



In vitro and *in vivo* characterisation of anti-murine IL-13 antibodies recognising distinct functional epitopes

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ABSTRACT

Interleukin-13 (IL-13) sequentially binds to IL-13R α 1 and IL-4R α forming a high affinity signalling complex. This receptor complex is expressed on multiple cell types in the airway and signals through signal transducer and activator of transcription factor-6 (STAT-6) to stimulate the production of chemokines, cytokines and mucus. Antibodies have been generated, using the UCB Selected Lymphocyte Antibody Method (UCB SLAM), that block either binding of murine IL-13 (mIL-13) to mIL-13R α 1 and mIL-13R α 2, or block recruitment of mIL-4R α to the mIL-13/mIL-13R α 1 complex. Monoclonal antibody (mAb) A was shown to bind to mIL-13 with high affinity (K_D 11 pM) and prevent binding of mIL-13 to mIL-13R α 1. MAb B, that also bound mIL-13 with high affinity (K_D 8 pM), was shown to prevent recruitment of mIL-4R α to the mIL-13/mIL-13R α 1 complex. *In vitro*, mAbs A and B similarly neutralised mIL-13-stimulated STAT-6 activation and TF-1 cell proliferation. *In vivo*, mAbs A and B demonstrated equipotent, dose-dependent inhibition of eotaxin generation in mice stimulated by intraperitoneal administration of recombinant mIL-13. In an allergic lung inflammation model in mice, mAbs A and B equipotently inhibited *muc5ac* mucin mRNA upregulation in lung tissue measured two days after intranasal allergen challenge. These data support the design of therapeutics for the treatment of allergic airway disease that inhibits assembly of the high affinity IL-13 receptor signalling complex, by blocking the binding of IL-13 to IL-13R α 1 and IL-13R α 2, or the subsequent recruitment of IL-4R α .

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1. Introduction

Interleukin-13 (IL-13) has biological activity that identifies it as a therapeutic target for the treatment of asthma [1]. IL-13 stimulates goblet cell metaplasia and mucus hypersecretion [2], IgE production from B-cells from atopic patients [3], airway fibrosis [4], activates airway smooth muscle directly [5] and increases airway hyperreactivity to inhaled spasmogens [6]. Furthermore, anti-IL-13 neutralising monoclonal antibodies (mAb) are efficacious in experimental models of asthma in mouse [2,7], sheep [8] and non-human primates [9].

The predominant biological effects of IL-13 are driven through a heterodimeric receptor complex composed of IL-13R α 1 and IL-4R α subunits. IL-13 initially binds to IL-13R α 1 with low affinity, forming a complex that subsequently leads to recruitment of the IL-4R α subunit to form a high affinity receptor capable of signalling through STAT-6 [10,11]. IL-13 also binds with high affinity to a second receptor, IL-13R α 2. IL-13R α 2 can function as both a decoy receptor [12] and

possibly as a signalling receptor [13]. The requirement for the assembly of a heterodimeric cell surface IL-13R α 1/IL-4R α receptor signalling complex offers at least two points of intervention for mAbs designed to neutralise the activity of IL-13. This has been demonstrated for the human receptor system by Kasaian et al. who have described the pharmacology of two neutralising anti-human IL-13 mAb that block either IL-13 binding to IL-13R α 1, or the recruitment of IL-4R α by the IL-13/IL-13R α 1 complex [14].

In this paper we describe the receptor binding characteristics, *in vitro* neutralisation profile in cellular assays and *in vivo* pharmacological profile of two UCB Selected Lymphocyte Antibody Method (UCB SLAM)-derived anti-murine IL-13 (mIL-13) antibodies that differentially interrupt the assembly of the IL-13 receptor signalling complex.

2. Materials and methods

2.1. Antibody generation

Rabbits were immunised with cells expressing murine IL-13. A panel of mIL-13-specific antibodies was isolated using a modified version of SLAM [15]. B cells were cultured in 96-well tissue culture plates and the

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supernatants screened for mIL-13 binding and neutralisation in a TF-1 cell proliferation assay [16]. Instead of using a haemolytic plaque assay as described in the original SLAM process, a fluorescence-based method to identify the antigen-specific B cells from a positive well was used. Immunoglobulin-secreting B cells from a positive well were mixed with

streptavidin beads coated with biotinylated murine IL-13 (R and D Systems, UK) and a goat F(ab')₂ anti-rabbit Fcγ fragment-specific FITC conjugate (Jackson ImmunoResearch, USA). After incubation at 37 °C for 1 h, antibody secreted from antigen-specific B cells was captured on beads in the vicinity of that B cell. The presence of the FITC conjugate

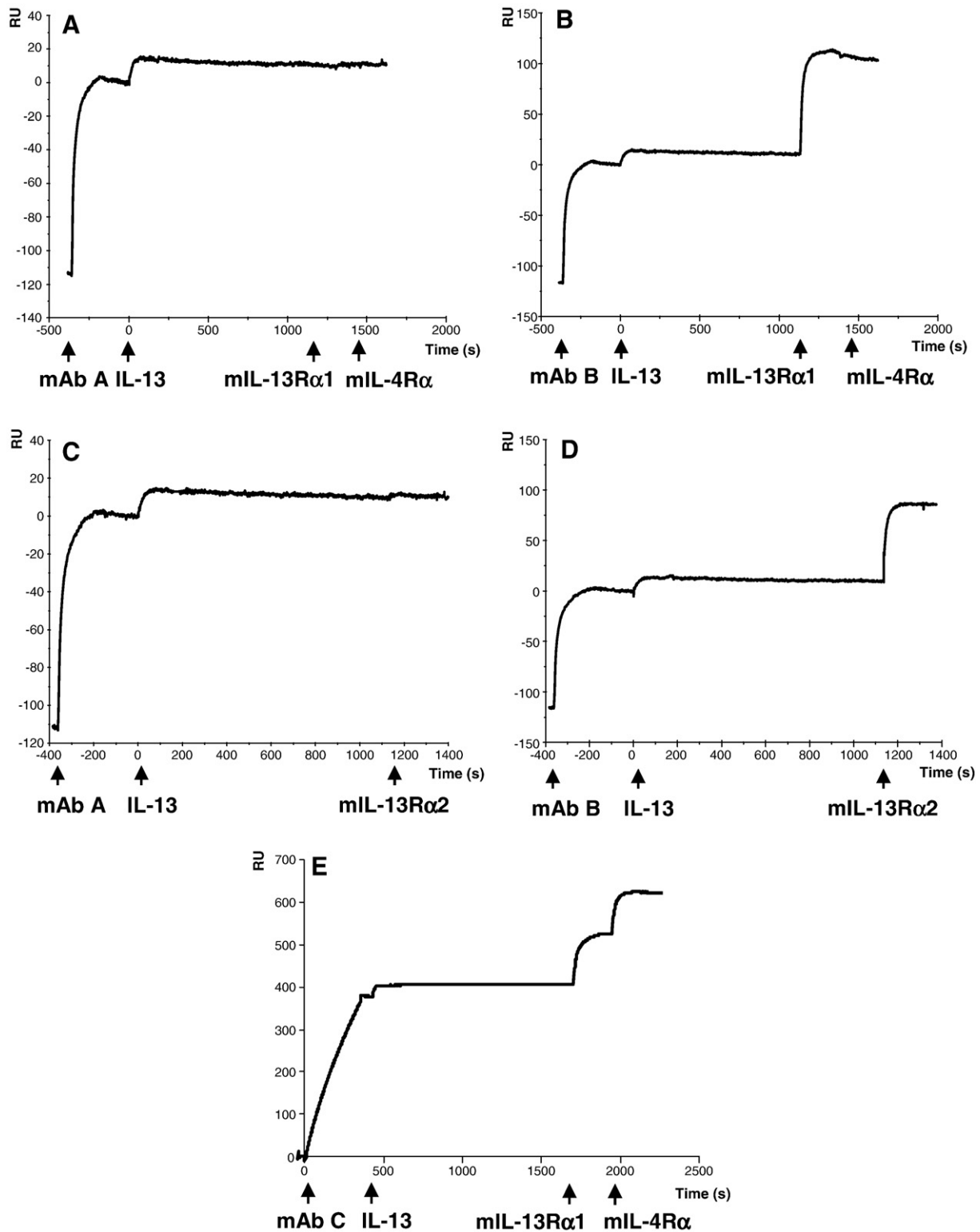


Fig. 1. (A–D) Effect of mAb A and mAb B on binding of mIL-13 to mIL-13Rα1, mIL-13Rα2 and mIL-4Rα measured using surface plasmon resonance. mAb A (A, C) or mAb B (B, D) was captured onto the surface of a CM5 sensor chip with anti-mouse Fc IgG. mIL-13, mIL-13Rα1 and mIL-4Rα (A, B) or mIL-13 and mIL-13Rα2 (C, D) were sequentially injected and their binding measured as Response Units (RU). (E) Sequential binding of mIL-13Rα1 and mIL-4Rα to mIL-13. A non-neutralising mIL-13 mAb (mAb C) was captured onto the surface of a CM5 sensor chip with anti-Fc IgG. mIL-13, mIL-13Rα1 and mIL-4Rα were sequentially injected and their binding measured as Response Units (RU).

resulted in the labelling of antibody-coated beads and the formation of fluorescent foci around the antigen-specific B cells. These individual B cells, identified using a fluorescence microscope, were then picked with a micromanipulator and deposited into a PCR tube. Antibody variable region genes were recovered from single cells by reverse transcription (RT)-PCR. Rabbit variable region genes were murinised and expressed as mouse IgG₁ antibodies in a HEK-293 transient expression system. Recombinant antibodies were processed to >99% purity using Protein A followed by gel filtration chromatography. Neutralisation activity with recombinant antibodies was confirmed in a reporter assay using HEK-BLUE™ STAT-6 cells (Invivogen, UK). Neutralising antibodies were further characterised by surface plasmon resonance to determine affinity and mechanism of neutralisation.

2.2. Determination of anti-mIL-13 mAb affinity and interruption of mIL-13 receptor complex assembly

Surface plasmon resonance was performed using a BIAcore 3000 (BIAcore AB, Sweden) [17]. Affinipure F(ab')₂ fragment goat anti-mouse Fc (Jackson ImmunoResearch, USA) was immobilised on a CM5 Sensor Chip via amine coupling chemistry to a capture level of ~5000

Response Units (RU). HBS-EP buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, BIAcore AB) was used as the running buffer with a flow rate of 10 µL/min. A 10 µL injection of antibody was used for capture by the immobilised anti-IgG-Fc. A 90 µL injection of mIL-13 was titrated from 5 nM to 0.3 nM over the captured antibody at 30 µL/min. The surface was regenerated by a 30 µL injection of 40 mM HCl, followed by a 5 µL injection of 5 mM NaOH.

Background subtraction binding curves were analysed using the BIAevaluation software (version 3.2) following standard procedures. Kinetic parameters were determined from the fitting algorithm.

The mechanism by which mAbs A and B inhibited assembly of the mIL-13 receptor complex was determined by capturing the antibody with the immobilised anti-IgG-Fc. A 30 µL injection of mIL-13 at 50 nM was flowed over the surface followed by 30 µL of the mIL-13Rα1, mIL-13Rα2 or mIL-4Rα at 150 nM (R and D Systems, UK). To demonstrate assembly of the mIL-13 signalling complex, a non-neutralising IL-13 mAb was captured with anti-IgG-Fc. A 30 µL injection of mIL-13 at 50 nM was followed sequentially by 30 µL mIL-13Rα1 and mIL-4Rα at 150 nM. The cross-blocking of mAb A with mAb B was tested by capturing mAb B or B and adding the Fab fragment of mAb A at 10 µg/mL in the soluble phase. The surface was regenerated as above.

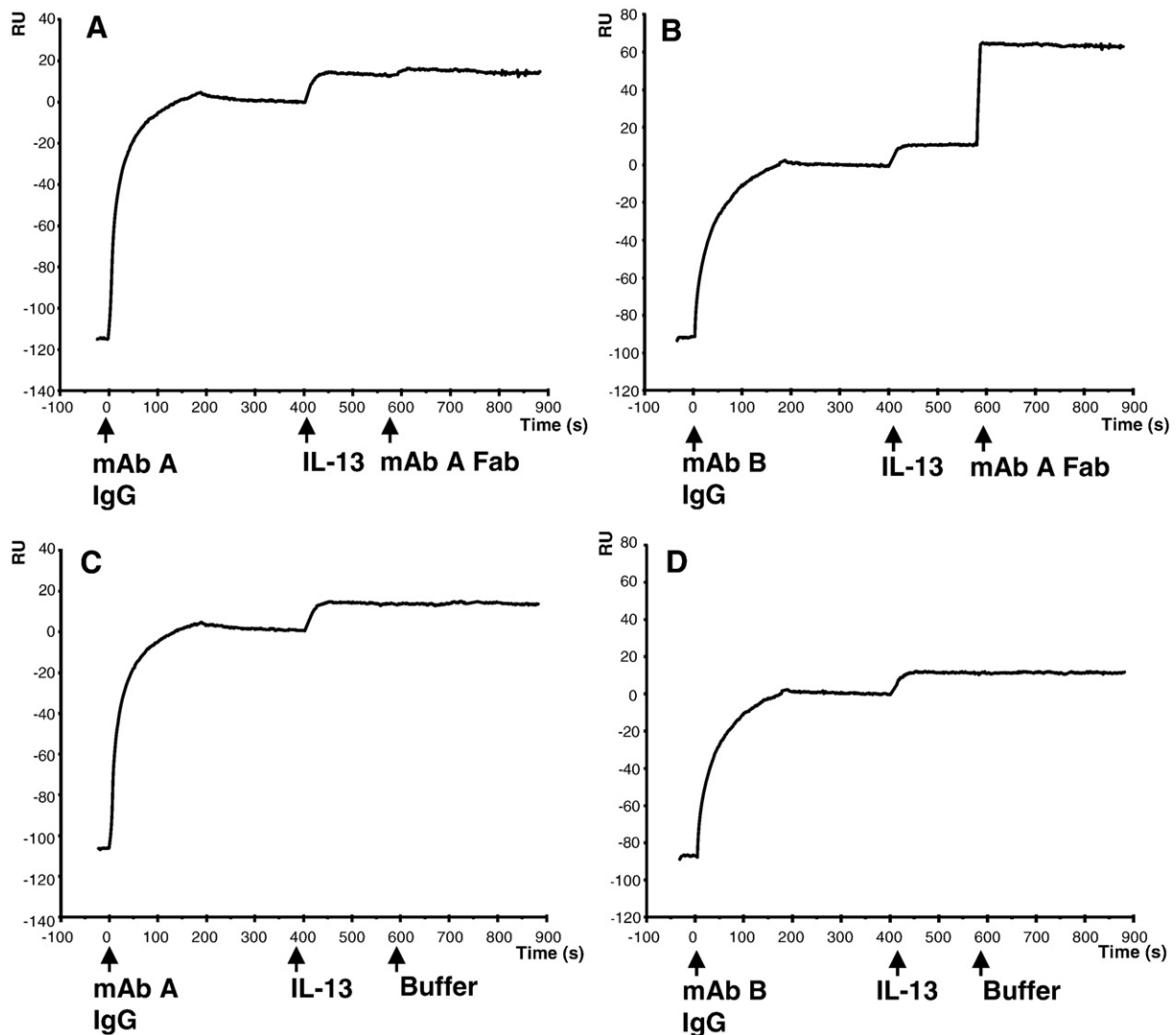


Fig. 2. Effect of mAb B on the binding of mAb A to mIL-13 measured using surface plasmon resonance. mAb A (A, C) or mAb B (B, D) was captured onto the surface of a CM5 sensor chip with anti-mouse Fc IgG. mIL-13 followed by Fab fragment of mAb A (A, B) or buffer (C, D) was sequentially injected and their binding measured as Response Units (RU).

2.3. *In vitro* neutralisation of mIL-13 bioactivity

HEK293 cells stably expressing human STAT-6 and stably expressing secreted embryonic alkaline phosphatase (SEAP) under the control of the IFN- β minimal promoter fused to four STAT-6 binding sites (HEK-BLUE™ STAT-6, Invivogen, UK) were incubated together with 1 ng/mL mIL-13 and mAb A or mAb B at 37 °C, 5% CO₂. After 24 h 40 μ L aliquots of supernatant were aspirated and transferred to equivalent wells of flat-bottomed 96-well plate. 160 μ L/well of QUANTI™-blue (Invivogen, UK) was mixed with cell supernatants and colour allowed to develop; optical density was measured at 630 nm.

TF-1 human erythroleukemic cells (ATCC, USA) have been shown to proliferate in response to IL-13 [16]. TF-1 cells were maintained in RPMI-1640 supplemented with 10% (v/v) FCS, 1% (w/v) sodium pyruvate, 2 mM L-glutamine, penicillin (5000 U/mL), streptomycin (100 μ g/mL) and 2 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (R and D Systems, UK); GM-CSF was excluded and FCS reduced to 5% (v/v) for proliferation assays. TF-1 cells were incubated with 1 ng/mL mIL-13 and mAb A or mAb B for 72 h at 37 °C, 5% CO₂. Proliferation was measured as tritiated thymidine incorporation during the last 6 h of culture.

NIH-3T3 cells were maintained in DMEM supplemented with 10% (v/v) FCS, 2 mM L-glutamine, penicillin (5000 U/mL) and streptomycin (100 μ g/mL). Cells were detached from adherent monolayer by trypsinisation and washing in culture medium. Cells were incubated with 1 ng/mL mIL-13 and mAb A or mAb B for 30 min at 37 °C, 5% CO₂. Active STAT-6 in cell lysates was detected by mouse active STAT-6 transcription factor assay (R and D Systems, UK) according to the manufacturer's instructions.

2.4. Mice

Male Balb/c mice (20–25 g) were obtained from Harlan (UK). All mice were housed in standard cages and provided with food and water *ad libitum*.

2.5. Stimulation of eotaxin generation by mIL-13 *in vivo*

Mice were treated with either mAb A or mAb B (0.1, 1 or 10 mg/kg subcutaneously, s.c.) or vehicle (100 μ L PBS) 10 min prior to intraperitoneal (i.p.) administration of mIL-13. Four hours after administration of mIL-13, mice were killed by cervical dislocation and peritoneal lavage was performed (3 mL PBS (Sigma, UK) containing 0.25% (w/v) BSA (Sigma, UK), 12 mM HEPES (Life technologies, UK), and 5 mM EDTA (Sigma, UK)). The concentration of eotaxin-1 was determined by enzyme-linked immunosorbent assay (ELISA) (R and D Systems, UK).

2.6. Allergic lung inflammation model

Mice were sensitised with 30 μ g ovalbumin (Sigma, UK) in aluminium hydroxide (Reheis, Ireland) on days 0 and 7 s.c. Mice were subsequently challenged intranasally (i.n.) with 30 μ g ovalbumin (OVA) on day 21. Mice were treated with either mAb A or mAb B (0.1, 1 or 10 mg/kg s.c.) or vehicle (100 μ L PBS) 10 min prior to i.n. OVA challenge.

2.7. Measurement of muc5ac gene expression

The lower right lung of mice sensitised and challenged with OVA was removed 48 h post OVA challenge and stored in RNAlater (Ambion, UK). Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, UK). Genomic DNA was eliminated using the gDNA eliminator columns included in the RNeasy Plus Mini Kit. 1 μ g of total RNA was reverse transcribed with random hexamers in a 50 μ L reaction using TaqMan Reverse Transcription Reagents (ABI, UK). A pre-designed

probe to the *muc5ac* sequence was obtained from Applied Biosystems (Assay I.D. Mm01276725_g1). GAPDH was used as the internal calibrator. 10 μ L real-time PCR reactions were performed using the 7900HT Fast Real-Time PCR system (ABI, UK). The relative *muc5ac* expression was calculated using the comparative C_T method ($\Delta\Delta C_T$).

2.8. Statistical analysis

Data are presented as mean $\pm$ SEM. One-way analysis of variance (ANOVA) followed by Dunnett's post-test was used for comparison of

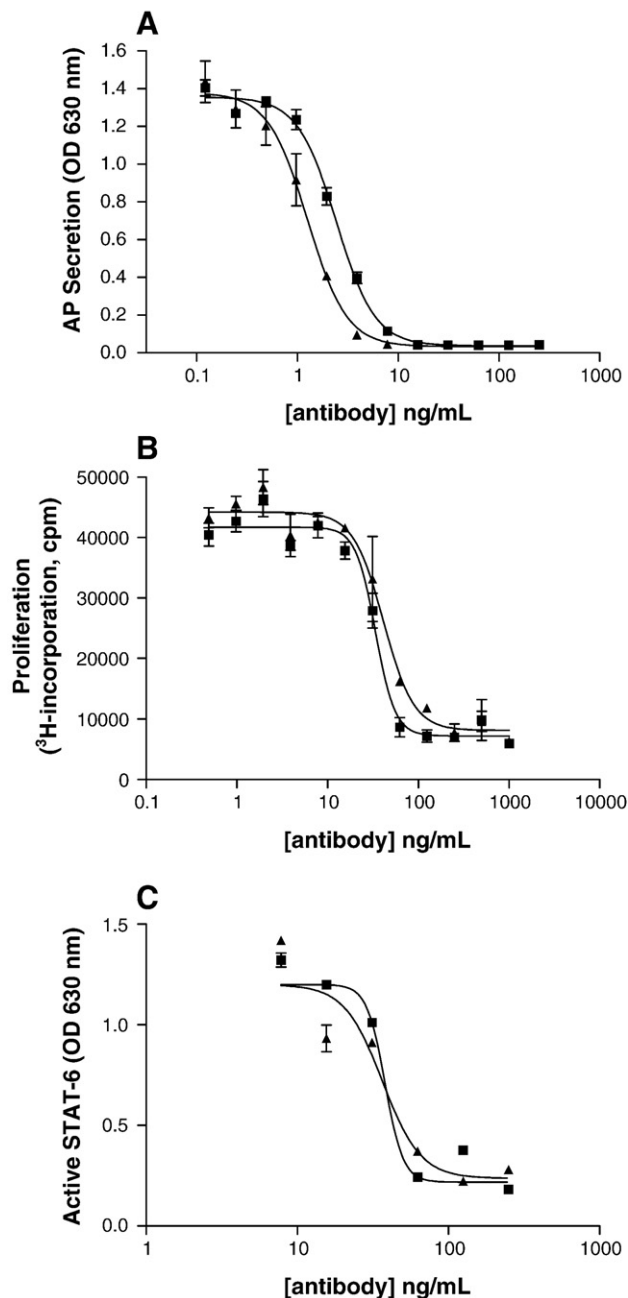


Fig. 3. (A) Effect of anti-mIL-13 mAbs A and B on mIL-13 bioactivity on human HEK-BLUE™ STAT-6 cells. Data show neutralisation of mIL-13-stimulated STAT-6 activity by mAb A (■) and mAb B (▲). Mean $\pm$ SEM, $n=6-12$ in triplicate. (B) Effect of anti-mIL-13 mAbs A and B on mIL-13 bioactivity on human TF-1 cells. Data show neutralisation of mIL-13-stimulated TF-1 cell proliferation by mAb A (■) and mAb B (▲). Mean $\pm$ SEM, $n=12$ in triplicate. (C) Effect of anti-mIL-13 mAbs A and B on mIL-13 bioactivity in mouse 3T3 fibroblasts. Data show neutralisation of mIL-13-stimulated STAT-6 activation by mAb A (■) and mAb B (▲). Mean $\pm$ SEM, $n=6$ in triplicate.

multiple test groups with a control group. $P < 0.05$ was considered significant.

3. Results

3.1. Inhibition of mIL-13 binding to mIL-13R α 1, mIL-13R α 2 and recruitment of mIL-4R α

Surface plasmon resonance (BIAcore 3000) was used to investigate the ability of neutralising mAbs generated using the UCB SLAM process to interrupt assembly of the high affinity IL-13 receptor complex. Anti-mIL-13 mAb A or B was captured on the surface of a CM5 sensor chip coated with F(ab')₂ goat anti-mouse Fc IgG. Recombinant mIL-13 was passed over the chip to allow capture by mAb A or B. Kinetic analysis revealed mAb A and mAb B bound to mIL-13 with high affinity, with dissociation constants (K_D) of 11 pM and 8 pM respectively. Binding of captured mIL-13 to mIL-13 receptor subunits added under flow, was subsequently measured. As shown in Fig. 1A, mAb A prevented binding of mIL-13R α 1-Fc to mIL-13. In contrast, mAb B allowed binding of mIL-13R α 1-Fc to mIL-13 but prevented subsequent binding of mIL-4R α -Fc (Fig. 1B). In addition to blocking mIL-13 binding to mIL-13R α 1, mAb A blocked mIL-13 binding to mIL-13R α 2 (Fig. 1C). In contrast, mAb B did not prevent binding of mIL-13 to mIL-13R α 2 (Fig. 1D). Sequential binding of IL-13R α 1 and IL-4R α to IL-13 captured by a non-neutralising mAb is shown in Fig. 1E. Studies were performed to investigate whether mAb B could block binding of mAb A to mIL-13. As expected, mAb A blocked binding of a Fab fragment of mAb A to mIL-13 (Fig. 2A). In contrast, mAb B did not block binding of a Fab fragment of mAb A to mIL-13 (Fig. 2B). These results demonstrate that mAbs A and B bind to distinct epitopes on mIL-13, and differentially interrupt assembly of the high affinity IL-13 receptor signalling complex.

3.2. In vitro neutralisation of recombinant murine IL-13 bioactivity

The neutralisation potency of mAbs A and B was determined using three different cell assay formats. Using the human HEK-BLUE™ STAT-6 cell line it was shown that mAbs A and B both neutralised the bioactivity of mIL-13 in a concentration-dependent manner; mAb A EC₅₀ 2.4 ng/mL, mAb B EC₅₀ 1.2 ng/mL (Fig. 3A). In addition, mAbs A and B inhibited proliferation of the human erythroleukemic cell line TF-1 stimulated by mIL-13; mAb A EC₅₀ 33.8 ng/mL, mAb B EC₅₀ 41.4 ng/mL (Fig. 3B). Finally, it was shown that mAbs A and B inhibited STAT-6 activation stimulated by mIL-13 in mouse NIH-3T3 fibroblasts; mAb A EC₅₀ 38.1 ng/mL, mAb B EC₅₀ 36.6 ng/mL (Fig. 3C). These results demonstrate that mAbs A and B

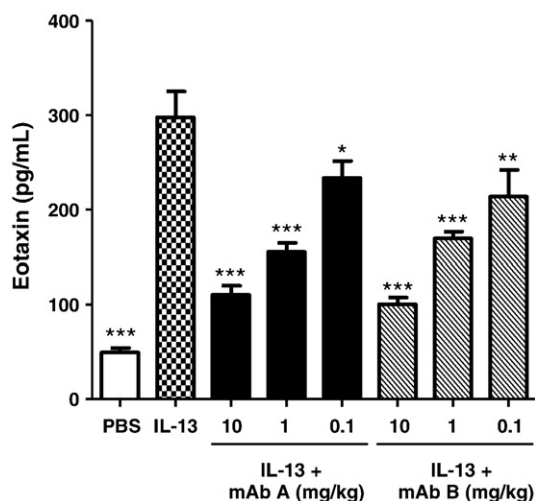


Fig. 4. Effect of anti-mIL-13 mAbs A and B on eotaxin generation stimulated by intraperitoneal mIL-13. Mean $\pm$ SEM, $n = 6-8$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to 0.3 μ g mIL-13, one-way ANOVA with Dunnett's post-test.

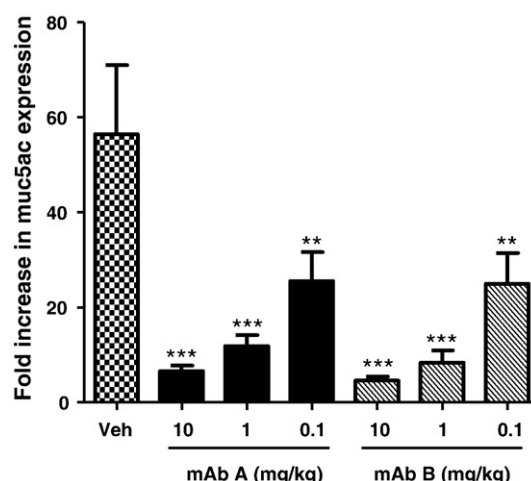


Fig. 5. Effect of anti-mIL-13 mAbs A and B on *muc5ac* mRNA upregulation in lung tissue following intranasal allergen challenge of sensitised mice. Mean $\pm$ SEM, $n = 9-11$. ** $P < 0.01$, *** $P < 0.001$ compared to vehicle, one-way ANOVA with Dunnett's post-test.

similarly neutralise the bioactivity of mIL-13, when signalling through either the mouse or human IL-13 receptor complex.

3.3. In vivo neutralisation of recombinant murine IL-13 bioactivity

The efficacy of mAbs A and B was investigated in an *in vivo* bioassay driven by mIL-13. IL-13 has been shown to stimulate production of the eosinophil chemoattractant eotaxin [18]. Intraperitoneal (i.p.) administration of 0.3 μ g mIL-13 stimulated an increase in eotaxin levels in the peritoneal cavity measured after 4 h. Mice were treated with mAb A or B (10, 1, 0.1 mg/kg), or vehicle (100 μ L PBS), intravenously (i.v.) immediately prior to i.p. administration of mIL-13. As shown in Fig. 4, mAbs A and B dose-dependently inhibited the increase in peritoneal eotaxin levels stimulated by mIL-13 (mAb A ED₇₅ 10.2 mg/kg; mAb B ED₇₅ 6.81 mg/kg).

3.4. In vivo neutralisation of endogenous IL-13 in a mouse allergic lung inflammation model

A model of allergic lung inflammation was used to determine the *in vivo* efficacy of mAbs A and B in a system driven by endogenous IL-13. IL-13 has been demonstrated to increase expression in the airways of the mucin-forming glycoprotein muc5ac [19]. Mice were sensitised to ovalbumin (days 0 and 7, s.c.) and subsequently challenged with ovalbumin on day 21 via the intranasal route. Levels of *muc5ac* mRNA in lung tissue were measured on day 23 using Taqman® RT-PCR. Mice were treated with mAb A or B (10, 1, 0.1 mg/kg) or vehicle (100 μ L PBS) s.c. immediately prior to intranasal challenge with ovalbumin. Intranasal ovalbumin challenge stimulated upregulation of *muc5ac* mRNA measured on day 23. MAbs A and B dose-dependently and equipotently inhibited upregulation of lung *muc5ac* mucin mRNA as shown in Fig. 5 (ED₇₅ mAb A 0.62 mg/kg; ED₇₅ mAb B 0.41 mg/kg). MAbs A and B did not have any effect on the number of inflammatory cells measured in the bronchial alveolar lavage fluid (BALF) at this time point (data not shown).

4. Discussion

The sequential assembly of the IL-13 receptor signalling complex offers at least two sites for pharmacological intervention. IL-13 initially binds to IL-13R α 1 with low affinity, forming the IL-13/IL-13R α 1 complex. The IL-4R α chain is subsequently recruited to form a high affinity receptor capable of signalling through STAT-6 [20]. To investigate this further, anti-murine IL-13 mAbs were generated using the UCB

SLAM process that by surface plasmon resonance, inhibited either mIL-13 binding to mIL-13R α 1 and mIL-13R α 2 (mAb A) or that inhibited binding of the mIL-13–mIL-13R α 1 complex to mIL-4R α (mAb B). Studies to assess the ability of mAbs A and B to neutralise mIL-13 demonstrated similar potencies in three types of *in vitro* cell assay, using both murine and human cells.

IL-13 is proposed to play a key role in the pathogenesis of severe asthma [1]. A key biological activity of IL-13 is to stimulate mucus cell hyperplasia and mucus secretion *in vitro* and *in vivo* [21,22]. It was therefore important to establish how the *in vitro* pharmacology of mAbs A and B translated into *in vivo* efficacy in a model system of allergic lung inflammation driven by endogenous IL-13. Allergen challenge of sensitised mice stimulated upregulation of *muc5ac* mucin mRNA in lung tissue that was dose-dependently inhibited by mAbs A and B. The *in vivo* data in a mouse model of allergic lung inflammation and from mice injected with recombinant mIL-13 to induce eotaxin generation, reported in this paper are consistent with the previously reported data in a non-human primate model of allergic lung inflammation [9,14]. Together these data suggest that key biological activities of IL-13 can be neutralised by blocking assembly of the high affinity IL-13 receptor signalling complex by two different mechanisms. Kasaian et al. [14] have described two anti-human IL-13 mAbs that interrupt assembly of the human IL-13 receptor signalling complex in an apparently identical manner as the anti-mouse mAbs A and B described herein. Combining these results, suggests that the mechanism of IL-13 receptor assembly is conserved between the murine and human systems.

IL-13R α 2 has been described as both a decoy receptor, inhibiting the activity of IL-13 in the mouse lung [23], and as a putative pro-fibrotic signalling receptor [13]. Soluble IL-13R α 2 is readily detectable in mouse plasma [24], but is not detectable in the plasma or bronchoalveolar lavage fluid from healthy or asthmatic human patients [25]. Interestingly, cell surface IL-13R α 2 on human fibroblasts has been shown to inhibit signalling of both IL-13 and IL-4 [26]. However, in mice IL-13R α 2 has also been proposed to mediate pro-fibrotic effects of IL-13 in the colon [13]. The role of IL-13R α 2 therefore remains to be conclusively defined, but there is clear evidence that it is differentially regulated in mice and humans. Using mAbs that differentially block IL-13 binding to IL-13R α 2, we observed no difference in their pharmacological profile using *in vivo* model systems of chemokine generation or mucus mRNA upregulation. Our results are consistent with those reported by Munitz et al. [27] who demonstrated that mucus gene upregulation in a mouse model of allergic lung inflammation is dependent on IL-13R α 1. Further studies to investigate the role of IL-13R α 2 in allergic lung inflammation models driven by house dust mite, an aeroallergen shown to cleave IL-13R α 2 from the cell surface [28], and that stimulates robust fibrosis, angiogenesis and airways hyperreactivity in mice [29], are planned.

In summary, we have directly compared the *in vitro* and *in vivo* pharmacological profiles of two anti-mIL-13 antibodies that neutralise mIL-13 and disrupt the assembly of the high affinity IL-13 receptor complex by two distinct mechanisms. These high affinity neutralising antibodies have similar *in vitro* potency as well as identical efficacy *in vivo* in mice, in models driven by recombinant and endogenous murine IL-13. These data support the design of therapeutic antibodies for the treatment of allergic airway diseases such as asthma, that inhibit assembly of the high affinity IL-13 receptor signalling complex, irrespective of whether they block binding of IL-13 to IL-13R α 1 and IL-13R α 2, or the subsequent recruitment of IL-4R α . It remains to be seen how these preclinical observations translate into clinical efficacy in patients with allergic lung inflammation.

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