A novel class of anti-IL-12p40 antibodies
Potent neutralization via inhibition of IL-12–IL-12Rβ2 and IL-23–IL-23R

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Abbreviations: ADCC, antibody dependent cellular cytotoxicity; CDC, complement dependent cytotoxicity; CDRs, complementarity determining regions; H/D, hydrogen/deuterium; PHA-P, Phytohemagglutinin; SPR, surface plasmon resonance

While current therapeutic antibodies bind to IL-12 and IL-23 and inhibit their binding to IL-12Rβ1, we describe a novel antibody, termed 6F6, that binds to IL-12 and IL-23 and inhibits the interaction of IL-12 and IL-23 with their cognate signaling receptors IL-12Rβ2 and IL23R. This antibody does not affect the natural inhibition of the IL-12/23 pathway by the antagonists monomeric IL-12p40 and IL-12p80 respectively, which suggests that a dual antagonist system is possible. We have mapped the epitope of 6F6 to domain 3 of the p40 chain common to IL-12 and IL-23 and demonstrate that an antibody bound to this epitope is sufficient to inhibit engagement of the signaling receptors. Antibodies with this unique mechanism of inhibition are potent inhibitors of IL-12 induced IFNγ production and IL-23 induced IL-17 production in vitro, and in an in vivo model of psoriasis, treatment with a humanized variant of this antibody, h6F6, reduced the inflammatory response, resulting in decreased epidermal hyperplasia. We believe that this new class of IL-12/23 neutralising antibodies has the potential to provide improved potency and efficacy as anti-inflammatory agents, particularly in diseases characterized by an overproduction of IL-12.

Introduction

The pro-inflammatory cytokines IL-12 and IL-23 share a common IL-12p40 subunit that binds the common IL-12Rβ1 (Fig. 1A).1-4 Signaling specificity is determined by the unique p35 (IL-12) and p19 (IL-23) subunits that bind IL-12Rβ2 and IL-23R respectively.5-7 The monomeric IL-12p40 and IL-12p80 subunits have been shown to be natural antagonists for IL-12 and IL-23 by competing for binding to IL-12Rβ1.8-10

Both IL-12 and IL-23 have been implicated in human autoimmune diseases and inflammatory conditions11 and several therapeutic strategies have been designed to inhibit IL-12 and/or IL-23 activity. An anti-IL-12p40 antibody has been described that is specific for amino acid residues 1–88 of IL-12p40 that specifically inhibits the interaction of IL-12 and IL-23 with IL-12Rβ1 (Fig. 1B).12 Such an antibody (ustekinumab) is marketed for the treatment of plaque psoriasis and has demonstrated impressive efficacy in the clinic.12 Another anti-IL-12p40 antibody (ABT-874) in development has been described in the literature as inhibiting the IL-12/23 interaction with IL-12Rβ1.13 Both antibodies have been efficacious in clinical trials for the treatment of plaque psoriasis,12,14 however ustekinumab failed to meet the primary endpoint of efficacy relative to placebo in patients with Crohn disease15 and multiple sclerosis,16,17 indicating that there is still a need for therapeutics with enhanced efficacy for treatment of these autoimmune diseases.

This study describes antibodies that inhibit the IL-12/23 receptor—ligand complex via a novel mechanism of action involving the selective neutralization of the IL-12/IL-12Rβ2 and the IL-23/IL-23R interaction (Fig. 1C). They differ from previously described antibodies in that they do not neutralize the binding of IL-12/23 to IL-12Rβ1. We demonstrate that antibodies with this novel mechanism of action are potent inhibitors of IL-12 and IL-23 in vitro and in vivo and may provide improved potency over existing therapies. Additionally, these antibodies could have improved efficacy as they do not inhibit monomeric IL-12p40 or IL-12p80, the natural antagonists of IL-12 and IL-23. Lastly, we demonstrate that antibodies of this class are potent in a model of psoriasis.
In receptor-neutralization assays we further demonstrated that c6F6 neutralized the binding of IL-12 to IL-12Rβ2 (Fig. 2A) and the binding of IL-23 to IL-23R (Fig. 2B). This result defined a new class of anti-IL-12/23 antibodies, one that is able to bind to IL-12p40 and specifically inhibit bioactivity associated with the secondary subunits of IL-12 and IL-23. Furthermore, m6F6 and c6F6 showed no neutralization of the binding of IL-12 or IL-23 to IL-12Rβ1 (Fig. 2C and D) or of binding of monomeric IL-12p40 or IL-12p80, to IL-12Rβ1 (Fig. 2E and F).

Using a stably transfected Jurkat cell line overexpressing IL-12Rβ1 (Fig. 3A) and negative for IL-12Rβ2 and IL-23R expression (data not shown), we demonstrated binding of monomeric IL-12p40, IL-12 and IL-23 to the IL-12Rβ1 positive Jurkat cell line (Fig. 3D). In an experiment in which c6F6 and c16E7 were titrated with monomeric IL-12p40 (Fig. 3C), IL-12 (Fig. 3D) or IL-23 (Fig. 3E), c16E7 demonstrated dose-response inhibition of cytokine binding to the IL-12Rβ1-expressing Jurkat cell line. In contrast, c6F6 did not appreciably neutralize binding of IL-12, IL-23 or the monomeric IL-12p40 chain alone to the IL-12Rβ1 positive Jurkat cell line. This further demonstrated that c6F6 does not inhibit the binding of IL-12, IL-23 and monomeric IL-12p40 to IL-12Rβ1.

Because c6F6 can bind to the IL-12p40 subunit without affecting its interaction with the IL-12Rβ1 chain, we investigated whether antibody-cytokine-receptor subunit complexes could form on the cell surface. Complexes of c6F6, but not c16E7, with IL-12p40 (Fig. 3F), IL-12 (Fig. 3G) and IL-23 (Fig. 3H) were identified.

In vitro activity of humanized 6F6. The murine antibody 6F6 was humanized to generate h6F6 and further affinity matured to generate h6F6A. These antibodies, h6F6 and h6F6A, displayed high affinity binding to IL-12 as measured by surface plasmon resonance (SPR). h6F6 and its chimeric parent c6F6 showed significantly more potent neutralization of IL-12 in an NK-92 cell-line-based assay and IL-23 in a mouse splenocyte-based assay than did c16E7 (Fig. 4A and C). In a human PBMC-based assay in which IL-12 and Phytohemagglutinin (PHA-P) induced IFNγ secretion, h6F6 was able to inhibit this response (Fig. 4B).

Epitope characterization. Epitope-mapping studies were undertaken to characterize the binding site of h6F6 on IL-12p40. Initially, hydrogen-deuterium (H/D) exchange mass

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**Figure 1.** Diagrammatic representation of IL-12 and IL-23 receptor-ligand interactions and neutralizing antibody-binding mechanisms. (A) IL-12 binds to IL-12Rβ1 and IL-12Rβ2; IL-23 binds to IL-12Rβ1 and IL-23R; monomeric IL-12p40 and IL-12p80 bind to IL-12Rβ1. (B) Hereetofore described therapeutic antibodies achieve their method of action by inhibiting IL-12 and IL-23 from binding to IL-12Rβ1, but they also inhibit the binding of monomeric IL-12p40 and IL-12p80 to IL-12Rβ1. (C) Antibodies described in this work, by contrast, act via inhibition of binding of IL-12 to IL-12Rβ2 and of IL-23 to IL-23R and do not prevent binding by monomeric IL-12p40 and IL-12p80 to IL-12Rβ1.

**Results**

We generated a panel of antibodies from which two IL-12p40-specific monoclonal antibodies, m16E7 and m6F6 were isolated after screening for binding to IL-12, IL-23 and monomeric IL-12p40. The sequences of the variable heavy and light chain regions were identified and grafted onto human IgG1 and kappa constant regions generating chimeric antibodies. The chimeric version of m16E7 was termed c16E7 and the chimeric version of m6F6 was termed c6F6.

**Specificity of c6F6.** Antibody c6F6 and its parental murine counterpart m6F6 bound equally well to human IL-12, IL-23, monomeric IL-12p40 and IL-12p80 in a dose-dependent manner (Suppl. Fig. 1A–D). These data demonstrate that c6F6 binds to the IL-12p40 subunit of IL-12/23.
The levels of binding of h6F6 to D265A and R266A were dramatically reduced (Fig. 5B) and were further analyzed in an ELISA format where h6F6, c16E7 or IL-12Rβ1 and IL-23R were coated on the plate. The mutant R266A displayed low levels of binding to IL-12Rβ1, IL-23R, h6F6 and c16E7 indicating that mis-folding of the mutant protein had occurred (Fig. 5C). The mutant, D265A, bound poorly to h6F6, thus identifying D265A as an important residue on IL-12p40 to which h6F6 binds. D265A bound c16E7, IL-12Rβ1 and IL-23R at comparable levels to that of IL-23 wild type, indicating that it had folded correctly and was capable of functional binding (Fig. 5C). Based on these results, we proposed that the binding site of h6F6 on IL-12p40 protein is the stretch of residues within the IL-12p40 D3 domain from 253 to 286, which corresponds to the sequence VQVQGKSKREWKKDRVFTDKTSATVICRNATKANASISV (Fig. 5A). Based on these results, targeted mutations in this sequence were introduced into the IL-12p40 subunit of IL-23. Additionally, as h6F6 does not bind to murine IL-12p40, the number of mutations that needed to be screened was narrowed down by including only residues that differ between the two species. The binding level (Response Units) of several of the mutants was appreciably lower than that of IL-23 wild-type, indicating that residues in this region of IL-12p40 contribute to the interaction of IL-12p40 with h6F6 (Fig. 5B). The levels of binding of h6F6 to D265A and R266A were dramatically reduced (Fig. 5B) and were further analyzed in an ELISA format where h6F6, c16E7 or IL-12Rβ1 and IL-23R were coated on the plate. The mutant R266A displayed low levels of binding to IL-12Rβ1, IL-23R, h6F6 and c16E7 indicating that mis-folding of the mutant protein had occurred (Fig. 5C). The mutant, D265A, bound poorly to h6F6, thus identifying D265A as an important residue on IL-12p40 to which h6F6 binds. D265A bound c16E7, IL-12Rβ1 and IL-23R at comparable levels to that of IL-23 wild type, indicating that it had folded correctly and was capable of functional binding (Fig. 5C). Based on these results, we proposed that the binding site of h6F6 on IL-12p40 protein is the stretch of residues within the IL-12p40 D3 domain from
Q253 to C286 that extends from the middle of β-strand 3 to the end of β-strand 5, with a critical interaction occurring at D265. Modeling this interaction revealed an antibody that binds to the IL-12p40 subunit, in close proximity to the p19 (IL-23) or p35 (IL-12) subunits (Fig. 5D).

Amelioration of skin inflammation induced by intradermal IL-23 administration. 16E7 and 6F6 are antibodies with specificity for human and primate IL-12/23, but show no cross-reactivity with the murine cytokines. This creates issues when testing the in vivo efficacy of these antibodies in mice. As antibodies against IL-12p40 have been effective in the treatment of psoriasis, we investigated an IL-23-induced inflammatory murine model that is characterized by epidermal hyperplasia and parakeratosis similar to that of human psoriasis. This model is based on published studies using murine IL-23,21 and because h6F6 is only capable of binding to human and primate IL-12p40, the model was modified to use human IL-23 (which is bound by the murine IL-23R complex as demonstrated in the murine splenocyte assay). We chose not to investigate 16E7 in this model due to its poor neutralization of IL-23 in the cell-based murine splenocyte assay (Fig. 4C).

Intradermal treatment of C57Bl/6J mice with human IL-23 for 6 days induced a dose-dependent localized inflammatory response characterized by erythema and induration (Suppl. Fig. 2A), with histological evidence of epidermal hyperplasia, parakeratosis and localized inflammatory infiltrate. Antibodies were tested for their ability to decrease the inflammatory response at a single dose on the day before cytokine treatment commenced. The h6F6-treated group had a reduced clinical score from day 5 onwards relative to the isotype control-treated group (Fig. 6A). In a therapeutic model, in which antibody was administered 2 days after the first administration of human IL-23, the...
h6F6-treated animals had a reduction of the total clinical score by 50% compared to that of the isotype-treated group after 7 days (Fig. 6B). In both models, h6F6 caused a statistically significant decrease in epidermal thickness (Fig. 6C–E). Additionally, skin sections from h6F6-treated animals showed fewer areas of severe lymphocyte infiltration and more areas showing little or no infiltrate when compared with the isotype control-treated animals (Fig. 6F).

Neutralization of IFN-γ induced by human p40/murine p35 in C57Bl/6j mice. Human IL-12 is only weakly active in inducing IFNγ production by murine splenocytes. A heterochimeric IL-12 molecule [human p40/murine p35], however, was capable of inducing an IFNγ response from murine splenocytes at levels similar to that seen for murine IL-12. C57Bl/6j mice treated with 0.1 mg/kg human p40/murine p35 for five consecutive days displayed a robust serum IFNγ response (Suppl. Fig. 2B). A single dose of h6F6 at 5 mg/kg was sufficient to reduce serum IFNγ in human p40/murine p35-treated mice (0.1 mg/kg for five days) to baseline detectable levels (Fig. 6G). h6F6 was tested for its ability to neutralize the human p40/murine p35 and hence abrogate the IFNγ response at a dose of 5 mg/kg with either a single dose on the first day of cytokine treatment or three doses on alternate days, concurrent with cytokine treatment.

Discussion

The interactions of IL-12 and IL-23 with their cognate receptors form part of a large and complicated regulatory network. At the core of the network are many competing interactions. Both IL-12 and IL-23, in addition to the antagonists monomeric IL-12p40 and IL-12p80, compete for binding to the IL-12Rβ1 receptor. To date, anti-IL-12/23 therapeutic antibodies have successfully been targeted to the common IL-12p40 subunit, to a region of IL-12p40 that is crucial for IL-12Rβ1 binding. While antibodies can act by inhibition of receptor-cytokine interaction, they also bind to their target molecule and effectively clear these cytokines from circulation.22 In this study, we demonstrated IL-12/IL-23 blockade through a novel mechanism, i.e., selective inhibition of IL-12 binding to IL-12Rβ2 and IL-23 binding to IL-23R, and show that this mechanism of inhibition is therapeutically relevant and the antibody has several advantages over current therapies that may prove to be clinically advantageous.

Monomeric IL-12p40 has been shown to compete directly with IL-12 for binding to IL-12Rβ1,23 and neutralizing IL-12p40-specific antibodies that act via inhibition of IL-12/23 binding to IL-12Rβ1 have been characterized. In contrast, an antibody specific for heterodimeric IL-12, but not for its individual subunits, was able to inhibit the binding of IL-12 to IL-12Rβ2.23,24 It has been postulated that the binding of IL-12 to IL-12Rβ2 occurs at the IL-12p35 subunit or the heterodimeric interface.25 Likewise, the binding of IL-23 to IL-23R is postulated to occur at the IL-23p19 subunit or the heterodimeric interface. It was therefore surprising that in receptor-neutralization assays we demonstrated that c6F6, an antibody specific for IL-12p40, neutralized the binding of IL-12 to IL-12Rβ2 and the binding of IL-23 to IL-23R. This result defined a new class of anti-IL-12/23 antibodies, one that neutralizes both IL-12 and IL-23, which may provide a therapeutic advantage over current treatments.
and large excesses of monomeric IL-12p40 and IL-12p80 are present, an antibody that blocks IL-12 and IL-23 activity, but does not interfere with the natural antagonism of IL-12 and IL-23 by the monomeric IL-12p40 and IL-12p80 subunits, could contribute to a dual antagonistic system. The antibody inhibits IL-12- and IL-23-mediated biological activity by direct blockade and additionally, secondary blockade can occur by monomeric IL-12p40 and IL-12p80 acting as natural antagonists. This could lead to enhancement of potency and greater clinical efficacy compared to antibodies that block binding of these natural antagonists to IL-12Rβ1.

It was demonstrated that 6F6 can also bind to IL-12/23 bound to IL-12Rβ1 on the cell surface. When an antibody forms complexes on the surface of cells, as demonstrated for c6F6 in that is able to bind to IL-12p40 and specifically inhibit bioactivity associated with the secondary subunits of IL-12 and IL-23.

Furthermore, 6F6 showed no neutralization of the binding of IL-12 or IL-23 to IL-12Rβ1 or of binding of monomeric IL-12p40 or IL-12p80, natural antagonists of IL-12 and IL-23, to IL-12Rβ1 in either receptor-neutralization experiments or cell-based receptor binding assays. Current antibodies in development targeting IL-12p40 also inhibit binding of monomeric IL-12p40 and IL-12p80 to IL-12Rβ1 and prevent these molecules from acting as natural antagonists of the IL-12 and IL-23 network. In some cases the amount of IL-12p40/80 produced by monocytes can be 5- to 500-fold higher than the amount of heterodimer,26 and has demonstrated antagonist activity. Thus, in a complex network in which IL-12, IL-23,
these experiments, it may trigger antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). As IL-12Rβ1 is widely expressed on activated T cells, NK cells and B cells, it is feasible that, in a disease setting, antibodies with the binding profile of c6F6 could form complexes with IL-12/23 on these activated cells and trigger ADCC or CDC, thereby dampening IL-12- or IL-23-driven inflammation by (i) depleting IL-12- and IL-23-responsive cell populations.

Figure 6. Antibody h6F6 reduces inflammation in an animal model of psoriasis and inhibits IL-12-induced IFNγ production in vivo. (A) h6F6, given one day before the start of cytokine injection, significantly ameliorated the clinical signs of skin inflammation induced by daily intradermal IL-23 injection. Asterisks indicate significant differences between h6F6 treatment and an isotype control antibody (*p < .05; **p < .01; ***p < .001; two way ANOVA, n = 7 per group). (B) h6F6A, given two days after the first cytokine injection, reduced the clinical signs of skin inflammation induced by daily intradermal IL-23 injection. Asterisks indicate significant differences between h6F6 treatment and an isotype control antibody (*p < .05; **p < .01; ***p < .001; two way ANOVA, n = 7 per group). Representative H&E-stained skin sections from isotype control (C) or h6F6 (D) treated animals. Skin from isotype control-treated animals (C) was characterized by epidermal hyperplasia, parakeratotic scale (open arrows) and extensive leukocyte infiltration (solid arrowheads). These characteristics were decreased or absent in h6F6-treated animals (D). (E) h6F6 significantly decreased epidermal hyperplasia induced by IL-23. (p < 0.05, Mann-Whitney U-test, n = 7 per group). (F) Skin sections from h6F6-treated animals had no areas of severe lymphocyte infiltration and more areas showing little or no infiltrate when compared to the isotype control-treated animals. (G) In an IL-12 based animal model, h6F6 abrogated the serum IFNγ response to human p40/murine p35 given i.p. daily for five days at 100 μg/kg (p < 0.005, Mann-Whitney U-test, n = 7 per group).
and (ii) removing a subset of IL-12- and IL-23-producing cell populations.

A humanized version of 6F6 was then examined for potency in inhibiting the IL-12 and IL-23 cellular effects. Surprisingly, this antibody potently neutralized the biological effects of IL-12 above expectation, when compared to conventional antibodies. This may be due to the kinetics of the receptor-cytokine interactions. IL-12Rβ1 is a moderate affinity receptor for IL-12, whereas the affinity of IL-12Rβ2 for IL-12 is low.5 In conducting these experiments, we also produced a Jurkat cell line strongly over-expressing IL-12Rβ2, and no binding of IL-12 to this cell line could be detected by flow cytometry (data not shown). It is thus probable that complex formation of IL-12 with IL-12Rβ2 requires the presence of IL-12Rβ1. In a complex network, targeting the weakest link or interaction (IL-12/II-12Rβ2) with a high affinity antibody appears to be a more potent and efficient way of inhibiting the IL-12R complex compared to targeting the IL-12/II-12Rβ1 interaction.

6F6, both in the form of a chimeric and humanized antibody, also potently inhibited IL-23, indicating that targeting the IL-23/II-23R interaction via this novel mechanism is an effective way of neutralizing IL-23 bioactivity. While strong potency is observed via this mechanism of IL-23 inhibition, the affinity of IL-23 for IL-23R is comparable to that of IL-23 for IL-12Rβ1. Further work is required to determine if there is an improvement in IL-23 potency compared to conventional IL-12p40 antibodies.

Seeking to translate in-vitro potency to in-vivo efficacy we tested the antibody in a murine model of psoriasis driven by direct administration of human IL-23. Similar studies using murine IL-23 were characterised by a TNF-driven inflammatory response, with upregulated IL-19 and IL-24. The IL-20 cytokine signaling pathway was also shown to be critical.21 We have demonstrated that directly targeting IL-23 is sufficient to ameliorate the psoriasis-like response, presumably by inhibition of these downstream effects. In addition, we demonstrated blockade of IL-12 induced IFNγ in a murine model in which a human p40/murine p35 was administered repeatedly. At a 5 mg/kg dose of 6F6 the IFNγ response was reduced to baseline. This confirms that the strong potency of 6F6 against IL-12 translates to in-vivo potency. Further work is required to delineate just how potent this antibody is relative to existing therapies.

Using epitope mapping studies, we modeled the interaction of 6F6 and revealed an antibody that binds to the IL-12p40 subunit, in close proximity to the p19 (IL-23) or p35 (IL-12) subunits. An analysis of the binding sites of various cytokine-receptor complexes has provided insights into the potential binding site for IL-23R on IL-23. This result confirms that the interaction of IL-23 with IL-23R, and likely IL-12 with IL-12Rβ2, mirrors the interaction of IL-2 with IL-2Rβ, IL-6 with IL-6Rβ and G-CSF with G-CSFR.23 We postulate that h6F6 binds to a location on IL-12p40 that prevents engagement of the p19 or p35 subunits, with or without involvement of IL-12p40, with their cognate receptors, IL-23R or IL-12Rβ2.

In summary, our data describe a new class of antibodies specific for IL-12p40, capable of selective inhibition of the binding of IL-12 to IL-12Rβ2 and IL-23 to IL-23R. In the case of IL-12, where the interaction with IL-12Rβ2 is weak, specifically targeting this interaction may prove particularly beneficial. These IL-12Rβ2 and IL-23R inhibitory antibodies differ from existing therapeutic antibodies, in that they do not inhibit the binding of monomeric IL-12p40 and IL-12p80, natural antagonists in this network, to IL-12Rβ1. This combination of targeted inhibition of pro-inflammatory signals, without interrupting natural antagonism, in conjunction with clearance of the cytokine-antibody complexes may lead to improved potency and clinical efficacy. In cell-based assays, antibodies with this novel method of action have the net effect, on the IL-12 and IL-23 networks, of potently suppressing downstream cytokine production. In a model driven by IL-12, a humanized antibody with this method of action demonstrated efficacy, and in a model of psoriasis, the same antibody was able to significantly reduce the inflammatory response. Whether this observed efficacy in animal models translates into improved therapeutic efficacy in a human disease setting will be further investigated and defined as this new class of antibodies progresses into the clinic.

Materials and Methods

Antibody generation and expression. Generation of hybridoma cell lines, expression and purification. BALB/c mice were immunized with human IL-12 (Peptotech) and anti-IL2p40 antibodies were generated using standard hybridoma technology. Hybridoma supernatants were screened for binding to monomeric IL-12p40, IL-12 and IL-23 and only hybridomas that bound to all three were selected for further analysis.

DNA sequencing. RNA was isolated from hybridomas using TRI reagent (Sigma-Aldrich) according to the manufacturer’s protocol. cDNA was synthesized from 10–200 ng RNA using the AccuScript® High Fidelity 1st Strand cDNA Synthesis Kit (Stratagene®), then used as a template in the following PCR reaction. Primers from the Novagen Murine IgG Primer Set for heavy-chain and for light-chain, respectively, were mixed with cDNA and Pfu II Mastermix® (Stratagene®) and then run in a thermocycler (Eppendorf Mastercycler®) according to the instructions provided with the Novagen Murine Primer Set. The PCR product was gel-purified with the DNA Gel Extraction Kit (QIAGen®), A-tagged using dATP and Taq-polymerase (Invitrogen™) at 72°C for 15 minutes, ligated into pGEM-T-Vector (Promega®) and transformed into TOP10 competent cells (Invitrogen™). Clones were screened for a ~500 bp insert. Plasmids from positive clones were isolated (Miniprep, QIAGen®) and sequenced. The nucleotide sequence was then translated into primary amino acid sequence in silico.

Construction of vectors expressing chimeric antibodies. The variable domains determined via DNA sequencing from the hybridoma cells m16E7 and m6F6 were expressed with human constant regions (IgG1 for heavy chain, kappa for light chain) by back translation of amino acid sequences into DNA sequences (GeneArt, Regensburg) and subcloned into the pTT5 heavy or light chain vector.27 The resulting chimeric antibodies were designated c16E7 and c6F6, respectively.
Expression and purification of recombinant antibodies via transient transfection. Heavy and light chain DNA was mixed with FuGENE® (Roche) and transfected into HEK293E cells according to the FuGENE protocol. After 7 days of culture, the supernatants were harvested and purified over a protein A column (HiTrap® GE Healthcare). The eluted antibody was desalted using Zeba® Desalting columns (Pierce®), analyzed by SDS-PAGE and gel filtration HPLC and the concentration determined using a BCA® assay (Pierce®).

Characterization of anti-IL-12/23 antibodies. Anti-IL-12/23 ELISA. IL-12 (Peprotech), IL-23 (Ebioscience), IL-12p40 (Ebioscience) or IL-12p80 (Peprotech), were used as capture antigens at a concentration of 1 μg/mL in a sandwich ELISA. The test antibody was serially diluted to generate a dose-response curve and was detected using a goat anti-human immunoglobulin G (H + L) antibody-HRP conjugate (Zymed®). Goat anti-murine immunoglobulin antibody-HRP conjugate (Dako) was used to detect bound murine antibody.

Receptor-neutralization assays. The extracellular domains of Fc chimeras (IL-12β1/Fc, IL-23/Fc or IL-12β2/Fc, R&D Systems®) were used as capture antigens. Cytokines and test antibodies were pre-incubated for 2 h then added to the ELISA plate. Bound cytokine to receptor was detected with biotinylated anti-human IL-12 antibody (Peprotech) at 0.5 μg/mL followed by detection with Strepavidin-HRP (Zymed). Color development reaction was performed with TMB and the absorbance was determined at 450 nm (ref. 620 nm).

Generation of a stably transfected cell line expressing the IL-12β1 chain. The amino acid sequence of IL-12β1 was back translated into DNA sequences which were optimized for mammalian cell chain.

Detection of cytokine binding to IL-12β1/Jurkat cells. HIS-tagged IL-23 or IL-12p40 or FLAG-tagged IL-12 was co-incubated with IL-12β1+ cells. Binding of cytokine to cells was detected with anti-6xHIS tag-PE (Abcam®) or anti-FLAG-FITC (Sigma-Aldrich®) using a flow cytometer.

Detection of inhibition of cytokine binding to IL-12β1/Jurkat cells. HIS-tagged IL-23 or IL-12p40 or FLAG-tagged IL-12 was co-incubated with antibody then IL-12β1+ cells were added and incubated. Cells were washed thoroughly in PBS/10% FBS and cell-bound cytokine was detected with anti-6xHIS tag-PE (Abcam®) or anti-FLAG-FITC (Sigma-Aldrich®) using a flow cytometer.

Detection of antibody-cytokine complexes on the surface of IL-12β1/Jurkat cells. FLAG-tagged IL-12 or HIS-tagged IL-23 or monomeric IL-12p40 was co-incubated with IL-12β1+ cells then antibody was added and cultures were incubated for an hour. Cells were washed thoroughly in PBS/2% FBS and antibody bound to cell-bound cytokine was detected with FITC-conjugated Rabbit anti-human IgG (Dako) using a flow cytometer.

In vitro IL-12/23 inhibition studies. Murine splenocyte assay. Concanavalin A (Sigma) at 1 μg/ml and recombinant human IL-13 (rhIL-23) at 25 ng/ml was added to test antibodies that had been titrated across a microtitre plate. Murine splenocytes were then added. A typical assay had three to five biological replicates per test antibody, and a starting test antibody concentration of 1–10 μg/mL. Cultures were incubated at 37°C, 5% CO2, for 3 days then supernatants were collected and assayed for IL-17 using an IL-17 ELISA kit (R&D Systems) as per manufacturer’s instructions.

NK92 IFNγ release assay. NK92 cells (ATCC, CRL-2407) were cultured under standard conditions with human IL-2 (Peprotech Asia) 200 U/ml. Cells were washed to remove cytokines and cultured overnight prior to assay. Cells were then stimulated with IL-12 and test antibodies were serially diluted. Cultures were incubated at 37°C with 5% CO2 for 24 h then supernatants were harvested and assayed for IFNγ using a human IFNγ ELISA kit (R&D Systems®). Human PBMC IL-12-induced IFNγ assay. Human PBMC were harvested from human buffy coat using lymphoprep separation and cultured with PHA-P (Sigma-Aldrich) 10 μg/mL for 3 days. Human IL-2 (R&D Systems®) 50 U/mL was added and the culture incubated for 24 hours and then plated out in 96-well plates. Human IL-2 (R&D Systems®) 1 ng/ml and human IL-12 1 ng/ml were added to each well and test antibodies were serially diluted. Cultures were incubated at 37°C with 5% CO2 for 2 days then supernatants were harvested and assayed for IFNγ as described above.

Hydrogen/deuterium exchange experiments. Epitope mapping by hydrogen/deuterium exchange mass spectrometry was carried out by the ExSAR corporation (www.exsar.com) as per their standard protocol24 using monomeric IL-12p40 and h6F6.

Creation of mutant IL-23 constructs. A gene was synthesized encoding wild-type IL-23 fused to a C-terminal FLAG tag. After analysis of the X-ray crystal structures of IL-23, e.g., PDB entry 3DB5, amino acids with exposed side chains were selected for mutation to an alanine residue via site-directed mutagenesis. All genes were cloned into the pTT5 vector and transfected into HEK293E cells using OptiMEM® (Invitrogen™) and FuGENE® (Roche) and transfected into HEK293E cells according to the FuGENE protocol. After analysis of the X-ray crystal structures of IL-23, e.g., PDB entry 3DB5, amino acids with exposed side chains were selected for mutation to an alanine residue via site-directed mutagenesis. All genes were cloned into the pTT5 vector and transfected into HEK293E cells using OptiMEM® (Invitrogen™) and FuGENE® (Roche) and transfected into HEK293E cells using OptiMEM® (Invitrogen™) and FuGENE® (Roche) as per the FuGENE protocol. The cultures were grown for 6 days and supernatant was harvested and filtered for analysis.

ELISA screening of mutant IL-23 constructs. IL-12β1/Fc Chimera (R&D Systems), IL-23R/Fc Chimera (R&D Systems), c16E7 and h6F6 were used as capture antigens at a concentration of 1 μg/mL in a dose response sandwich ELISA. Culture supernatant was serially diluted to generate a titration curve and added to 96-well ELISA plates. Bound IL-23 constructs were detected using Anti-FLAG® M2-HRP (Sigma-Aldrich®). Modeling. Computational epitope mapping of antibody and the D3 domain of IL-12p40 (PDB entry 3DUH) was performed using the Rosetta ensemble docking algorithm by Rosetta Design Group (www.rosettadesigngroup.com/index.php).
were undertaken with adherence to the ethical frameworks prescribed for the performance of experiments on animals according to the country where the studies were performed.

Male C57Bl/6J mice were depilated on a test area of the back two days prior to the commencement of injections, then given daily intradermal injections of either PBS or rhIL-23 in two locations on the back at a total of 3 or 10 μg/mouse/day. Both regimes gave some indication of inflammation but the dose of 10 μg/day gave a robust response with high levels of erythema. In a later study, mice were given 10 μg of rhIL-23/day for a total of 10 days to determine the full kinetics of the inflammatory response. The response was detectable from the third day of treatment, peaked at days six to seven then started to resolve. In a later study, mice were given 10 μg of rhIL-23/day for a total of 10 days to determine the full kinetics of the inflammatory response. The response was detectable from the third day of treatment, peaked at days six to seven then started to resolve.

**Antibody testing in vivo.** Male C57Bl/6J mice were treated with 10 μg/day of rhIL-23 as described above for 6 days. One day before the start of cytokine injection or 2 days after the first cytokine injection, they were given a single intraperitoneal injection of h6F6 or an isotype control antibody at a dose of 10 mg/kg. Mice were scored daily for erythema and induration in the test area. All treatments and observations were performed blinded. At the termination of the study, skin samples were collected from each mouse and were Haematoxylin and Eosin (H&E) stained.

Values for epidermal thickness were estimated blinded by two independent observers measuring thickness at 3 locations per skin section.

**Neutralization of serum IFNγ response to human p40/murine p35.** Human p40/murine p35 production and bioactivity. Human IL-12p40 fused to murine IL-12p35 were produced in HEK-293E cells as described above and purified using Ni-NTA chromatography. Responsiveness of mouse T cells to the human p40/murine p35 was compared in vitro with mouse and human IL-12. Human p40/murine p35 was capable of inducing IFNγ secretion from mouse splenocytes over a range of concentrations.

**Pilot range-finding study.** Male C57Bl/6J mice were treated with either PBS, mouse IL-12 at 0.03 mg/kg, or human p40/murine p35 at 0.03, 0.1 or 0.3 mg/kg by intraperitoneal injection for five days. On day six, terminal blood samples were collected and the sera assayed for IFNγ using a high-sensitivity IFNγ ELISA kit (Ebioscience) as per manufacturer’s instructions.

**Antibody testing in vivo.** Male C57Bl/6J mice were treated with human p40/murine p35 at 0.1 mg/kg by intraperitoneal injection for five days. They were also treated with either a single dose of h6F6 (5 mg/kg) on day 1, 30 minutes before dosing with human p40/murine p35, or with 3 doses of h6F6 or an isotype control on days 1, 3 and 5 of cytokine injection, 30 min before dosing with human p40/murine p35. On day six, terminal blood samples were collected and serum IFNγ determined as described above.

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