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ARTICLE OPEN

Synergistic activation of pro-inflammatory type-2 CD8$^+$ T lymphocytes by lipid mediators in severe eosinophilic asthma

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Human type-2 CD8$^+$ T cells are a cell population with potentially important roles in allergic disease. We investigated this in the context of severe asthma with persistent airway eosinophilia—a phenotype associated with high exacerbation risk and responsiveness to type-2 cytokine-targeted therapies. In two independent cohorts we show that, in contrast to Th2 cells, type-2 cytokine-secreting CD8$^+$CRTH2$^+$ (Tc2) cells are enriched in blood and airways in severe eosinophilic asthma. Concentrations of prostaglandin D_2 (PGD_2) and cysteinyl leukotriene E_4 (LTE_4) are also increased in the airways of the same group of patients. In vitro PGD_2 and LTE_4 function synergistically to trigger Tc2 cell recruitment and activation in a TCR-independent manner. These lipids regulate diverse genes in Tc2 cells inducing type-2 cytokines and many other pro-inflammatory cytokines and chemokines, which could contribute to eosinophilia. These findings are consistent with an important innate-like role for human Tc2 cells in severe eosinophilic asthma and suggest a potential target for therapeutic intervention in this and other diseases.

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INTRODUCTION

Type-2 cytokines (interleukin (IL)-4/5/9/13) orchestrate allergic inflammation, driving type-2 CD4$^+$ T helper (Th2) cell differentiation, IgE production, mucus hypersecretion and airway hyperresponsiveness (AHR). Specifically, IL-5 activates and is chemotactic to eosinophils and prolongs their survival. Anti-type-2 cytokine therapies, notably mepolizumab, an anti-IL-5 antibody, are effective in severe eosinophilic asthma by reducing circulating eosinophils and asthma exacerbations.[1–3] The major sources of such type-2 cytokines are Th2, group 2 innate lymphoid cells (ILC2)[4] and type-2 CD8$^+$ T cells (Tc2). Of these, most attention has been paid to CD4$^+$ T cells and more recently ILC2s, especially in human disease. Although, it has been known that type-2 CD8$^+$ T-cell populations exist, their overall functionality, transcriptional machinery and the mechanisms by which they are triggered have not been defined. This is important to address as recent data in other contexts have revealed previously overlooked functional diversity of human CD8$^+$ T cells in inflammatory diseases.[5]

Eosinophilic asthma constitutes an important clinical phenotype, defined by increased airway eosinophils,[6,7] which release granule-derived basic proteins, lipid mediators, cytokines and chemokines, driving inflammation and exacerbations.[8,9] In some patients with severe asthma, airway eosinophils persist despite use of high-dose inhaled corticosteroids, suggesting relative steroid-insensitivity.[10] This phenotype is commonly associated with co-morbid rhinosinusitis, nasal polyposis and aspirin-induced bronchoconstriction.[11] Eosinophilic asthma is commonly considered as a Th2 disorder based on human data in mild asthma[12,13] and animal models.[14] Recently, ILC2s have been implicated in murine airway inflammation,[15] and increased ILC2s are reported in human asthma.[16,17] In contrast, although some data exist for overall involvement of CD8$^+$ cells in asthma in both human[18–20]—in which CD8$^+$ cell frequencies correlated with disease severity and asthma mortality—and murine[21] studies, which suggest bystander activation, the specific functional role of Tc2 cells remains largely unexplored, particularly in defined asthma phenotypes. Improved understanding of the pathogenic roles of Tc2 in this specific phenotype is important for therapeutic advances.

All type-2 cytokine-producing cells highly express chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), a receptor for prostaglandin D_2 (PGD_2).[4,22] Through CRTH2, PGD_2 elicits chemotaxis, type-2 cytokine production and suppresses apoptosis in Th2 and ILC2s.[23–25] The clinical efficacy of CRTH2 antagonists varies, being greatest in severe eosinophilic asthma.[26,27] We have previously shown synergistic enhancement of PGD_2 with cysteinyl leukotrienes (CysLTs) in activating Th2 and

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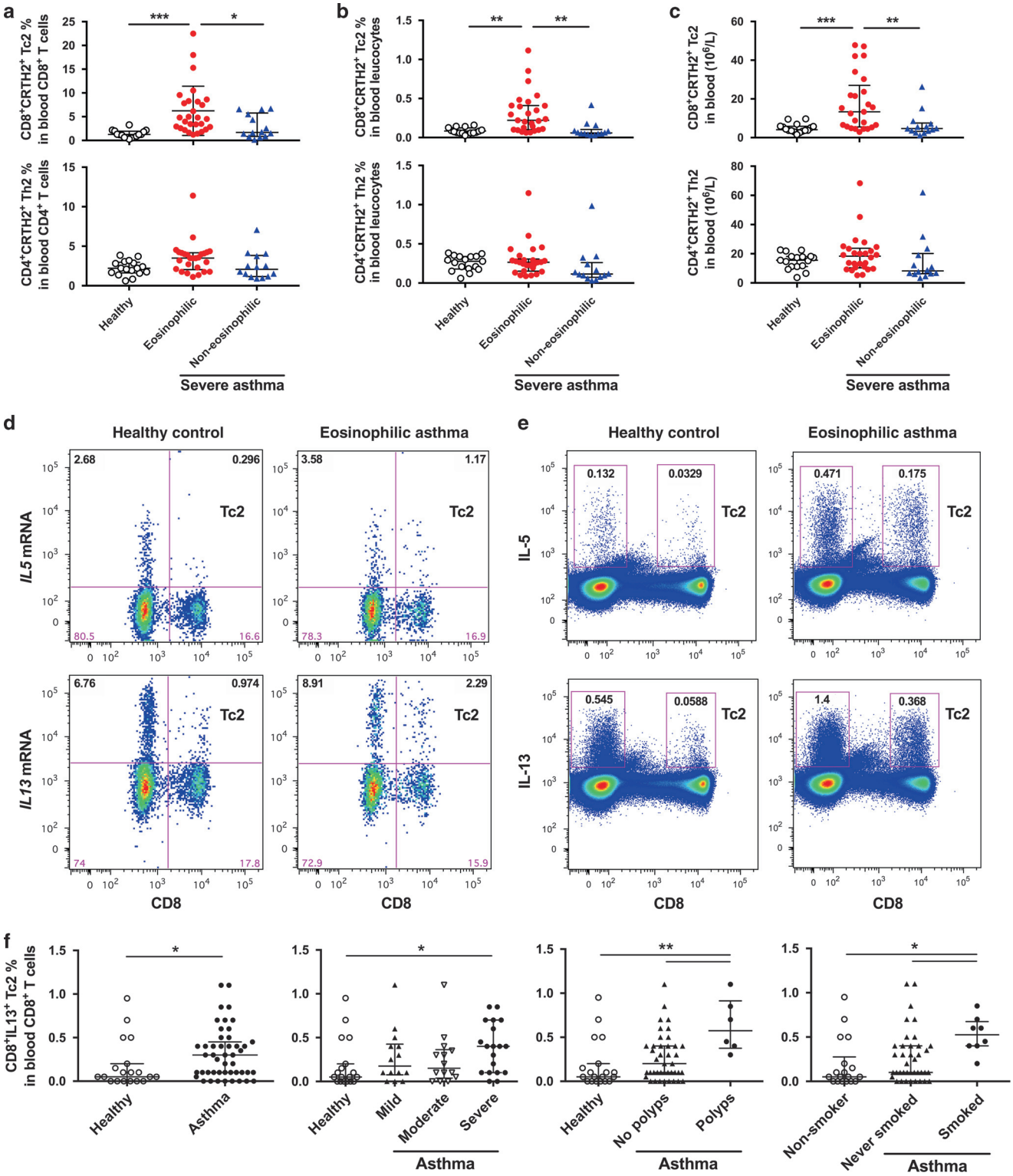

Fig. 1 Tc2 cells are enriched in peripheral blood in severe eosinophilic asthma. **a**–**c** Frequencies of $CD3^+CD8^+CRTH2^+$ Tc2 and $CD3^+CD4^+CRTH2^+$ Th2 cells in $CD4^+$ or $CD8^+$ T cells (**a**), in total leucocytes (**b**) or numbers of these cells in peripheral blood (**c**) were compared between healthy controls and severe asthma patient groups (eosinophilic and non-eosinophilic) of the Oxford cohort with flow cytometry. **d**, **e** IL-5- and IL-13-producing Tc2 cells in blood detected with PrimeFlow (**d**) or ICS after stimulation with 25 ng/ml PMA and 1 µg/ml ionomycin (**e**) were compared between healthy control and severe eosinophilic asthma. **f** Frequencies of IL-13-secreting $CD3^+CD8^+$ T cells in peripheral blood determined by flow cytometry with intracellular cytokine staining were increased in asthmatic patients from the Southampton cohort (left panel), which was associated with asthma severity (left middle panel), history of nasal polyposis (right middle panel) and history of smoking (right panel). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (data in **d** and **e** are representative of three and five independent experiments)

Table 1. Study subjects (mean ± SD)

Characteristic	Oxford cohort			*p*-Value	Southampton cohort			*p*-Value
	Control (*n* = 16)	Severe asthma			Control (*n* = 22)	asthma		
		Non-eosinophilc (*n* = 14)	Eosinophilic (*n* = 26)			Non-eosinophilc (*n* = 42)	Eosinophilic (*n* = 10)	
Age (y)	36.6 ± 12.6	62.3 ± 9.1	53.7 ± 13.7	0.0001	31.7 ± 11.9	38.4 ± 15.1	43.8 ± 15.4	0.1
Sex (male%)	15	67	50		64	38	80	0.03
Atopy (%)	23	33	58		0	81	100	<0.0001
BMI	22 ± 2.7	27.7 ± 3.0	31.9 ± 7.2	0.0001	25.5 ± 4.9	29.2 ± 7.8	28.12 ± 5.2	0.1
FEV1 (% pred)	104.2 ± 10.5	76.8 ± 23.3	75.7 ± 23.9	0.0007	107.4 ± 13.8	88.6 ± 22.0	69.6 ± 22.3	<0.0001
Sputum eosinophils (%)	0.25 ± 0.12	1.4 ± 0.8	28.7 ± 24.0	<0.0001	0.25 ± 0.65	0.72 ± 0.67	10.4 ± 10.2	<0.0001
FeNO (ppb)	26 ± 12	23 ± 9.0	38 ± 37	0.6	17 ± 7.1	42 ± 45	63 ± 48	0.001
Blood eosinophils (10^9/l)	0.14 ± 0.08	0.24 ± 0.27	0.57 ± 0.3	<0.0001	0.15 ± 0.07	0.21 ± 0.15	0.37 ± 0.28	<0.05
Serum IgE (IU/ml)	33 ± 53	271 ± 367	487 ± 820	0.001	37 ± 30	251 ± 401	541 ± 698	0.0002
ICS dose (BDP equivalent µg)	0	1329 ± 574	1758 ± 486	<0.0001	0	762 ± 794	1452 ± 1172	<0.0001
Leukotriene receptor antagonist use, *n* (%)	0 (0)	4 (29)	10 (38)		0 (0)	11 (26)	4 (40)	

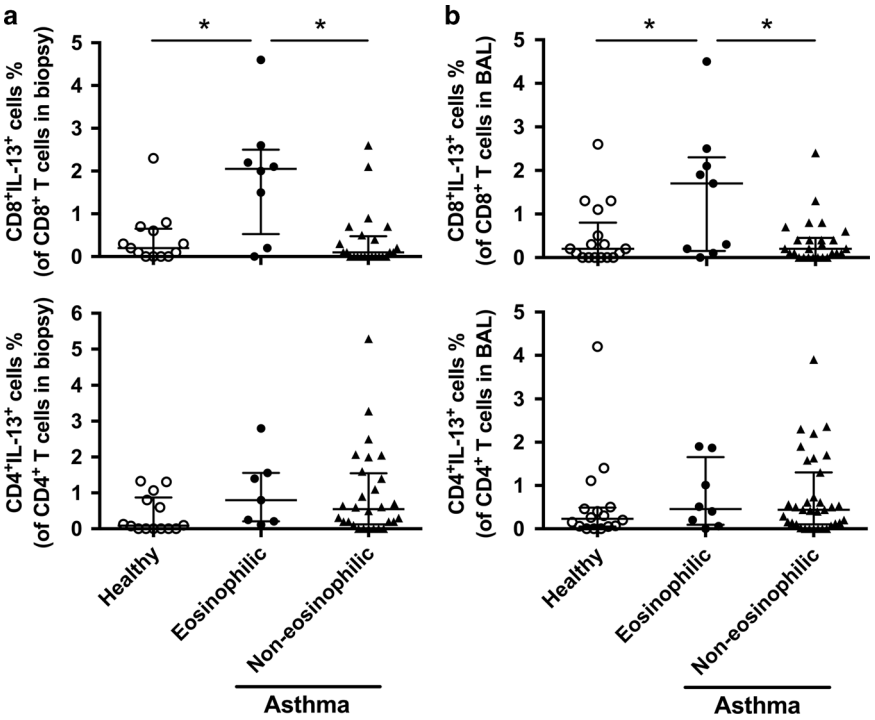

Fig. 2 Tc2 cells are increased in lung in eosinophilic asthma of the Southampton cohort. Frequencies of $CD3^+CD8^+IL\text{-}13^+$ and $CD3^+CD4^+IL\text{-}13^+$ T cells in BB (**a**) and BAL (**b**) were compared between healthy controls and asthma groups (eosinophilic and non-eosinophilic) with ICS. *$p < 0.05$

ILC2s.[28,29] These lipid mediators and their receptors have not been studied in relation to $CD8^+$ cells.

To investigate this, we first analysed type-2 $CD8^+$ T-cell frequencies and functional profiles in blood, bronchoalveolar lavage (BAL) and bronchial biopsies (BB) in well-defined patient cohorts, and further evaluated whether the airway environment is conducive to Tc2 activation via CRTH2 by measuring airway PGD_2 and leukotriene E_4(LTE_4). We then defined the activity of these lipids on Tc2 cells in vitro and investigate a mechanistic link between Tc2 cell activation and airway eosinophilia. Our observations provide compelling evidence of innate-like activation of Tc2 cells by pro-inflammatory lipids, a diverse range of functions of this cell population, and a potential role in severe eosinophilic asthma.

RESULTS

Tc2 cells are enriched in eosinophilic asthma

CRTH2 is highly expressed on type-2 cytokine-producing human peripheral blood $CD8^+$ T lymphocytes (described here as Tc2 cells)

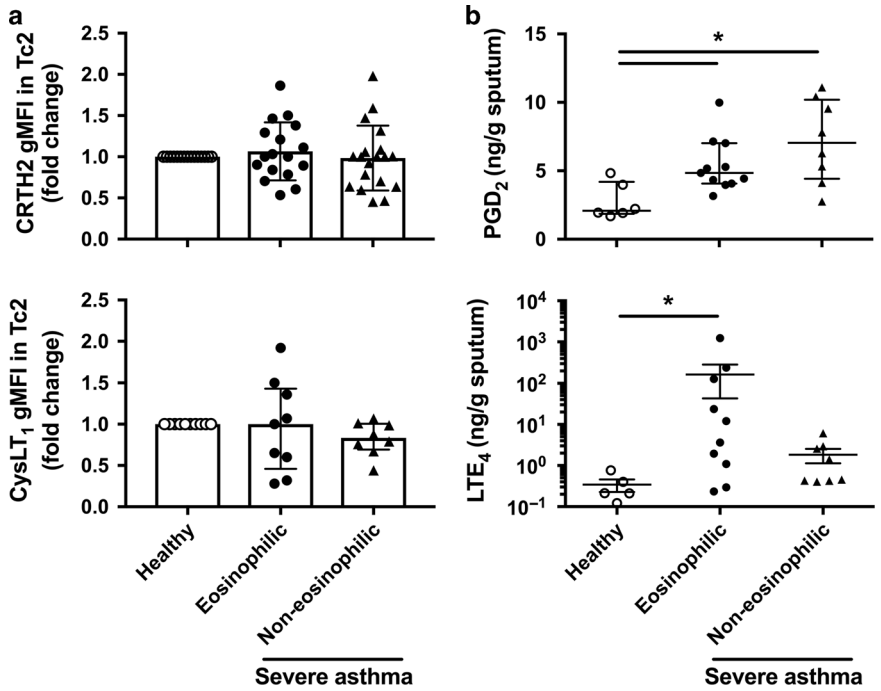

Fig. 3 Eicosanoid mediators but not their receptors are increased in lung in severe eosinophilic asthma in the Oxford cohort. **a** Expressions of CRTH2 (upper panel) and $CysLT_1$ (bottom panel) in individual Tc2 cells were paired compared between healthy controls and severe asthma groups with flow cytometry. **b** Levels of PGD_2 (upper panel) and LTE_4 (bottom panel) in sputum determined with ELISA were compared between healthy controls and severe asthma groups. *$p < 0.05$

(Supplementary Fig. 1a).[22] We therefore first analysed human Tc2 cells using the phenotypic expression of CRTH2 on $CD8^+$ T cells to define the Tc2 population in blood (Supplementary Fig. 1b). In a cohort of 56 participants from Oxford, UK, peripheral blood $CD3^+CD8^+CRTH2^+$ Tc2 cells were substantially higher in patients with severe eosinophilic asthma (~6.24 ± 5.18% of CD8, $n = 26$) than in severe non-eosinophilic asthma (~2.93 ± 2.46% of CD8, $n = 14$, $p < 0.05$) or health (~1.32 ± 0.79% of CD8, $n = 16$, $p < 0.001$; Fig. 1a; Table 1). Findings were similar when blood Tc2 cells were expressed as a percentage of total leucocytes or in absolute numbers (Fig. 1b, c; Supplementary Table 1). Conversely, the frequencies of $CD3^+CD4^+CRTH2^+$ Th2 cells (Supplementary Fig. 1b) did not differ significantly (Fig. 1a–c). Analysis of functional IL-5- and IL-13-producing $CD8^+$ T cells ex vivo detected with PrimeFlow assays at mRNA level (Fig. 1d) and intracellular cytokine staining (ICS) at protein level (Fig. 1e; Supplementary Fig. 2) also supported Tc2 enrichment in severe eosinophilic asthma, although only small numbers of IL-5-/IL-13-positive cells were detected by ICS in samples without stimulation (Supplementary Fig.2b). Furthermore, Tc2 cells were also found in the BAL and sputum in severe eosinophilic asthma (Supplementary Fig 3a). $CD8^+$ T cells were detected abundantly in BB from the same group of patients (Supplementary Fig. 3b).

We sought to confirm the validity of these findings in a second independent cohort of 74 participants from Southampton, UK (Table 1),[30] using a different and complementary cell gating strategy, ICS (Supplementary Fig. 4a), which allows us to test whether the findings obtained using phenotypic staining were recapitulated using a functional assay. We found $CD3^+CD8^+IL\text{-}13^+$ cells were increased in peripheral blood in asthma (~0.3% of $CD8^+$ T-cells, $n = 47$) compared with health (~0.05%, $n = 19$, $p = 0.04$; Fig. 1f, left panel). This increase correlated with asthma severity (Fig. 1f, middle-left panel; $p = 0.01$), with the presence of nasal polyps (Fig. 1f, middle-right panel; $p = 0.008$) and with previous smoking history (Fig. 1f, right panel; $p = 0.008$), co-morbidities associated with severe asthma. By contrast, frequencies of $CD3^+CD4^+IL\text{-}13^+$ cells were significantly increased in mild (steroid-naïve) asthma (0.5%, $n = 14$) compared with health (0.19% $n = 22$, $p < 0.01$), but not in steroid-treated moderate or severe asthma (Supplementary Fig. 5). IL-4 expression in sputum T cells was associated positively with peripheral blood $CD3^+CD8^+IL\text{-}13^+$ cell frequencies ($r_s = 0.537$, $p = 0.006$), but negatively with peripheral blood $CD3^+CD4^+IL\text{-}13^+$ cell frequencies ($r_s = -0.442$, $p = 0.03$) (Supplementary Fig. 6a).

As with peripheral blood (Fig. 1 and Supplementary Fig. 1b, c), $CD3^+CD8^+IL\text{-}13^+$ cells (Supplementary Fig. 4b,c) were strikingly increased in BB and BAL in eosinophilic asthma (~2.05%, $n = 8$ for BB; ~1.5%, $n = 9$ for BAL amongst $CD8^+$ T cells) compared with non-eosinophilic asthma (~0.1%, $n = 24$ for BB; ~0.2%, $n = 26$ for BAL) and health (~0.2%, $n = 13$ for BB; ~0.2%, $n = 17$ for BAL; $p < 0.005$) in the Southampton cohort (Fig. 2; Supplementary Table 1). Frequencies of $CD8^+IL\text{-}13^+$ cells in BB were correlated significantly with frequencies in BAL ($r_s = 0.385$, $p = 0.007$) (Supplementary Fig. 6b). Again, $CD3^+CD4^+IL\text{-}13^+$ cells in BB or BAL were not significantly increased in eosinophilic or non-eosinophilic forms. Amongst asthmatics, high frequencies of BB $CD3^+CD8^+IL\text{-}13^+$ cells were associated with high bronchodilator reversibility (Supplementary Fig. 6c, $p < 0.007$).

Stimulatory eicosanoid mediators but not their receptors are enriched in eosinophilic asthma

Since Tc2 cells highly express CRTH2 together with $CysLT_1$, a leukotriene receptor (Fig. 1a; Supplementary Fig. 7a), we compared their expression and levels of their ligands in the airways between asthma phenotypes (Fig. 3). The expression levels of CRTH2 and $CysLT_1$ in individual Tc2 cells by flow cytometry were not significantly changed in the Oxford cohort (Fig. 3a). PGD_2 assessed in sputum supernatants from asthma was significantly increased compared with health (2.76 ± 0.54 ng/g, $n = 6$, $p < 0.01$) although no significant difference between eosinophilic (5.4 ± 0.59 ng/g, $n = 11$) and non-eosinophilic groups (7.2 ± 1.46 ng/g, $n = 6$, $p = 0.14$) was detected (Fig. 3b). Pulmonary LTE_4

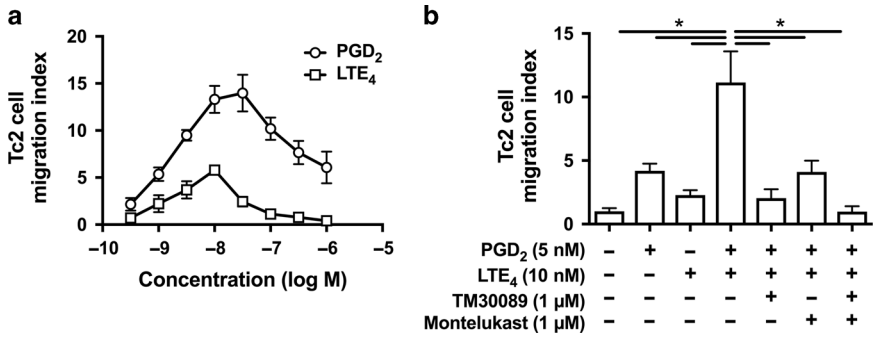

Fig. 4 Tc2 cells (cultured) migrate in response to PGD_2 and LTE_4. **a** Cell migration in response to various concentrations of PGD_2 and LTE_4 in chemotaxis assays. **b** Cell migration in response to the combination of PGD_2 and LTE_4 in the absence or presence of TM30089 and montelukast. *$p < 0.0001$ ($n = 3$)

was only significantly increased in patients with severe eosinophilic asthma (164.5 ± 52 ng/g, $n = 10$) but not in non-eosinophilic patients (1.84 ± 0.65 ng/g, $n = 8$), compared with health (0.34 ± 0.15 ng/g, $n = 5$, $p = 0.1$; Fig. 3b).

Tc2 cell migration induced by PGD_2 and LTE_4

To explore the potential pathogenic role of Tc2 cells and these lipid mediators, we isolated and cultured human Tc2 cells for further in vitro investigation (Supplementary Fig. 7b). These cells are $CCR7^{-}CD62L^{-}$ effectors (Supplementary Fig. 7c) and showed higher baseline type-2 gene expression signatures (*IL17RB*, *GPR44*, *CLECL1*, *IL9R*, *NAMPT*, *AF208111*, *HPGDS*, *P2RY14*, *RG4*, *IRS2* and *GATA3*) but lower type-1 (*IFNG*, *AIF1*, *LTA*, *TXK* and *IL18RAP*) and killer cell gene signatures (*KIR2DL1*, *KIR2DL4*, *KIR2DL5A*, *KLRF1*, *CD160* and *TYPOBP*) compared with other $CD8^{+}$ cells (Supplementary Fig. 7d).

PGD_2 and CysLTs are chemotactic agents for many types of immune cells.[25,28] To investigate airway Tc2 cell recruitment, we examined the effect of these lipids in chemotaxis assays. Both lipids caused cell migration in a typical bell-shaped dose-dependent manner, peaking at ~30 nM for PGD_2 and ~10 nM for LTE_4 (Fig. 4a). The maximum response induced by PGD_2 was higher (2.4-fold) than that by LTE_4. Cell migration was synergistically enhanced by combined stimulation (Fig. 4b). The contribution of PGD_2 and LTE_4 on the cell migration was blocked by the CRTH2 antagonist TM30089 and the $CysLT_1$ antagonist montelukast.

Enhancement of type-2 cytokine production in Tc2 cells by PGD_2 and LTE_4

We investigated the effects of PGD_2 or LTE_4 on type-2 cytokine production by Tc2 cells (Fig. 5). After treatment with PGD_2 or LTE_4, IL-5 and IL-13 production was elevated at both transcriptional (Fig. 5a) and translational (Fig. 5b) levels in a dose-dependent manner with half maximal effective concentration (EC_{50}) = 17.3 nM (6.1 ng/ml) at mRNA or 17.9 nM (6.3 ng/ml) at protein on IL-5, and 21.1 nM (7.4 ng/ml) at mRNA or 16 nM (5.6 ng/ml) at protein on IL-13 for PGD_2; and $EC_{50} = 4.5$ nM (2 ng/ml) at mRNA or 13.5 nM (5.9 ng/ml) at protein on IL-5, and 7.4 nM (3.3 ng/ml) at mRNA or 9 nM (4 ng/ml) at protein on IL-13 for LTE_4, which were close to the concentrations of these lipids detected in patients' sputa (Fig. 3b). Responses to PGD_2 were significantly stronger than to LTE_4. Compared with Th2 cells,[23,31] the effect of PGD_2 and LTE_4 on Tc2 is much more potent (Fig. 5c).

We then further examined type-2 cytokine production by Tc2 cells in response to the lipids alone or combination (Fig. 5d, e). Both PGD_2 and LTE_4 increased type-2 cytokine production. Their combination enhanced the response synergistically. Using PrimeFlow assays to analyse IL-5/IL-13 mRNA at individual cell level confirmed these data (Fig. 5f; Supplementary Fig. 7e). IL-5-/13-positive cells were increased from 2.88% (control) to 23.17%, 12.23% or 28.8% after treatment with PGD_2, LTE_4 or their combination, respectively. Among these positive cells, some expressed IL-5 dominantly, some IL-13 dominantly and only some produced IL-5/13 simultaneously (Supplementary Fig. 7e), although >90% of these cells were capable of producing both following Phorbol-12-myristate 13-acetate (PMA)/ionomycin stimulation (Supplementary Fig. 7f).

Effect of PGD_2 and LTE_4 on the gene expression profile of Tc2 cells

Using microarrays we investigated Tc2 transcriptional responses to PGD_2 and LTE_4. In all, 1104, 3360 and 4593 gene transcriptions were significantly modulated ($p < 0.05$) (including up/downregulation) by LTE_4, PGD_2 or their combination, respectively (Supplementary Fig. 8a). The effect of PGD_2 was much broader and stronger than that of LTE_4, and the effect of the combination treatment was mainly contributed by PGD_2 (Supplementary Fig. 8b).

We next focused on the genes encoding cytokines, chemokines, their receptors and CD molecules (Fig. 6a; Supplementary Table 2). About 90 such genes were significantly modulated, mostly upregulated, and most obviously cytokines (*IL3*, *IL5*, *IL8*, *IL13*, *IL22*, *CSF2*, *TNF* and *XCL1*). Although a few of these were induced by LTE_4 alone, most were driven by PGD_2 alone or the combination. Some transcriptional changes were regulated only by the combination treatment (eg, *IL21*, *IL22*, *LASS1*, *CCL3*, *CCL4*, *IL1RL1*, *CD1E* and *CCL21*). Microarray data were largely confirmed by PCRarray on human common cytokines although some significant effects (*IL9* and *CSF1*) were detected only in PCRarray (Fig. 6b).

For verification, we assayed selected cytokines by quantitative real-time (RT) PCR (qPCR; Fig. 6c) and Luminex (Fig. 6d). At mRNA level, most of the genes (except *CSF1*) showed synergistic effects of PGD_2 and LTE_4. At protein level, effects of LTE_4 were marginal in some cytokines (IL-3, IL-21 and IL-22), while effects of PGD_2 were obvious in all the genes, particularly significant in IL-8, IL-22, granulocyte-macrophage colony-stimulating factor (GM-CSF), CSF1, tumour necrosis factor-α (TNFα) and CCL4. Combination treatment either additively (CSF1) or synergistically (other genes) enhanced cytokine production. IL-2 enhanced the effects of the lipids on some cytokine (IL-5/8/13 and GM-CSF) production, particularly in PGD_2 stimulation (Supplementary Fig. 9). The effects of PGD_2 and LTE_4 were inhibited by TM30089 and montelukast, respectively (Fig. 6e).

Tc2 cells expressed cytotoxic proteins perforin and granzymes (GZMA, GZMB and GZMK) without stimulation (Supplementary Fig. 10). PGD_2 and LTE_4 had no significant effect, although activation of the cells by PMA/ionomycin downregulated the expression of the cytotoxic proteins.

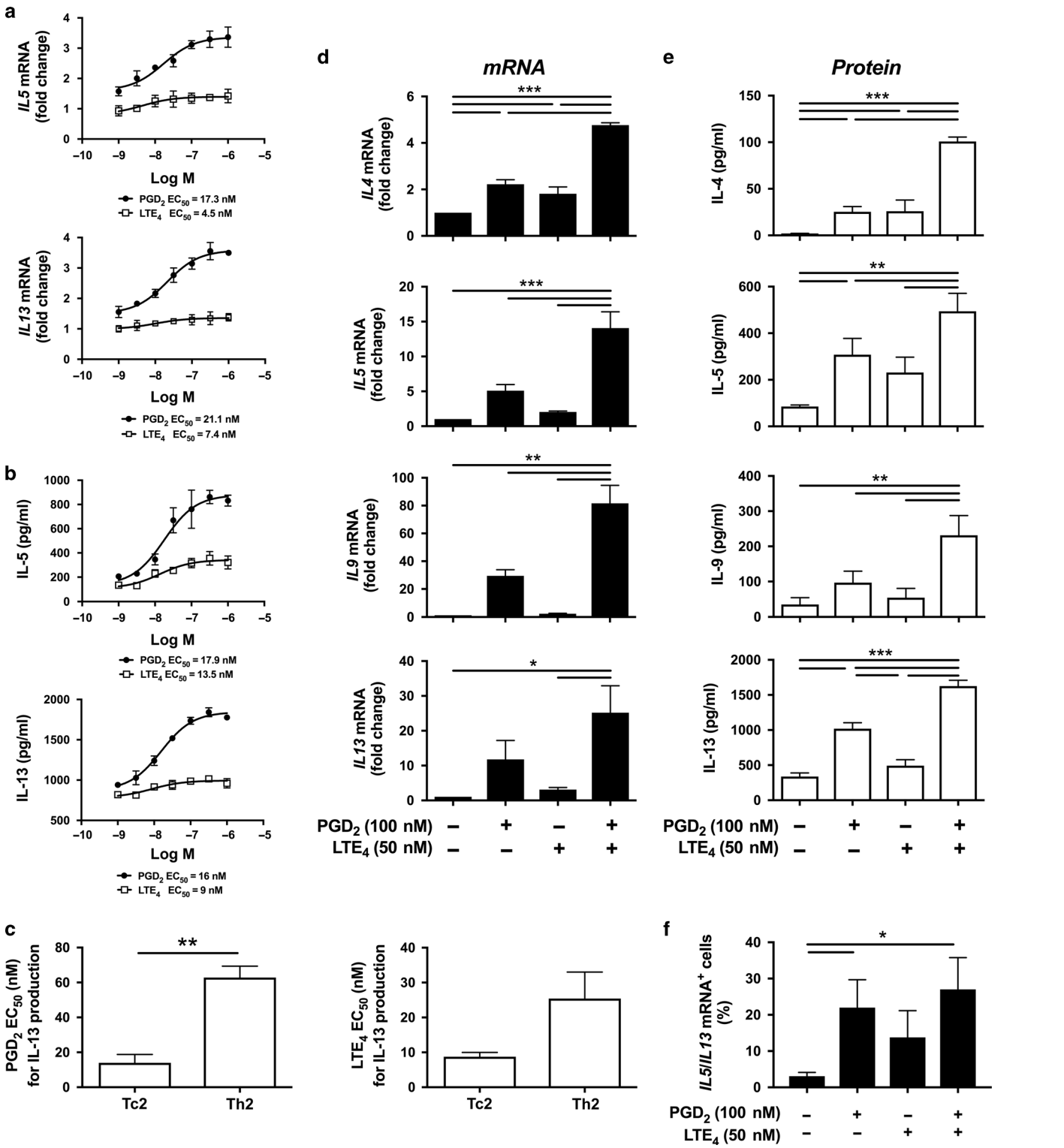

Fig. 5 PGD_2 and LTE_4 promote type-2 cytokine production in cultured Tc2 cells. **a**, **b** mRNA levels by qPCR in Tc2 cells (**a**) and protein levels by ELISA in the cell supernatants (**b**) for IL-5 and IL-13 after treatment with various concentration of PGD_2 and LTE_4. **c** Half maximal effective concentration (EC_{50}) of PGD_2 or LTE_4 for IL-13 production in Tc2 cells compared with that in Th2 cells. **d**, **e** mRNA levels in Tc2 cells measured by qPCR (**d**) and protein levels in the cell supernatants measured by Luminex (**e**) for type-2 cytokines after treatment with PGD_2 and LTE_4 alone or their combination. The mRNA levels in control samples were treated as onefold. **f** Increase of *IL5*- and *IL13*-mRNA-positive Tc2 cells after treatment with PGD_2 and LTE_4 alone or their combination detected by using PrimeFlow RNA assay. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ ($n = 3$ for **a**–**c** and **f**; $n = 6$ for **d** and **e**)

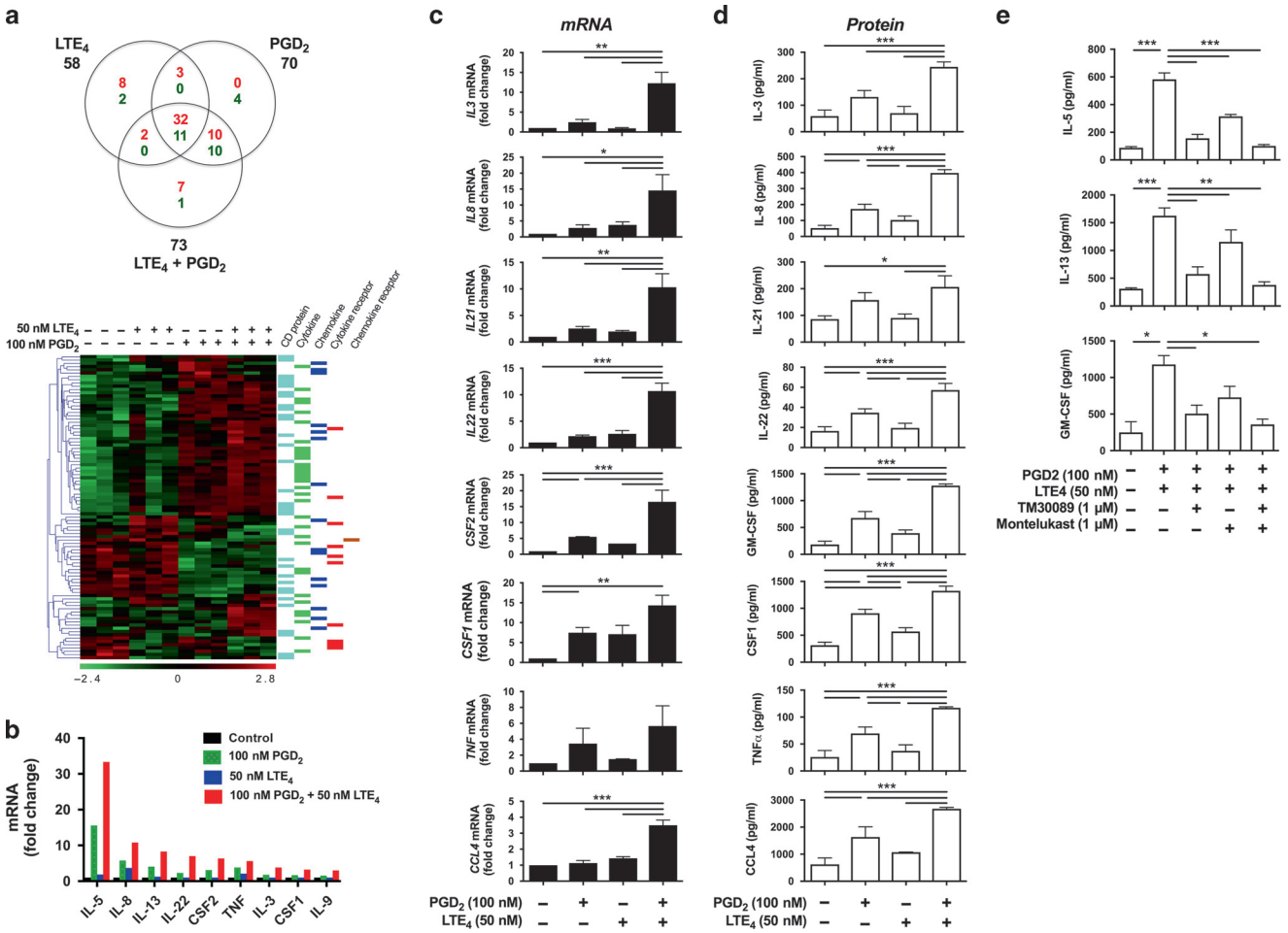

Fig. 6 PGD_2 and LTE_4 modulated gene transcription and production of cytokines, chemokines and surface receptors in cultured Tc2 cells. **a** Venn diagram and heat map showing significantly ($p < 0.05$) regulated genes for cytokines, chemokines and surface receptors (red for upregulation and green for downregulation) in Tc2 cells detected by microarray after treatments with PGD_2, LTE_4 or their combination. Three experimental replicates were prepared for each condition. **b** Upregulated cytokine genes determined by using a PCRarray after the same treatments as those in (**a**). **c**, **d** The change of mRNA levels in cells measured with qPCR (**c**) and protein levels in the cell supernatants detected by using Luminex assays (**d**) for selected cytokines and chemokine after the same treatments as above. The mRNA levels in control samples were treated as onefold. **e** The cytokine production induced by PGD_2 and LTE_4 was inhibited by TM30089 and montelukast. $*p < 0.05$, $**p < 0.005$, $***p < 0.001$ ($n = 1$ for **a**; $n = 3$ for **b** and **c**; $n = 6$ for **d**; $n = 4$ for **e**)

Effect of mast cell-derived PGD_2 and LTE_4 on the activation of Tc2 cells

To investigate the mechanism of Tc2 activation under more physiological conditions, we evaluated the impact of endogenously synthesised eicosanoids on Tc2 function. In vitro-derived human mast cells were stimulated with IgE followed by cross-linking using an anti-IgE antibody (Fig. 7a). Only low levels of PGD_2 (~0.1 ng/ml) and LTE_4 (~6 ng/ml) were detected in mast cell supernatants before stimulation (supernatant UM). Supernatant from activated mast cells (supernatant SM) contained high levels of PGD_2 (~11.5 ng/ml) and LTE_4 (~86.8 ng/ml), which were similar to concentrations in sputum from asthmatic patients (Fig. 3b). Supernatant SM induced Tc2 cell migration (Fig. 7b) and marked cytokine (IL-5/13) production (Fig. 7c). Both effects were reduced by TM30089 and montelukast, and completely inhibited by their combination.

The role of Tc2 in eosinophilia

Tc2-conditioned media were used to investigate the potential role of Tc2 cells in eosinophilia (Fig. 8). Anti-CD3/CD28 Tc2 stimulation increased IL-5 and GM-CSF secretion from ~600 and ~1300 pg/ml respectively in unstimulated supernatants (supernatant UT) to ~1290 and ~2900 pg/ml in stimulated cell supernatants (supernatant 3/28; Fig. 8a).

Recombinant human IL-5 (rhIL-5) induced eosinophil shape change, a biomarker of eosinophil migration, in fresh blood in a dose-dependent manner (Supplementary Fig. 11a), which was inhibited by anti-IL-5 neutralizing antibody. Supernatant 3/28 more potently induced eosinophil shape change ex vivo than supernatant UT (Fig. 8b). The reaction was inhibited partially by IL-5 antibody, implicating IL-5 and other eosinophil-active factors.

We examined the influence of GM-CSF released by Tc2 cells on eosinophil survival by measuring protection from serum starvation-induced apoptosis (Fig. 8c). The increase of annexin V-positive (apoptotic) eosinophils after serum withdrawal was inhibited by rhGM-CSF, which was reversed by anti-GM-CSF neutralizing antibody (Supplementary Fig. 11b). Supernatant 3/28 provide similar protection, which was reduced by anti-GM-CSF antibody (Fig. 8c).

Eotaxins play a critical role in airway eosinophil recruitment in asthma.[32] Bronchial epithelial cells produce eotaxins, which is regulated by IL-4/13.[33] We investigated effects of IL-4/13 derived

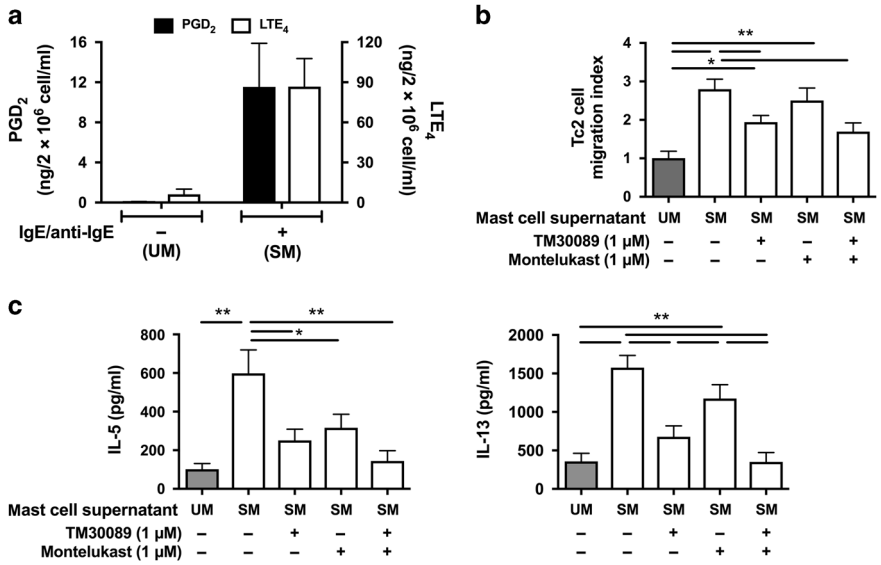

Fig. 7 Tc2 cell activation in response to mast cell supernatant containing endogenous PGD_2 and LTE_4 is mediated by receptors sensitive to TM30089 and montelukast, respectively. **a** Levels of PGD_2 (black bars) and LTE_4 (white bars) were increased in supernatants from mast cells stimulated with IgE and anti-IgE antibodies (supernatant SM) compared with supernatants from cells without stimulation (supernatant UM). **b** More Tc2 cell migration to supernatant SM (white bars) than to supernatant UM (grey bar) in a chemotaxis assay was reduced by TM30089 and montelukast. **c** IL-5 and IL-13 productions in Tc2 cells were significantly increased in response to supernatant SM (white bars) compared with that to the supernatant UM (grey bars), and were inhibited by TM30089 and montelukast. *$p < 0.05$, **$p < 0.005$ ($n = 3$ for **a** and **b**; $n = 4$ for **c**)

from Tc2 cells on eotaxin production in A549, a human alveolar epithelial cell line, and human bronchial epithelial cells (HBECs; Fig. 8d–f). Eotaxin generation, particularly eotaxin-2 (CCL24) and eotaxin-3 (CCL26), by A549 cells was induced by rhIL-4/13 (Supplementary Fig. 12a), and reversed by neutralizing antibodies against IL-4/13 (Supplementary Fig. 12b). The eotaxins induced by IL-4/13 in A549 cells were functionally able to activate eosinophils ex vivo (Supplementary Fig. 12c). Tc2 supernatant UT also upregulated eotaxin production except eotaxin-1 (CCL11) in A549 (Fig. 8e). Supernatants from Tc2 cells activated by anti-CD3/CD28 antibodies (3/28) or PGD_2/LTE_4 (P/L) contain more IL-4/13 than supernatant UT (Fig. 8d), and showed higher capacities to induce eotaxins (Fig. 8e). These observations were confirmed in HBECs, although eotaxin-1/2 levels were not detected in these cells (Fig. 8f). Eotaxin induction by Tc2 media was significantly inhibited by anti-IL-4/13 neutralizing antibodies.

DISCUSSION

Tc2 cells are type-2 cytokine-secreting $CD8^+$ T lymphocytes that highly express CRTH2. We found enrichment of Tc2 cells in both peripheral blood and airways specifically in patients with severe asthma and persistent corticosteroid-insensitive eosinophilia in two independent cohorts. Importantly, the same conclusion was achieved using different methodologies, either surface CRTH2 expression or type-2 cytokine production as markers identifying Tc2 cells. Airway PGD_2 and LTE_4 were also increased in eosinophilic asthma. The interaction of Tc2 cells with these lipids induced strong activation of Tc2 cells, leading to cell migration and pro-inflammatory protein production, which in turn promoted eosinophil recruitment and survival directly or indirectly, suggesting an important role for Tc2 cells in the pathogenesis of eosinophilic asthma and their potential contribution to airway eosinophilia.

Research has long focussed on Th2 cells in asthma[13] although blood Th2 cells are not associated with eosinophilia,[34] and bronchial Th2 cells are elevated in mild, but not severe asthma.[30] We found no significant differences in Th2 numbers between eosinophilic and non-eosinophilic asthma and healthy controls. Conversely, Tc2 cells were significantly enriched, and associated with asthma severity, nasal polyposis, AHR and airway type-2 cytokines. This is consistent with reports in atopic asthma, chronic rhinosinusitis with nasal polyps and non-atopic eosinophilic diseases,[18,35,36] of peripheral blood $CD8^+$ T-cell activation in severe asthma[37] and observed associations between submucosal bronchial $CD8^+$ cells and eosinophilia.[38] Our findings are also consistent with immunohistochemical findings of increased activation and IL-4 secretion by submucosal $CD8^+$ T cells in fatal, virus-associated acute asthma.[19] Onset of severe eosinophilic asthma commonly follows a viral respiratory tract infection, which could promote $CD8^+$ T-cell activation, including Tc2 cells leading to enhanced type-2 immunity in this phenotype. A subset of $IFN\gamma^+IL\text{-}4^{low}$ $CD8^+$ T cells were also detected in the airway of children with severe asthma.[20] Furthermore, in murine models Th2 cell-derived IL-4 can reprogramme virus-specific $CD8^+$ T cells to secrete type-2 cytokines.[21,39] Other murine data suggest preventing Tc2 cell differentiation reduces development of allergic airway disease.[40] The effector phenotype of our cultured Tc2 cells further emphasises their potential role in airway inflammation, as reported in mouse models where effector $CD8^+$ T cells mediated airway inflammation and AHR.[41] Therefore, our findings strongly support the hypothesis that Tc2 cells, a previously unappreciated lymphocyte population, are key drivers contributing to type-2 immunity, particularly in severe eosinophilic asthma and should stimulate further investigation of these mechanisms.

Airway eosinophilia is associated with asthma exacerbations[42] and airway remodelling.[43] In asthma, eosinophil granule proteins are cytotoxic and disrupt the protective pulmonary epithelial barrier triggering inflammation.[44] The importance of eosinophilic inflammation in asthma is best informed by response to therapies. Sputum eosinophilia is associated with responsiveness to corticosteroid therapy.[45] Other strategies such as anti-IgE, anti-IL-5 and CRTH2 antagonism aimed to normalise sputum eosinophils reduced exacerbation frequency and severity in clinical trials in asthma.[2,3,26,27,42] Our in vitro data have indicated that Tc2 activation can contribute to airway eosinophilia. Tc2-derived IL-5 and GM-CSF promote eosinophil migration and survival. Other Tc2

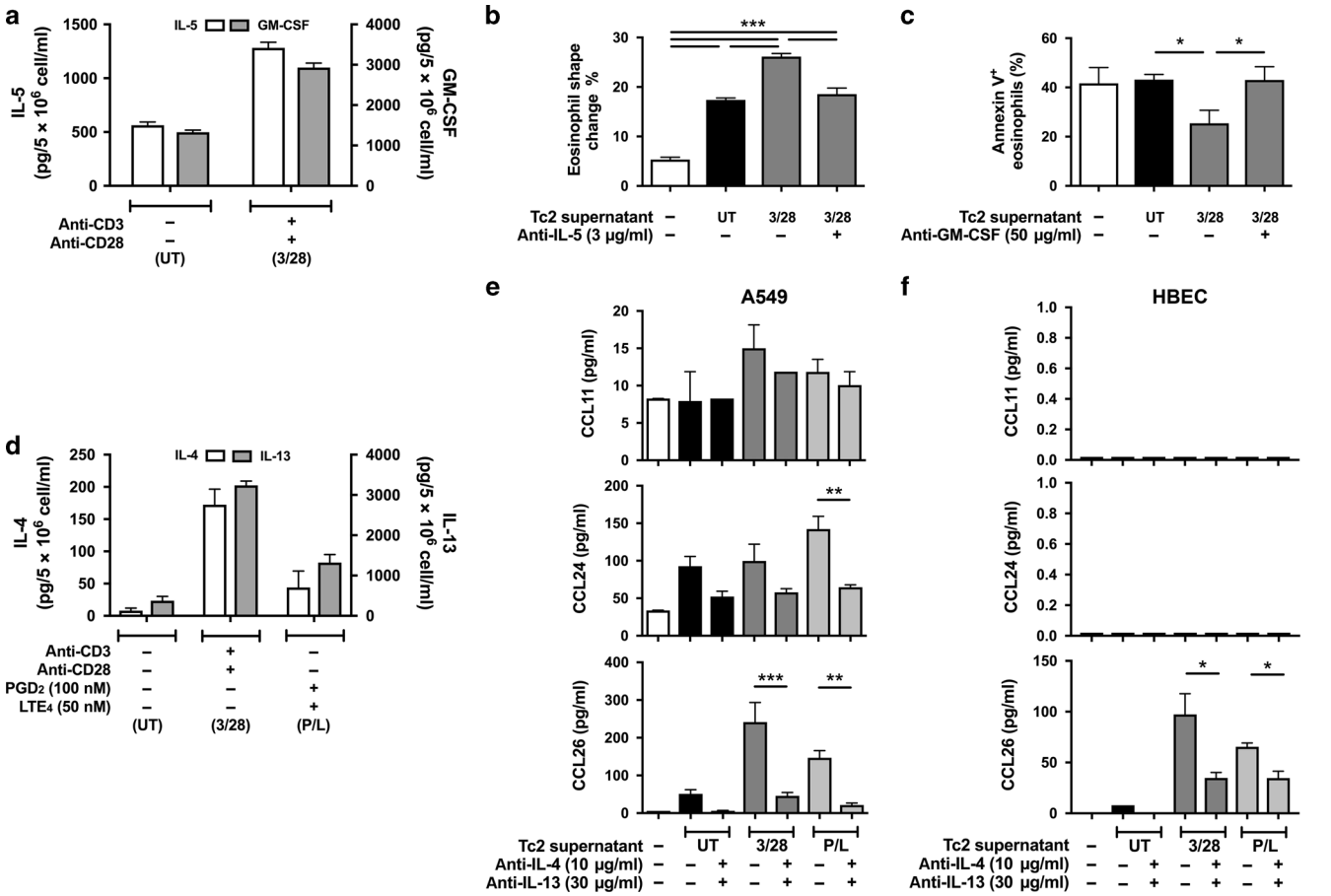

Fig. 8 Cytokines released by human Tc2 cells promote eosinophil shape change and survival, and induce eosinophil chemokine production from airway epithelial cells. **a** Concentrations of IL-5 (white bars) and GM-CSF (grey bars) determined by ELISA were increased in the supernatants from Tc2 cells treated with anti-CD3/CD28 antibodies (supernatant 3/28) compared with the supernatants from the cells without treatment (supernatant UT). **b** Supernatant 3/28 induced more eosinophil shape change in fresh blood than supernatant UT, which was reversed by adding anti-IL-5 antibody. **c** Supernatant 3/28 reduced annexin V-positive eosinophils induced by serum withdrawal in the culture, which was reversed by adding anti-GM-CSF antibody. **d** Concentrations of IL-4 (white bars) and IL-13 (grey bars) were increased in the supernatants of Tc2 cells treated with anti-CD3/CD28 antibodies (supernatant 3/28) or 100 nM PGD_2/50 nM LTE_4 (supernatant P/L) compared with untreated cells (supernatant UT). **e**, **f** Tc2 supernatants, particularly from treated cells, induced CCL11/24/26 (eotaxin-1/2/3) productions in A549 cells (**e**) or CCL26 in primary human bronchial epithelial cells (**f**) measured by Luminex, which were inhibited significantly by neutralising antibodies against IL-4 and IL-13. *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$ ($n = 3$ for **a** and **f**; $n = 4$ for **b**–**e**)

cytokines, including IL-4/13 induce airway epithelial cells to produce eotaxins, the dominant chemokines for eosinophils in severe asthma, acting via CCR3.[46] Furthermore, in murine models, $CD8^+$ T or virus-specific Tc2 cells mediate virus-induced lung eosinophilia and AHR.[21,47] All this evidence suggests an important role of Tc2 cells in eosinophil-mediated lung inflammation.

PGD_2 and CysLTs are predominantly derived from mast cells in an IgE-dependent mechanism, which are abundant at sites of allergic responses, promoting airway inflammation and deterioration in lung function.[48] We observed increased airway PGD_2 in eosinophilic and non-eosinophilic severe asthma, while LTE_4 was enriched only in severe eosinophilic asthma, consistent with other reports.[49] Since many patients with severe eosinophilic asthma are non-atopic with low levels of IgE,[6] the key source of these lipids remains unclear. Others have previously reported an asthma-associated enrichment in BAL of IL-13-producing $CD8^+$ cells expressing the leukotriene B_4 receptor BLT1.[50] We previously demonstrated synergistic pro-inflammatory effects of PGD_2 and LTE_4 in human Th2 and ILC2s, which inhibited by TM30089 and montelukast.[28,29,31] This also occurs in Tc2 cells, indicating that these CD8 cells can be activated by lipid mediators in an antigen-independent manner. Of relevance, we observed higher potency of PGD_2 and LTE_4 on Tc2 than on Th2 cells. Synergistic effects of PGD_2 and LTE_4 in Tc2 were replicated using endogenous lipid mediators from activated mast cells. These findings provide a potential mechanistic insight into the clinical efficacy of CRTH2 antagonism in severe eosinophilic asthma.[27]

Transcriptional analysis suggested a broad range of effects of these lipids beyond type-2 cytokine induction in Tc2 cells, with >2000 upregulated transcripts, including genes for bioactive cytokines and chemokines for eosinophils: IL-3, IL-5 and GM-CSF promote eosinophil differentiation and migration[51]; TNFα delays eosinophil apoptosis[52]; overexpression of IL-25 resulted in eosinophilia in murine models[53]; and CCL3, CCL4 and CCL7 are chemokines for eosinophils. One notable finding was the impact of these lipids on IL-5 expression by Tc2 cells. The EC_{50} for PGD_2-induced IL-5 production was 17.9 nM, compared to 63 nM for Th2 cells.[23] Thus, Tc2 cells stimulated in this way generate a strong and early IL-5 response, which could be relevant to the clear clinical benefit of anti-IL-5 in severe eosinophilic asthma.[3,42]

In conclusion, Tc2 represent an important cell type with innate-like characteristics and substantial pro-inflammatory potential. In vivo there is significant enrichment of Tc2 cells in both peripheral blood and airways in eosinophilic asthma, particularly

in severe disease, associated with increases in airway PGD_2 and LTE_4. These lipid mediators function potently and synergistically to recruit and activate Tc2 cells to produce type-2 and multiple other pro-inflammatory cytokines and chemokines, which sufficient to contribute to airway eosinophilia. Taken together these data suggest that Tc2 cells constitute potentially novel biomarkers and important therapeutic targets in severe eosinophilic asthma.

METHODS

Reagents

The following were used: PGD_2 and LTE_4 (Enzo Life Science); TM30089 and montelukast (Cayman Chemical); rhIL-5, anti-CD3/28 antibodies, human $CD8^+$ T-cell isolation kit and anti-human CD294 or CD16 MicroBeads (Miltenyi Biotec Ltd); BD FACS Lysing Solution (BD Biosciences); X-VIVO-15 medium and Bronchial Epithelial Cell Growth Medium (Lonza); AIM-V medium (Invitrogen); Ficoll-PaqueTM Plus (GE Healthcare); RNeasy Mini kit and Omniscript reverse transcription kit (Qiagen); Real time quantitative PCR Master Mix and probes (Roche); Primers (Eurofins MWG Operon); rhIL-6/SCF and anti-IL-4/5/13 neutralizing antibodies (Bio-techne); goat anti-human CD3 (Santa Cruz); rabbit anti-human CD8 (Abcam); peroxidase polymer-conjugated anti-rabbit and peroxidase polymer-conjugated anti-goat antibodies (Vector Laboratories); Fluorescein-tyramide and Cy5-tyramide (PerkinElmer); human myeloma IgE (Calbiochem); anti-human GM-CSF antibody, Annexin V-APC and Human GM-CSF ELISA kit (BioLegend); IC fixation buffer, Permeabilisation Buffer, human IL-4/5/13 ELISA kits (eBioscience); and rhIL-2/4/GM-CSF (PeproTech) and other chemicals (Sigma-Aldrich).

Human clinical samples

For the Oxford cohort, patients meeting the American Thoracic Society/European Respiratory Society definition of severe asthma[10] with a sputum eosinophil count of >3% (eosinophilic, $n = 26$) or <3% (non-eosinophilic, $n = 14$), and 16 healthy control subjects were recruited from Churchill Hospital, Oxford (Table 1). For the Southampton cohort, 22 healthy participants, 10 with eosinophilic asthma (sputum eosinophil count >3%) and 42 non-eosinophilic asthma (Table 1) were enroled from NIHR Southampton Respiratory Biomedical Research Unit and outpatient clinics at University Hospital Southampton.[30]

Peripheral blood was collected and used directly for flow cytometry. Sputum was induced with nebulised saline solution (3–5%) after pre-treatment with salbutamol. Selected sputum plugs were dispersed with 0.2% dithiothreitol (DTT), filtered for cells for flow cytometry analysis or microarray, and supernatants for enyme-linked immunosorbent assay (ELISA) analysis. BB and BAL were collected under bronchoscopy.[30] Biopsies were dispersed with collagenase for 1 h, and BALs were treated with 0.1% DTT and filtered for flow cytometry. For microarray, $CD3^+$ sputum cells were flurescence-activated cell sorting (FACS)-sorted using a FACS Aria II^{TM} cell sorter (BD Biosciences).

Human $CD8^+CRTH2^+$ Tc2 cell preparation and treatment

Human Tc2 cells were isolated from CD Leucocyte Cones (National Blood Service, UK). Briefly, $CD8^+$ cells were isolated from peripheral blood mononuclear cells (PBMCs) using MACS $CD8^+$ T-cell isolation kit, followed by CRTH2-positive selection using anti-human CD294 MicroBeads, and further amplified in X-VIVO 15 medium containing 10% human serum and 50 U/ml rhIL-2.

For gene or protein analysis, Tc2 cells were treated with conditions as indicated in the results for 4 h. Cell supernatants were collected for ELISA or Luminex assays, and cell pallets for qPCR, RNAarray or microarray.

For Tc2-conditioned supernatants, cells were treated with immobilised anti-CD3/anti-CD28 antibodies or PGD_2 (100 nM)/LTE_4 (50 nM) for 4 h, and then supernatants were harvested for ELISA, or used as Tc2-conditioned media for the treatment of fresh blood, eosinophils, A549 cells or primary HBECs.

Human mast cell culture and treatment

Human mast cells were cultured and treated as described previously.[31] Briefly, $CD34^+$ progenitor cells were isolated from human cord blood (National Blood Service, Oxford, UK) by using a human CD34 MicroBead kit. The cells were cultured with Iscove's modified Dulbecco's medium containing 10% human serum, 0.55 µM 2-ME, penicillin/streptomycin, human recombinant stem cell factor (100 ng/ml) and human rIL-6 (50 ng/ml) for 14–15 weeks. Half the culture medium was replaced twice weekly with fresh medium containing the same concentration of cytokines. The cells were pre-treated with 5 mg/ml purified human myeloma IgE for 4 days, washed and then sensitised passively with fresh IgE (5 mg/ml) for 2 h. After washing, the cells were incubated with medium or challenged with goat anti-human IgE (1 µg/ml) for 1 h. The supernatants of the cells were collected and measured for PGD_2 and LTE_4, and used as mast cell supernatants for the treatment of Tc2 cells.

Human bronchial epithelial cell culture and treatment

A549 cells (American Type Culture Collection) were cultured in RPMI 1640 medium with 10% fetal calf serum (FCS). Primary HBECs were obtained from bronchial brushings collected under bronchoscopy, and cultured with Bronchial Epithelial Cell Growth Medium containing 50 µg/ml gentamicin and 1× penicillin/streptomycin. Both cell types were treated with rhIL-4 and rhIL-13 in RPMI 1640 (5% FCS) or Tc2-conditioned media in the presence or absence of anti-IL-4 and IL-13 neutralizing antibodies as indicated in the results for 16 h. The supernatants of the cells were harvested for Luminex assay for eotaxins.

PrimeFlow RNA assay

The levels of transcription for IL-5 and IL-13 in individual Tc2 cells in whole blood or Tc2 cultures were analysed with a PrimeFlow RNA Assay kit (eBioscience) according to the manufacturer's instructions. Briefly, fresh blood or purified Tc2 cells were treated with conditions indicated for 4 h, and then were stained with the antibodies (Supplementary Table 3) and viability dye followed by fixation and permeabilisation. Then the cells were hybridised with RNA probes for IL-5 and IL-13. The signals were amplified and labelled with fluorescent probes. The results were analysed with a BD LSRFortessa flow cytometer (BD Biosciences).

Microarrays

For $CD3^+$ cells sorted from sputa, RNAs were isolated using an Absolutely RNA Nanoprep Kit (Agilent), and microarrays were performed using Affymetrix HT HG-U133 + PM GeneChips by Janssen Research & Development (Springhouse, Pennsylvania). For cultured Tc2 and $CRTH2^-CD8^+$ T cells, RNAs were extracted with an RNeasy Mini kit, and microarrays were conducted using an Illumina HumanHT-12v4 Expression Beadchip at the Transcriptomics Core Facility, The Jenner Institute, University of Oxford. Preprocessing data analysis was performed using R language (www.R-project.org) and Bioconductor packages (www.bioconductor.org/). Genes significant at a $p < 0.05$ were selected by using Limma Bioconductor package. Heat maps and gene hierarchical clustering were generated by using *tmev* microarray software suite.

Enzyme-linked immunosorbent assay

The levels of IL-4, IL-5, IL-13 and GM-CSF in the Tc2 supernatants were assayed with ELISA kits, and the concentrations of PGD_2 and LTE_4 in the mast cell supernatants were measured with a PGD_2–MOX enzyme immunoassay kit and LTE_4 enzyme immunoassay kit (Cayman Chemicals) respectively according to the manufacturer's instructions. The results were measured in an EnVision Multilable Reader (PerkinElmer).

Luminex assays
The concentrations of cytokines and eotaxins were measured using a Luminex Screening Assay kit (Bio-techne) as per the manufacturer's instructions. Results were obtained with a Bio-Plex 200 System (Bio-Rad).

PCRarray
mRNA levels were assessed with PCRarray by using an RT^2 Profiler PCRarray Human Common Cytokines PCR Array kit (Qiagen) in a LightCycler 480 Real-Time PCR System (Roche).

Quantitative RT-PCR
qPCR was conducted as described previously.[31] Briefly, total RNA of the cells was extracted using an RNeasy Mini kit. cDNA was prepared using a Omniscript RT kit. qPCR was conducted using Master Mix and Probe in a LightCycler 480 Real-Time PCR System (Roche). *GAPDH* was used as control gene. Primers and probes (Roche) used are listed in Supplementary Table 4.

Flow cytometry
For blood samples, fresh blood was labelled using antibodies (Supplementary Table 3) followed by red blood cell lysis with a BD FACS Lysing Solution. For cells from blood, sputum, BAL or BB, the cells were stained with antibody cocktail and live/dead dye (Supplementary Table 3). The protocols have been optimised with intraclass correlation coefficient = 0.86 for Tc2 and 0.71 for Th2, and most of the experiments were paired between asthmatic patients and healthy controls. For ICS in the Oxford cohort, PBMCs from fresh blood were treated without or with 25 ng/ml PMA and 1 µg/ml ionomycin in the presence of 5 µg/ml brefeldin A for 6 h. In the Southampton cohort, the cells were rested overnight in AIM-V medium and stimulated with 25 ng/ml PMA and 500 ng/ml ionomycin in the presence of 2 µM monensin for 5 h. After surface marker staining, the cells were fixed with IC fixation buffer (Oxford) or 1% formaldehyde (Southampton) and then treated with a Permeabilisation Buffer followed by incubation with anti-IL-5 and IL-13 antibodies. Fluorescence-minus-one (CRTH2, IL-5 and IL-13) controls (Oxford) or unstimulated controls (Southampton) were included in each experiment. The samples were analysed with a BD LSRFortessa flow cytometer at Oxford or a BD FACS Aria II cell sorter at Southampton.

Chemotaxis assays
Tc2 cells were resuspended with RPMI 1640 media; 25 ml of cell suspension and 29 ml test compounds as indicated in the results or mast cell supernatants were applied to upper and lower chambers, respectively, in a 5-µm pore-sized 96-well ChemoTx plate (Neuro Probe). After incubation (37 °C, 60 min), the migrated cells in the lower chambers were collected and measured with a Cell Titer-Glo Luminescent Cell Viability Assay (Promega) and quantified by using an EnVision Multilable Reader.

Immunohistochemistry
Paraffin-embedded sections of BB were prepared by Oxford Centre for Histopathology Research. After deparaffinization and rehydration, the sections were boiled in a Target Retrieval Solution (Dako), followed by incubation with peroxidase blocking reagent (Bio-Rad) and normal horse serum. The sections were then labelled with primary (goat anti-human CD3 and rabbit anti-human CD8) and secondary (peroxidase polymer-conjugated anti-rabbit followed by Cy5-tyramide) antibodies. After treatment with peroxidase blocking reagent again, the sections were further incubated with another secondary antibody (peroxidase polymer-conjugated anti-goat followed by fluorescein-tyramide) and then 5 µg/ml 4′,6-diamidino-2-phenylindole solution. Images were acquired on an Olympus FV1200 inverted confocal microscope, and processed with ImageJ.

Eosinophil shape change assay
Fresh blood was incubated with an equal volume of Tc2 or A549 cell conditioned media in the presence or absence of anti-IL-5 neutralizing antibody or other reagents as indicated for 1 h. The samples were treated with a Cytofix Fixation Buffer (BD Biosciences) followed by RBC Lysis Solution (Gentra Systems). Eosinophil forward scatter was analysed on a BD LSRFortessa flow cytometer.

Eosinophil apoptosis assay
From human blood, the erythrocyte/granulocyte pellet was collected after Ficoll-Hypaque gradient, incubated with 3% dextran saline solution for sedimentation. The granulocytes from the supernatant of the sedimentation were further purified by lysis of the remaining erythrocytes with 0.6 M KCl hypotonic water. Eosinophils were negatively selected with anti-CD16 microbeads. After treatments with diluted Tc2-conditioned media in the presence or absence of anti-GM-CSF neutralizing antibody or other reagents as indicated for 12 h, eosinophils were labelled with annexin V and then analysed with a BD LSRFortessa.

Statistics
Data for clinical samples in Figs. 1–3, and Supplementary Fig. 5, 6 are presented as medians with interquartile range and other data are presented as means with SEM. Data were analysed using one-way analysis of variance followed by the Tukey's test or Student's *t*-tests. Groups ranked according to disease severity were tested for linear trends using Jonckheere-Terpstra tests. Values of $p < 0.05$ were considered statistically significant.

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AUTHOR CONTRIBUTIONS

B.H., T.S.C.H., L.S. and E.M. performed the experiments and collected, analysed and discussed the data; M.S., R.S., W.L., W.C., J.L., S.G., J.C., T.P., S.T., A.K., T.L., J.M., C.C., C.B., M.B. and C.W. were involved in part of the experiments, patient recruitment and patient sample collection; A.R. conducted microarray; T.S.C.H., R.D. and G.O. designed the experiments, and were involved in the manuscript writing; I.P., P.K. and L.X. conceived, designed and managed the project and drafted the manuscript.

ADDITIONAL INFORMATION

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Ethical approval The studies were approved by Leicestershire, Nottinghamshire Rutland Ethics Committee, UK (08/H0406/189) and the Southampton and South West Hampshire Research Ethics Committee B, UK (10/H0504/2).

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