Inter-Regulation of Th17 Cytokines and the IL-36 Cytokines *In Vitro* and *In Vivo*: Implications in Psoriasis Pathogenesis

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Accumulating evidence indicates that IL-1 family members and Th17 cytokines have a pathogenic role in psoriasis. We investigated the regulatory interactions of the IL-1-like IL-36 cytokine family and the Th17 cytokines in the context of skin inflammation. We observed increased gene expression of all three IL-36 cytokines in a Th17-dominant psoriasis-like animal model. The induction was downregulated by neutralizing IL-22. Expression of the IL-36s was also induced in cultured primary human keratinocytes (KC) by IL-17A and tumor necrosis factor (TNF)-α, and IL-22 synergized with IL-17A and TNF-α. Furthermore, the IL-36s directly induced their own expression and the production of proinflammatory mediators (TNF-α, IL-6, IL-8) in KC. These functions were markedly enhanced with the addition of IL-17A or TNF-α to the cultures. Similarly, IL-36α and IL-36β augmented IL-17A-mediated induction of antibacterial peptides. Finally, we show that the increased gene expression of IL-36 correlated with Th17 cytokines in the lesions of psoriatic patients. Our results indicate that the IL-36 cytokines are not only regulated by Th17 cytokines, but that they themselves can regulate the expression and enhance the function of Th17 cytokines. We propose that a feedback loop between the IL-36 and Th17 cytokines is involved in driving cytokine expression in psoriatic tissues.

Journal of Investigative Dermatology (2011) **131,** 2428–2437; doi:10.1038/jid.2011.234; published online 1 September 2011

INTRODUCTION

IL-36 is a newly named cytokine family that comprises three members: IL-36α, IL-36β, and IL-36γ, previously designated IL-1F6, IL-1F8, and IL-1F9, respectively (Dinarello *et al.*, 2010), as a result of their homology to IL-1 (Kumar *et al.*, 2000; Smith *et al.*, 2000). Similar to the classical IL-1 cytokines, IL-36 cytokines are also involved in the initiation or regulation of immune responses (Sims and Smith, 2010). All IL-36 cytokines bind IL-1 receptor (IL-1R)-related protein 2, which in turn recruits the signal component IL-1RAcp, the accessory protein (Acp) that is also shared by IL-1α and IL-1β, and activates a signaling pathway similar to IL-1α and IL-1β (Sims, 2002; Towne *et al.*, 2004). Different from IL-1α and IL-1β, IL-36s are expressed in a restricted manner, primarily in the skin and other epithelial tissues, whereas the receptors are more widely expressed (Kumar *et al.*, 2000; Smith *et al.*, 2000; Sims *et al.*, 2006). Transgenic overexpression of IL-36α in basal KC in mice results in cutaneous alterations in both the epidermis and dermis, which is independent of T-cell involvement (Blumberg *et al.*, 2007). In addition, increased IL-36α levels have been observed in psoriasis vulgaris biopsies, indicating that the IL-36 cytokines may have a proinflammatory role in this disease (Blumberg *et al.*, 2007).

Psoriasis vulgaris is a common chronic inflammatory skin disorder mediated by T-cell interactions with KC and other types of skin-infiltrating immune cells. Recent discoveries have highlighted the involvement of Th17 T-helper cells and the cytokines they produce in psoriasis (Wilson *et al.*, 2007; Pene *et al.*, 2008). However, the mechanisms by which Th17 cytokines contribute to the pathogenesis of psoriasis still remain unclear. Here, we explored the inter-regulation of the IL-36 cytokines and the Th17 cytokines, and elucidated the contributions of this family of innate immune cytokines in the cytokine network of human psoriasis.

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Abbreviations: IL-1R, IL-1 receptor; KC, keratinocyte; TNF-α, tumor necrosis factor-α

Received 14 December 2010; revised 17 May 2011; accepted 4 June 2011; published online 1 September 2011

RESULTS

Increased gene and protein expression of IL-36 cytokines in psoriasis-like mouse skin lesions

We have previously described a psoriasis-like mouse model, which involves the adoptive transfer of naive $CD4^+$ $CD45RB^{hi}CD25^-$ cells into CB17 *scid/scid* mice (Ma *et al.*,

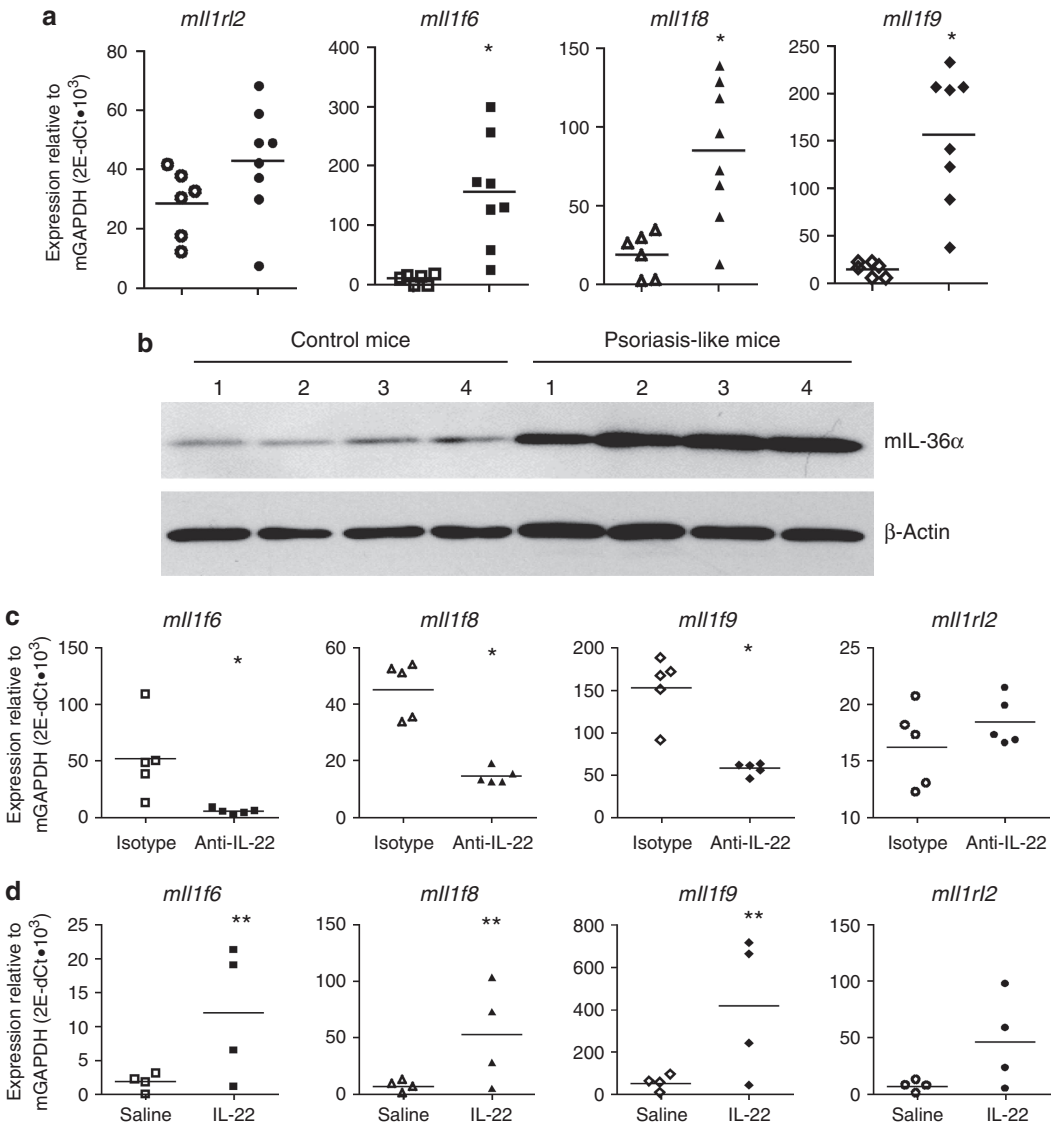

Figure 1. Regulation of IL-36 cytokines by IL-22 in mouse psoriasis-like skin. Tissue lysates from *scid* mice that received either naive T cells or saline (control) (**a**) and that co-received antibodies (isotype control or anti-IL-22) and naive T cells (**c**) were analyzed for the mRNA expression of mouse *Il1f6, Il1f8, Il1f9,* and *Il1rl2* by quantitative PCR. In addition, ear tissues with or without psoriatic-like plaques were analyzed for the expression of IL-36α protein by western blot (**b**). (**d**) Ear tissues from naive BALB mice that received saline (control) or rmIL-22 were also subjected to the same gene expression analysis. One of two (**a**, total $n=8$, $^*P<0.005$), one of three (**c**, $n=5$ per group, $^*P<0.01$), and one of two (**d**, $n=4$ per group, $^{**}P<0.1$) independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2008). The transferred T cells elicit a Th17-dominant immune response and induce scaly and raised skin plaques with certain microscopic characteristics resembling human psoriasis. In the ear samples that contain these psoriasis-like plaques, we detected significantly elevated gene expression of all three IL-36 cytokines (mRNA of *Il1f6*, *Il1f8*, and *Il1f9*) when compared with control mice that did not receive T-cell transfer, hence without skin lesions (Figure 1a). The expression of their unique receptor IL-1R-related protein 2 (gene symbol: *Il1rl2*) was increased slightly, but did not reach the level of statistical significance. Markedly elevated IL-36α protein was also detected in the ear biopsies with psoriatic-like plaques (Figure 1b), indicating that the protein level was consistent with the observed increased gene expression.

IL-22 regulates the expression of IL-36 cytokines in normal and psoriasis-like mouse skin

We have previously demonstrated that Th17 cells are major effectors driving disease progression in this T-cell-adoptive transfer psoriasis-like model, and that IL-22 neutralization alone is sufficient to prevent disease progression (Ma *et al.*, 2008). Using the same neutralizing regimen in this model, we found that the diminished symptoms in the recipients of anti-IL-22 antibody correlated with reductions in *Il1f6* (~9-fold),

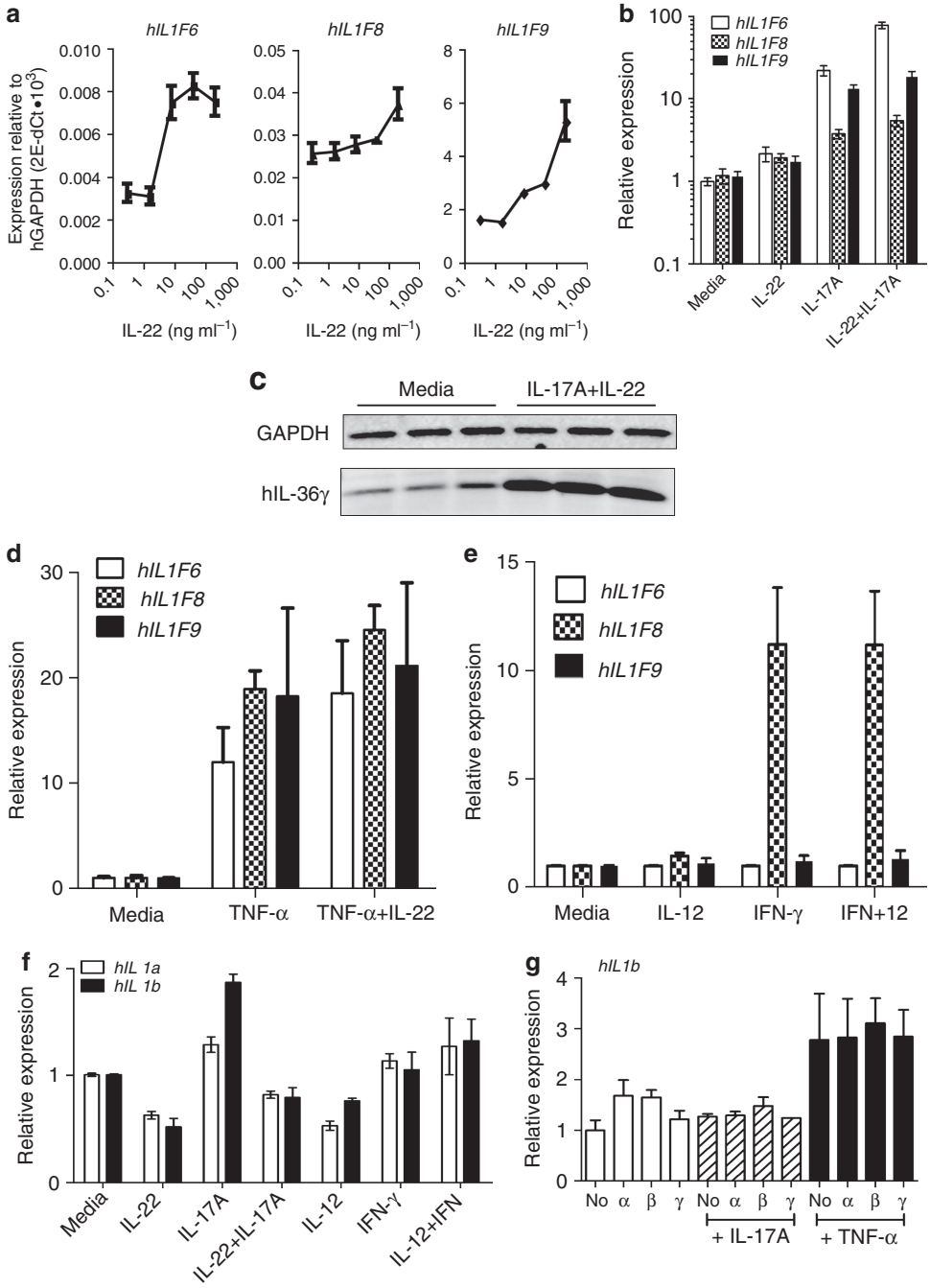

Figure 2. Induction of IL-36 gene expression in human primary keratinocytes (KC) *in vitro*. KC were incubated with (**a**) 0.32, 1.6, 8, 40, or 200 ng ml^{-1} of IL-22, (**b**, **c**) 200 ng ml^{-1} of IL-22 in combination with IL-17A, (**d**) IL-22 with tumor necrosis factor (TNF)-α, (**e**) IL-12 and IFN-γ, (**f**) Th1 or Th17 cytokines alone or in combination, as indicated, and (**g**) 1 μg ml^{-1} of IL-36 in the presence of IL-17A or TNF-α, all for 24 hours. Relative expression levels of indicated genes are reported as mean ± SD of triplicates of a representative donor. (**c**) IL-36γ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins detected by western blot analysis. The protein level of IL-36γ was ~10 ng in 15 μg of cell extract as judged using recombinant IL-36γ as standards. Shown is one of four (**a**), six (**b**), two (**c**), six (**d**), three (**e**, **f**), and two (**g**) experiments with a total of eight donors.

Il1f8 (~2.3-fold), and *Il1f9* (~2.2-fold), but not *Il1rl2* transcripts (Figure 1c). In contrast, repeated injection of recombinant IL-22 protein in the normal skin of wild-type BALB/c mice induced gene expression of all IL-36s (Figure 1d). These data suggest that the Th17 cytokine IL-22 can regulate gene expression of IL-36s, but not the receptor, at the site of inflammation.

IL-22, IL-17A, and tumor necrosis factor-α induce the *ex vivo* production of all three IL-36 by human KC, whereas IFN-γ selectively induces IL-36β

To further delineate the effects of IL-22 on the expression of IL-36s, we used a primary human epithelial KC culture system and studied the effects of IL-22 when added to this culture system. Despite a vast difference in the relative

transcript quantity of each member, a dose-dependent increase in all three, *IL1F6*, *IL1F8*, and *IL1F9*, transcripts was observed in KC from all donors tested (Figure 2a).

We have previously demonstrated that IL-22 and IL-17A are coexpressed by Th17 cells, and that these two cytokines have a synergistic effect in enhancing the production of innate inflammatory ''danger signals'', such as β-defensin 2 and S100 proteins, by human KC (Liang *et al.*, 2006). Here we examined the regulation of IL-36s by IL-22, IL-17A, and IL-17F, separately or in combination, in primary human KC cultures. In contrast to the modest effects of IL-22, transcripts of all IL-36s were profoundly induced in response to IL-17A, with *IL1F6* displaying the strongest increase (Figure 2b). Addition of IL-22 further enhanced the induction of *IL1F6*, and to a lesser extent, the induction of *IL1F8* and *IL1F9* by IL-17A, indicating that IL-22 and IL-17A also have synergistic or additive effects in the regulation of IL-36 cytokines. The amount of IL-36γ protein was increased ~7-fold in KC that were cocultured with IL-22 and IL-17A (Figure 2c), confirming the correlation between gene and protein expression. Protein induction of IL-36γ was ~100-fold greater than IL-36α, and protein level of IL-36β was below detection limits (data not shown). IL-17F alone or in combination with IL-22 had no effect on the gene expression of IL-36s and their receptor (data not shown).

Tumor necrosis factor (TNF)-α is another important proinflammatory cytokine that is produced by Th17 cells. Clinical studies have demonstrated that blocking the TNF pathway is an effective treatment in psoriasis patients (Gottlieb *et al.*, 2005). TNF-α stimulation of the KC resulted in a 10- to 20-fold increase in all three IL-36 mRNA levels compared with cells cultured with media alone (Figure 2d). TNF-α-mediated IL-36 induction could be further boosted with the addition of IL-22 to the culture. Although IL-17A and TNF-α induced all three IL-36 in the KC, IL-17A most strongly induced the expression of IL-36α, and TNF-α strongly induced IL-36β.

The induction and progression of tissue damage in psoriasis has been traditionally linked to Th1 T cells and their signature cytokines IFN-γ and IL-12 (Lowes *et al.*, 2007). Incubation with IFN-γ alone induced about a 10-fold increase of *IL1F8* transcript while having minimal effect on *IL1F6* and *IL1F9* mRNA (Figure 2e). IL-12 had no effect either alone or in combination with IFN-γ. These data demonstrate that in our *in vitro* cultures, the expression of IL-36 in KC is predominantly regulated by Th17 cytokines with some contributions by Th1 cytokines.

We also examined the regulation of classic IL-1α and IL-1β by Th1, Th17, and IL-36 cytokines in KC cultures. A slight increase in the gene expression of *IL1b* was induced by IL-17A (<2-fold, Figure 2f) and TNF-α (3-fold, Figure 2g) in KC. However, IL-22, IFN-γ, IL-12, and all three IL-36 showed minimal effects, either alone or in combination. The lack of regulation of IL-1α/β by Th17 cytokines in our KC cultures suggests that the regulation of IL-36s by Th17 cytokines is relatively specific, and there is distinct spatial and temporal expression of the two branches of the IL-1 family.

IL-36 cytokines synergize with IL-17A and TNF-α to increase their own expression

To elucidate the functions of the IL-36 family of cytokines in local tissues, we first examined the ability of the IL-36s to regulate their own expression, as related IL-1 cytokines are able to do so. Primary KC were incubated with all three IL-36s in a range of 0–10 $\mu g\,ml^{-1}$ concentrations. As shown in Figure 3a, both IL-36α and IL-36β induced the gene expression of themselves and other family members in a dose-dependent manner. IL-36α peaked around 3 $\mu g\,ml^{-1}$ and IL-36β may not reach plateaus even at a concentration of 10 $\mu g\,ml^{-1}$. This ''self-induction'' was significantly enhanced with the addition of IL-17A (at constant concentration of 20 $ng\,ml^{-1}$) in the cultures. All three IL-36 had synergistic effects with IL-17A and the ''self-induction'' all peaked at around a concentration of 1–3 $\mu g\,ml^{-1}$. In fact, IL-36γ, which hardly induced the gene expression of itself and other members, was able to stimulate the expression of all three members in the presence of IL-17A in KC cultures. Synergistic effects with TNF-α in self-induction were also observed when IL-36s were used at concentrations of 1 $\mu g\,ml^{-1}$ (Figure 3b) and also in cross-induction of *IL1F6* and *IL1F9* by IL-36β (Figure 3c). These data suggest that all three members of the IL-36 family are capable of self-regulation in KC *in vitro* cultures, with IL-36β having the strongest effects and IL-36γ the weakest. IL-17A and TNF-α not only induce IL-36 cytokines but also enhance their function in a selective manner.

IL-36α and IL-36β induce proinflammatory mediators in human KC *in vitro*

Next, we tested IL-36 for the ability of inducing proinflammatory cytokines in KC. We found that IL-36α and IL-36β, but not IL-36γ, directly induced TNF-α mRNA and protein synthesis in KC (Figure 4a). IL-17A had little effect by itself, but strongly enhanced IL-36α- and IL-36β-mediated inductions of TNF-α. Interestingly, although TNF-α induced its own expression, addition of IL-36α or IL-36β further strengthened *TNFa* gene expression, indicating that IL-36α and IL-36β could regulate TNF-α directly in KC. In addition, we observed direct regulation of IL-8 and IL-6 by IL-36α and IL-36β, which synergized with IL-17A and TNF-α (Figure 4b and c). In testing the regulation of β-defensin (gene symbol *DEF4*) and s100A7, two proteins previously shown to be elevated in psoriatic lesions, all three IL-36 showed minimal direct effects. However, when paired with IL-17A, IL-36α and IL-36β further increased IL-17A-induced *DEF4* mRNA by 2- and 6-fold, respectively (Figure 4d). Interestingly, similar synergistic effects were observed between IL-17A and IL-36β and IL-36γ in *S100a7* induction (Figure 4e). In addition, we studied the effects of IL-36s on KC proliferation by examining the gene expressions of keratin 16 and 17 (gene symbol: *KRT16* and *KRT17*), which are expressed at elevated levels by KC in psoriasis (Leigh *et al.*, 1995). After incubation of KC with IL-36, either alone or in combination with IL-17A or TNF-α, levels of *KRT16* and *KRT17* expression were fluctuated within 1.0- to 1.5-fold for the 6–72 hour culture period that was tested (Figure 4f and g, shown at 24 hours),

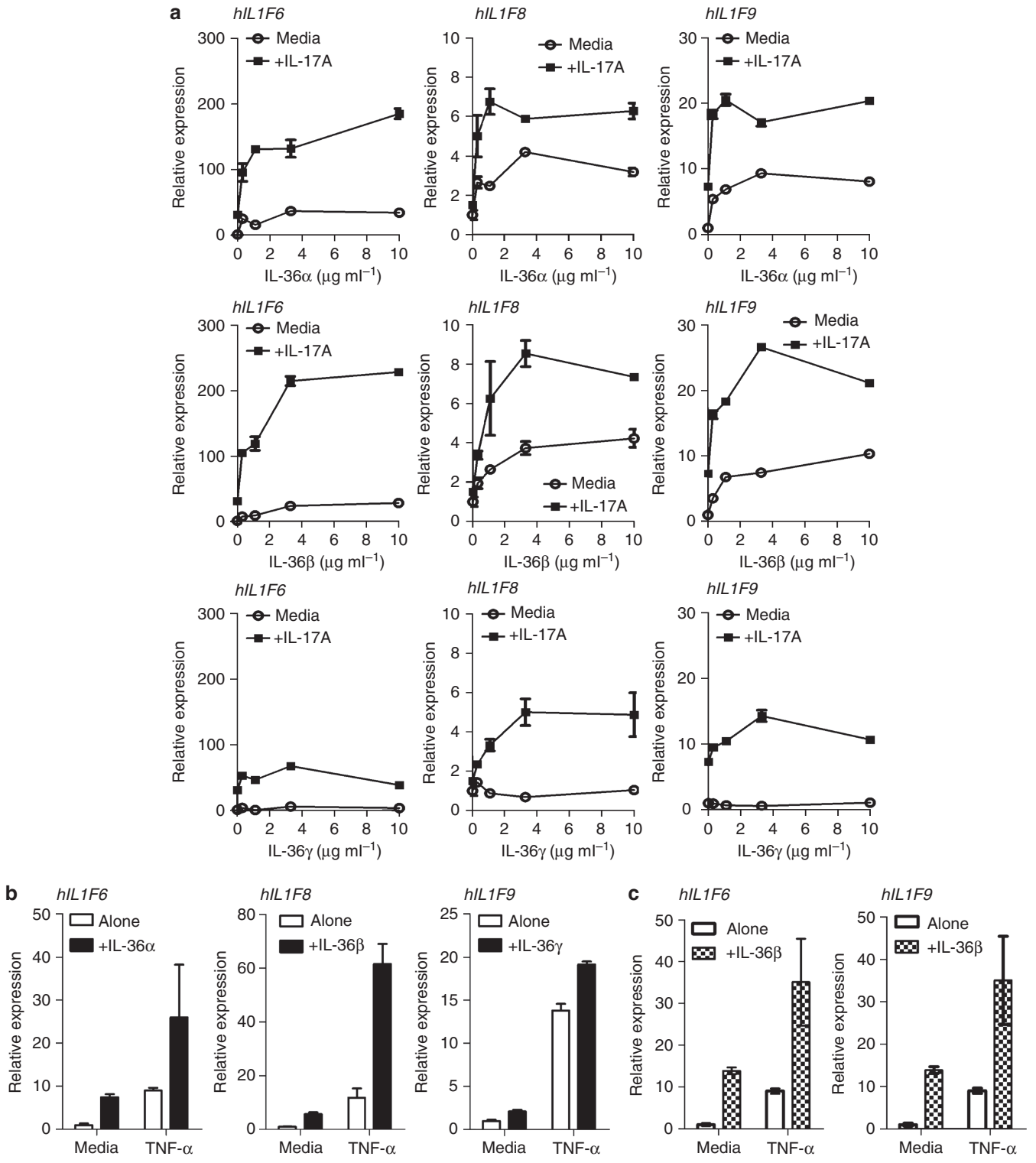

Figure 3. Self- or cross-regulation of gene expression of IL-36 cytokines in keratinocytes (KC) *in vitro*. Gene expression of human *IL1F6, IL1F8*, and *IL1F9* in KC after 24 hours of incubation with 0, 0.3, 1.1, 3.3, and 10 μg ml^{-1} of IL-36α, IL-36β, and IL-36γ in the absence or presence of 20 ng ml^{-1} of IL-17A (**a**), or after incubation with 1 μg ml^{-1} of IL-36 alone or in combination with 20 ng ml^{-1} of tumor necrosis factor (TNF)-α (**b**, **c**). Data are reported as mean ± SD of triplicates of one representative donor out of five donors.

indicating that IL-36s do not regulate K16 and K17 directly. Together, these data suggest that IL-17A and TNF-α-induced IL-36 in KC may enhance the pathogenic function of IL-17A and TNF-α locally, thus contributing to the establishment and perpetuation of a proinflammatory environment in tissues.

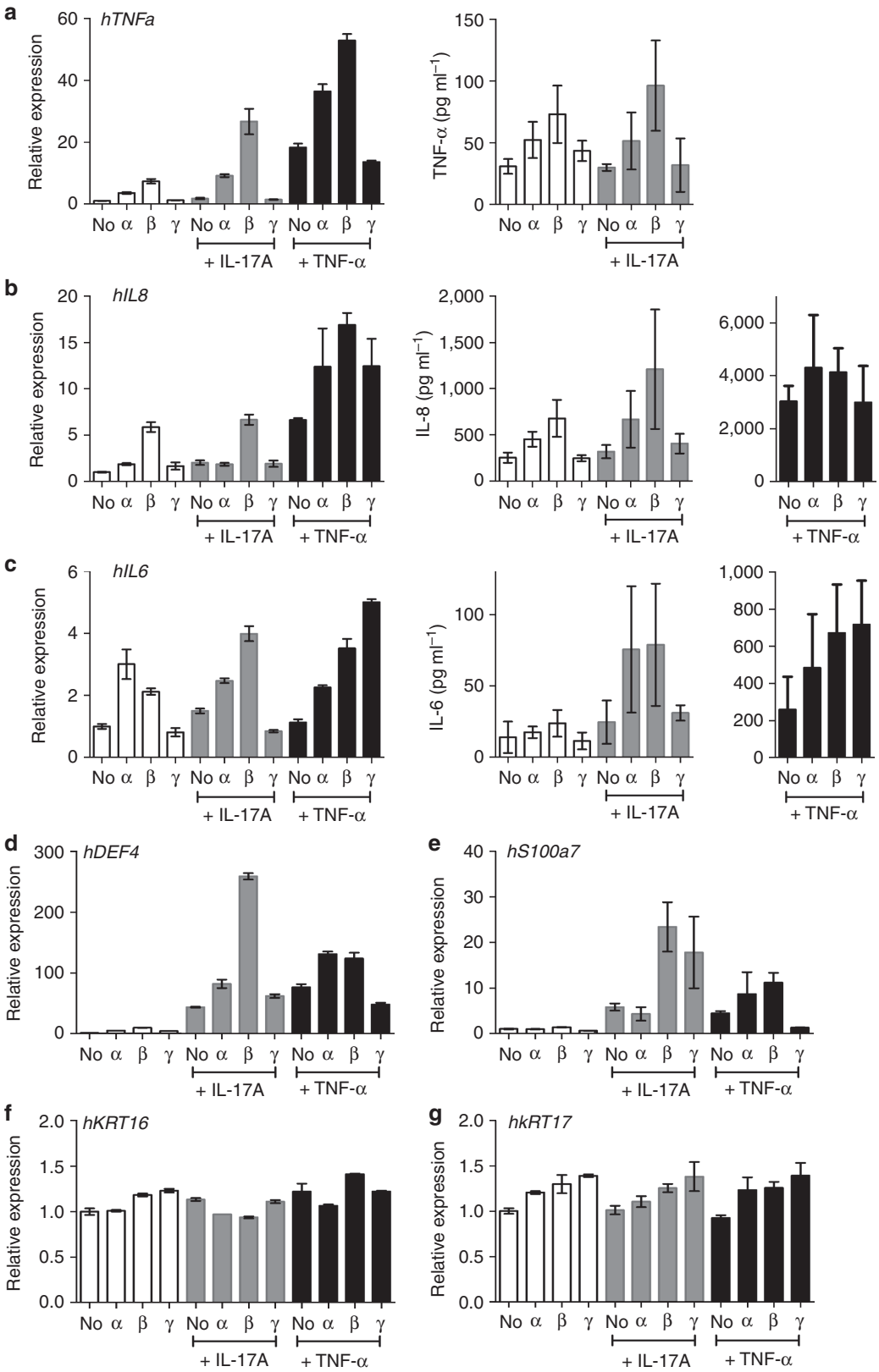

Figure 4. Functions of IL-36 cytokines in keratinocytes (KC) *in vitro*. Induction of tumor necrosis factor (TNF)-α (**a**), IL-8 (**b**), and IL-6 (**c**) were detected in KC as mRNA transcripts (2 hours) or as proteins in the supernatant (6 hours) after incubation with either IL-36 cytokines alone or in combination with IL-17A or TNF-α. TNF-α protein was not measured in TNF-α-treated cultures. Gene expression levels of human *DEF4* (**d**, 48 hours), *S100a7* (**e**, 48 hours), *KRT16* (**f**, 24 hours), and *KRT17* (**g**, 24 hours) are reported as mean ± SD of triplicates of one representative donor out of five donors.

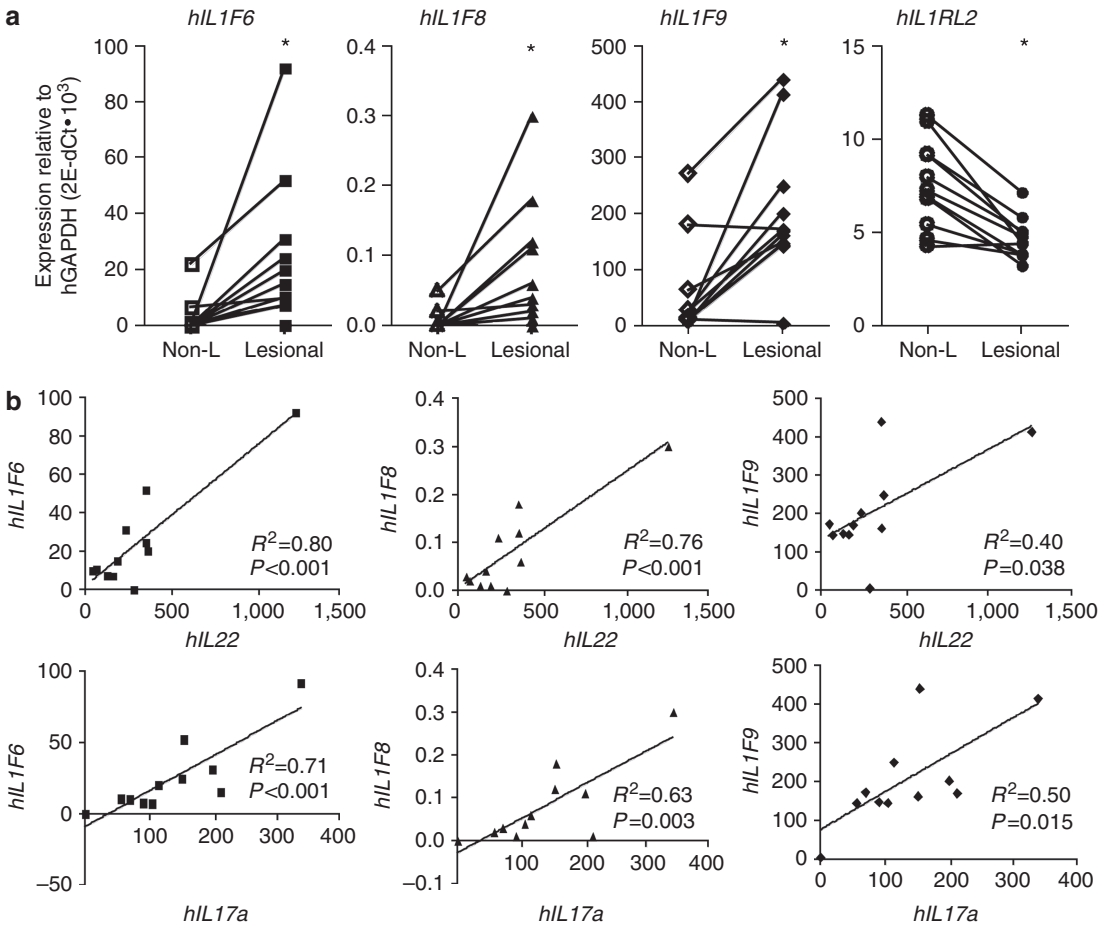

Figure 5. Correlation between IL-36 and Th17 cytokine gene expression in the human psoriasis skin lesions. (**a**) Transcript levels of human *IL1F6, IL1F8, IL1F9,* and *IL1RL2* in non-lesional (non-L) or lesional skin biopsy pairs from human psoriasis patients ($n=11$); *$P<0.02$. (**b**) Comparison of human *IL1F6, IL1F8,* and *IL1F9* with *IL22* and *IL17a* transcripts (units on *x* and *y* axis are in relative units normalized to transcripts of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)) in the lesional skin biopsies. Statistical correlations were determined according to Pearson's value correlation test, and differences between pairs of cytokines were determined by two-tailed Student's *t*-test.

Increased expression of IL-36 cytokines correlates with expression of IL-17A, IL-22, TNF-α, and IFN-γ in human psoriasis skin lesions

Gene expression of a panel of proinflammatory cytokines was examined in paired non-lesional and lesional skin samples obtained from psoriatic patients. All patients had increased expressions of *IL1F6* (~20-fold), *IL1F8* (~100-fold), and *IL1F9* (~4-fold) in the psoriatic lesions, either as a group or as individual patients (Figure 5a). This increase strongly correlated with the expression of Th17 cell-secreted cytokines IL-22, IL-17A, and TNF-α, but not IL-17F (Figure 5b and Table 1). In addition, elevated IFN-γ expression was also detected in the lesions and correlated well with the expression of IL-36s, indicating a possible interplay between Th1 and Th17 cytokines in psoriatic skin lesions. This is in agreement with a very recent report showing that IFN-γ synergizes with IL-17 in the production of antimicrobial and chemotactic proteins by psoriatic KC, suggesting that Th1 and Th17 cytokines may all contribute to human psoriasis (Kryczek *et al.*, 2008).

DISCUSSION

This study characterizes the interplay between the systemic proinflammatory cytokines IL-17A, IL-22, and TNF-α, which are predominantly produced by immune cells, and the IL-36 cytokines, which are expressed in a restrictive manner, primarily in the skin and epithelium. We demonstrate that the IL-36s are not only regulated by Th17 cytokines, but that they themselves can regulate the expression and enhance the function of Th17 cytokines. We propose that the collaboration between IL-36 and Th17 cytokines augments the downstream function of both IL-36 and Th17 cytokines.

The relationship between the IL-36 and Th17 cytokines is illustrated in our study first by the effect of IL-22 on their *in vivo* expression in a Th17-dominant psoriatic-like mouse model (Ma *et al.*, 2008). Blockade of IL-22 signaling downregulated the gene expression of all IL-36 cytokines and skin inflammation in the psoriatic ear tissues, whereas injection of recombinant IL-22 protein into the ears of naive mice had opposite results (Figure 1; Ma *et al.*, 2008). Using an *in vitro* primary human KC culture system, we further

Table 1. Correlation of cytokine expression profiles in human psoriatic lesions

Gene symbol	*IL1F6*	*IL1F8*	*IL1F9*	*IL1RL2*
IL12P35				
r	−0.324	−0.324	−0.524	0.154
P	0.331	0.332	0.098	0.651
R^2	0.105	0.105	0.274	0.024
IL17a				
r	0.848	0.795	0.705	−0.037
P	0.001	0.004	0.015	0.913
R^2	0.719	0.632	0.497	0.001
IL17f				
r	0.491	0.562	0.237	−0.351
P	0.125	0.072	0.482	0.290
R^2	0.241	0.316	0.056	0.123
IL22				
r	0.894	0.873	0.629	−0.103
P	0.000	0.001	0.038	0.764
R^2	0.799	0.761	0.395	0.011
IL23				
r	0.588	0.600	0.790	0.000
P	0.057	0.051	0.004	1.000
R^2	0.346	0.360	0.624	0.000
IFNg				
r	0.863	0.810	0.862	−0.171
P	0.001	0.003	0.001	0.615
R^2	0.745	0.656	0.742	0.029
TNFa				
r	0.618	0.677	0.676	0.162
P	0.043	0.022	0.023	0.635
R^2	0.382	0.459	0.457	0.026

Comparison of human *IL1F6, IL1F8, IL1F9,* and receptor *IL1RL2* gene transcripts with those of the other cytokines in the lesional skin biopsies. Statistical correlations were determined according to Pearson value correlation test. The correlation coefficient, *r*, quantifies the direction and magnitude of correlation. The quantity of the coefficient of determination, R^2, is the fraction of the variance that is ''shared'' between two variables. *P*-values were determined by two-tail Student's *t*-test.

demonstrated that IL-36 can be induced by Th17 cytokines. IL-17A and TNF-α are strong inducers of IL-36s, whereas IL-22, a weaker one by itself, has synergistic/additive effects with IL-17A or TNF-α. This is in agreement with our previous report that IL-22 has a synergistic/additive effect on the induction of antimicrobial peptides by IL-17A or IL-17F (Liang *et al.*, 2006). Interestingly, we found that IL-36 cytokines also have a synergistic/additive effect on the induction of antimicrobial peptides by IL-17A or TNF-α, indicating that Th17 cytokines and IL-36 can reinforce similar responses in KC. Levels of IL-20, a cytokine closely related to IL-22, are elevated in psoriatic lesions and contribute to KC differentiation (Wolk *et al.*, 2009). It is possible that, similar to IL-22, IL-20 may have a role in IL-36 expression in KC.

Although psoriasis is one of the most common T-cell-mediated autoimmune diseases in humans, a role for the innate immune system in triggering the T-cell cascade has been proposed (Boyman *et al.*, 2004; Nestle *et al.*, 2005). TNF-α is an important proinflammatory cytokine that initiates and maintains inflammatory responses in the skin (Aggarwal, 2003), and clinical studies have demonstrated that blocking the TNF pathway is an effective treatment in psoriasis patients (Gottlieb *et al.*, 2005). Here we show that the production of TNF-α can be induced by either IL-36α or IL-36β in primary human KC. The presence of IL-17A and TNF-α in the cytokine milieu further augments the function of IL-36, driving the production level of TNF-α even higher. In turn, TNF-α is a potent inducer of all three IL-36s in KC. This amplification loop involves cytokines produced by and functioning on KC and can occur in the absence of T cells. In addition to the induction of TNF-α, IL-36 induces proinflammatory mediators that are regulated by NF-κB (Towne *et al.*, 2004), including IL-6, IL-8, and the antimicrobial peptides. Our findings are consistent with a recent report that IL-36 cytokines contribute to the pathogenesis of psoriasis by inducing proinflammatory cytokines and a range of antimicrobial peptides and matrix mettalloproteinases from reconstituted human epidermis (Johnston *et al.*, 2011). It is possible that in cooperation with TNF-α, the innate immune IL-36 cytokines are capable of initiating skin inflammation in psoriasis in the absence of adaptive immunity. This may partially explain the observation that transgenic overexpression of IL-36α protein in basal level KC can induce psoriasis-like inflammation in lymphocyte-deficient Rag2 knockout mice (Blumberg *et al.*, 2007).

The regulation of IL-36 by Th17 cytokines we observed in *in vitro* KC cultures and in the psoriasis-like mouse model is consistent with gene expression levels seen in non-lesional and lesional biopsy samples from psoriasis patients. Increased expression of IL-36 is correlated with increased expression of cytokines that are known to be involved in the pathogenesis of psoriasis (IL-17A, IL-22, TNF-α, IL-23, and IFN-γ, but not IL-12 and IL-17F). We did not directly measure the relationship between IL-36 and IL-23 in our *in vitro* assays, as IL-23 is not made by nor does it directly affect KC. However, recent genome-wide association studies have implicated *IL23R* genetic variants as conferring risk to psoriasis, suggesting an important role of the IL-23 pathway in the disease pathogenesis (Nair *et al.*, 2009). We speculate that the correlation between IL-23 and IL-36 is a result of the role of IL-23 in generation of Th17 cells. This is consistent with the observation that IL-36s induce IL-6 and TNF-α in KC that

directly contribute to Th17 differentiation and maintenance. However, this does not exclude a more direct effect of IL-36 on Th17 generation, in a manner similar to that of IL-1β, which is important for the differentiation and growth of Th17 cells in humans (Acosta-Rodriguez *et al.*, 2007). Whether or not the IL-36 family members have similar function as IL-1β in this context awaits future investigation. The involvement of IL-36 in the adaptive immunity and this mutual induction between IL-36 and Th17 cytokines have also been demonstrated in a recent study using a psoriatic-like mouse model induced by application of an irritant on the skin of mice overexpressing IL-36α transgene (Blumberg *et al.*, 2010).

Both Th1 and Th17 cells have been verified as major T-cell components of psoriasis (Lowes *et al.*, 2008). It is still not clear which cell type has a predominant role, although a greater number of Th17 and Th22 cells are identified in the circulations of psoriasis patients than Th1 cells (Kagami *et al.*, 2009). In this study, we show regulation of IL-36 by both Th17 and Th1 cytokines. Although only the transcription of IL-36β is stimulated by IFN-γ in KC cultures, the gene expressions of all three IL-36 are correlated with that of IFN-γ in the psoriatic plaques from patients. Interestingly, IL-36β is a robust inducer of IL-36 family members in KC cultures, indicating that increasing IL-36β expression could result in increased expression of other members. Therefore, it is possible that IL-36 cytokines are involved in mediating effects from both Th1 and Th17 cytokines in psoriasis. It is puzzling that although all members of IL-36 family bind to their common receptor IL-1R-related protein 2, they clearly convey different signals and thus may have different activities. The differential effects of IL-36 cytokines may be explained by the role of the N-terminal region of the proteins on activity as it has been suggested that N-terminal cleavage increases the activity of the IL-36 cytokines (Blumberg *et al.*, 2010), although the mechanism of such cleavage *in vivo* is unclear. However, it is also possible that the functional differences of IL-36 members may reflect intrinsic diversity of these molecules and possible non-redundant roles played by each member at the site of inflammation.

In summary, we have shown here the connection between the Th17 cytokine network and the local expression of IL-36 in primary human KC, in a psoriatic-like mouse model and in the lesions of psoriasis patients. Our data suggest that IL-36s are not only effector cytokines downstream of their induction by Th17 cytokines in the skin, but also inducers of many proinflammatory mediators (cytokine, chemokine, and anti-microbial peptide) that regulate the cellular and soluble components of the local inflammatory milieu. The cross talk between the Th17 cytokines and IL-36 family suggests that levels of both cytokine classes can be amplified in a disease setting with a resultant amplification in their activity, and could perhaps have a role in the chronic autoimmune inflammation observed during psoriasis. These findings further characterize an innate immune pathway and its role as a trigger of a common human autoimmune disease, and suggest that IL-36 cytokines and their receptor represent potential early targets for the treatment of psoriasis.

MATERIALS AND METHODS

Mice

Female BALB/cBy (donor) and C.B-17/prkdc *scid/scid* (recipient) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in a specific pathogen-free environment at a Pfizer animal facility and were used between 6 and 8 weeks of age. All protocols were approved by the Pfizer Animal Care and Use Committee.

Human sample

Human skin samples were obtained as described before (Bhawan *et al.*, 2004). An institutional approval was obtained for the biopsies from each participating site with adherence to the Declaration of Helsinki Principles. Patients provided written, informed consent.

Reagents

Anti-IL-22 neutralizing and isotype control antibodies, recombinant mouse and human IL-22, and human IL-17A and IL-17F were generated internally. Recombinant human IL-36α, IL-36β, IL-36γ, TNF-α, IFN-γ and IL-12, and anti-mouse IL-36α and anti-human IL-36γ antibodies were purchased from R&D Systems (Minneapolis, MN). All proteins are with endotoxin level $<1.0\,\mathrm{EU\,mg^{-1}}$ of cytokine. We carefully tested and eliminated the effect of lipopolysaccharide (LPS) in our culture systems with exogenous LPS and LPS antagonist polymyxin B.

Treatment of mice

Induction of psoriasis-like lesions in mice, treatment with anti-IL-22 antibody and intradermal injection of recombinant IL-22 were described previously (Ma *et al.*, 2008). Briefly, psoriatic-like skin lesions were induced in C.B-17/Prkdc *scid/scid* mice after adoptive transfer of naive $CD4^+CD45RB^{hi}CD25^-$ T cells isolated from BALB/cBy mice. Tissues were harvested on day 70 for analysis. Alternatively, mice that received naive T cells were also treated with anti-IL-22 or the isotype control antibodies twice a week for 70 days. To evaluate the effects of IL-22 in normal mouse tissue, naive BALB/cBy mice were injected intradermally every other day for 2 weeks with saline (left ear) or rmIL-22 (right ear). Ears were harvested and subjected to analysis of gene or protein expression.

Keratinocyte cultures

Primary human epithelial KC (ScienCell, Carlsbad, CA) were cultured in KC-conditioned medium on poly-D-lysine-coated plates (BD Biosciences, Bedford, MA). Cells were passed at 80% confluency and all experiments were conducted between passages 1 and 4. In all, 30,000 cells per well (24-well plate) were seeded and allowed to adhere for 48 hours before cultured with cytokines. IL-22 and IL-12 were used at $200\,\mathrm{ng\,ml^{-1}}$, IL-17A, TNF-α, IFN-γ at $20\,\mathrm{ng\,ml^{-1}}$, and IL-36s at $1\,\mu\mathrm{g\,ml^{-1}}$, unless indicated in the graph.

Quantitation of cytokine transcripts

RNA was isolated using the QIAGEN RNeasy kit (Qiagen, Valencia, CA) and subjected to quantitative reverse transcription-PCR using prequalified primers and probes from Applied Biosystems (Carlsbad, CA). The ΔδCt method was used to normalize transcripts to *GAPDH*, and reported as either unit relative to *GAPDH* or relative expression to levels detected in controls.

Western blotting

Cells were lysed in RIPA buffer with COMPLETE protease inhibitors (Boerhinger, Ingelheim am Rhein, Germany). Protein concentration was determined using the Pierce BCA kit (Rockford, IL) and 15 μg of protein was loaded per well. Chemiluminescence data from western blots were collected and quantitated using a Kodak IS2000NM Imager (Rochester, NY).

Statistics

Two-tailed Student's *t*-test was used to calculate statistical significance for differences between groups. $P \leqslant 0.05$ was considered statistically significant.

CONFLICT OF INTEREST

All authors were full-time employees of Pfizer during the period of this study.

ACKNOWLEDGMENTS

We thank Dr Ullrich Schwertschlag for providing human samples.

REFERENCES

Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A *et al.* (2007) Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 8:942–9

Aggarwal BB (2003) Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 3:745–56

Bhawan J, Bansal C, Whren K *et al.* (2004) K16 expression in uninvolved psoriatic skin: a possible marker of pre-clinical psoriasis. *J Cutan Pathol* 31:471–6

Blumberg H, Dinh H, Dean C Jr *et al.* (2010) IL-1RL2 and its ligands contribute to the cytokine network in psoriasis. *J Immunol* 185:4354–62

Blumberg H, Dinh H, Trueblood ES *et al.* (2007) Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. *J Exp Med* 204:2603–14

Boyman O, Hefti HP, Conrad C *et al.* (2004) Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor-alpha. *J Exp Med* 199:731–6

Dinarello C, Arend W, Sims J *et al.* (2010) IL-1 family nomenclature. *Nat Immunol* 11:973

Gottlieb AB, Chamian F, Masud S *et al.* (2005) TNF inhibition rapidly down-regulates multiple proinflammatory pathways in psoriasis plaques. *J Immunol* 175:2721–9

Johnston A, Xing X, Guzman AM *et al.* (2011) IL-1F5, -F6, -F8, and -F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. *J Immunol* 186: 2613–22

Kagami S, Rizzo HL, Lee JJ *et al.* (2009) Circulating Th17, Th22, and Th1 cells are increased in psoriasis. *J Invest Dermatol* 130:1373–83

Kryczek I, Bruce AT, Gudjonsson JE *et al.* (2008) Induction of IL-17+ T cell trafficking and development by IFN-gamma: mechanism and pathological relevance in psoriasis. *J Immunol* 181:4733–41

Kumar S, McDonnell PC, Lehr R *et al.* (2000) Identification and initial characterization of four novel members of the interleukin-1 family. *J Biol Chem* 275:10308–14

Leigh IM, Navsaria H, Purkis PE *et al.* (1995) Keratins (K16 and K17) as markers of keratinocyte hyperproliferation in psoriasis *in vivo* and *in vitro*. *Br J Dermatol* 133:501–11

Liang SC, Tan XY, Luxenberg DP *et al.* (2006) Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* 203:2271–9

Lowes MA, Bowcock AM, Krueger JG (2007) Pathogenesis and therapy of psoriasis. *Nature* 445:866–73

Lowes MA, Kikuchi T, Fuentes-Duculan J *et al.* (2008) Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells. *J Invest Dermatol* 128:1207–11

Ma HL, Liang S, Li J *et al.* (2008) IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. *J Clin Invest* 118:597–607

Nair RP, Duffin KC, Helms C *et al.* (2009) Genome-wide scan reveals association of psoriasis with IL-23 and NF-kappaB pathways. *Nat Genet* 41:199–204

Nestle FO, Conrad C, Tun-Kyi A *et al.* (2005) Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *J Exp Med* 202:135–43

Pene J, Chevalier S, Preisser L *et al.* (2008) Chronically inflamed human tissues are infiltrated by highly differentiated Th17 lymphocytes. *J Immunol* 180:7423–30

Sims J, Towne J, Blumberg H (2006) 11 IL-1 family members in inflammatory skin disease. *Ernst Schering Res Found Workshop* 56:187–91

Sims JE (2002) IL-1 and IL-18 receptors, and their extended family. *Curr Opin Immunol* 14:117–22

Sims JE, Smith DE (2010) The IL-1 family: regulators of immunity. *Nat Rev Immunol* 10:89–102

Smith DE, Renshaw BR, Ketchem RR *et al.* (2000) Four new members expand the interleukin-1 superfamily. *J Biol Chem* 275:1169–75

Towne JE, Garka KE, Renshaw BR *et al.* (2004) Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF-kappaB and MAPKs. *J Biol Chem* 279:13677–88

Wilson NJ, Boniface K, Chan JR *et al.* (2007) Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* 8:950–7

Wolk K, Witte E, Warszawska K *et al.* (2009) The Th17 cytokine IL-22 induces IL-20 production in keratinocytes: a novel immunological cascade with potential relevance in psoriasis. *Eur J Immunol* 39: 3570–81