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Identification of potent antagonist antibodies against mouse IL-13R α 1 using novel bioassays

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ABSTRACT

Interleukin-13 (IL-13) is a cytokine implicated in airway diseases such as asthma and idiopathic pulmonary fibrosis. IL-13 signals through a heterodimeric receptor complex consisting of IL-13R α 1 and IL-4R α , known as the type II IL-4R. IL-4 also signals through this receptor and as such many of the biological effects of IL-13 and IL-4 are similar. Here we describe the development of two sensitive bioassays to determine the potency of antagonists of the mouse type II IL-4R. Both IL-13 and IL-4 dose-dependently induce CCL17 production from J774 mouse monocytic cells and CCL11 production from NIH3T3 mouse fibroblasts in the presence of TNF α . The assays were optimized to minimize TNF α concentration, cell number and incubation time whilst retaining a suitable signal-to-background ratio. Anti-cytokine antibodies or recombinant soluble receptors completely neutralized IL-13 or IL-4 activity in these bioassays. The J774 assay was used to screen a panel of anti-mIL-13R α 1 antibodies for neutralizing activity against this receptor. We report the identification of the first monoclonal antibodies that bind mouse IL-13R α 1 and neutralize both IL-13-induced and IL-4-induced cellular function. These antibodies should prove useful for determining the effects of neutralizing IL-13R α 1 in mouse models of disease. In addition, these bioassays may be used for measuring the bioactivity of mouse IL-13 and IL-4 and for the discovery of additional antagonists of the mouse IL-13R α 1/IL-4R α complex.

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Abbreviations: CCL, CC chemokine ligand; IL, interleukin; IL-4R α , interleukin-4 receptor α ; IL-13R α 1, interleukin-13 receptor α 1; IL-13R α 2, interleukin-13 receptor α 2; mAb, monoclonal antibody; HRP, horseradish peroxidase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; γ c, common-gamma chain; TNF α , tumor necrosis factor α ; TGF β , transforming growth factor β .

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1. Introduction

Interleukin-13 (IL-13) and IL-4 are closely related cytokines that share a common heterodimeric receptor, termed the type II IL-4R, consisting of IL-13R α 1 and IL-4R α . As IL-13 and IL-4 can signal through the same receptor many of the functions of these cytokines are shared. However, IL-4 can also signal through a unique receptor termed the type I receptor (IL-4R α and the common γ -chain (γ c)) which is not activated by IL-13.

Many non-hematopoietic cells express the type II receptor, including fibroblasts, smooth muscle cells and mucus secreting goblet cells (Grünig et al., 1998; Wills-Karp et al., 1998; Zhu et al., 1999). Although these cells respond to both IL-4 and IL-13, it appears that IL-13 is the main effector

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cytokine acting *in vivo* as neutralizing IL-13 but not IL-4 blocks airway hypersensitivity, mucus production and fibrosis in mouse models of human Th2 inflammatory disease (Chiaramonte et al., 1999; Corry et al., 1996; Grünig et al., 1998; Wills-Karp et al., 1998; Zhu et al., 1999). Macrophages and blood monocytes express both type I and type II receptors and stimulation of these cells with either IL-4 or IL-13 induces an “alternative” activation state (Doherty et al., 1993; Doyle et al., 1994; Stein et al., 1992). Alternative activation of macrophages leads to the production of various chemokines involved in the recruitment of cells associated with a Th2 response and to expression of the low affinity IgE receptor (CD23) on the cell surface. In addition, it results in the production of proteins that contribute to defense against helminths and fungal infections and that mediate tissue remodeling and anti-inflammatory responses (Van Dyken and Locksley, 2013). In mice, T cells express both the IL-4R α chain and the γ chain but lack expression of IL-13R α 1. Therefore, IL-4 but not IL-13 signaling through the type I receptor can promote differentiation of naïve CD4⁺ T cells into T helper type 2 (Th2) cells.

Genome wide association studies in humans have identified polymorphisms in the genes encoding both IL-13 and the IL-13R that are associated with asthma (Graves et al., 2000; Howard et al., 2002; Ober et al., 2000). Many studies in mice have also implicated IL-13 as a major cytokine regulating airway hyper-reactivity. Recently, mice with genetic deletions of IL-13R α 1 have been described that define distinct roles for the type II IL-4R in mediating features of human asthma in mouse models of airways disease such as airway hyperreactivity, mucus hypersecretion, chemokine (CCL2, CCL11, CCL17 and CCL24) and TGF β production, as well as IgE production (Munitz et al., 2008; Ramalingam et al., 2008). The scientific evidence that implicate the IL-13 pathway in airways disease has prompted the development of new monoclonal antibody therapeutics that target the IL-13 pathway, including anti-IL-13, anti-IL-4, anti-IL-4R α and anti-IL-13R α 1 antibodies (Caruso et al., 2013). Phase 2 clinical trials of anti-IL-13 antibodies have demonstrated efficacy in asthmatic patients, with better responses found in patients with either elevated serum IgE levels and eosinophil counts or elevated serum periostin, a molecule whose expression can be directly regulated by IL-13 (Corren et al., 2011). Correspondingly, in phase 2 trials with an anti-IL-4R α monoclonal antibody there was an ~80% reduction in asthma exacerbations compared to placebo when controller medications were withdrawn (Wenzel et al., 2013). We have developed an anti-hIL-13R α 1 monoclonal antibody that neutralizes the activity of both human IL-13 and IL-4 signaling through the type II receptor (PCT Patent Publication WO 2008/060813; Redpath et al., 2013). However, this antibody does not cross react with the mouse IL-13R α 1, which prevents the investigation of neutralizing this receptor in mouse models of disease.

In order to screen for antibodies that target mouse IL-13R α 1 and neutralize both mouse IL-13 and IL-4 signaling through the type II IL-4R, we developed bioassays in mouse cell lines that endogenously express IL-13R α 1 and IL-4R α and that are relevant to airway disease. The mouse J774 macrophage and NIH3T3 fibroblast cell lines were found to produce CCL17 (TARC) and CCL11 (eotaxin) respectively in response to both

mouse IL-13 and IL-4 when co-treated with TNF α . These assays were used to determine the potency of known antagonists of mouse IL-4 and IL-13 activity and to identify novel anti-mIL-13R α 1 neutralizing monoclonal antibodies.

2. Materials and methods

2.1. Reagents

J774 and NIH3T3 cell lines were purchased from ATCC (cat. no.: TIB-67 and CRL-1658) and were maintained in RPMI (Sigma, cat. no.: R0883) with 10% fetal calf serum (Thermo Scientific, cat. no.: SV30176.03), 1 X Penicillin/Streptomycin (GIBCO®, Life Technologies, cat. no.: 15140-122), 1 X glutamax (GIBCO®, Life Technologies, cat. no.: 35050-061) at 37 °C, 5% CO₂. FreeStyle™ 293-F (FS293F) cells and the mammalian expression vector pcDNA3.1 were obtained from Invitrogen. FS293F cells were cultured in FreeStyle™ 293 Expression Medium (Life Technologies) supplemented with penicillin/streptomycin/fungizone reagent (GIBCO®, Life Technologies) and cells were maintained at 37 °C in incubators with an atmosphere of 8% CO₂. Mouse CCL11 duoset (cat. no.: DY420) and mouse CCL17 duoset (cat. no.: DY529) ELISA kits were from R&D Systems. Mouse IL-4, IL-13, TNF α and anti-IL-4 (clone 30340) and anti-IL-13 (clone 38213) antibodies were from R&D Systems.

2.2. J774 and NIH3T3 bioassays

Cells were de-attached from tissue culture flasks with accutase® (Sigma), counted and plated at 1×10^5 cells per well (unless stated otherwise) in 96-well flat bottom tissue culture plates (TPP). Cells were incubated at 37 °C, 5% CO₂ for 4 h to allow cells to adhere prior to stimulation with TNF α and IL-4 or IL-13 as indicated for 24 h unless otherwise stated. For J774 assays where the potency of monoclonal antibodies or soluble receptor-Fc fusion constructs were determined cells were pre-incubated with Fc-Block™ (Miltenyi Biotec) for 10 min prior to addition of test article. Supernatants were collected and assayed for either CCL11 or CCL17 by ELISA.

2.3. CCL11 and CCL17 enzyme-linked immunosorbent assays

CCL11 and CCL17 ELISAs were performed using duoset ELISA kits (R&D Systems, cat. no.: DY320 and DY364) according to manufacturer's instructions. Briefly, maxisorb 96 well plates (Nunc) were coated overnight with capture antibody in PBS (Thermo Scientific) then blocked in 1% BSA (Miltenyi Biotec) before standards or test cell culture supernatants were added. Captured chemokine was detected by addition of biotinylated detection antibody followed by streptavidin-HRP and TMB substrate solution (KPL, cat. no.: 50-76-00). Reactions were stopped with 2 N H₂SO₄ and the absorbance at 450 nm measured on a plate reader (Envision, Perkin Elmer).

2.4. Expression and purification of recombinant receptor-Fc proteins

The cDNAs encoding the extracellular domains of mouse interleukin-13 receptor alpha 1 (Mu-sIL13R α 1 aa1-331; GenBank Accession no. NP_598751), mouse interleukin-13

receptor alpha 2 (Mu-sIL13R α 2 aa1-332; NP_032382) and mouse interleukin-4 receptor alpha (Mu-sIL4R α aa1-232; NP_001008700) were fused in-frame to a cDNA encoding aa 103-324 of mouse IgG1 heavy chain (mFc; GenBank Accession no. AAK53870), then cloned into the mammalian expression vector pcDNA3.1 (Invitrogen) and the nucleotide sequences validated by DNA sequence analysis. The resulting constructs were designated Mu-sIL13R α 1-mFc, Mu-sIL13R α 2-mFc and Mu-sIL4R α -mFc. Recombinant receptor-Fc proteins were affinity-purified from transiently-transfected FS293F supernatants using HiTrap MabSelect SuRe (GE Healthcare Life Sciences) chromatography resins followed by desalting with a HiPrep™ 26/10 Desalting column (GE Healthcare Life Sciences) on an ÄKTAexpress high throughput chromatography system (GE Healthcare Life Sciences). Purity was assessed by SDS-PAGE and recombinant receptor-Fc concentration determined chromatographically by comparison to control standards.

2.5. Isolation of anti-mIL-13R α 1 mAbs

A library was screened for Fab fragments that bound the recombinant extracellular domain of recombinant mouse IL-13R α 1/Fc Chimera (R&D Systems, cat no: 491-IR-200) immobilized on Dynabeads® M-280 Streptavidin (Invitrogen Life Technologies) by biotin-anti-human Fc antibody capture (Jackson ImmunoResearch Laboratories). The selection was done following methods described previously (Hoet et al., 2005). Positive clones were PCR-amplified and sequenced essentially as described (Hoet et al., 2005). Competitive ELISA was used to screen for high affinity clones. Unique antibody fragment-displaying clones were reformatted to express full-length IgG4 antibodies with the serine 241 to proline hinge region mutation (Angal et al., 1993) and either a lambda or kappa light chain by cloning the entire light chain (variable and constant domains) and the variable domain of the heavy chain from the selected Fab constructs into mammalian expression vectors (Jostock et al., 2004). Full-length IgG4 mAbs were affinity-purified from the supernatants of transiently-transfected FS293F cells as described above.

2.6. Phospho-STAT6 assay

Cells were treated as described with various antibodies then incubated for 30 min, 37 °C prior to stimulation with cytokine for 20 min, 37 °C. Cells were washed with FACS Wash (PBS, 0.1% BSA) then fixed with 200 μ l 2% formaldehyde in PBS, 37 °C for 10 min. Cells were washed again with FACS Wash then permeabilized with 200 μ l ice-cold 90% methanol on ice for 30 min. Cells were washed $\times$ 2 with FACS Wash and 10 μ l anti-pSTAT6-PE antibody (BD Biosciences) added. Cells were incubated at room temperature for 1 hr, washed with FACS Wash and analysed by flow cytometry (Fortessa, BD Biosciences).

2.7. Cell viability assay

J774 cells were plated at 1×10^4 cells per well in 96-well flat bottom tissue culture plates (TPP). Cells were stimulation as described then incubated at 37 °C, 5% CO₂ for 24 h and the number of viable cells remaining was determined using the

ViaLight®Plus Cell Proliferation and Cytotoxicity BioAssay Kit (Lonza).

3. Results

3.1. Identification of mouse cell lines that respond to IL-13 and IL-4

Mouse cell lines that endogenously express the IL-13R were sought to establish bioassays for the detection of mIL-13R α 1 antagonists. Both J774 monocytic cells and NIH3T3 fibroblasts have previously been shown to respond to mIL-13 (Mijatovic et al., 1997; Junttila et al., 2008). Others have shown that addition of TNF α to human cell lines allows hIL-13 to induce a dose-dependent increase in chemokine production (Miller et al., 2008). We cultured J774 and NIH3T3 cells with mIL-13 in the presence or absence of 20 ng/ml mouse TNF α for 24 h then assayed the supernatants for CCL11 and CCL17 production. Mouse IL-13 induced a dose-dependent increase in CCL11 production from NIH3T3 cells (Fig. 1a) and a dose-dependent increase in CCL17 production from J774 cells (Fig. 1b). J774 cells did not produce CCL11 and NIH3T3 cells did not produce CCL17 at any concentration of mIL-13 tested (Fig. 1a, b). IL-13-induced chemokine production by both cell lines required TNF α ; however, TNF α alone was not sufficient to induce either CCL17 or CCL11 (Fig. 1a, b).

3.2. Optimizing the J774 and NIH-3 T3 bioassays

To optimize the concentration of TNF α required for IL-13-induced chemokine production we titrated TNF α against increasing concentrations of IL-13 on both J774 and NIH3T3 cells and determined CCL17 and CCL11 production by ELISA. Titration of TNF α resulted in a dose-dependent increase in IL-13-induced chemokine production for both cell lines (Fig. 2a and supplementary fig. 1a). As IL-13 and IL-4 share a receptor system, consisting of IL-13R α 1 and IL-4R α , we also determined the ability of mouse IL-4 to induce CCL17 and CCL11 from J774 and NIH3T3 cells respectively. Like IL-13, IL-4-induced chemokine release from both J774 (Fig. 2b) and NIH3T3 cells (supplementary fig. 1b) was enhanced by TNF α . Addition of TNF α did not shift the dose responses of IL-13 or IL-4 in either cell line but increased the amount of chemokine produced in response to set concentration of cytokine. We chose to further develop these assays at 10 ng/ml TNF α to maintain a suitable signal-to-background ratio whilst minimizing cytokine requirement. IL-4 was a more potent inducer of chemokine production than IL-13 in both J774 and NIH3T3 cells as maximal chemokine production was observed at lower concentrations of IL-4 compared to IL-13 (Fig. 2).

We next determined the optimal cell number for these assays. Cells were plated at 1×10^5 cells per well for all previous experiments, therefore we expanded the range of cell densities tested by titrating cells from 1×10^4 cells per well to 2×10^5 cells per well in 96 well flat bottom plates followed by stimulation with either IL-13 or IL-4 in the presence of TNF α (10 ng/ml). Both IL-13 and IL-4 induced CCL17 and CCL11 production to > 250 pg/ml compared to no production in untreated cells when cells were plated at

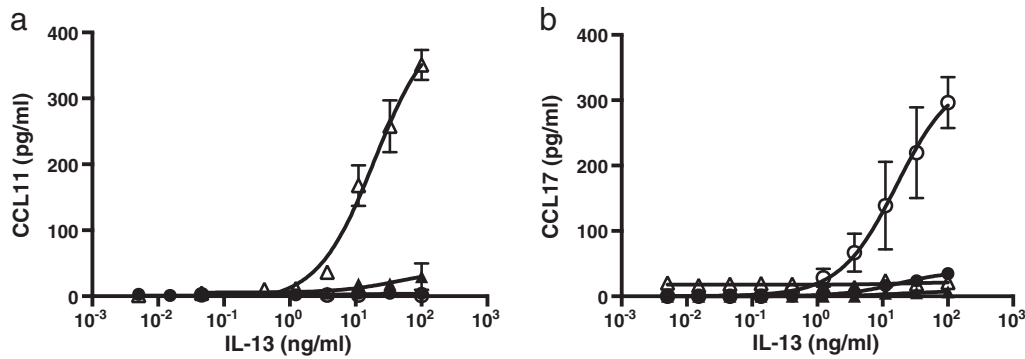


Fig. 1. J774 and NIH3T3 cells produce CCL17 or CCL11 in response to murine IL-13 in the presence of TNF α . J774 (circles) or NIH3T3 (triangles) cells were cultured with increasing concentrations of IL-13 in the presence (open symbols) or absence (closed symbols) of TNF α (20 ng/ml) for 24 h. Supernatants were collected and assayed for CCL11 (a) or CCL17 (b) production by ELISA. Means of triplicate wells $\pm$ standard deviation shown. These experiments were repeated twice with representative data shown.

concentrations of 1×10^5 per well and above (Fig. 2c and d and supplementary fig. 1c and d). However, we noted that J774 cells consistently produced much higher levels of

chemokine than NIH3T3 cells at comparable cell densities and cytokine concentrations. This difference in chemokine production between the two cell lines was more pronounced

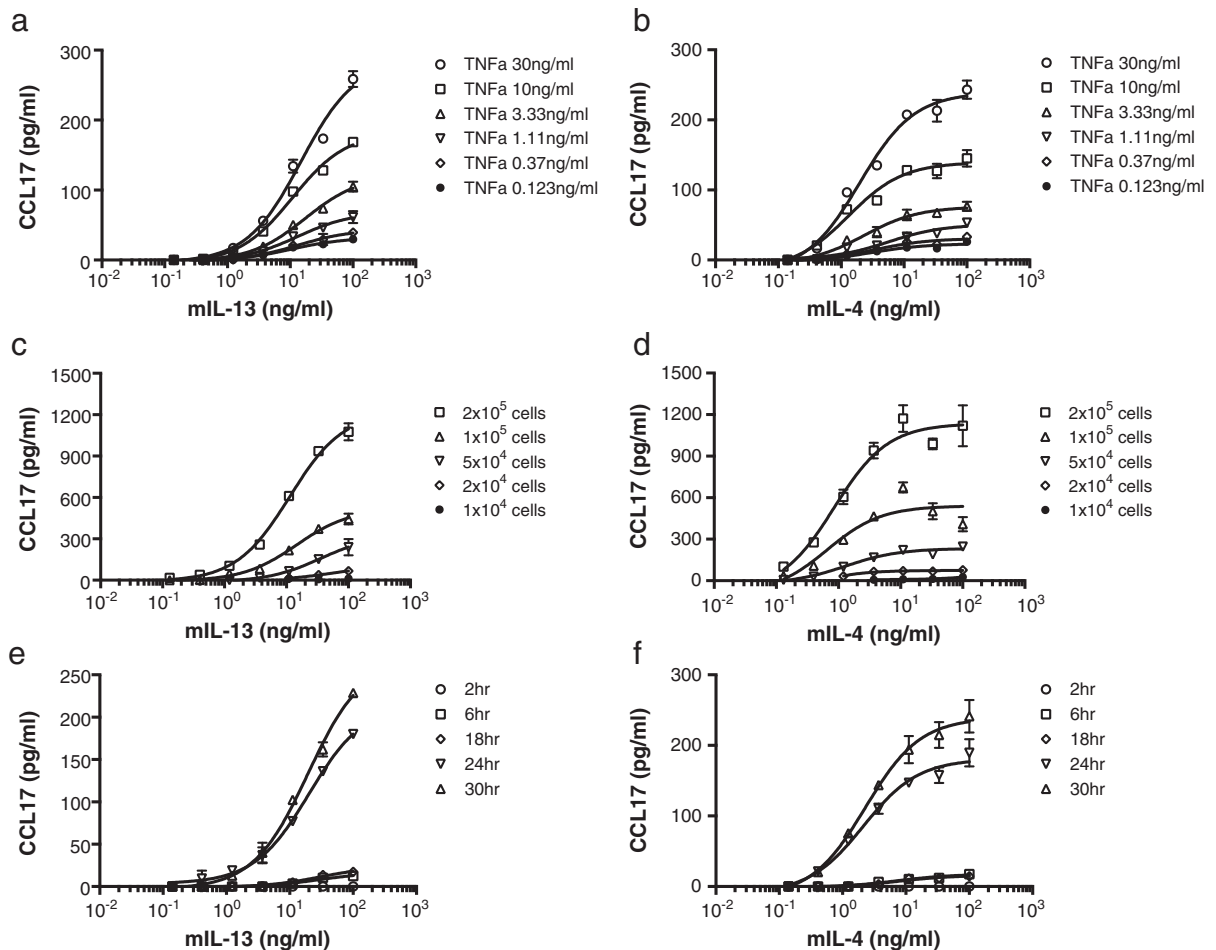


Fig. 2. Optimization of IL-13- and IL-4-induced CCL17 production from J774 cells. J774 cells were cultured with increasing concentrations of TNF α (a, b) or with increasing cell numbers (c, d) or for increasing incubation times (e, f) in the presence of IL-13 (a, c and e) or IL-4 (b, d and f). Supernatants were collected and assayed for CCL17 production by ELISA. Means of triplicate wells $\pm$ standard deviation shown. These experiments were repeated twice with representative data shown.

when cells were stimulated with IL-4 than IL-13. We chose to further optimize the assay at 1×10^5 cells/well as this seeding density allowed us to limit the number of cells required per plate while maintaining a good assay range and signal-to-background ratio.

The optimal cell stimulation time for chemokine production was determined by incubating J774 and NIH3T3 cells with either IL-4 or IL-13 in the presence of TNF α from 2–30 h. Chemokine production in response to IL-13 and IL-4 had been determined at 24 h post stimulation for all previous assays. A significant increase in either CCL17 or CCL11 production from J774 and NIH3T3 cells respectively was not observed until 24 h post cytokine stimulation (Fig. 2e and f and supplementary fig. 1e and f). Only a marginal increase in CCL11 production from J774 cells was observed when cells were treated with either IL-13 or IL-4 for 30 h instead of 24 h, indicating that a 24 h stimulation provides optimal time for chemokine production and detection in these assays.

For our purposes we established assay conditions where a signal above background was maintained over a broad range of IL-13 or IL-4 concentrations (0.3 ng/ml $\rightarrow$ 100 ng/ml), while minimizing cell and cytokine requirements. These conditions consisted of plating cells at a density of 1×10^5 cell/well in the presence of 10 ng/ml TNF α then stimulating them with IL-13 or IL-4 for 24 h prior to assaying supernatants for chemokine production. The EC₅₀s for IL-13 and IL-4 for both cell lines in these assay conditions were $\sim$ 5 ng/ml and $\sim$ 1 ng/ml respectively. Further increases in cell density,

TNF α concentration and assay time did not expand the range of the assay but simply increased the signal above background.

3.3. Determining potency of mouse IL-13 and IL-4 antagonists

We next used the above conditions of the J774 and NIH3T3 bioassays to determine the potency of commercial anti-mouse IL-13 and IL-4 monoclonal antibodies. J774 and NIH3T3 cells were stimulated with either IL-13 or IL-4 at their EC₅₀s in the presence of increasing concentrations of anti-IL-13 (Fig. 3a and c) and anti-IL-4 mAbs (Fig. 3b and d). The antibodies completely neutralized IL-13- or IL-4-induced CCL17 (Fig. 3a and b) and CCL11 (Fig. 3c and d) production from either cell type, with IC₅₀ of $\sim$ 0.14–1.5 μ g/ml (1–10 nM) for anti-IL-13 antibodies and $\sim$ 0.02–0.03 μ g/ml (0.15 nM) for anti-IL-4 antibodies.

We also generated Fc-fusion proteins of the extracellular domains of mouse IL-13R α 1, IL-13R α 2 and IL-4R α then used these soluble receptors to neutralize either IL-13 or IL-4 signaling in J774 cells. The soluble receptors were added to assays in fixed amounts of 10, 1 and 0.1 μ g/ml and then either IL-13 or IL-4 was titrated. As expected, both IL-13R α 1-Fc and IL-13R α 2-Fc protein inhibited IL-13 induced CCL17 release from J774 cells (Fig. 4a and b), with the IL-13R α 2-Fc protein being more potent. The IL-4R α -Fc protein had no effect on IL-13-induced chemokine production (Fig. 4c). In addition, we demonstrated that titration of

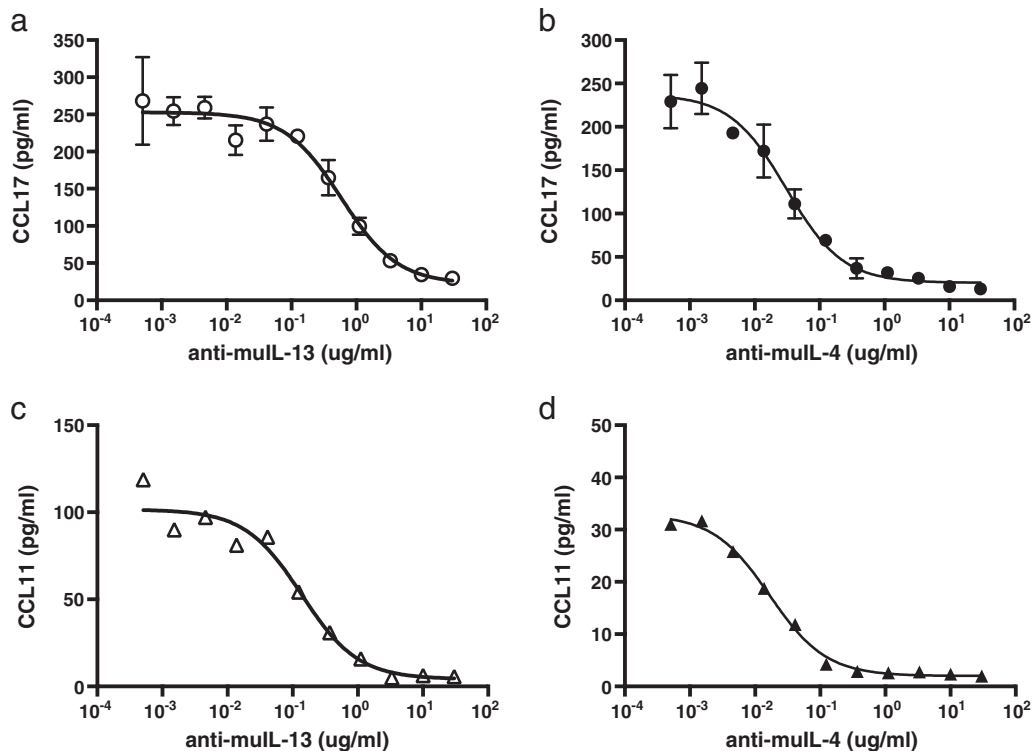


Fig. 3. Anti-IL-13 and anti-IL-4 monoclonal antibodies specifically neutralize IL-13 and IL-4-induced chemokine production in J774 and NIH3T3 cells. J774 (a, b) or NIH3T3 (c, d) cells were pre-treated with increasing concentrations of anti-mIL-13 or anti-mIL-4 monoclonal antibodies then stimulated with TNF α (10 ng/ml) in the presence of 5 ng/ml mIL-13 (a, c) or 1 ng/ml mIL-4 (b, d) for 24 h. Supernatants were collected and assayed for CCL17 or CCL11 production by ELISA. Means of triplicate wells $\pm$ standard deviation shown. These experiments were repeated twice with representative data shown.

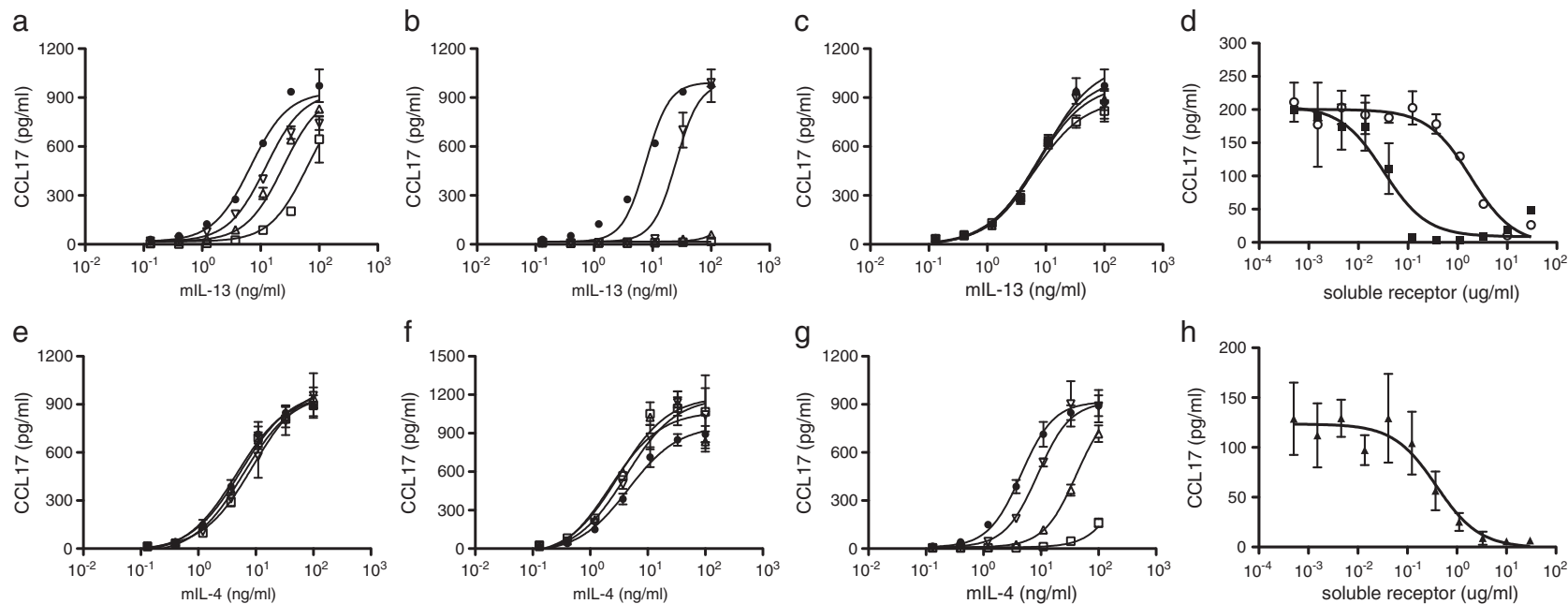


Fig. 4. Soluble IL-13 and IL-4 receptors specifically neutralize IL-13 and IL-4-induced chemokine production in J774 cells. J774 cells were stimulated with TNF α (10 ng/ml) and increasing concentrations of mIL-13 (a–c) or increasing concentrations of mIL-4 (e–f) alone (closed circles) or with mIL-13R α 1-Fc (a, e), mIL-13R α 2-Fc (b, f) or mIL-4R α -Fc (c, g) proteins at 0.1 μ g/ml (inverted triangles), 1.0 μ g/ml (triangles) or 10 μ g/ml (squares) for 24 h. J774 cells were also stimulated with TNF α and 5 ng/ml mIL-13 (d) or 1 ng/ml mIL-4 (h) in the presence of increasing concentrations of mIL-13R α 1-Fc (open circles), mIL-13R α 2-Fc (closed squares) or mIL-4R α -Fc (closed triangles) proteins for 24 h. Supernatants were collected and assayed for CCL17 production by ELISA. Means of triplicate wells $\pm$ standard deviation shown. These experiments were repeated twice with representative data shown.

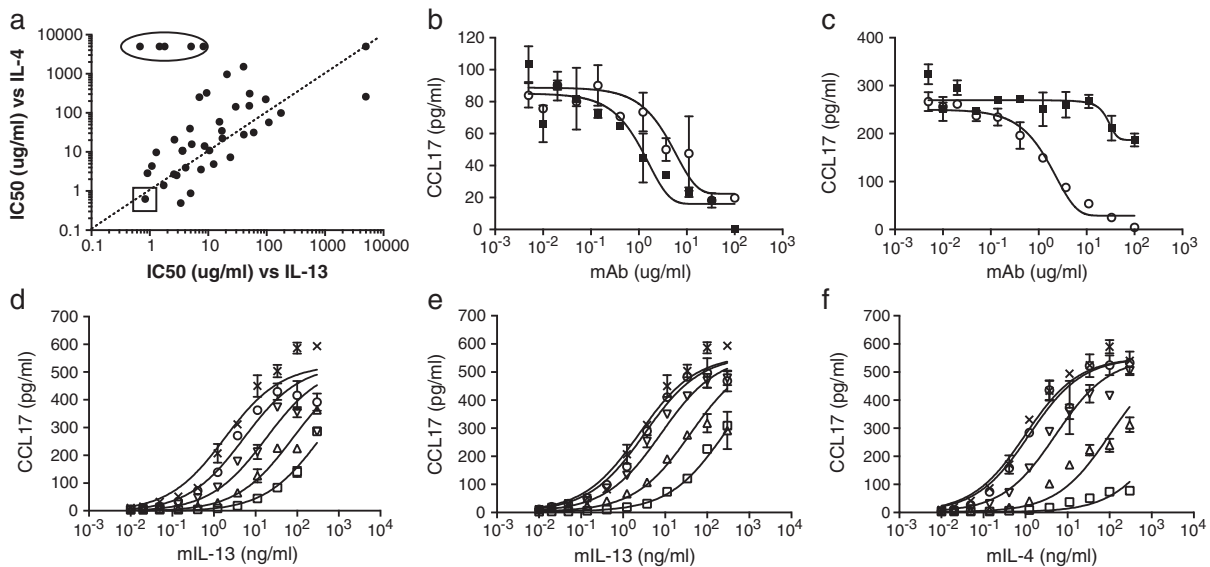


Fig. 5. Identification of neutralizing anti-mIL-13R α 1 monoclonal antibodies. (a) J774 cells were stimulated with TNF α (10 ng/ml) and 5 ng/ml mIL-13 or 1 ng/ml mIL-4 in the presence of increasing concentrations of phage display-derived anti-mIL-13R α 1 antibodies for 24 h. Supernatants were collected and assayed for CCL17 production by ELISA and IC50s for each antibody determined. For each antibody the IC50 for IL-13 neutralization was plotted against the IC50 for IL-4 neutralization. Antibodies that neutralize IL-13 but not IL-4 activity are circled; the most potent inhibitory antibody is boxed and the dotted line represents a correlation of $r = 1$. IL-13 potency showed a positive correlation with IL-4 potency (Spearman $r = 0.412$, $p < 0.01$; excluding antibodies that do not neutralize IL-4 $r = 0.757$, $p \leq 0.0001$). (b) J774 cells were stimulated with TNF α (10 ng/ml) and mIL-13 (5 ng/ml) in the presence of increasing concentrations of 4B3 (open circles) or 4D9 (closed squares). (c) J774 cells were stimulated with TNF α (10 ng/ml) and mIL-4 (1 ng/ml) in the presence of increasing concentrations of 4B3 (open circles) or 4D9 (closed squares). (d–f) J774 cells were stimulated with TNF α (10 ng/ml) and increasing concentrations of mIL-13 (d, e) or increasing concentrations of mIL-4 (f) alone (crosses) or with 4D9 (d) or 4B3 (e, f) at 0.1 μ g/ml (open circles), 1.0 μ g/ml (inverted triangles), 10 μ g/ml (triangles) or 100 μ g/ml (squares) for 24 h. Supernatants were collected and assayed for CCL17 production by ELISA. Means of triplicate wells $\pm$ standard deviation shown. These experiments were repeated twice with representative data shown.

both IL-13R α 1-Fc and IL-13R α 2-Fc fusion proteins could dose-dependently neutralize a single concentration of mIL-13-induced CCL17 production from J774 cells (Fig. 4d). Neither the IL-13R α 1-Fc or IL-13R α 2-Fc proteins inhibited IL-4-induced CCL17 release from J774 cells (Fig. 4e and f); however, the IL-4R α -Fc protein was a potent inhibitor of IL-4-induced chemokine production (Fig. 4g). Similar results were observed with the NIH3T3 bioassay (data not shown). Dissociation constants for antagonist binding (K_B) were determined by applying Gaddum/Schild EC50 shifts (Gaddum, 1957; Schild, 1957); IL-13R α 1-Fc has a K_B of ~ 2.4 nM, IL-13R α 2-Fc has a K_B of ~ 0.89 nM and IL-4R α -Fc has a K_B of ~ 2.15 nM in these experiments, which are similar to previously reported values (Donaldson et al., 1998; Hilton et al., 1996). Similar to the effect of the IL-13R α 1-Fc and IL-13R α 2-Fc fusion proteins against IL-13, titration of IL-4R α -Fc could dose-dependently neutralize a single concentration of mIL-4-induced CCL17 production from J774 cells (Fig. 4h). These data demonstrate that mIL-13 and mIL-4 signal specifically through IL-4R α and IL-13R α 1 to mediate chemokine production in these cell lines.

3.4. Identification of neutralizing anti-mIL-13R α 1 monoclonal antibodies

To identify antibodies that neutralize the mouse type II IL-4R we screened a library for antibodies that bound

specifically to soluble mouse IL-13R α 1-Fc fusion protein. We isolated 190 clones from two separate selections and tested these for binding to soluble mIL-13R α 1-Fc by ELISA. We found 148 clones (77%) that bound to mIL-13R α 1. Of these clones 42 were found to be unique, as determined by sequencing their CDR VH and VL domains, and were subsequently cloned as full-length human IgG4 mAbs. These 42 mAbs were expressed, purified and screened for neutralizing activity against mIL-13 and mIL-4 in the J774 bioassay described above. We determined the potency of the antibodies for mIL-13 and mIL-4 neutralization through analysis of CCL17 production. Of the 42 antibodies screened only two did not show neutralizing activity against IL-13 while 6 had no potency against IL-4-induced CCL17 production (Fig. 5a). The potency of these antibodies against IL-13 positively correlated with their potency against IL-4 (Spearman $r = 0.412$, $p < 0.01$; excluding antibodies that did not neutralize IL-4 $r = 0.757$, $p \leq 0.0001$).

The most potent IL-13-neutralizing antibody, 4D9, had an IC50 of 0.67 μ g/ml (~ 4.5 nM) against mIL-13 but was only a partial inhibitor of IL-4 in the J774 assay (Fig. 5b and c). The partial inhibition of IL-4 activity was expected as J774 cells express the gamma common (γ c) receptor (data not shown) thus allowing residual IL-4 signaling through the type I receptor in the presence of an IL-13R α 1 neutralizing mAb. Interestingly, the next most potent antibody, clone 4B3, demonstrated complete neutralizing activity against both

mIL-13 and mIL-4 (Fig. 5b and c) in the J774 assay with IC₅₀s of 0.83 µg/ml (~5.6 nM) and 0.62 µg/ml (~4.0 nM) respectively. 4B3 also completely inhibited IL-13- and IL-4-induced STAT6 activation of J774 cells demonstrating that the effect of this antibody on preventing chemokine production was at the level of receptor activation (supplementary fig. 2a–c). We confirmed that the neutralizing effect of 4B3 on chemokine production was not due to cytotoxic effects by incubating J774 cells with up to 100 µg/ml of the antibody for 24 h with no impact on viable cell number, either without cytokine or in the presence of IL-13 or IL-4 (supplementary fig. 2d).

As J774 cells are monocytic IL-4 should signal through both the type II and the type I IL-4R; therefore, the ability of 4B3 to completely neutralize IL-4 activity in these cells was unexpected. We therefore determined the neutralizing effects of 4D9 and 4B3 on mIL-13 and mIL-4 signaling in primary mouse peripheral blood cells isolated from C57/B6 mice. In whole blood flow cytometry assays mIL-13 and mIL-4 induced STAT6 phosphorylation in cells gated on monocytes (supplementary fig. 3a) but only mIL-4 induced STAT6 phosphorylation in cells gated on lymphocyte populations (supplementary fig. 3b). Both 4D9 and 4B3 inhibited mIL-13-induced STAT6 activation in cells gated on monocytes (supplementary fig. 3c) and 4D9 showed the expected partial inhibition of IL-4 in this cell population (supplementary fig. 3d). However, 4B3 showed complete inhibition of IL-4-induced signaling in primary mouse monocytes (supplementary fig. 3d), consistent with results obtained in the J774 assay. Moreover, 4B3 also inhibited mIL-4-induced phosphorylation of STAT6 in primary mouse lymphocytes, which only express the type I IL-4R, whereas the mIL-13Rα1-specific mAb 4D9 had no effect in these cells (supplementary fig. 3e). The data suggest that 4B3 may cross react with a component of the type I IL-4R and therefore inhibit IL-4 signaling through both the type I and type II IL-4Rs. We tested 4B3 and 4D9 for binding to mIL-13Rα1 and mIL-4Rα in transiently transfected FS293F cells by flow cytometry. Staining of mIL-4Rα-transfected FS293F cells with 4B3 and 4D9 demonstrated that 4B3 could bind mIL-4Rα, whereas 4D9 or an isotype-control antibody could not (supplementary fig. 3f). As expected, both 4B3 and 4D9 showed binding to FS293F cells expressing mIL-13Rα1 (supplementary fig. 3g). These results show that 4D9 is a specific mIL-13Rα1 neutralizing mAb, whereas 4B3 binds both mIL-13Rα1 and mIL-4Rα. This cross-reactivity with mIL-4Rα provides a mechanism by which 4B3 completely neutralizes IL-13 and IL-4 function in mouse monocytes and IL-4 activity in mouse lymphocytes.

We next applied Gaddum/Schild regression analysis to determine an equilibrium dissociation constant for both 4B3 and 4D9 binding to cell surface expressed receptor. Antibody was added to assays in fixed amounts of 10, 1 and 0.1 µg/ml and either IL-13 or IL-4 was titrated (Fig. 5c and d). Applying Gaddum/Schild EC₅₀ shift analysis to the IL-13 dose response in the presence of 4D9 (Fig. 5d) or to the IL-13 and IL-4 dose responses in the presence of 4B3 (Fig. 5e and f) K_B values of 0.26 nM were obtained for 4D9 and 2.45 nM and 2.3 nM were obtained for 4B3. The affinity and potency of these antibodies against both mIL-13 and mIL-4 will enable them to be used to research the effects of neutralizing signaling through the IL-13Rα1 and IL-4Rα in mice.

4. Discussion

Here we report the generation of potent antibody antagonists of the mouse type II IL-4R (IL-13Rα1 and IL-4Rα) using antibody library screening combined with sensitive and optimized bioassays. To date, no reliable assay has been described to determine mIL-13 and mIL-4 activity in mouse cells. Although both mIL-13 and mIL-4 are active on human cell lines such as TF-1 (Zurawski et al., 1993) and A-549 (Miller et al., 2008), these cell lines do not allow identification of inhibitors of the mouse type II IL-4R subunits (IL-13Rα1 or IL-4Rα). Identification and characterization of such inhibitors is useful for investigating the effects of neutralizing the type II IL-4R in mouse models of disease and to support the clinical development of human monoclonal antibody therapeutics directed against this pathway that do not cross-react with mouse orthologues of the type II IL-4R.

We chose to develop bioassays for IL-13 and IL-4 activity in cell lines relevant to human airways disease. Monocytes and macrophages contribute to the inflammatory response by producing various cytokines and chemokines, such as IL-12, IL-23, TNFα, CCL17 and CCL22 that recruit and activate inflammatory cells. IL-4 and IL-13 induce an alternative activation state in macrophages that results in a characteristic gene expression profile, where genes such as TNFα and IL-12 are down regulated and others such as arginase, inos, CCL17, CCL22, CCL26 and CD23 are upregulated (Van Dyken and Locksley, 2013). Fibroblasts and epithelial cells also respond to IL-4 and IL-13 by producing chemokines that promote Th2 cell recruitment and by producing factors that induce fibrosis and tissue remodeling such as periostin and TGFβ. The mouse monocytic cell line, J774 and the mouse fibroblast cell line, NIH3T3 have both been shown to respond to mouse IL-13 and IL-4 as evidenced by an inhibition of LPS-stimulated TNFα production from J774 cells (Mijatovic et al., 1997) and an increase in STAT6 activation in NIH3T3 cells (Junttila et al., 2008). The human lung epithelial cells line A-549 has been used to establish a bioassay for human IL-13 activity where these cells produce CCL17 in response to IL-13 when treated in the presence of TNFα. We show that the mouse cell lines J774 and NIH3T3 also release chemokines, CCL17 and CCL11 respectively, in response to IL-4 and IL-13 and like the human A-549 cells these responses were dependent on the presence of TNFα. Another mouse cell line that is responsive to mIL-4 and hIL-13, derived from the B9 hybridoma (B9-1-3), has been described (Bouteiller et al., 1995). This cell line is dependent on IL-6 for growth and can become factor independent, which makes it difficult to establish robust cellular assays that rely on cytokine-induced proliferation as an endpoint. The J774 and NIH3T3 cell lines are convenient cells to use for bioassays as they do not require any special media for their growth, are factor-independent and have a fast doubling time. IL-13 and IL-4 have EC₅₀s in these assays of ~1–5 ng/ml (~0.1–0.5 nM), which is similar to previously reported responses (Bouteiller et al., 1995; Junttila et al., 2008).

We have previously described a novel fully human therapeutic antibody directed against the human IL-13Rα1 subunit that neutralizes both IL-4 and IL-13 (PCT Patent Publication WO 2008/060813; Redpath et al., 2013). This

antibody was shown to neutralize human and non-human primate IL-13R α 1 activation but does not cross react with mouse IL-13R α 1. Mice are used extensively in pre-clinical development of therapeutics including determining the pharmacokinetic and pharmacodynamic properties of molecules, understanding potential toxicology associated with the drug and for proof of concept in models of human disease. To date, experiments in mice designed to elucidate the roles of IL-4 and IL-13 in models of human disease (particularly airways disease) have relied upon genetic deletion of various components of the IL-4/IL-13 signaling pathway or on soluble receptor antagonists that neutralize signaling through both the type I and type II IL-4Rs. No antibodies that bind mouse IL-13R α 1 and neutralize mIL-13 and mIL-4 have previously been described.

To identify antibodies that neutralize the mouse type II IL-4R we initially screened a library for Fab fragments that bound the ecto-domain of mouse IL-13R α 1. Positive clones were reformatted as full IgG4 antibodies then tested for neutralizing activity in the J774 bioassay. Using this assay we identified a series of antibodies that had a range of neutralizing activity against mouse IL-13 and/or IL-4. Several antibodies were identified that had complete neutralizing activity against both IL-13 and IL-4 with IC50s of approximately 0.5–15 μ g/ml (~5–100 nM). The most potent IL-13 neutralizing antibody, 4D9, was only a partial IL-4-antagonist in the J774 assay suggesting it is a selective type II IL-4R inhibitor. We confirmed these results by showing that 4D9 could inhibit mIL-13-induced STAT6 activation in primary murine monocytes but only partially inhibit mIL-4-induced responses in the same cells. As expected, 4D9 had no effect on IL-4-induced STAT6 activation in primary mouse lymphocytes. Surprisingly one antibody, 4B3, had complete neutralizing activity against both IL-13 and IL-4 in J774 cells with an IC50 of approximately 4–6 nM and affinity of 2.3–2.5 nM as determined by Gaddum/Schild shifts. Although this result was unexpected we showed that 4B3 could also completely inhibit IL-4-induced activation of STAT6 in primary mouse monocyte and lymphocyte populations. We subsequently demonstrated that this antibody cross-reacts with IL-4R α providing a mechanism by which it inhibits both type I and type II IL-4R activation.

We believe that the bioassays described will be valuable tools to test human therapeutic compounds for their ability to also inhibit IL-13 and IL-4 pathways in the mouse. These assays may also be used to identify novel inhibitors of the mouse type II IL-4R signaling pathway that can subsequently be tested for efficacy in mouse models of disease relevant to human conditions. In addition, the identification of monoclonal antibodies that neutralize IL-13 and IL-4 activation of the type II IL-4R and/or type I IL-4R in mouse cells will enable the effects of inhibiting this receptor to be elucidated in models of airway inflammation.

Appendix A. Supplementary data

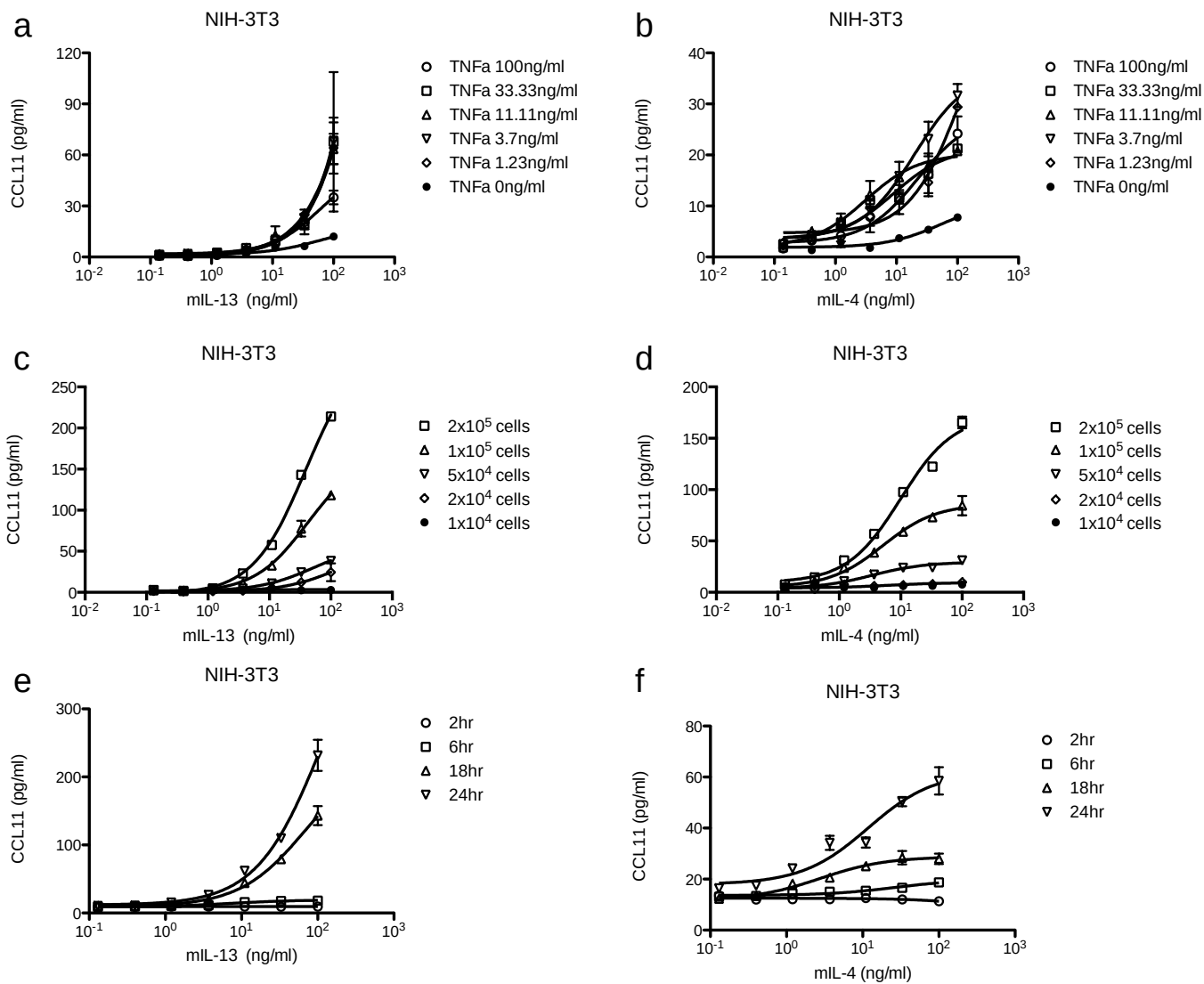
Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jim.2014.03.019>.

References

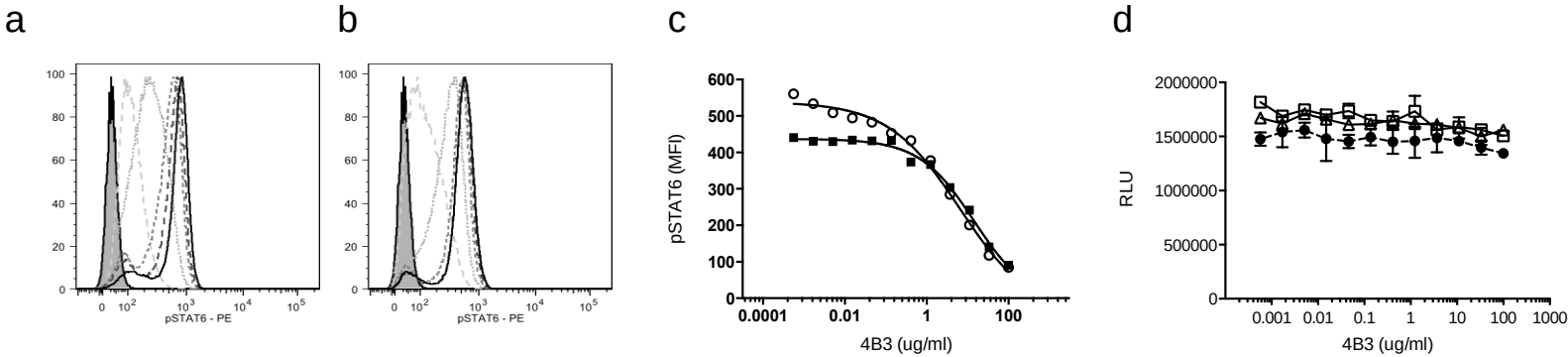
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Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.

