

T1/ST2 expression on Th2 cells negatively regulates allergic pulmonary inflammation

Niamh E. Mangan¹, Ayan Dasvarma², Andrew N. J. McKenzie² and Padraic G. Fallon¹

¹ Institute of Molecular Medicine, Trinity College Dublin, St James's Hospital, Dublin, Ireland

² MRC Laboratory of Molecular Biology, Hills Road, Cambridge, UK

The transmembrane form of T1/ST2 (ST2) is a specific marker on murine Th2 cells that have been generated *in vitro*, or isolated from sites of allergic type 2 inflammation. Despite the association of ST2 with Th2 cells, to date no obligate role for ST2 in type 2 responses *in vivo* has been described. We have specifically addressed the role of ST2 on T cells by generation of ST2^{-/-} mice crossed with ovalbumin (OVA) T cell receptor-transgenic mice. OVA-specific ST2^{-/-} cells had normal cytokine responses to T cell activation after *in vitro* Th2 differentiation, but OVA stimulation of IL-5 was increased. Transfer of OVA-specific ST2^{-/-} Th2 cells into BALB/c mice caused exacerbated pulmonary inflammation with occluded airways, elevated airway hyper-responsiveness and increased susceptibility to methacholine challenge that was associated with mortalities of recipient mice. The increased pulmonary inflammation in OVA-specific ST2^{-/-} Th2 cell recipients was associated with selective differences in pulmonary levels of Th2 cytokines compared with OVA-specific ST2⁺ Th2 cell recipients. Recipients of OVA-specific ST2^{-/-} Th2 cells had a significant increase in eosinophils and a significant reduction in F4/80^{hi} macrophages in the lungs. This is the first demonstration of a role for ST2 expression on Th2 cells down-regulating pulmonary inflammation. These data have major implications for the targeting of ST2 as a therapy for allergic airway disorders.

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Introduction

Allergic asthma is an inflammatory condition of the lungs due to a breakdown in immune regulation [1]. Asthmatics develop airway hyper-responsiveness (AHR) that is associated with inflammation of the airways,

pulmonary eosinophilia and mucus hyperplasia. In humans and animal models, AHR is associated with an increase in type 2 cytokine responses, with elevated secretion of interleukin (IL)-4, IL-5, IL-9 and IL-13, that induce the eosinophilia, mucus production and airways remodelling seen in allergic pulmonary inflammation. One approach to develop new therapies to treat asthma is to selectively eliminate CD4⁺ T cells of the T helper (Th) 2 type that are a specific source of IL-4, IL-5 and IL-13 in allergic lung inflammation [1].

One such therapeutic target on Th2 cells is the transmembrane form of T1/ST2, referred to hereafter in these studies as ST2. Although ST2 is expressed on several cell types, including mast cells and macrophages,

Correspondence: Dr. Padraic Fallon, Institute of Molecular Medicine, Trinity College Dublin, St James's Hospital, Dublin 8, Ireland

Fax: +353-1-896-4040

e-mail: pfallon@tcd.ie

Abbreviations: AHR: airway hyper-responsiveness ·

FSC: forward scatter · Mch: methacholine · Penh: enhanced pause · SSC: side scatter · ST2: transmembrane form of T1/ST2

it is specifically expressed on Th2, but not Th1 cells [2, 3]. Elevated levels of the soluble form of T1/ST2 have been detected in patients with various inflammatory diseases including asthma, where this elevation correlates with the severity of disease [4, 5]. Early studies have highlighted a role for ST2 in allergic inflammation [3, 6]. However, studies by three different groups utilising ST2-deficient or transgenic (Tg) mice all demonstrated that deficient mice had no substantial impairment in the generation of effective Th2 responses [7–9]. Therefore it is not currently clear whether there is an obligate role for ST2 expression in Th2 cell-mediated immune responses and its biological role in allergic responses is also as yet not clearly defined [10].

A confounding factor in studies to date addressing the role of ST2 expression on Th2 cell responses using ST2-deficient or Tg mice is the normal expression of ST2 on macrophages and mast cells. Indeed ST2 expression on macrophages has been implicated in tolerance of mice to LPS [11]. In this study we have generated a model system for a definitive examination of the mechanism of action of ST2 expression on Th2 cells in a type 2 pulmonary inflammation model. We have defined a distinct role for ST2 on Th2 cells in the regulation of *in vivo* allergic responses, demonstrating that in the absence of ST2, airway inflammation is significantly exacerbated, resulting in mortality. These studies are of particular importance for the potential use of anti-ST2 therapies in the treatment of airway inflammation.

Results and discussion

Potential role for ST2 in cell recruitment in OVA-induced airway inflammation

It has been known for almost 10 years that ST2 is a selective surface marker for Th2 cells, and ST2 has thus been postulated to be involved in the effector function and regulation of Th2 cells [2, 3]. To address a functional role for ST2-expressing cells in Th2 cell-mediated immune responses, we used ST2-deficient (ST2^{-/-}) and wild-type (WT) mice in an OVA-induced allergic pulmonary inflammation model [12, 13]. OVA sensitisation of ST2^{-/-} and WT mice induced AHR, with dose-dependent increases in enhanced pause (Penh) values in response to methacholine (Mch) challenge (Fig. 1A). There was a significant increase ($p < 0.005$ – 0.0001) in the airway reactivity of both WT and ST2^{-/-} mice that received OVA compared with PBS treatment. In three separate experiments, however, although there were marginally greater Penh responses in OVA-sensitised ST2^{-/-} mice compared to WT mice, the increase was not statistically significant.

To confirm these data we also measured lung resistance (GL) on restrained mice while under anesthesia [14, 15]. While OVA sensitisation of mice induced significant changes in GL compared to PBS treatment ($p < 0.05$ – 0.0001), there were no significant differences in the Mch-induced GL between ST2^{-/-} and WT mice (Fig. 1B). Scoring of histology sections also showed no significant differences in airway mucus hyperplasia, pulmonary fibrosis or peribronchial cell inflammation (data not shown). There were also no differences between OVA-sensitised ST2^{-/-} and WT mice in cytokine responses following restimulation of mediastinal lymph nodes with OVA (Supporting Information Fig. S1), or levels of cytokines detected in the lungs (data not shown).

There was a significant increase ($p < 0.05$) in the numbers of eosinophils present in the bronchoalveolar lavage fluid (BALF) of OVA-treated ST2^{-/-} mice compared with WT animals, as shown by detection of eosinophils [(CD4, CD8, CD19)⁻, CCR3⁺, side scatter (SSC)^{hi}] by flow cytometry (Fig. 1C). In contrast, there were reduced numbers ($p < 0.05$) of mononuclear [(CD4, CD8, CD19)⁻, SSC^{hi}, forward scatter (FSC)^{hi}, autofluorescent] [16] cells in the BALF of untreated (PBS) ST2^{-/-} mice compared to WT mice, with a significant ($p < 0.0001$) reduction in mononuclear cells induced by OVA in ST2^{-/-} mice compared to WT mice (Fig. 1D). Further characterization of these pulmonary mononuclear cells identified no differences in dendritic (CD11c⁺) cells present (data not shown), but alterations in the levels of highly autofluorescent F4/80^{hi} macrophages (Fig. 1E) that were also CD11b⁺ (data not shown), with the absence of ST2 in mice leading to a significant ($p < 0.0001$) defect in the OVA-induced F4/80^{hi} macrophage infiltration of the lung (Fig. 1E). The reduced presence of pulmonary macrophages in unprimed ST2^{-/-} PBS-treated mice indicates a role for ST2 in macrophage location and possibly infiltration in the tissue. These data are further corroborated by the lack of F4/80^{hi} macrophages in the lungs of ST2^{-/-} mice upon OVA challenge (Fig. 1E).

The partial and selective defects in OVA-induced pulmonary inflammation in ST2^{-/-} mice highlights the absence of convincing data using these mice for an obligate role for ST2 in Th2 responses *in vivo*, as reported in other studies [7–9]. Although there were moderate effects in type 2-induced lung inflammation in ST2^{-/-} mice, interpretation of the data in the context of Th2 cells may be confounded by the additional absence of expression of ST2 on other cells including macrophages and mast cells.

Generation of OVA-Tg/ST2^{-/-} mice to assess the function of ST2 on CD4⁺ T cells

ST2^{-/-} and DO-11.10 (OVA-Tg) mice were crossed to generate OVA-Tg/ST2^{-/-} mice. After *in vitro* Th2 differentiation of CD4⁺ T cells from OVA-Tg and OVA-Tg/ST2^{-/-} mice both sets of Th2 cells were >98% positive for the OVA TCR (KJ1-26⁺), with OVA-Tg/ST2^{-/-} cells not expressing ST2 while OVA-Tg Th2 cells showed high expression of ST2 (Fig. 2A). Similar to previous studies [1, 8] following anti-CD3 mAb activation *in vitro*, there were no differences in production of Th2 cytokines or IL-10, TGF- β and IFN- γ by ST2 in intact *versus* ST2^{-/-}

Th2-differentiated cells (Fig. 2B). However, following *in vitro* restimulation with OVA peptide, OVA-Tg/ST2^{-/-} Th2 cells had comparable IL-4, IL-13 and IL-10 production as OVA-Tg Th2 cells, but significantly increased production of IL-5 ($p < 0.05$; Fig. 2B). There were no significant differences in production of IFN- γ and TGF- β between groups (Fig. 2B). Additionally, real-time PCR analysis of Th2-differentiated cells also showed consistent elevation in IL-5 mRNA in OVA-Tg/ST2^{-/-} Th2 cells, but comparable levels of a range of other relevant genes, including IL-4, IL-9, IL-10, IL-13, IL-33, T-bet and GATA-3 (data not shown).

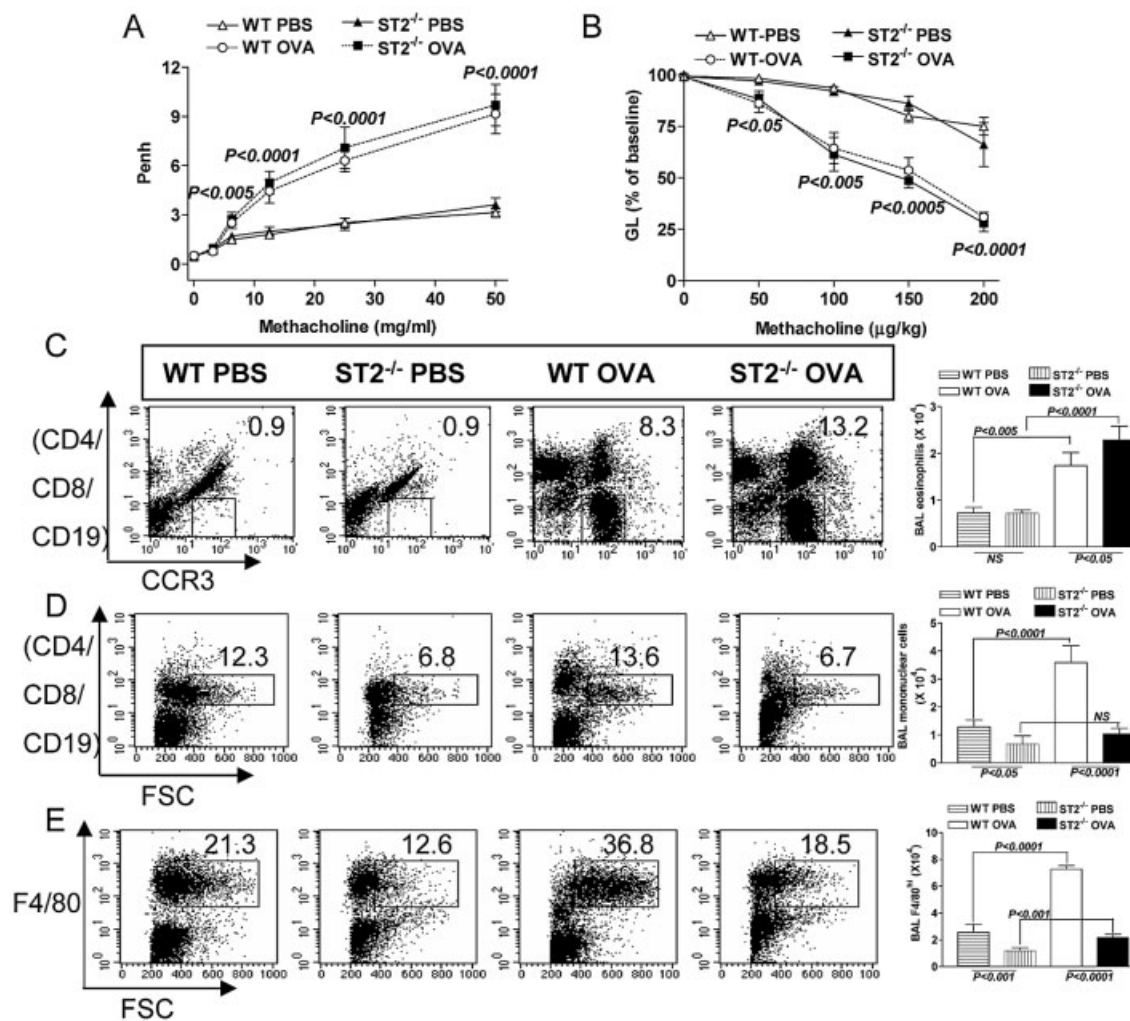


Figure 1. ST2 has a role in OVA-induced airways inflammation in mice. (A) Measurement by whole-body plethysmography of airway responsiveness of PBS-treated or OVA-sensitized ST2^{-/-} and WT mice to Mch (0–50 mg/mL) challenge as assessed by Penh. *p* values represent Student's *t*-test for statistical difference at each Mch dose between WT or ST2^{-/-} mice $\pm$ OVA. (B) Measurement of lung resistance (GL) on restrained, unconscious, sensitized ST2^{-/-} or WT mice to Mch (3.125–250 µg/kg). Statistical analysis is as described for (A). (C) Elevated numbers of eosinophils [(CD4, CD8, CD19)⁻, CCR3⁺, SSC^{hi}] in the BALF of OVA-sensitized ST2^{-/-} mice compared to WT mice as assessed by flow cytometry. (D, E) Reduced presence of (D) mononuclear cells [(CD4, CD8, CD19)⁻, SSC^{hi}, FSC^{hi}, autofluorescent] [16] (D) and F4/80^{hi} macrophages (E) in the BALF of both PBS- and OVA-treated ST2^{-/-} mice compared to WT mice. Graphs in (C–E) are presented as mean $\pm$ SEM of total numbers of cells in the BALF of four to five individual mice in each group. Student's *t*-test was used for statistical analysis. Results are pooled from four separate experiments with $n = 12$ –22 per group. NS, not significant.

In an examination of differences in cell surface markers expressed on Th2 cells generated from OVA-Tg/ST2^{-/-} mice or OVA-Tg mice, there were no significant differences in the surface expression of a range of T cell regulatory (CD25, Foxp3, CD103, glucocorticoid-in-

duced TNFR), inhibitory (CTLA-4), activation (CD25, CD62L) or migratory (CCR7, CD137) markers (data not shown). However, alterations were noted with elevated CD45RB^{hi} expression, a characteristic of pathogenic CD4⁺ cells [17], and a reduction in the expression of inducible co-stimulator on Th2 cells differentiated from OVA-Tg/ST2^{-/-} mice compared with OVA-Tg mice (data not shown).

These data thus indicate that in the absence of ST2, OVA-specific CD4⁺ cells differentiate *in vitro* under antigen-specific Th2 conditions into cells with normal IL-4 and IL-13, but elevated IL-5 production. These data are in contrast to those by others where ST2 expression on CD4⁺ T cells is associated with a greater production of IL-5 than from ST2^{-/-} CD4⁺ T cells [18]. However, differences in methods used to induce Th2 differentiation between studies may account for the alterations in cytokine expression in the absence/presence of ST2 on Th2 cells.

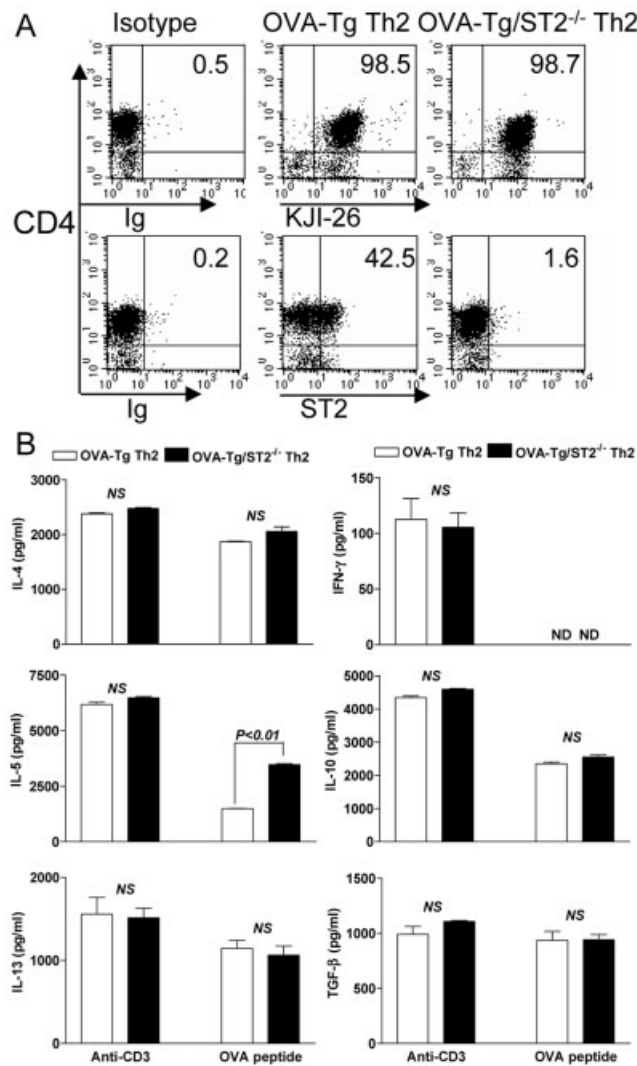


Figure 2. OVA-Tg/ST2^{-/-} CD4⁺ T cells differentiate *in vitro* to Th2 cells normally but have increased production of OVA-specific IL-5. (A) *In vitro* differentiated Th2 cells from OVA-Tg and OVA-Tg/ST2^{-/-} mice had comparable expression of OVA TCR as confirmed by flow cytometry staining for the OVA TCR clonotypic receptor KJI-26 (top panel). The absence of ST2 on OVA-Tg/ST2^{-/-} Th2 cells was also confirmed by flow cytometry (lower panel), with >40% of OVA-Tg Th2 cells positive for ST2. Quadrant gates were set on isotype controls and results are representative of at least three separate repeat experiments. (B) Following two rounds of *in vitro* differentiation, Th2 cells were restimulated with anti-CD3 (100 ng/mL) or OVA peptide (100 ng/mL) for 24 h and cytokines in the culture supernatants were assayed by ELISA. *p* values are from analysis by Student's *t*-test. Data are representative of three separate experiments. IL-5 was the only cytokine consistently different (*p* < 0.01) between groups in all experiments. ND, not detected; NS, not significant.

ST2 expression on OVA-specific Th2 cells down-regulates AHR in mice

OVA-Tg or OVA-Tg/ST2^{-/-} mice responded to OVA challenge in a similar manner to ST2^{-/-} and WT mice (Fig. 1 and data not shown). Therefore we used a Th2 cell transfer model to address the function of ST2⁺ versus ST2⁻ Th2 cells, generated as in Fig. 2, in OVA-induced lung inflammation. OVA-primed BALB/c mice were injected with *in vitro* differentiated Th2 cells generated from either OVA-Tg or OVA-Tg/ST2^{-/-} mice to induce AHR in recipient mice. Strikingly, mice that received OVA-Tg/ST2^{-/-} Th2 cells developed severe pulmonary distress when exposed to Mch, with mice starting to die after 12.5 mg/mL of Mch, and all mice dead at concentrations of 25 mg/mL (Fig. 3A). At the Mch dose of 12.5 mg/mL, OVA-Tg/ST2^{-/-} Th2 recipients had significantly enhanced Penh values compared with OVA-sensitised mice that had received ST2⁺ Th2 cells (*p* < 0.001; Fig. 3B). Similarly, following invasive plethysmography, OVA-Tg/ST2^{-/-} Th2 cell recipients also died upon Mch challenge (data not shown).

Death of Mch-treated mice injected with OVA-Tg/ST2^{-/-} Th2 cells was not associated with degranulation of mast cells, with these mice having comparable serum levels of mast cell protease-1 as OVA-Tg Th2 cell recipients (4.49 ± 0.61 versus 3.83 ± 0.52 ng/mL, respectively). There were also no differences in the numbers of mast cell or basophils, as determined by flow cytometry, detected in the lungs of either group (data not shown). The lungs of ST2-deficient Th2 cell recipient mice had increased lung inflammation compared to mice injected with ST2^{+/+} Th2 cells, with enhanced peribronchial and perivascular cell infiltration (Fig. 3C). Furthermore, these mice had extensive goblet

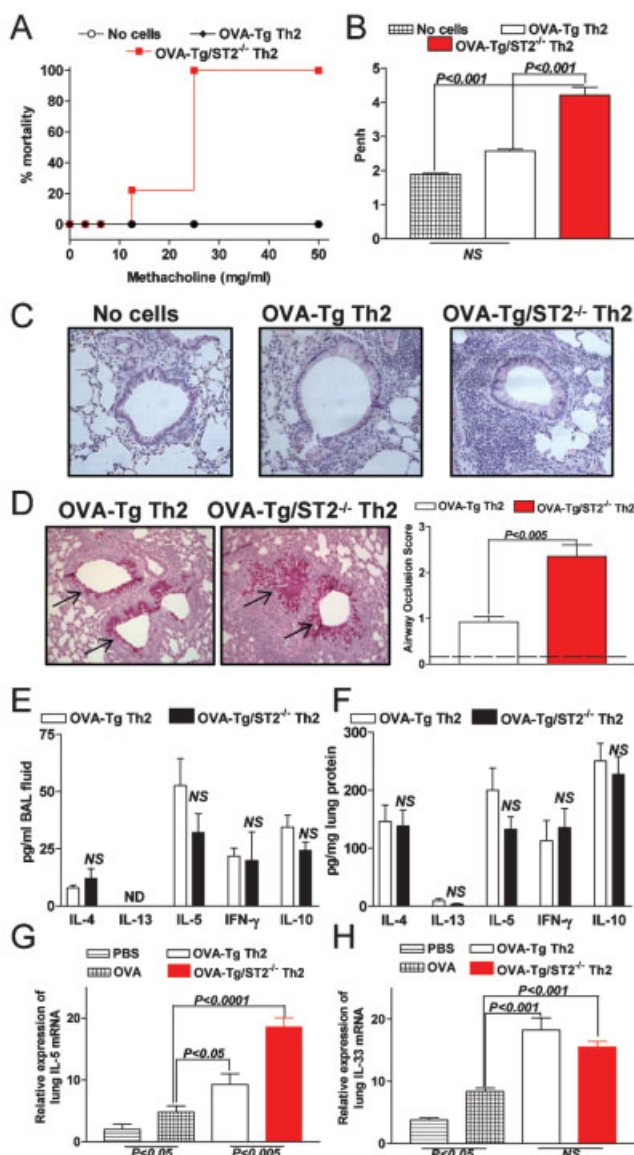


Figure 3. Transfer of OVA-Tg/ST2^{-/-} Th2 cells induces elevated AHR and deaths in recipient OVA-primed mice. (A) Mortality curve following exposure to Mch of mice that received OVA-Tg/ST2^{-/-} Th2 cells versus recipients of OVA-Tg Th2 cells. (B) Transfer of OVA-Tg/ST2^{-/-} Th2 cells induced pronounced AHR in recipient mice compared with mice receiving OVA-Tg Th2 cells. Penh values are at 12.5 mg/mL Mch. (C) Hematoxylin and eosin-stained sections of lungs from OVA-treated mice not given cells (No cells) or transferred with Th2 cells. (D) Airway occlusion on periodic acid-Schiff-stained sections of lungs from OVA-treated mice that were transferred with Th2 cells. Airways are indicated with arrows. Figure shows scores of occluded airways, measured as described in the *Materials and methods*. Statistical analysis was determined using Mann-Whitney test. (E, F) Detection of cytokines in BALF (E) or lung homogenates (F) from mice. (G, H) Relative expression of IL-5 (G) and IL-33 (H) mRNA in lung tissue from six to ten individual mice per group following detection by real-time RT-PCR. Student's t-test was used to assess statistical differences between groups in (B, E, F) and ANOVA to assess differences in (G, H). Results are presented as mean + SEM, from five to ten mice per group, and are representative of three separate experiments. NS, not significant.

further significant increase ($p < 0.005$) in IL-5 mRNA in OVA-Tg/ST2^{-/-} Th2 cell recipients compared with mice that received OVA-Tg Th2 cells (Fig. 3G).

Recently, Schmitz and colleagues [19] identified the new Th2 cytokine, IL-33, as a ligand for ST2 and illustrated, consistent with a potential role for ST2 in type 2 inflammation, that *in vivo* administration of IL-33 to mice evoked type 2 lung inflammation. Indeed, sensitisation with OVA alone increased IL-33 mRNA expression in the lungs of mice compared with PBS-treated mice ($p < 0.05$; Fig. 3H). Interestingly, the expression of IL-33 mRNA in the lungs of recipients of both ST2⁺ and ST2⁻ Th2 cells was increased significantly ($p < 0.001$) when mice were challenged with OVA compared with mice that did not receive Th2 cells (Fig. 3H). There was no difference in IL-33 mRNA between recipients of ST2⁺ or ST2⁻ Th2 cells (Fig. 3H). The relevance, if any, of IL-33-ST2 interactions in the increased lung pathology after transfer of OVA-Tg/ST2^{-/-} Th2 cells will require verification by detection of IL-33 protein, when reagents are available. In addition, we have not assessed in these studies the previously noted role of soluble T1/ST2 as a pro-inflammatory mediator [20]. However, further investigation on the functions of the various forms of T1/ST2 is required to elucidate potential differences in their mechanism of action.

In both the BALF and lungs of mice that received OVA-Tg/ST2^{-/-} Th2 cells there was a significant ($p < 0.01$ and $p < 0.05$, respectively) increase in infiltrating OVA-specific (KJ1-26⁺) T cells compared to mice that had received OVA-Tg Th2 cells (Supporting Information Fig. S2). Additionally, there were increased numbers of

cell hyperplasia, with significantly more airways occluded than mice injected with ST2⁺ Th2 cells ($p < 0.005$; Fig. 3D).

Despite the increased AHR and death of mice, there were no significant differences in Th2 cytokines (IL-4, IL-5, IL-13) and IFN-γ or IL-10 detected in the BALF (Fig. 3E) or lung homogenates (Fig. 3F) of mice that received Th2 cells generated from OVA-Tg/ST2^{-/-} or OVA-Tg cells. OVA-stimulated mediastinal lymph nodes from both groups of mice also had comparable production of cytokines (data not shown). An examination of IL-4, IL-13, IFN-γ and IL-10 mRNA expression in the lungs of mice that received Th2 cells confirmed the data from detection of cytokine protein in the lungs (data not shown). In contrast, while OVA treatment alone increased the relative levels of IL-5 mRNA in the lungs of mice, the additional transfer of OVA-Tg Th2 cells significantly ($p < 0.05$) elevated this expression, with a

KJI-26⁺ cells in the mediastinal lymph nodes of OVA-Tg/ST2^{-/-} Th2 cell recipient mice compared to mice that received OVA-Tg Th2 cells (data not shown). These data collectively highlight an imperative *in vivo* role for ST2 expression in regulation of Th2 cell effector function, with transfer of allergen-primed ST2^{-/-} Th2 cells causing exacerbated lung inflammation (Fig. 3).

Increased eosinophil recruitment but reduced mononuclear cells in the lungs

Mice that received OVA-Tg/ST2^{-/-} Th2 cells had significantly ($p < 0.005$) more cells in BALF relative to mice injected with OVA-Tg Th2 cells (Fig. 4A). Mice that received Th2 cells (OVA-Tg or OVA-Tg/ST2^{-/-}) in the absence of OVA challenge, *i.e.* PBS-treated cell recipient

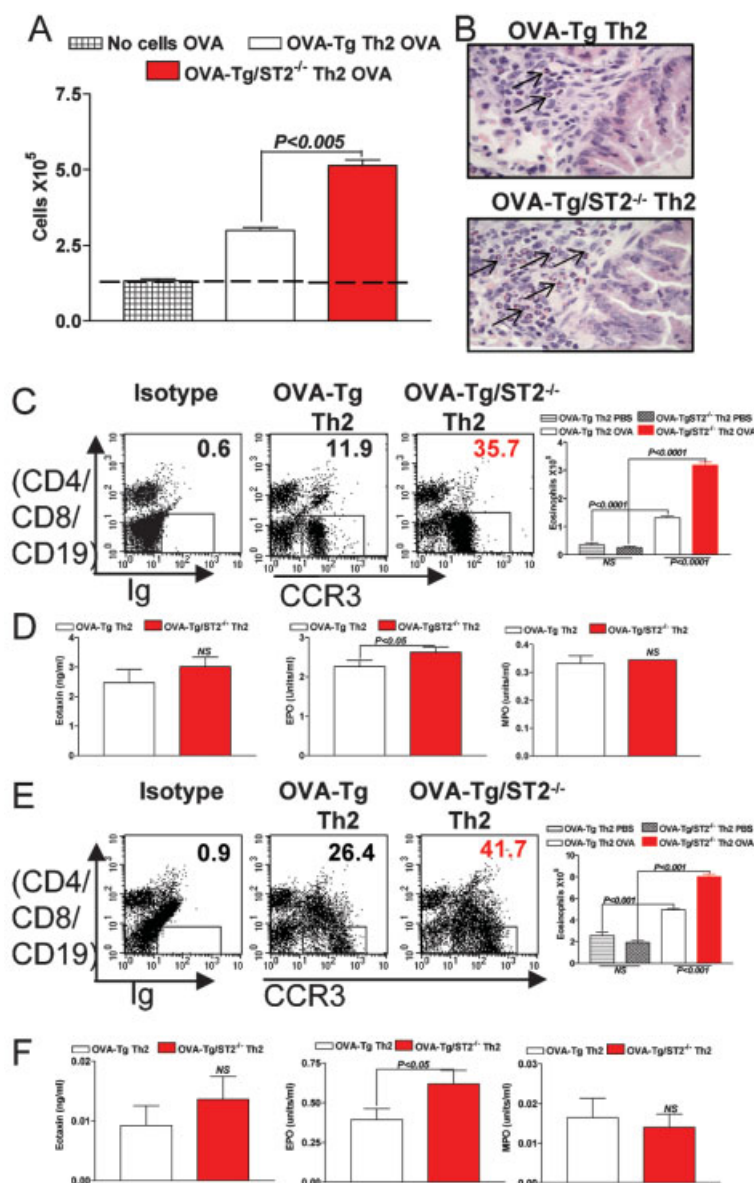


Figure 4. Increased eosinophils in the lungs of mice that received OVA-Tg/ST2^{-/-} Th2 cells. (A) Increase in total cell numbers recovered in the BALF of OVA-primed mice that received OVA-Tg or OVA-Tg/ST2^{-/-} Th2 cells. The hatched line indicates the basal mean level of cell infiltration into the lungs of mice treated with OVA alone. (B) Hematoxylin and eosin-stained sections of lungs from OVA-treated mice transferred with Th2 cells, demonstrating eosinophil-rich peribronchial inflammation in OVA-Tg/ST2^{-/-} Th2 cell recipients. Examples of eosinophils are indicated with arrows. (C, E) Detection by flow cytometry of eosinophils ((CD4, CD8, CD19)⁻, CCR3⁺, SSC^{hi}) in the BALF (C) and in the lungs (E) of OVA-primed Th2 cell recipients. Representative images are shown and graphs at the end of panels indicate actual eosinophil numbers present in the BALF and lungs, accordingly. (D, F) Levels of eotaxin, eosinophil peroxidase and myeloperoxidase activity in the BALF (D) and lung digests (F) of mice transferred Th2 cells. Experiments were repeated three times with data presented as mean + SEM ($n=10-15$). Student's *t*-test was used to assess statistical differences between groups. NS, not significant.

mice, did not have increased inflammatory cell infiltrates in the lung (data not shown). Consistent with the elevated BALF counts in OVA-challenged mice, the increased peribronchial inflammation in OVA-Tg/ST2^{-/-} Th2 cell recipients described earlier (Fig. 3C), was associated with marked eosinophil infiltration (Fig. 4B).

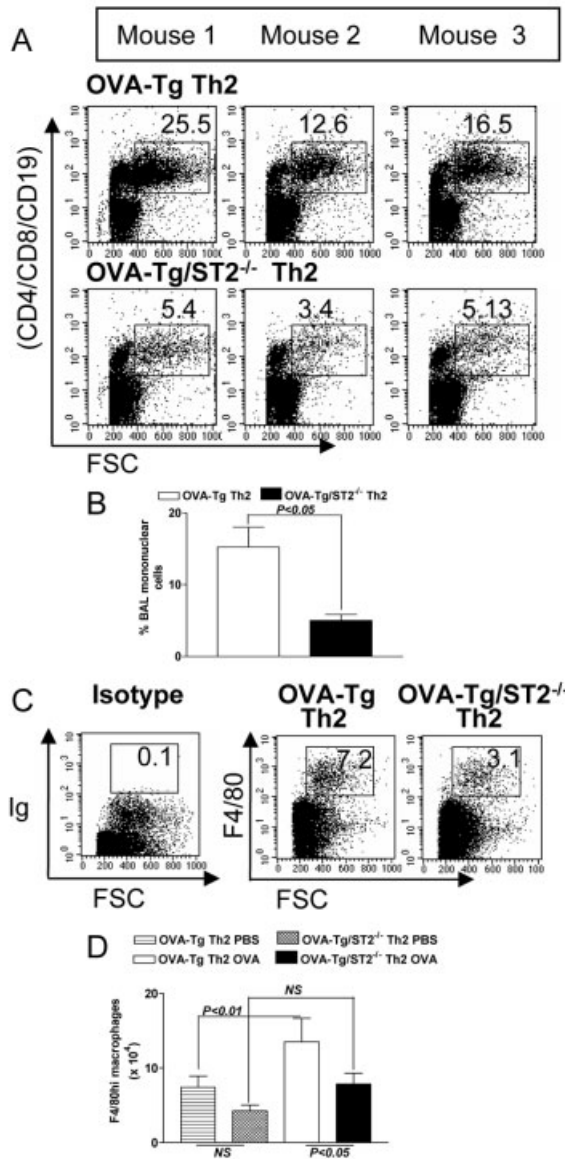


Figure 5. Transfer of OVA-specific ST2^{-/-} Th2 cells leads to a reduction in the frequencies of macrophages in lungs of recipient mice. (A) Three representative images of individual mice showing reduction in the frequency of mononuclear cells present in the BALF of mice that received OVA-Tg/ST2^{-/-} Th2 cells. (B) Percentages of mononuclear cells in BALF of recipient mice. Results are presented as mean ± SEM (n=15–20). (C) The mononuclear population absent in BALF of mice transferred with OVA-Tg/ST2^{-/-} Th2 cells was an F4/80^{hi} macrophage population. (D) F4/80^{hi} cell numbers in the BALF of PBS- or OVA-treated Th2 cell recipients with a significant reduction in F4/80^{hi} cells in BALF of mice that were given OVA-Tg/ST2^{-/-} Th2 cells. NS, not significant.

Mice that received OVA-Tg/ST2^{-/-} Th2 cells had a striking increase in numbers of eosinophils in BALF ($p < 0.0001$; Fig. 4C) and lungs ($p < 0.001$; Fig. 4E). In correlation with the increase in eosinophils in the lungs of mice with OVA-Tg/ST2^{-/-} cells, these mice had a significant ($p < 0.05$) increase in eosinophil peroxidase in BALF (Fig. 4D) and lungs (Fig. 4F). As the transferred Th2 cells had greater IL-5 production (Fig. 2B), this may explain the increased eosinophilia present at the site of inflammation in the lung. As there were no significant differences in the levels of eotaxin in the BALF and lung digests of cell recipients (Fig. 4D, F), it would be of interest to assess levels of other eosinophil chemo-attractants [21].

A marked feature of the altered lung inflammation in ST2^{-/-} mice was the reduction in mononuclear cells in the lungs (Fig. 1D), which could be due to ST2 expression on macrophages. In the lungs of OVA-Tg/ST2^{-/-} Th2 cell recipients, there was also a significant ($p < 0.05$) reduction in the proportion of mononuclear cells (Fig. 5A, B). More specifically, the numbers of F4/80^{hi} macrophages detected in the BALF from these mice were significantly reduced ($p < 0.05$) compared to lungs from mice with OVA-Tg Th2 cells (Fig. 5C, D). We did not find any differences in the levels of eosinophils in the lungs of basophils (non-B, non-T, c-kit⁻, FcεRIα⁺, SSC^{lo} [22, 23]; non-B, non-T c-kit⁺ FcεRIα⁻ cells [24]) in either transfer group (data not shown). Therefore the ST2-expressing Th2 cells may mediate a selective regulation of the lung cell infiltrate via an uncharacterized Th2 cell-macrophage interaction resulting in control of eosinophil recruitment. In support of a potential role for pulmonary macrophages in regulating eosinophil-rich inflammation, depletion of alveolar macrophages in OVA-sensitized mice has been reported to lead to exacerbated type 2 lung inflammation, including significantly elevated pulmonary eosinophilia [25].

The dynamic differential cell recruitment to the lungs induced by ST2^{-/-} Th2 cell transfer occurred independently of lymphocytes in recipients as was shown by transfer of Th2 cells to RAG-1^{-/-} mice (Fig. 6, 7). In the lungs of OVA-primed RAG-1^{-/-} mice injected with OVA-Tg/ST2^{-/-} Th2 cells there was a significant ($p < 0.01$) increase in eosinophils (Fig. 6), and a reduction in F4/80⁺ macrophages ($p < 0.05$; Fig. 7).

In both BALB/c and RAG^{-/-} mice in the absence of OVA priming before cell transfer there was a reduction in the influx of KJ1-26⁺ Th2 (ST2^{+/+} or ST2^{-/-}) cells in the lungs of mice compared to the numbers of KJ1-26⁺ cells infiltrating the lungs of mice that received OVA i.p. prior to cell transfers (data not shown). Furthermore, this reduction in the numbers of OVA-specific Th2 cells in the lungs correlated with a decrease in the magnitude of the inflammatory response described in Fig. 3–7. Nevertheless, the transfer of OVA-Tg/ST2^{-/-} Th2 cells into

BALB/c or RAG^{-/-} mice that were not primed with OVA led to exacerbated lung pathology and differential eosinophil and macrophage recruitment to the lung compared to recipients of ST2^{+/+} Th2 cells (data not shown).

Mice that received either ST2-expressing or -deficient Th2 cells had no detectable differences in the frequencies of infiltrating neutrophils (data not shown), pulmonary levels of IFN- γ (Fig. 3E, F) or myeloperoxidase (Fig. 4D, F). This would indicate that the increased lung inflammation in mice injected with OVA-Tg/ST2^{-/-} Th2 cells was not due to exacerbated type 1 inflammation in recipients. In contrast, in a previous study, treatment of mice with an antibody against ST2 enhanced Th1 responses possibly by removing the Th2 cells [2]. While selective *in vivo* depletion of ST2⁺ Th2 cells may lead to increased Th1 responses [2], our studies, where the Th2 cells are present in the absence of ST2 expression on their surface and sustain Th2 cytokine production, do not lead to elevated Th1 responses.

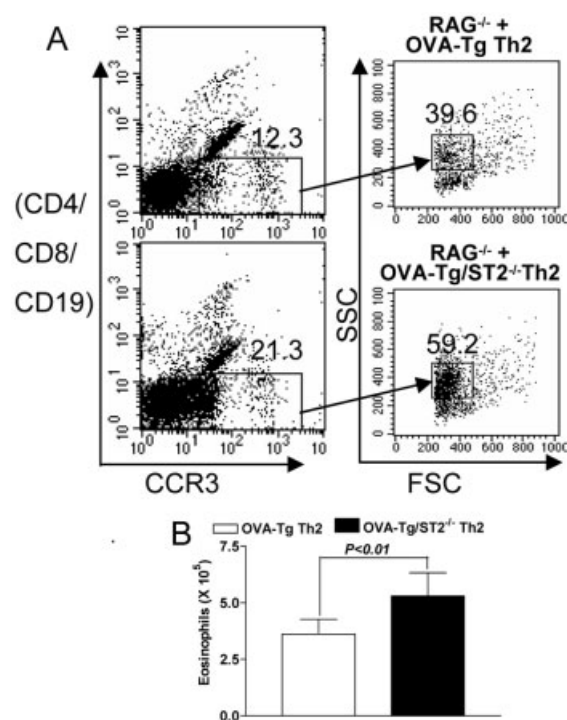


Figure 6. Increased eosinophilia in the BALF of RAG-1^{-/-} mice with OVA-Tg/ST2^{-/-} Th2 cells. (A) Detection by flow cytometry of eosinophils characterised as (CD4, CD8, CD19)⁻, CCR3⁺ (left panel) that are also SSC^{hi} (right panel showing eosinophil gate from the left panel) in the BALF of OVA-treated RAG-1^{-/-} mice following transfer of OVA-specific Th2 cells. (B) Total numbers of eosinophils in the BALF of RAG-1^{-/-} recipients of ST2^{+/+} Th2 cells, illustrating significantly elevated infiltration of eosinophils in BALF following OVA-Tg/ST2^{-/-} Th2 cell transfer. Data are from three separate experiments; n=10–15 mice per group with statistical analysis performed using Student's t-test.

Concluding remarks

In this study we show that transfer of Th2 cells that do not express ST2 increases lung inflammation in allergen-primed recipient mice. The exacerbated pulmonary inflammation in ST2^{-/-} Th2 cell recipients was characterised by elevated AHR and occluded airways, with an altered pulmonary cell infiltration involving significantly increased eosinophils and reduced F4/80⁺ macrophages. The increase in antigen-specific production of IL-5 by ST2^{-/-} Th2 cells may account for the eosinophils in recipient mice. These studies illustrate a potential new function for ST2 in regulation of macrophage recruitment to allergic lungs. As we have illustrated the complexity of ST2 biological activity, this study highlights concerns with targeting ST2 as a therapeutic in type 2 allergic diseases, including airways inflammation [6, 26], arthritis [20] and even atopic dermatitis in humans [27]. Our data demonstrate a potential role for ST2 expression on Th2 cells as a negative regulator in the homeostasis of an *in vivo* allergic inflammatory response. This study opens future avenues of research to investigate the implications of ST2 expression on Th2 cells functioning to down-regulate allergic inflammation.

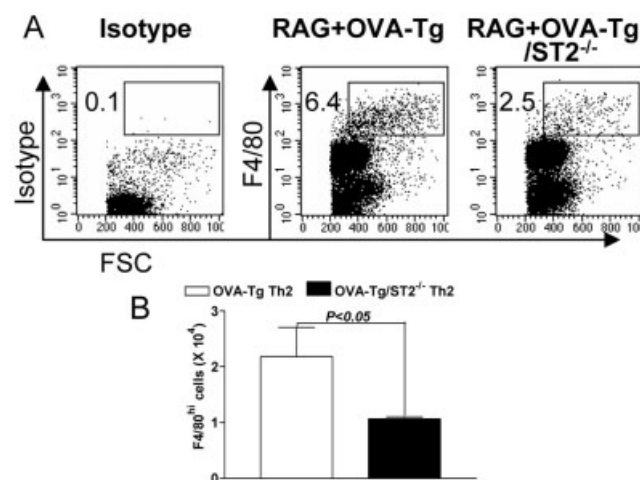


Figure 7. OVA-treated RAG-1^{-/-} mice have reduced F4/80^{hi} cells in the lungs following transfer of OVA-Tg/ST2^{-/-} Th2 cells. Reduced F4/80^{hi} autofluorescent macrophages in the BALF of OVA-primed RAG-1^{-/-} mice injected with OVA-Tg/ST2^{-/-} Th2 cells as assessed by flow cytometry (A) and by actual cell numbers of cells in individual mice (B) compared with RAG-1^{-/-} mice that were given OVA-Tg Th2 cells. Experiments were repeated two to three times with data presented as mean + SEM (n=8–15). Student's t-test was used to assess statistical differences between groups.

Materials and methods

Mice

ST2-deficient (ST2^{-/-}) mice [8] were backcrossed for more than seven generations onto a BALB/c background. DO-11.10 Tg (OVA-Tg) and RAG-1^{-/-} mice were obtained from Jackson Laboratories and maintained on a BALB/c background. OVA-Tg mice were crossed with ST2^{-/-} mice to generate mice that were homozygous positive for the transgene and homozygous negative for ST2; these were called OVA-Tg/ST2^{-/-}. Mice were housed in individually ventilated and filtered cages under positive pressure (Tecniplast, Kettering, UK). Food and water were supplied *ad libitum*. Sentinel mice were screened to ensure specific pathogen-free status. All animal experiments were performed in compliance with Irish Department of Health and Children regulations.

OVA- or Th2 cell-induced lung inflammation and measurements of AHR

ST2^{-/-} and WT mice were sensitised with OVA and airways challenged with OVA to induce pulmonary allergic inflammation. AHR and lung resistance measurements were assessed as described previously [15]. AHR was induced by Th2 cell adoptive transfer [28], with modifications. In brief, BALB/c or RAG-1^{-/-} mice were left unprimed or sensitised i.p. on day 0 with 2 µg OVA in 2 mg alum (Pierce). OVA was prepared under endotoxin-limiting conditions (<0.5 endotoxin units per mg protein) as described previously [15]. The OVA-primed mice were injected i.v. with 5 × 10⁶ Th2-differentiated cells (see section *Th2 cell differentiation*), on day 7. Mice were challenged from days 8 to 14 with 2% OVA aerosol in endotoxin-free Dulbecco's PBS for 20 min.

AHR was assessed by barometric whole-body plethysmography (Emka Technologies, Paris, France) in response to different doses of Mch challenge on day 15 using unrestrained plethysmography to quantify pulmonary reactivity as Penh, or using the restrained method on anaesthetised mice for functional lung conductance measurements, as described [12, 14, 15]. Penh values were expressed as the fold increase for each concentration of Mch compared with Penh values after PBS challenge. Twenty-four hours later mice were sacrificed by terminal anaesthesia and samples taken for further immunological analysis. For functional parameters the maximum resistance (GL as inverse of resistance) was taken after each Mch dose with saline challenge and expressed as a percentage over baseline. As mice were anaesthetised for this procedure and killed afterwards, no immunological assessment was performed on these mice.

Lung immunology and histology

Bronchoalveolar lavage

BALF were collected by cannulating the trachea and lavaging the lungs two times with 1.0 mL of ice-cold PBS. The BALF cells were pelleted, washed, and counted. BALF was stored at -80°C for cytokines/chemokine ELISA. BALF cells were used on cytopins or flow cytometry. For cytopins, the numbers of

eosinophils, neutrophils, macrophages and lymphocytes were determined by performing a differential count on at least 400 cells/slide of Giemsa-stained cytocentrifuge preparations.

Lung homogenates and digests

Whole lungs were removed from the mice and either snap-frozen, placed in 10% formaldehyde-saline for histology, or digested with collagenase (Roche) for flow cytometry analysis, as described previously [15]. In some experiments the left lobes of the lungs were removed for assessment of tissue cytokines and chemokines (IL-4, IL-5, IL-13, IL-10, IFN-γ and eotaxin). Eosinophil peroxidase and myeloperoxidase levels in the BALF and lung digests of mice were determined based on a method previously described [29]. Eosinophil peroxidase or myeloperoxidase activity was expressed as units per mL.

Lung histology

Lungs from mice were taken and fixed in 10% formaldehyde-saline for histology. Paraffin-embedded tissue sections were stained with hematoxylin and eosin and eosinophil infiltration was counted on Giemsa-stained sections. Airway mucus occlusion was analyzed on periodic acid-Schiff-stained lung sections. A semi-quantitative score was used for airway occlusion as follows: 0, 0–10% occlusion; 1, 10–30% occlusion; 2, 30–60% occlusion; 3, 60–90% occlusion; 4, 90–100% occlusion, as described [30]. For each mouse the mean score was determined from at least ten individual airways.

Th2 cell differentiation

In vitro Th2 differentiation of naive CD4⁺ T cells was modified from previous methods [28]. CD4⁺ T cells were isolated from the spleens of OVA-Tg and OVA-Tg/ST2^{-/-} mice using CD4⁺ cell enrichment columns (R&D Systems, Abingdon, UK). Irradiated accessory cells were prepared from spleen cells by depletion of B and T cells, as described previously [31]. CD4⁺ T cells were stimulated for two rounds of differentiation with OVA peptide (Cambridge Research Biochemicals, Cleveland, UK; <0.01 endotoxin units per mg protein) present in all polarising cultures. To confirm successful polarisation following two rounds of differentiation, Th2 cells were washed and restimulated with anti-CD3 (100 ng/mL) or OVA peptide (100 ng/mL) for 24 h, and cytokines (IL-4, IL-5, IL-13, IL-10, TGF-β, IFN-γ) in the culture supernatants were assayed by ELISA. Furthermore, in some experiments differentiated Th2 cells were assessed by RT-PCR for levels of T cell cytokine (IL-4, IL-5, IL-9, IL-10, IL-13, IL-33, T-bet and GATA-3) mRNA.

Flow cytometry

Cell surface marker expression was assessed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Fluorescence-labelled antibodies were obtained from either BD Pharmingen, Caltag or eBiosciences, and were used at 0.5–1 µg/10⁶ cells. For immunofluorescence staining in the lung, the various cell populations in digests were identified as described [15, 16]. Lungs cells were first gated on CD19, CD4 and CD8 *versus* FSC. Lymphocytes were identified as FSC^{lo},

SSC^{lo} CD19⁺, CD4⁺, CD8⁺. Eosinophils were distinguished as SSC^{hi}, CD19⁻, CD4⁻, CD8⁻, CCR3⁺, non-autofluorescent granulocytes that stained positive for CCR3. Mononuclear cells were characterised as large (FSC^{hi}), granular (SSC^{hi}), autofluorescent cells that are CD4⁻, CD8⁻ and CD19⁻, i.e. not in the lymphocyte gate. Macrophages were characterized as large mononuclear cells that were highly autofluorescent, and confirmed to be CD11b (Mac-1)⁺ or F4/80^{hi}. See online Supporting Information for sources and clones of antibodies.

Cytokine/chemokine ELISA

Sandwich ELISA were performed to quantify levels of specific cytokines in the supernatants from lung tissue, BALF and *in vitro* cell stimulation cultures. Reagents for quantification of IL-4, IL-5 and IL-13 were from BD PharMingen, and for IL-10 and IFN- γ were purchased as a DuoSet ELISA development system from R&D Systems. Eotaxin detection reagents were also purchased from R&D Systems. Mouse mast cell protease-1 was measured in sera (Moredun Scientific Ltd., Penicuik, Scotland).

RT-PCR

Harvested cells were pelleted and stored at -80°C. RNA was extracted from cells using TRIzol reagent (Invitrogen), according to manufacturer's instructions. cDNA was reverse-transcribed from DEPC water-eluted RNA using Superscript reverse transcriptase II (Stratagene). The cDNA was amplified and quantified using an Applied Biosystems 7900 Real Time PCR system. Levels of the following genes were quantified for each sample: IL-4, IL-5, IL-9, IL-10, IL-13, IL-33, T-bet and GATA-3. 5'-FAM/3'-TAMRA-labelled probes were used. The primers and probe sequences are detailed in Supporting Information Table 1.

Statistical analysis

Statistical analysis was performed with GraphPad Prism and GraphPad Instat software. Results represent the mean + SEM unless stated otherwise. Differences were considered significant when $p < 0.05$ as assessed by Student's *t*-test or Mann-Whitney non-parametric analysis where indicated.

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