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Mahil, SK, Catapano, M, Di Meglio, P et al. (10 more authors) (2017) An analysis of IL-36 signature genes and individuals with IL1RL2 knockout mutations validates IL-36 as a psoriasis therapeutic target. Science Translational Medicine, 9 (411). eaan2514. ISSN 1946-6234

https://doi.org/10.1126/scitranslmed.aan2514

Science Translational Medicine AAAS

Submitted Manuscript: Confidential template updated: February 28 2012

Title: An analysis of IL-36 signature genes and individuals with *IL1RL2* knockout mutations validates IL-36 as a psoriasis therapeutic target

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One Sentence Summary: IL-36 receptor (IL36R) blockade ameliorates psoriatic inflammation and should be well tolerated, since IL36R knockout mutations are benign.

Abstract: Interleukin (IL)-36α, -β and –γ are innate mediators of acute epithelial inflammation. Here we sought to demonstrate that these cytokines are also required for the pathogenesis of plaque psoriasis, a common and chronic skin disorder, caused by abnormal TH17 cell activation. To investigate this possibility, we first defined the genes that are induced by IL-36 cytokines in primary human keratinocytes. This enabled us to demonstrate a significant IL-36 signature among the transcripts that are up-regulated in plaque psoriasis and the susceptibility loci associated with the disease in genome-wide studies. Next, we investigated the impact of *in-vivo* and *ex-vivo* IL-36 receptor blockade using a neutralizing antibody or a recombinant antagonist. Both inhibitors had marked anti-inflammatory effects on psoriatic skin, demonstrated by statistically significant reductions in IL-17 expression, keratinocyte activation and leukocyte infiltration. Finally, we explored the potential safety profile associated with IL-36 blockade by phenotyping twelve individuals carrying knockout mutations of the IL-36 receptor gene. We found that normal immune function was broadly preserved in these individuals, suggesting that IL-36 signaling inhibition would not substantially compromise host defenses. These observations, which integrate the results of transcriptomics and model system analysis, pave the way for early-stage clinical trials of IL-36 antagonists.

Introduction

Interleukin (IL)-36α, -β and –γ are a group of IL-1 family cytokines that signal through a common dimeric receptor, composed of the IL-36R and IL-1RAcP subunits. The activation of this complex triggers Nuclear Factor Kappa-light-chain-enhancer of activated B cells (NF-κB) and Mitogen Activated Protein Kinase (MAPK) signaling, leading to the production of pro-inflammatory molecules such as IL-1α/β, IL-6 and IL-8 (*1*). Conversely, binding of IL-36R by the IL-36 receptor antagonist (IL-36Ra, encoded by the *IL36RN* gene) blocks downstream signal transduction (*1*). The importance of this immunomodulatory mechanism was underscored by the discovery of loss-of-function *IL36RN* mutations in patients with generalized pustular psoriasis (GPP) (*2*). This is a severe autoinflammatory disorder, which is characterized by recurrent episodes of neutrophilic skin pustulation and systemic inflammation (manifesting as an acute phase response with neutrophilia) (*3*). Of note, the majority of GPP patients also present with plaque psoriasis (hence psoriasis), a chronic and disfiguring skin disease that affects 1-3% of the population (*3*).

Research carried out in the last decade has demonstrated that the abnormal activation of T helper 17 (TH17) lymphocytes is a major disease driver in psoriasis (*4*). At the same time, several lines of evidence suggest that IL-36 cytokines are also likely to have a pathogenic role. First, individuals with GPP show elevated IL-36 expression in skin (*5*) and present a risk of psoriasis that is ten times higher than that observed in the general population (*6*). Second, the gene encoding the IL-36 receptor (*IL1RL2*) maps to a locus showing genome-wide significant association with psoriasis (*7*). Finally, work carried out in animal models has demonstrated that IL-36 cytokines can activate dendritic cells and thus promote TH17 lymphocyte polarization (*8*). Since the IL-17 molecules released by TH17 cells can in turn up-regulate IL-36 production, it

has been proposed that the two cytokines drive a powerful feedback loop, which effectively propagates skin inflammation (*9*).

Taken together, the above observations suggest that germline *IL36RN* mutations cause widespread up-regulation of IL-36 signaling and severe autoinflammation (GPP), while localized, TH17-dependent over-expression of IL-36 cytokines amplifies cutaneous immune responses in psoriasis. Thus, we propose that IL-36 is a shared disease driver, which contributes to the onset of plaque and pustular psoriasis through different molecular mechanisms.

Given that the role of IL-36 in GPP is well established, here we have investigated IL-36 signaling as a pathogenic mediator and therapeutic target in psoriasis. By following-up the results of IL-36 transcription profiling with targeted inhibition experiments, we demonstrated that *in-vivo* and *ex-vivo* IL-36 blockade can reverse the inflammatory phenotype of psoriasis skin lesions. We also showed that mutations that abolish IL-36 signaling do not substantially compromise human immune function, suggesting that pharmacological IL-36 blockade is likely to have an acceptable safety profile.

Results

IL-36 signature genes are up-regulated in psoriatic skin lesions and enriched within psoriasis susceptibility loci

To explore the role of IL-36 in psoriasis, we first assessed the presence of an IL-36 signature in the disease transcriptome. To date, IL-36 target genes have only been profiled in a single microarray study, based on the stimulation of keratinocyte cultures with unprocessed IL-36 cytokines (*10*). As these precursor molecules were later found to have minimal biological

activity (*11*), the power to detect differential expression was limited. Here, we sought to generate a reference IL-36 transcriptome through the RNA sequencing of healthy primary keratinocytes treated with bio-active IL-36α, -β or -γ (table S1). The principal component analysis of our dataset demonstrated a complete separation between treated (n=3 for each cytokine) and untreated samples (n=3), but also an overlap between the profiles of keratinocytes stimulated with individual cytokines (Fig. 1A). This pattern was confirmed by unsupervised hierarchical clustering (Fig. 1B), suggesting important similarities between the three IL-36 transcriptomes.

We found that 4096, 4459 and 3468 genes were differentially expressed (False Discovery Rate (FDR) <0.05) upon treatment with IL-36α, IL-36β and IL-36γ respectively (Fig. 1C). Of these, 207, 352 and 229 were up-regulated by at least two-fold (Fig. 1C, table S2). In keeping with the results of the principal component analysis and hierarchical clustering, we observed a substantial overlap between these datasets, as 182 genes (hereafter referred to as the *IL36_182 set*) were induced by all three cytokines (Fig. 1C). These included previously described IL-36 dependent loci (*CCL20, CXCL1, IL8, DEFB4A, STEAP4*) (*5, 10*), as well as novel targets such as the chemotactic proteins *S100A7, S100A8, S100A9* and the phospholipase *PLA2G4D*, which generates the lipid antigens presented by Langerhans cells (*12*) (Fig. 1C, table S2).

In keeping with the notion that IL-36 is a dominant cytokine in the GPP skin transcriptome, we found 58 *IL36_182* genes among the 500 that were most significantly up-regulated in a recently described (*5*) GPP cohort (Fig. 1D). We then re-analyzed a publicly available psoriasis dataset (*13*) and found 68 *IL36_182* loci among the 500 most over-expressed genes (Fig. 1D). The overlap between the *IL36_182 set* and the genes up-regulated in psoriasis or GPP was highly significant ($P<10^{-30}$ for both diseases), but the same did not apply to the gene set

induced by IL-4 (P>0.03 for both GPP and psoriasis), a Th2 cytokine that was analyzed as a negative control (fig. S1).

To complement the above observations, we also examined the overlap between IL-36 target genes and psoriasis susceptibility intervals previously detected in genome-wide association studies (GWAS). We found that the *IL36_182 set* was significantly enriched among the genes that lie within psoriasis associated regions (P=9.1x10^{-4}), but not among those that map to unrelated susceptibility loci, linked to autism or schizophrenia (P>0.05 for both diseases) (Fig. 1E). Thus, our analyses identified a clear IL-36 gene signature in GPP and psoriasis.

IL-36 signature genes cluster to pathways implicated in the pathogenesis of psoriasis

To further explore the biological significance of our differential expression findings, we undertook a gene set enrichment analysis of the IL-36α, IL-36β and IL-36γ transcriptomes. This demonstrated a marked over-representation of inflammatory processes implicated in the pathogenesis of psoriasis. In fact, the pathway showing the most significant enrichment in our dataset was *Role of IL-17A in psoriasis*, with FDR<10^{-10} observed for all three cytokines. In keeping with this result, a re-analysis of a published IL-17 transcriptome (*14*) revealed that 26/46 (56%) of the genes that are up-regulated by IL-17 in keratinocytes are part of the *IL36_182 set* (table S3).

Very significant FDRs were also detected for pathways related to the infiltration of inflammatory elements into lesional skin (*Granulocyte adhesion and diapedesis* and *Agranulocyte adhesion and diapedesis*; all FDR<10^{-6}) and the activation of p38 MAPK signaling (FDR≤10^{-5}) (Fig. 2A, table S4). Importantly, there was a marked overlap between the processes

driven by IL-36α, -β and γ, with 45 pathways enriched in all three transcriptomes ($P<10^{-30}$ for all pairwise comparisons) (Fig. 2B).

We next extended our gene set enrichment analysis to the disease datasets. As expected, we found a substantial overlap between the pathways that were driven by IL-36 and those that were enriched in the GPP transcriptome ($P= 9.4x10^{-31}$; fig. S2). We then examined the psoriasis dataset, where we identified 71 enriched pathways (FDR<0.05), 37 of which (52%) were shared with the IL-36 transcriptome ($P=5.8x10^{-35}$) (Fig. 2C, table S4). In keeping with the results of the differential expression analysis, we found that the genes underlying these enrichment signals were all part of the *IL36_182 set* (table S4).

To validate the effects of IL-36 cytokines on genes that are relevant to the pathogenesis of psoriasis, we stimulated keratinocyte cultures obtained from additional healthy donors (n=3). We focused our attention on a representative set of loci (highlighted in red font in Fig. 1C) that were markedly up-regulated in psoriatic skin and mapped to pathways that were enriched in the disease transcriptome. These included *IL36G, S100A7* and *LCN2* (*Role of IL-17A signaling in psoriasis*), *CCL20, IL8* and *MMP9* (*Granulocyte/agranulocyte adhesion and diapedesis*) as well as *IRAK2* and *PLA2G4D* (*p38 MAPK signaling*). Real-time PCR analyses confirmed that these genes were all strongly up-regulated by IL-36α, IL-36β and IL-36γ (Fig. 3A). The induction was especially robust for *S100A7* (all fold changes>50) and *IL36G* (fold changes>8.5). The latter result was also validated at the protein level (Fig. 3B) suggesting the presence of a feed-forward loop that sustains IL-36 expression in inflamed skin. , These results indicate that IL-36 cytokines (produced upon infection or trauma (fig. S3)) amplify keratinocyte inflammatory responses by up-regulating their own expression and that of molecules which attract TH17 cells (CCL20) and directly or indirectly potentiate IL-17 signaling (S100A7, LCN2, PLA2G4D) (fig. S4).

In-vivo IL-36 receptor blockade improves the phenotypic appearance of murine psoriasiform dermatitis

Having demonstrated that IL-36 cytokines drive immune pathways that are critical to the pathogenesis of psoriasis, we proceeded to assess the anti-inflammatory effects of IL-36 signaling inhibition. We first investigated the potential of IL-36 blockade in imiquimod-induced psoriasiform dermatitis, an IL17-mediated mouse model of the disease, which is widely used in preclinical studies (*15, 16*). As expected, we found that imiquimod administration resulted in an inflammatory skin phenotype characterized by increased acanthosis (epidermal thickness) (Fig. 4, A and B), abundant leukocyte infiltration (Fig. 4, C and D), up-regulation of IL-17 producing T cells (Fig. 4E and fig. S5) and IL-17 mRNA (Fig. 4F). These pathological manifestations, however, were substantially mitigated by 48h pre-treatment with an anti-IL36R neutralizing antibody.

First of all, the mice that had received the IL-36R blocking-antibody presented with less severe skin lesions compared to littermates treated with an IgG2a isotype control (Fig. 4A). IL-36 inhibition was also associated with a ~30% reduction in acanthosis, which was accompanied by a comparable decrease in scale thickness (Fig. 4A). This was consistent with the observed up-regulation of *Krt10* (a marker of keratinocyte terminal differentiation) and the reduced expression of *Krt16* (a marker of keratinocyte hyper-proliferation) (Fig. 4B).

Secondly, flow cytometry analyses of skin cell suspensions demonstrated a substantial decrease in neutrophil infiltration in mice treated with the anti-IL36R antibody (Fig. 4C). These animals also showed a substantial decline in the numbers of γδ T cells (Fig. 4D), which was especially pronounced for the IL-17 producing sub-population (Fig. 4E). This decrease, which

was mirrored by a reduced accumulation of IL-17$^+$ αβ T lymphocytes (fig. S5), was especially noteworthy, given the key pathogenic role of IL-17$^+$ γδ T cells in imiquimod-induced dermatitis (*17*).

Finally, and in keeping with the diminished infiltration of inflammatory elements, real-time PCR analysis of lesional skin showed reduced cytokine (*Il36g*, *Il17a*, *Il22*, *Tnf*, *Csf2* and *Csf3*) and chemokine (*Cxcl1*, *Cxcl2*, *Cxcl5*) expression in mice treated with the anti-IL36R antibody (Fig. 4F). Taken together, these results show that *in-vivo* IL-36 signaling inhibition has powerful anti-inflammatory effects on psoriasiform dermatitis.

Ex-vivo IL-36 receptor blockade improves the inflammatory phenotype of human psoriasis skin

To further investigate the therapeutic potential of IL-36 blockade, we investigated a validated *ex-vivo* model of human psoriasis, based on the short-term culture (24h) of paired skin biopsies, obtained from lesional and non-lesional patient skin (*18*). This system reflects the complexity of the disease and effectively recapitulates the pathogenic cross-talk between keratinocytes and immune cells. Here, real-time PCR analysis of cultures obtained from 5 independent patients confirmed the up-regulation of *IL36G* and *IL17A* in lesional skin (Fig. 5A). Importantly, supplementation of the culture medium with recombinant IL-36Ra markedly reduced the over-expression of these cytokines, alongside that of chemokines such as *IL8* and *CCL20* (Fig. 5A). The treatment also reduced the levels of *KRT16*, while increasing *KRT10* expression (Fig. 5A). Parallel ELISA measurements confirmed that IL-36Ra supplementation inhibited the release of IL-17 and IL-36γ into the culture medium (Fig. 5B), thus validating the gene expression results at the protein level.

Finally, immunofluorescence microscopy demonstrated a highly significant (albeit not absolute) reduction in the number of T lymphocytes and dendritic cells in IL-36Ra treated lesional skin (Fig. 5C). Given the decreased expression of chemokines such as CCL20, this is likely to reflect a reduced retention of immune cells within the tissue.

To complement these findings, we sought to investigate the mechanisms underlying the effects of IL-36Ra on psoriatic lesions. We first examined the expression of the IL-36 receptor gene (*IL1RL2*) in disease relevant cell types. While transcript levels were low in normal skin-resident T lymphocytes, *in-vitro* differentiated macrophages and *in-vitro* differentiated dendritic cells, *IL1RL2* was very strongly expressed in keratinocytes (Fig. 5D). This suggests that IL-36Ra treatment mostly affects the latter cell type, where it inhibits IL-36R dependent chemokine and cytokine production. To further explore this model, we examined the impact of IL-36Ra on MAPK signaling, one of the main mediators of IL36-driven cytokine induction. We found that p38 phosphorylation, which was examined as a representative readout of MAPK activation, based on the results of pathway enrichment analysis, was markedly reduced in IL-36Ra treated skin (Fig. 5E). These experiments demonstrate that *ex-vivo* IL-36 inhibition reduces cytokine expression and leukocyte infiltration in psoriatic skin lesions, most likely through an effect on keratinocyte MAPK signaling.

Phenotyping of individuals carrying IL1RL2 knockout mutations supports the safety of IL-36 signaling blockade

Having demonstrated the anti-inflammatory effects of IL-36 signaling inhibition in different experimental systems, we sought to establish whether pharmacological IL-36 blockade would compromise human immune function. We hypothesized that naturally occurring

mutations in *IL1RL2* (the gene encoding the IL-36 signaling receptor) would mimic the effects of IL-36 signaling blockade. We therefore proceeded to ascertain a sample of individuals with homozygous *IL1RL2* loss-of-function alleles.

To maximize the likelihood of identifying subjects carrying *IL1RL2* knockout mutations, we focused our attention on a population displaying high rates of parental relatedness. We therefore queried the exome profiles of 2,162 unrelated British-Pakistani individuals recruited by Born in Bradford, a community based project which aims to investigate health outcomes in Bradford, UK (*19*). We found that 28 unrelated study participants (1.3%) harbored low-frequency homozygous changes in *IL1RL2*. Twelve of these individuals carried likely knockout mutations, i.e. homozygous missense alleles classified as damaging by multiple algorithms (table S5). Of note, the most prevalent change was a p.Ala471Thr substitution, found in 9/12 subjects and previously characterized *in-vitro* as a deleterious allele (*20*).

To investigate the impact of these damaging *IL1RL2* changes on immune function, we first reviewed the medical records of the 12 individuals harboring damaging homozygous mutations. One had always lived in the United Kingdom, whereas the other 11 had grown up in rural Pakistan and only left the country as young adults. We found that all were in good health and none had suffered from cardiovascular disease, cancer or immune-mediated conditions. Importantly none of the 12 subjects had a history of recurrent infections. While IL-36 signaling has been implicated in host defense against *M. tuberculosis* (*21*), none of the 12 individuals had contracted tuberculosis, a condition that is endemic in Pakistan (*22*). All showed normal leukocyte counts (table S6).

To further explore these findings, we recalled 6 subjects with deleterious *IL1RL2* mutations, 5 of whom were p.Ala471Thr homozygotes (the sixth was homozygous for a

p.Ile229Met deleterious change). In parallel we also recruited 4 age- sex- and ethnicity matched controls, who carried wild-type *IL1RL2* sequences (Fig. 6, A and B). Clinical examination confirmed that none of the individuals with *IL1RL2* mutations presented with anomalies of the skin, oral mucosa or lymph nodes. Serology tests showed that total IgA, IgG and IgM levels were normal. The same applied to *Aspergillus* IgG levels, which were investigated as IL-36 signaling has been implicated in the response to this particular pathogen (*23*). Finally, we found that all individuals with *IL1RL2* mutations had circulating antibodies against varicella zoster virus and tetanus toxoid, showing that they had mounted an adequate immune response following antigen exposure (table S7).

To investigate in more detail the immune phenotype associated with deleterious *IL1RL2* alleles, we obtained Peripheral Blood Mononuclear Cells (PBMCs) from the six individuals with homozygous knockout mutations and the four control subjects. We first demonstrated that *IL1RL2* and *IL1RAP* (the gene encoding the IL-36R co-receptor) are both expressed in these cells (fig. S6). Next, we stimulated PBMCs with a representative IL-36 cytokine (IL-36α). We found that this treatment failed to up-regulate downstream gene expression in the individuals with *IL1RL2* knockout mutations (Fig. 6, C and D), despite a normal response to a control stimulation with phorbol myristate acetate (PMA)/ionomycin (Fig. 6E). This confirmed that the p.Ala471Thr and p.Ile229Met alleles abolish IL-36 receptor activity and formally demonstrated that the individuals who harbor these mutations lack a functional IL-36 receptor.

Next, we treated PBMCs with Infanrix, a vaccine mix conferring protection against diphtheria, tetanus, pertussis, polio and *Haemophilus influenzae* type B (HiB). We then measured the number of IL-17+ T-lymphocytes, so as to investigate the effect of *IL1RL2* mutations on adaptive responses to bacterial and viral antigens. We found that the induction of T-cell mediated

IL-17 production was comparable in individuals with knockout changes and control subjects (Fig. 6F). We obtained similar results when we stimulated PBMCs with *C. albicans* extracts, showing that the response to this fungal pathogen was also unaffected (Fig. 6F). Finally, we treated PBMCs with poly(I:C), a synthetic dsRNA which has been reported to up-regulate IL-36γ, suggesting an involvement of IL-36 cytokines in anti-viral defenses (*24*). Again, we found that the induction of anti-viral (*IFIT1*, *RSAD2*) and anti-inflammatory gene products (IL-8) was comparable in control and knockout cells (Fig. 6, G and H).

These observations demonstrate that normal immune function is largely preserved in the presence of *IL1RL2* knockout mutations, suggesting that IL-36 blockade is likely to be well tolerated.

Discussion

In this study, we have investigated the IL-36 signaling pathway as a shared pathogenic driver for plaque and pustular psoriasis. We initially examined the genes that are up-regulated by IL-36 treatment in skin keratinocytes. This is the cell type where the expression of the IL-36 receptor is highest and the effects of its stimulation are likely to be most pronounced. In fact, keratinocyte RNA sequencing allowed us to define a robust IL-36 signature, which is readily detectable among the genes that are over-expressed in psoriasis and those that map to disease susceptibility intervals. Our pathway enrichment analyses support a pathogenic model whereby IL-36 dependent genes drive several inter-connected feedback loops which potentiate IL-17 signaling and leukocyte chemotaxis

IL-36 is initially produced upon exposure to viral infection (*24*) and skin trauma, two well-known triggers of psoriasis exacerbation (*3*). IL-36 cytokines can then induce the expression of *IL36G* in an autocrine and paracrine fashion. Our real-time PCR and ELISA data indicate that this is a very robust up-regulation, which is likely to play an important role in the propagation of cutaneous inflammatory responses. Interestingly, the induction of *IL36A* and *IL36B* is much weaker (table S2). This suggests a prominence of IL-36γ in early disease pathogenesis that could potentially be exploited for targeted therapeutic intervention.

IL-36 cytokines also drive the production of molecules that attract neutrophils (e.g. IL-8 and CXCL1), dendritic cells (e.g. CCL20) and T lymphocytes (CXCL10 and CCL20) to sites of inflammation. The strongest effect was demonstrated for S100A7, a potent inducer of T-cell chemotaxis (*25*), which is up-regulated more than 100-fold by IL-36β and IL-36γ. The expression of LCN2, a molecule promoting neutrophil activation and infiltration (*26*), was also markedly increased by IL-36 treatment.

In parallel, we showed that IL-36 can up-regulate the PLA2G4D phospholipase. This enzyme, repeatedly found to be over-expressed in psoriasis (*27*), generates lipid antigens for presentation to CD1a reactive T lymphocytes (*12*). Given that the activation of the latter cells results in the production of IL-17 and IL-22 (*12*), *PLA2G4D* induction is another mechanism whereby IL-36 potentiates TH17 activity. It is also possible that PLA2G4D might, like other molecules that are chronically over-expressed in psoriatic skin (*28*), be recognized as an auto-antigen by HLA-restricted T cells.

This *in-silico* derived pathogenic model is in keeping with the experimental characterization of IL-36γ and PLA2G4D as psoriasis biomarkers (*27, 29*). It is also borne out by

the results of our *ex-vivo* and *in-vivo* studies, where IL-36 blockers reduced IL-17 and IL-36 expression in lesional skin, while also decreasing chemokine induction and T-cell infiltration.

Importantly, the *in-vivo* work was carried out in a single animal model (albeit a widely used one), where IL-36 signaling inhibition could not completely reverse the consequences of imiquimod administration. While this is an important limitation, the anti-inflammatory effects of the treatment were supported by multiple experimental readouts and further validated by *ex-vivo* studies of patient skin, as recommended by the latest guidelines on the analysis of imiquimod-induced dermatitis (*15*). In this context, the incomplete resolution of inflammation may simply reflect the short-term nature of the treatment (7 days for *in-vivo* and 24h for *ex-vivo* assays). Thus, the analysis of experimental systems enabling prolonged IL-36 blockade (e.g. 3D organotypic skin models) may be needed to fully validate the therapeutic potential of IL-36 antagonists.

Real-time PCR analysis of cells obtained from healthy donors showed that *IL1RL2* levels were low in macrophages, dendritic cells and skin resident T lymphocytes. Although *IL1RL2* transcripts were clearly present in neutrophils, the significance of this observation is unclear, given that IL-36R surface expression is not detectable in these cells (*30*). Thus, the anti-inflammatory effects of IL-36 blockade are likely mediated by a disruption of keratinocyte activation, resulting from the binding of IL-36Ra to an abundantly expressed receptor.

Having demonstrated the *in-vivo* and *ex-vivo* efficacy of IL-36 signaling inhibition, we sought to investigate its long-term effects on human immune function. We undertook the phenotyping of individuals carrying *IL1RL2* knockout mutations, as the analysis of loss-of-function alleles can generate invaluable insights into the effects of targeted pharmaceutical

inhibition. For instance, the safety of cholesterol-lowering PCSK9 inhibitors was supported by the observation that *PCSK9* mutations do not affect human health (*31*).

Here, we identified 12 individual harboring *IL1RL2* knockout changes through an analysis of the Born in Bradford cohort (*19*). Although all the mutations present in this relatively small dataset were missense variants, pathogenicity predictions and IL-36 stimulations showed that these were deleterious alleles, which had a profound impact on receptor function. Conversely, clinical examinations did not uncover any evidence for impaired immune function.

Serology tests also failed to detect any anomalies. Of particular interest, *Aspergillus* IgG levels were unremarkable, responses to *C. albicans* stimulation were normal, and none of the individuals with *IL1RL2* knockout mutations presented with Candidiasis, even though IL-36 signaling has been implicated in anti-fungal defenses (*23*). Although the size of our dataset does not allow us to exclude the possibility that IL-36 inhibition may confer vulnerability to rare infections, the data presented here indicates that IL-36 blockade is broadly compatible with normal immune function.

Our observations have important translational implications. Although biologics targeting the IL-23/TH17 axis (especially IL-17 blockers) have transformed the treatment of psoriasis (*32*), disease management can still be problematic in real world settings, where the response to existing cytokine blockers does not match the efficacy levels observed in clinical trials (*33*). The efficiency of biologic therapies is even lower in pustular forms of psoriasis, where the need for an effective treatment is especially pressing (*3*).

In this context, the findings obtained in our model systems support the development of IL-36 blockade as a therapeutic strategy for plaque and pustular psoriasis. While the phenotyping of individuals harboring *IL1RL2* knockout mutations suggests a favorable safety profile for IL-36

antagonists, we cannot exclude the possibility that these subjects may have developed compensatory immune mechanisms in early life. It is also possible that the effects of *IL1RL2* mutations may have been mitigated by genetic modifiers occurring at high frequency within the British Pakistani population. In spite of these limitations, the immune compromised phenotype of individuals suffering from other receptor deficiencies suggests that IL-36 signaling may indeed be dispensable to host defenses and warrants further characterization of IL-36 antagonists in Phase I clinical trials.

Materials and Methods

Study design

The aim of this study was to demonstrate that IL-36 cytokines play a pathogenic role in psoriasis and to validate the IL-36 receptor as a therapeutic target. The first objective was pursued by demonstrating that IL-36 dependent genes identified in keratinocyte RNA-seq experiments are enriched in the psoriasis skin transcriptome and in disease associated loci. The second objective was investigated in *in-vivo* and *ex-vivo* studies of IL-36 receptor blockade. Deep phenotyping of individuals who lack a functional IL-36 receptor (IL-36R) owing to *IL1RL2* knockout mutations, was also undertaken, in order to assess the long-term effects of IL-36 signaling inhibition.

The RNA-seq experiment was carried out in triplicate, to maximize the power of detecting differentially expressed genes at a coverage exceeding $2x10^7$ reads per sample (*34*). *In-vivo* studies were carried out in two groups of five mice, based on previous experience with comparable work (*18*).

For the remaining experiments, sample sizes were dictated by the availability of psoriasis patients and *IL1RL2* knockouts matching our strict inclusion criteria (see Supplementary Materials). mRNA levels, cytokine concentrations and leukocyte numbers were investigated as pre-defined end points. Immunofluorescence and ELISPOT images were quantified in a blinded fashion. No randomization of animal groups was necessary. Although drug administration to imiquimod-treated mice was not blinded, epidermal and scale thickness measurements were assessed in a blinded fashion. Primary data are located in table S10.

Details of sampling and experimental replicates are provided in each figure legend.

Human subjects

This study was performed in accordance with the declaration of Helsinki and was approved by National Research Ethics Service Committee London - Chelsea. Written informed consent was obtained from all participants. Adult patients with psoriasis (n=10, table S8) were ascertained by dermatologists at Guy's and St Thomas' NHS Foundation Trust (see the Supplementary Materials for further details). Discarded skin was obtained from 4 healthy donors undergoing plastic surgery at Guy's and St Thomas' NHS Foundation Trust. Primary keratinocyte cultures were then established as described elsewhere (*35*).

RNA sequencing

Sequences were aligned to the Ensembl Human GRCh37 transcriptome using Bowtie 2-2.2.2 (*36*) and the number of reads that mapped to each gene was quantified with htseq-count (*37*).

Raw expression data for psoriatic skin, the IL-4 and IL-17 keratinocyte transcriptomes were downloaded from the Gene Expression Omnibus database (GSE67785, GSE59275 and GSE12109 respectively) and differential expression was computed using DESeq2 (*38*). Genes that were up-regulated by individual IL-36 cytokines (fold change ≥2, FDR <0.05) were selected for pathway enrichment analysis, which was implemented with Ingenuity Pathway Analysis (QIAGEN Bioinformatics).

In-vivo IL-36 signaling blockade

Mice were bred in the Francis Crick Institute animal facility under specified pathogen-free conditions, where experiments were performed in accordance with institutional guidelines and UK Home Office regulations. Female BALB/cJ mice, 8 weeks old, treated for 5 consecutive days with 5% imiquimod (Meda AB) received daily intra-peritoneal injections (150 μg) of anti-IL36R antibody (M616, Amgen) or rat IgG2a isotype control (Bio X Cell), starting on day -2 of imiquimod administration. Full thickness skin biopsies were collected on day 7 for histological staining, RNA extraction and flow-cytometry (see the Supplementary Materials for further details).

Statistical analyses

The significance of the overlap between the up-regulated genes (or enriched pathways) represented in Venn diagrams was calculated using the hyper-geometric distribution (phyper function in R). Fisher's exact test was used to assess the enrichment of IL-36 signature genes within non-MHC intervals associated with psoriasis, schizophrenia and autism. The NHGRI-EBI

Catalog of published genome-wide association studies (https://www.ebi.ac.uk/gwas, March 2017 update) was queried using the search terms “psoriasis”, “schizophrenia” and “autism”, to generate three lists of disease associated single nucleotide polymorphisms (SNPs). A window of ±50kb was added to the lead SNP at each locus and the genes mapping to the resulting regions were extracted using BioMart (http://www.ensembl.org/biomart).

Fisher’s test and hyper-geometric distribution *P* values were adjusted for multiple testing using the Bonferroni correction. Unless otherwise indicated, experimental data are presented as means +/- SEM. Differences between groups were assessed using the unpaired Student's t test or one-way ANOVA with Dunnett's post-test, as appropriate.

Supplementary Materials

Materials and Methods

Fig. S1. IL-4 target genes are an appropriate negative control for IL-36 enrichment analyses, as there is no overlap between IL-4 and IL-36 signature genes.

Fig. S2. The pathways that underlie the pathogenesis of GPP are enriched within the IL-36 transcriptome.

Fig. S3. Mechanical trauma up-regulates IL-36 production by cultured keratinocytes.

Fig. S4. Proposed pathogenic model illustrating the role of IL-36 as an amplifier of TH17 signaling in psoriasis.

Fig. S5. *In vivo* IL-36 signaling blockade reduces the infiltration of IL-17 producing αβ T-cells in imiquimod-induced psoriasiform dermatitis.

Fig. S6. The genes encoding the IL-36 receptor subunits are expressed in PBMCs.

Fig. S7. Dose and time dependent effects of IL-36 cytokines in primary keratinocytes.

Fig. S8. Complete western blots for figure 5E.

Table S1. RNA-seq coverage statistics.

Table S2. Keratinocyte genes that are up- or down-regulated by IL-36 treatment.

Table S3: Overlap between the *IL36_182 set* and keratinocyte genes up-regulated by IL-17.

Table S4. Results of pathway enrichment analysis.

Table S5. Rare homozygous *IL1RL2* variants observed in the Born in Bradford cohort.

Table S6. Full blood counts of individuals with homozygous *IL1RL2* mutations and controls.

Table S7. Immune serology results.

Table S8. Skin donor demographics.

Table S9. Oligonucleotide primers used in the study.

Table S10. Raw data for *in-vivo* and *ex-vivo* experiments.

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Acknowledgments: We are grateful to the patients and volunteers who took part in this study.

We thank Brigitta Stockinger for her support and the Francis Crick Institute Biological Research Facility for expert breeding and maintenance of the mice, the Flow Cytometry facility, the Histopathology Facility and Ms Ying Li for their assistance with histology

and quantification. We thank Amgen for providing the M616 anti-IL-36R neutralizing antibody. **Funding**: We acknowledge support from the Department of Health via the National Institute for Health Research (NIHR) BioResource Clinical Research Facility and comprehensive Biomedical Research Centre award to Guy's and St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust (guysbrc-2012-1). This paper presents independent research funded by the NIHR under its Collaboration for Applied Health Research and Care (CLAHRC) for Yorkshire and Humber. Core support for Born in Bradford is also provided by the Wellcome Trust (WT101597). This work was funded by a Medical Research Council (MRC) Stratified Medicine award (MR/L011808/1) and was supported in part by a Wellcome Trust Strategic Award (WT 102627) to RCT. SKM is supported by a MRC Clinical Training Fellowship (MR/L001543/1). MC's PhD studentship is funded by the Psoriasis Association. The animal work was conducted at the Francis Crick Institute, which receives its core funding from Cancer Research UK, the MRC and the Wellcome Trust. **Author contributions**: FCa designed the study and supervised the experimental work. SKM carried out the experiments, with input from MP. PDM performed the animal work. MC, ND, IMC, HA, FCi implemented the RNAseq and the downstream data analysis. JNB, CHS, RCT and JW coordinated the recruitment of patients and healthy volunteers. FCa wrote the manuscript with input from SKM, PDM, ND, CHS, FCi and JNB. **Competing interests**: FCa and JNB have received funding from Boehringer-Ingelheim. **Data and materials availability**: The RNA-seq data reported in this paper are archived at the Gene Expression Omnibus Repository (www.ncbi.nlm.nih.gov/geo; accession no. GSE96601).

Figure Legends

Fig. 1. Identification of IL-36 signature genes in human keratinocytes. (**A**) Principal Component Analysis of RNA-seq data from IL-36 treated and untreated keratinocytes. Each dot represents an experimental replicate. (**B**) Heatmap generated from unsupervised clustering of differentially expressed genes (FDR<0.05) transcript levels. (**C**) Volcano plots show the genes that are most strongly induced by IL-36 treatment. Black dots refer to genes showing a log2|fold change| > 1. Labels indicate the ten most significantly up-regulated genes in each dataset (black font) and the loci selected for real-time PCR validation (red font). The Venn diagram displays the intersection of up-regulated genes (FDR<0.05, fold change ≥ 2) across the IL-36α, -β and -γ transcriptomes. The number of up-regulated genes for each dataset is reported in parentheses. (**D**) The Venn diagrams show the intersection of the core IL-36 transcriptome (*IL36_182 set*) with the genes over-expressed in GPP or psoriasis (Ps) skin lesions. The 10 IL-36 signature genes that are most significantly up-regulated in the psoriasis and GPP datasets are listed beside each diagram. (**E**) Bar chart showing the over-representation of the *IL36_182 set* among genes lying within psoriasis susceptibility regions detected in GWAS. Schizophrenia and autism susceptibility intervals were analyzed as negative controls.

Fig. 2. The pathways that underlie the pathogenesis of psoriasis are enriched within the IL-36 transcriptome. (**A**) Pathways displaying the most significant enrichment in the IL-36α, -β and -γ transcriptomes. The red dotted line shows the –log(FDR) level corresponding to an FDR<0.05. (**B**) The Venn diagram shows the intersection of the pathways that are enriched (FDR<0.05) in the three IL-36 transcriptomes. The number of enriched pathways for each dataset is reported in parentheses. (**C**) The Venn diagram shows the intersection of the pathways

that are enriched in psoriasis (Ps) and in the three IL-36 transcriptomes. The 10 most significantly enriched pathways are reported on the right.

Fig. 3. IL-36 cytokines amplify immune responses implicated in psoriasis pathogenesis. (**A**) Validation of IL-36 responsiveness in genes that contribute to the pathogenesis of psoriasis. Primary keratinocytes from 3 independent healthy donors were treated with IL-36α, -β, –γ or vehicle (-). Gene expression was compared in treated vs. untreated keratinocytes, using real-time PCR. Data for each individual donor was normalized to transcript levels in untreated cells and is presented as the mean +/- standard error of the mean (SEM) of measurements obtained in triplicate stimulations. (**B**) Effect of IL-36 cytokines on the secretion of IL-36γ by keratinocytes. IL-36γ release was quantified by ELISA following stimulation of primary keratinocytes with IL-36α, -β, –γ. Data for each individual donor are presented as the mean +/- SEM. *P* values were calculated by Student's t-test. **P* < 0.05, ***P* < 0.01, ****P* ≤ 0.001, *****P* ≤ 0.0001.

Fig. 4. *In-vivo* IL-36 signaling blockade ameliorates imiquimod-induced psoriasiform dermatitis. Mice received an anti-IL36R or isotype-matched control antibody (n=5 per group) for 7 days, starting 2 days prior imiquimod treatment. The experiment was carried out twice with comparable results. (**A**) Representative photographs (left) and haematoxylin/eosin staining (centre) of imiquimod-treated mice receiving either anti-IL36R or an isotype antibody. Untreated skin is shown as a reference. Measurements of epidermis and scale thickness are plotted on the right. Scale bar: 100μm. (**B**) Real-time PCR analysis of skin differentiation (*Krt10*) and hyperproliferation (*Krt16*) markers, in imiquimod-treated mice receiving either an isotype antibody or anti-IL-36R. (**C**) Quantification of neutrophils infiltrating the skin of untreated *vs*. imiquimod-treated mice receiving either an isotype antibody or anti-IL-36R. Representative FACS plots of $CD11b^{+}Ly6G^{+}$ neutrophils gated within live CD45+ cells are also shown. (**D**)

Quantification of dermal γδ T-cells infiltrating the skin of untreated *vs*. imiquimod-treated mice receiving either an isotype antibody or anti-IL-36R. (**E**) Representative FACS plot (left) and quantification (right) of IL-17 and IL-22 producing dermal γδ T-cells gated within live CD45+ cells in the skin of untreated *vs.* imiquimod-treated mice receiving either an isotype antibody or anti-IL-36R. (**F**) Real-time PCR of genes encoding chemokines and cytokines relevant to psoriasis in untreated *vs*. imiquimod-treated mice receiving either an isotype antibody or anti-IL-36R. All data were normalized to the transcript levels observed in untreated mice and are presented as means +/- SEM. *P* values were calculated with one-way ANOVA and Dunnett's post-test. **P* <0.05, ***P* <0.01, ****P* ≤0.001, *****P* ≤0.0001.

Fig. 5. ***Ex-vivo*** **IL-36 receptor blockade reduces inflammation in psoriasis skin lesions.** (**A** and **B**) Lesional (LS) skin biopsies were cultured for 24h in the presence or absence of IL-36Ra. For each patient, a paired non-lesional (NL) sample was analyzed as a reference. Cytokine expression was assessed by real-time PCR (**A**) (n=5 patients, each represented by one line per graph), while IL-17 and IL-36 release was quantified by ELISA (**B**) (n=3 patients, data presented as mean +/- SEM). All *P* values were calculated by one-way Repeated Measures ANOVA with Greenhouse-Geisser correction. (**C**) Representative confocal microscopy images of CD3+ T-cells and CD11c+ dendritic cells in lesional skin samples treated with IL-36Ra (n=3) (left). The number of positive cells per field, quantified in ≥ 5 images per sample, is presented as mean +/- SEM (right). *P* values were calculated by one-way ANOVA followed by Dunnett's post-test. **P* < 0.05, ***P* < 0.01, *****P* ≤ 0.0001. Scale bar: 50μm. The dermal-epidermal junction is highlighted by a dotted line. (**D**) Real-time PCR of *IL1RL2* expression in disease relevant cell types. Each bar shows the median and range of expression values in 2-5 healthy donors. (**E**) Western blotting (left, loading control: hsc70) and densitometric analysis of p-p38/p38 ratios

(right) after IL-36Ra treatment of lesional skin. The data are representative of results from 2 independent skin samples.

Fig. 6. Individuals with homozygous *IL1RL2* mutations show no signs of abnormal immune function. (**A**) Demographics and mutation status of the Born in Bradford participants analyzed in the study. Bold font denotes individuals recalled for further phenotyping. (**B**) Representative chromatograms confirming the genotypes of recalled subjects. The nucleotide changes are highlighted by red asterisks. (**C** and **D**) PBMCs obtained from individuals with *IL1RL2* knockout mutations (n=6) and control (n=4) subjects were treated for 7h with IL-36α. Cytokine production was determined by real-time PCR (**C**, data presented as the mean of triplicate stimulations) and ELISA (**D**, data presented as the mean of duplicate stimulations). (**E** to **H**) PBMCs obtained from individuals with *IL1RL2* knockout mutations and control subjects were treated with PMA/Ionomycin (**E**), Infanrix 5-in-1 vaccine (**F**), *Candida albicans* extracts (**F**) or poly(I:C) (**G** and **H**). Gene expression was measured by real-time PCR (**E** and **G**, data presented as the mean of triplicate stimulations), numbers of IL-17-secreting T cells were quantified by ELISPOT (**F**, data presented as the mean stimulation index observed in triplicate stimulations; example photomicrographs are shown) and cytokine release was determined by ELISA (**H**, data presented as the mean of duplicate stimulations). All *P* values were calculated by Student's t-test. $^{**}P < 0.01$; ns, not significant. KMutH, knockout mutation homozygotes; Ctr, controls.