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

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Supplementary Information

SUPPLEMENTARY TABLES

Supplementary Table 1. Frequencies of Tc2 cells in both Oxford and Southampton cohorts

	Tc2 frequency (mean ± SD)	Control (n=16)	Asthma		*p*-value* <
			Eosinophilic (n=26)	Non-eosinophilic (n=14)	
Blood	% in CD8$^+$ T cells	1.32 ± 0.79	6.24 ± 5.18	2.93 ± 2.46	0.05
	% in leukocytes	0.084 ± 0.04	0.309 ± 0.26	0.096 ± 0.10	0.0003
	x10^6/L in blood	4.87 ± 2.29	17.87 ± 14.2	6.81 ± 6.57	0.0003
		(n=17)	**(n=8)**	**(n=24)**	
BB	% in CD8$^+$ T cells	0.2 ± 0.63	2.05 ± 1.44	0.1 ± 0.56	0.005
BAL	% in CD8$^+$ T cells	0.2 ± 0.71	1.5 ± 1.40	0.2 ± 0.52	0.005

* *p*-values were obtained from analysis with one-way ANOVA followed by the Tukey's test.

Supplementary Table 2. List of cytokine, chemokine, their receptor and CD molecule genes regulated by PGD_2, LTE_4 or their combination in Tc2 cells detected by microarray*

Up-regulation				Down-regulation			
	Sample treatment				Sample treatment		
Gene	PGD_2	LTE_4	PGD_2 + LTE_4	Gene	PGD_2	LTE_4	PGD_2 + LTE_4
IL3	+	+	+++**	*BMP8B*	-	-	-
IL5	+	+	++	*CYFIP2*	-	-	-
IL8	+	+	+++	*FLT3LG*	-		-
IL10	+	+	+	*GDF11*	-		-
IL13	+		++	*LASS1*	-		
IL16	+	+	+	*TNFSF13*	-		-
IL21			+	*TSLP*	-	-	-
IL22			++	*CCL2*		-	
IL24	+	+		*CCL21*			-
IL25	+	+	+	*IL3RA*	-		
IL26	+	+	+	*IL6R*	-		-
BMP1	+		+	*IL7R*	-	-	-
CSF2	+	+	++	*IL11RA*	-		
GDF11		+		*CCK1*	-	-	-
TNF	+	+	++	*CNTFR*	-	-	-
TNFSF8	+	+	+	*LEPR*	-	-	-
TNFSF11	+	+	+	*CMKLR1*	-		-
TNFSF13		+		*CD2*	-	-	-
TNFSF14	+	+	+	*CD14*	-		-
LASS1			+	*CD40*	-	-	-
LIF	+	+	+	*CD46*	-		-
LTA	+	+	+	*CD58*		-	
NAMPT	+		+	*CD99*	-		-
XCL1	+	+	++	*CD79A*	-		
FASLG	+	+	++	*CD79B*	-	-	-
CCL2	+		+	*CD99L2*	-		-
CCL3			+	*CD300LG*	-	-	-
CCL4			+	*GPR44*	-		-
CCL7		+					
CCL21	+	+					
CCL22	+	+	+				
CCL25	+		+				
CD2BP2	+	+	+				
CMTM4	+		+				
PF4V1	+		+				
PPBP	+	+	+				
IL1RL1			+				
IL2RB	+	+	+				
IL2RG	+	+	+				
IL3RA		+	+				
IL6R		+					
IL11RA	+	+					
CD1E			+				
CD7	+	+	+				
CD14		+					
CD28	+	+	+				
CD40LG	+	+	+				
CD44	+		+				
CD46		+					
CD53	+		+				
CD55	+	+	+				
CD58	+		+				
CD59	+	+	+				
CD69	+	+	+				
CD79A		+	+				
CD82	+	+	+				
CD99		+					
CD99L2		+					
CD109	+	+	+				
CD151	+	+	+				
CD164	+	+	+				
CD226	+	+	+				

* The concentrations of PGD_2 and LTE_4 were 100 nM and 50 nM respectively.
** ++ indicates fold change ≥3; +++ indicates fold change ≥6.

Supplementary Table 3. Antibodies used for flow cytometry and PrimeFlow RNA assays

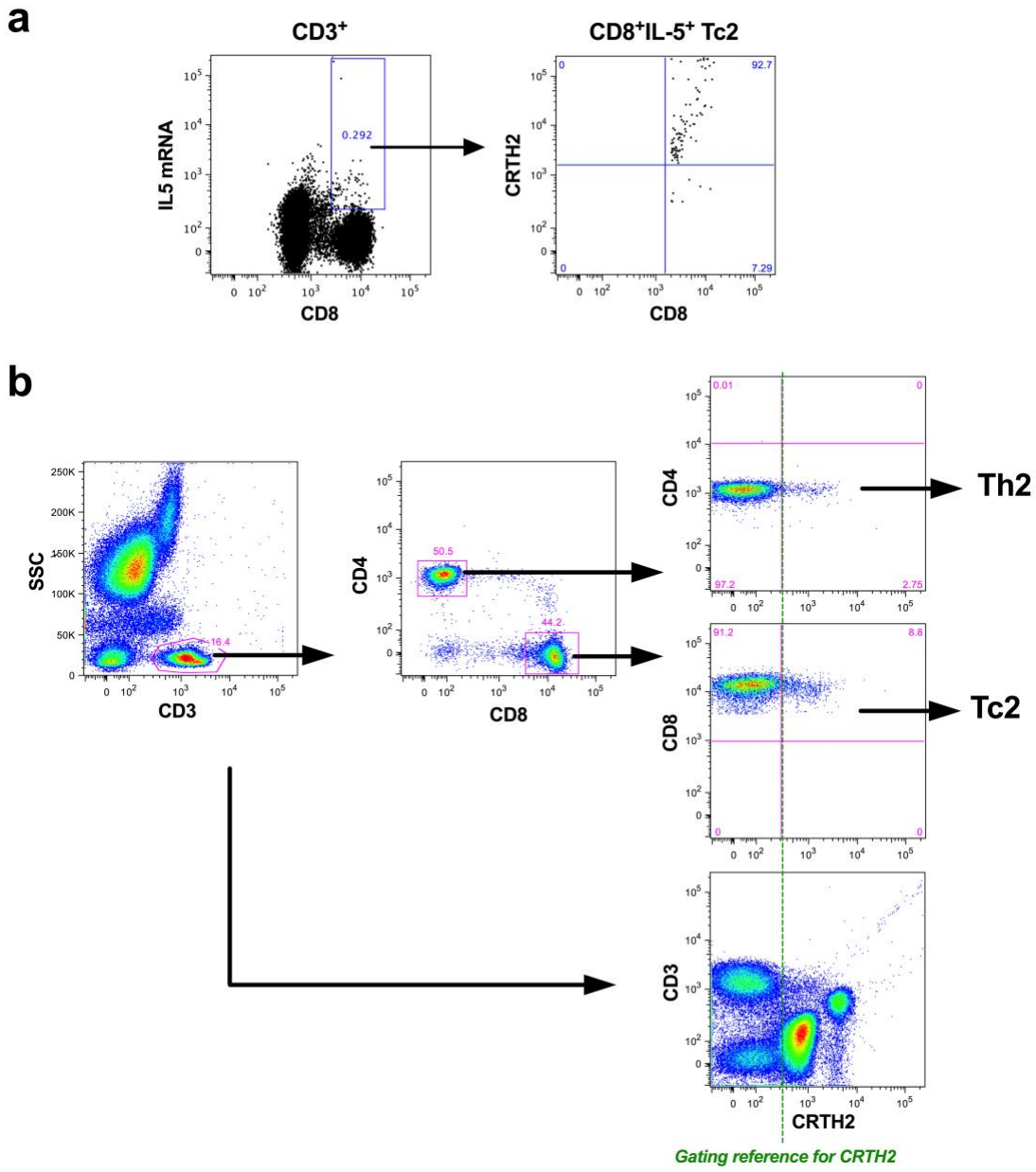
Antigen	Clone	Supplier	Used for
CCR7	3D12	eBioscience	Flow cytometry
CD3	SK7	BioLegend	Flow cytometry
CD3	UCTH1	eBioscience	Flow cytometry, PrimeFlow
CD4	L200	BD Biosciences	Flow cytometry
CD4	OKT4	BioLegend	Flow cytometry, PrimeFlow
CD8	SK1	BioLegend	Flow cytometry, PrimeFlow
CD62L	DREG-56	eBioscience	Flow cytometry
CRTH2	BM16	Miltenyi Biotec	Flow cytometry, PrimeFlow
$CysLT_1$	polyclonal	Novus Biologicals	Flow cytometry
Granzyme A	356412	R&D Systems	Flow cytometry
Granzyme B	GB11	BD Biosciences	Flow cytometry
Granzyme K	24C3	Immunotools	Flow cytometry
IL-5	JES1-39D10	BioLegend	Flow cytometry
IL-13	85BRD	eBioscience	Flow cytometry
IL-13	11711	R&D Systems	Flow cytometry
perforin	B-D48	BioLegend	Flow cytometry

Supplementary Table 4. Primers and probes used for q-PCR

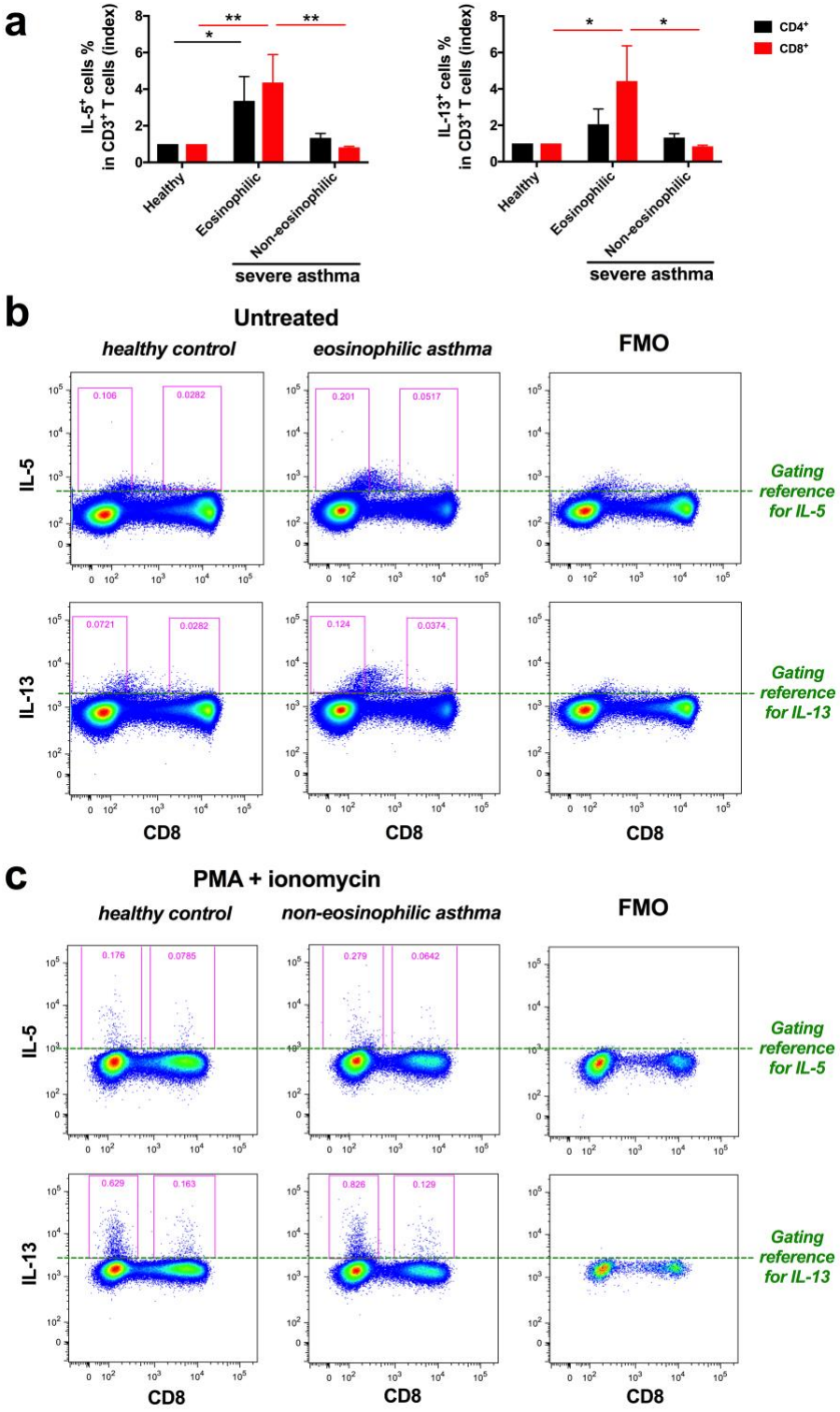
Gene	Primer	Probe No.
CSF1	5'-GCAAGAACTGCAACAACAGC-3' 5'-ATCAGGCTTGGTCACCACAT-3'	19
CSF2	5'-TCTCAGAAATGTTTGACCTCCA-3' 5'-GCCCTTGAGCTTGGTGAG-3'	1
CYSLT1	5'-ACTCCAGTGCCAGAAAGAGG-3' 5'-GCGGAAGTCATCAATAGTGTCA-3'	29
CYSLT2	5'-CTAGAGTCCTGTGGGCTGAAA-3' 5'-GTAGGATCCAATGTGCTTTGC-3'	48
DP1	5'-CCTGGAGGAGCTGGATCA-3' 5'-GCTCCATAGTAAGCGCGATAAA-3'	18
GAPDH	5'-AGCCACATCGCTCAGACAC-3' 5'-GCCCAATACGACCAAATCC-3'	60
GPR44	5'-CCTGTGCTCCCTCTGTGC-3' 5'-TCTGGAGACGGCTCATCTG-3'	43
GPR99	5'-CAACCTGATTTTGACTGCAACT 5'-GGATAATCGTGGTATAGCAAAGTG	16
IL3	5'-TTGCCTTTGCTGGACTTCA-3' 5'-CTGTTGAATGCCTCCAGGTT-3'	60
IL4	5'-CACCGAGTTGACCGTAACAG-3' 5'-GCCCTGCAGAAGGTTTCC-3'	16
IL5	5'-GGTTTGTTGCAGCCAAAGAT-3' 5'-TCTTGGCCCTCATTCTCACT-3'	25
IL8	5'-AGACAGCAGAGCACACAAGC-3' 5'-ATGGTTCCTTCCGGTGGT-3'	72
IL9	5'-CTTCCTCATCAACAAGATGCAG-3' 5'-AGAGACAACTGGTCACATTAGCAC-3'	59
IL13	5'-AGCCCTCAGGGAGCTCAT-3' 5'-CTCCATACCATGCTGCCATT-3'	17
IL21	5'-AGGAAACCACCTTCCACAAA-3' 5'-GAATCACATGAAGGGCATGTT-3'	7
IL22	5'-CAACAGGCTAAGCACATGTCA-3' 5'-ACTGTGTCCTTCAGCTTTTGC-3'	6
P2Y12	5'-TTTGCCTAACATGATTCTGACC-3' 5'-GGAAAGAGCATTTCTTCACATTCT-3'	27
TNF	5'-CAGCCTCTTCTCCTTCCTGAT-3' 5'-GCCAGAGGGCTGATTAGAGA-3'	29

SUPPLEMENTARY FIGURES

Supplementary Fig. 1



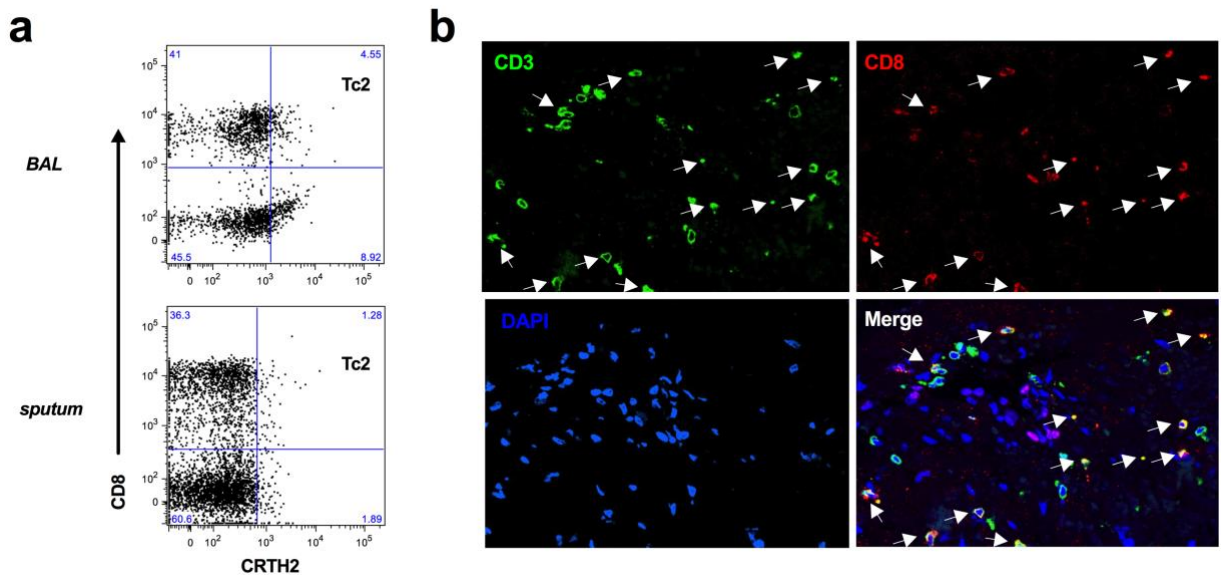
Supplementary Fig. 1 Tc2 cells are $CD3^+CD8^+CRTH2^+$ T lymphocytes. **a** IL-5-producing $CD3^+CD8^+$ Tc2 cells in human peripheral blood *ex-vivo* are CRTH2 positive detected with PrimeFlow RNA assay. **b** Gating strategies for Tc2 and Th2 cells in peripheral blood from the Oxford cohort. Fresh bloods were stained with a mixture of antibodies and analysed with flow cytometry. Tc2 cells were gated as $CD3^+CD4^-CD8^+CRTH2^+$ cells and Th2 cells were gated as $CD3^+CD4^+CD8^-CRTH2^+$ cells. CRTH2 negative cell groups were used as gating reference.



Supplementary Fig. 2 Type-2 cytokine producing $CD8^+$ Tc2 cells are enriched in the blood from severe eosinophilic but not non-eosinophilic asthma. PBMCs isolated from fresh blood were treated with (**a, c**) or without (**b**) 25 ng/ml PMA and 1 µg/ml ionomycin for 6 h, and then

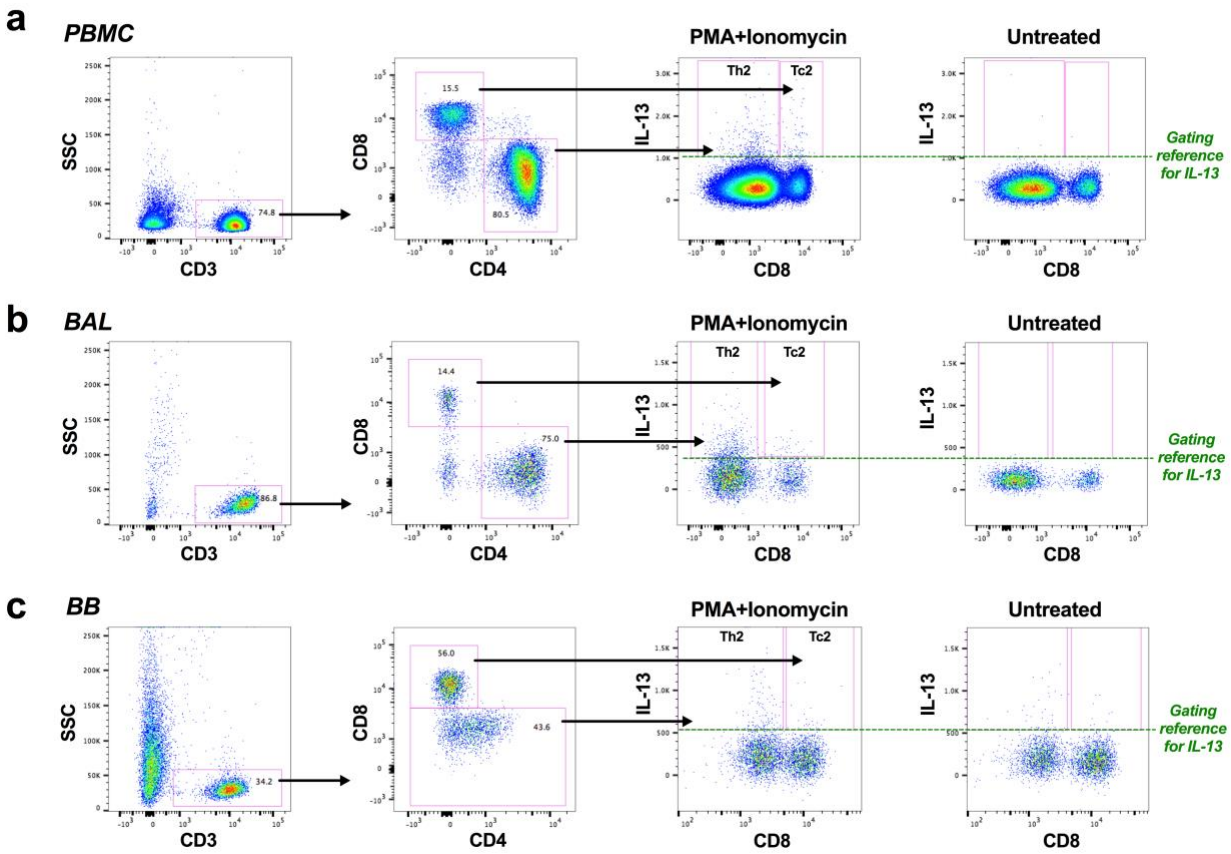
the frequencies of IL-5 or IL-13 secreting $CD3^+CD8^-$ and $CD3^+CD8^+$ T-cells were detected by flow cytometry with ICS. $IL\text{-}5^+CD8^+$ or $IL\text{-}13^+CD8^+$ T-cells were increased in the blood from patient with severe eosinophilic (**a, b**) but not non-eosinophilic (**c**) asthma. For comparison in **a**, the frequencies of IL-5/IL-13 secreting cells in healthy control samples were treated as 1. Fluorescence-minus-one (FMO) for IL-5 or IL-13 were used as gating reference. $*p < 0.05$, $**p < 0.01$; (n=5 for **a**; Data in **b** and **c** are representative of 5 or 3 independent experiments respectively).

Supplementary Fig. 3



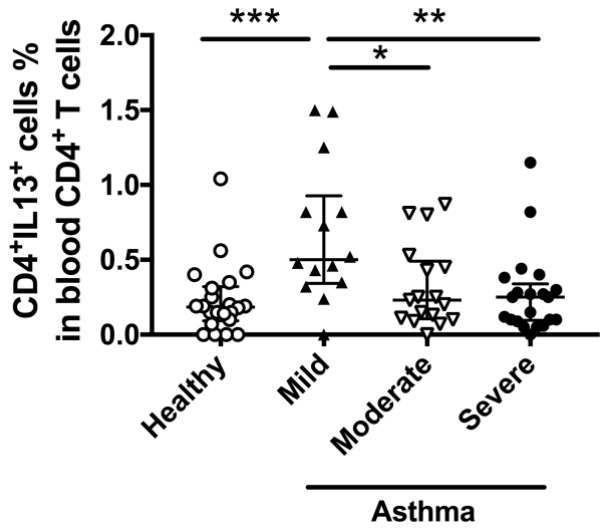
Supplementary Fig. 3 Tc2 cells are detectable in lung in severe eosinophilic asthma. **a** $CD3^+CD8^+CRTH2^+$ Tc2 cells detected in BAL and sputum from severe asthma patients by flow cytometry. **b** $CD3^+CD8^+$ T-cells (arrows) detected in BB by immunohistochemistry. Data in **a** and **b** are representative of >10 and 3 independent experiments respectively.

Supplementary Fig. 4



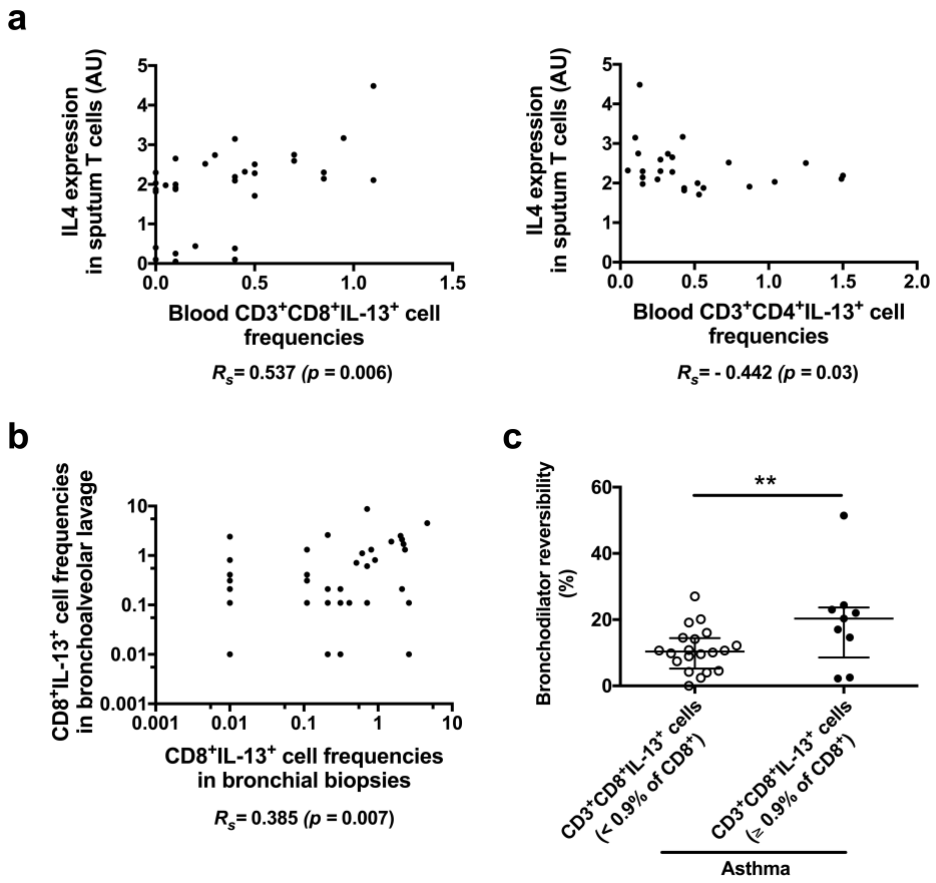
Supplementary Fig. 4 Enumeration of type-2 cytokine producing $CD8^+$ Tc2 and $CD4^+$ Th2 cells in the blood and airways from Southampton cohort. **a, b, c** PBMCs isolated from fresh blood (**a**), bronchoalveolar lavage (BAL) cells (**b**) and endobronchial biopsies (BB) (**c**) were treated with 25 ng/ml PMA and 0.5 µg/ml ionomycin for 5 h. Biopsies were then dispersed with collagenase I at 1 mg/ml for 1 hour, and frequencies of IL-13 secreting $CD3^+CD4^+$ (PBMC and BAL) or $CD3^+8^-$ (biopsies) (Th2) and $CD3^+CD8^+$ (Tc2) T-cells were detected by flow cytometry with ICS. Untreated samples were used as control for gating reference.

Supplementary Fig. 5



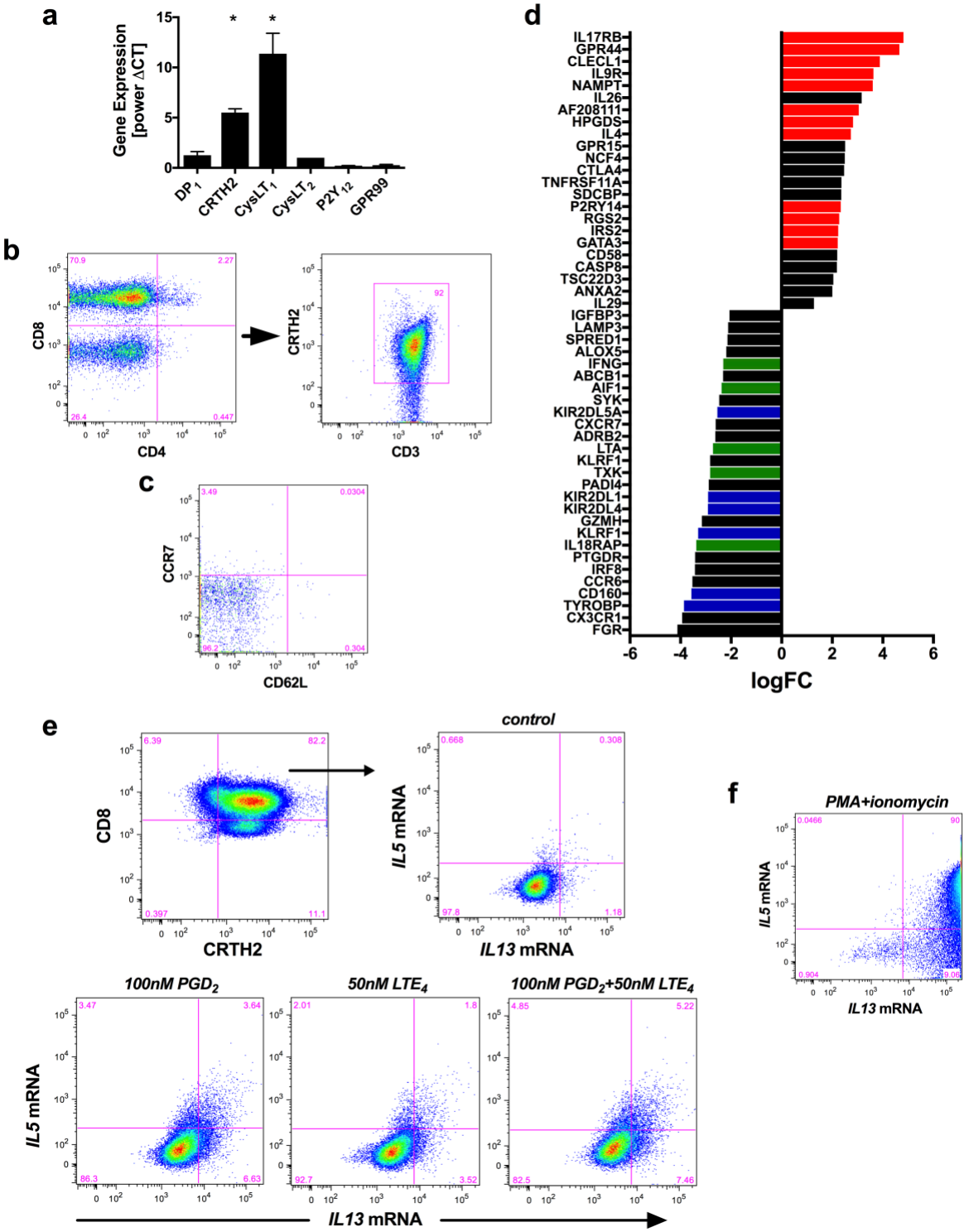
Supplementary Fig. 5 Frequencies of IL-13 secreting $CD3^+CD4^+$ T cells in blood were increased in patients with mild asthma but not in moderate or severe asthma from the Southampton cohort. The frequencies of IL-13 secreting cells determined with ICS were compared based on asthma severity according to global physician assessment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Supplementary Fig. 6



Supplementary Fig. 6 Frequencies of Tc2 but not Th2 are correlated with lung inflammation in the Southampton cohort. **a** Relative expression of *IL4* in T-cells measured by microarray in FACS sorted $CD3^+$ cells from induced sputum from the Southampton cohort, is correlated positively with peripheral blood $CD3^+CD8^+IL\text{-}13^+$ (Tc2) frequencies (% in $CD8^+$ T-cells), but negatively with peripheral blood $CD3^+CD4^+IL\text{-}13^+$ (Th2) frequencies (% in $CD4^+$ T-cells). Significant correlations were not observed with sputum IL-5 or IL-13 (not shown). **b** Tc2 frequencies in BB is correlated with that in BAL. **c** High $CD3^+CD8^+IL\text{-}13^+$ (Tc2) frequencies were arbitrarily defined as ≥ 0.9% in BB and/or ≥ 0.7% in blood, being equivalent to, or above, the top decile of Tc2 frequencies observed in healthy controls. High Tc2 frequencies are associated with high salbutamol bronchodilator reversibility in asthmatic subjects. *Rs* = Spearman's correlation coefficient. ** $p < 0.007$.

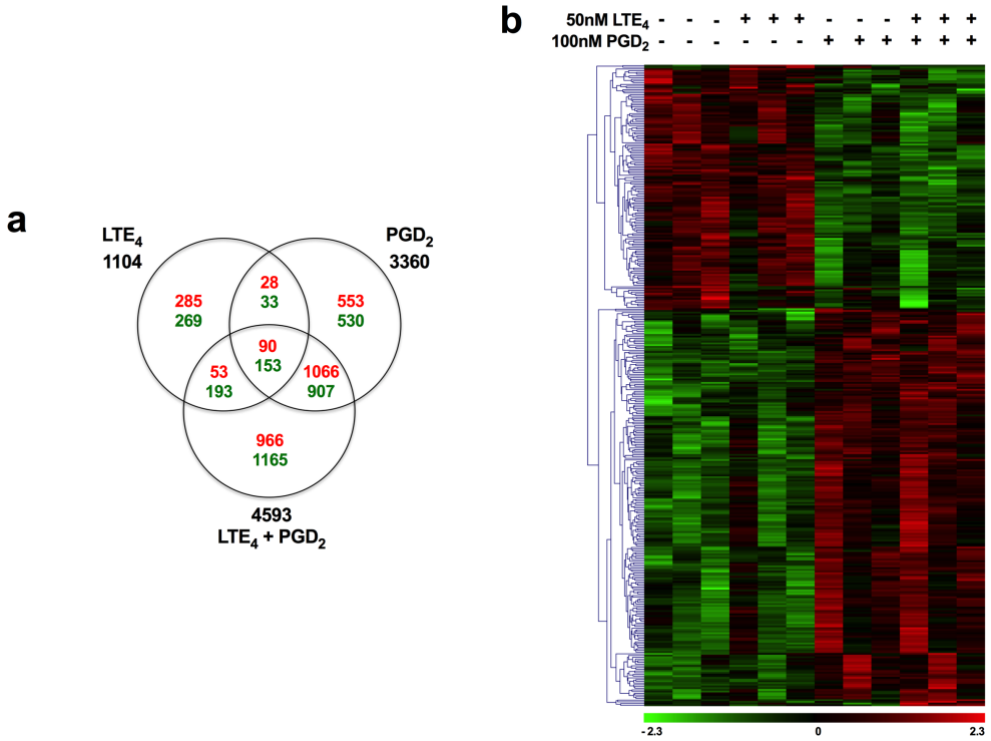
Supplementary Fig. 7



Supplementary Fig. 7 Tc2 cells highly express CRTH2 and $CysLT_1$, show high type-2 immunity related gene signature, and are capable of producing type-2 cytokines. **a** The levels

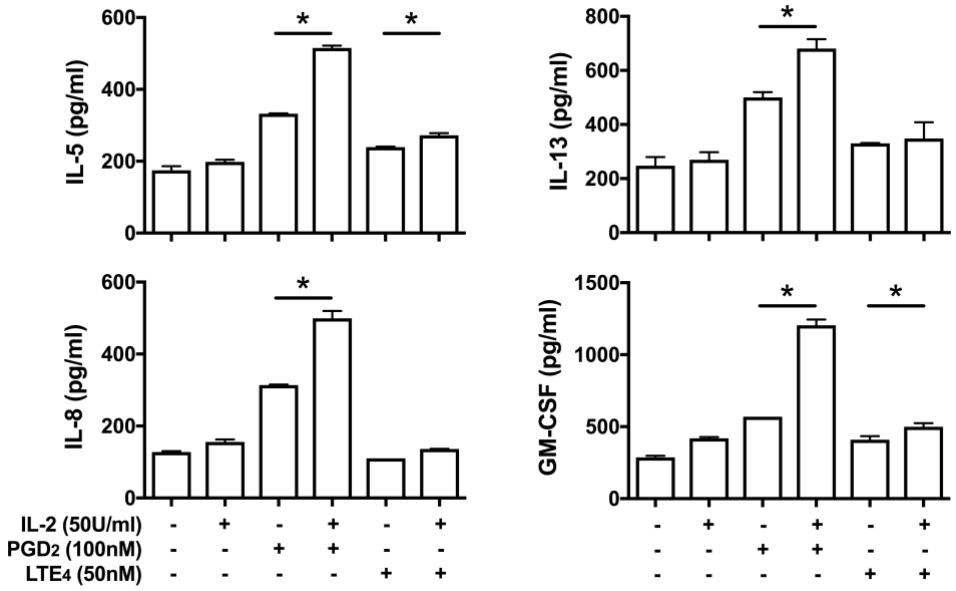
of mRNA for the receptors for PGD_2 (DP_1 and CRTH2) and for cysteinyl leukotrienes ($CysLT_1$, $CysLT_2$, $P2Y_{12}$ and GPR99) in Tc2 cells were compared by using qPCR. All the genes were normalized with GAPDH, and $CysLT_2$ was treated as 1. The levels of CRTH2 and $CysLT_1$ are significantly higher than other receptors. **b, c** Cultured Tc2 cells were (**b**) >70% CD8 positive and >90% CRTH2 positive, and (**c**) ~100% CCR7 and CD62L double negative. **d** Significant ($p < 0.05$) differences in gene transcriptions between Tc2 cells ($CD8^+CRTH2^+$) and other $CD8^+$ cells ($CD8^+CRTH2^-$) was detected by using microarray. $CD8^+CRTH2^+$ Tc2 cells express higher type-2 immunity related genes (red) but lower type-1 (green) and killer cell related genes (blue) than other $CD8^+$ cells. **e**, **f** Cultured Tc2 cells are capable to produce type-2 cytokines. *IL5*- and *IL13*-mRNA positive Tc2 cells were increased after treatment with PGD_2 and LTE_4 alone or their combination detected by using PrimeFlow RNA assay (**e**), and more than 90% of cells showed *IL5* and *IL13* double positive after stimulation with 25 ng/ml PMA and 750 ng/ml ionomycin (**f**). *$p < 0.05$; data in **c**, **e** and **f** are representative of 3 independent experiments; (n=8 for **a**; n=3 for **d**).

Supplementary Fig. 8



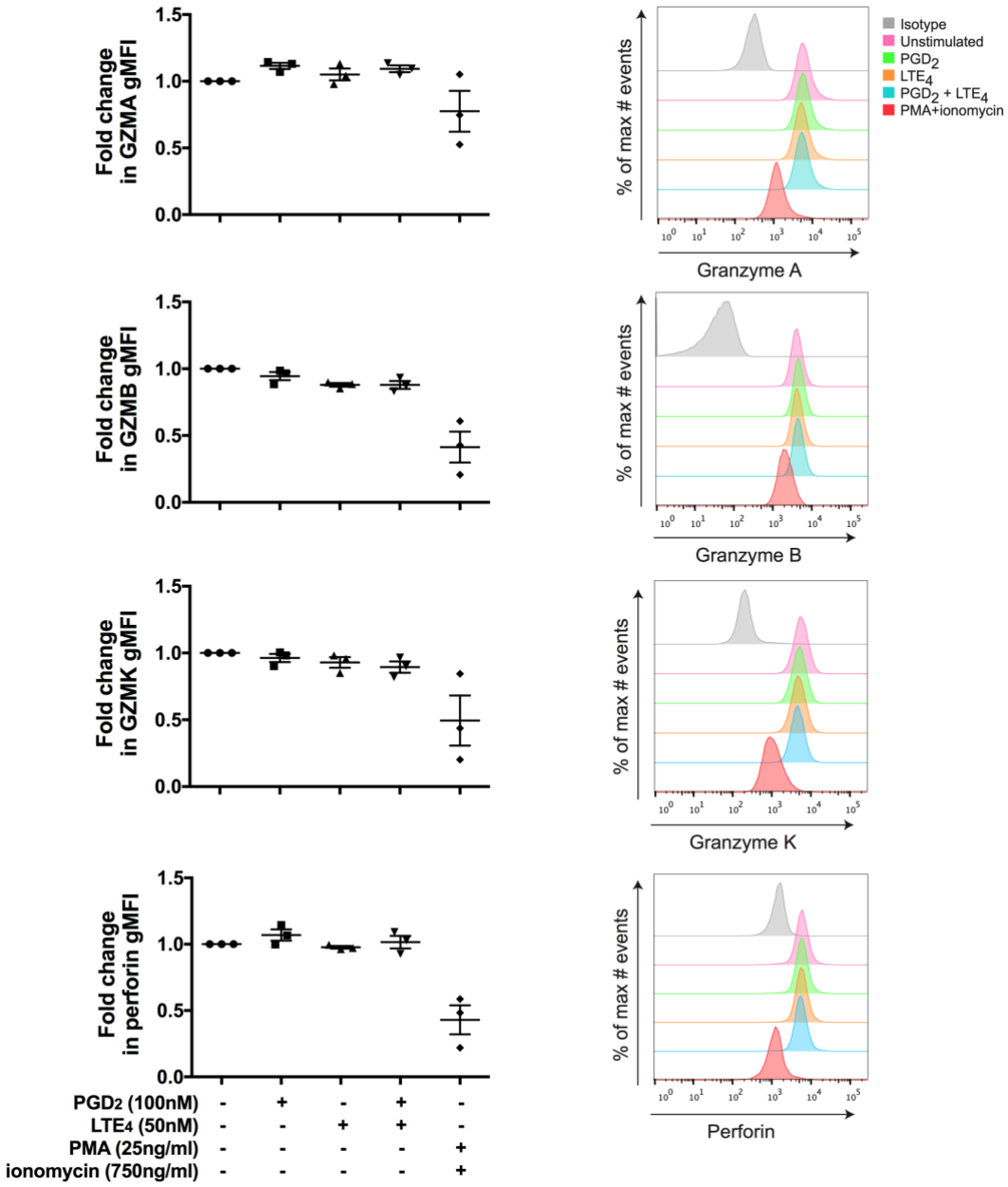
Supplementary Fig. 8 Large numbers of gene transcripts in Tc2 cells are modulated by PGD_2 and LTE_4. **a** Venn diagram and **b** het map representing total numbers of genes significantly regulated ($p < 0.05$), including up-regulations (red) and down-regulations (green), by 50 nM LTE_4, 100 nM PGD_2 or their combination for 4 h in Tc2 cells detected with microarray. (n=3).

Supplementary Fig. 9



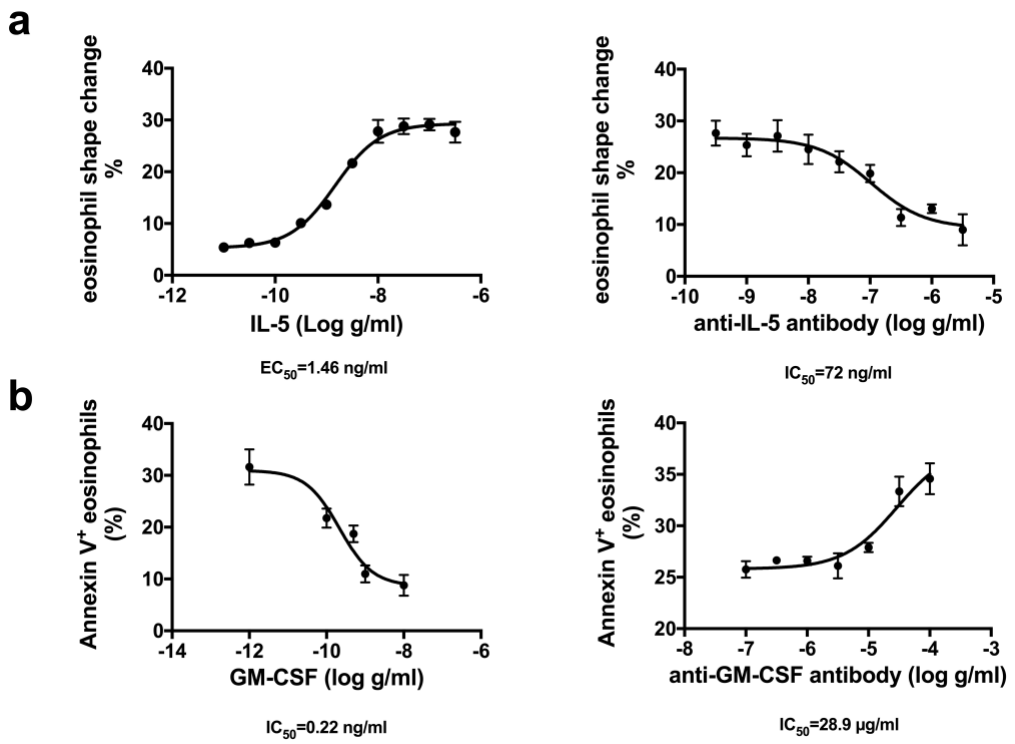
Supplementary Fig. 9 IL-2 enhances cytokine production in Tc2 cells in response PGD_2 and LTE_4. Release of IL-5, IL-8, IL-13 and GM-CSF by Tc2 cells in response to 100 nM PGD_2 or 50 nM LTE_4 detected with Luminex, particularly to PGD_2, were increased in the presence of 50 U/ml IL-2. * $p < 0.05$, (n=3).

Supplementary Fig. 10



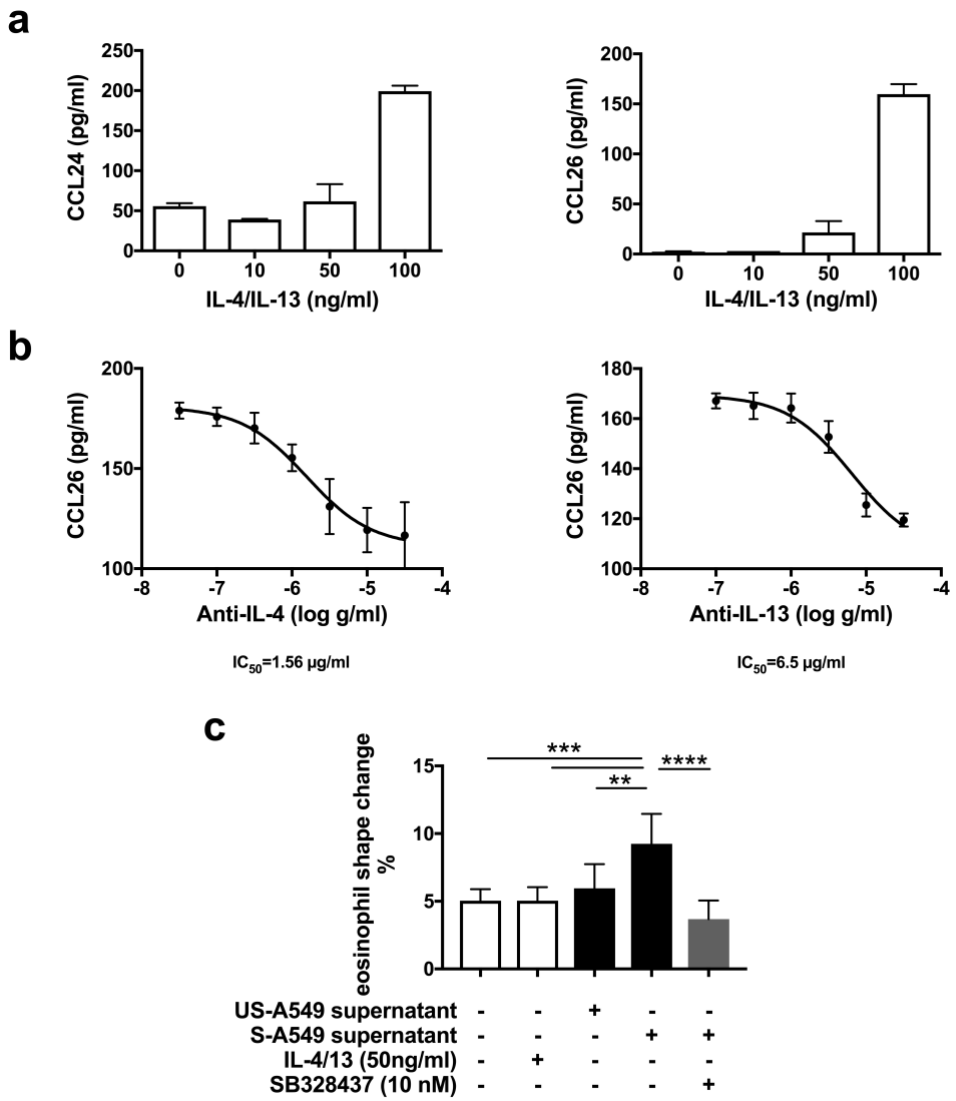
Supplementary Fig. 10 Production of cytotoxins in Tc2 cells are not affected by PGD_2 and LTE_4. The expression levels of GZMA, GZMB, GZMK and perforin in Tc2 cells determined with flow cytometry were not changed by treatment with 100 nM PGD_2, 50 nM LTE_4 or their combination for 8 h. The reduction of the cytotoxin levels in Tc2 cells by 25 ng/ml PMA/750 ng/ml ionomycin was used as positive controls. Histogram shows a representative experiment for these assays (n=3).

Supplementary Fig. 11



Supplementary Fig. 11 Effects of IL-5 and GM-CSF on eosinophil shape-change and apoptosis are inhibited by the neutralizing antibodies against these cytokines. **a** Fresh blood was incubated with various concentrations of rhIL-5 (left panel) or with 10 ng/ml rhIL-5 in the presence of various concentrations of anti-IL-5 neutralizing antibody (right panel) for 1 h, and then the shape change of eosinophils in the blood was measured using flow cytometry. The percentage shape change was increased with the concentration of IL-5, which was reversed by anti-IL-5 antibody in a dose-dependent manner. **b** Eosinophil apoptosis was induced by serum deprivation in the presence of various concentrations of rhGM-CSF (left panel) or 1 ng/ml GM-CSF and various concentrations of anti-GM-CSF neutralizing antibody (right panel), and then cell apoptosis was determined with Annexin V staining by flow cytometry. Annexin V^+ eosinophils were reduced with increasing concentrations of GM-CSF, which was inhibited by anti-GM-CSF antibody in a dose-dependent manner. (n=10 for **a** left panel; n=3 for **a** right panel and **b**).

Supplementary Fig. 12



Supplementary Fig. 12 Eotaxins induced by IL-4 and IL-13 in A549 cells were able o activate eosinophils. **a** A549 cells were incubated with or without increased concentrations of rhIL-4 and rhIL-13 overnight. Concentrations of CCL24 and CCL26 in cell culture supernatants of the cell culture were measured by Luminex assay. CCL24 and CCL26 were increased with increasing concentrations of IL-4 and IL-13. **b** A549 cells were treated with 100 ng/ml rhIL-4 and 100 ng/ml rhIL-13 in the presence of various concentrations of anti-IL-4 or anti-IL-13 neutralizing antibodies. The production of CCL26 induced by the cytokines was partially inhibited by the antibodies in a dose-dependent manner. **c** Fresh blood was incubated with

supernatants from A549 cells treated without (US-A549) or with 100 ng/ml rhIL-4 and 100 ng/ml rhIL-13 (S-A549) in the presence or absence of CCR3 antagonist SB328437 (10 nM). Activation of eosinophils in the blood was measured with eosinophil shape change assay. Normal medium and IL-4/13 were used as control. The supernatant from IL-4/13-activated A549 cells increased eosinophil shape change that was inhibited by SB328437. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, (n=3).