

Murine IL-4 Δ 2 splice variant down-regulates IL-4 activities independently of IL-4R α binding and STAT-6 phosphorylation



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ABSTRACT

IL-4 is a pleiotropic cytokine that is highly Th2 polarizing. The ratio of IL-4 and its splice variant IL-4 Δ 2 observed in human health and disease suggests a role for both isoforms. In the present study, the biological function of murine IL-4 Δ 2 and the potential mechanism of action were studied. We report for the first time the generation of a functional, recombinant murine IL-4 Δ 2 form which is suggestive of its possible biological role in this species. Recombinant murine IL-4 Δ 2 inhibited IL-4 mediated cellular processes in macrophages and lymphocytes. Specifically, (i) it reversed IL-4 mediated inhibition of IFN- γ induced nitric oxide release by macrophages, (ii) inhibited IL-4 mediated induction of T cell proliferation, and (iii) prevented IL-4 stimulation of IgE synthesis by B cells. However, IL-4 Δ 2 did not compete with IL-4 for IL-4R α binding and did not interfere with the downstream STAT-6 phosphorylation in T cells, suggesting an alternative mechanism for its antagonism of specific IL-4-driven effects. These findings suggest that the mouse is a suitable experimental model for studies of the biology of IL-4 and its alternative splice variant.

1. Introduction

IL-4 is a pleiotropic cytokine with a wide range of functions [1]. It can induce proliferation of activated B cells [2,3], T cells [4], thymocytes and natural killer cells [5] and it also has mast cell stimulating activities [3,4]. It has regulatory functions in B cells [6], T cells [7], macrophages [8] and other haematopoietic and non-haematopoietic cells [9]. IL-4 is known to up-regulate the expression of IL-4 receptor (IL-4R) [10] and it also controls proliferation, apoptosis and expression of an array of genes in a range of cell types such as lymphocytes, macrophages, fibroblasts and endothelial cells [11–17]. However, arguably the most prominent biological function of IL-4 is the role it plays in the Th phenotypic balance of lymphocytes [18], specifically in promoting Th2 responses [11–17,19–21], whilst inhibiting Th1 [19] and Th17 responses [22].

The IL-4 gene comprises four exons, with exon 2 being the shortest containing 48 base pairs that encode 16 amino acids. The full-length native protein is encoded by all four original exons [23,24]. In 1996, it was shown by direct sequencing of the human IL-4 transcript that removal of exon 2 by RNA splicing could also occur [25]. The identified transcript and resultant protein were termed IL-4 Δ 2 [25–28]. The IL-4 Δ 2 splice variant is found in lymphocytes from humans, chimpanzees, cynomolgus macaques, rabbits, woodchucks [29], cattle [30] and mice

[31]. Though the mRNA is only quantifiable in the spleen and bone marrow of mice [31], it is suggested that the IL-4 Δ 2 protein is functional in this species [32,33]. The fact that IL-4 Δ 2 has been identified across a number of mammalian species suggests that this molecule may play an important regulatory role, albeit one that is poorly understood at present.

The main studies investigating IL-4 Δ 2 in the context of disease have focused on its role in asthma, scleroderma and tuberculosis (TB) [34], but differences between IL-4 and IL-4 Δ 2 mRNA ratios in patients and healthy controls have also been identified in *Fasciola hepatica* [30] and *Helicobacter pylori* [35,36] infections and in sepsis [37]. However, only in asthma studies was the expressed protein of both variants assessed [34].

A number of studies have elucidated the role of human IL-4 Δ 2 by means of cellular assays using recombinant protein [25–27,38] and *in vivo* gene delivery [32,33]. In cell culture assays, recombinant IL-4 Δ 2 had no autonomous effect on T cell colony expansion; however it was capable of inhibiting IL-4 mediated proliferation. It had no effect on human monocytes with regards to the production of IFN- γ , IL-1, IL-6, IL-8, MCP-1, CD14, CD23, CD16 or TLR2 [27]. However, IL-4 Δ 2 was shown to down-regulate production of full-length IL-4 by Th2 differentiated cells [1] and reversed human IL-4 inhibition of LPS-induced COX-2 expression and consequent PGE2 secretion by monocytes. It also

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blocked IL-4 mediated expression of CD23 and secretion of IgE by B cells [26].

Here, we set out to investigate the role of the murine version of the IL-4Δ2 molecule, to establish whether it too antagonises the full-length IL-4 mediated processes. Furthermore, the target receptor subunit and downstream signalling were interrogated to identify potential mechanisms of its action. Demonstration of a functional IL-4Δ2 molecule in mice would provide a further insight into the biology of this molecule and facilitate studies of its role in disease pathogenesis and the potential for therapeutic applications.

2. Material and methods

2.1. Recombinant IL-4Δ2

Recombinant murine IL-4Δ2 (rm-IL-4) was produced in *E. coli* and supplied by Biobasic (Canada). The reported molecular weight was 11.8 ± 1 kDa, the preparation was > 95% pure and the endotoxin level was < 0.1 EU/μg. The product was lyophilized from a 0.2 μm filtered concentrated solution in 0.1% TFA, 40% acetonitrile and reconstituted in appropriate buffers prior to use.

2.2. Protein analysis – SDS-PAGE & Western blot

Recombinant protein samples plus Laemmli buffer were run on Invitrogen 12%, NUPAGE Bis-Tris gels with MOPS (Invitrogen) running buffer. Instant Blue (Sigma) was used for staining the gel or the proteins were transferred onto nitrocellulose for Western blot analysis as previously described [39]. Briefly, the membrane was blocked with Tris-buffered saline, pH 7.5 (TBS) 5% (v/v) milk/0.1% (v/v) Tween₂₀, and incubated with anti-mouse IL-4 (mIL-4) goat antibody (Polyclonal, IgG, AF404R & D Systems, Minneapolis, USA) at 1/200 dilution, for 1 h at room temperature. The secondary antibody, polyclonal anti-goat IgG (whole molecule) – peroxidase conjugated antibody produced in rabbit, (A5420, Sigma) was used at a 1/1000 dilution and applied to the membrane for 30 min at room temperature. After washing and addition of peroxidase substrates (ECL, Amersham), the membrane was visualised using the Syngene camera and software (Syngene).

2.3. Macrophage nitric oxide assay

J774 and MHS cells (ATCC) were grown in tissue culture flasks in DMEM and RPMI (Invitrogen), respectively, supplemented with 10% FBS, 2 mM L-glutamine and Penicillin (100 units/ml)/Streptomycin (100 μg/ml) at 37 °C, 5 % CO₂, until 70–90% confluent. Cells were seeded in a 96-well flat bottom tissue culture plates (Falcon, Becton Dickinson) at 5.5×10^4 cells/well and incubated overnight. Cells were then incubated with either medium alone, IL-4 (Sigma) or IL-4Δ2, or IL-4 + IL-4Δ2 for 48 h prior to the transfer of 100 μl of supernatant to an ELISA plate, and the addition of an equal volume of Griess Reagent (Sigma). For this and all other cell culture assays the antagonist was added first to the culture. Plates were read at 560 nm using the Promega GloMax Multi+.

2.4. T-cell proliferation assay

HT2 murine T cell line (ATCC) was grown in RPMI-1640 medium supplemented with 5 ng/ml IL-2 and 50 μM beta-mercaptoethanol (GIBCO) (R10). 96-well tissue culture plates (Falcon, Becton Dickinson) were seeded at 2×10^5 cells/well and following addition of different cytokine combinations. Cells were pulsed with 1 mCi of ³H-thymidine (PerkinElmer, Wallac, UK) for 24 h. Cells were harvested with the Harvester 96 (TomTec Life Sciences, USA) onto Printed Filtermat paper (PerkinElmer) prior to the addition of melted wax (MeltiLex TM A, PerkinElmer) onto each scintillation sheet. Radioactive counts per minute (cpm) were measured using a scintillation counter

(PerkinElmer). As a control, cells cultured with IL-2 (R & D Systems) were co-incubated with increasing concentrations of IL-4Δ2 and the viability assessed by trypan blue staining and counting with a haemocytometer.

2.5. B-cell culture and IgE assay

Spleens from Balb/c mice were aseptically recovered into sterile R10 medium, placed into a 70 μm cell strainer and homogenized with a syringe plunger. T cells were removed by using anti-mouse CD90 (Thy-1) (eBioscience,) at 2.5 μg/ml and baby rabbit complement (Bio-Rad), followed by separation of B cells on Lympholyte M. B-cell purity was assessed by CD19 expression and determined to be > 84%. Cells were seeded at 10^6 /ml in tissue culture plates before stimulation. Where IL-4Δ2, this was the first stimuli to be added to the cells. IgE in the supernatant was measured by ELISA (Ready Set Go, eBioscience).

2.6. Surface plasmon resonance assays

Surface plasmon resonance assays were performed on a Biacore X100 instrument (GE). Protein A (Sigma) was immobilised on both cells of a CM5 sensor chip using amine-coupling method (GE Healthcare). Protein A was used to capture the IL-4Rα Fc Chimera (R & D Systems) ligand on flow cell 2, while flow cell 1 containing protein A only served as a reference surface. Murine IL-4 (Biolegend) at 20, 40 and 80 nM were tested in a multi-cycle assay with a flow rate of 100 μl/s, a contact time of 40 s and a dissociation time of 180 s. Recombinant murine IL-4Δ2 was also tested as an analyte in a separate assay and was used at concentrations of 1500, 3000 and 6000 nM in a multi-cycle assay with a flow rate of 60 μl/s, a contact time of 80 s and a dissociation time of 180 s. For the competition assay, murine IL-4 (Biolegend) at 30 nM was pre incubated at room temperature for 30 min with increasing concentrations of IL-4 Δ2: 30, 1500, 3000 and 6000 nM. The pre-mixed analytes or IL-4 on its own at 30 nM as a control were then applied over immobilized IL-4Rα. The flow rate was 30 μl/s with a contact time of 120 s and a dissociation time of 300 s. Regeneration of the chip was achieved with 10 mM glycine pH 2.0. The samples were dissolved in running buffer (10 mM HEPES, 150 mM NaCl, 0.005% P20, pH 7.4) and the interaction was studied at a temperature of 25 °C. Obtained responses were corrected by the subtraction of the blank and the reference signal from a flow channel without captured receptor (double referencing), and analysed with Biacore™ X100 Evaluation Software Version 2.0.1.

2.7. IL-4Rα binding ELISA

ELISA plates (Nunc Maxisorp) coated overnight with 5 μg/ml recombinant mouse IL-4Rα Fc Chimera (R & D Systems) were blocked with 300 μl of PBS 5% milk for a 1 h 30 min. IL-4 and IL-4Δ2 were added at 1 μg/ml initial concentration with 1/10 serial dilutions and incubated for 1 h 30 min at 37 °C. Biotinylated anti-IL-4 antibody, (BVD6-24G2, Biolegend), at 1 μg/ml was used as detection antibody. After washing, 100 μl of 0.2 μg/ml of streptavidin peroxidase (Sigma) was added. The plate was then washed for a final time, and the peroxidase reaction developed by the addition of 100 μl of SIGMAFAST™ OPD substrate to each well. Plates were incubated in the dark for 15 min and read using a Tecan plate reader at 450 nm.

2.8. Intracellular measurement of phosphorylated STAT-6 by flow cytometry

Viability of HT2 cells was determined by the addition of trypan blue and the cell counts performed with a dual chamber counting slide (Bio-Rad) and read on the TC 20™ automated cell counter (Bio-Rad). 1×10^6 live HT2 cells were transferred to 5 ml polystyrene round bottom tubes. Cells were subjected to one of the following treatments:

IL-4, IL-4Δ2, IL-4 with IL-4Δ2, or medium alone. Cells were incubated at 37 °C for 15 min before the addition of 1.5% paraformaldehyde (Sigma) and a further 15 min incubation. Cells were then centrifuged at 400g for 5 min and placed on ice. Ice-cold 90% methanol was then slowly added to a total volume of 1 ml and the suspension was incubated for 30 min on ice. Cells were washed with 2 ml of cold PBS two times and re-suspended in 100 μl PBS 2.5% FBS. Anti-mouse STAT-6 (pY641) PE Clone 18/P-STAT-6 (BD Biosciences Phosflow™) antibody was added to the cells at 1/100 dilution and incubated in the dark at 4 °C for 45 min. Suspensions were topped up with 2 ml flow cytometry buffer (PBS 0.5 % BSA 0.01% Na-azide) and centrifuged at 400g for 5 min. Cells were re-suspended in flow cytometry buffer and acquired on a BD FACSCalibur.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software Inc.) software. Details of the tests used and significance levels are detailed in the results.

3. Results

3.1. Recombinant IL-4Δ2 generation

The recombinant murine IL-4Δ2 generated in *E. coli* was analysed by SDS-PAGE with Coomassie staining, and Western blotting (Fig. 1). Recombinant murine IL-4 (Sigma) was used for comparison, and was detected as a 16 kDa protein band. IL-4Δ2 was visualised as a single 12 kDa protein band consistent with the missing exon 2. Confirmation of the specificity of the band was carried out by a Western blot probed with an anti-IL-4 antibody, revealing both IL-4 and IL-4Δ2. Interestingly, both IL-4 and IL-4Δ2 were detected more weakly under non-reducing than reducing conditions, possibly due to presence of higher molecular forms.

3.2. Recombinant murine IL-4Δ2 antagonises IL-4-induced nitric oxide production

We next carried out a number of cellular assays to evaluate the role of recombinant murine IL-4Δ2, and more specifically, its ability to antagonise full length murine IL-4 as previously reported for the human molecule [26,27]. Firstly, IL-4Δ2 was tested for its ability to antagonise

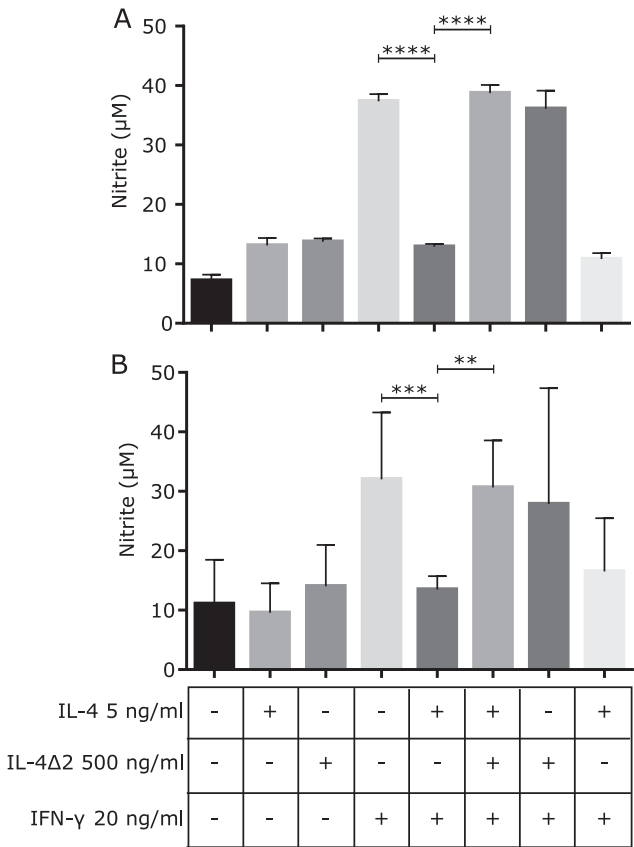


Fig. 2. IL-4Δ2 reverses the IL-4 mediated inhibition of IFN-γ induced nitric oxide in murine macrophage cells. (A) MHS and (B) J774 cells were stimulated with either IL-4 (A) or IL-2 (B) and the amount of nitric oxide produced measured. The bars represent the mean nitric oxide concentration for each treatment + SEM (n = 6). Data was analysed using a one-way ANOVA and a Tukey's multiple comparison test and significance levels are indicated as follows: * P ≤ 0.05, ** P ≤ 0.001, *** P ≤ 0.0001 and P ≤ 0.00001. A number of less important comparisons were omitted to improve clarity. Experiment was repeated three times with a similar result.

processes mediated by full-length IL-4 in macrophages. Stimulation of the murine alveolar macrophage cell line MHS with 20 ng/ml of IFN-γ induced high levels of nitric oxide as measured by the addition of Griess

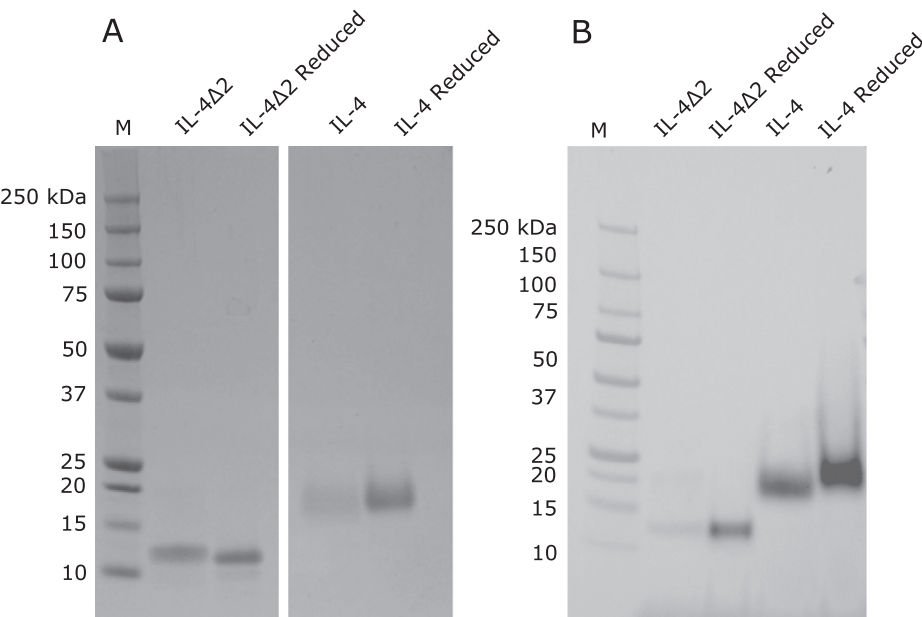


Fig. 1. Analysis of *E. coli* produced murine IL-4Δ2. Samples were run on an Invitrogen NUPAGE Bis-Tris 12 % SDS-PAGE gel showing from left to right, *E. coli* derived murine IL-4Δ2, and murine IL-4 (Sigma) used as a control. All samples were run under non-reducing and reducing conditions. (A) Coomassie stain; each sample contained 3 μg of protein. (B) Anti-IL-4 Western blot analysis of IL-4Δ2; samples (200 ng) were transferred to a nitrocellulose membrane and probed with anti-IL-4 antibody. M, molecular weight standards.

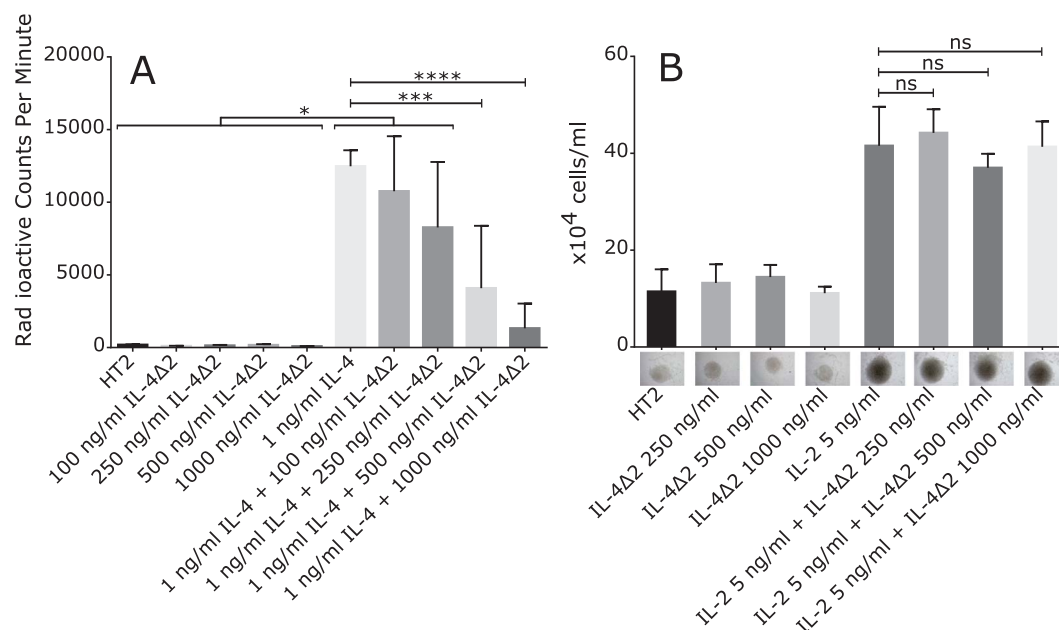


Fig. 3. IL-4Δ2 inhibits IL-4 induced HT2 cell proliferation. (A) HT2 proliferation assay dose response. HT2 cells were stimulated with 1 ng/ml of IL-4 and an increasing concentration of IL-4Δ2, 100–1000 ng/ml, and the proliferation levels measured by means of tritiated thymidine uptake expressed as radioactive counts per minute. The bars represent the mean radioactive counts per minute for each culture + SEM (n = 6). Data were analysed using a one-way ANOVA and a Tukey's multiple comparison test. Cultures showing significant differences are indicated * $P \leq 0.05$, *** $P \leq 0.001$ and **** $P \leq 0.0001$ P. Experiment was repeated five times with a similar outcome. (B) IL-4Δ2 has no effect on IL-2 mediated proliferation. HT2 cells were stimulated with 5 ng/ml of IL-2 and between 250 and 1000 ng/ml IL-4Δ2 after which the cells were counted. The bars represent the mean cell count for each group + SEM (n = 4). Below each bar is an image of the cell cluster present in a representative well for each experimental parameter. Data were analysed using a one-way ANOVA and a Tukey's multiple comparison test; ns = non-significant. A number of comparisons have been omitted for clarity.

reagent to the supernatant (Fig. 2A; mean $37.4 \mu\text{M} \pm 0.5$ SEM). The addition of 5 ng/ml IL-4 inhibited the IFN- γ induced nitric oxide to close to background levels (mean $12.9 \mu\text{M} \pm 0.2$ SEM). However, the addition of recombinant murine IL-4Δ2 reversed the IL-4 mediated inhibition and restored IFN- γ induced nitric oxide production in a dose-dependent manner, although amounts of the order of 100-fold excess were required to induce suppression. Treatment with IL-4Δ2 alone had no effect on cellular nitric oxide production. A similar pattern was observed with the murine macrophage cell line J774 (Fig. 2B).

3.3. Recombinant murine IL-4Δ2 antagonises IL-4-induced T- cell proliferation

Secondly, the effect of recombinant murine IL-4Δ2 on IL-4 induced T cell proliferation was investigated (Fig. 3A). As expected [40] stimulation of HT2 cells with 1 ng/ml of IL-4 induced significant proliferation (mean $12476 \pm \text{SEM } 550.6$). The addition of IL-4Δ2 inhibited IL-4 dependent proliferation in a dose dependent manner, with statistically significant reductions observed with high doses of IL-4Δ2, whereas none of the tested concentrations of recombinant murine IL-4Δ2 alone had any effect on the proliferation of HT2 cells.

To exclude induction of apoptosis or cytotoxicity by IL-4Δ2 as alternative explanations of this effect on cell proliferation, cells were cultured for 24 h with IL-2 and IL-4Δ2, at concentrations up to 1000 ng/ml (Fig. 3B). The mean viable cell count of untreated HT2 cells (8×10^4 cells/ml) was not significantly changed following addition of IL-4Δ2 at concentrations 250–1000 ng/ml. However, the cell number more than tripled when stimulated with 5 ng/ml of IL-2; this could not be reversed by the addition of increasing concentrations of IL-4Δ2, consistent with the absence cytotoxic or pro-apoptotic effect of IL-4Δ2 on these cells.

3.4. Recombinant murine IL-4Δ2 antagonises IL-4-induced B cell IgE production

Having observed apparent IL-4Δ2 antagonism of IL-4 mediated processes on macrophages and T cells, we next interrogated the potential of IL-4Δ2 to antagonise B cell IL-4 induced IgE synthesis [26]. Splenocytes from Balb/c mice were B cell enriched using anti-mouse CD90 (Thy-1) and baby rabbit complement in order to reduce confounding effects resulting from the presence of other cell types. In addition to IL-4, another secondary synergising B cell signal is required for IgE class switching, so cell cultures were supplemented with anti-CD40 FGK45.5 IgG2a (Miltenyi Biotec). Cells were then stimulated with or without IL-4Δ2 and the supernatant harvested for quantification of IgE by ELISA (Fig. 4). When cultured with media alone or supplemented with anti-CD40 mAb alone, B cell splenocytes failed to produce any detectable IgE in the culture supernatant. Similarly, cells stimulated with IL-4Δ2 up to 1000 ng/ml did not produce any quantifiable IgE. In contrast, supernatants from cells stimulated with IL-4 and anti-CD40 mAb were found to contain substantial levels of IgE. The addition of IL-4Δ2 at 500 ng/ml or 1000 ng/ml reduced the levels of IL-4 induced IgE by approximately 50 %, at three different dilutions of culture supernatant (Fig. 4).

3.5. Mechanisms of interaction of IL-4Δ2 with IL-4 and IL-4R α

Having observed that IL-4Δ2 could antagonise IL-4 mediated cellular processes *in vitro*, experiments were conducted to elucidate the mechanism of its action. IL-4 binds to the IL-4 receptor complex with high affinity almost entirely facilitated by the IL-4R α chain. It has been suggested that human IL-4Δ2 specifically interacts with the α chain of IL-4R, competing with IL-4 for the binding sites [41] without inducing signal transduction. The capacity of mIL-4R α to bind mIL-4 or mIL-4Δ2 was assessed by Biacore assay.

The experimental set up relied on non-covalent attachment of IL-4R α on a protein A coated CM5 chip. This capture strategy utilised the

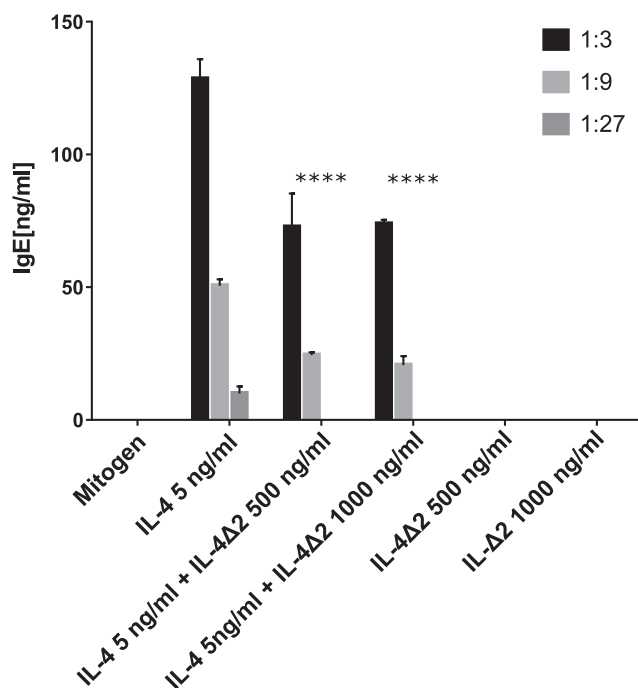


Fig. 4. IL-4Δ2 reduces the levels of IgE in primary B cells cultures following IL-4 stimulation. Isolated splenic B cells were stimulated with IL-4 and IL-4Δ2 in the presence of a CD40 agonist. Culture supernatants were recovered and 3-fold IgE titres measured by an ELISA. The bars correspond to the mean + SEM (n = 3), each shade represents a dilution, 1:3, 1:9 and 1:27. The datasets were compared using a one-way ANOVA and a Tukey's multiple comparison test; relevant treatment groups showing significant differences to the control (IL-4 5 ng/ml) are indicated with ****, $P \leq 0.0001$. Experiment was performed twice with similar results.

Fc chimera region on the recombinant protein. The flat line prior to the zero time point on the sensorgram indicated that the protein A-receptor complex was stable (Fig. 5A). Both IL-4 variants were then flowed over the chip as analytes. High affinity binding of mIL-4 at 20 nM concentration was observed as expected (Fig. 5A), with a K_d of 6.8×10^{-10} , while IL-4Δ2 at the same concentration failed to bind, and only showed measurable binding at concentrations of > 3000 nM (Fig. 5B). The observed response at high concentrations of IL-4Δ2 was characterised by very fast association and dissociation kinetics resulting in a modelled K_d of 4.028×10^{-5} M. However, due to the high concentrations of analyte used in this assay, this low affinity interaction could have been also due to nonspecific protein aggregation. To further test this, an IL-4Rα capture ELISA was performed, where an interaction with IL-4 was recorded but no interaction was detected between IL-4Δ2 and IL-4Rα (Fig. 5D). A competition assay (Fig. 5C), showed that IL-4Δ2 at up to 200-fold excess did not disrupt binding of 30 nM IL-4 to the immobilized receptor. Increasing concentrations of IL-4Δ2 (30, 1500, 3000 and 6000 nM) were pre-incubated with 30 nM IL-4 prior to applying over immobilized 4Rα but no disruption of IL-4 binding was observed. Only 6000 nM IL-4Δ2 slightly altered the binding curve but this could be due to non-specific surface binding of IL-4Δ2, leading to steric hindrance of IL-4 binding and affecting the primarily on-rate.

Therefore, although the Biacore assay showed some evidence of binding, the data taken together do not provide a conclusive evidence for the interaction between IL-4Δ2 and IL-4Rα, making it unlikely that IL-4Δ2 exerts its antagonistic effects primarily or only through competition for receptor binding.

3.6. STAT-6 phosphorylation

We therefore looked for alternative mechanisms of action of IL-4Δ2. As several cytokine receptors recruit the common gamma chain and signal through the JAK-STAT pathway, we next examined if exposure to

IL-4Δ2 could modulate the IL-4 induced levels of phosphorylated STAT-6 in murine HT2 cells. HT2 cells in culture were subjected to a range of cytokine treatments and Tyr641 phosphorylation was measured after 15 min. Background levels of STAT-6 phosphorylation in unstimulated cultures were determined to be less than 1% positive cells (Fig. 6, upper left panel). The addition of 0.5 ng/ml of IL-4 increased the percentage of STAT-6 phosphorylated cells to 44.5%. The increase of IL-4 concentration to 20 ng/ml only marginally increased that percentage to 50.3% (upper panels). The presence of increasing concentrations of IL-4Δ2 had no effect on the phosphorylation of STAT-6 in HT2 cells. The recorded % of STAT-6 phosphorylated cells in the presence of 1000 ng/ml of IL-4Δ2 was 1.03%. The addition of IL-4Δ2 to IL-4 stimulated cells did not result in a significant reduction in the percentage of STAT-6 phosphorylated cells (41.4 and 41.5%, respectively).

4. Discussion

Several previous reports have demonstrated that recombinant human IL-4Δ2 is capable of inhibiting IL-4 mediated processes *in vitro* [26,27,41]. Here we provide evidence that the murine counterpart may have a similar role [42,43]. Specifically, we show that murine IL-4Δ2 was able to antagonise the inhibitory effect of IL-4 on IFN-γ-mediated nitric oxide production in murine macrophages. These results parallel those reported by Arinobu et al. with human IL-4Δ2 where it was demonstrated that rhIL-4Δ2 was capable of antagonising IL-4 mediated inhibition of LPS induction of prostaglandin E2 (PGE2) [26].

IL-4 is known to be a strong inducer of Th2 polarization and induces proliferation of these cells. To test whether mIL-4Δ2 is able to antagonise IL-4 mediated Th2 processes, the HT2T-cell proliferation assay was performed. In a direct measurement of IL-4Δ2 activity, murine IL-4Δ2 was shown to antagonise IL-4-induced proliferation of these cells. No significant effect was observed with IL-2 induced proliferation, indicating the specificity of the effect. This antagonism of IL-4 was dose sensitive and relatively high amounts of IL-4Δ2 were needed to observe inhibition. Again, these observations mirrored those seen in studies with human IL-4Δ2 [27]. The function of IL-4 in B cell differentiation to IgE phenotype has been studied previously [44]. In the present study, production of IgE was also shown to be stimulated by IL-4 in a dose dependent manner. IL-4Δ2 did not induce detectable amounts of IgE itself but antagonised IL-4 mediated secretion at concentrations about 100-fold in excess of IL-4.

The antagonistic effects of IL-4Δ2 observed here and reported previously for the human version [26,27] suggest a regulatory role for IL-4Δ2 in IL-4 signalling. It has been proposed that IL-4Δ2 antagonises IL-4 by competitively binding to a shared receptor. Studies using iodinated IL-4Δ2 showed that the molecule was capable of binding to human T and B cells, their interaction being dose dependent and unlabelled IL-4Δ2 in excess inhibited binding of the labelled molecule. Moreover, titrated doses of unlabelled IL-4 were able to inhibit binding of labelled IL-4Δ2, indicating that these two molecules may share a common receptor [27]. Similarly, it was shown that binding of radiolabelled IL-4Δ2 to human monocytes was inhibited in a dose dependent manner by unlabelled IL-4 [26].

In the light of the apparent biological effects of IL-4Δ2 in the cellular assays described above, the next step in this study was to question the mechanism by which this molecule acts. To do so, we first studied the interactions at a receptor level. We performed Biacore™ measurements of murine IL-4 and IL-4Δ2 binding to IL-4Rα. As observed for human IL-4 [45], murine IL-4 associated at exceedingly high rate with IL-4Rα, and despite the use of high flow rates (100 ul/min), significant mass transport limitations were still encountered. In contrast, no strong interaction between IL-4Δ2 and IL-4Rα chain was observed. A possible low affinity interaction characterised by rapid association and rapid dissociation was reflected in a visible shift in the sensorgram curve when 3000 and 6000 nM IL-4Δ2 were applied over the channel. Analysis in the BIAeval software suite yielded a steady state affinity K_D

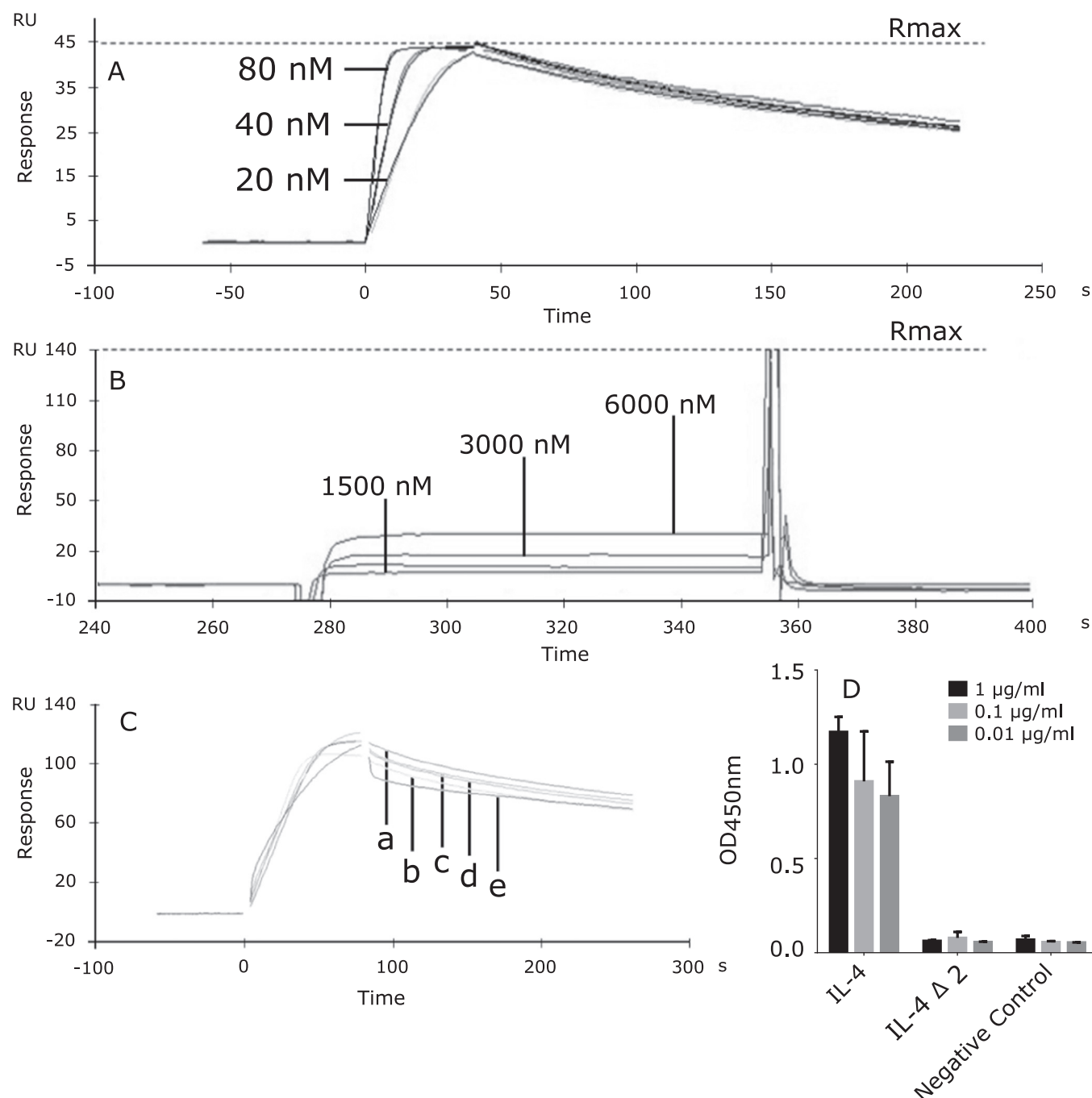


Fig. 5. IL-4Rα interactions with recombinant rmIL-4 and rmIL-4Δ2. (A) Kinetics of IL-4 binding to IL-4Rα by Biacore. Overlay plot showing the IL-4Rα binding of IL-4 (20, 40 and 80 nM). The 'y' axis depicts the response on the sensorgram presented as relative units (RU) and the 'x' axis corresponds to the injection time in seconds (s). A 1:1 steady state affinity binding model was fitted. (B) Biacore analysis of IL-4Rα interactions with rmIL-4Δ2. Interactions of IL-4Δ2 (3000, 6000 and 12000 nM) with IL-4Rα captured by a protein A coated CM5 chip. The dotted line represents maximal expected binding based on the amount of the receptor immobilised. Experiment was performed twice with similar results. (C) Biacore analysis of IL-4 binding to IL-4Rα. Overlay plot showing the IL-4Rα binding of IL-4 (a; 30 nM) and IL-4 (30 nM) pre-incubated with increasing concentrations of IL-4Δ2 (b; 30 nM, c; 1500 nM, d; 3000 nM and e; 6000 nM). (D) IL-4Rα capture ELISA of IL-4 and IL-4Δ2. Detection levels of IL-4 and IL-4Δ2 captured by an IL-4Rα coated ELISA plate and detected with the anti-IL-4 biotinylated BVD6-24G2. The cytokines were assayed at 1, 0.1 and 0.01 μg/ml; PBS was used as a negative control. The bars represent mean absorbance for each measurement at 450 nm + SEM (n = 3). Experiment was performed twice with similar results.

however, the used IL-4Δ2 concentrations were substantially lower than the calculated K_D , rendering the estimation potentially unreliable. Notwithstanding this experimental limitation, the low level affinity binding detected in the assay is within the range of other similar studies, where, for example, it was reported that a synthetic peptide QR8 (comprised of a sequence recognized by a neutralizing murine IL-4 antibody, 11B11) bound to murine IL-4Rα with an affinity constant of 8.77×10^{-4} M [46]. The concentration range tested in Yao et al. study

[46] was much greater than could be tested in the present study due to formulation limitations and artefacts caused by high concentrations of protein in the Biacore assays. We also generated data that suggested that the anti-mouse-IL-4 clone BVD6-24G2 can bind both isoforms whilst the 11B11 clone detected only the full-length molecule. The identification of a discriminating antibody could prove to be useful clinically and would allow for more accurate studies investigating the presence of both proteins in their native form.

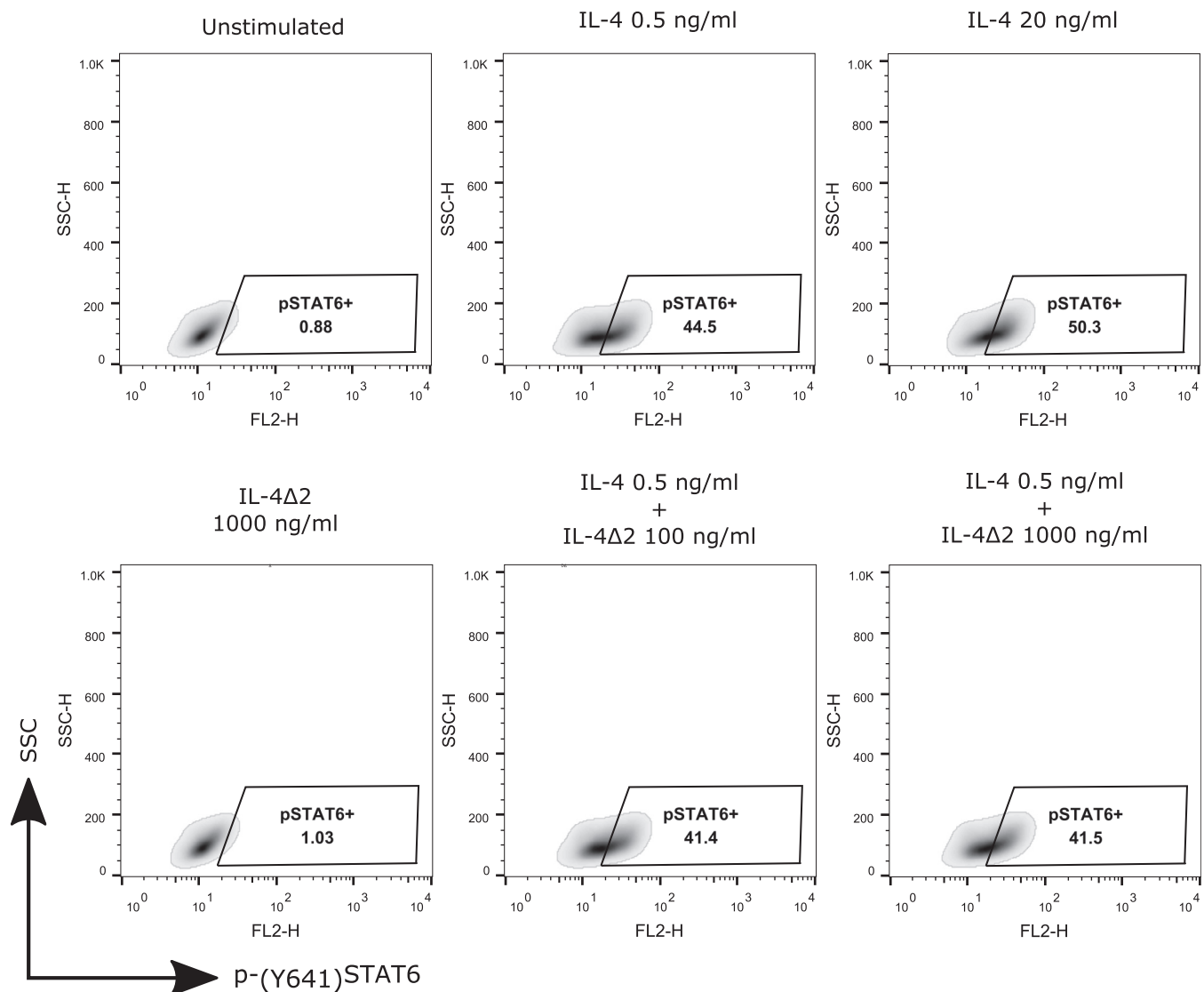


Fig. 6. The effect of IL-4 and rIL-4Δ2 on p-(Y641)STAT6 in HT2 cells. HT2 cells were stimulated for 15 min with 0.5–20 ng/ml IL-4, 1000 ng/ml IL-4Δ2 or 1 ng/ml IL-4 + IL-4Δ2 100–1000 ng/ml. Cells were then fixed and assessed for intracellular STAT-6-Y641 phosphorylation by flow cytometry. SSC is shown plotted against p-(Y641)STAT6; gates for pSTAT+ (positive phosphorylation events) were determined by an unstained control. Experiment was performed twice with similar results.

The interaction of both isoforms of human IL-4 with IL-4Rα has been studied by using the M1 antibody [32], which binds to IL-4Rα [47]. The authors of this study suggested that IL-4Δ2 had lower affinity to IL-4Rα than IL-4 based on competition with the M1/IL-4Rα interaction in a flow cytometry assay. On close inspection, full length IL-4 hindered M1 antibody binding to IL-4Rα whereas IL-4Δ2 did not [32]. This is more suggestive of the absence of binding of IL-4Δ2 to IL-4Rα or a very low affinity interaction, which nevertheless could be capable of inducing biological activity in cellular or *in vivo* assays but could not be detected in a flow cytometry assay. Furthermore, *in vivo* studies utilising IL-4Rα knockout mice demonstrated that a number of IL-4Δ2 mediated processes were, to some degree, IL-4Rα dependent, in that observations seen in wild-type mice were not seen in the knockout counterparts [32]. In summary, more comprehensive and in-depth studies using more sensitive assays are needed to fully understand how IL-4Δ2 signals at a cellular receptor level.

In keeping with previous studies [48], IL-4 was readily able to induce saturating levels of STAT-6 phosphorylation in HT2 cells. Importantly, IL-4Δ2 was unable to inhibit this process, or even induce STAT-6 phosphorylation when administered independently. Our findings therefore corroborate those of Luzina et al. who demonstrated that

IL-4Δ2 does not signal through STAT-6 *in vitro* [33]. However, IL-4 does not exclusively signal through STAT-6 phosphorylation, so the involvement of IL-4Δ2 may be elsewhere in the signal transduction cascade or in non-canonical signalling pathways. A previous study suggested that IL-4Δ2 had little effect in cell culture assays, only affecting cytokine expression to a very small degree [34]. Consequently, little is known about signal transduction in relation to the splice variant IL-4Δ2, which is exacerbated by the fact that the receptor complex involved in IL-4Δ2 binding has not been identified. IL-4Δ2 induced signal transduction can more precisely be studied *in vivo* as this molecule appears to be significantly more active in this setting. Paradoxically, *in vivo* studies comparing wild type animals and STAT-6 knockouts showed that IL-4Δ2 mediated processes were partially, but not exclusively, STAT-6 reliant [32].

The question remains as to what receptor or receptor complex IL-4Δ2 engages, as well as what role it might play in downstream signalling. The cytoplasmic tail of IL-4Rα has five important tyrosines; Y1 is central to an insulin-IL-4R motif and interacts with IRS proteins, Dok-1, 2 and Shc. The residues 2, 3 and 4 are docking sites for SH2 domain comprising proteins such as STAT-6 [49]. Tyrosine number 5 is located in the consensus motif called ITIM, and it interacts with SH2 domains of

SHP-1, SHP-2 and SHIP-1 according to the cell type and is associated with a tyrosine motif which mediates inhibition [1]. Although the IL-4R complexes do not have any kinase potential of their own, the cytoplasmic regions are connected to a member of the JAK family, such as JAK1, JAK2, JAK3 and TYK2 [50], as well as Fes [51] and Syk [52]. JAK1 interacts with IL-4R [53] while other JAKs may interact with other receptors. With the existence of such a large number of receptor complexes and signalling molecules relevant to the IL-4 pathway, it is feasible that IL-4Δ2 mediates its effect through a pathway not dependent on STAT-6.

Recent studies have suggested that IL-4Δ2 plays a more complex role than simple antagonism of IL-4 expression and mediated processes. IL-4Δ2 was thus shown to induce the production of IFN- γ , IL-6, IL-10, MCP-1 and TNF- α by primary human T lymphocytes [34]. *In vivo* gene delivery techniques demonstrated that IL-4Δ2 was autonomously functional *in vivo* and caused immune inflammation [32,33]. The activity differed from that of IL-4 and it was not seen as an antagonist in respect to the parameters analysed. The combined effect of both cytokines was synergistic of their independent effects, although contrasting effects between the two isoforms have also been recorded; IL-4 promoted pulmonary eosinophilia and goblet hyperplasia whereas IL-4Δ2 did not. Of particular interest is the fact that the delivery of the IL-4Δ2 gene influenced the cytokine milieu gene expression differently from IL-4, notably increasing IFN- γ expression, an effect not seen with the full-length molecule [32]. The consistently observed ability of IL-4Δ2 to induce the production and expression of IFN- γ , either in recombinant protein stimulation assays [34] or *in vivo* gene delivery experiments, further reinforces the notion that this molecule is a cytokine which promotes a Th1 response [32].

The mode by which IL-4Δ2 exerts its antagonistic actions is still unclear although, as demonstrated here, it is unlikely to be due or solely due to competitive binding to high affinity IL-4R α . IL-4 is itself capable of binding to alternative cell surface receptors which results in differential intracellular downstream signal transduction [1]. There has been a growing realisation regarding the complex interplay in the IL-4/IL-13 activities and interactions, including the IL-4 splice variants [1]. IL-4 is believed to differentially interact with two distinct forms of receptors, IL-4R complex I and II, made up of different subunits [1]. This complex involvement of multiple subunits and signalling pathways opens up further options for IL-4Δ2 to mediate the effects of IL-4.

In comparison to the full length molecule, it has been shown that secretion of IL-4Δ2 by activated T cells occurs at later time points coinciding with the drop in levels of IL-4. It is therefore reasonable to hypothesise that the shift in expression of IL-4 to IL-4Δ2 coincides with the end or dampening of the Th2 immune response. This bears the hallmarks of a regulatory mechanism, where, to avoid a detrimental uncontrolled Th2 response, IL-4Δ2 acts as a Th1-directing cytokine [1]. Therefore when studying or targeting the full length molecule one must take into consideration the splice variants. It is imperative that IL-4Δ2 is considered when developing novel therapeutics which interact with IL-4 or IL-4 receptors. In the case of certain disease states where excess IL-4 is considered unfavourable, therapeutic interventions blocking IL-4 could be desirable. However, these approaches should be designed to avoid also inhibiting the regulatory effects on IL-4 and other autonomous functions mediated by IL-4Δ2, as the net therapeutic effect could be lost or diminished.

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Conflict of interest

None declared.

Author contributions

RR, PWD and DCM conceived and designed the study and co-wrote the paper. GRD performed majority of experiments, with assistance from AS. CVD helped with genetic constructs; MJP and SS planned the Biacore strategy and helped with interpretations; PH and AC provided assistance with the cellular assays and helped design the STAT-6 phosphorylation assay.

References

- [1] I.G. Luzina, A.D. Keegan, N.M. Heller, G.A. Rook, T. Shea-Donohue, S.P. Atamas, Regulation of inflammation by interleukin-4: a review of "alternatives", *J. Leukocyte Biol.* 92 (4) (2012) 753–764.
- [2] C.M. Snapper, W.E. Paul, Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production, *Science* 236 (4804) (1987) 944–947.
- [3] T. Yokota, T. Otsuka, T. Mosmann, J. Banchereau, T. DeFrance, D. Blanchard, J.E. De Vries, F. Lee, K. Arai, Isolation and characterization of a human interleukin cDNA clone, homologous to mouse B-cell stimulatory factor 1, that expresses B-cell and T-cell-stimulating activities, *Proc. Natl. Acad. Sci. U. S. A.* 83 (16) (1986) 5894–5898.
- [4] T.R. Mosmann, M.W. Bond, R.L. Coffman, J. Ohara, W.E. Paul, T-cell and mast cell lines respond to B-cell stimulatory factor 1, *Proc. Natl. Acad. Sci. U. S. A.* 83 (15) (1986) 5654–5658.
- [5] H. Spits, H. Yssel, Y. Takebe, N. Arai, T. Yokota, F. Lee, K. Arai, J. Banchereau, J.E. de Vries, Recombinant interleukin 4 promotes the growth of human T cells, *J. Immunol.* 139 (4) (1987) 1142–1147.
- [6] V.A. Illera, C.E. Perandones, L.L. Stunz, D.A. Mower Jr., R.F. Ashman, Apoptosis in splenic B lymphocytes. Regulation by protein kinase C and IL-4, *J. Immunol.* 151 (6) (1993) 2965–2973.
- [7] K.K. Comfort, J.M. Haugh, Combinatorial Signal Transduction Responses Mediated by Interleukin-2 and -4 Receptors in a Helper TH2 Cell Line, *Cell. Mol. Bioeng.* 1 (2–3) (2008).
- [8] R. Appelberg, I.M. Orme, M.I. Pinto de Sousa, M.T. Silva, In vitro effects of interleukin-4 on interferon-gamma-induced macrophage activation, *Immunology* 76 (4) (1992) 553–559.
- [9] J.W. Lowenthal, B.E. Castle, J. Christiansen, J. Schreurs, D. Rennick, N. Arai, P. Hoy, Y. Takebe, M. Howard, Expression of high affinity receptors for murine interleukin 4 (BSF-1) on hemopoietic and nonhemopoietic cells, *J. Immunol.* 140 (2) (1988) 456–464.
- [10] K. Ikizawa, K. Kajiura, T. Koshio, N. Matsuura, Y. Yanagihara, Inhibition of IL-4 receptor up-regulation on B cells by antisense oligodeoxynucleotide suppresses IL-4-induced human IgE production, *Clin. Exp. Immunol.* 100 (3) (1995) 383–389.
- [11] P. Chomarat, J. Banchereau, Interleukin-4 and interleukin-13: their similarities and discrepancies, *Int. Rev. Immunol.* 17 (1–4) (1998) 1–52.
- [12] A. Kelly-Welch, E.M. Hanson, A.D. Keegan, Interleukin-13 (IL-13) pathway, Science's STKE: signal transduction knowledge environment 2005(293) (2005) cm8.
- [13] A.E. Kelly-Welch, E.M. Hanson, M.R. Boothby, A.D. Keegan, Interleukin-4 and interleukin-13 signaling connections maps, *Science* 300 (5625) (2003) 1527–1528.
- [14] S.L. LaPorte, Z.S. Juo, J. Vacklavikova, L.A. Colf, X. Qi, N.M. Heller, A.D. Keegan, K.C. Garcia, Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system, *Cell* 132 (2) (2008) 259–272.
- [15] T.D. Mueller, J.L. Zhang, W. Sebald, A. Duschl, Structure, binding, and antagonists in the IL-4/IL-13 receptor system, *Biochim. Biophys. Acta* 1592 (3) (2002) 237–250.
- [16] T. Murata, N.I. Obiri, R.K. Puri, Structure of and signal transduction through interleukin-4 and interleukin-13 receptors (review), *Int. J. Mol. Med.* 1 (3) (1998) 551–557.
- [17] K. Nelms, A.D. Keegan, J. Zamorano, J.J. Ryan, W.E. Paul, The IL-4 receptor: signaling mechanisms and biologic functions, *Annu. Rev. Immunol.* 17 (1999) 701–738.
- [18] J. Zhu, W.E. Paul, CD4 T cells: fates, functions, and faults, *Blood* 112 (5) (2008) 1557–1569.
- [19] S.L. Constant, K. Bottomly, Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches, *Annu. Rev. Immunol.* 15 (1997) 297–322.
- [20] L.H. Glimcher, K.M. Murphy, Lineage commitment in the immune system: the T helper lymphocyte grows up, *Genes Dev.* 14 (14) (2000) 1693–1711.
- [21] M. Kopf, G. Le Gros, M. Bachmann, M.C. Lamers, H. Bluethmann, G. Kohler, Disruption of the murine IL-4 gene blocks Th2 cytokine responses, *Nature* 362 (6417) (1993) 245–248.
- [22] Y. Bi, G. Liu, R. Yang, Reciprocal modulation between TH17 and other helper T cell lineages, *J. Cell. Physiol.* 226 (1) (2011) 8–13.
- [23] N. Arai, D. Nomura, D. Villaret, R. DeWaal Malefijt, M. Seiki, M. Yoshida, S. Minoshima, R. Fukuyama, M. Maekawa, J. Kudoh, et al., Complete nucleotide sequence of the chromosomal gene for human IL-4 and its expression, *J. Immunol.* 142 (1) (1989) 274–282.
- [24] T. Otsuka, D. Villaret, T. Yokota, Y. Takebe, F. Lee, N. Arai, K. Arai, Structural

- analysis of the mouse chromosomal gene encoding interleukin 4 which expresses B cell, T cell and mast cell stimulating activities, *Nucl. Acids Res.* 15 (1) (1987) 333–344.
- [25] W.J. Alms, S.P. Atamas, V.V. Yurovsky, B. White, Generation of a variant of human interleukin-4 by alternative splicing, *Mol. Immunol.* 33 (4–5) (1996) 361–370.
- [26] Y. Arinobu, S.P. Atamas, T. Otsuka, H. Niino, K. Yamaoka, H. Mitsuyasu, Y. Niho, N. Hamasaki, B. White, K. Izuhara, Antagonistic effects of an alternative splice variant of human IL-4, IL-4delta2, on IL-4 activities in human monocytes and B cells, *Cell. Immunol.* 191 (2) (1999) 161–167.
- [27] S.P. Atamas, J. Choi, V.V. Yurovsky, B. White, An alternative splice variant of human IL-4, IL-4 delta 2, inhibits IL-4-stimulated T cell proliferation, *J. Immunol.* 156 (2) (1996) 435–441.
- [28] V.P. Zav'yalov, A.I. Denesyuk, B. White, V.V. Yurovsky, S.P. Atamas, T. Korpela, Molecular model of an alternative splice variant of human IL-4, IL-4 delta 2, a naturally occurring inhibitor of IL-4-stimulated T cell proliferation, *Immunol. Lett.* 58 (3) (1997) 149–152.
- [29] I. Gautherot, N. Burdin, D. Seguin, L. Aujame, R. Sodoier, Cloning of interleukin-4 delta2 splice variant (IL-4delta2) in chimpanzee and cynomolgus macaque: phylogenetic analysis of delta2 splice variant appearance, and implications for the study of IL-4-driven immune processes, *Immunogenetics* 54 (9) (2002) 635–644.
- [30] A.S. Waldvogel, M.F. Lepage, A. Zakher, M.P. Reichel, R. Eicher, V.T. Heussler, Expression of interleukin 4, interleukin 4 splice variants and interferon gamma mRNA in calves experimentally infected with *Fasciola hepatica*, *Vet. Immunol. Immunopathol.* 97 (1–2) (2004) 53–63.
- [31] O.P. Yatsenko, M.L. Filipenko, E.A. Khrapov, E.N. Voronina, V.A. Kozlov, S.V. Sennikov, Alternative splicing of mRNA of mouse interleukin-4 and interleukin-6, *Cytokine* 28 (4–5) (2004) 190–196.
- [32] I.G. Luzina, V. Locketell, N.W. Todd, K. Highsmith, A.D. Keegan, J.D. Hasday, S.P. Atamas, Alternatively spliced variants of interleukin-4 promote inflammation differentially, *J. Leukocyte Biol.* 89 (5) (2011) 763–770.
- [33] I.G. Luzina, V. Locketell, N.W. Todd, A.D. Keegan, J.D. Hasday, S.P. Atamas, Splice isoforms of human interleukin-4 are functionally active in mice in vivo, *Immunology* 132 (3) (2011) 385–393.
- [34] I.G. Luzina, V. Locketell, S. Lavania, E.M. Pickering, P.H. Kang, Y.N. Bashkatova, S.M. Andreev, S.P. Atamas, Natural production and functional effects of alternatively spliced interleukin-4 protein in asthma, *Cytokine* 58 (1) (2012) 20–26.
- [35] B. Orsini, B. Ottanelli, A. Amedei, E. Surrenti, M. Capanni, G. Del Prete, A. Amorosi, S. Milani, M.M. D'Elia, C. Surrenti, *Helicobacter pylori* cag pathogenicity island is associated with reduced expression of interleukin-4 (IL-4) mRNA and modulation of the IL-4delta2 mRNA isoform in human gastric mucosa, *Infect. Immun.* 71 (11) (2003) 6664–6667.
- [36] B. Orsini, J.R. Vivas, B. Ottanelli, A. Amedei, E. Surrenti, A. Galli, S. Milani, P. Pinzani, G. Del Prete, C. Surrenti, C.T. Baldari, E. Touati, D.E. MM, Human gastric epithelium produces IL-4 and IL-4delta2 isoform only upon *Helicobacter pylori* infection, *Int. J. Immunopathol. Pharmacol.* 20 (4) (2007) 809–818.
- [37] H.P. Wu, C.L. Wu, C.K. Chen, K. Chung, J.C. Tseng, Y.C. Liu, D.Y. Chuang, The interleukin-4 expression in patients with severe sepsis, *J. Crit. Care* 23 (4) (2008) 519–524.
- [38] S.P. Atamas, V.V. Yurovsky, R. Wise, F.M. Wigley, C.J. Goter Robinson, P. Henry, W.J. Alms, B. White, Production of type 2 cytokines by CD8+ lung cells is associated with greater decline in pulmonary function in patients with systemic sclerosis, *Arthritis Rheumat.* 42 (6) (1999) 1168–1178.
- [39] I. Pepponi, E. Stylianou, C. van Dolleweerd, G.R. Diogo, M.J. Paul, P.M. Drake, J.K. Ma, R. Reljic, Immune-complex mimics as a molecular platform for adjuvant-free vaccine delivery, *PLoS one* 8 (4) (2013) e60855.
- [40] K. Izuhara, N. Harada, Interleukin-4 (IL-4) induces protein tyrosine phosphorylation of the IL-4 receptor and association of phosphatidylinositol 3-kinase to the IL-4 receptor in a mouse T cell line, HT2, *J. Biol. Chem.* 268 (18) (1993) 13097–13102.
- [41] A.M. Vasiliev, R.N. Vasilenko, N.L. Kulikova, S.M. Andreev, I.O. Chikileva, G.Y. Puchkova, I.V. Kosarev, A.V. Khodyakova, V.S. Khlebnikov, L.R. Ptitsyn, G.Y. Shcherbakov, V.N. Uversky, L.M. DuBuske, V.M. Abramov, Structural and functional properties of IL-4delta2, an alternative splice variant of human IL-4, *J. Proteome Res.* 2 (3) (2003) 273–281.
- [42] S.L. Abramson, J.I. Gallin, IL-4 inhibits superoxide production by human mononuclear phagocytes, *J. Immunol.* 144 (2) (1990) 625–630.
- [43] G. Bhaskaran, A. Nii, S. Sone, T. Ogura, Differential effects of interleukin-4 on superoxide anion production by human alveolar macrophages stimulated with lipopolysaccharide and interferon-gamma, *J. Leukocyte Biol.* 52 (2) (1992) 218–223.
- [44] F.D. Finkelman, J. Holmes, J.F. Urban Jr., W.E. Paul, I.M. Katona, T help requirements for the generation of an in vivo IgE response: a late acting form of T cell help other than IL-4 is required for IgE but not for IgG1 production, *J. Immunol.* 142 (2) (1989) 403–408.
- [45] B.J. Shen, T. Hage, W. Sebald, Global and local determinants for the kinetics of interleukin-4/interleukin-4 receptor alpha chain interaction. A biosensor study employing recombinant interleukin-4-binding protein, *Eur. J. Biochem.* 240 (1) (1996) 252–261.
- [46] G. Yao, W. Chen, H. Luo, Q. Jiang, Z. Xia, L. Zang, J. Zuo, X. Wei, Z. Chen, X. Shen, C. Dong, B. Sun, Identification of core functional region of murine IL-4 using peptide phage display and molecular modeling, *Int. Immunol.* 18 (1) (2006) 19–29.
- [47] M.P. Beckmann, K.A. Schooley, B. Gallis, T. Vanden Bos, D. Friend, A.R. Alpert, R. Raunio, K.S. Prickett, P.E. Baker, L.S. Park, Monoclonal antibodies block murine IL-4 receptor function, *J. Immunol.* 144 (11) (1990) 4212–4217.
- [48] F. Sheikh, H. Dickensheets, J. Pedras-Vasconcelos, T. Ramalingam, L. Helming, S. Gordon, R.P. Donnelly, The Interleukin-13 receptor-alpha1 chain is essential for induction of the alternative macrophage activation pathway by IL-13 but not IL-4, *J. Innate Immun.* 7 (5) (2015) 494–505.
- [49] H.Y. Wang, W.E. Paul, A.D. Keegan, IL-4 function can be transferred to the IL-2 receptor by tyrosine containing sequences found in the IL-4 receptor alpha chain, *Immunity* 4 (2) (1996) 113–121.
- [50] C. Schindler, D.E. Levy, T. Decker, JAK-STAT signaling: from interferons to cytokines, *J. Biol. Chem.* 282 (28) (2007) 20059–20063.
- [51] K. Izuhara, R.A. Feldman, P. Greer, N. Harada, Interaction of the c-fes proto-oncogene product with the interleukin-4 receptor, *J. Biol. Chem.* 269 (28) (1994) 18623–18629.
- [52] J. Ennaciri, D. Girard, IL-4R(alpha), a new member that associates with Syk kinase: implication in IL-4-induced human neutrophil functions, *J. Immunol.* 183 (8) (2009) 5261–5269.
- [53] T. Yin, M.L. Tsang, Y.C. Yang, JAK1 kinase forms complexes with interleukin-4 receptor and 4PS/insulin receptor substrate-1-like protein and is activated by interleukin-4 and interleukin-9 in T lymphocytes, *J. Biol. Chem.* 269 (43) (1994) 26614–26617.