Dual Inhibition of Interleukin-23 and Interleukin-17 Offers Superior Efficacy in Mouse Models of Autoimmunity


ABSTRACT

Therapies targeting either interleukin (IL)-23 or IL-17 have shown promise in treating T helper 17 (Th17)–driven autoimmune diseases. Although IL-23 is a critical driver of IL-17, recognition of nonredundant and independent functions of IL-23 and IL-17 has prompted the notion that dual inhibition of both IL-23 and IL-17 could offer even greater efficacy for treating autoimmune diseases relative to targeting either cytokine alone. To test this hypothesis, we generated selective inhibitors of IL-23 and IL-17 and tested the effect of either treatment alone compared with their combination in vitro and in vivo. In vitro, using a novel culture system of murine Th17 cells and NIH/3T3 fibroblasts, we showed that inhibition of both IL-23 and IL-17 completely suppressed IL-23–dependent IL-22 production from Th17 cells and cooperatively blocked IL-17–dependent IL-6 secretion from the NIH/3T3 cells to levels below either inhibitor alone. In vivo, in the imiquimod induced skin inflammation model, and in the myelin oligodendrocyte glycoprotein peptide–induced experimental autoimmune encephalomyelitis model, we demonstrated that dual inhibition of IL-17 and IL-23 was more efficacious in reducing disease than targeting either cytokine alone. Together, these data support the hypothesis that neutralization of both IL-23 and IL-17 may provide enhanced benefit against Th17 mediated autoimmune diseases and provide a basis for a therapeutic strategy aimed at dual targeting IL-23 and IL-17.

Introduction

The IL-23/IL-17–mediated axis of inflammation is pivotal to the development of T cell–driven autoimmune diseases, including rheumatoid arthritis, inflammatory bowel disease, ankylosing spondylitis, multiple sclerosis (MS), psoriasis, and psoriatic arthritis (Chen et al., 2006; Uyttenhove and Van Snick, 2006; Gaffen et al., 2014). IL-23/IL-17–mediated pathology is associated with a subset of CD4+ effector T cells called T helper 17 (Th17) (Harrington et al., 2006). The pathogenic function of Th17 cells is driven in part by IL-23, a heterodimeric cytokine consisting of a p19 subunit coupled to the p40 subunit that is also shared by IL-12. IL-23 promotes Th17 pathogenesis by driving expression of the hallmark Th17 cytokine, IL-17, as well as other cytokines, such as IL-22 and granulocyte monocyte colony-stimulating factor (GM-CSF), and by suppressing IL-10 (Murphy et al., 2003; Langrish et al., 2005; McKenzie et al., 2006; McGeachy et al., 2007; McGeachy, 2011). IL-17 (or IL-17A) is the founding member of a family of six structurally related cytokines named IL-17A through F (Aggarwal and Gurney, 2002; Weaver et al., 2007). The most closely related IL-17 members, IL-17A and IL-17F, can be secreted as IL-17A/A and IL-17F/F homodimers as well as an IL-17A/F heterodimer (Chang and Dong, 2007). All three are produced by Th17 cells but, arguably, IL-17A/A and IL-17A/F are the more proinflammatory members (Ishigame et al., 2009). In addition to Th17 cells, IL-17 can be produced by CD8+ T cells, γδ T cells, and invariant natural killer T (iNKT) cells and by lymphoid tissue inducer cells (Cua and Tato, 2010). IL-17 triggers
fibroblasts, epithelial cells, endothelial cells, and macrophages to secrete cytokines, chemokines, and growth factors, including IL-6, IL-8, CXCL1, granulocyte colony-stimulating factor, and GM-CSF, which recruit and expand neutrophils as well as monocytes and enhance the local inflammatory milieu (Aggarwal and Gurney, 2002). Acknowledgment of the critical involvement of IL-23 and IL-17 in autoimmune disorders has motivated the discovery of therapeutics for targeting IL-23 and IL-17 pathways.

Many pharmaceutical companies have now developed antibodies against IL-23 or IL-17 that have been largely successful in clinical trials, but they have also presented some unexpected results as well. Antibodies against the common p40 subunit shared by IL-12 and IL-23 and IL-23–specific antibodies were remarkably efficacious in psoriasis patients and showed promising results in Crohn’s disease. However, in MS patients, the trials with anti-p40 antibodies failed (Segal et al., 2008; Longbrake and Racke, 2009; Sandborn et al., 2012; Kimball et al., 2013; Papp et al., 2013a; Gaffen et al., 2014). Agents that block IL-17 were impressively efficacious in psoriasis, psoriatic arthritis, and ankylosing spondylitis, and elicited disease improvement in rheumatoid arthritis (Leonardi et al., 2012; Papp et al., 2012a,b, 2013b; Kimball et al., 2013; Papp et al., 2013a; Gaffen et al., 2014). However, trials with IL-17 inhibitors in Crohn’s disease were prematurely terminated owing to a lack of efficacy and in some cases an exacerbation of the disease (Hueber et al., 2012). These mixed outcomes highlight the complexity of the IL-23–IL-17 pathways and underscore the fact that IL-23 and IL-17 are not entirely redundant targets.

Although IL-23 drives IL-17 production from Th17 cells, IL-23 and IL-17 can function independently of each other. IL-23 induces additional proinflammatory cytokines such as IL-22 and GM-CSF, which have been shown to be more critical than IL-17 to the pathology of certain animal disease models (Liang et al., 2006; Zheng et al., 2007; van der Fits et al., 2009; Codarri et al., 2011; El-Behi et al., 2011). IL-17 can be induced independently of IL-23 by transforming growth factor (TGF)-β and IL-6 in mice (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006) and by T-cell receptor activation in human cells (unpublished observation). Other cell types can also be stimulated to produce IL-17 without IL-23, such as iNKT cells by α-galactosylceramide, and mast cells with complement component 5a, lipopolysaccharide, tumor necrosis factor-α or IgG complexes (Yoshiga et al., 2008; Hueber et al., 2010).

Neutralization of IL-17 alone might block all IL-17 regardless of its source, but other IL-23–dependent cytokines such as IL-22 and GM-CSF would be unaltered and could contribute to disease. Blockade of IL-23 alone would be expected to inhibit all IL-23–dependent cytokines including much of the Th17-produced IL-17. However, not all pathogenic IL-17 would be inhibited since it can be produced without IL-23 in certain situations. Therefore, dual inhibition of IL-23 and IL-17 could neutralize the IL-23–dependent cytokines as well as the effects of IL-23–independent IL-17 leading to a comprehensive blockade of the IL-23/IL-17 pathway and enhanced efficacy in treating disease.

Targeting a more complete neutralization of the IL-23/IL-17 pathway has therefore been of interest. Although agents targeting both IL-17 and IL-23 have been reported, a complete understanding of the potential for improved efficacy with dual inhibition of IL-23 and IL-17 is lacking (Mabry et al., 2010). To investigate if greater efficacy could be achieved with dual blockade, we generated potent and selective inhibitors of IL-23 and IL-17A. These tools enabled us to explore the impact of IL-23 and IL-17 inhibition alone and in combination in vitro and in vivo. We show in a novel in vitro culture system, where the impact of IL-23 and IL-17 blockade could be tested within the same setting, that dual inhibition caused a stronger suppression of downstream cytokines than blockade of either cytokine alone. Furthermore, combination treatment with both IL-23 and IL-17 inhibitors in vivo offered superior efficacy compared with treatment with either inhibitor alone in the imiquimod (IMQ)-induced skin inflammation and the myelin oligodendrocyte glycoprotein (MOG) peptide–induced experimental autoimmune encephalomyelitis (EAE) mouse models.

Materials and Methods

Mice. BALB/c mice were purchased from either Charles River Laboratories (Wilmington, MA) or Harlan Laboratories (Indianapolis, IN) and C57BL/6 mice were purchased from Charles River, Harlan Laboratories, or The Jackson Laboratories (Bar Harbor, ME). The strain, sex, age and vendor of the mice used for each in vivo protocol is indicated within the description of each procedure below. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee.

Generation of IL-23p19 Mouse Adnectin. The ATI-1221 anti-mouse IL-23p19 adnectin was generated using established methods (Mitchell et al., 2014). Briefly, murine IL-23 (eBioscience, San Diego, CA; chemically biotinylated at Bristol-Myers Squibb (BMS; Princeton, NJ)) was presented to large synthetic libraries of adnectins using murine anti-p40 mAbs (clone C17.8; eBioscience). In this way, the IL-23–specific p19 subunit was displayed for adnectin binding. To generate the pegylated version of the adnectin, ATI-1249, purified ATI-1221, was conjugated with a maleimide derivative of a 40-kDa 2-branched polyethylene glycol moiety (mPEG2-MAL) via standard maleimide chemistry at the penultimate C-terminal cysteine residue according to methods described previously (Mitchell et al., 2014).

Generation of Mouse IL-17RA-Fc Decoy Receptor. Mouse IL17RA-Fc (mIL-17RA-Fc) was overexpressed in transiently transfected HEK293-4E cells. Conditioned medium was loaded to protein A column. Captured protein was eluted by 50 mM sodium glycol moieties (mPEG2-MAL) via standard maleimide chemistry at the penultimate C-terminal cysteine residue according to methods described previously (Mitchell et al., 2014).

Generation of Mouse IL-17 Monoclonal Antibody. BALB/c mice (Charles River Laboratories) were immunized intraperitoneally and subcutaneously four times with recombinant murine (rm) IL-17A (R&D Systems/Bio-Techne, Minneapolis, MN) (2 μg/mouse per injection) in Ribi adjuvant (Sigma-Aldrich, St. Louis, MO). Serum samples were evaluated for reactivity to the immunogen by enzyme-linked immunosorbent assay (ELISA) using rmIL-17 (R&D Systems). The mice received two final boosts of 2 μg/mouse per injection of rmIL17 on days 3 and 2 before fusion. B cells from spleens harvested from mouse #230891 were then fused (ratio of 1:1) with mouse ×3A.Ag6.653 myeloma cells (CRL-1581; American Type Culture Collection, Manassas, VA) by electrofusion (Hybriimmune, ECM 2001; Harvard Apparatus, Holliston, MA). The resulting hybridomas were plated out in flat-bottom 96-well plates (200 μl/well), and incubated for 7 days in hypoxanthine-aminopterin-thymidine (HAT) selection medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 5% BM-Condensed H1 Hybridoma Cloning Supplement (Roche, Atlanta, GA), and 1× HAT) and an

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additional 7 days in 1× sodium hypoxanthine and thymidine selection medium (same medium supplemented with 1× sodium hypoxanthine and thymidine instead of HAT). Hybridoma supernatants were then screened by ELISA for antigen specificity (see below). Antigen-specific clones were subcloned by limiting dilution to achieve stability and monoclonality. Clones of interest were expanded and antibodies were purified from culture supernatant over protein A column. Hybridoma clones were preserved by freezing in 10% DMSO freezing media (Sigma-Aldrich).

ELISA Screening for Antigen-Specific Detection of Anti-mIL-17A mAbs. Recombinant mouse IL-17 (R&D Systems) was coated on a 96-well plate at 2 µg/ml overnight at 4°C (50 µl/well). The plate was blocked with 1× PBS solution with Tween (PBST) containing 1% bovine serum albumin (200 µl/well) for 1 hour at room temperature, then washed three times with 1× PBST. Undiluted hybridoma supernatants were added to each well (50 µl/well). Plates were washed three times with 1× PBST before addition of anti-mouse IgG-Fc–specific horseradish peroxidase–conjugated antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:2000 in 1× PBST with 1% bovine serum albumin (50 µl/well). Plates were incubated with the secondary antibody for 1 hour at room temperature. The plates were washed six times prior to addition of 100 µl/well of ABTS substrate (Moss Inc., Pasadena, MD), and absorbance was read at 450 nm and referenced at 620 nm. Rat anti-mouse IL-17 antibodies (clone 50104 and clone 50101; R&D Systems) were used as positive control in the ELISA assay along with irrelevant rat IgG (Sigma-Aldrich) and mouse IgG (Sigma-Aldrich) as isotype controls.

Mouse IL-23-Dependent Phospho-STAT3 Induction in Kit225 Cells. Kit225 cells were seeded into 96-well plates at 4 × 10⁵ cells/well and were rendered quiescent in the absence of fetal bovine serum and IL-2 for 3 hours at 37°C. For inhibition dose responses for ATI-1221 and ATI-1249, 10 pM mIL-23 (generated at BMS), or 10 pM mIL-23 preincubated with a range of concentrations of ATI-1221 and ATI-1249 antagonist for 1 hour, was applied to the cells and the cells returned to the incubator for 15 minutes at 37°C to allow the phosphorylation of STAT3 (abbreviated as pSTAT3). Each test condition was assayed in duplicate in 96-well plates. Stimulation was used at the plateau, by the use of both the IL-2 and IL-23 in this model, GM-CSF, and IL-6 (eBioscience) along with 20 ng/ml recombinant mouse IL-6 (R&D Systems) and 5 ng/ml rmIL-2 (eBioscience) along with 2 µg/ml anti-IL-4 (clone 11B11; eBioscience) and 2 µg/ml anti–IFN-γ (clone XM16.2; eBioscience) neutralizing antibodies for 4 days. The conditioned media from these cultures were harvested and assayed for IL-17A, IL-22, and IL-6 concentrations by ELISA (R&D Systems). To test the impact of ATI-1221 and mIL-17RA-Fc for inhibiting cytokine responses, each inhibitor at 20 nM concentration was added either alone or in combination during stimulation with rmIL-2 and rmIL-23. One-way analysis of variance (ANOVA) and Bonferroni’s post-test using GraphPad Prism software were performed on cytokine results to test for statistical significance of differences observed.

IL-23-Dependent In Vivo Pharmacodynamic Model. Female C57BL/6 mice (Charles River Laboratories) at 8–9 weeks of age were acclimated for 3 days and were randomly assigned in treatment groups based on body weight. In this model, animals exposed to mIL-2 (eBioscience) and mIL-23 (generated at BMS) increased their serum levels of mIL-17 as a response from polyclonally activated T cells. A priming dose with mIL-2 was administered to all groups (except the control group) 24 hours before mIL-23 administration. When coadministered, mIL-2 and mIL-23 were diluted in PBS for a final 100 µl intraperitoneal administration per mouse. Two hours before the start of the challenge doses of mIL-2 plus mIL-23, a single dose of ATI-1249 formulated in PBS was administered subcutaneously at the doses indicated. Challenge doses of mIL-2 plus mIL-23 were given at 0, 7, and 23 hours. Optimal amounts of mIL-2, when coadministered with mIL-23, were determined experimentally and were used to maintain mIL-17 induction throughout the evaluation period. Thus, at time points 0 hours and 23 hours, 5 µg/mL was used, whereas 10 µg/mL was used at the 7-hour time point. A constant amount of 0.2 µg/mouse of mIL-23 was coadministered at all the indicated time points. After 30 hours the priming dose was experimentally determined to be the highest peak for mIL-17 induction in the absence of anti–IL-23 blockers. Thus, all treatment groups were anesthetized at 30 hours using 2.5% isoflurane inhalation and blood was terminally collected via cardiac puncture. Serum was separated by centrifugation, and serum samples were stored at −80°C analysis. Serum mIL-17 cytokine levels were evaluated by ELISA (R&D Systems), following the manufacturer’s instructions. Serum mIL-22 cytokine levels were evaluated using EMD Millipore (Billerica, MA) Luminox Milliplex kit, following the manufacturer’s instructions. One way ANOVA and Dunnett’s multiple comparison tests using GraphPad Prism software were performed on cytokine results to test for statistical significance of observed differences between treatments.

IL-17-Dependent In Vivo Pharmacodynamic Model. Female BALB/c mice (Harlan Laboratories) at 8–10 weeks of age were dosed subcutaneously with the anti–IL-17 antibody at various doses. Eighteen
hours later, mice were challenged with rmlIL-17A (150 μg/kg) (generated at BMS) or rmlIL-17A/F (150 μg/kg) (R&D Systems) subcutaneously in 0.2 ml of PBS. Two hours later, serum samples were collected and the levels of CXCL were measured by ELISA (R&D Systems). One-way ANOVA and Dunnett’s multiple comparison tests using GraphPad Prism software were performed on cytokine results to test for statistical significance of the differences observed between treatment.

**Mouse Imiquimod-Induced Skin Inflammation Model.** Female BALB/c mice (Harlan Laboratories) at 6–9 weeks of age were randomly assigned to treatment groups (n = 8). Three days before initiation of the study, mice were shaved along their backs (from shoulder to hip) with electric clippers, and a depliation lotion (Nair; Church & Dwight Co., Ewing, NJ) was applied for complete removal of hair. Anesthetized mice (2.5% isoflurane inhalation) received a dose of 62.5 mg/mouse of imiquimid (IMQ Cream 5%); Graceway Pharmaceuticals, Bristol, TN) applied with a wooden applicator to the shaved skin. In the prophylactic study design beginning 18 hours before IMQ treatment, mice received either PBS, anti–IL-17 antibody (20 mg/kg s.c.), ATI-1249 (20 mg/kg s.c.), or a combination of the two therapeutics. Skin thickness was measured daily using a Mitutoyo dial caliper (model no. 2412F; Mitutoyo, Kawasaki, Japan). The dorsal skin was visually assessed for erythema and scaling daily using the following criteria: 0, none; 1, slight; 2, moderate; 3, marked; 4, severe. Skin thickness was calculated as the percent change in thickness from baseline measurement taken before the start of IMQ application for each animal. The results are expressed as mean ± S.E.M. for each group. Data calculations were performed in Microsoft Excel (Redmond, WA), while statistical analysis (one-way ANOVA with Bonferroni’s post-test) and graphics were performed using GraphPad Prism software. After seven applications of IMQ using 2.5% isoflurane inhalation, all treatment groups were anesthetized and blood was collected via cardiac puncture. Serum was separated by centrifugation and samples were stored at −80°C until analysis. Serum cytokine levels were evaluated using Luminex multiplex kits (EMD Millipore) following the manufacturer’s instructions. One-way ANOVA and Bonferroni’s post-test using GraphPad Prism software was performed on cytokine results to test for statistical significance of differences observed between treatment groups.

**Histology for Mouse IMQ-Induced Skin Inflammation Model.** Dorsal skin from five animals per group was collected at necropsy, fixed in 10% neutral buffered formalin, routinely processed to paraffin, and sectioned at 3 μm for H&E and 6 μm for toluidine blue assessment of inflammation. кожи embedded tissues were sectioned at 3 μm for H&E, and 6 μm for toluidine blue staining for severity of inflammation and demyelination. A histologic score of 0 to 5 was assigned separately for each lesion characteristic for all three transverse sections of spinal cord evaluated per animal. A score of 0 indicates no lesions/infiltrate; 1, minimal; 2, mild; 3, moderate; 4, marked; and 5, severe. Average scores of inflammation and demyelination were calculated and reported.

**Results**

The Anti-Murine IL-23–Specific Adnectins ATI-1221 and ATI-1249 Potently Inhibit IL-23–Dependent Responses In Vitro. To selectively and specifically neutralize murine IL-23, a murine IL-23p19–specific adnectin was generated using mRNA display (Roberts and Szostak, 1997). An adnectin is a recombinant protein derived from the tenth type III domain of fibronectin that has been modified to bind specifically to a selected target. The functional potency of the adnectins was evaluated for inhibition of murine IL-23–dependent pSTAT3 signaling in the human cell line Kit225. As a result of the 70% homology between mouse and human IL-23, stimulation of Kit225 cells with murine IL-23 induced a robust 8- to 9-fold induction of pSTAT3 above background (Oppmann et al., 2000). Using this assay we obtained a series of mIL-23 binding adnectins. To improve the affinity of these molecules against mIL-23, an optimization-selection was carried out using increased selective pressure, including lowering target concentration. From this optimization process, ATI-1221 was identified as a potent IL-23–specific inhibitor with subnanomolar binding affinity to IL-23 (Supplemental Table 1). In the Kit225 assay, ATI-1221 dose dependently inhibited the IL-23–induced activation of pSTAT3 with an IC<sub>50</sub> of 1.5 nM (Fig. 1, A and B).

To test ATI-1221 for activity against IL-23–dependent responses in primary cells, an assay was established for inducing IL-23–driven cytokine secretion from differentiated mouse Th17 cells. T cells were polarized to a Th17 phenotype by incubation with TGF-β1 and IL-6 for 6 days. The Th17 cells were harvested and recultured with IL-2 and IL-23 to induce IL-17A and IL-22. The addition of IL-2 was required to maintain cell viability and enable robust cytokine production but did not strongly induce IL-17A or IL-22 by itself. Addition of IL-23 to IL-2 typically caused a 2- to 3-fold induction of IL-17A and at least a 15-fold induction of IL-22. Addition of a range of concentrations of ATI-1221 during stimulation of Th17 cells with IL-2 and IL-23 caused potent inhibition of both IL-23–dependent IL-17A and IL-22, with comparable IC<sub>50</sub> values of 1.6 and 1.9 nM, respectively (Fig. 1, A, C, and D).

To increase the pharmacokinetic properties of ATI-1221, the adnectin was reformatted as a PEGylated molecule and renamed ATI-1249. To ensure that PEGylation did not affect
potency, it was tested for functional activity in vitro in the IL-23–induced pSTAT3 assay in Kit-225 cells and also the primary Th17 cell assay. Similar to the unformatted adnectin, ATI-1249 bound IL-23 with subnanomolar affinity and inhibited pSTAT3 in a dose-dependent manner with an IC50 of 2.6 nM (Supplemental Table 1; Fig. 1, A and B). In the primary Th17 cell assay, ATI-1249 also inhibited IL-23–dependent cytokine secretion from Th17 cells with an IC50 of 1.9 nM for inhibition of IL-17A and 2.3 nM for inhibition of IL-22 (Fig. 1, A, C, and D). Therefore, PEGylation of ATI-1249 did not adversely affect the potency of the anti–IL-23 adnectin. The specificity of ATI-1221 and ATI-1249 for IL-23 over IL-12 was confirmed by demonstrating that ATI-1221 and ATI-1249 had no effect on IL-12–dependent mouse Th1 differentiation and secretion of IFN-γ compared with an anti-p40 antibody (Supplemental Fig. 1). Together these data established ATI-1221 and ATI-1249 as potent and selective inhibitors of IL-23–dependent responses in vitro.

The mIL-17RA-Fc and Anti-Mouse IL-17 Antibody Potently Inhibit IL-17–Dependent CXCL1 In Vitro. IL-17 acts on various cell types, including epithelial cells, endothelial cells, and fibroblasts to induce numerous cytokines and chemokines, such as IL-6, granulocyte colony-stimulating factor, GM-CSF, IL-1β, TGF-β, tumor necrosis factor-α, IL-8, and CXCL1 (Aggarwal and Gurney, 2002; Weaver et al., 2007). To test the impact of IL-17 neutralization on cellular responses in vitro we generated a soluble decoy receptor mouse IL-17RA-Fc as well as a monoclonal antibody against mouse IL-17. To measure the activity and selectivity of mIL-17RA-Fc and the anti-mouse IL-17 antibody for inhibition of IL-17–dependent responses, these agents were tested for inhibition of murine IL-17A/A and IL-17A/F–dependent induction of CXCL1 from NIH/3T3 fibroblast cells. Stimulation of NIH/3T3 cells for 24 hours with mouse IL-17A/A or IL-17A/F elicited a greater than 15- or 200-fold induction of CXCL1, respectively. Treatment with mIL-17RA-Fc caused a dose-dependent inhibition of IL-17A/A–induced activity with an IC50 of 1.8 nM (Fig. 2, A and B), and IL-17A/F–induced activity with an IC50 of 2.6 nM (Fig. 2, A and C). The anti–IL-17 antibody was slightly more potent against IL-17A and IL-17A/F with an IC50 of 0.9 nM and 1.2 nM, respectively (Fig. 2). These data confirmed that both the mIL-17RA-Fc and the anti–IL-17 antibody were potent and selective inhibitors of mouse IL-17A/A and IL-17A/F in vitro.

**Dual Inhibition of IL-23 and IL-17 In Vitro Offers More Complete Suppression of Cytokine Secretion.** To extend our findings with IL-23 and IL-17 inhibitors in distinct IL-23– and IL-17–dependent assays, we sought to test the impact of dual inhibition of IL-23– and IL-17–dependent responses within the same assay system. To test for potential cooperative effects of inhibiting both IL-23 and IL-17 in vitro, a method was developed in which polarized mouse Th17 cells were cocultured with NIH/3T3 cells. In this system, Th17 cells, providing an additional readout of IL-23 function, were tested for inhibition of murine IL-17A/A and IL-17A/F elicited a greater than 15- or 200-fold induction of CXCL1, respectively. Treatment with mIL-17RA-Fc caused a dose-dependent inhibition of IL-17A/A–induced activity with an IC50 of 1.8 nM (Fig. 2, A and B), and IL-17A/F–induced activity with an IC50 of 2.6 nM (Fig. 2, A and C). The anti–IL-17 antibody was slightly more potent against IL-17A and IL-17A/F with an IC50 of 0.9 nM and 1.2 nM, respectively (Fig. 2). These data confirmed that both the mIL-17RA-Fc and the anti–IL-17 antibody were potent and selective inhibitors of mouse IL-17A/A and IL-17A/F in vitro.
In this coculture system, stimulation with IL-2 alone led to an increase in IL-17A and IL-22 consistent with the ability of IL-2 to act as a T-cell survival and proliferation factor promoting increased levels of T-cell cytokines in the culture. Importantly, the low levels of IL-6 produced by the coculture system were also increased with the addition of IL-2, consistent with the increases in T-cell cytokines in this system. Of note, blockade of IL-23 did not affect the modestly increased IL-17A or IL-22–cytokine production driven by IL-2 alone, suggesting that production of these cytokines was not dependent on IL-23 (Fig. 3A) (Liang et al., 2006). Blockade of IL-23 with the anti–IL-23 adnectin ATI-1221 alone caused a greater than 95% inhibition of IL-17A (Fig. 3A) and 92% inhibition of IL-22 (Fig. 3B). Production of IL-22 driven either by IL-2 or IL-2/IL-23 was not affected by the inhibition of IL-17 and was blocked similarly by either the IL-23 adnectin or the combination of the IL-23 adnectin with mIL-17RA-Fc (Fig. 3B). These data suggest minimal or no contribution of the IL-17 neutralization in the combination treatment, which might have been expected considering the exclusive dependence of IL-22 on IL-23 (Liang et al., 2006).

In addition, IL-6 was detected in these cultures at baseline (media alone) and was further increased by the addition of IL-2 or IL-2/IL-23 (Fig. 3C). IL-23 blockade had no effect on IL-6 in the baseline or IL-2-stimulated cultures. The IL-6 levels in cultures with IL-2 alone was modestly but significantly augmented approximately 25% by the addition of IL-23 (Fig. 3C). While the IL-6 levels seem to be very dependent on IL-17 levels detected in the culture, the dramatically increased IL-17 produced in the presence of IL-23 caused only a modest enhancement of IL-6 production from the NIH/3T3 cells. Consistent with this notion, IL-23 neutralization had only a modest effect for blocking IL-6 driven by IL-2/IL-23 but did reduce the IL-6 levels to the level induced by IL-2 alone. However, the amount of IL-6 in the culture was still markedly elevated in the presence of IL-23 blockade. Notably, IL-17 production was not completely eliminated in the presence...
of IL-23 neutralization (Fig. 3A). The level of IL-17 that persisted was actually comparable to the amount observed with IL-2 alone, suggesting that IL-17 was produced independently of IL-23. Clearly, this amount of IL-17 was sufficient to drive robust IL-6 secretion even in the presence of IL-23 neutralization. However, the IL-6 production under all conditions, including baseline, was inhibited by IL-17 blockade, consistent with the hypothesis that IL-6 production in the NIH/3T3 cells was driven by IL-17 expressed either at baseline or further induced by IL-2 and IL-2/IL-23, with an 85% inhibition of IL-6 at baseline, 67% inhibition of IL-6 after IL-2 treatment alone, and 55% inhibition in the IL-2/IL-23 costimulation observed (Fig. 3C). Of note, neutralization of IL-17, however, did not block the levels of IL-6 in the presence of IL-2/IL-23 to the level observed with IL-2 alone. These data suggest that the IL-17 inhibition alone was insufficient to reduce the IL-6 levels that were augmented with IL-23. Together, these results demonstrate that IL-23 blockade alone was ineffective for complete inhibition of IL-17-dependent IL-6 production, and IL-17 blockade alone was also inadequate for total inhibition of IL-23-dependent responses, including production of IL-22.

When both ATI-1221 and mIL-17RA-Fc were added together in cultures stimulated with IL-23 and IL-2, the dual inhibition further reduced IL-6 levels more than 69% (Fig. 3C). This reduction of IL-6 was 37% lower than the level observed in the presence of IL-17 blockade alone and was comparable to the level induced by IL-2 alone (Fig. 3C). The augmented suppression of IL-6 levels suggest that the IL-23 inhibition abolished the IL-23-dependent IL-17 as observed with ATI-1221 alone, and the mIL-17Fc further blocked any residual IL-17 secreted independently of IL-23. This comprehensive neutralization of IL-17 with dual inhibition led to the more profound reduction of IL-6. Despite the fact that the IL-23 adnectin was virtually ineffective for reducing IL-6 levels on its own, a reduction was observed when it was combined with IL-17 neutralization, indicating that better blockade of the IL-17–IL-23 axis could be achieved by dual inhibition. Collectively, these data from the coculture system demonstrate that dual targeting of both IL-23 and IL-17 not only completely inhibited IL-23–dependent IL-22 but also had a more dramatic effect by blocking IL-6 levels as well.

The PEGylated Anti-Murine IL-23–Specific Adnectin ATI-1249 Inhibits IL-23–Dependent Activity In Vivo.

With the aim of ultimately testing the impact of IL-23 and IL-17 dual inhibition in vivo, we validated our inhibitors in IL-23– and IL-17–dependent pharmacodynamic (PD) models. First, we evaluated the activity of the PEGylated anti–IL-23 adnectin ATI-1249 for inhibiting IL-23–dependent responses in vivo. The PD model used to test the functional activity of ATI-1249 against IL-23–dependent responses in vivo involved administering mIL-2 and mIL-23 to mice according to a specific regimen (Fig. 4A). IL-2 was injected alone 24 hours before three consecutive treatments with IL-2 and IL-23 at 0, 7, and 23 hours. Three challenges of IL-2/IL-23 were required to detect robust cytokine levels in the serum. At 30 hours, the mice were bled and serum was processed for measuring IL-17 and IL-22 concentrations by ELISA. Challenge with IL-2 alone or mouse IL-23 alone had minimal effects on cytokine secretion, but the combination of IL-23 with IL-2 resulted in robust production of IL-17A and IL-22 (Fig. 4, B and C). To test the activity of ATI-1249 for inhibition of these IL-23–dependent cytokines, mice were treated with a range of ATI-1249 concentrations 2 hours before the first challenge with IL-2/IL-23 (Fig. 4A). In these mice, a dose response was observed, with maximal reduction of both IL-17

![Fig. 4. Mouse anti–IL-23 adnectin ATI-1249 inhibits IL-23–dependent cytokines in vivo. (A) Experimental design of the IL-23–dependent PD assay, (B) ELISA measurements of IL-17A, and (C) IL-22 in serum collected from mice challenged by intraperitoneal injection with one dose of IL-2 alone and three subsequent doses of IL-2/IL-23 at 24, 31, and 47 hours after the first treatment with IL-2. To test the impact of IL-23 inhibition, mice were dosed by subcutaneous injection with a range of ATI-1249 concentrations 2 hours before the first challenge with IL-2/IL-23. A terminal bleed was performed and serum was processed for analysis at 56 hours. The results are expressed as the mean IL-17 or IL-22 level in serum measured in five mice per group. Error bars represent S.E.M. *P < 0.05 by one-way ANOVA with Dunnett’s multiple comparison test for IL-2/IL-23 versus either IL-2+PBS or the IL-23+PBS controls. #P < 0.05 by one-way ANOVA with Dunnett’s multiple comparison test for ATI-1249–treated groups versus untreated IL-2+IL-23 control.](image)
and IL-22 obtained at 5 mg/kg of ATI-1249, corresponding to 98 and 88% reductions, respectively (Fig. 4, B and C). These data demonstrate that ATI-1249 caused potent inhibition of IL-23–dependent cytokine responses in vivo and suggest that good pharmacodynamic coverage of IL-23 was obtained during the 32 hours of the experiment post-adnectin dosing.

The Mouse Anti–IL-17 Antibody Inhibits IL-17A/A and IL-17A/F–Dependent Activity In Vivo. Next, we tested our anti–IL-17 inhibitors for activity in vivo. Because the anti–IL-17 antibody was slightly more potent against IL-17A/A and IL-17A/F activity than the mIL-17-RA decoy receptor in vitro, we tested the activity of anti–IL-17 antibody for inhibition of IL-17A/A and IL-17A/F responses in vivo. The PD model established for investigating IL-17–dependent responses in vivo involved subcutaneous challenge of mice with mouse IL-17A/A or IL-17A/F followed by blood collection at 2 hours after treatment. Serum was subsequently processed and tested for CXCL1 by ELISA. Mice challenged with either IL-17A/A or IL-17A/F exhibited a robust secretion of CXCL1 into the serum (Fig. 5, A and B). Administering a range of anti–IL-17 antibody concentrations 18 hours before either IL-17A/A or IL-17A/F challenge caused significant inhibition of CXCL1. With IL-17A/A challenge, the anti–IL-17 antibody caused a dose-dependent reduction of CXCL1, in which the highest dose tested, 1.0 mg/kg, blocked 92% of the untreated control (Fig. 5A). The anti–IL-17 antibody was less potent in blocking IL-17A/F–induced CXCL1 in vivo but still significantly inhibited secretion at the highest doses tested, both the 0.2 and 1.0 mg/kg doses showing approximately 50% inhibition of CXCL1 production (Fig. 5B). These data demonstrated that the anti–IL-17 antibody had potent activity against both IL-17A/A and IL-17A/F activity in vivo, with good pharmacodynamic coverage through the 20 hours of the experiment.

Dual Inhibition of Mouse IL-23 and IL-17 Offers Superior Efficacy in the Imiquimod-Induced Skin Inflammation Model. Treatment of mouse skin with IMQ leads to skin inflammation, including increases in skin thickness and scaling, that has been reported to be dependent on IL-17 and IL-23 on the basis of the protection from these symptoms in mice deficient for IL-23p19 and IL-17 receptor (van der Fits et al., 2009). Therefore, we tested whether inhibition of both IL-23 and IL-17 might offer superior efficacy compared with inhibition of either alone in the IMQ skin inflammation model. In this model, IMQ was applied to the skin on backs of shaved mice for 7 consecutive days. Successive IMQ treatments caused a rapid increase in skin thickness in the first 4 days with a greater than 70% change from baseline at day 4 (Fig. 6A). Skin scaling also increased, reaching a score of 3 by day 7 (Fig. 6B). Histologic examination of the skin showed that there were minimal-to-moderate epidermal hyperplasia (acanthosis) and moderate-to-marked mixed-cell infiltrates of the dermis in the back skin of IMQ-induced mice (Fig. 6, C, G, and H).

Preventative dosing of both inhibitors in this model was performed at doses expected to give robust suppression of the target cytokines. On the basis of the nearly maximal activity of this antibody at 1 mg/kg in the IL-17–dependent PD model, complete neutralization of IL-17A would be expected at the high dose (20 mg/kg) used in this model. Similarly, neutralization of IL-23 by 20 mg/kg ATI-1249 would be expected on the basis of the near maximal suppression of IL-23–mediated effects at 5 mg/kg in the IL-23–dependent PD model described earlier. Treatment with the anti–IL-17 antibody 1 day before IMQ application and every other day thereafter had no significant effect on preventing skin thickening or scaling (Fig. 6, A and B). In contrast, treatment with a similar regimen of ATI-1249 caused reduced thickness starting at day 5 and was 38% lower than the vehicle control at day 7 but did not reach statistical significance compared with the vehicle control. However, ATI-1249 did have a significant effect for reducing skin scaling by day 6 (Fig. 6B). Dosed simultaneously, the combination of ATI-1249 and anti–IL-17 antibody caused an effect similar to that of ATI-1249 alone for reducing skin thickening initially and by day 7 had a superior effect. Notably, only the dual treatment induced a statistically significant reduction in skin thickening compared with the vehicle control. A more dramatic effect of the combination dosing was observed on the skin scaling score where no increase in scaling was observed beyond 4 days with combination treatment. Moreover, although treatment with ATI-1249 alone and together with anti–IL-17 antibody caused significant reductions in the scaling score, the combination therapy was still comparatively superior (Fig. 6B). The suboptimal efficacy of the monotherapies and the superiority of the combination treatment were further supported by histologic evaluation of skin collected from treated mice (Fig. 6, C–F). In the presence of ATI-1249 or the anti–IL-17 antibody alone, there was no significant effect on the acanthosis score. The combination treatment, on the other hand, reduced acanthosis 55% compared with the control or either of the monotherapies but did not reach statistical significance (Fig. 6G). For the dermal infiltrate analysis, the group mean scores were reduced 31% by ATI-1249 alone and 38% by anti–IL-17 antibody alone compared with the vehicle control group (Fig. 6H). With the combination treatment, the infiltrates were significantly reduced by 69% compared with vehicle and over 50% compared with either ATI-1249 or anti–IL-17 antibody alone.

Analysis of serum cytokines in IMQ challenged mice showed that several IL-23 and IL-17–associated cytokines were increased, including IL-17A, IL-22, and IL-6 (Fig. 7, A–C). However, owing to the local nature of the inflammation...
in the skin in this model, the overall levels of the cytokines remained low and exhibited considerable interanimal variability. Nonetheless, a reduction of cytokine levels was observed in the serum from treated animals, which were in line with expectations from the in vitro experiments. In mice treated with ATI-1249 alone, the group mean concentration for IL-17A

**Fig. 6.** Dual inhibition of IL-23 and IL-17 offers superior efficacy in the IMQ-induced skin inflammation model. Three days before IMQ challenge mice were anesthetized with isoflurane and their back hair was removed. One day before IMQ application and every other day thereafter animals were dosed subcutaneously with PBS, 20 μg/kg ATI-1249 alone, 20 μg/kg anti–IL-17 antibody alone, or both combined. Imiquimod was applied every day from days 0 to 6. (A) Skin thickness and (B) scaling was measured daily. Results are expressed as the mean values for eight animals per cohort. Error bars represent S.E.M. *P < 0.05 by one-way ANOVA with Bonferroni’s post-test for multiple comparisons for any treatment versus vehicle, (#) anti–IL-17 antibody versus ATI-1249, or (+) anti–IL-17 antibody versus the combination treatment. ***P < 0.001 by one-way ANOVA with Bonferroni’s post-test for multiple comparisons for the combination treatment versus vehicle. At necropsy, back skin was prepared for histology and analyzed for acanthosis, and dermal infiltrate by neutrophils, eosinophils, plasma cells, lymphocytes, and melanocytes. Representative images were scored as follows: (C) PBS—acanthosis, 3; dermal infiltrate, 4; (D) ATI-1249—acanthosis, 2; dermal infiltrate, 2; (E) anti–IL-17 Ab—acanthosis, 2; dermal infiltrate, 3; (F) combination—acanthosis: 1; dermal infiltrate, 1. From five animals in each treatment group the average histology score was calculated for (G) acanthosis and (H) dermal infiltrate. Error bars represent S.E.M. *P < 0.01 between the combination treatment versus PBS by Kruskal-Wallis test with Dunn’s post-test for multiple comparisons.
and IL-22 but not IL-6 was reduced. The anti–IL-17 antibody did not reduce IL-22 or IL-6. However, treatment with the combination of ATI-1249 and the anti–IL-17 antibody caused decreases in both IL-22 and IL-6 serum levels (Fig. 7, B and C). These effects on serum cytokines in this in vivo model corroborate the cooperative effects of the combination treatment observed in vitro. Collectively, data from the analysis of the skin thickness, scaling, histology, and serum cytokine levels all suggest that dual inhibition of IL-23 and IL-17 in the IMQ skin inflammation model offered superior efficacy compared with neutralization of either IL-23 or IL-17 alone.

**Dual Inhibition of Mouse IL-23 and IL-17 Offers Superior Efficacy in the MOG Peptide-Induced EAE Model.** Similar to the IMQ-induced skin inflammation model, the IL-23–IL-17 axis has been shown to be central to inhibition of disease development in models of central nervous system (CNS) inflammation (Hofstetter et al., 2005; Langrish et al., 2005; Chen et al., 2006; Kroenke et al., 2008; Ishigame et al., 2009; Hu et al., 2010; El-Behi et al., 2011; Kap et al., 2011). To test whether dual inhibition of IL-23 and IL-17 offers superior efficacy compared with either inhibitor alone for treatment of CNS inflammation, we tested ATI-1249 and the anti–IL-17 antibody dosed alone or in combination in a MOG peptide–induced EAE model. In this model, 25 mg/kg of each agent was used. On the basis of IL-23 and IL-17 challenge models, the dose of inhibitors used was 5- and 25-fold over the maximal efficacious dose for good pharmacodynamic coverage in mice, respectively.

Induction of EAE in control animals resulted in rapid weight loss starting at day 9 along with onset of clinical symptoms that reached maximum severity by 12–14 days (Fig. 8, A and B). Treatment with ATI-1249 or the anti–IL-17 antibody alone administered just before disease onset at day 7 caused a modest reduction in disease score compared to vehicle-treated animals. Either inhibitor dosed by itself also had a modest effect for preventing weight loss (Fig. 8, A and B). Whereas the protection by the anti–IL-17 antibody was maintained to day 20, protection waned in mice treated with ATI-1249 alone. When both ATI-1249 and the anti–IL-17 antibody were administered together at day 7, the combination treatment caused a more dramatic reduction in the severity of clinical disease and prevented weight loss more significantly than either treatment alone (Fig. 8, A and B).

Histologic examination of the spinal cords from EAE mice treated with ATI-1249 and the anti–IL-17 antibody alone or in combination corroborated the superiority of the dual treatment for preventing disease (Fig. 8, C–F). Dosed alone, ATI-1249 or the anti–IL-17 antibody provided partial protection on the basis of inflammation and demyelination scores (Fig. 8, G and H). In contrast, the combination of ATI-1249 or the anti–IL-17 antibody yielded a more significant effect in reduction of spinal cord inflammation and demyelination scores (Fig. 8, G and H). These data demonstrated that in the MOG peptide–induced EAE model the dual inhibition of IL-23 and IL-17 offered a superior effect compared with either inhibitor alone for reduction of overall disease severity by all measures: clinical scores, body weight loss, spinal cord inflammation, and demyelination.

**Discussion**

The generation of potent and specific neutralizing molecules against mouse IL-23p19 and IL-17 enabled us to probe the impact of IL-23 and IL-17 inhibition alone and in combination and, for the first time, demonstrate a cooperative effect of dual inhibition on cytokine production in vitro and enhanced efficacy in vivo. These findings are remarkable given the established connection between IL-23 and IL-17 that might suggest that these cytokines are redundant (Murphy et al., 2003; Langrish et al., 2005; Lugger et al., 2008). Yet, IL-23 induces additional cytokines, such as IL-22, that have distinct functions (Liang et al., 2006). Consistent with the biology of IL-23 in mice, we showed that committed Th17 cells stimulated with IL-23 secreted robust levels of IL-17A and IL-22. Notably, the secretion of IL-22 is unique to mouse Th17 cells polarized with TGF-β1 and IL-6. In human T cells, the addition of TGF-β has been shown to inhibit IL-22 but drive production of IL-26, which is related to IL-22 and is also induced by IL-23 (Wilson et al., 2007; Manel et al., 2008). Both IL-22 and IL-26 have been associated with human diseases, especially in psoriasis, in which both cytokines are elevated in lesional psoriatic skin (Wilson et al., 2007). In our studies, the production of IL-22 coincident with IL-17A in the mouse Th17 cells afforded two readouts that could be used to measure IL-23 function. Incubation with an unformatted anti–IL-23p19-selective adnectin, AT1-221, as well as a PEGylated form, AT1-249, potently and comparably inhibited both IL-23–dependent cytokines, IL-17A and IL-22. In vivo, dosing ATI-1249 into IL-23–challenged mice also blocked production of IL-17 and IL-22. These data confirmed the in vitro and in vivo activity of these adnectins and the role for IL-23 in driving not only IL-17 but also IL-22.

In a similar way, IL-17 can act independently of IL-23. In mice, IL-17 can be produced in the absence of IL-23 by Th17...
cells during differentiation, as well as by iNKT and mast cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006; Yoshiga et al., 2008; Hueber et al., 2010). To assess the impact of IL-17 blockade, two inhibitors of IL-17 were generated, a mouse decoy receptor, mIL-17RA-Fc, and a monoclonal antibody against murine IL-17A. Both agents

Fig. 8. Dual inhibition of IL-23 and IL-17 offers superior efficacy in the MOG peptide–induced EAE model. Mice were immunized with MOG peptide emulsified with incomplete Freund’s adjuvant and two injections of pertussis toxin. Mice were treated subcutaneously with PBS (every other day), ATI-1249 (25 mg/kg every other day), anti–IL-17 antibody (25 mg/kg once per week), or combination of ATI-1249 and anti–IL-17 antibody (the same regimen used in the monotherapy groups). Dosing was initiated the day before immunization. Superior efficacy is demonstrated with the combination of ATI-1249 and the anti–IL-17 antibody based on (A) clinical scores and (B) body weight. Results are expressed as the mean values for 14 animals per cohort. Error bars represent S.E.M. *P < 0.05 by one-way ANOVA with Bonferroni post-test for multiple comparisons for any treatment versus vehicle, (#) anti–IL-17 antibody versus ATI-1249 (^) ATI-1249 versus the combination treatment or (+) anti–IL-17 antibody versus the combination. Representative images are transverse sections of lumbar spinal cord from mice induced with MOG35–55 EAE, stained with luxol fast blue, and H&E counterstain to demonstrate reduction in inflammation (arrow), active demyelinating lesions (arrow head), and tissue damage (*) comparing treatment with (C) PBS, (D) ATI-1249, (E) anti–IL-17 Ab, and (F) combination therapy. Scale bar, 100 μm. A cumulative score was calculated for (G) inflammation and (H) demyelination for each treatment group. Results are expressed as the mean values for seven animals per cohort. Error bars represent S.E.M. *P < 0.01 between combination treatment compared with PBS by Kruskal-Wallis test with Dunn post-test for multiple comparisons.
blocked IL-17A/A– and IL-17A/F–induced responses in NIH/3T3 cells in vitro. Moreover, in an IL-17–dependent PD model in vivo, dosing the anti–IL-17 antibody into mice before challenge with IL-17A/A and IL-17A/F inhibited CXCL1 production by either cytokine. Together, these data confirmed the activity of our key tool inhibitors for neutralizing IL-17A activity in vitro and in vivo and enabled us to assess the impact of any IL-23–independent functions of IL-17.

Using these inhibitors of IL-23 and IL-17, the relative effects of each agent alone and in combination was tested in a coculture system where exogenous IL-23 drove IL-17 production from Th17 cells, which in turn induced cytokine production from NIH/3T3 cells. Here, we chose to measure IL-6 rather than CXCL1 induction from NIH/3T3 cells on the basis of the demonstrated role for IL-6 in autoimmune disease (Yao et al., 1995; Hwang et al., 2004; Yen et al., 2006; Chiricuzzi and Krueger, 2013). Using this system, we confirmed the key functional role of IL-23 for parallel induction of IL-22 and IL-17 and the key functional role of IL-17 in the induction of IL-6, but we also revealed some critical limitations of neutralizing either IL-23 or IL-17 alone. Blocking IL-23 only partially inhibited IL-17 produced by the cytokine and, therefore, had a modest nonsignificant effect on the IL-6 produced in the system. Conversely, inhibiting IL-17 was more efficacious for blocking IL-6 but did not inhibit IL-22 produced by IL-23. The combination treatment showed good neutralization of IL-22 and, interestingly, greater inhibition of IL-6 than either treatment alone, perhaps reflecting that when the level of IL-17 is decreased by IL-23 blockade, inhibition of the cytokine production by the residual IL-17 is more easily inhibited. Remarkably, even the basal IL-6 produced in the coculture system was extremely sensitive to IL-17 blockade, with a near-complete suppression of IL-6 by the anti–IL-17 antibody. The profound inhibition of proinflammatory cytokines with dual inhibition suggested that this comprehensive blockade might offer enhanced efficacy for treating disease.

To investigate whether dual inhibition of IL-17 and IL-23 might lead to improved efficacy in vivo, we tested the anti–IL-23 adnectin ATI-1249 and the anti–IL-17 antibody in the IMQ-induced skin inflammation model. Mice deficient in IL-23p19 or the IL-17 receptor are protected from IMQ-induced scaling and skin thickening, demonstrating a pivotal role for the IL-23/IL-17 axis in this model (van der Fits et al., 2009). Follow-on studies using pharmacologic inhibitors of IL-17A and IL-17F have shown reduced disease in various EAE models (Hofstetter et al., 2005; Langrish et al., 2005; Chen et al., 2006; Kroenke et al., 2008; Ishigame et al., 2009; Hu et al., 2010; El-Behi et al., 2011; Kap et al., 2011). Consistent

Nevertheless, neutralizing both IL-23 and IL-17 caused a cooperative effect to reduce IMQ-induced pathology, especially in disease scores for scaling and histologic scores for cellular infiltrate. These findings suggest that, although blocking IL-23 alone might significantly inhibit cytokine secretion and downstream responses, the added inhibition of IL-17 leads to a more robust blockade of cytokine, including any from IL-23–independent sources. Notably, the serum IL-6 and IL-22 levels induced in IMQ-challenged animals in the presence of inhibitors of both IL-23 and IL-17 were reduced comparably or more than either inhibitor alone. These in vivo data are remarkably consistent with our in vitro data demonstrating a similar cooperative effect of these inhibitors for reducing IL-6 and IL-22 levels. Taken together, our data confirm the role of IL-23 and IL-17 in IMQ-induced skin inflammation and demonstrate that dual blockade of both IL-23 and IL-17 offers enhanced efficacy against disease.

The involvement of IL-23 and IL-17 in skin inflammation has now been well validated by clinical trials in psoriasis, where treatments with antibodies against either IL-23 or IL-17 demonstrated impressive efficacy in patients. In phase II studies, antibodies against IL-23p19, IL-17, and the IL-17RA receptor subunit, all caused >75% disease clearance (Psoriasis Area and Severity Index (PASI) 75) in 70–80% of patients. Almost half also reached PASI 100 or 100% disease clearance (Papp et al., 2012a,b, 2013a,b; Rich et al., 2013; Gaffen et al., 2014). Our data showing efficacy from targeting IL-23 alone in the IMQ-induced model substantiate the role of IL-23 in both mouse and human skin inflammation. Yet, the lack of efficacy from neutralizing IL-17 alone in the IMQ model highlight potential differences between the animal and human pathology. Antibodies against IL-17A specifically are highly efficacious for treating psoriasis, suggesting a significant role for IL-17A in human but not mouse disease (Papp et al., 2012a,b; Patel et al., 2013; Rich et al., 2013). It is probable that, although IL-23 and IL-17 levels are known to be elevated in the lesional skin of psoriasis patients, the relative involvement of each in the development of the disease in mice and humans is different (Nogales et al., 2008; Johansen et al., 2009; Martin et al., 2013). Because the mouse model does not precisely parallel human disease, the direct applicability of our findings to psoriasis remains uncertain. Nevertheless, the cell types, cytokines, and mechanisms involved in the IMQ model are comparable to those in human disease. Therefore, our data suggest that blocking IL-23 and IL-17 simultaneously might offer superior anti-inflammatory activity against some elements of skin inflammation. Still, the magnitude of the response to all the mechanisms targeting the IL-23/IL-17 pathway in clinical trials raises the question of whether there is room in human psoriasis for increased efficacy through a more comprehensive targeting of these pathways.

Beyond skin pathology, a critical role for both IL-23 and IL-17 has also been described in models of CNS inflammation, leading us to investigate whether dual targeting might offer an enhanced efficacy in the MOG peptide–induced EAE model. Mice that are genetically deficient for IL-23p19 or IL-17, or receive treatment with IL-23p19 or IL-17 neutralizing agents, have shown reduced disease in various EAE models (Hofstetter et al., 2005; Langrish et al., 2005; Chen et al., 2006; Kroenke et al., 2008; Ishigame et al., 2009; Hu et al., 2010; El-Behi et al., 2011; Kap et al., 2011). Consistent
with these data, treatment of MOG peptide–challenged mice with either the anti–IL-23 adnectin alone or the anti–IL-17 antibody alone significantly abrogated disease compared with controls as measured by decreased clinical score, maintenance of body weight, and histology readouts of reduced inflammation and demyelination. Even though each of the inhibitors alone demonstrated a reduction in the disease, the combination treatment dramatically reduced all disease manifestations to levels significantly lower than the suppression by either ATI-1249 or the anti–IL-17 antibody alone. This outcome further supports our assertion that a dual blockade of IL-23 and IL-17 offers better efficacy than blocking either cytokine alone in murine models of CNS inflammation.

Though clinical trials testing neutralizing agents against either IL-23 or IL-17 yielded uniformly positive outcomes in psoriasis patients, the results from these inhibitors in MS patients have been mixed. Despite the universal effect of IL-23 or IL-17 neutralization in reducing EAE, only the IL-17 antibodies have shown early positive data in clinical trials of MS, and trials with agents against IL-12p40 failed to demonstrate any efficacy (Segal et al., 2008; Longbrake and Racke, 2009). These findings may be surprising, given the significant impact of both anti–IL-23p19 and anti–IL-17 antibodies for preventing CNS inflammation in mice, but they highlight again the discrepancies between the mouse models and human disease (Chen et al., 2006; Uyttenhove and Van Snick, 2006). Yet, IL-23 and IL-17 brain expression levels have been associated with MS, in which IL-23p19 is expressed in active MS lesions and IL-17A is overexpressed in patient brain biopsies (Li et al., 2007; Tesmer et al., 2008). As with the IMQ model, the cell types and cytokines involved in the EAE model are comparable to those in human disease. Therefore, our data demonstrating a remarkably suppressive effect of dual IL-23 and IL-17 neutralization of EAE disease may suggest that a similar combination could lead to more promising efficacy in MS relative to the modest or no efficacy seen clinically with the individual agents.

Collectively, our findings demonstrate that comprehensive blockade of both IL-23- and IL-17-dependent cytokines through dual blockade of IL-23 and IL-17 leads to enhanced efficacy for reducing disease in the IMQ skin inflammation and MOG peptide–induced EAE disease models. Comparison of our data with clinical data in psoriasis and MS for anti–IL-23 and anti–IL-17 therapies, as well as the published data in murine models of skin and CNS inflammation, suggests that the balance of the role of IL-17 and IL-23 may be different in animal models compared with human disease. However, assessment of the dual targeting of IL-17 and IL-23 in these murine models does provide proof of principle and suggests that, even given the potential human-to-murine difference, the more complete blockade obtained with dual targeting will provide better efficacy in human disease. These data provide a basis for pursuing a dual targeting strategy for IL-23 and IL-17, either through separate dosing of neutralizing agents or perhaps with a bispecific molecule. The generation of a stable bispecific molecule with specificity for IL-23 and IL-17 has been described and while the function of each IL-23 and IL-17 neutralizing arm was demonstrated in separate assays there was no validation that the combined effect of neutralizing both IL-17 and IL-23 was more efficacious than monotherapies either in vitro or in vivo (Mabry et al., 2010). Our data from both in vitro and in vivo models suggest that a more complete blockade of IL-23- and IL-17–dependent responses can be achieved through dual inhibition and that this profound inhibition of Th17 responses might offer enhanced benefit for treating certain autoimmune disorders.

**Authorship Contributions**

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