A Toll-Like Receptor 7, 8, and 9 Antagonist Inhibits Th1 and Th17 Responses and Inflammasome Activation in a Model of IL-23-Induced Psoriasis

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Psoriasis is a chronic inflammatory skin disease that involves the induction of T-helper 1 (Th1) and T-helper 17 (Th17) cell responses and the aberrant expression of proinflammatory cytokines, including IL-1β. Copious evidence suggests that abnormal activation of Toll-like receptors (TLRs) contributes to the initiation and maintenance of psoriasis. We have evaluated an antagonist of TLR7, 8, and 9 as a therapeutic agent in an IL-23-induced psoriasis model in mice. Psoriasis-like skin lesions were induced in C57BL/6 mice by intradermal injection of IL-23 in the ear or dorsum. IL-23-induced increase in ear thickness was inhibited in a dose-dependent manner by treatment with antagonist. Histological examination of ear and dorsal skin tissues demonstrated a reduction in epidermal hyperplasia in mice treated with the antagonist. Treatment with antagonist also reduced the induction of Th1 and Th17 cytokines in skin and/or serum, as well as dermal expression of inflammasome components, NLRP3 and AIM2, and antimicrobial peptides. These results indicate that targeting TLR7, 8, and 9 may provide a way to neutralize multiple inflammatory pathways that are involved in the development of psoriasis. The antagonist has the potential for the treatment of psoriasis and other autoimmune diseases.

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INTRODUCTION

Psoriasis is a chronic inflammatory skin disease characterized by hyperplasia of the epidermis, infiltration of leukocytes in dermis and epidermis, and dilation and growth of blood vessels (Nickoloff and Nestle, 2004). It affects approximately 1–2% of the Caucasian population (Christophers, 2001). Almost 90% of individuals with psoriasis are affected by the most common form of the disease known as psoriasis vulgaris or plaque psoriasis (Nestle *et al.*, 2009). Many patients with psoriasis have other medical issues. The frequency of seronegative arthritis in individuals with psoriasis is approximately 6–42%. Comorbidities observed in patients with psoriasis include type 2 diabetes, metabolic syndrome, obesity, poor quality of life, and depression (Christophers, 2001; Gelfand *et al.*, 2006; Azfar and Gelfand, 2008; Davidovici *et al.*, 2010; Mehta *et al.*, 2010; Nijsten and Stern, 2012).

Psoriatic skin lesions are infiltrated with immune cells, predominantly $CD3^+$ T cells and $CD11c^+$ dendritic cells (DCs; Chamian *et al.*, 2005; Lowes *et al.*, 2005). Infiltrating T-helper 1 (Th1) and T-helper 17 (Th17) cells and activated DCs produce proinflammatory cytokines, such as tumor necrosis factor-α, IFN-γ, IL-17, IL-22, IL-23, IL-12, and IL-1β. These cytokines contribute to the pathogenesis of psoriasis by activating keratinocytes and other resident cutaneous cells, and by inducing abnormal expression of antimicrobial peptides and other defensin genes (Zaba *et al.*, 2009).

Treatment options for psoriasis range from topical agents for mild disease and phototherapy for moderate disease to systemic administration of drugs such as methotrexate, cyclosporine, or retinoids for severe disease. Several monoclonal antibodies and a decoy receptor that target tumor necrosis factor-α are used for the treatment of psoriasis, as are monoclonal antibodies used for targeting the p40 subunit of IL-12 and IL-23. Although these agents effectively alleviate the symptoms, they do not cure the disease and are often associated with underlying toxicities (Laws and Young, 2012). Agents with better therapeutic efficacy are still needed.

Toll-like receptors (TLRs) contribute to the pathogenesis of psoriasis (Hari *et al.*, 2010). TLRs are transmembrane receptors that recognize pathogen-associated molecular patterns and regulate innate immune defense against pathogens. TLR3, 7, 8, and 9 are located in the endosome. TLR3, TLR7, and TLR8 bind RNA, whereas TLR9 binds DNA containing unmethylated CG dinucleotides (Kawai and Akira, 2010). The expression and activity of TLR7, 8, and 9 are regulated by cross talk between these receptors. Deletion of TLR7

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Abbreviations: DC, dendritic cell; Th, T-helper; TLR, Toll-like receptor; pDC, plasmacytoid DC

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attenuates the pathology in mouse models of lupus, whereas deletion of either TLR9 or TLR8 enhances the pathology in these models (Christensen *et al.*, 2006; Demaria *et al.*, 2010; Nickerson *et al.*, 2010).

TLR7 and 9 are expressed on plasmacytoid DCs (pDCs) and B cells, whereas TLR8 is expressed on myeloid DCs. Immune complexes formed by self-nucleic acids and LL-37, an antimicrobial peptide overexpressed in psoriatic lesions, activate both pDCs and myeloid DCs (Lande *et al.*, 2007; Ganguly *et al.*, 2009). The interaction between immune complexes and TLRs induces the production of type I IFN, supports autoreactive T-cell activation, and leads to psoriatic skin lesions, in a self-sustaining feedback loop (Farkas and Kemény, 2012). Imiquimod, a TLR7/8 agonist, exacerbates psoriasis in patients and causes psoriatic lesions in mice. Induction of psoriatic disease in rodents by this TLR agonist is also dependent on the IL-23/Th17 pathway (van der Fits *et al.*, 2009; Patel *et al.*, 2011). Inhibition of TLR-mediated immune responses may provide another approach to the treatment of psoriasis.

Synthetic oligonucleotides that act as antagonists of TLR7 and/or TLR9 exert therapeutic effects in preclinical models of autoimmune diseases such as lupus, experimental autoimmune encephalitis, arthritis, and uveitis (Zeuner *et al.*, 2002; Ho *et al.*, 2003; Dong *et al.*, 2004; Barrat *et al.*, 2007; Fujimoto *et al.*, 2009; Lenert 2010). We have reported the development of a class of synthetic oligonucleotide-based compounds that inhibits TLR7 and TLR9 activation. We designed these compounds based on structure–activity relationship studies of immune-stimulatory oligonucleotides, in which nucleotide and backbone modifications affected their activity (Wang *et al.*, 2009; Yu *et al.*, 2009). Now we have created a class of oligonucleotide-based antagonist compounds that inhibit the induction of cytokine production mediated by TLR7, 8, and 9 in human cell-based assays and in cynomolgus monkeys (Kandimalla *et al.*, 2013).

We have investigated the effect of an antagonist of TLR7, 8, and 9 on the induction of the Th1 and Th17 pathways and inflammasome activation in a mouse model of psoriasis. Treatment with the antagonist regulated inflammatory gene expression and reduced skin lesions. These studies demonstrate the therapeutic potential of the TLR7, 8, and 9 antagonist for the treatment of psoriasis and other inflammatory skin diseases.

RESULTS

Antagonist specifically inhibits TLR7-, 8-, and 9-mediated immune responses

The antagonist specifically inhibits TLR7-, 8-, and 9-, but not TLR3- and 4-, mediated NF-κB activation in HEK293 cells expressing TLRs (Supplementary Figure S1 online). In mice, where TLR8 is not functional (Sarvestani *et al.*, 2012), antagonist inhibited TLR7- and 9-mediated immune responses (Supplementary Figure S2 online).

Antagonist reduces skin lesions in IL-23-induced psoriasis

IL-23 is a key cytokine that contributes to the propagation of the Th17 response and induces psoriasis-like skin lesions in mice (Chan *et al.*, 2006; Zheng *et al.*, 2007); thus, we used an IL-23-induced psoriasis mouse model to evaluate the effect of the TLR antagonist on psoriasis.

Skin lesions were induced in mice by intradermal injection of IL-23 into the left ear. Groups of IL-23-injected mice were treated with increasing doses of antagonist administered subcutaneously at the base of the ear. Measurement of gross ear thickness showed that psoriatic mice had thicker ears than did naive mice. Antagonist-treated mice had less thickening than did those that received control oligo, and, importantly, the antagonist reduced ear thickness in a dose-dependent manner (Figure 1a). Hematoxylin and eosin staining of ear sections showed that psoriatic mice had epidermal hyperplasia and increased lymphocyte infiltration within the dermis when compared with naive mice. Treatment with antagonist decreased epidermal hyperplasia in a dose-dependent manner (Figure 1b and c). Further, hematoxylin and eosin staining appeared to show that lymphocyte infiltrate decreased with the administration of the antagonist, although this was not quantified (Figure 1c). Control oligonucleotide had no effect on ear thickness or skin histology changes.

Treatment with antagonist inhibits Th17 and Th1 responses

IL-23 stimulates Th17 cells to produce IL-17, IL-21, and IL-22 (Di Cesare *et al.*, 2009). Development of psoriatic-like lesions in the IL-23 mouse model requires induction of Th17 cytokines, mainly IL-17A and IL-22 (Zheng *et al.*, 2007; Rizzo *et al.*, 2011). To assess the effect of antagonist on the induction of Th1 and Th17 responses, mice were injected intradermally with IL-23 in the back, as it allows collection of greater quantity of tissue samples than the ear. The duration of the study was shortened to 6 days, as all histological changes were clearly detectable by this time (data not shown). IL-23-injected mice were treated with phosphate-buffered saline or $15\,mg\,kg^{-1}$ of antagonist subcutaneously at a distal site. Tissue staining confirmed that IL-23 injection resulted in epidermal hyperplasia and infiltration of inflammatory cells, whereas antagonist inhibited these alterations. A control oligo was tested in a similar protocol at $15\,mg\,kg^{-1}$ that showed minimal impact on epidermal hyperplasia (Figure 2).

IL-23 injection induced a Th1 response as demonstrated by the increase in serum levels of IL-6 and IL-12. The treatment with antagonist resulted in a decrease in serum levels of IL-6 and IL-12, respectively (Supplementary Figure S3a online). In agreement with serum cytokine levels, skin homogenates also showed an increase in IL-6 levels in psoriatic mice and a 53% reduction in IL-6 levels in animals treated with antagonist (Supplementary Figure S3b online).

The expression of IL-17A, IL-17F, and IL-21 mRNA was higher in skin biopsies of psoriatic mice than in skin biopsies from naive mice. Interestingly, IL-23p19 mRNA was upregulated, suggesting that a feedback mechanism controls its expression. Importantly, treatment with antagonist inhibited the expression of IL-21 by 67%, IL-23p19 by 31%, IL-17A by 40%, and IL-17F by 48% (Figure 3a). Protein levels of IL-17 were elevated at the IL-23 injection site and were reduced by

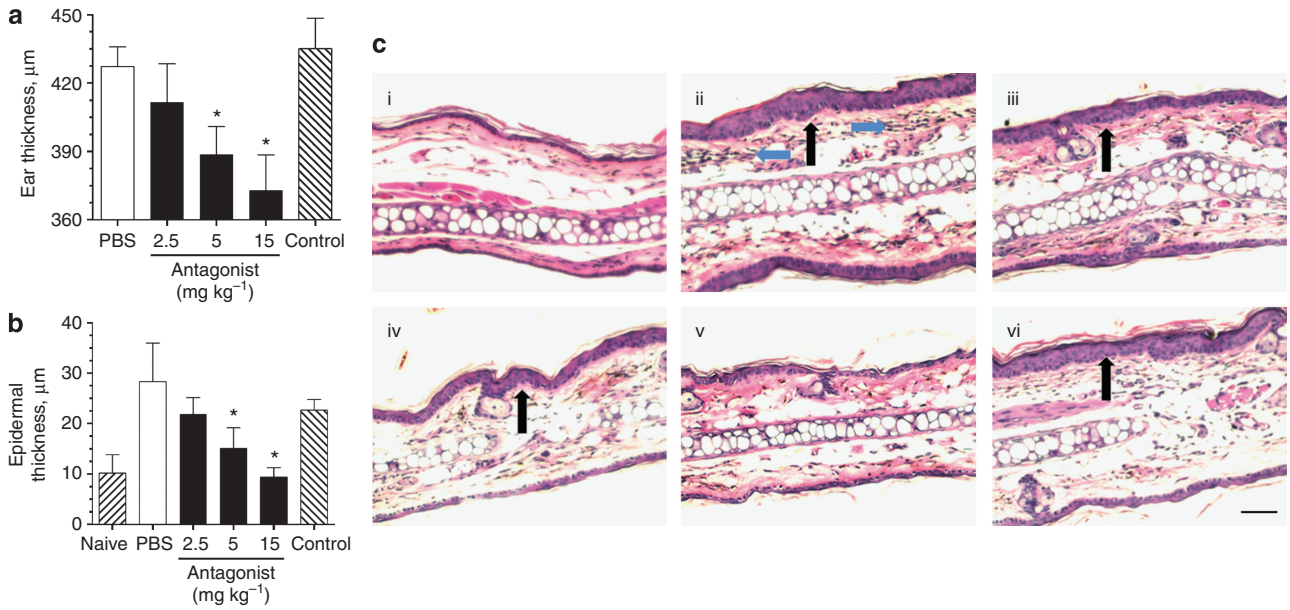

Figure 1. Antagonist inhibits development of IL-23-induced psoriasis in mice. (**a**) Ear thickness of mice at day 16. Each bar represents the mean ear thickness of seven mice. (**b**) Epidermal hyperplasia was quantified by measuring thickness in more than 10 fields, at intervals of 100 μm in each section. (**c**) Ear histopathological changes: (i) naive mice, (ii) IL-23-injected mice treated with phosphate-buffered saline (PBS), (iii) IL-23-injected mice treated with: 2.5, (iv) 5, and (v) 15 mg kg^{-1} of antagonist, and (vi) 15 mg kg^{-1} control oligo. Microscopy photographs display representative changes in the ears for each group. Black arrows show epidermal hyperplasia; blue arrows indicate inflammatory cell infiltration. Bar = 50 μm. *P<0.05 versus PBS group. Error bar depicts SD.

30% upon treatment with the antagonist (Figure 3b). In addition, IL-23 injection induced an increase in IFN-γ expression in the skin (Supplementary Figure S4 online). Treatment with antagonist reduced IFN-γ expression with minimal impact on IL-4 or IL-10 expression.

We observed the upregulation of mRNA for defensin B4, LL-37, S100A4, and S100A7a in the skin of psoriatic mice. Expression of these antimicrobial peptides has been observed in skin lesions of psoriatic patients (Madsen *et al.*, 1991; Nestle *et al.*, 2009; Zibert *et al.*, 2010; Suárez-Fariñas *et al.*, 2012). Treatment with the antagonist resulted in a decrease in mRNA expression of defensin B4 (90%; Figure 4a), S100A4 (23%; Figure 4c), and S100A7a (76% Figure 4d); LL-37 mRNA levels were also lower but the reduction did not reach statistical significance (Figure 4b).

Antagonist inhibits the expression of IL-1β, NLRP3, and AIM2

IL-1β has a central role in cutaneous inflammation in psoriasis (Nestle *et al.*, 2009). Its expression is affected by the activation of inflammasomes, including NLRP3 and AIM2 (Sims *et al.*, 2010). In psoriatic mice, IL-1β protein levels in skin were higher than in naive controls. The mRNA levels for IL-1β, NLRP3, and AIM2 were also higher. Treatment with the antagonist resulted in a 77% decrease in IL-1β protein (Figure 5a) and reduction of AIM2 (34%; Figure 5b), NLRP3 (57%; Figure 5c), and IL-1β mRNA (72%; Figure 5d) present in injected tissue. A control oligo had minimal impact on IL-23-induced gene expression, whereas antagonist reduced IL-23-induced expression of IL-17A, IFN-γ, NLRP3, and IL-1β (Supplementary Figure S5).

Antagonist specifically inhibits TLR7- and TLR9-mediated inflammasome activation in J774 cells

The activation of NLPR3 and of pro-IL-1β is regulated by NF-κB transcription factor-activating stimuli, including TLR7 and 9 (Bauernfeind *et al.*, 2012). We assessed the effects of the antagonist on NLRP3 and IL-1β mRNA expression and IL-1β secretion *in vitro* using mouse J774 cells. Cells were treated with TLR9, TLR7, or lipopolysaccharide (TLR4 agonist) alone or in combination with the antagonist. ATP was added to cells as a stimulus for inflammasome assembly after treatment with the agonist/antagonist combination. Treatment of J774 cells with lipopolysaccharide or with agonist of TLR9 or TLR7 resulted in a significant induction of NLRP3 (Figure 6a) and IL-1β (Figure 6b) mRNA expression. mRNA levels of both NLRP3 and IL-1β were significantly reduced when the antagonist was added with the TLR9 or TLR7 agonist. The induction of NLRP3 and IL-1β mRNA by lipopolysaccharide was not affected by the addition of the antagonist, in agreement with its specificity for TLR7 and TLR9. Secretion of IL-1β in the cell supernatant was dependent on the addition of ATP, in combination with the TLR agonist. However, low levels of IL-1β protein were detected in the supernatant of cells treated with agonist of TLR9 or TLR7, in combination with the antagonist (Figure 6c).

DISCUSSION

We have evaluated an antagonist of TLR7, 8, and 9 in a mouse model of psoriasis induced by IL-23. The results demonstrate that the antagonist hinders the development of skin lesions, reduces the Th1 and Th17 response, and inhibits inflammasome activation.

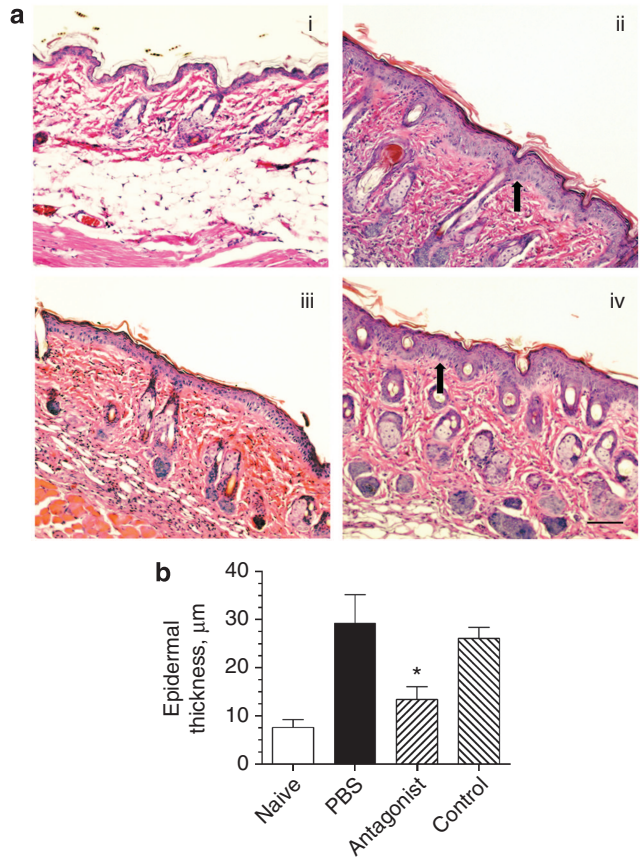

Figure 2. Antagonist inhibits IL-23-induced dorsal skin epidermal hyperplasia. C57BL/6 mice ($n=4$) were injected with either phosphate-buffered saline (PBS) or IL-23 intradermally in the dorsal skin, according to the protocol shown in Supplementary Figure S3 online. PBS, 15 mg kg^{-1} antagonist, or 15 mg kg^{-1} control oligo was injected subcutaneously at a distal site. (**a**) Dorsal skin histology changes: (i) naive mice received daily PBS injection for 4 days (Naive), (ii) IL-23-injected treated with PBS (PBS), (iii) IL-23-injected mice treated with 15 mg kg^{-1} antagonist (Antagonist), and (iv) IL-23-injected mice treated with 15 mg kg^{-1} control oligo (Control). (**b**) Epidermal hyperplasia was quantified by measuring thickness in more than 10 fields, at intervals of 100 μm in each section. Bar = 50 μm. *$P<0.05$ versus PBS group. Error bar depicts SD.

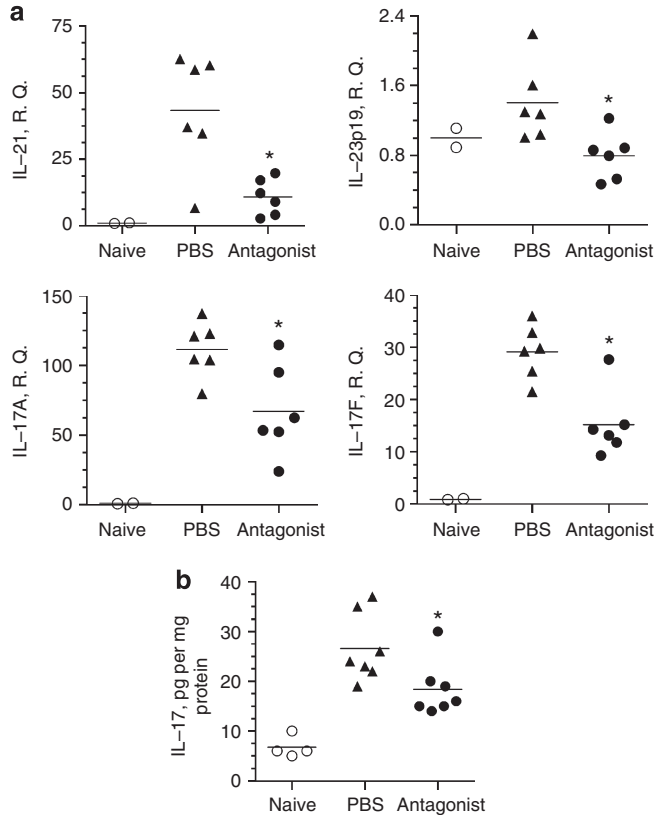

Figure 3. Decreased expression of IL-23 and T-helper 17 (Th17) cytokines in skin samples of antagonist-treated mice. IL-23-injected mice were treated with antagonist or phosphate-buffered saline (PBS). (**a**) Dorsal skin samples were collected and the mRNA expression of IL-21, IL-23, IL-17A, and IL-17F was analyzed by real-time quantitative PCR. (**b**) Dorsal skin samples taken at the site of IL-23 injection were homogenized, centrifuged, and IL-17 content in supernatants was measured by ELISA. *$P<0.05$ versus PBS group. R.Q., relative quantity.

Injection of IL-23 into mice produces some of the clinical features associated with psoriasis. Hyperproliferation of keratinocytes and thickened epidermis with infiltration of mononuclear cells were evident in the IL-23-injected tissues, similar to the histological changes detected in psoriatic patients (Figure 1; Chan *et al.*, 2006; Zheng *et al.*, 2007; Heidrick *et al.*, 2009). Increased mRNA levels for defensin B4, S100A7a, S100A4, and LL-37 detected in the injected skin were similar to those observed in psoriatic patients (Figure 4; Madsen *et al.*, 1991; Nestle *et al.*, 2009; Zibert *et al.*, 2010; Suárez-Fariñas *et al.*, 2012). Treatment of mice with the antagonist reduced skin lesions and expression of the antimicrobial peptides, suggesting that blocking TLR7 and TLR9 has therapeutic potential.

The observed therapeutic effect exerted by the antagonist is associated with the inhibition of multiple cytokines that are important for disease development. The inhibition of IL-6, IFN-γ, IL-12, IL-23, IL-21, and IL-17 demonstrate that the antagonist inhibits both Th1 and Th17 responses (Figure 3, Supplementary Figures S3 and S4 online). The inhibition of T-cell responses hinges on the antagonist's ability to suppress TLR7 and TLR9 activation, and probably affects the induction of IL-12 and IL-6 by cells such as pDCs that express both of these receptors. These cells may contribute to disease development in mice by interacting with immune complexes carrying self-nucleic acids. Indeed, the antimicrobial peptide LL-37 is overexpressed in the IL-23-injected mice and in human psoriatic lesions (Figure 4), and has been shown to bind to DNA and RNA and activate pDCs *in vitro* (Lande *et al.*, 2007; Ganguly *et al.*, 2009). Keratinocytes show increased TLR9 expression when exposed to LL-37 and have increased production of type I IFN after subsequent treatment with a TLR9 agonist (Morizane *et al.*, 2012). In addition, IL-23 injection leads to an increase in TLR7 and 9 expression in skin (data not shown). Furthermore, treatment with the antagonist inhibits development of epidermal hyperplasia in mouse dermis, following injection of LL-37 (data not shown). These data suggest that the antagonist inhibits IL-6 and IL-12

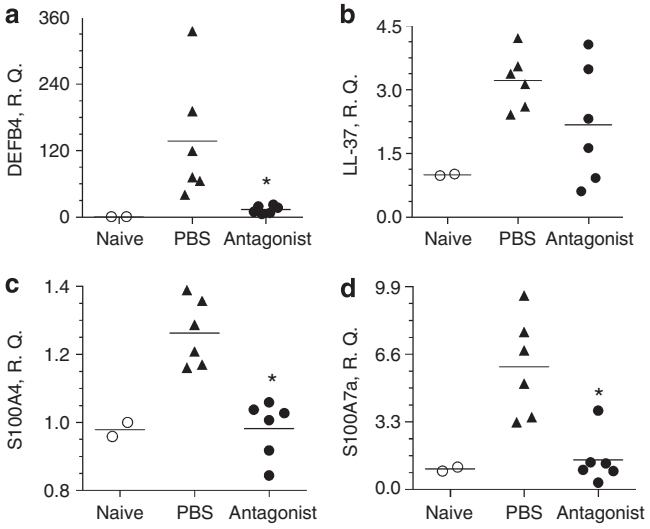

Figure 4. Treatment with the antagonist decreases the expression of antimicrobial peptides mRNA. IL-23-injected mice were treated with antagonist or phosphate-buffered saline (PBS). Dorsal skin samples were collected and the expression of defensin B4 (DEFB4; **a**), LL-37 (**b**), S100A4 (**c**), and S100A7a (**d**) mRNA was analyzed by real-time quantitative PCR. *$P<0.05$ versus PBS group. R.Q., relative quantity.

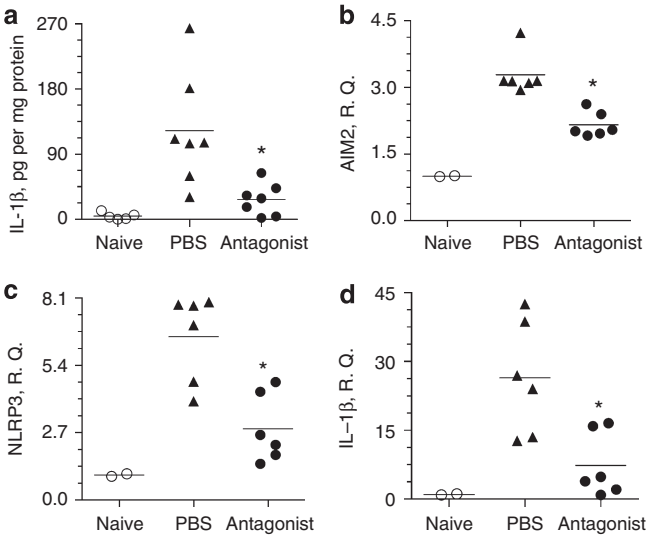

Figure 5. Antagonist inhibits inflammasome activation. (**a**) Dorsal skin homogenates were analyzed for IL-1β levels by ELISA. Values of IL-1β were normalized to total protein. The expression of AIM2 (**b**), NLRP3 (**c**), and IL-1β mRNA (**d**) was analyzed by real-time quantitative PCR. *$P<0.05$ versus phosphate-buffered saline (PBS) group. R.Q., relative quantity.

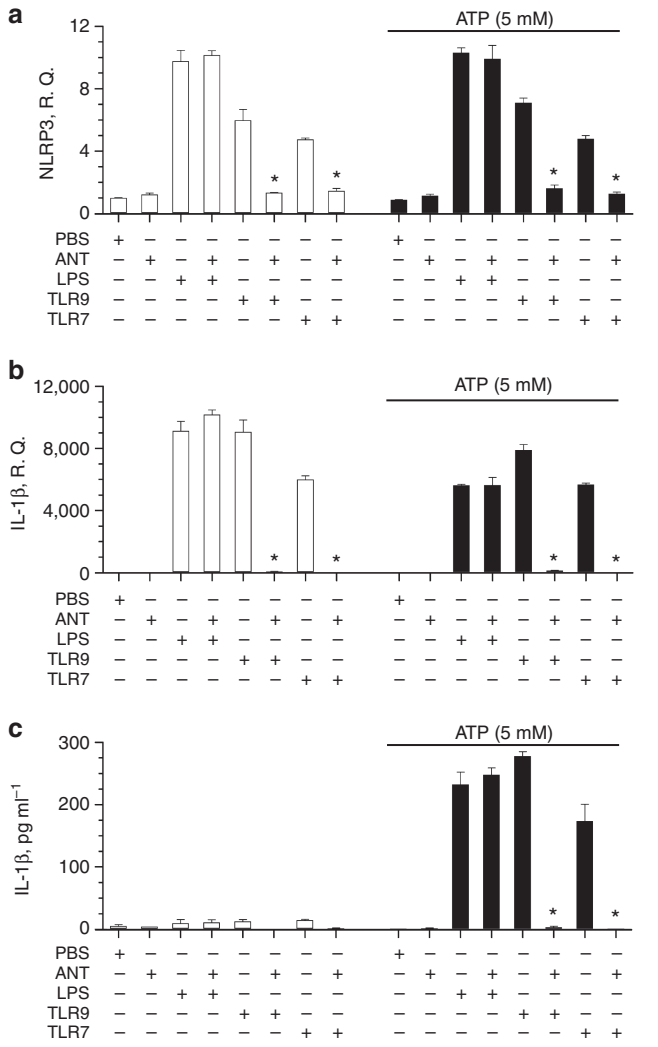

Figure 6. Antagonist specifically inhibits Toll-like receptor 9 (TLR9) and Toll-like receptor 7 (TLR7) agonist-induced inflammasome activation in J774 cells. J774 cells were primed with lipopolysaccharide (LPS; 100 ng ml^{-1}), TLR9 (1 μg ml^{-1}), or TLR7 (50 μg ml^{-1}) agonist in the presence or absence of antagonist (10 μg ml^{-1}) for 4 hours. Agonist-primed or resting (phosphate-buffered saline; PBS) cells were then stimulated with ATP (5 mM) for 1 hour. Cell lysates were processed for RNA isolation and quantitative real-time PCR analysis for NLRP3 (**a**) and IL-1β (**b**) gene expression, whereas culture supernatants were analyzed for IL-1β secretion (**c**). Data are representative of three independent experiments. *$P<0.05$ versus agonist treated. R.Q., relative quantity.

expression by preventing the interaction between these receptors and immune complexes.

Injection of IL-23 in mouse epidermis triggers gene expression signals that involve DCs and keratinocytes, and contributes to the propagation of disease by inducing the production of several proinflammatory cytokines (Di Cesare *et al*, 2009). The upregulation of IL-23 mRNA is observed in the IL-23-injected tissues and may be explained, in part, by a feedback mechanism that involves DCs (Figure 3). This class of antagonists suppresses TLR7- and TLR9-mediated cytokine induction in human pDCs and in mice (Wang *et al.*, 2009; Yu *et al.*, 2009; Kandimalla *et al.*, 2013). It is possible that a similar transcriptional regulatory effect is exerted in mice, following the administration of the antagonist. The inhibition of IL-12, IL-23, and IL-17 is an important aspect of psoriasis therapy. Indeed, antibodies targeting the p19 subunit of IL-23, IL-17, and IL-17R are currently in clinical development for the

treatment of patients with moderate-to-severe plaque psoriasis (Johnson-Huang *et al.*, 2012).

The inhibition of IL-1β, AIM2, and NLRP3 expression by antagonist treatment suggests that the antagonist may extend its inhibitory effects beyond the regulation of Th1 and Th17 pathways to inflammasome activation (Figure 5). Inflammasomes contribute to inflammatory response by regulating IL-1β maturation and secretion (Schroder *et al.*, 2010). IL-1β influences Th17 maturation and cytokine production. Dysregulation of inflammasome expression and IL-1β secretion is associated with inflammatory diseases, including psoriasis (Mills and Dunne, 2009). NLRP3 and AIM2 inflammasomes are expressed in keratinocytes and their expression is upregulated in IL-23-injected mice and in psoriatic lesions. In cultured keratinocytes, IFN-γ induces AIM2 expression (Dombrowski *et al.*, 2011). The decreased expression of AIM2 observed in the treated mice may be due to the inhibition of inflammatory cytokine that ultimately affects AIM2 expression.

Conversely, the expression of NLPR3 and of IL-1β precursor is regulated by NF-κB transcription factor-activating stimuli. NF-κB activation is in itself controlled, in part, by TLR activation (Bauernfeind *et al.*, 2012). The inhibitory effect exerted by the antagonist on TLR7 and TLR9 activation may affect the expression of the inflammasome components, as well as that of IL-1β precursor. This conclusion is supported by *in vitro* studies using mouse J774 cells in which transcription of NLRP3 and IL-1β mRNA was induced by the exposure of the cells to either a TLR7 or a TLR9 agonist and could be controlled by the antagonist with a concomitant inhibition of IL-1β secretion (Figure 6).

The data collected suggest that the antagonist may exert a therapeutic effect by blocking the activation of TLR7 and 9. The impact of TLR8 inhibition on this disease model is unclear, because TLR8 has a limited role in the regulation of innate immune responses in mice. Nonetheless, in view of the cross talk described between these receptors, it is likely that an antagonist capable of blocking these three receptors has significant therapeutic potential in humans.

The antagonist of TLR7, 8, and 9 is a therapeutic agent that has distinct characteristics from other therapeutic approaches. The antagonist blocks TLR activation, thereby affecting the signaling cascade that controls the expression of multiple cytokines. This inhibition ultimately results in the simultaneous blockade of many cellular events. In addition, blockade of TLR activation is not expected to perturb the triggering of downstream signals mediated by other cellular events. Thus, the mechanism of action of the antagonist is significantly different from that of immunosuppressants or monoclonal antibodies utilized for the treatment of psoriasis and other autoimmune diseases. The former induce a global inhibitory effect on the immune system, whereas the latter are limited to the inhibition of a preselected target and are designed to completely neutralize its activity. Thus, although the clinical implications of the differences between the antagonist and other therapeutic agents are not clear at this time, the unique mechanism of action of this agent opens new opportunities for the treatment of several diseases. We have initiated a Phase-2 randomized, placebo-controlled clinical trial in patients with moderate-to-severe psoriasis to evaluate IMO-3100, an antagonist of TLR7 and TLR9.

The therapeutic effects exerted by the antagonist reinforce the notion that the activation of TLR7 and TLR9 is an important component of the multifactorial process involved in the development of psoriasis. The data suggest that by targeting these receptors it is possible to neutralize multiple inflammatory pathways that are intimately involved in the pathology of inflammatory and autoimmune diseases. Thus, the TLR7, 8, and 9 antagonist described herein may prove advantageous over therapies that target individual cytokines for the treatment of autoimmune diseases.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice 6–8 weeks old were purchased from The Jackson Laboratory (Bar Habor, ME). Mice were housed at the Idera Pharmaceuticals, Cambridge, MA, animal facility. All protocols were approved by the Idera Institutional Animal Care and Use Committee.

Induction of disease

Skin lesions were induced in groups of mice ($n=7$) by intradermal injection into the left ear with 0.5 μg of mouse IL-23 (eBioscience, San Diego, CA) in 20 μl phosphate-buffered saline on day 0, 2, 4, 6, 10, 12, and 14. IL-23-injected mice were treated with subcutaneous injection of 2.5, 5, or 15 mg kg^{-1} of antagonist on day 3, 6, 9, 12, and 15. One group of IL-23-injected mice was treated with 15 mg kg^{-1} of a control oligonucleotide and another with phosphate-buffered saline, following the same dosing schedule as for the antagonist. The IL-23-induced increase in ear thickness was monitored using a Peacock dial thickness gauge, and ear samples were collected on day 18.

The induction of lesions on dorsal skin was achieved by daily intradermal injection of IL-23 (1 μg) from day 0 to 3 ($n=7$). IL-23-treated mice were injected subcutaneously at a distal site with 15 mg kg^{-1} of antagonist, control oligo, or with phosphate-buffered saline on days 3, 4, and 5. All mice were killed on day 6, and samples were collected for evaluation (Supplementary Figure S6 online).

Synthesis and purification of TLR7, 8, and 9 antagonist

The antagonist oligonucleotide, 5′-CTATCTGUC*G*TTCTCTGU-3′ (C* is 5-methyl-dC, G* is 7-deaza-dG, underlined nucleotides are 2′-*O*-methyl-ribonucleotides, and all others are 2′-deoxynucleotides), and the control oligonucleotide, 5′-CTATCTCACCTTCTCTGT-3′, were synthesized and purified as described earlier (Wang *et al.*, 2009; Yu *et al.*, 2009). The molecular weight of the antagonist and control was 5,801 and 5,624, respectively, and contained <0.075 EU mg^{-1} of endotoxin by the Limulus assay (Bio-Whittaker, Walkersville, MD).

Measurement of cytokine content

Cytokine levels in serum and skin samples were measured by ELISA. Skin cytokine level was calculated based on protein concentrations measured with Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). All cytokines were measured by ELISA kits purchased from R&D Systems (Minneapolis, MN), except IL-6 and IL-12, which were

measured using paired antibodies purchased from BD Pharmingen (San Jose, CA).

Quantitative real-time PCR analysis

Skin total RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA). RNA (1 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed using StepOnePlus PCR system (Invitrogen), and the relative amount of gene expression was calculated using hypoxanthine-guanine phosphoribosyltransferase as endogenous control. Relative quantity of mRNA was calculated as respect to the mean of naive group, which is set to 1. Primer probe sets were purchased from Invitrogen (details included in Supplementary Material online).

Histology

Formalin-fixed ear samples were processed and stained with hematoxylin and eosin by Mass Histology Service (Worcester, MA). Epithelial hyperplasia and leukocyte infiltration were assessed using an Olympus I × 81 light microscope. Epidermal hyperplasia was quantified by measuring thickness in more than 10 fields, at intervals of 100 μm in each section.

Inflammasome activation in J774 cells

Murine cells J774A.1 (ATCC, Rockville, MD) were cultured in DMEM supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific, Waltham, MA) and antibiotics (penicillin G/streptomycin). Cells were plated overnight in medium, primed with lipopolysaccharide (100 ng ml^{-1}), TLR9 (1 μg ml^{-1}), or TLR7 (50 μg ml^{-1}) agonist in the presence or absence of antagonist (10 μg ml^{-1}) for 4 hours. Agonist-primed or resting cells were stimulated with ATP (5 mM, Invivogen, San Diego, CA) for 1 hour. Culture supernatants were analyzed for IL-1β secretion by ELISA. Total RNA was isolated using the RNeasy Mini kit.

Statistical analysis

Student's unpaired *t*-test was used to determine the statistical significance between means of experimental group and control. $P<0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors are/were employees of Idera Pharmaceuticals and hold stock options.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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