

Allergy / Early View

LETTER |  Full Access

T regulatory cells from atopic asthmatic individuals show a Th2-like phenotype

Kirstin Jansen, Pattraporn Satitsuksanoa, Oliver F. Wirz, Stephan R. Schneider, Willem van de Veen, Ge Tan, Milena Sokolowska, Simon D. Message, Tatiana Kebabdz, Nicholas Glanville ... [See all authors](#) ▾

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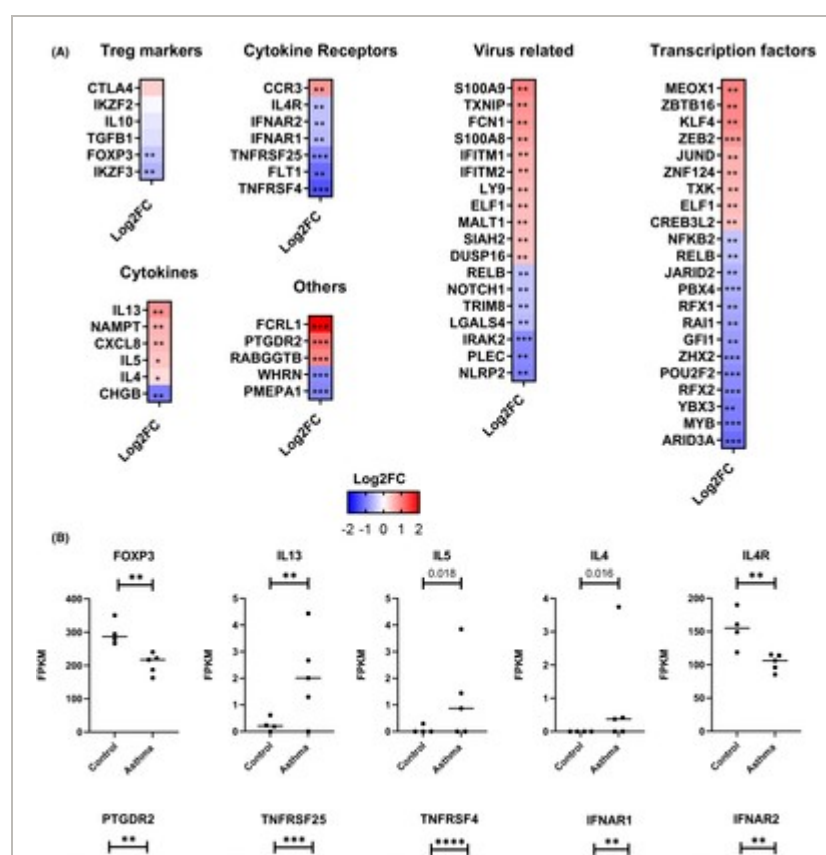
To the editor,

Asthma is the most common chronic inflammatory disease of the lung, characterized by wheezing, shortness of breath and variable airflow obstruction. It is a heterogeneous disease that can be classified into different endotypes of which T2-high - allergic asthma is one of the most common forms, especially in children. Allergic asthma is characterized by increased IgE and type-2 cytokines, including IL-5, IL-4 and IL-13.¹ Thus far, it is not completely understood why these type-2 responses are poorly controlled in asthma. T regulatory cells (Treg cells) are key mediators in controlling type 2 responses. However, under certain conditions, Treg cells can display a pathogenic and proinflammatory phenotype and contribute to disease pathogenesis.² Treg cells of food allergic children

showed a T helper 2 (Th2)-like phenotype. Whether this Th2-like phenotype of Treg cells is also present in asthmatic individuals is unknown.

Therefore, in this exploratory study, we compared the gene-expression profile of Treg cells from allergic-asthmatic individuals to non-allergic non-asthmatic controls. We isolated peripheral blood mononuclear cells (PBMCs) from asthmatic individuals ($n = 5$) and controls ($n = 4$) (Table S1) and sorted $CD3^+CD4^+D25^{hi}CD127^{low}$ Treg cells with flow cytometry (Figure S1). Then, we isolated RNA from the sorted Treg cells and performed RNA-sequencing (see Appendix S1 for detailed methods). In total, 369 genes were differentially expressed between Treg cells from asthmatic individuals and controls ($p < .01$; Figure S2).

We clustered the genes into different groups: Treg cell markers, cytokine receptors, virus related, transcription factors, cytokines and others (Figure 1A). We found that the expression of *FOXP3* was reduced in Treg cells from asthmatic individuals (Figure 1B). This is in line with a previous study that observed a lower expression of *FOXP3* in Treg cells from asthmatic individuals.³ *FOXP3* expression inversely correlated with the IgE levels found in the serum (Figure S3A), supporting the finding that Treg cells can suppress IgE production⁴ Similarly the IL-4 receptor alpha transcript *IL4RA* was significantly reduced in asthmatic individuals and also strongly correlated with the levels of IgE in the serum (Figure S3B). Previously, it was shown in mice that IL-4 receptor signaling is essential in controlling Th2 responses and airway inflammation.⁵ Our data suggest a similar role of *IL4RA* in humans. Likewise, we observed a downregulation of TNF receptor superfamily member 25 (*TNFRSF25*), which was shown to contribute to preventing allergic lung inflammation⁶ and downregulation of OX40 (*TNFRSF4*) and the type 1 IFN receptors *IFNAR1/2* in asthmatic individuals (Figure 1B).



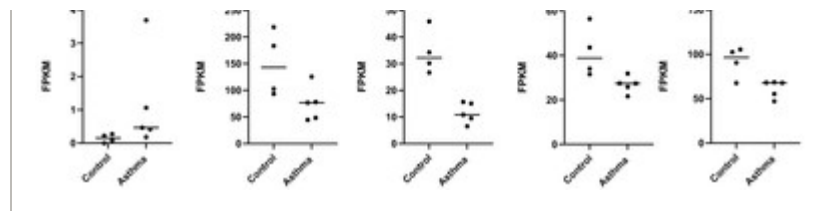
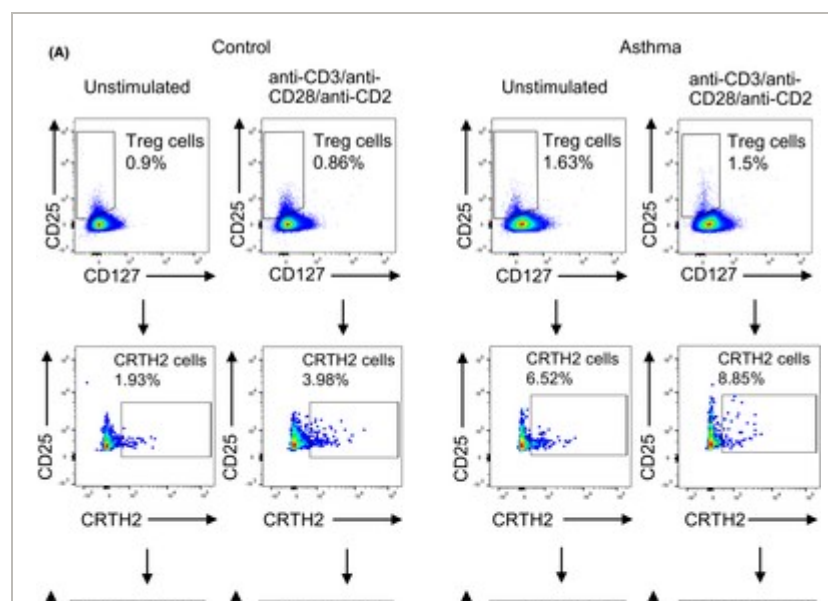


FIGURE 1

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Tregs from asthmatic individuals show a distinct phenotype compared to controls. (A) Genes that are significantly changed in Tregs cells from asthmatic individuals compared to controls (log 2 ratio) – clustered in the groups: Treg markers, cytokine receptors, virus related, transcription factors, cytokines and others. (B) Fragments per kilo base per million mapped reads (FPKM) values of genes of interest (FOXP3, IL13, IL5, IL4, IL4R, PTGDR2, TNFRSF25, TNFRSF4, IFNAR1, IFNAR2) of all donors. $N = 4$ (healthy), 5 (asthma). *** $p < .001$, ** $p < .01$, * $p < .05$

In addition, we found a significant upregulation of *IL13* mRNA expression and a trend to increased expression of *IL4* and *IL5* mRNAs in Tregs from asthmatic individuals, indicating a Th2-like phenotype as was reported in Tregs from children with food allergies.² Furthermore, we found an upregulation of the prostaglandin D2 receptor (*PTGDR2*) or CRTH2, in line with a previous study that reported an increased amount of CRTH2⁺ Tregs in asthmatic individuals.⁷ To confirm these results, we stimulated PBMCs from asthmatic and controls from a separate cohort with medium or anti CD3/CD2/CD28 overnight and assessed the expression of CRTH2 and production of IL-4 with an intracellular cytokine staining. We showed that the expression of CRTH2 and IL-4 on Treg cells is higher in asthmatic individuals compared to controls (Figure 2A). Cumulative data of 4 asthmatic and 6 controls showed that there is a higher CRTH2 expression in asthmatic individuals compared to controls (Figure 2B). IL-4 was not expressed in Treg cells from control individuals and significantly higher produced in Treg cells from asthmatic individuals (Figure 2C), confirming that Treg cells from atopic asthmatic individuals have a more Th2-like phenotype.



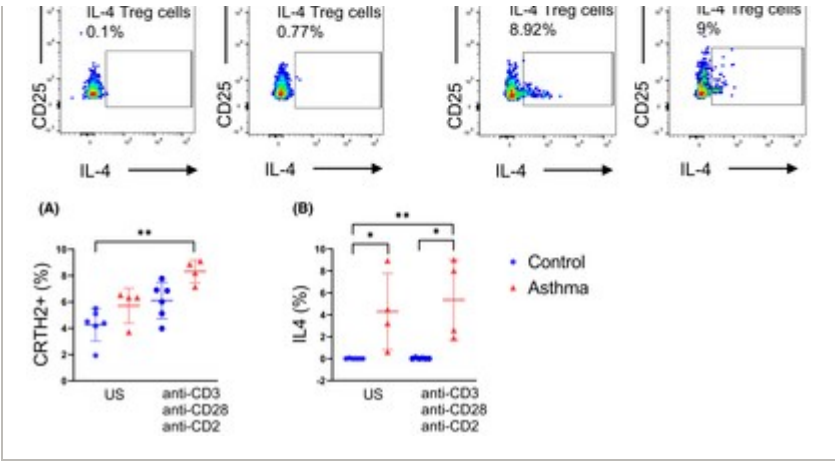


FIGURE 2

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Tregs from asthmatic individuals have increased Th2 characteristics. (A) Gating strategy of IL4+ and CRTH2+ Treg cells of PBMCs from asthmatics and controls after stimulation with CDmix and PMA/ionomycin and brefeldin A, including one representative donor for asthmatics and controls). (B) Cumulative data of the % of cells positive for CRTH2 for all donors. (C) Cumulative data of the % of cells positive for IL-4 for all donors. $N = 6$ (controls) and $N = 4$ (asthma) $**p < .01$, $*p < .05$

Lastly, we performed a pathway analysis to address whether the genes that were differentially expressed between Tregs from asthmatic individuals and controls belonged to known pathways. Using MetaCore™ we identified several pathway maps and process networks relevant to T cell immune responses (Table 1). In addition, we looked at GO processes and found that many of the genes upregulated in Treg cells from asthmatics were related to epigenetic regulation, including nucleosome organization, nucleosome assembly and chromatin organization. Using the tool STRING, which can map known interactions between genes, we identified a cluster of histone genes upregulated in Tregs from asthmatics (Figure S4). So far, there is no data reporting the function of histone genes in Tregs or related to asthma, but perhaps this finding could be related to changes in epigenetics. It was reported that in asthmatic individuals Tregs have increased CpG methylation of the *FOPX3* locus compared to non-asthmatic individuals.³

TABLE 1. Differentially expressed pathways maps, process networks and Go processes in Tregs from asthmatic and healthy subjects

Pathway maps	Genes	pValue
Immune response IFN alpha/beta signalling via PI3K and NF-kB pathways	CCND3, MAPK3, IFI17, IFITM1, IFNAR1, IFNAR2, IRS1, NFKB2	1.209e-6
Immune response OX40L/ OX40 signalling pathway	IL13, MALT1, NFKB2, FOXP3, TNFRSF4,	7.611e-6
Th2 cytokine- and TNF-alpha-induced inflammatory response in asthmatic airway	CXCL8, MAPK3, IL13, ITGA5, NFKB2, IL4RA	1.097e-5
Process networks		
Inflammation_IL-10 anti-inflammatory response	CCND3, CCNG1, CXCL8, IRAK2, NFKB2, RELB	9.770e-4
Inflammation_IL-4 signaling	MAPK3, HLA-DQA1, IL13, CXCL8, IL4RA, IRS1, NFKB2	1.517e-3
Inflammation_Interferon signaling	MAPK3, IFITM1, IFITM2, KLF4, IFNAR1, IFNAR2	4.336e-3
Go processes		
Nucleosome Assembly	HIST1H1C, HIST1H1D, HIST1H1E, HIST1H2AB, HIST1H2AD, HIST1H2AE, HIST1H2AK, HIST1H2AM, HIST1H2BC, HIST1H2BD, HIST1H2BG, HIST1H2BI, HIST1H2BN, HIST1H3B, HIST1H3D, HIST1H3D, HIST1H3H, HIST1H3I, HIST1H3J, HIST1H4, HIST1H4E, HIST1H4F, HIST2H2AB, HIST2H2AC, HIST2H2BF, HIST4H4, SMYD3, PTY2d1, TSPYL1, MPP7	1.314e-20
Nucleosome Assembly		3.906e-17
Chromatin Assembly		4.434e-17

Red colour indicates a higher expression in asthmatic individuals, while blue indicates a higher expression in healthy individuals.

Note

Red color indicates a higher expression in asthmatic individuals, while blue indicates a higher expression in healthy individuals.

In conclusion, Treg cells from atopic asthmatic individuals show increased expression of Th2 characteristics that could lead to a reduced control of allergic airway inflammation. Further studies are needed to confirm these findings in an independent and larger population and investigate their contribution to disease pathology. Additionally, it should be assessed if this Th2 signature is mostly related to the atopic status of the individuals, by comparing atopic asthmatics with atopic non-asthmatic individuals and what the effect of atopy is by comparing non-atopic asthmatic individuals with atopic asthmatic individuals.

CONFLICT OF INTEREST

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Programme “CURE”, Novartis Research Institutes and AstraZeneca. Took part in the advisory board and received research grants from GlaxoSmithKline, Sanofi/Regeneron, SciBase, and Novartis. He is Editor-in-Chief of Allergy. MM received grant from European Commission Horizon 2020 Programme "COFUND" and received lecture fees from AstraZeneca, GlaxoSmithKline, Novartis, Berlin-Chemie/Menarini, Chiesi, Takeda, Shire, Lek-AM Polska, and Celon Pharma. SJ reports personal fees from Aquarius Bio, AstraZeneca, Bayer, Bioforce, Boehringer Ingelheim, Enanta, Gerson Lehrman Group, Kaleido, Lallemand Pharma, Myelo Therapeutics GmbH, Novartis, resTORbio and Virtus Respiratory Research, outside the submitted work. In addition, SJ is an author on patents for the use of interferons for exacerbations of airway diseases. KN reports grants from National Institute of Allergy and Infectious Diseases (NIAID), National Heart, Lung, and Blood Institute (NHLBI), National Institute of Environmental Health Sciences (NIEHS), and Food Allergy Research & Education (FARE); stock options from IgGenix, Seed Health, ClostraBio, and ImmunelD; is Director of World Allergy Organization (WAO), Advisor at Cour Pharma, Consultant for Excellergy, Red tree ventures, Eli Lilly, and Phylaxis, Co-founder of Before Brands, Alladapt, Latitude, and IgGenix; and National Scientific Committee member at Immune Tolerance Network (ITN), and National Institutes of Health (NIH) clinical research centers, outside the submitted work; patents include, “Mixed allergen composition and methods for using the same”, “Granulocyte-based methods for detecting and monitoring immune system disorders”, “Methods and Assays for Detecting and Quantifying Pure Subpopulations of White Blood Cells in Immune System Disorders,” and “Methods of isolating allergen-specific antibodies from humans and uses thereof.” MA has received research grants from Swiss National Science Foundation, Sean Parker Allergy Center Seed grant and European Commission Horizon 2020 Framework Programme “CURE”. All other authors declare no conflicts of interest in relation to this work.

Supporting Information



Filename	Description
all15193-sup-0001-AppendixS1.docx Word 2007 document , 1.5 MB	Appendix S1

Please note: The publisher is not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing content) should be directed to the corresponding author for the article.

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Supplemental data: T regulatory cells from asthmatic individuals show a Th2-like phenotype

Supplemental material:

RNA sequencing

Peripheral blood mononuclear cells (PBMCs) were collected at stable baseline from atopic asthmatic (n=5) and healthy non-atopic non-asthmatic subjects (n=4) during a previously reported study¹ As previously reported, atopic status was assessed by skin prick testing to common aeroallergens: six grass pollen mix; house dust mite; cat; dog; *Aspergillus fumigatus*; *Cladosporium herbarum*; *Alternaria alternata*; birch, three trees, and nettle pollen. One wheal 3 mm greater than negative control or total IgE > 110 units/mL was considered diagnostic of atopy. Measurements of serum IgE, lung function testing including peak expiratory flow (PEF), forced expiratory volume in 1 s (FEV₁), forced vital capacity (FVC), and histamine challenge were performed. All subjects were non-smokers, and for at least 6 weeks prior to and during the study asthmatic subjects were only taking short-acting β 2 agonists if needed¹. Clinical and demographic details of the subjects studied herein are in **(Table S1)**.

Cryopreserved PBMC were thawed and washed with complete RPMI (cRPMI) 1640 medium supplemented with L-glutamine (2 mmol/l), MEM vitamin, penicillin (100 U/ml), streptomycin (100 lg/ml), kanamycin, nonessential amino acids, sodium pyruvate (Life Technologies, Carlsbad, CA, USA), and 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA) and rested for 30 min at 37°C on a roller at low speed in cRPMI. Afterwards PBMCs were washed and resuspended in staining buffer (PBS, 0.5% BSA). PBMC were stained with viability dye, CD3, CD4, CD25, CD127, CD14, CD19 **(Table S2)**. Cells were incubated for 15 min at 4°C, washed and filtered prior to sorting. Tregs were sorted using a BD ARIA III (Becton Dickinson, Franklin Lanes, NJ) and Treg % and purity after sorting was measured. **(Table S1)** Sorted cells were lysed immediately using RLT plus buffer (QIAGEN, Hilden, Germany). Cell lysates were stored at -80°C until total RNA was isolated using RNeasy Plus Micro Kit (QIAGEN) according to the manufacturer's protocol. RNA was stored at -80°C until it was analysed.

The quantity and quality of the isolated RNA from the in vivo experiment was determined with a Qubit® (1.0) Fluorometer (Life Technologies, California, USA) and an automated electrophoresis solution (Tapestation, Agilent, Waldbronn, Germany). The SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian (Clontech Laboratories, Inc., A Takara Bio Company, California, USA) was used in the

succeeding steps. Briefly, total RNA samples (0.25–10 ng) were reverse-transcribed using random priming into double-stranded cDNA in the presence of a template switch oligo (TSO). When the reverse transcriptase reaches the 5' end of the RNA fragment, the enzyme's terminal transferase activity adds non-templated nucleotides to the 3' end of the cDNA. The TSO pairs with the added non-templated nucleotide, enabling the reverse transcriptase to continue replicating to the end of the oligonucleotide. This results in a cDNA fragment that contains sequences derived from the random priming oligo and the TSO. PCR amplification using primers binding to these sequences can now be performed. The PCR adds full-length Illumina adapters, including the index for multiplexing. Ribosomal cDNA is cleaved by ZapR in the presence of the mammalian-specific R-Probes. Remaining fragments are enriched with a second round of PCR amplification using primers designed to match Illumina adapters.

The quality and quantity of the enriched libraries were validated using Qubit® (1.0) Fluorometer and the TapeStation (Agilent, Waldbronn, Germany). The libraries were normalized to 10nM in Tris-Cl 10 mM, pH8.5 with 0.1% Tween 20. The TruSeq SR Cluster Kit HS4000 or TruSeq PE Cluster Kit HS4000 (Illumina, Inc, California, USA) was used for cluster generation using 8 pM of pooled normalized libraries on the cBOT. Sequencing was performed on the Illumina HiSeq 4000 paired-end at 2 X126 bp or single end 126 bp using the TruSeq SBS Kit v4-HS (Illumina, Inc, California, USA).

Adapters and low-quality tails were trimmed. STAR aligner (v2.5.3a)² was used to align the RNA-seq dataset to Ensembl genome build GRCh.p10 (Release 89). Gene expression counts were calculated with feature counts from Bioconductor package R Subread aligner (v1.26.0)³. A gene was considered expressed if, in at least one group, it had more than 10 counts in more than half of the replicates in that group. Differential expressed genes were detected using generalized linear model with quasi-likelihood test implemented in Bioconductor package edgeR (v3.18.1).⁴ Genes with p-value < 0.01 were considered significant. Protein interaction network was prepared using STRING (v10)⁵ and furthered processed with Cytoscape (v3.2.1). Networks and pathway enrichment were generated using MetaCore version 6.3 (Thomson Reuters).

Intracellular cytokine staining

For the intracellular cytokine staining, PBMCs were sorted from non-asthmatic non-atopic in house donors that served as controls (n=6) and from a separate cohort of atopic mild asthmatic individuals (n=4). For at least 6 weeks prior to and during the study, asthmatic subjects were only taking short-acting β_2 agonists if needed. No skin prick test, FEV1 or histamine measurements were performed for these subjects. PBMCs were cultured in cRPMI in a density of 10^6 cells/ml and stimulated over night with anti-

CD3/anti-CD28/anti-CD2 mAbs. Four hours before flowcytometric analysis, cells were stimulated with PMA and ionomycin and in the last 2 hours brefeldin A was added. PBMC were stained with a viability dye, CD3, CD4, CD25 and CD127 (**Table S2**), fixed with FIX/perm buffer and stained with IL-4 and CRTH2 antibodies (**Table S2**).

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Subject no.	Age	Sex	IgE units /ml	Skin prick tests positive	FEV1 % predicted	Histamine PC ₂₀ , mg/mL *	Histamine PC ₁₀ , mg/mL	Allergy	Treg % of CD4 ⁺	Purity after sort %
Control (non-asthmatic, non-atopic)										
32	24	F	22	Nil	99	> 32 (17.1)	5.78	Nil	4.63	93.62
38	18	M	6	Nil	109	12.4	10.9	Nil	5.43	96.3
42	23	F	11	Nil	85	> 16 (10.3)	15.26	Nil	7.03	95.6
49	18	F	16	Nil	86	> 16 (12.9)	11.3	Nil	4.59	95.2
Median	20.5		13.5		92.5	12.4	11.1		5.03	95.4
Atopic asthmatics										
26	24	F	229	d(3)latex(6)	83	0.228	0.139	A, E	4.11	97
39	19	M	162	Nil	105	2.83	0.794	A	8.24	95.4
53	22	F	212	h(5)	118	7.4	4.37	A	6.27	95.4
59	21	F	374	g(4)h(4)a(3)b(3) 3t(3)c(6)d(4)	104	1.37	0.886	A, R	4.84	97.6
62	22	F	355	h(5)c(2)	136	3.93	2.16	A	4.56	95
Median	22		229		105	2.83	0.886		4.84	95.4

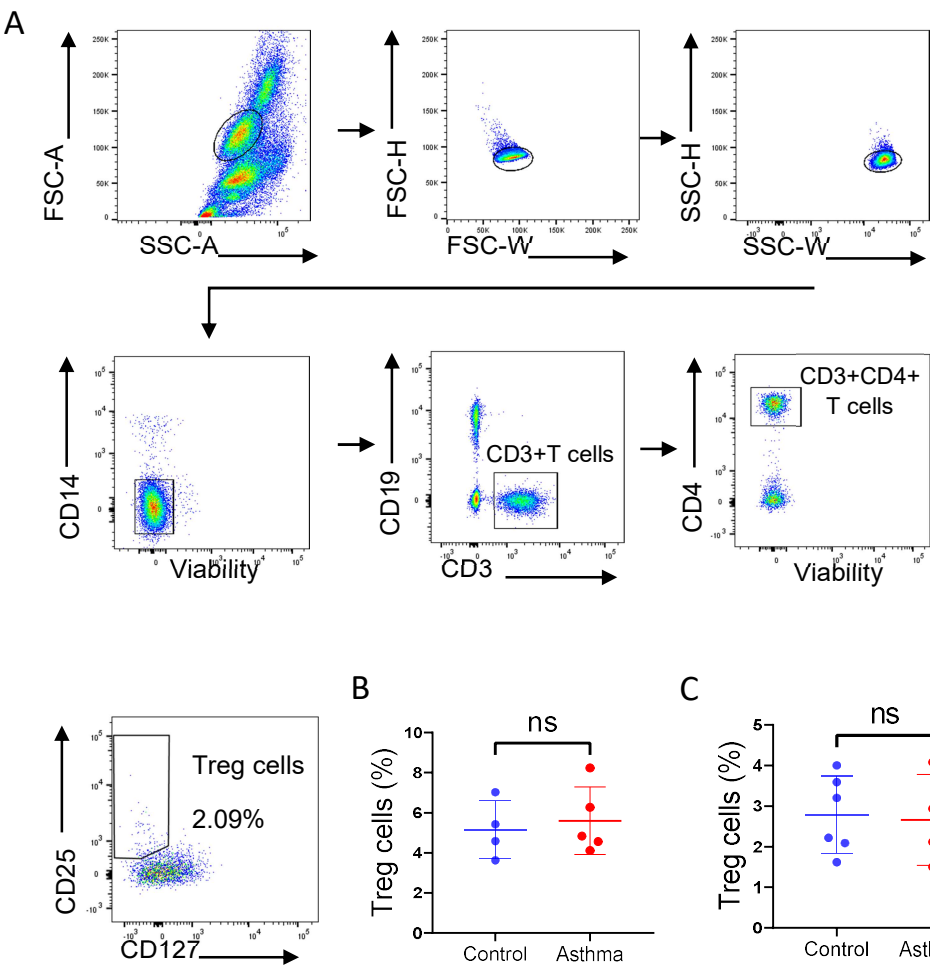
Table S1: Characteristics of asthmatic and control subjects of the sequencing data.

g, grass pollen; h, house mite; cl, cladosporium; a, aspergillus; b, birch; 3t, 3 trees; n, nettle; c, cat; d, dog; alt, alternaria; A, asthma; R, rhinitis; E, eczema. *One healthy subject had a histamine PC₂₀ > 32 mg/mL (the maximum concentration used in this study) and two were unable to tolerate a dose of histamine greater than 16 mg/mL. Where this was the case the percentage fall in FEV₁ is shown in parentheses alongside the maximum histamine concentration used.

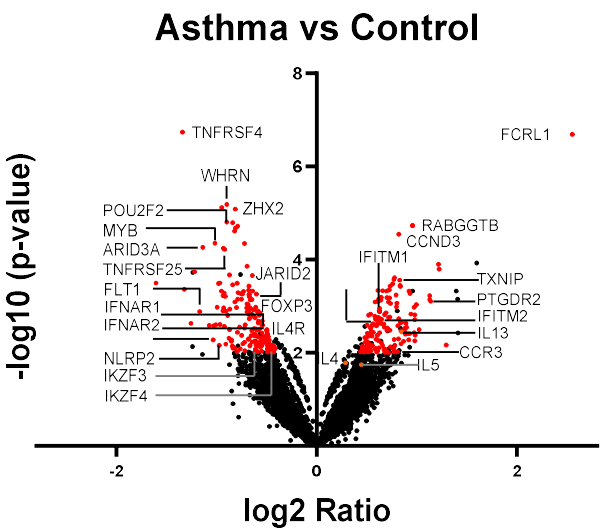
Table S2: Antibodies used for sorting and intracellular cytokine staining.

Treg sorting experimental + intracellular cytokine staining				
Marker	Dye	Catalog number		Used for experiment
Viability	Ef780	eBioscience	65-0865-14	Treg sorting + Intracellular cytokine staining
CD3	Bv510	Biolegend	300448	Treg sorting + Intracellular cytokine staining
CD4	PE-CF594	BD	562281	Treg sorting + Intracellular cytokine staining
CD14	AF700	Biolegend	325614	Treg sorting + Intracellular cytokine staining
CD19	PE-Cy7	eBioscience	25-0199-42	Treg sorting + Intracellular cytokine staining
CD25	APC	Biolegend	302610	Treg sorting + Intracellular cytokine staining
CD127	PE	Biolegend	351304	Treg sorting + Intracellular cytokine staining

IL4	AF488	Biolegend	500710	Intracellular cytokine staining
CRTH2	PE-Cy7	Biolegend	350118	Intracellular cytokine staining



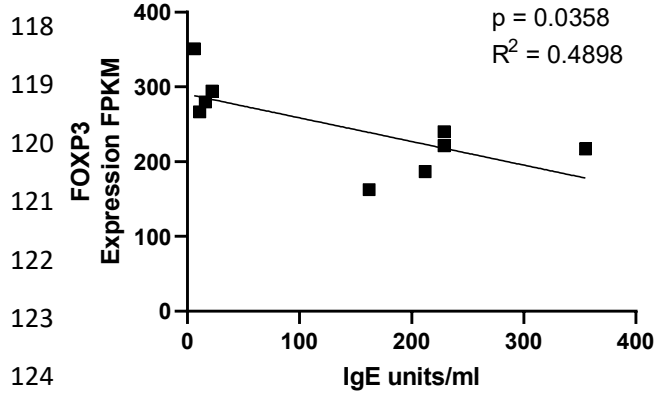
Supplementary Figure 1: Gating strategy for sorting. (A) Gating strategy for CD25+CD127- Treg cells from PBMC. (B) Cumulative data of the percentage of Tregs from the donors used for the RNA sequencing data. (C) Cumulative data of the percentage of Tregs from the donors used for the protein analysis.



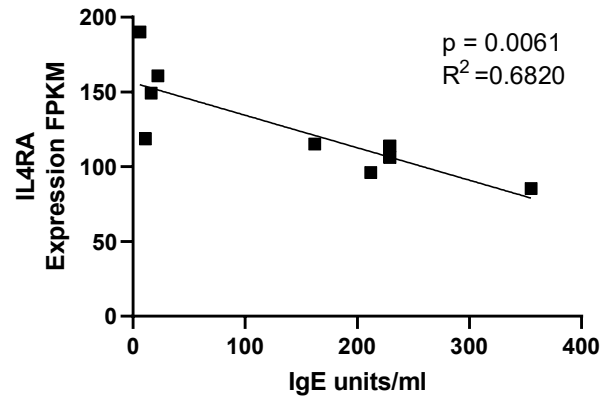
Supplementary Figure 2: Volcano plot showing differentially expressed genes ($p < 0.01$) in asthmatic subjects compared to controls. Log2 ratio < 0 means gene is expressed higher in healthy individuals, while log2 Ratio > 0 the gene is higher expressed in asthmatic individuals. Significant genes are shown in red, IL-4 and IL-5 in orange.

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A

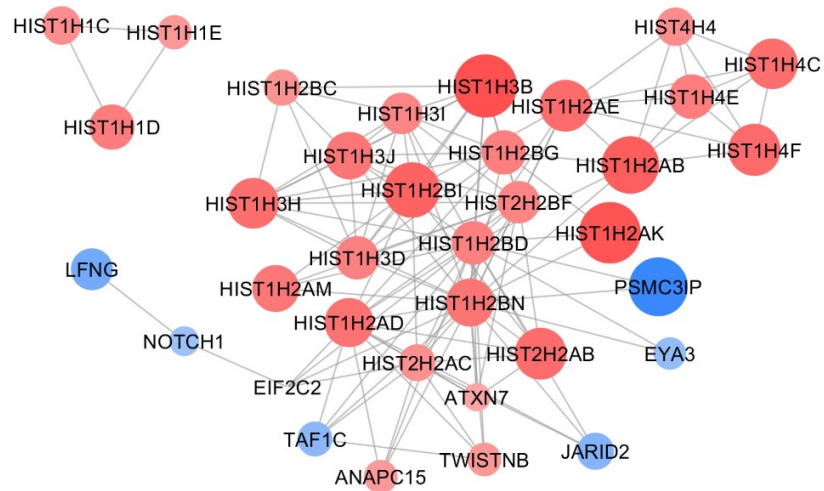
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B



Supplementary Figure 3: Correlation between expression of FOXP3 (A) and IL4RA (B) with IgE serum levels.



Supplementary Figure 4: Satellite plot showing a cluster of known interactions related to nucleosome assembly. Genes higher expressed in asthmatic individuals are shown in red, and lower expression in blue.