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# Muramyl Dipeptide Induces Th17 Polarization through Activation of Endothelial Cells

Michela Manni, Wanhong Ding, Lori L. Stohl, and Richard D. Granstein

Endothelial cells (ECs) express the nucleotide-binding oligomerization domain (Nod) receptor 2, which recognizes the bacterial derivative muramyl dipeptide (MDP). MDP stimulation of these cells enhances their IL-6 production and may thus contribute to the immune and inflammatory activities in the skin. However, whether ECs are capable of influencing the development of T cell priming and its polarization remains unknown. We report that in vitro the murine bEnd.3 EC line induces, following MDP stimulation, a Th17 polarization at the expense of Th1 and Th2 polarization in the setting of Langerhans cell (LC) Ag presentation to responsive T cells as assessed by IL-17, IL-6, IFN- $\gamma$ , and IL-4 production. Interestingly, IL-22 production, which has been associated with Th17 priming, was not influenced by MDP-treated bEnd.3 cells, illustrating differential regulation of this cytokine from IL-17. Additional analysis confirmed a significantly increased percentage of IL-17<sup>+</sup>CD4<sup>+</sup> T cells by flow cytometry and an increased mRNA level of the specific Th17 transcription factor retinoic acid-related orphan receptor  $\gamma$ t in cocultures of LCs and responsive T cells in the presence of activated bEnd.3 cells. Experiments using the RNA interference technique to knockdown IL-6 in bEnd.3 cells confirmed that IL-6 produced by bEnd.3 cells stimulated by MDP is at least partially involved in Th17 polarization. Our data suggest that activated ECs are capable of influencing LC Ag processing and presentation to T cells and induce a Th17 polarization. These results are important for the understanding of Th17-related disorders of the skin such as psoriasis. *The Journal of Immunology*, 2011, 186: 3356–3363.

Dermal microvascular endothelial cells (DMECs) participate in immune and inflammatory activities in the skin through release of cytokines and chemokines, which modulate immune cell functions, and by expression of adhesion molecules involved in transmigration of leukocytes out of the vasculature. Several studies have demonstrated that endothelial cells (ECs) express different pattern recognition receptors (PRRs), such as TLRs and nucleotide-binding oligomerization domain (Nod) proteins, and may respond to the correlated pathogen-associated molecular patterns (PAMP) (1). The Nod2 receptor is a member of the family of Nod proteins and recognizes the peptidoglycan derivative muramyl dipeptide (MDP) (2). Mutations in the *Nod2* gene have been correlated with the development of Blau syndrome (3) and Crohn's disease (4). Th17 cells are a recently discovered lineage of effector CD4<sup>+</sup> T cells characterized by the production of IL-17, IL-21, and IL-22 and by the expression of the transcription factor retinoic acid-related orphan receptor  $\gamma$ t (ROR $\gamma$ t). In mice, polarization to a Th17 immune response is dependent on the presence of the proinflammatory cytokines IL-6

and TGF- $\beta$ 1, whereas it is suppressed by IFN- $\gamma$ , IL-4, and IL-12 (5). Additionally, the Th17 response is amplified by IL-23 (6). In humans, Th17 development is promoted by IL-6, TGF- $\beta$ 1, and IL-1 $\beta$  (7). Recent studies have also proposed the existence in humans of a new lineage of effector CD4<sup>+</sup> T cells producing IL-22 but not IL-17 (Th22). These cells appear to develop in an IL-6- and IL-23-rich environment and are inhibited by the presence of TGF- $\beta$ 1 (8, 9). Interestingly, it has been reported that in human ECs, IL-6 production is enhanced following MDP stimulation (10). In addition, Nod2 activation in human dendritic cells (DCs) promotes IL-17 production by memory T cells through induction of IL-23 and IL-1 expression, suggesting a role of this PRR in Th17 development (11). Th17 cells participate in defense against extracellular bacteria and fungi. However, these cells may also play a role in tumor immunity (12), autoimmune disorders (13), graft rejection, and host versus graft disease (14, 15). Recent studies have reported the presence in psoriatic lesional skin of enhanced IL-23 and IL-17 expression together with an increased population of Th17 cells (16, 17). Moreover, IL-6, which is necessary for Th17 priming, has previously been found to be overexpressed in lesions of psoriasis (18, 19). In rheumatoid arthritis patients, a Th17 cytokine profile expression is correlated with cartilage and bone destruction (20), whereas IL-6 and IL-17 expression is significantly increased in multiple sclerosis lesions (21). IL-22 and Th22 cells have also been proposed to be implicated in psoriasis development and to play a major role in atopic dermatitis (22, 23). Langerhans cells (LCs) are professional APCs resident in the epidermis and characterized by expression of langerin and birbeck granules (24). LCs have classically been thought to be involved in the initiation of cutaneous immune response by processing microbial Ag for presentation to effector T cells (25). However, studies using LC-deficient mice suggest that in the steady state, LCs may induce tolerance (26). In this study, we hypothesize that DMECs may participate in the skin immune response by modulating LC's Ag processing and presentation. In particular, we show in an in vitro model that the murine bEnd.3 ECs line may respond

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Abbreviations used in this article: CM, complete medium; cOVA, chicken OVA; DC, dendritic cell; DMEC, dermal microvascular endothelial cell; EC, endothelial cell; Kera, keratinocyte; LC, Langerhans cell; MDP, muramyl dipeptide; Nod, nucleotide-binding oligomerization domain; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; ROR $\gamma$ t, retinoic acid-related orphan receptor  $\gamma$ t; siRNA, short interfering RNA.

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to microbial stimuli, such as MDP, by increasing IL-6 production and consequently influencing the LCs and T cells microenvironment during Ag presentation. In this scenario, the increased IL-6 concentration biases T cell development to a Th17-type immune response with inhibition of Th1- and Th2-type immunity as assessed by IL-17, IFN- $\gamma$ , and IL-4 production in this study. These data were confirmed by ROR $\gamma$ t mRNA expression and flow cytometric analysis of IL-17<sup>+</sup>CD4<sup>+</sup> T cells. These results are important for the understanding of Th17-related pathologies, especially in the skin, where IL-6 and IL-17 production along with Th17 cells have been correlated with the development of autoimmune diseases such as psoriasis.

## Materials and Methods

### Mice

BALB/c (H-2<sup>d</sup>) inbred mice and transgenic DO11.10 (C.Cg-Tg[DO11.10]10D10/J, H-2<sup>d</sup>) mice (BALB/c background) were purchased from The Jackson Laboratory (Bar Harbor, ME). DO11.10 mice express T cell  $\alpha$  and  $\beta$  receptor transgenes, which recognize a fragment of chicken OVA (cOVA<sub>323–339</sub>) peptide. All mice were maintained in the Weill Cornell Medical College animal facility under specific pathogen-free conditions, food and water ad libitum, and under a standard 12 h photoperiod at a constant temperature of 21°C. Seven- to 10-wk-old female mice were used for all experiments. All experiments were approved by the Institutional Animal Care and Use Committee of the Weill Cornell Medical College.

### Media and cell lines

The bEnd.3 cell line (27) was obtained from the American Type Culture Collection (Manassas, VA). This cell line is an EC line established from the cerebral cortex of BALB/c mice and has many characteristics of freshly isolated ECs including expression of von Willebrand factor (28), ICAM-1 (29), and VCAM-1. The PAM212 cell line is a transformed BALB/c murine keratinocyte (Kera) cell line provided by Stuart Yuspa (National Cancer Institute, Bethesda, MD).

bEnd.3 and PAM212 cells were cultured in DMEM (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated FBS (Gemini Bio-Products, Sacramento, CA), 100 U/ml penicillin (Mediatech), 100  $\mu$ g/ml streptomycin (Mediatech), and 2 mM L-glutamine (Mediatech).

LCs and T cells were cultured in complete medium (CM): RPMI 1640 (Mediatech) supplemented with 10% FBS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.1 mM nonessential amino acids (Mediatech), 0.1 mM essential amino acids (Mediatech), 2 mM L-glutamine, 1 mM sodium pyruvate (Mediatech), and 10 mM HEPES buffer (Mediatech).

### Primary cells

Primary mouse dermal ECs were obtained from Celprogen (San Pedro, CA) and maintained in dermal EC complete growth medium (Celprogen) in precoated flasks (Celprogen).

Primary mouse Kera were isolated from the negative fraction of LC purification on the basis of a previously described protocol (30). Briefly, epidermal cells depleted of LC were treated with a dead cell removal kit (Miltenyi Biotec, Auburn, CA) following the manufacturer's instruction to eliminate dead cells. The Kera obtained were plated in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin (Mediatech), 100  $\mu$ g/ml streptomycin (Mediatech), and 2 mM L-glutamine (Mediatech) and used for the experiments the next day.

### Cell stimulation

To investigate the effect of Nod2 and TLR-4 activation on the bEnd.3 and PAM212 cell lines and on primary dermal ECs and primary Kera, cells were plated at a concentration of  $0.2 \times 10^6$  cells/well in a 12-well plate in the presence or absence of 2  $\mu$ g/ml MDP (InvivoGen, San Diego, CA) or 0.1  $\mu$ g/ml LPS (Invitrogen, Carlsbad, CA). The supernatants were collected 3, 6, 9, and 24 h later and analyzed for cytokine content by ELISA.

### LC purification

LCs were obtained following a modified standard protocol (31, 32). Briefly, the truncal skin of mice was shaved and chemically depilated. The s.c. fat and panniculus carnosus were removed by blunt dissection. The skin was then floated dermis side down in PBS (Mediatech) containing 0.5 U/ml

dispase (Mediatech) and 0.25% trypsin (Mediatech). Epidermal sheets were collected by gentle scraping, washed, and dissociated by continuous mild agitation in HBSS (Mediatech) supplemented with 2% FBS. Epidermal cells were then filtered through a 40- $\mu$ m cell strainer (BD Biosciences, San Jose, CA). LCs were enriched by incubation with mouse anti-mouse I-A<sup>d</sup> Ab (AMS 32.1) (BD Biosciences) followed by incubation with goat anti-mouse IgG Ab conjugated to magnetic microspheres (Dynabeads; Invitrogen). LCs were isolated by placing the tube in a magnet (Invitrogen), discarding the supernatant, and washing the bead-bound cells three times with HBSS containing 2% FBS. This procedure yields to a cell population of ~95% LCs as shown by flow cytometry.

### RNA interference

Twenty thousand bEnd.3 or PAM212 cells per well were plated in 96-well round-bottom plates (BD Falcon, San Jose, CA) in antibiotic-free medium and incubated at 37°C until adherence. The appropriate amount of ON-TARGET plus SMARTpool Mouse IL-6 short interfering RNA (siRNA; Dharmacon, Lafayette, CO) target sequences (5'-CCUAGUGCGUUAUG-CCUAA-3'; 5'-UUACACAUGUUCUCUGGGA-3'; 5'-GGACCAAGAC-CAUCCAAUU-3'; and 5'-CUACCAACUGGAUUAUAAU-3') or the corresponding ON-TARGET plus nontargeting pool were diluted in Opti-MEM medium (Invitrogen) to obtain a final concentration of 100 nM. Lipofectamine 2000 (Invitrogen) was also diluted following the manufacturer's instruction in OptiMEM. The diluted siRNA and Lipofectamine 2000 were mixed and incubated for 20 min at room temperature for complex formation. Twenty-five microliters complexed siRNA was added to each well. After overnight incubation at 37°C, the medium was replaced with CM and cells used for the in vitro Ag presentation experiments.

### In vitro Ag presentation to DO11.10 T cells

To evaluate possible involvement of ECs in the skin immune response,  $0.5 \times 10^5$  bEnd.3 or  $0.25 \times 10^5$  PAM212 cells/well were plated in 96-well, round-bottom plates and incubated overnight at 37°C. The following day, cells were treated with 2  $\mu$ g/ml MDP or medium alone for 3 h at 37°C. Cells were then washed extensively, and  $1 \times 10^4$  purified LCs in CM were added to each well. T cells were isolated from the spleens of DO11.10 transgenic mice using the previously described nylon-wool protocol (33). Briefly, spleens were collected and mechanically disrupted to obtain a single-cell suspension. After erythrocyte lysis by exposure to hypotonic medium, spleen cells were filtered through a 70- $\mu$ m cell strainer (BD Falcon) and extensively washed before application to a nylon-wool column. After 1 h incubation at 37°C, T cells were eluted from the column, and  $2 \times 10^5$  T cells in CM were added to each well along with 10  $\mu$ M cOVA<sub>323–339</sub> (Peptides International, Louisville, KY). Supernatants were harvested 48 h later and analyzed by sandwich ELISA for cytokine content.

### ELISA

To quantitate protein production in culture supernatants, IL-1 $\beta$ , IL-4, IL-6, IL-10, and IL-17 DuoSet ELISA kits (R&D Systems, Minneapolis, MN), IL-22 kits (InvivoGen), IL-12, IL-23p19, and TGF- $\beta$ 1 kits (eBioscience, San Diego, CA), and IFN- $\gamma$  kits (BD Biosciences) were used following the manufacturer's instructions. OD was determined at 450 nm in a Versamax microplate reader (Molecular Devices, Sunnyvale, CA) and data analyzed with the Softmax software.

### Real-time RT-PCR

For gene expression analysis, T cells and LCs were gently collected from mixed culture wells after 24 h incubation, and total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from 1  $\mu$ g total RNA using random hexamer primer and Superscript III (Invitrogen). ROR $\gamma$ t expression was analyzed by real-time PCR using Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and the ABI 7900HT instrument (Applied Biosystems). GAPDH expression was used as internal control, and melting curve analysis was performed to assess the specificity of the amplification. Data were analyzed using the REST 2008 v2.0.7 software from Corbett Life Science (Valencia, CA) (34).

### Staining and FACS

Cells were cocultured for 48 h as described above and then restimulated with 10 ng/ml PMA and 1  $\mu$ g/ml ionomycin for 3 h prior to enrichment of IL-17-secreting cells using the mouse IL-17 secretion assay cell enrichment and detection kit (Miltenyi Biotec) following the manufacturer's instruction. This kit allows for enrichment and staining of IL-17-secreting cells with a PE dye. Cells were then also stained with FITC-conjugated rat

anti-mouse CD4 (H129.19) Ab (BD Biosciences). The corresponding isotype, FITC rat IgG2a, $\kappa$  (BD Biosciences), was used as a negative control. Lymphocytes were gated on the basis of forward and side scatter properties prior to analysis. Samples were analyzed using the BD FACScan cytometer and BD CellQuest software (BD Biosciences).

### Statistical analyses

Differences between groups were analyzed using the Statview software and the repeated measure ANOVA test or Student *t* test. Real-time PCR data were analyzed using the REST 2008 v2.0.7 software from Corbett Life Science.

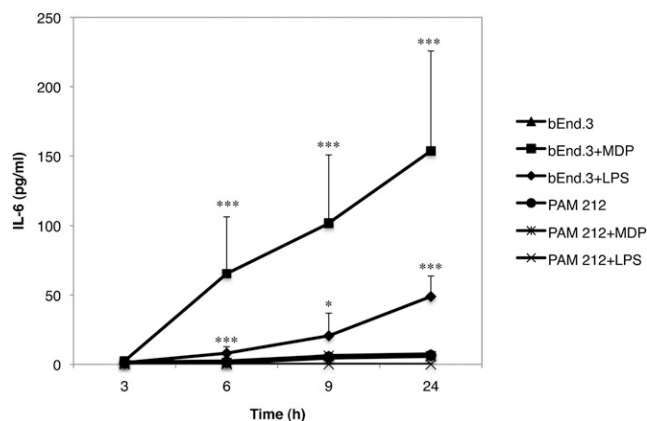
## Results

### *Nod2 and TLR4 activation in bEnd.3 cells increases IL-6 production*

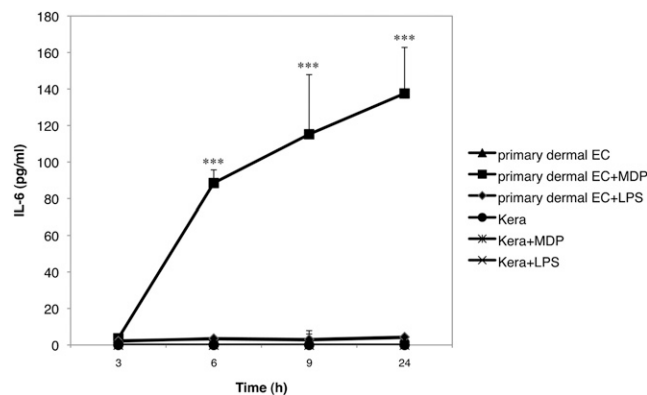
It has been previously reported that human ECs express the intracellular Nod2 receptor and increase IL-6 secretion following MDP stimulation (10). To examine the effect of the Nod2 agonist MDP on cytokine production in the murine transformed EC line bEnd.3 and the Kera cell line PAM212,  $0.2 \times 10^6$  cells were incubated in the presence or absence of 2  $\mu\text{g/ml}$  MDP. In some wells, cells were stimulated with the TLR4 agonist LPS as a control for the specificity of MDP. The supernatants were collected 3, 6, 9, and 24 h later and analyzed for cytokine content by ELISA. IL-6 was significantly higher in supernatants of bEnd.3 cells treated with MDP compared to untreated cells, whereas PAM212 cells did not produce IL-6 with or without MDP stimulation (Fig. 1). Interestingly, LPS was also able to induce IL-6 production in bEnd.3 cells but only after 24 h incubation and in a much lower amount than MDP. None of the other cytokines tested (IL-1 $\beta$ , IL-10, IL-12, IL-17, and IL-23) were detected in supernatants conditioned by stimulated bEnd.3 or PAM212 cells. TGF- $\beta$ 1 was found in the supernatants of both cell lines but was not modulated by MDP (data not shown).

### *Nod2 but not TLR4 activation in primary dermal ECs increases IL-6 production*

To verify that the EC line bEnd.3 behaves in a similar way as primary dermal ECs, primary cells were plated and treated under the same conditions as described above, and supernatants were analyzed for IL-6 content. Freshly isolated Kera were used as a control. MDP induced IL-6 production in primary ECs in the



**FIGURE 1.** Nod2 and TLR4 activation of bEnd.3 ECs promotes IL-6 production. bEnd.3 or PAM212 cells were incubated in the presence or absence of 2  $\mu\text{g/ml}$  Nod2 ligand MDP or 0.1  $\mu\text{g/ml}$  LPS. Supernatants were collected 3, 6, 9, and 24 h later and analyzed for cytokine content by ELISA. The graph represents the mean of three independent experiments  $\pm$  SD. \* $p < 0.05$ , \*\*\* $p < 0.0001$ .



**FIGURE 2.** Nod2 but not TLR4 activation in primary ECs promotes IL-6 production. Primary dermal ECs or primary Kera were incubated in the presence or absence of 2  $\mu\text{g/ml}$  MDP or 0.1  $\mu\text{g/ml}$  LPS. Supernatants were collected 3, 6, 9, and 24 h later and analyzed for cytokine content by ELISA. The graph represents the mean of three independent experiments  $\pm$  SD. \*\*\* $p < 0.0001$ .

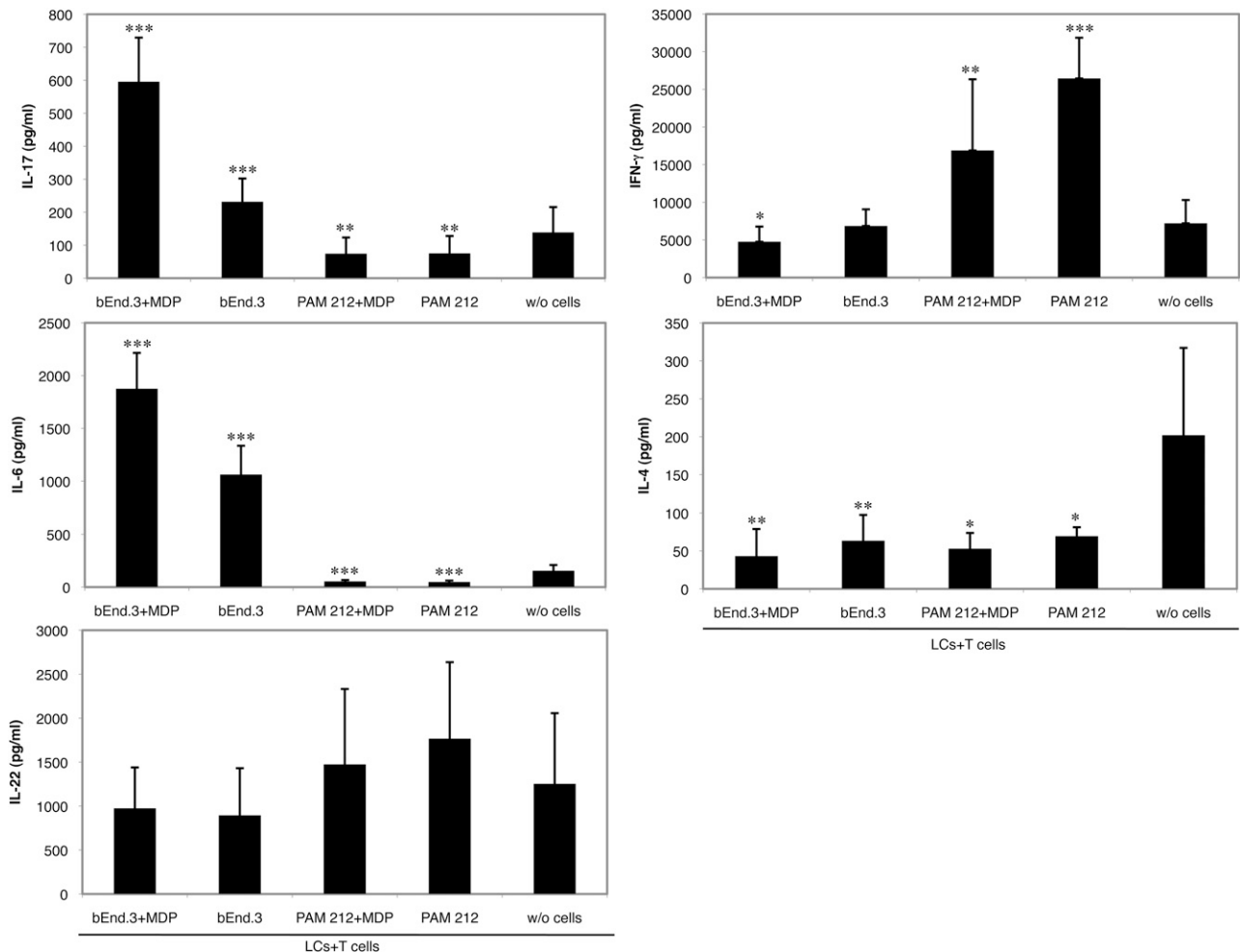
same manner as in the bEnd.3 cell line (Fig. 2). Accordingly, freshly isolated Kera did not produce IL-6 when stimulated with either MDP or LPS. Interestingly, LPS failed to induce IL-6 production in primary dermal ECs in contrast to the results obtained with the cell line.

### *Nod2 activation of bEnd.3 cells polarizes LC Ag presentation to an IL-17 immune response*

To determine whether IL-6 production by ECs activated by MDP may influence the skin immune response to an immunogenic Ag, we developed an in vitro model in which MDP-treated or untreated bEnd.3 cells were cultured together with a mixed population of LCs and T cells from DO11.10 transgenic mice in the presence of cOVA<sub>323–339</sub>. The bEnd.3 cell line, which we established responds to MDP in the same way as primary ECs, was used as a surrogate for dermal ECs. After 48 h incubation, supernatants were harvested and analyzed for cytokine content by ELISA. We evaluated IL-6 along with IL-17, IL-22, IL-4, and IFN- $\gamma$  production. Fig. 3 shows that in the presence of activated bEnd.3 cells, IL-6 and IL-17 production was significantly higher compared with the wells in which LCs and T cells were cultured alone, whereas IL-4 and IFN- $\gamma$  were inhibited. The resulting cytokine pattern in these wells suggests Th17 polarization with inhibition of Th1- and Th2-type immunity. Interestingly, IL-22 production, which is often correlated to Th17 priming, was not influenced by the presence of activated bEnd.3 cells. This suggests that in our in vitro model, IL-17 and IL-22 production are differentially regulated.

Similar results were obtained when nonactivated bEnd.3 cells were used. However, the amount of IL-17 produced was significantly lower compared with wells with MDP-treated bEnd.3 cells, and IFN- $\gamma$  was not inhibited, signifying that Nod2 activation in ECs is necessary for a marked Th17 polarization. Although we previously reported that nonactivated bEnd.3 cells produce a low amount of IL-6, we observed an increased concentration of this cytokine in wells in which LC and T cells were cultivated in the presence of bEnd.3 cells. This likely resulted from the cross talk among the three cell types in the wells. When PAM212 cells with or without MDP stimulation were used instead of bEnd.3 cells, the resulting cytokine pattern was suggestive of a Th1 polarization at the expense of Th17 and Th2. Indeed, IL-17 and IL-4 were both inhibited, whereas IFN- $\gamma$  production was strongly induced in supernatants of wells containing PAM212 compared with those of wells without bEnd.3 or PAM212 cells, regardless of whether





**FIGURE 3.** Nod2 activation of bEnd.3 ECs promotes Th17-related cytokine release in LC and T cell Ag presentation cultures. bEnd.3 or PAM212 cells were preincubated in the presence or absence of the Nod2 ligand MDP for 3 h. After extensive washing, LCs and T cells from transgenic mice were added along with cOVA<sub>323–339</sub>. Supernatants were harvested 48 h later for cytokine content analysis by ELISA. The graphs represent the mean of four independent experiments  $\pm$  SD. \* $p < 0.02$ , \*\* $p < 0.002$ , \*\*\* $p < 0.0001$ .

PAM212 cells were stimulated with or without MDP. These data confirm that MDP activation in bEnd.3 cells is required for inducing Th17 development.

We attempted to repeat this experiment with primary murine DMECs. However, primary ECs are difficult to use in coculture experiments due to stringent media requirements, such as the necessity for growth factors, which may affect the other cells in the coculture and the experiment final outcome. Indeed, when the coculture experiment was carried out in the primary EC line medium, no effective Ag presentation was observed even in the absence of ECs (data not shown). In contrast, when the Ag presentation assay was performed in CM, we observed a significant increase in cell death (as assessed by release of lactate dehydrogenase) in wells in which ECs were present, which also resulted in unsuccessful Ag presentation (data not shown).

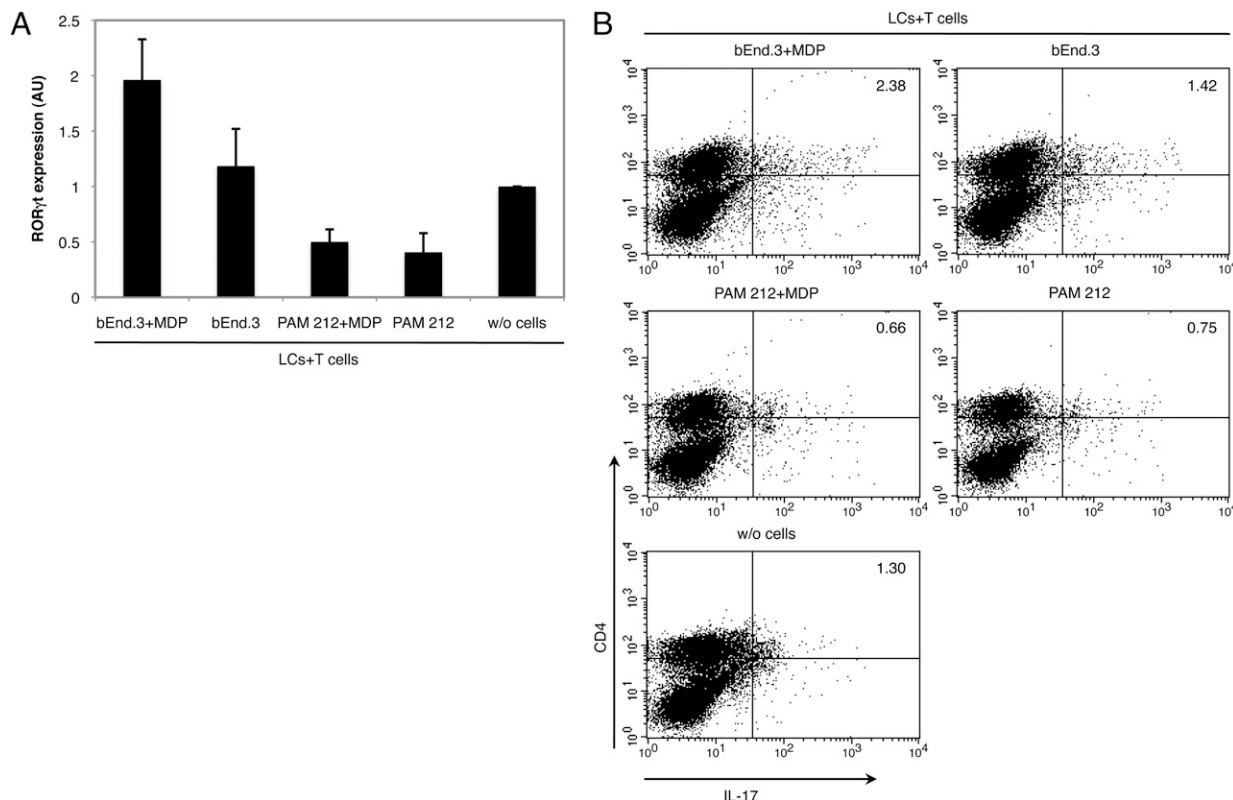
#### *Nod2 activation of bEnd.3 cells induces ROR $\gamma$ t expression and drives Th cell polarization to a Th17 type*

To confirm the ability of activated bEnd.3 cells to drive Th17 polarization, we analyzed the expression of the Th17-specific transcription factor ROR $\gamma$ t in LCs and T cells cultivated in the presence or absence of bEnd.3 or PAM212 cells cultured with or without MDP. We found that ROR $\gamma$ t expression was induced in the MDP-stimulated bEnd.3 cells group, whereas lower mRNA expression was detected in cells cultivated with untreated bEnd.3

cells (Fig. 4A). In contrast, no significant difference in ROR $\gamma$ t mRNA expression was found in wells containing MDP-stimulated bEnd.3 cells compared with the control group. These results were confirmed by flow cytometric analysis of IL-17<sup>+</sup>CD4<sup>+</sup> T cells (Fig. 4B). In wells in which LCs and T cells were cultivated in the presence of activated bEnd.3 cells, the percentage of IL-17<sup>+</sup>CD4<sup>+</sup> T cells was higher than in wells in which LC and T cells were cultured alone. Addition of untreated bEnd.3 cells to LC and T cell culture was also able to induce the differentiation of IL-17<sup>+</sup>CD4<sup>+</sup> T cells, though this increase was much lower than in the bEnd.3 plus MDP group. With regard to cells cultivated in the presence of MDP-stimulated or not PAM212 cells, the percentage of IL-17<sup>+</sup>CD4<sup>+</sup> T cells was even lower than the control group in which LC and T cells were cultured alone. These results correlate with cytokine production in supernatants and confirm the ability and specificity of activated bEnd.3 cells to induce Th17 differentiation.

#### *IL-6 knockdown in bEnd.3 cells inhibits Th17 polarization*

To investigate the mechanism by which activated ECs induce Th17 polarization, we set up an experiment in which bEnd.3 cells were pretreated with IL-6 siRNA to knockdown the production of this cytokine prior to treatment with MDP and cultivation with LCs and T cells. IL-6, along with TGF- $\beta$ 1, is known to promote Th17 differentiation in mice (5), and, as shown above, Nod2 activation



**FIGURE 4.** Nod2 activation in bEnd.3 ECs promotes Th17 cell differentiation. bEnd.3 or PAM212 cells were preincubated in the presence or absence of the Nod2 ligand MDP for 3 h. After extensive washing, LCs and T cells from transgenic mice were added along with cOVA<sub>323–339</sub>. Cells were collected 24 h later for RNA extraction or 48 h later for CD4 and IL-17 staining. **A**, Expression of RORγt was quantitated by real-time RT-PCR using GAPDH as an internal control. In every experiment, RORγt was analyzed using the REST 2008 software. The bars represent the mean of three independent experiments  $\pm$  SD. In all three experiments, RORγt was found to be significantly increased in cells cultivated in the presence of activated bEnd.3 cells compared with cultures of LCs and T cells without bEnd.3 cells. **B**, Cells were restimulated after 48 h incubation with 10 ng/ml PMA and 1  $\mu$ g/ml ionomycin for 3 h prior to enrichment with the mouse IL-17 secretion assay cell enrichment and detection kit, which simultaneously enriches and stains IL-17 cells with a PE dye. Cells were then also stained with FITC-conjugated rat anti-mouse CD4 (H129.19) Ab and analyzed by flow cytometry. IL-17<sup>+</sup>CD4<sup>+</sup> cells were significantly increased in cells cultivated in presence of activated bEnd.3 cells. The figure shows one representative experiment out of three independent significant experiments.

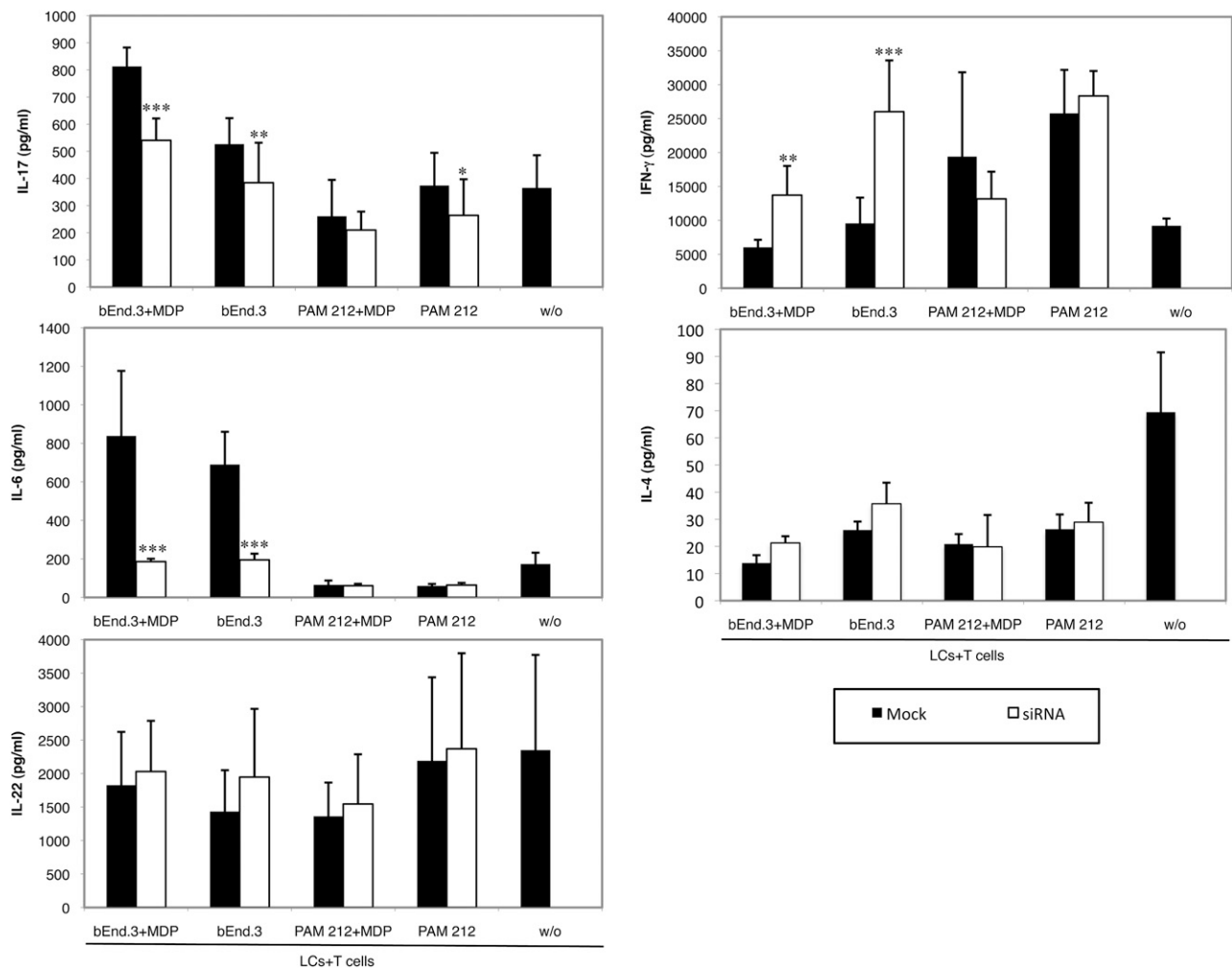
in bEnd.3 cells but not in PAM212 cells stimulates IL-6 production.

Pretreatment of bEnd.3 cells with IL-6 siRNA significantly inhibited IL-6 release into supernatants of LCs and T cells cultured in the presence of bEnd.3 cells treated with or without MDP (Fig. 5). The portion of IL-6 resulting from bEnd.3 production following MDP treatment and cross talk between the cells in the wells was totally inhibited, and the final amount of IL-6 in these wells was comparable to the amount found in wells of LCs and T cells cultured alone. The IL-6 inhibition in these groups was also reflected in the IL-17 production, which was significantly lower when bEnd.3 cells were treated with IL-6 siRNA. In contrast, siRNA treatment significantly boosted IFN- $\gamma$  production and had no effect on IL-4 in these wells. Interestingly, IL-22 production was not affected by siRNA treatment, confirming that IL-17 and IL-22 production are differentially regulated. These results suggest that IL-6 produced by bEnd.3 cells is responsible for the Th17 polarization and may at the same time inhibit the Th1 immune response. siRNA treatment of PAM212 cells had no influence on the final amount of IL-6, IL-22, IL-4, and IFN- $\gamma$  produced. In wells in which LCs and T cells were cultured along with PAM212 cells, there was a small, although significant, inhibition of IL-17 production. However, this inhibition did not correlate with increased IFN- $\gamma$  induction as in wells containing MDP-stimulated bEnd.3 cells.

## Discussion

Although it is generally believed that the first responder to pathogens are often infected nonimmune cells rather than innate immune cells, very little is known about their active contribution to the innate immune response and their subsequent influence on the adaptive immune response. ECs are among the first nonimmune cells in the body to directly contact Ags and PAMPs, such as TLR and Nod agonists, during skin infection, and they may contribute to the innate and adaptive immune response by producing inflammatory cytokines and chemokines. In this study, we demonstrated that ECs likely have an important role in the modulation of the development of the adaptive immune response.

We found that Nod2 activation of the bEnd.3 EC line, but not of the PAM212 Kera cell line, resulted in an increased production of IL-6. These data are consistent with the previous report that activation of human ocular ECs by MDP resulted in increased IL-6 secretion (10). TLR4 activation in bEnd.3 cells was also able to induce IL-6 production, but in a much lower amount than Nod2. In addition, TGF- $\beta$ 1 was also present in the supernatant of bEnd.3 cells, but, in contrast to IL-6, its concentration was not influenced by MDP. The data concerning Nod2 activation were further confirmed by substituting the cell lines with primary dermal ECs and primary Kera. This experiment established that the bEnd.3 and PAM212 cell lines behave as the corresponding primary cells when stimulated with MDP. Interestingly, when primary ECs were



**FIGURE 5.** IL-6 downregulation in bEnd.3 ECs inhibits Th17 polarization. bEnd.3 or PAM212 cells were cultured overnight with 100 nM mouse IL-6 siRNA or the corresponding nontargeting control siRNA. Cells were then stimulated with or without the Nod2 ligand MDP for 3 h. After extensive washing, LCs and T cells from transgenic mice were added along with cOVA<sub>323–339</sub>. Supernatants were harvested 48 h later for cytokine content analysis. The graphs represent the mean of three independent experiments  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ .

stimulated with LPS, there was no increase of IL-6 production, suggesting that the cell line response to TLR4 stimulation diverges from that of primary ECs. However, it should be noted that the amount of IL-6 produced by LPS-treated bEnd.3 cells was minimal. IL-6 and TGF- $\beta$ 1 are known to be implicated in the polarization of the adaptive immune response toward a Th17 type (5). Therefore, we further questioned whether Nod2-activated ECs might be implicated in Th17 development.

In our experiments, we used the bEnd.3 cell line as a surrogate for primary microvascular ECs. We determined that when LCs and the responsive T cells were cultured in the presence of MDP-activated bEnd.3 cells with cOVA<sub>323–339</sub>, the resulting supernatant cytokine profile was suggestive of Th17 polarization. Indeed, IL-17 and IL-6 were both significantly induced compared with wells in which LCs and T cells were cultured alone or in presence of untreated bEnd.3 cells. In addition, IFN- $\gamma$  and IL-4, a Th1- and a Th2-type cytokine, respectively, were both inhibited in accordance with the paradigm that Th17 cell expansion inhibits Th1- and Th2-type immunity (35).

To better characterize the cells responsible for the increased IL-17 concentration seen in the in vitro model, we analyzed by FACS the IL-17<sup>+</sup>CD4<sup>+</sup> T cell content in the wells after the period of Ag presentation. We found that in wells in which LCs and T cells

were cultured in presence of activated bEnd.3 cells, the percentage of IL-17<sup>+</sup>CD4<sup>+</sup> T cells was almost doubled compared with control wells. In addition the expression of IL-17 was much more intense in bEnd.3 plus MDP wells as shown by the shift of IL-17<sup>+</sup> cells toward the right of the FACS dot plot. These data further confirmed the idea that activated bEnd.3 cells induce Th17 polarization as did increased expression of mRNA for the Th17-associated transcription factor ROR $\gamma$ t. In contrast, when untreated bEnd.3 cells were used, the percentage of IL-17<sup>+</sup>CD4<sup>+</sup> T cells and the expression of ROR $\gamma$ t mRNA were much lower than wells containing MDP-stimulated bEnd.3 cells, correlating well with the ELISA results and suggesting that activation of ECs by MDP and the consequent IL-6 production are crucial for Th17 priming. In contrast, when PAM212 cells were used as a control, the cytokine profile in the supernatants was suggestive of a Th1 polarization, a decrease of IL-17<sup>+</sup>CD4<sup>+</sup> T cells was observed in the FACS experiment, and there was an inhibition of ROR $\gamma$ t mRNA expression. These results demonstrate the specificity of ECs in stimulating a Th17 immune response and suggest that Kera might have a similar ability to favor a Th1 immune response. To further investigate the mechanism by which ECs induce Th17 polarization, we blocked IL-6 production in bEnd.3 and PAM212 cells using the siRNA technique prior to stimulation with MDP

and coculture with LCs and T cells. IL-6 blockade of bEnd.3 cells partially, although significantly, impaired IL-17 secretion in wells containing MDP-stimulated bEnd.3 cells while allowing for significantly induced IFN- $\gamma$  production. These results further validate the ability of MDP-treated bEnd.3 cells to promote Th17 cells expansion while inhibiting Th1 immunity and confirmed the importance of IL-6 in the Th17 development as previously described in others studies (5, 36, 37). However, the inhibition of IL-17 found in our experiments was only partial, suggesting the presence of other bEnd.3-derived factors involved in the generation of Th17 cells. Interestingly, IL-22 production was not affected by the MDP treatment of bEnd.3 cells or the IL-6 blockade. This demonstrates differential regulation of IL-17 and IL-22 production in our in vitro model. Given the recent reports of the existence of a distinct subset of CD4<sup>+</sup>T cells, different from Th17 cells, producing IL-22 but not IL-17 (Th22) (8, 9, 38), one could speculate that MDP-activated bEnd.3 cells favor the development of T cells that produce predominantly IL-17 but not those that produce only IL-22 (Th22 cells).

We also attempted to reproduce these findings with primary murine DMECs. Unfortunately, we faced a number of technical difficulties. First, when we performed the assay in CM as in the original experiment, effective Ag presentation did not occur in wells in which ECs were present. As discussed above in *Results*, we discovered that the percentage of dead cells in these wells was significantly higher compared with wells in which LC and T cells were cultured alone or in presence of the bEnd.3 cell line, as assessed by release of lactate dehydrogenase. These results suggested that carrying out the Ag presentation assay in CM did not provide a suitable environment for primary EC survival with cell death and a consequent toxic environment for Ag presentation. Next, we replaced the CM with the primary EC medium in an attempt to provide better conditions for primary EC survival. However, under these conditions, Ag presentation was also inhibited, possibly due to the presence of hydrocortisone in the EC medium. Thus, we have been unable to perform the experiment using primary ECs. We realize that the use of primary cells in these experiments would further support our hypothesis. Nonetheless, we believe our results using bEnd.3 cells as a surrogate for primary microvascular ECs is highly suggestive that activated ECs in vivo are capable of biasing local immune responses toward the Th17 pole.

Although primary ECs do not produce IL-6 following LPS stimulation, we repeated the Ag presentation experiment using bEnd.3 cells stimulated with LPS, and we obtained similar results to the previous experiment in which MDP was used (data not shown). These results suggest that Th17 polarization does not specifically require Nod2 activation in ECs but necessitates the production of IL-6 from these cells. IL-6 production may be induced by a different type of PAMPs, such as TLR agonists, depending on the expression of the corresponding PRRs on ECs. Our experiments are most likely going to be reproducible using other PAMPs able to induce IL-6 production in ECs.

The skin is the largest organ of the body and plays a central role in the host defense. The resident APCs of the skin are LCs, which reside in the epidermis, and dermal DCs. Our data suggest that in vivo MDP-activated ECs may influence the LC microenvironment during Ag processing and presentation by increasing IL-6 production and subsequently modulate the development of the adaptive immune response by enhancing the Th17 polarization. Possibly, the use of other PAMPs to activate ECs and induce IL-6 production from these cells would result in a similar effect. In this regard, as LCs traffic through lymphatics to regional lymph nodes, they would be ideally situated to be exposed to factors produced by

microvascular ECs. Preliminary results using bone marrow-derived DCs as an alternative of LCs showed that MDP-activated bEnd.3 cells failed to induce an IL-17–type immune response in our in vitro model. These results agree with previous studies that showed a discrete ability of skin-resident APCs in inducing Th17 priming (39) and suggest that LCs possess a distinctive ability in promoting Th17 differentiation.

Interestingly, it has been reported that in vivo MDP treatment failed to induce resistance to *Mycobacterium leprae* and *Mycobacterium marinum* in mice (40). In addition, in vitro treatment of T cells with murabutide, an MDP analog, reversed T cell anergy to *M. leprae* Ags (41), suggesting that in vivo, the inability to mount an effective response to MDP may play a role in the exacerbation of the infection. Our findings, along with the fact that multi-bacillary leprosy is highly angiogenic (42), suggest that activation of the MDP pathway in vivo might be useful in the treatment of this disease.

In conclusion, our results may be of great significance for a more complete understanding of the mechanisms underlying Th17-related disorders of the skin, such as psoriasis and other autoimmune diseases, in particular multiple sclerosis, which have been correlated with high IL-6 and IL-17 production along with the presence of Th17 cells. In the future, study of signaling inhibitors that block or modulate the IL-6–ROR $\gamma$ t–IL-17 pathway may be of great interest in the development of potential treatments of such diseases.

## Disclosures

The authors have no financial conflicts of interest.

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