our *ex vivo* experiments because of the reduced viability of skin-derived LC in cell culture. However, epidermal LC secreted IL-6 and IL-1β and acquired the capacity to differentiate Th17. Because in murine macrophages, TLR2 has been shown not to recognize PGN in purified preparations [37], we investigated PGN ligands for intracellular receptors and performed blocking experiments as well. MurNAc-L-Ala-γ-D-Glu-mDAP and muramyl dipeptide (MDP), ligands for NOD1/NOD2 and NOD2, respectively, failed to induce IL-6, IL-1β, and IL-23 in CD1c$^+$ MoLC (data not shown). Thus, neither minimal bioactive motives nor degradation products of PGN, targeting intracellular receptors, could elicit Th17-driving cytokines in CD1c$^+$ MoLC. In addition to cytokine secretion we recently found marked elevation of maturation-associated molecules after stimulation by PGN that could be inhibited by anti-TLR2 [23].

Measuring Th17-driving cytokines in supernatants of TLR2-stimualted CD1c$^+$ MoLC we further questioned if these cells indeed could differentiate Th17 cells from CD4$^+$ T cells. The major finding of our study is that at stimulation with agonists of TLR2 the presence of CD1c$^+$ MoLC is required for Th17 differentiation. In contrast, CD4$^+$ T cells alone did not shift to Th17 cells when stimulated with agonists of TLR2. We therefore assume induction of Th17 cells from naive T cells, but could not exclude a minor IL-17 contribution of a small subpopulation of already polarized central memory and effector memory CD4$^+$ T cells as recently reported [25]. The requirement of the presence of DC is further supported by studies with murine bone marrow-derived DC. DC stimulated by curdlan, a dectin-1 agonist, were found to effectively promote Th17 differentiation from CD4$^+$ T cells *via* an autonomous pattern recognition pathway [38]. However, to our knowledge the induction of Th17 by TLR-activated skin-DC, in our study substituted for methodical reasons by CD1c$^+$ MoLC, has not been reported before.

On investigation, both cytokines IL-17 and IFN-γ were found to be coexpressed, whereas IL-4 was not induced in CD4$^+$ T cells cocultured with PGN-stimulated CD1c$^+$ MoLC/MoDC. Comparing PGN-stimulated MoLC (TGF-DC) to PGN-stimulated MoDC (IL-4-DC) the higher ratio of IL-17 to IFN-γ was also observed in T cells expanded by IL-2 [18]. In cocultures of unsorted MoLC (TGF-DC) or MoDC (IL-4-DC) with T cells, however, weak induction of IL-17 was detected. It therefore, again, seems to be likely that the release of IL-17 in CD4$^+$ T cells is connected to the CD1c$^+$ phenotype of MoDC/MoLC because CD1c-sorted circulating blood-DC also induced higher amounts of IL-17 in this study [18].

Extending our analysis, we found that the supplement of external IL-6 and TGF-β to the cocultures reduced the potential to secrete IL-17 in T cells. These results may be explained by the observation that TGF-β at higher concentrations generates and expands induced Treg in *ex vivo* cell cultures [39]. Thus, we propose that external TGF-β supplemented in our 5-day cell cultures may induce Treg arising from CD4$^+$CD25$^-$ T cells. Induced Treg on their part can release further TGF-β which could silence or suppress effector Th cell differentiation and proliferation [40], including Th17 cells *via* induction of transcription factor Foxp3 and inhibition of ROR-γt [41].

By performing blocking experiments, we found that anti-IL-1β and anti-TGF-β, not single antibody against IL-6 but combinations of anti-IL-6 and anti-IL-23, abrogated secretion of IL-17. In monocytes anti-TGF-β and anti-IL-23 could not inhibit secretion of IL-17 [18], indicating different cytokine profiles in PGN-stimulated monocytes and CD1c$^+$ MoLC. Of interest, mice studies investigating cytokine-driven Th17 polarization in the absence of DC demonstrated a negligible influence of IL-6 in combination with TGF-β and IL-23 [42]. Taken together, data from our ELISA and FACS analyses indicate that generation of Th17 cells by CD1c$^+$ MoLC stimulated with agonists of TLR2 is mainly dependent on TGF-β and IL-1β. It could be speculated that higher IL-17-levels detected by ELISA compared with results from flow cytometry for blocking TGF-β and IL-23 rely on different kinetics of IL-17 release out of the cells caused by altered intracellular cytokine accumulation after treatment with brefeldin A in IL-23-blocked cells.

To determine if activation of LC/DC *via* TLR2 influences Th17-cell differentiation, we finally performed experiments blocking TLR2 and found impaired secretion of IL-17. Consequently, CD1c$^+$ MoLC-expressing TLR2 were the initiators of IL-17 secretion in CD4$^+$ T cells. Moreover, assuming no blocking effect of T2.5 antibody on TLR2 expressed by T cells that were set in culture after removement of unbound antibody from the wells with CD1c$^+$ MoLC, we could deduce only a minor influence of induced Treg on polarization of Th17 in our cultures. However, a possible induction of Th17 cells could also rely on a direct effect mediated by superantigens in PGN preparations from gram-positive bacteria [31]. Thus, by data from experiments blocking TLR2 on CD1c$^+$ MoLC, direct effects on TCR, mediated by contaminig components of PGN preparations, could be excluded as there were only low amounts of IL-17 measured in supernatants of these situations.

In addition to our study there were further reports suggesting a role for PGN in Th17 polarization. In DC of Crohn's disease patients that are deficient for detection of intracellular PGN derivate and with a lack of sensing MDP, diminished IL-17 was detected [43]. In line with these results it was previously observed that Th17 phenotype could be induced by human monocytes stimulated by hyphae of *C. albicans* [25], compounds of which are discussed as TLR2 ligands.

In this study we present data demonstrating that LC, activated by TLR2, can indeed induce Th17 differentiation. By blocking experiments with neutralizing antibody for TLR2 we could show that the activation of MoLC/MoDC by bacterial ligands for TLR2 and, as a consequence, generation of Th17 cells is

specifically inhibited. Furthermore, we are convinced that our data may be of high importance at future strategies for treating immune disorders of human skin, *i.e.* psoriatic lesions and LC histiocytosis.

Materials and methods

Isolation of epidermal LC, generation and isolation of CD1c$^+$ MoDC/MoLC

Human skin was obtained from healthy volunteers by plastic surgery (reduction mammoplasty) and prepared as recently described [44]. In short, epidermal sheets were detached from the dermis after incubation with dispase I (2 U/mL, Roche, Mannheim, Germany). After trypsin (0.25%; Biochrom, Berlin, Germany), LC were positively selected from single-cell suspensions by anti-CD1c antibody (AD5-8E7, IgG2a) and anti-biotin-antibody coated to microbeads (Bio3-18E7, IgG1, Miltenyi Biotec, Bergisch Gladbach, Germany). Buffy coats were obtained from the German Red Cross blood donation service, Berlin. All work with human primary cells was performed in adherence to the Helsinki guidelines. Human monocytes were magnetically isolated from PBMCs by depletion (monocyte isolation kit II, Miltenyi Biotec). MoDC and MoLC were generated from same donor blood in RPMI 1640 supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10% v/v heat-inactivated FBS (Biochrom) by 6-day culture with human recombinant GM-CSF (100 ng/mL), IL-4 (10 ng/mL [32]), and TGF-β1 (for MoLC only, 10 ng/mL, R&D Systems, Wiesbaden-Nordenstadt, Germany [22]). Then CD1c$^+$ cells were positively selected by CD1c-MACS as described above for epidermal LC.

Stimulation and blocking of TLR

CD1c$^+$ MoDC and CD1c$^+$ MoLC were cultured at 10^6 cells/mL for 48 h in 24-well tissue culture plates (Greiner, Frickenhausen, Germany). Cells were stimulated by 1 µg/mL ultrapure LPS from *Salmonella minnesota*, 100 ng/mL flagellin from *B. subtilis*, 5 µg/mL endotoxin-free DNA from *Escherichia coli* K12, 5 µg/mL polyI:C, 1 µg/mL imidazoquinoline compound Gardiquimod, 100 ng/mL *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-(*R*))-Cys-Ser-(Lys)$_4$ (Pam$_2$CSK$_4$), 5 µg/mL MurNAc-L-Ala-γ-D-Glu-mDAP, and MDP (InvivoGen, San Diego, USA), 20 µg/mL PGN from *S. aureus* (Fluka, Buchs, Switzerland), 1 µg/mL *N*-pamitoyl-*S*-[2,3-bis (palmitoyloxy)-(2*RS*)-propyl]-(*R*)-Cys-Ser-(Lys)$_4$ (Pam$_3$CSK$_4$), 100 ng/mL MALP2, 5 µg/mL polyU, 5 µg/mL CpG oligodeoxynucleotide 2395 (Axxora, Lörrach, Germany). In some experiments, cells were incubated with TLR2-blocking T2.5 (IgG1, eBioscience, San Diego, USA) or isotype control (MOPC-21, BD Biosciences, Heidelberg, Germany) and extensively washed after stimulation as recently reported [23].

Isolation of CD4$^+$ T cells

Untouched CD4$^+$ T cells (mean of 96% CD4$^+$ and 99% CD3$^+$, detected by flow cytometry) were purified from density gradient-enriched fraction of PBMC by depletion of contaminating cells using CD4$^+$ T-cell isolation kit II (Miltenyi Biotec). Where indicated, CD4$^+$CD25$^+$ T cells were additionally positively enriched from the fraction of Th cells by anti-CD25 microbeads (Miltenyi Biotec).

Coculture of stimulated MoDC/MoLC with CD4$^+$ T cells

CD4$^+$ T cells were plated at 2.5×10^5 cells/well and stimulated with 2.5×10^4 allogeneic CD1c$^+$ MoDC or CD1c$^+$ MoLC. Antibodies neutralizing IL-6 (6708), IL-1β (8516), IL-23p19 (AF1716), and TGF-β (1D11, R&D Systems) were used at 5 µg/mL. As controls, CD4$^+$T cells were activated by 10^4 anti-CD3/anti-CD28-coated Dynabeads (Dynal-Invitrogen, Karlsruhe, Germany). In some experiments 20 ng/mL IL-6 and 5 ng/mL TGF-β (R&D Systems) *per* well were supplemented as indicated. After 5 days of coculture without external IL-2, supernatants and cells were harvested.

Flow cytometry

For the last 4 h of coculture, cells were restimulated by 5 ng/mL PMA and 500 ng/mL ionomycin (Sigma-Aldrich, Munich, Germany) in the presence of 1 µg/mL brefeldin A (BD Biosciences). Then cells were washed in Cytofix/Cytoperm solution (BD Biosciences) according to the manufacturer's instructions and stained for intracellular IL-17A and IFN-γ by specific antibodies eBio64DEC17-FITC (IgG1, eBioscience) and 25723.11-APC (IgG2b, BD Biosciences), respectively. After washing, cells were analyzed on FACSCalibur or LSRII (BD Biosciences) flow cytometers using CellQuestPro or FACSDiva software. The cell types enriched by magnetic isolation were routinely tested by 7-AAD, CD4-APC (RPA-T4), HLA-DR-APC (TÜ36, BD Biosciences), and Langerin-PE (CD207, DCGM4, Coulter, Krefeld, Germany).

Quantitative real-time PCR

Total RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using the oligo(dT) primer with Superscript II (Invitrogen, Groningen, The Netherlands) and analysed by quantitative PCR using LightCycler FastStart DNA Master SYBR Green I on the Lightcycler Instrument (Roche). Specificity of the amplification was confirmed by melting curve analysis. The starting quantity of the initial cDNA sample was calculated from primer-specific standard curves using gene-specific primers for human ROR-γt (5′-TTT TCC GAG GAT GAG ATT GC-3′; 5′-CTT TCC ACA TGC TGG CTA

CA-3′) and human beta-2-microglobulin (5′-TGGAGAGAGAA-TTGAAAAAGTGGAGC-3′; 5′-TTAAAAAGCAAGCAAGCAGAATTT-GG-3′). The expression of ROR-γt was normalized to the expression of β2-microglobulin.

Cytokine detection

By sandwich ELISA secretion of IL-6, IL-1β, TGF-β (DuoSet, R&D Systems) and IL-23p19 (Biosource-Invitrogen) was determined after 48 h in the supernatants of 1×10^6 CD1c$^+$ MoLC/DC/mL. The protein levels of IFN-γ, IL-4, and IL-17 (DuoSet, R&D Systems) were determined after 5 days in the wells of CD1c$^+$ MoLC/DC cocultured with CD4$^+$ T cells.

Statistical methods

Mean, SD, and statistical significance were calculated using SigmaPlot (Systat, Erkrath, Germany). For analysis of differences between experimental groups, Student's t test for unpaired data was used. Values of $p \leq 0.05$ were considered statistically significant.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **LC:** langerhans cell · **MALP2:** macrophage-activating lipopeptide 2 · **MDP:** muramyl dipeptide · **MoDC:** monocyte-derived DC · **MoLC:** monocyte-derived LC-like cells · **Pam$_3$CSK$_4$:** *N*-pamitoyl-*S*-[2,3-*bis*(palmitoyloxy)-(2RS)-propyl]-(R)-Cys-Ser-(Lys)$_4$ · **PGN:** peptidoglycan · **ROR-γβ:** retinoid orphan receptor γβ

Full correspondence: Dr. Matthias Peiser, Federal Institute for Risk Assessment, Experimental Research, Thielallee 88-92, D-14195 Berlin, Germany
Fax: +49-30-8412-3763
e-mail: matthias.peiser@bfr.bund.de