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IL-36 Promotes Myeloid Cell Infiltration, Activation, and Inflammatory Activity in Skin

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G lobal definition of the human transcriptome has revealed many new members of the IL-1 family, including IL-36α (formerly known as IL-1F6) (1), IL-36β (IL-1F8) (2, 3), IL-36γ (IL-1F9) (2, 4), and IL-36Ra (IL-1F5), which, along with the IL-36R (IL-1Rrp2), constitute an independent IL-1 signaling system analogous to IL-1α, -1β, -1Ra, and IL-1RI. We (5) and others (1, 4) have recently demonstrated that IL-36Ra, IL-36α, IL-36β, and IL-36γ mRNA and protein are elevated in skin plaques of the inflammatory disease psoriasis, and keratinocytes (KC) were identified as the predominant source (4, 5). Emerging evidence from mouse models indicates a critical role for the IL-36 system in skin inflammation (1, 6, 7), and a crucial role for human IL-36Ra was recently highlighted, when missense mutations in the IL-36Ra gene were shown to be associated with a form of generalized pustular psoriasis (8, 9).

The IL-1 family members IL-36α (IL-1F9) (2, 4), and IL-36Ra (IL-1F5) are potent drivers of allogeneic MLRs, demonstrating that IL-36 can stimulate the maturation and function of DC and drive T cell proliferation. These data indicate that IL-36 cytokines actively propagate skin inflammation via the activation of keratinocytes, APC, and, indirectly, T cells. The Journal of Immunology, 2014, 192: 000–000.

Abbreviations used in this article: DC, dendritic cell; GPP, generalized pustular psoriasis; KC, keratinocyte; mDC, myeloid dendritic cell; MO-DC, monocyte-derived DC; NHK, normal human keratinocyte; QRT-PCR, quantitative RT-PCR.

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every injection to ensure similar location between days. Prior to the first injection, the animal was anesthetized with isoflurane and shaved. Four hours after the last injection, animals were euthanized and each injection site was harvested. During tissue harvesting, each injection site (~circular in nature) was dissected from the rest of the dorsal skin and then bisected. Half of each site was put into either 10% neutral buffered formalin and tissue freezing medium for histological and immunostaining analyses, as previously described in detail (12). The other half was placed into tubes and snap frozen, and total RNA was extracted. Quantitative RT-PCR (QRT-PCR) was performed and data were normalized to the housekeeping gene 18S and expressed as fold change over BSA-treated controls (n = 4). H&E staining and immunohistochemistry using Abs specific for CD4, CD8, CD11b, CD11c (BD Biosciences, San Jose, CA), and F4/80 (eBioscience, San Diego, CA) were also completed, as described previously (12).

IL-36R expression

CD4+ and CD8+ T cells and CD14+ monocytes were prepared from PBMC by negative immunomagnetic selection (Miltenyi Biotec). Cells were typically >85% pure cultures, as determined by flow cytometry using Abs against CD3, CD4, CD8, and CD14, as detailed below. Human mDC were magnetically isolated from PBMC using two rounds of positive selection for CD14+ cells after negatively selecting CD19+ cells using the BDCA-1 microbead kit from Miltenyi Biotec. mDC were assessed to be 95% viable lineage (CD3, CD14, CD16, CD19, CD20, CD56) negative, HLA-DR, CD11c, CD123 phenotype by flow cytometry. Total RNA was isolated from CD4+ T cells, CD8+ T cells, mDC, and monocytes, and QRT-PCR for IL-1R1, IL-1RACP and IL-36R was carried out, as described below. Surface expression of IL-1R1, IL-1RACP and IL-36R was detected by incubating cells with biotinylated polyclonal goat anti-human IL-1R1 (BAF269), IL-1RACP (BAF676), and IL-36R (IL-1Rrp2, BAF872) Abs (all 50 μg/ml; R&D Systems) on ice for 30 min. After washing in FACS buffer, 25 μl allophycocyanin-labeled streptavidin (BD Biosciences), anti-CD3 (S41; Invitrogen), CD4 (S3.5; Invitrogen), CD8 (SK1; BD Biosciences), and CD14 (M5E2; BioLegend, San Diego, CA) were added for 30 min at 4°C in the dark, and, after two more washes, cells were analyzed with a LSRR2 flow cytometer (BD Biosciences).

T lymphocyte stimulation

CD3+ T cells were isolated from PBMC as above and incubated for 24 h with either unstimulated or 1 μg/ml CD3 and CD28 Abs (BD Biosciences), together with 10 ng/ml IL-1β or 100 ng/ml IL-36α, β, or γ. Cultures were subsequently prepared for QRT-PCR or flow cytometry. Cells were washed in FACS buffer (PBS + 0.5% BSA + 0.1% NaN3) and then stained with Abs against CD3 (clone S4.1; Invitrogen), CD4 (OKT-4; eBioscience), CD8 (SK1; BD Biosciences), CLA (HECA-452; BioLegend), CD103 (Ber-ACT8; BioLegend), CD25 (BC96; BioLegend), CD69 (FN50; BioLegend), CD54 (HCDC54; BioLegend), and appropriate isotype-matched control Abs for 30 min at 4°C in the dark. After two washes in FACS buffer, cells were analyzed using a BD LSRR2 flow cytometer gating on lymphocytes expressing CD3 and CD4 or CD8.

Neutrophil isolation and stimulation

Neutrophils were isolated from heparinized peripheral blood, as previously outlined (13). IL-1R1, IL-1RACP, and IL-36R expression was determined by FACS, as described above, gating on small, highly granular cells expressing CD11b. Neutrophil stimulations were performed with 10 ng/ml IL-1β; 100 ng/ml truncated IL-36α, β, or γ, 100 ng/ml IL-1α; or 100 ng/ml LPS (Sigma-Aldrich); or 50 ng/ml PMA (Sigma-Aldrich) for 4 h. Conditioned media were assayed for CXCL8 and TNF-α by ELISA (Duoset; R&D Systems).

APC isolation and culture

CD14+ monocytes and BDCA-1 mDC were prepared from PBMC as above and stimulated for 12 h in round-bottom 96-well culture plates in complete RPMI 1640 containing 10% FCS supplemented with GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) (R&D Systems). Cells were fed on day 4. On day 8, DC were seeded into poly (2-hydroxyethyl-methacrylate) (Sigma-Aldrich)-coated 12-well culture plates (Corning Costar) at a density of 1 million cells/well and stimulated for 2 d with a mixture containing IL-6 (10 ng/ml) PGE2 (0.1 μM), along with IL-1β (10 ng/ml), IL-36α, IL-36β, or IL-36γ (100 ng/ml in 500 μl complete RPMI 1640). DC phenotype was analyzed by flow cytometry, as described above, using Abs against CD86 (FUN-1; BD Biosciences), HLA-DR (LN3; eBioscience), CD1a (HI149; eBioscience), CD11c (3.9; eBioscience), CD123 (6H6; BioLegend), and appropriate isotype control Abs.

Allogeneic MLR

MO-DC were matured as above with 10 ng/ml IL-6, 0.1 μM PGE2, 10 ng/ml IL-1β, and 100 ng/ml IL-36α, and, on day 10, they were incubated with 200,000 allogeneic CD4+ T cells at ratios of 1:20 and 1:100 for 5 d in round-bottom 96-well culture plates (NUNC). In some cases, T cells were prelabeled with CFSE (Invitrogen) before culture, as directed by manufacturer. Cells were stained with anti-CD3 (HI1T3a; BioLegend) for 20 min at 4°C and then treated with 200 μl 1 μg/ml DAPI (Invitrogen) in PIPES buffer for 10 min at room temperature. Cells were analyzed by flow cytometry gating on lymphocytes with the DAPI detection channel set for linear detection.

Real-time QRT-PCR

Total RNA was isolated (RNeasy mini kit; Qiagen) and reverse transcribed (High Capacity cDNA Transcription Kit; Applied Biosystems, Foster City, CA), and transcripts were quantified using a 7900HT Fast Real-Time PCR system (Applied Biosystems) normalizing to the expression of the housekeeping gene ribosomal large, P0 (RPRL0). TaqMan primer sets were purchased from Applied Biosystems. IL1A Hs00174092_m1, IL1B Hs00174097_m1, IL6 Hs00171138_m1, CCL3 Hs00174575_m1, CCL4 Hs00171072_m1, CCL5 Hs00234140_m1, CCL7 Hs00234142_m1, CCL4 Hs00999948_m1, CCL5 Hs00174575_m1, CCL7 Hs00171147_m1, CCL20 Hs00355476_m1, IL-1R1 Hs00168392_m1, IL1RAP Hs00370506_m1, IL1Rl2 (IL36R) Hs00909276_m1, and RPLP0 Hs00999902_m1 were used in this study.

Statistical analysis

Data were tested for normality, and statistical significance was calculated using two-way Student t tests, Mann–Whitney U test, or one-way ANOVA with Dunner’s posttest, as appropriate, using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

Results

IL-36 induces chemokine expression by human keratinocytes

We have previously shown that human keratinocytes are an important source of antimicrobial peptides when exposed to IL-36 family cytokines (5). We now demonstrate that IL-36 cytokines induce the robust expression of chemokines that drive immune cell chemotaxis. Treatment of NHK with IL-36α, IL-36β, or IL-36γ led to significant increases in the macrophage (CCL3, CCL4, CCL5, CCL2, CCL17, and CCL22), T cell chemotaxatants (CCL20, CCL5, CCL2, CCL17, and CCL22), and the neutrophil chemokines (CXC8L, CCL20, and CXC8L1) (Fig. 1A). Moreover, all IL-36R agonists, but not IL-36Ra, dose dependently induced CXC1L, CCL5, CXCL8, and CCL20 mRNA expression and CXCL8 and CCL20 protein secretion by NHK (Fig. 1B–E), demonstrating that, following IL-36 exposure, KC are potent sources of macrophage, T cell, and neutrophil chemokines.

IL-36 induces myeloid cell infiltration of skin concomitant with chemokine and growth factor induction

Given that IL-36 induced chemokine expression by human KC in vitro, we sought to test whether this drove cell chemotaxis in vivo by injecting 5 μg murine rIL-36α or BSA intradermally into CD1 mice every other day for 10 d. By day 10, the mouse skin did not appear erythematous or thickened, but, histologically, a mild acanthosis and an increase in eosinophilic dermal collagen were evident along with a very pronounced leukocytic infiltrate (Fig. 2A).
FIGURE 1. IL-36 cytokines induce chemokine expression by keratinocytes. Four-day postconfluent NHK were stimulated for 24 h with recombinant truncated IL-36R ligands or IL-1β. Total RNA was extracted, mRNA transcripts were quantified by QRT-PCR relative to the housekeeping gene RPL-P0, and conditioned medium was assayed by ELISA. A total of 100 ng/ml IL-36α, IL-36β, and IL-36γ significantly induced T cell chemokine mRNA expression compared with untreated cells, mean ± SD (n = 3) (A). IL-36α, IL-36β, IL-36γ, and IL-1β, but not IL-36Ra, dose dependently induced CXCL1, CCL5, CXCL8, and CCL20 mRNA expression (B–E) and CXCL8 and CCL20 protein secretion (F and G) by keratinocytes. Mean ± SD (n = 3). Statistical significance indicated by *p < 0.05, **p < 0.01, or ***p < 0.001, Student t test.
The infiltrate was striking in its largely granulocytic character (Fig. 2B–D) with few T cells (Fig. 2E, 2F). QRT-PCR revealed that IL-36 treatment induced significant fold changes in a number of leukocyte chemokines, including CCL3, CCL4, and CXCL12 (Fig. 2G), as well as IL-1β and HB-EGF (Fig. 2H; all \( p, 0.05 \), fold change versus BSA control, \( n = 4 \)), further supporting a role for IL-36 in facilitating immunocyte recruitment to inflamed skin.

**APC, but not T cells, express IL-36R**

Given that KC expressed chemokines in response to IL-36 treatment and IL-36 injected intradermally led to leukocyte infiltration, we questioned whether T cells, neutrophils, and APC would also respond to IL-36 cytokines. First, we examined whether isolated blood CD4\(^+\) T cells, CD8\(^+\) T cells, monocytes, or mDC expressed IL-36R. After magnetic separation, giving >85% pure CD4\(^+\) T cells, CD8\(^+\) T cells, monocytes, and >95% mDC from PBMC, we isolated RNA and performed QRT-PCR. Along with monocytes, mDC, and primary keratinocytes (NHK), both subsets of T cells expressed the IL-1R1 (Fig. 3A) and IL-1RAcP (Fig. 3B) receptors. IL-36R transcripts were not detectable from CD4\(^+\) or CD8\(^+\) T cells (Fig. 3C); however, monocytes, mDC, and NHK expressed IL-36R mRNA (Fig. 3C). To demonstrate cell surface IL-36R expression, we stained T cells, monocytes, and mDC with Abs against IL-36R and used flow cytometry to show that IL-36R

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**FIGURE 2.** IL-36 induces myeloid cell infiltration of skin concomitant with chemokine and growth factor induction. A total of 5 \( \mu \)g murine IL-36α or BSA was injected intradermally into CD1 mice every other day for 10 d. Back skin was harvested, snap frozen, and processed for RNA and histochemistry. IL-36α treatment led to acanthosis and an increase in eosinophilic dermal collagen (A) and striking infiltration of granulocytes (CD11b) (B), macrophages (F4/80) (C), DC (CD11c) (D), CD4\(^+\) cells (E), but not CD8\(^+\) cells (F). These changes were accompanied by increases in chemokines (G) and cytokines/growth factors (H). Mean ± SEM (\( n = 4 \) mice). Statistical significance indicated by *\( p < 0.05 \) (two-tailed t test). Scale bar, 100 \( \mu \)m.
was most strongly expressed on the surface of mDC (Fig. 3D), which was ∼10-fold more than on monocytes (Fig. 3E) and absent from the surface of T cells (Fig. 3F). In contrast to myeloid cells, blood neutrophils showed no expression of IL-36R and failed to respond to IL-36 treatment (Supplemental Fig. 1). Likewise, neither resting nor CD3/CD28-activated CD4⁺ nor CD8⁺ T cells responded to IL-36 treatment (Supplemental Fig. 2).

IL-36 activates and induces APC to secrete IL-1 and IL-6

Given that monocytes and mDC expressed the prerequisite receptors for IL-36, we stimulated monocyte and mDC cultures with 100 ng/ml IL-36α, β, or γ, and, in contrast to T cells (Supplemental Fig. 2), monocytes were activated and significantly upregulated expression of IL-1A, IL-1B, and IL-6 mRNA (Fig. 4A–C) after 12 h of IL-36 treatment, which is supported by significantly increased IL-1β and IL-6 protein secretion into the conditioned medium (Fig. 4D, 4E). Likewise, mDC treated with IL-36 cytokines for 12 h significantly increased the proportion of cells with strong CD83, CD86, and HLA-DR expression, as determined flow cytometrically (Fig. 5A–C).

Moreover, when conditioned media were assayed by ELISA, both IL-1β and IL-6 were significantly elevated in IL-36α- and β-treated mDC cultures (Fig. 5D, 5E). Of note is that the responses of mDC to IL-36 were not mediated by the secreted IL-1β or IL-6 (Supplemental Fig. 3).

DC matured in the presence of IL-36 have increased activity

Given that primary blood mDC typically compose only 2% of the PBMC population isolated from blood, we sought to use in vitro MO-DC as a surrogate APC. We cultured MO-DC and found IL-36R was expressed 6-fold more abundantly by CD1a⁺CD14⁻ MO-DC than precursor CD1a⁺CD14⁺ monocytes (Fig. 6A). We next examined whether IL-36 could alter MO-DC phenotype during maturation. After 6 d in culture, immature MO-DC were stimulated for 48 h with a cytokine mixture that promoted DC maturation in an IL-1β-dependent manner. A combination of IL-6 and PGE₂ with IL-1β, IL-36α, IL-36β, or IL-36γ significantly activated DC, as illustrated by 3-fold increases in CD86 with IL-36α (p = 0.014; Fig. 6B), and significantly increased proportions...
of DC strongly expressing HLA-DR compared with IL-6 and PGE2 alone (p = 0.03; Fig. 6C).

Having established that IL-36 could alter DC phenotype, we began to assess whether this translated into altered DC function. Thus, we cocultured IL-36–matured DC with allogeneic T cells at DC:T cell ratios of 1:20 (Fig. 6D, 6E) and 1:100 (Fig. 6D) for 5 d and monitored T cell proliferation using CFSE and DAPI labeling of T cells. Compared with basal stimulation with IL-6 and PGE2 only, DC matured with IL-1β or IL-36α induced significant increases in T cell proliferation in the allogeneic MLR in terms of both CFSE dilution (Fig. 6D, 6E) and increased numbers of superdiploid cells (Fig. 6F).

**Discussion**

Skin inflammation such as that seen in psoriasis results from the multipartite interactions of, at least, KC, T cells, APC, fibroblasts, and endothelial cells (15, 16). In this scenario, KC are not passive and may initiate inflammatory cascades following physical stress,
UV irradiation, or infection (17, 18). KC are a major source of IL-36 cytokines, particularly during inflammation (1, 4, 5, 19, 20). We now demonstrate that KC, when treated with IL-36 cytokines, are potent sources of chemokines active upon T cells and APC (Fig. 1), and this activity was not mediated via IL-1β or IL-6 (Supplemental Fig. 3). In the current study, we focused on the use of the recently characterized, highly active truncated IL-36 cytokines, which have been shown to bind IL-36R and activated cells in the ng/ml range (11). The truncated IL-36α and β were at least 40-fold more potent than their full-length counterparts (11).
environment (25). The epidermal hyperproliferation in psoriasis is demonstrated that IL-36 cytokines induce APC expression of IL-1. DC treated with IL-36 promoted increased T cell proliferation (26). We show, however, that APC such as CD14+ monocytes, CD1a+CD14+ DC and incubated IL-36–matured DC with allogeneic CD4+ T cells. CD86 binds to CD28 on the T cell, providing the costimulatory second signal essential for priming naive T cells. We next investigated the functional consequences of IL-36 activity on DC and incubated IL-36–matured DC with allogeneic CD4+ T cells. DC treated with IL-36 promoted increased T cell proliferation (Fig. 6D–F), which strongly suggests that IL-36 may influence T cell function in skin via its effects on APC. Moreover, we demonstrate that IL-36 cytokines induce APC expression of IL-1β and IL-6 (Figs. 4, 5), which potentially contributes to a pro-Th17 environment (25). The epidermal hyperproliferation in psoriasis is now thought to be driven, at least in part, by IL-17– and IL-22–secreting T cells infiltrating the skin (26). In humans, naive CD4+ T cells differentiate into mature Th17 cells in vitro in response to IL-1β, IL-6, and/or IL-23 (26–28), and we have recently demonstrated that IL-1β and/or IL-23 can promote the survival and expansion of Th17 cells from the memory T cell pool (29). This is a particularly important observation, as memory T cells are likely to be the most important T cell subset for the maintenance of psoriasis (30, 31).

A form of generalized pustular psoriasis (GPP) has recently been associated with missense mutations in IL36RN (8, 9) that affect the structure and function of IL-36Ra protein, leading to unrestrained IL-36 agonist activity. Although GPP has a strong neutrophil component, we could not demonstrate IL-36R expression by neutrophils or direct activity of IL-36 on neutrophils (Supplemental Fig. 1). GPP is also associated with increased activation of Th17 and Th22 cells during the disease flare (32), and, interestingly, Th17 T cells, immature DC, γδ T cells, and neutrophils all express CCR6 (21, 33, 34); we show that IL-36 is a strong inducer of the CCR6 ligand CCL20 expression by KC (Fig. 1), consistent with a role for the IL-36–CCL20–CCR6 axis in driving psoriatic inflammation (7).

IL-1 family members have also been shown to act synergistically with other cytokines and growth factors (35), and, in this respect, IL-36α, β, and γ are no exception, with synergism reported with IL-17A, TNF-α (36), and IL-1β (37) extending the potential of IL-36 in the skin, particularly on psoriatic keratinocytes (38). This may be particularly relevant given that psoriasis is now considered a mixed Th1/Th17 disease and that IL-36 upregulates the expression of a number of IFN-γ–induced chemokines.

Our data presented in this study, together with our previous findings (5), data from others using mice (1, 7), and studies of the IL36RN mutations associated with pustular psoriasis (8, 9), all suggest an important role for the IL-36 system in skin inflammation: IL-36 induces KC expression of antimicrobial peptides, matrix metalloproteinases (5), and chemokines (Fig. 1), which recruit T cells and APC. IL-36 activates APC and biases their cytokine profile (Figs. 4–6) (27), which further drives the inflammatory response. Taken together, these data are consistent with the notion that IL-36 cytokines, which we have shown to be overexpressed in psoriasis (5), can influence the phenotype and function of DC, with subsequent changes in T cell activity.

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Disclosures

The authors have no financial conflicts of interest.

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