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Follicular helper T cells are responsible for IgE responses to Der p 1 following house dust mite sensitization in mice

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Summary

Background Th2 cells have long been considered responsible for the switching of B cells to production of IgE during cognate interaction, primarily due to their expression of CD40L and secretion of IL-4. This concept has been challenged by the more recent definition of follicular helper T cells (Tfh) as the key T cell subset in B cell isotype switching, due to their physical location at the boundary of T cell:B cell areas in lymphoid follicles and ability to express IL-4 and CD40L.

Objective To determine whether Tfh cells are responsible for IgE responses to Der p 1 allergen after house dust mite (HDM)-induced allergic sensitization.

Methods Mice deficient in Tfh cells were sensitized to HDM and Der p 1-specific IgE measured by ELISA.

Results Mice with a mutation in T cell-expressed IL-6R were unable to expand Tfh populations after HDM sensitization, and their anti-Der p 1 IgE, IgG1 and total IgE responses were reduced by 80–90% compared with wild-type mice. These animals displayed unaltered lung Th2 and eosinophilic responses after intranasal HDM challenge and normal IL-4 production, but B cell infiltration of the airways was abrogated.

Conclusions and Clinical Relevance Our data indicate that Tfh cells are largely responsible for switching B cells to IgE synthesis, most likely via an IgG1$^+$ intermediate. However, Th2 cells are the major source of IL-4 during HDM sensitization and this might contribute to IgE synthesis at a stage distal to Tfh-mediated isotype switching. The IL-6/follicular helper T cell pathway is a potential therapeutic target in allergic disease.

Keywords IgE, follicular helper T cell, Th2 cell, Der p 1, house dust mite
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Introduction

Mature B lymphocytes are not capable of producing IgE antibodies until a process of isotype switching, triggered by interaction with T cells recognizing the same allergen, has occurred. Th2 cells have long been considered the T cell subset responsible for the switching of B cells to IgE synthesis from IgM or IgG isotypes [1]. However, this paradigm has been challenged by the more recent characterization of follicular helper T cells (Tfh) as the key T cell involved in isotype switching, consistent with their presence in germinal centres of lymphoid tissues [2]. By contrast, Th2 cells may preferentially migrate to mucosal tissues exposed to allergens, driving allergic inflammation [3]. Tfh are CD4$^+$ T cells expressing CXCR5, the receptor for CXCL13 chemokine,

which mediates migration towards the boundary of T cell and B cell areas of lymphoid follicles where isotype switching occurs [4]. Furthermore, Tfh express CD40L and secrete IL-4 [2], the key factors responsible for IgE switching. They also produce IL-21, a cytokine that stimulates B cell growth but has been reported to suppress IgE synthesis [5]. Mice with reduced numbers of Tfh are deficient in IgG antibody responses [6], but the role of Tfh in IgE responses to common aeroallergens has not been definitively demonstrated. Indeed, it has been suggested that switching to IgE can occur in poorly organized germinal centres or in mucosal tissues by a direct IgM to IgE switch [7–9]. IgE switching in germinal centres is believed to occur via an IgG4$^+$ intermediate stage in humans or an IgG1$^+$ B cell in mice [10, 11].

To address the role of Tfh cells in IgE responses to house dust mite (HDM) allergen, we used mice genetically modified to lack IL-6 receptor signalling specifically within the T cell compartment. IL-6 is the key cytokine driving Tfh development via STAT3 signalling and expression of the bcl-6 transcription factor, in combination with IL-21, which is produced by Tfh cells [12–14]. This contrasts with the Th2 developmental pathway involving IL-4, STAT6 and GATA-3. We sensitized these animals with HDM and elicited allergic airway inflammation and anti-Der p 1-specific IgE antibody responses. Our data reveal a key role for Tfh in IgE switching of B cells responding to HDM.

Methods

Mice

Wild-type C57BL/6 mice were purchased from Harlan UK (Bicester, UK). $CD4^{Cre} \times gp130^{-/-}$ mice [15], which lack the IL-6R signalling component specifically on T cells, were on a C57BL/6 background and bred in our specific-pathogen-free facility. Male and female animals were used. Experimental groups were matched for age and sex and co-housed for several weeks before use at 8–12 weeks of age. Experimental procedures were performed under UK Home Office authorization with approval from our institutional committee and in accordance with EU directives.

House dust mite sensitization and challenge

Each mouse was immunized i.p. with 25 μg HDM extract, prepared by sonication of whole *Dermatophagoides pteronyssinus* (Allergon AB, Angelholm, Sweden) in PBS and adsorbed to 200 μL alum gel (Alu-Gel-S, Serva, Heidelberg, Germany), on days 0 and 14. On days 21,24,25,26 and 27, mice were challenged with 25 μg HDM in 50 μL PBS intranasally. Animals were killed 24 h after the final challenge, and bronchoalveolar lavage (BAL) was performed with 1 mL PBS. Left lungs were weighed and digested with collagenase (250 μg/mL, Sigma) for 45 min to extract cells for flow cytometry. Right lungs were prepared for histology as described [16]. Blood was collected from the tail on day 14 and by cardiac puncture on day 28. Serum was collected and stored at −20°C, and anti-coagulated blood (sodium citrate) was stained directly with mAbs. Respiratory (mediastinal) lymph nodes were excised for cell culture.

T cell culture

Lymph node cell suspensions were cultured at 5×10^6/mL in XVIVO-15 serum-free medium (Lonza, Blackley, UK), 37°C 5% CO_2. HDM (10 μg/mL) was added alongside diluent controls. After 4 days, cell supernatants were collected.

Flow cytometry

Staining was performed with 50 μL blood or BAL/lung cells and 0.1 μg antibodies in 100 μL PBS+1% FBS. Antibodies used were as follows: anti-CXCR5-APC (SPRCL5), anti-PD-1-PerCPeFluor710 (J43), anti-IL-33R (ST2)-PerCPeFluor710 (RMST2-2), anti-IFN-γ-PE/Cy7 (XMG1.2), anti-IL-4-PE (11B11), anti-IL-17A-eFluor660 (eBio17B7), anti-CD4-FITC (RM4-5), all from eBioscience (Hatfield, UK) and anti-CD8ß-APC (53-5.8, BioLegend, London, UK). Blood was then treated with 0.5 mL erythrocyte lysis buffer for 10 min before washing. Intranuclear staining for bcl-6 was performed as described [17] using anti-bcl-6-PE (BCL-DWN), eBioscience. BAL differential cell counts and BAL T cell cytokine staining were performed as described [16]. A FACScalibur™ flow cytometer and CellQuest Pro™ software (BD Biosciences, Oxford, UK) were used. To identify Tfh in spleen cell populations, an alternative 6-colour protocol was used using a lineage-negative gating approach and staining for CXCR5, PD-1 and ICOS to discriminate Tfh. Splenocytes were stained with fixable near-IR dead cell stain (Life Technologies), then washed and stained with anti-CD4-FITC, anti-PD-1-PerCPeFluor710, anti-CXCR5-APC, anti-ICOS/CD278-PE (C398.4A) and biotinylated antibodies to lineage markers CD19 (eBio1D3), CD8α (53-6.7), CD11b (M1/70), CD11c (N418) and FcεR1 (MAR-1), all eBioscience. Cells were washed and stained with streptavidin-BV421 (BioLegend) before analysis on a BD FACS Canto II flow cytometer. Live cells were gated for $CD4^+$ lineage-negative cells first, before quantifying $CXCR5^+PD-1^+ICOS^+$ cells. To assess IgA coating of intestinal microbes [18], fresh stools were collected from separate cages of untreated mice of each strain, resuspended in 1 mL PBS and debris was removed by centrifugation. About 5 μL supernatant was stained in PBS/FCS with SYBR Green (Life Technologies, $1/10^5$) and anti-IgA-PE (11-44-2, eBioscience). Controls contained extracts from wild-type mice and isotype control antibody. Cells were then analysed by flow cytometry.

ELISAs

Total IgE was measured by sandwich ELISA as described [19]. Der p 1-specific IgE was measured by class capture assay using anti-IgE capture mAb (LOME-3, AbD Serotec, Kidlington, UK) and detection with biotinylated natural Der p 1 allergen (Indoor Biotechnologies, Cardiff, UK). Biotinylation was performed with a Sulfo-NHS Biotinylation Kit (Thermo Scientific, Loughborough, UK) according to instructions. Streptavidin–peroxidase (eBioscience)

was used to reveal binding. Anti-Der p 1 IgGs were measured by standard ELISA using 1 μg/mL coating with Der p 1 and detection with anti-mouse IgG isotype-peroxidase conjugates (AbD Serotec). Antibody tires were expressed as arbitrary units (AU/mL) by reference to standard curves of positive control sera. IL-4 and IL-13 in BAL or lymph node cell supernatants were measured using eBioscience Ready-Set-Go™ kits according to instructions.

Statistical analysis

GraphPad Prism 5 software (GraphPad, San Diego, CA) was used to plot and analyse the data. Unpaired *t*-tests were used except where significant differences between variances were observed, in which case Mann–Whitney tests were employed. Graphs are annotated as follows: *: $P < 0.05$, **: $P < 0.005$, ***: $P < 0.0005$, ****: $P < 0.00005$.

Results

CD4^{Cre}xgp130$^{-/-}$ mice are Tfh-deficient

CD4^{Cre}xgp130$^{-/-}$ mice have been previously characterized in our laboratory and others as Th17-deficient animals due to their lack of the IL-6R signalling molecule gp130 in the T cell compartment [15, 16]. As it has recently been demonstrated that IL-6 is also a key cytokine responsible for development of Tfh cells [12], we compared CD4^{Cre}xgp130$^{-/-}$ mice with their wild-type counterparts for Tfh responses. To avoid the presence of Th17 cells confounding the interpretation of the data, we used a model of allergic disease in which Th17 cells are largely absent, and allergen induces purely Th2-type responses. In the C57BL/6 strain, this can be achieved using HDM/alum parenteral sensitization followed by HDM intranasal challenge. We used this protocol in both strains in parallel and detected Tfh cells by staining for CD4, CXCR5, PD-1 and ICOS (CD278) [14]. Clearly distinguishable Tfh populations expressing these markers were only seen in splenocytes after gating out dead cells and cells of lineages other than CD4 T cells (Figs 1a and b). In peripheral blood, however, Tfh-like populations were distinguishable using a simple 3-colour staining protocol (Fig. 1c). In both spleen and blood, the frequencies of Tfh were significantly increased after HDM sensitization in wild-type mice (Figs 1b and c). However, there was no significant increase in these cells in CD4^{Cre}xgp130$^{-/-}$ animals, indicating that they were Tfh-deficient.

To confirm the absence of Tfh cells in CD4^{Cre}xgp130$^{-/-}$ mice, we stained for intranuclear expression of bcl-6, the signature transcription factor of Tfh [13], and showed increased bcl-6 staining within CD4 T cells of HDM-sensitized wild type but not CD4^{Cre}xgp130$^{-/-}$ mice (Fig. 1d) compared with control animals. To evaluate a Tfh-dependent parameter that was not dependent on allergen sensitization, we assessed the IgA response to intestinal microbes, which is detectable by flow cytometry of fecal extracts of untreated mice [18]. The results (Fig. 1e) show that wild-type mice produce IgA to these environmental antigens naturally, but CD4^{Cre}xgp130$^{-/-}$ animals do not, consistent with their lack of Tfh cells.

CD4^{Cre}xgp130$^{-/-}$ mice are deficient in IgG1 and IgE responses to Der p 1.

Serum from HDM-sensitized animals was assayed for allergen-specific antibodies (Fig. 2). Only secondary responses, after the second HDM/alum immunization, were detected, and peak responses were observed on day 28. Total IgE levels were increased around 40-fold in HDM-sensitized wild-type animals at this time point, but this response was reduced by 80–90% in CD4^{Cre}xgp130$^{-/-}$ mice. Likewise, both IgE and IgG1 responses to the major protein allergen of HDM, Der p 1, were dramatically reduced in these animals. Der p 1-specific IgG2a and IgG3 antibodies, which are associated with Th1-like responses in mice, were not detectable (not shown).

Allergic airway inflammation in CD4^{Cre}xgp130$^{-/-}$ mice is unaltered except in the case of B cell infiltration

Flow cytometry of BAL cells was used to enumerate leucocyte infiltration of airways on day 28 (Fig. 3a). This showed highly eosinophilic infiltrates induced by HDM in both strains, with around 95% of BAL cells being identified as eosinophils. Very few neutrophils, associated with Th17 responses, were seen, but these were significantly reduced in CD4^{Cre}xgp130$^{-/-}$ mice. Interestingly, B cells in the BAL, which are not normally increased in ovalbumin-induced allergic airway disease in C57BL/6 mice, were dramatically increased in HDM-sensitized wild-type animals but not in the CD4^{Cre}xgp130$^{-/-}$ strain, where this response was reduced by 75%. To assess general lung inflammation, lung weights from mice killed on day 28 were compared (Fig. 3b). HDM challenge significantly increased lung weights in both strains, indicating similar levels of inflammation. To assess the level of Th2 response in the lung tissue, cells extracted from lung tissue were stained for CD4 and IL-33R (ST2), a surface marker specific for Th2 cells [20]. These results (Fig. 3b) showed a significant tissue infiltration by Th2-type cells in HDM-challenged animals, with no difference in levels of response between strains.

Total T cell numbers in BAL were also increased similarly in both strains, although there was a non-significant trend for fewer T cells in BAL of CD4^{Cre}xgp130$^{-/-}$ mice (Fig. 3c). To assess T cell
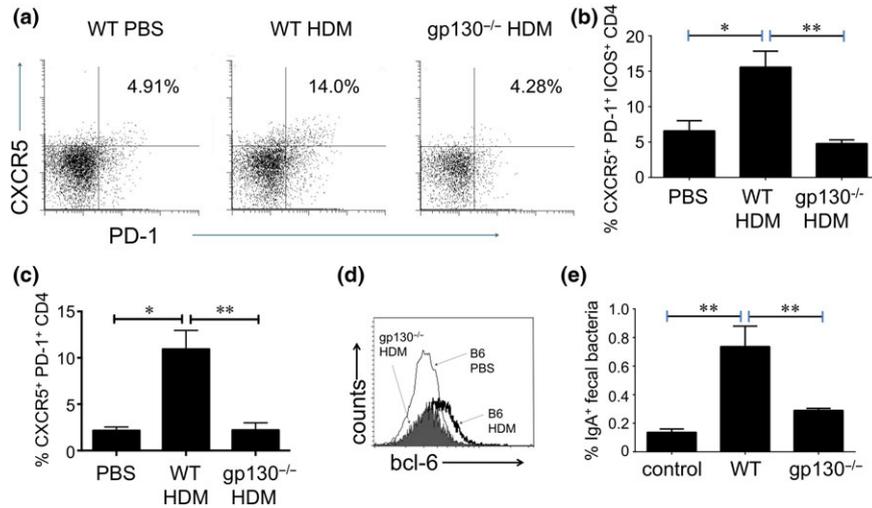
Fig. 1. CD4^{Cre}xgp130$^{-/-}$ mice are deficient in Tfh responses. (a) Staining for CXCR5 and PD-1 on gated lineage-negative, ICOS$^+$ CD4 cells from spleen of wild-type C57BL/6 or CD4^{Cre}xgp130$^{-/-}$ mice (gp130$^{-/-}$), sensitized and challenged with HDM (day 28) or PBS only. Double-positive cells were considered Tfh. (b) Pooled data showing proportions of CXCR5$^+$PD-1$^+$ICOS$^+$ CD4 splenocytes from mice as in a; $n = 5$, one of two independent experiments with similar results. (c) Staining for CXCR5 and PD-1 on peripheral blood CD4 T cells of mice as in b. (d) intranuclear staining for bcl-6 transcription factor in gated splenic CD4$^+$ T cells as in a; representative staining from two experiments is shown. (e) Flow cytometric detection of IgA coating on bacteria from fecal extracts of untreated wild-type C57BL/6 or CD4^{Cre}xgp130$^{-/-}$ mice. Samples were stained with SYBR Green DNA stain to identify bacteria and anti-mouse IgA or isotype control. %IgA-positive cells in DNA$^+$ events is shown; $n = 5$, one of two independent experiments with similar results.
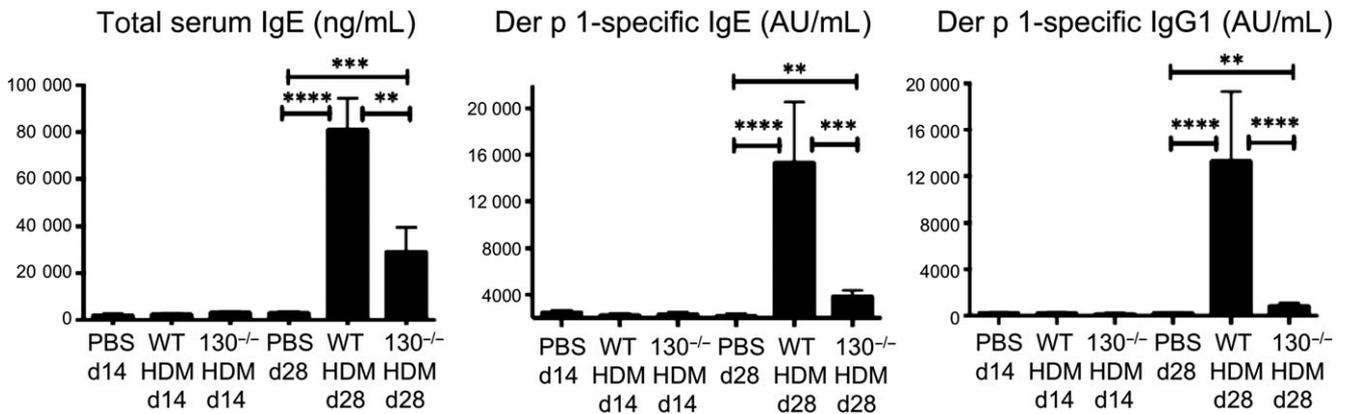

Fig. 2. CD4^{Cre}xgp130$^{-/-}$ mice are deficient in antibody responses to HDM. Animals sensitized as in Fig. 1 were bled on day 14 (primary response) and day 28 (secondary response) and total or Der p 1-specific IgE or IgG1 were measured as indicated in sera. $N = 8$–15 mice, pooled from three independent experiments.

cytokine profile, BAL T cells were isolated by magnetic selection, stimulated and intracellular cytokine staining for IL-4, IL-17 and IFN-γ performed in CD4 T cells (Fig. 3c). The response was highly Th2-skewed, with large increases in IL-4$^+$ T cells but not IL-17 or IFN-γ-producing cells after HDM challenge. The Th2 response was not significantly different in CD4^{Cre}xgp130$^{-/-}$ mice, but they did have significantly fewer Th17 cells than wild type despite the low numbers. Histological sections of the lung were examined (Fig. 3d) and showed inflammation of the airways in both strains of mice after allergen challenge, with fewer lymphocytes

(T or B cells) being apparent in CD4^{Cre}xgp130$^{-/-}$, consistent with the BAL cell analysis.

IL-4 and IL-13 production are unaltered in CD4^{Cre}xgp130$^{-/-}$ mice

As Tfh cells are reported to be a major source of IL-4 [21], we examined secretion of IL-4 in our HDM model (Fig. 4). We also measured IL-13, a Th2 cytokine not produced by Tfh cells [22]. Both cytokines showed the same pattern, being increased significantly in the BAL of both strains after HDM challenge. There were also no
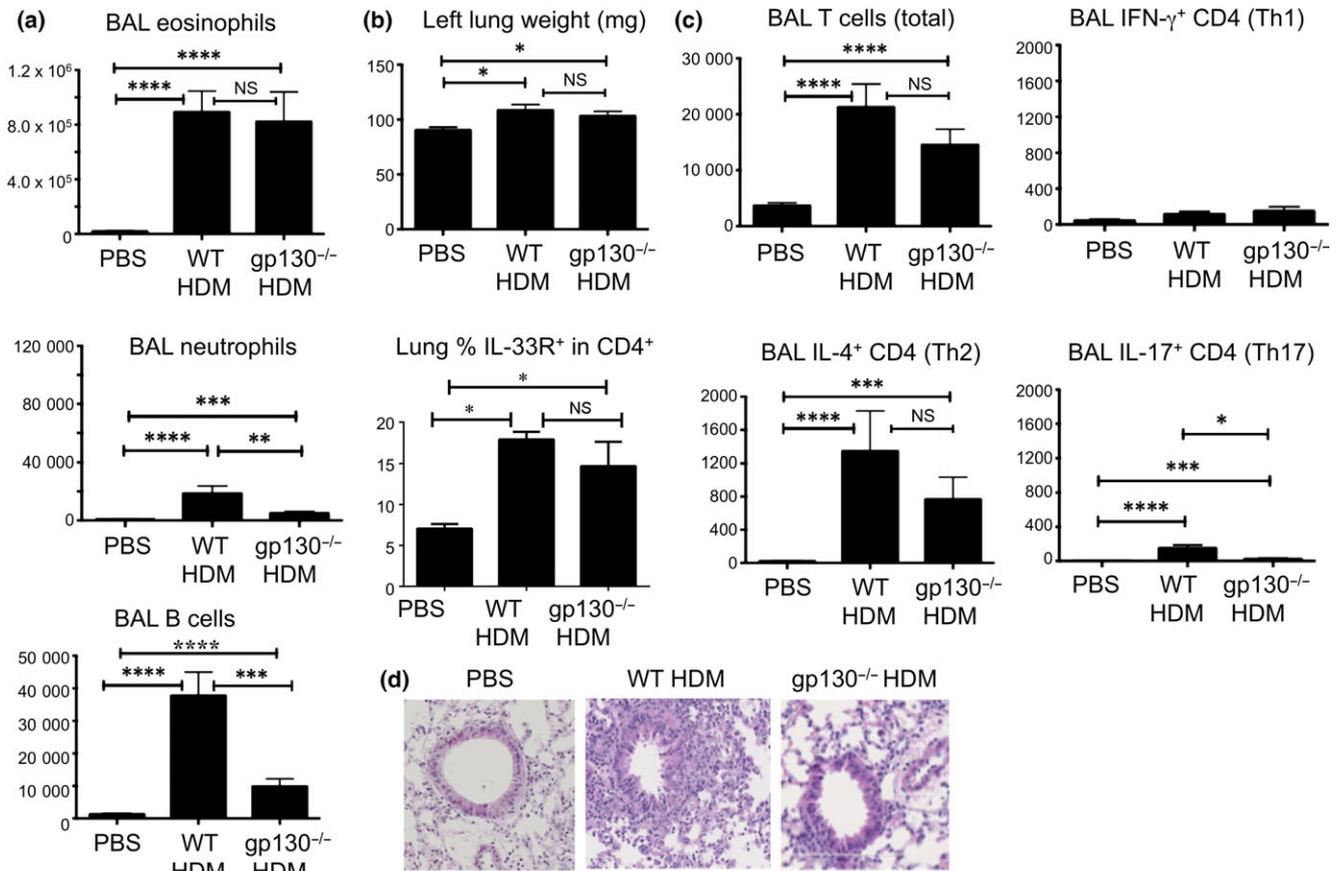
Fig. 3. CD4^{Cre}xgp130$^{-/-}$ mice exhibit normal allergic airway inflammation after HDM sensitization and respiratory challenge but lack B cell infiltration of the airways. Animals as in Fig. 1 were killed on day 28, and bronchoalveolar lavage cells were enumerated by flow cytometry and left lungs weighed. N = 9–15 mice, pooled from three independent experiments. (a) cell counts in BAL; (b) left lung weights and proportions of IL-33R$^+$ cells in CD4 T cells extracted from lung tissue; (c) BAL T cells were isolated and stimulated with anti-CD3/28 magnetic beads and stained for intracellular cytokines. Total cytokine producing CD4 cells are shown alongside total T cell counts in BAL; (d) haemotoxylin/eosin staining on histological sections from lungs, showing airways with lymphocytic infiltrates in allergen-challenged mice.

differences in the secretion of IL-4 and IL-13 in draining lymph node cells restimulated with HDM *in vitro*. These data suggest that Tfh cells were not the major source of IL-4 or IL-13 in the lung or lymph node.

Discussion

The role of the Th2 cell in allergic diseases such as asthma was first proposed over 20 years ago [3], and both intervening and recent data confirm the importance of this subset in driving allergic disease in humans [23]. However, the discovery of Tfh cells, which produce IL-4 but have distinct pathways of differentiation, trafficking and cytokine profile to Th2 cells, has thrown into question the role of Th2 in IgE synthesis. Tfh cells are responsible for isotype switching in B cells and, in a model of helminth infection, were shown to be the major source of IL-4 [24]. Switching of B cells to IgG and IgE isotypes occurs primarily at the boundary of T cell:B cell areas in lymphoid follicles, and

large-scale secretion of these antibodies requires plasma cell generation in germinal centres [25]. Nevertheless, it has been suggested that switching to IgE may occur in other sites, such as mucosal tissues, or in less organized areas of T cell:B cell interaction than classical germinal centres [25]. Due to the organization of immunoglobulin genes, the typical pathway for IgE switching is via an IgG1 (mice) or IgG4 (human) intermediate stage [10, 11], and both IL-4 and CD40L are critical in driving the switching process. However, direct switching from IgM to IgE synthesis has also been proposed, which might produce lower affinity IgE antibodies [25]. As Th2 cells typically express CCR4, CCR3 and CCR8 chemokine receptors and migrate to inflamed peripheral tissues such as the lung [26], while Tfh express CXCR5 and migrate towards germinal centres of lymphoid tissue [4], we asked whether Tfh were responsible for IgE switching in HDM-driven allergic disease.

Our results are the first to demonstrate a critical role for Tfh in IgE responses to an aeroallergen. We first
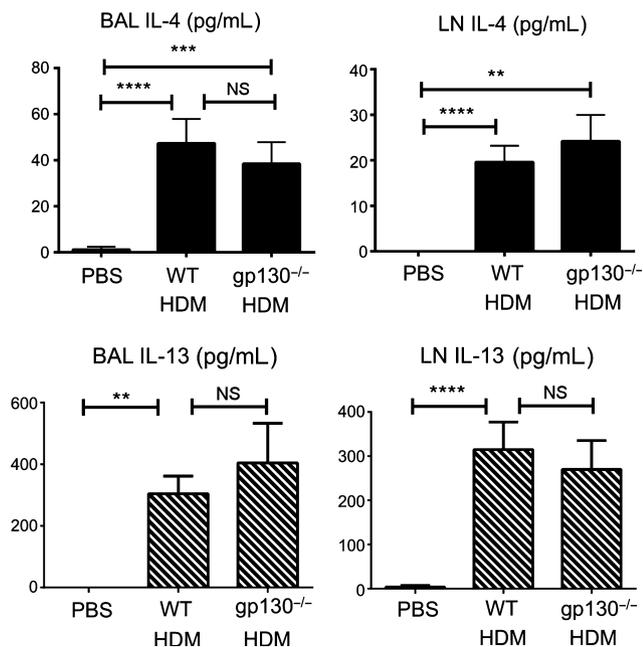
Fig. 4. Th2 cytokine levels in lung and lymph node supernatants are unaltered in CD4^{Cre}xgp130$^{-/-}$ mice. IL-4 and IL-13 levels were measured by ELISA in BAL fluid (left) or 4 days supernatants from HDM-stimulated lymph node cells (LN), from mice as in Fig. 1. N = 8–13 mice, pooled from three independent experiments.

demonstrated that CD4^{Cre}xgp130$^{-/-}$ mice are deficient in Tfh responses, due to the lack of gp130 signalling in T cells dependent on their lineage-specific genetic deletion. It should be noted that gp130 is a signalling component for several cytokine receptors in addition to IL-6 (the key differentiating factor for Tfh), including IL-27 and IL-11 [27]. Also IL-6 can induce Th17 as well as Tfh cells, dependent on the presence of TGF-ß [28]. Therefore, a lack of Tfh cells is not the only immunological abnormality in CD4^{Cre}xgp130$^{-/-}$ animals and this could alter the interpretation of our results. However, we chose a model with negligible Th17 response and previous work has shown that these animals are not immunodeficient, displaying T cell responses that are generally mildly skewed towards Th2 or regulatory T cell responses [15, 16]. This contrasts with a recently described model of Tfh deficiency in which bcl-6 is targeted in T cells [29]. These mice showed deficient Th2-type responses in lymph node cells after sheep red blood cell immunization. Thus, although our mouse model has limitations, it is the most suitable currently available strain to test our hypothesis.

Our data show a dramatic but not complete deficiency in antibody responses in CD4^{Cre}xgp130$^{-/-}$mice, including both IgG and IgE responses. We conclude that Tfh cells are largely responsible for switching B cells to IgG1 and IgE in the context of HDM sensitization leading to release of Der p 1 allergen-specific antibodies.

This conclusion is supported by the facts that (i) the genetic deficiency in IL-6R was limited to the T cell lineage by expression of the CD4 Cre transgene, excluding off-target effects of IL-6, (ii) no defect in Th2 responses was observed in CD4^{Cre}xgp130$^{-/-}$ animals, as shown by lung and lymph node responses and previous work [15, 16], (iii) the ability of CD4^{Cre}xgp130$^{-/-}$ animals to produce IgG and IgA antibodies of any isotype or exhibit B cell inflammation was greatly reduced, consistent with Tfh deficiency. Thus, based on current knowledge of T cell development, it is reasonable to conclude that the altered antibody responses were due to a lack of Tfh cells.

One limitation of our data was the identification of Tfh cells by flow cytometry using CXCR5 and PD-1 markers. Similar to other reports [30], we found this population quite difficult to resolve from other T cells in splenocyte populations and could not reliably identify them in lymph node cells (not shown). Unexpectedly, peripheral blood Tfh cell populations were most readily detectable. Tfh cells are present in the circulation, although they exhibit distinct characteristics to the classical Tfh [31, 32]. More definitive Tfh markers would be useful for cell sorting and adoptive transfer studies to detail the functions and trafficking of Tfh populations in allergic models.

Interestingly, allergic airway inflammation was essentially unaltered in Tfh-deficient mice after HDM challenge. CD4^{Cre}xgp130$^{-/-}$ mice are therefore useful to differentiate the Th2, cell-mediated pathway of allergic disease from humoral and IgE-dependent pathways which are not essential for eosinophilic inflammation [33]. IL-6 is essential for Tfh development *in vivo* by directing T cell differentiation in cooperation with IL-21 [12–14]. Tfh-deficient mice had normal levels of lung inflammation, eosinophilia and Th2 (IL-4-producing) CD4 T cells in their airways. Although there were on average fewer Th2 cells in BAL of CD4^{Cre}xgp130$^{-/-}$ mice, this was not significant and could be explained by the trend for lower numbers of total T cells in BAL rather than a shift in cytokine profile, as confirmed by the IL-33R staining data in lung tissue CD4 cells. The trend for lower levels of T cell inflammation was also not significant but might be related to reduced B cell or neutrophil recruitment in CD4^{Cre}xgp130$^{-/-}$ mice. As expected, the genetically altered strain was deficient in Th17 cells and neutrophils as previously shown in an ovalbumin model [16]; however, the level of this response was negligible in comparison to Th2-dependent eosinophilia. Unexpectedly, large numbers of B cells were seen to infiltrate the airways after HDM challenge of wild-type mice, a response that is not seen when using ovalbumin as the allergen which results in a mixed Th17/Th2 response [16]. This B cell migration was greatly reduced in Tfh-deficient mice. B cell

recruitment is therefore presumably dependent on both highly polarized Th2 cells in the lung and IgG1/IgE-switched B cell development. These factors may contribute to local IgE release and mast cell activation. The IL-4$^+$ CD4 T cells in the airways did not express CXCR5 and are therefore considered Th2 cells (not shown). Consistent with this, IL-4 and IL-13 levels in the BAL and secreted from allergen-stimulated lymph node cells were indistinguishable in Tfh-deficient vs. wild-type animals. IL-13 is secreted by Th2 cells but not Tfh cells [22]. These results suggest that in contrast to the response to helminth infection [24], Th2 cells and not Tfh are the major source of IL-4 in HDM/alum-driven sensitization. This was apparent even in draining lymph node cells where Tfh would be expected to accumulate in response to allergen.

Our data do not exclude a critical role for Th2 cells in IgE synthesis. The similar reduction in IgE and IgG1 responses in our model strongly suggests that Tfh cells are required for IgE switching by providing IgG1-switched B cells as an intermediate stage [11]. However, Th2 cells may be involved in later stages of IgE$^+$ B cell development after Tfh involvement and may also be responsible for the 10–20% of the IgG1/IgE response that remained in CD4^{Cre}xgp130$^{-/-}$ animals. Lastly, they could be responsible for the recruitment of Tfh-switched B cells to the airways during inflammation, driven by HDM exposure. Overall, our data demonstrate that IL-6-driven Tfh development is a critical stage in allergic sensitization which may be amenable to therapeutic intervention and may help to explain the patchy response to Th2-targeted therapies [34]. Biotherapeutics targeting IL-6 are in advanced development for inflammatory diseases and might prove useful in long-term treatment of IgE-mediated pathology.

Conflict of interest

The authors declare no conflict of interest.

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