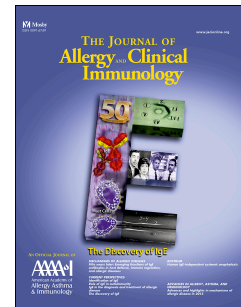


Accepted Manuscript

Allergen-specific immunotherapy modulates the balance of circulating Tfh and Tfr cells

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Title: Allergen-specific immunotherapy modulates the balance of circulating Tfh and Tfr cells

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Capsule summary:

Follicular helper T cells (T_{fh} cells) are a CD4 T cell subset essential for germinal center formation and B cell responses, although their role in allergy and allergen-specific immunotherapy (AIT) is unclear. Here, we show that AIT-treated patients have a higher ratio of regulatory T_{fh} cells (T_{fr}) to follicular T cells (T_{fh}) and hypothesize an IL-2 dependent mechanism.

Key words: T follicular helper cell, regulatory T follicular helper cell, Allergen-specific immunotherapy, Allergy, Interleukin-2

To the Editor:

Follicular helper T cells (Tfh cells) are a CD4 T cell subset specialized in providing help for the development and maintenance of B cell responses¹. Tfh cells are essential for germinal center formation and the development of effective humoral immunity². In humans, circulating resting memory Tfh cells are minimally defined as CD3⁺CD4⁺CD45RO⁺CXCR5⁺³. Follicular regulatory T cells (Tfr), a T cell subset believed to originate from thymic-derived FOXP3⁺ T cell precursors, have been shown to regulate and suppress germinal center reactions⁴. Several reports suggest that Tfh cells likely play a role in mediating allergic disease^{5, 6}, however, there is a lack of human studies of Tfh cell biology in the context of allergy^{7, 8} and allergen-specific immunotherapy (AIT). Given the critical role of Tfh and Tfr cells in regulation of IgE production in the context of allergic disease in murine models⁶, we hypothesized that AIT modulates Tfh and/or Tfr cells.

A total of 70 subjects were recruited for this study, including 25 Timothy grass allergic patients, 32 patients who received subcutaneous shots of AIT and were in treatment maintenance at the time of blood draw and 13 non-allergic healthy controls identified as having negative skin prick tests to a panel of 32 allergen extracts and no clinical history of allergy (Table E1, online repository).

The effect of AIT on Tfh cell frequency was assessed by flow cytometry, quantifying the total peripheral memory Tfh cell population (defined as CD3⁺CD4⁺CD45RO⁺CXCR5⁺) (Figure 1a). We found a significant reduction of Tfh cells in AIT-treated patients compared to untreated allergic donors (median 6.4% in allergics, 3.5% in AIT, $p=0.008$, Figure 1b). Reduction of Tfh cells after

AIT was also seen compared to non-allergic controls (median 5.1%) but this difference did not reach statistical significance ($p=0.13$, Figure 1b). To verify our manual gating analysis results, we additionally analyzed the data using a FLOCK-based automated gating strategy, which produced similar results (Figure E1).

Given that the reduction of Tfh cells in AIT patients is most prominent in the CXCR5^{hi} subset, we characterized gene expression in CXCR5^{hi} vs CXCR5^{lo} cells in five allergic donors with a predominant CXCR5^{hi} Tfh population (Figure 1a, red box) and five AIT-treated donors with a predominantly CXCR5^{lo} Tfh population (Figure 1a, blue box). CXCR5^{hi} and CXCR5^{lo} cells were sorted and high quality RNA (RIN > 7.5) was obtained for subsequent transcriptomic analysis. As expected, transcription of CXCR5 was significantly reduced in CXCR5^{lo} cells compared to CXCR5^{hi} (Figure 1c). Paired comparisons of CXCR5^{hi} vs. CXCR5^{lo} samples identified 26 genes with significantly different expression levels (multiple hypothesis adjusted p -value <0.05, Figure 2d). Examination of the 26 differentially expressed genes revealed that several had known immunological functions, including regulatory activity (*FOXP3*, *CCR8*, *LAG3*, *CD200*, *LRRC32* and *CD70*), inflammation (*CCL5*, *LGALS3* and *ENC1*), cytotoxicity (*GZMA*) and proliferation (*MKI67*). Unsupervised hierarchical clustering of samples based on the expression pattern of the 26 genes resulted in separation of CXCR5^{hi} from CXCR5^{lo} cells independent of the AIT status of the donor from which the samples were derived (Figure 2e). Thus the differences observed are intrinsic properties of CXCR5^{hi} vs CXCR5^{lo} cells and are independent of AIT treatment status.

82 To determine how the identified differences in mRNA levels between CXCR5^{hi} vs
83 CXCR5^{lo} cell populations impacted protein expression, we performed FACS
84 analyses for proteins from seven out of the 26 differentially expressed genes,
85 based on antibody availability (Figure 2a). In addition, we also included
86 antibodies against Helios because transcription of this gene was borderline
87 significant in our analysis ($p_{\text{adj}}=0.16$, Table E2) and Helios has been shown to
88 be important for regulatory T cells function⁹. Significantly higher expression in
89 CXCR5^{lo} vs CXCR5^{hi} cells was detected for FOXP3, Helios and Granzyme A.
90 Comparing the frequency of Helios and Granzyme A expression in
91 CXCR5^{lo}FOXP3⁺ vs CXCR5^{lo}FOXP3⁻ cells revealed an enrichment of
92 FOXP3⁺Helios⁺ cells, which was significantly higher than FOXP3⁺Granzyme A⁺
93 cells (median 7.0 and 0.5, respectively, $p<0.0001$) (Figure 2b), suggesting that
94 Helios and FOXP3 are frequently co-expressed on a single cell level, whereas
95 Granzyme A is expressed by a different subpopulation within the CXCR5^{lo} cells.
96 Given the upregulation of Foxp3 and Helios in CXCR5^{lo} cells, we analyzed the
97 median fluorescence intensity of CXCR5 in classical Tfh (CXCR5⁺FOXP3⁻Helios⁻)
98 vs Tfr (CXCR5⁺FOXP3⁺Helios⁺) cells (Figure 2c). A pronounced difference in
99 CXCR5 expression ($p>0.0001$) was observed in the Tfr subset compared to Tfh
100 cells, indicating that Tfr cells fall within the CXCR5^{lo} fraction of Tfh cells in our
101 gating strategy. Thus, the increase in CXCR5^{lo} cells observed in AIT-treated
102 patients may be associated with a relative increase of Tfr cells. To assess the
103 functionality of the stimulated Tfr cells, we performed intracellular cytokine
104 staining which revealed that IL-10 production is more than 6 fold higher in Tfr

cells compared to Tfh, a fold difference higher than any other cytokine measured (Figure E2).

Finally, we wanted to determine if the observed changes in CXCR5⁺ cells in the AIT cohort are associated with an induction of the Tfr phenotype (FOXP3⁺Helios⁺), and if they can be induced directly by TCR stimulation, which is associated with IL-2 production. Purified Tfh cells (CD45RO⁺CXCR5⁺) from eight donors were cultured for 5 days in medium alone or in the presence of TCR stimulation (anti-CD3/38 beads) with or without anti-IL-2. Flow cytometric analysis revealed a significant reduction of CXCR5 expression in the presence of anti-CD3/28 stimulation (median 77.7%) compared to culture in medium alone (median 94.9%), which was largely rescued in the presence of anti-IL-2 (median 86.4%) (Figure 2d). Tfr cells (CXCR5⁺FOXP3⁺Helios⁺) were significantly increased in the presence of anti-CD3/28 stimulation (median 8.9%) compared to medium alone (median 1.5%). Addition of anti-IL-2 partially reversed Tfr induction (median 5.0%) after TCR stimulation (Figure 2e). Based on these data, we speculate that TCR stimulation and/or the presence of IL-2 during *in vitro* culture may have agonistic effects on the development/survival of Tfr cells over CXCR5^{hi} Tfh cells.

In the context of AIT immunotherapy, we hypothesize that repeated administration of allergen extracts elicits IL-2 production from allergen-specific T cell responses, which globally impairs CXCR5 expression in memory Tfh cells and induces/retains Tfr cell populations. This could be an important mechanism contributing to the induction of tolerance during AIT.

Word count: 1,019 words.

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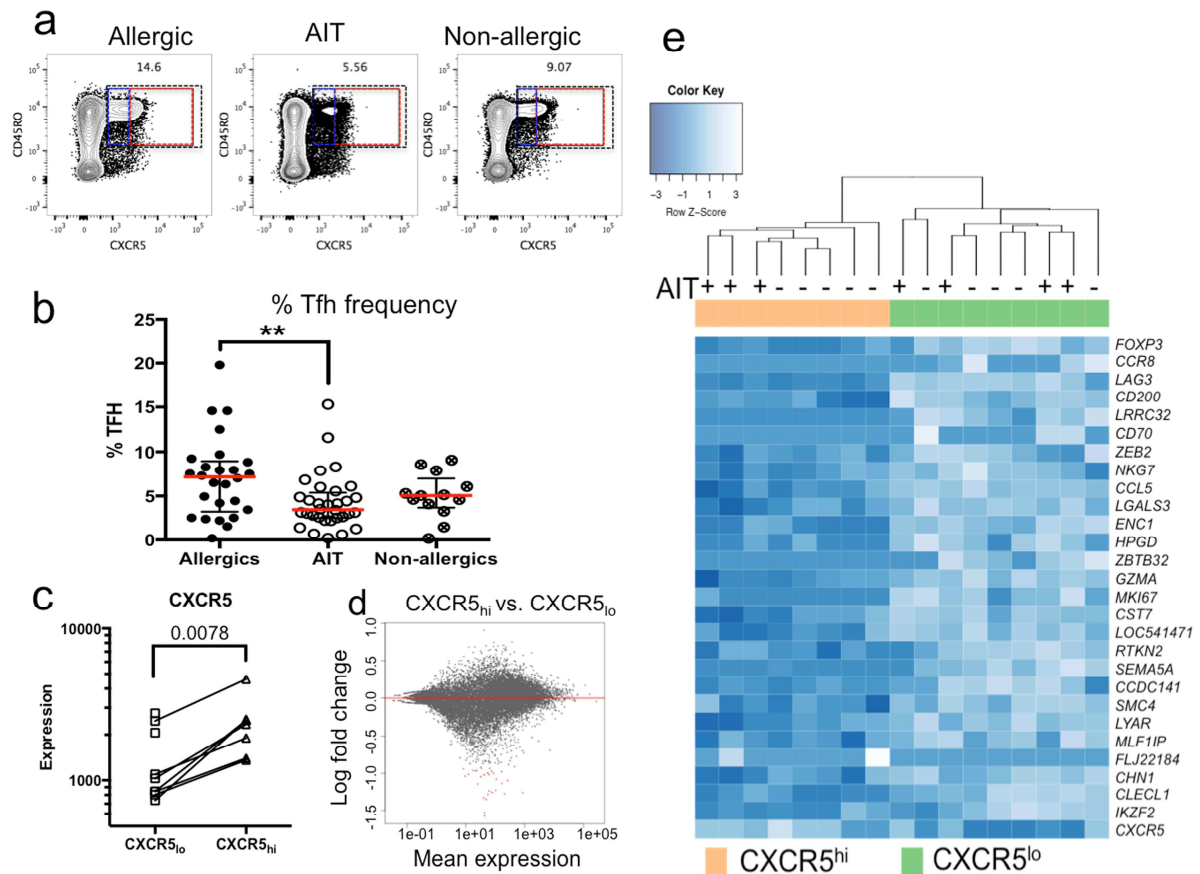
⁴National Jewish Health, Denver, CO, USA.

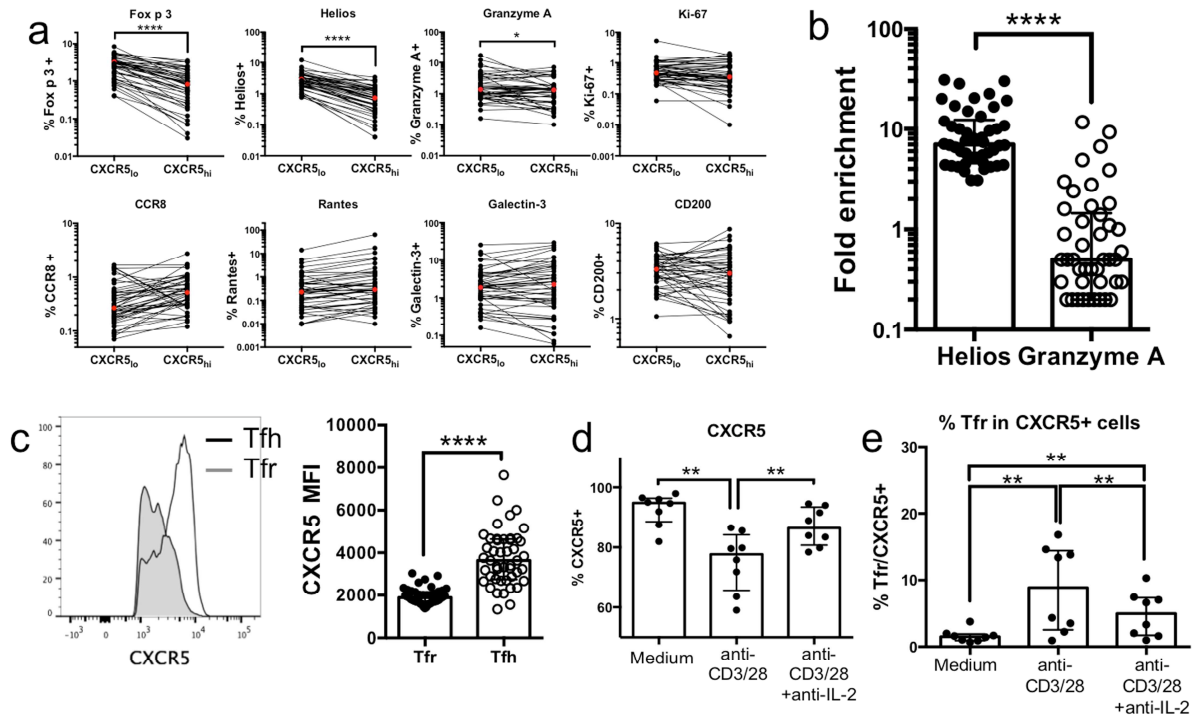
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Figure 1: AIT-treated patients exhibit reduced Tfh frequencies compared to untreated allergic and non-allergic controls. a: Representative Tfh cell staining in an allergic, AIT-treated and non-allergic patient. Dashed boxes indicate the total Tfh population (black), CXCR5^{lo} (blue) and CXCR5^{hi} (red) populations. b: Tfh cell percentage in an allergic (n=25), AIT-treated (n=32) and non-allergic (n=13) patients. c: Quantification of CXCR5 gene expression in CXCR5^{hi} and CXCR5^{lo} sorted cells. d: MA plots comparing fold changes of gene expression between CXCR5^{hi} and CXCR5^{lo} cells vs. average gene expression. Genes with significant adjusted p-values are colored in red e: Heatmap of differentially expressed genes in CXCR5^{hi} vs CXCR5^{lo} samples from allergic and AIT-treated individuals.

Figure 2: Protein expression profiles of selected markers in CXCR5^{hi} vs CXCR5^{lo} cells and the effect of TCR stimulation on CXCR5 expression. a: Quantification of protein expression of markers shown to be differentially expressed in CXCR5^{hi} vs CXCR5^{lo} cells as determined by RNAseq. b: Fold change of Helios and Granzyme A expression in CXCR5^{lo}FOXP3⁺ vs CXCR5^{lo}FOXP3⁻ cells. c: Median fluorescent intensity (MFI) for CXCR5 expression in Tfr and Tfh cells. d: CXCR5 expression in sorted Tfh cells after 5 days of culture in medium alone or in the presence of anti-CD3/28 beads with or without anti-IL-2. e: Percent of Tfr cells (Foxp3+Helios+) in total CXCR5⁺ cells after culture in medium or with anti-CD3/28 beads with or without anti-IL-2. Statistical analysis was done by Wilcoxon matched-pairs signed rank test (one-sided). (n=46), *-p<0.05; ****-p<0.0001





1 Online repository

2 **Materials and methods**

4 **Study participants**

5 Patients were recruited following Institutional Review Board approval (La Jolla
6 Institute for Allergy and Immunology, La Jolla, CA) (Federal Wide Assurance no.
7 00000032). All patients enrolled in this study provided written consent. For the
8 allergic cohort Timothy grass (TG)-allergic donors with skin prick test wheal of ≥ 3
9 mm in diameter to TG and a clinical history consistent with seasonal grass pollen
10 allergy were recruited (n=25). The second cohort consisted of TG-allergic
11 subjects who receive subcutaneous shots of allergen-specific immunotherapy
12 (AIT) for a minimum of 6 months and were in treatment maintenance at the time
13 of the blood draw (n=32). Allergen extracts used were from Alk America. Select
14 allergens were from Greer (primarily molds) and Hollister-Stier (AP dog). The
15 patients received one shot per month. Due to concerns about increased risk for
16 anaphylaxis after a large volume blood draw, patients were recalled on another
17 day outside the shot day for blood sample collection. Blood was drawn within a
18 month of the last allergy shot. Non-allergic, healthy control donors were identified
19 as having negative skin prick tests to a panel of 32 allergen extracts and no
20 clinical history of allergy (n=13). A complete summary of all donors recruited for
21 this study is provided in supplemental table 1.

22 **Flow cytometry**

23 To assess Tfh cell frequency, PBMCs from all 3 donor cohorts were thawed,
24 washed and stained with an antibody cocktail for CD4 (eBioscience, San Diego,
25 CA, clone RPA-T4), CD3 (BD, Franklin Lakes, NJ, clone UCHT1), CD45RO

(eBioscience, clone UCHL1), CXCR5 (BD, clone RF8B2), and CD19 (BD, clone HIB19), CD14 (BD, clone M5E2), CD8 (BD, clone RPA-T8) and live/dead aqua fixable viability dye for exclusion. Cells were stained for 20 minutes at room temperature, washed and analyzed by flow cytometry. Frequency and CXCR5 median fluorescence intensity of Tfh cells was determined by gating on the CD4+CD3+CD45RO+CXCR5+ population.

Expression of markers of interest identified by RNA-Seq analysis was assessed by surface staining PBMC *ex vivo* with the panel described above, except CXCR5 (Biolegend, clone J252D4), which performed better during fixation, in addition to CCR8 (R&D, clone 191704) (panel 1) and CD200 (Biolegend, clone OX-104) (panel 3). Subsequently cells were treated with FOXP3 Fixation/Permeabilization Kit (eBioscience) and intracellular staining was performed for FOXP3 (ebioscience, clone 236A/E7), Helios (eBioscience, clone 22F6) and Ki-67 (Biolegend, clone Ki-67) (Panel 1); FOXP3 and RANTES (Biolegend, clone VL1) (Panel 2) and FOXP3, Galectin-3 (Biolegend, clone M3/38) and Granzyme A (Biologend, clone CB9) (Panel 3). All data acquisition was performed using a BD LSR II flow cytometer and data was analyzed using FlowJo software (TreeStar, Ashland, OR). All data acquisition was performed blinded.

Tfh cell sorting for RNA-Seq

PBMCs from five allergic and 5 AIT-treated patients were stained with the antibody cocktail for CD4, CD3, CD45RO, CXCR5, and CD19, CD14, CD8 and live/dead aqua for exclusion as described above. Tfh cells were gated as

CD4+CD3+CD45RO+CXCR5+ and directly sorted into 750 µl of Trizol LS (Invitrogen). Data acquisition and cell sorting was performed using a FACS Aria II flow cytometer (BD, Two out of five AIT-treated donors had insufficient numbers of CXCR5^{hi} cells for RNA-Seq analysis. One of the AIT CXCR5 samples was excluded from analysis due to low quality mRNA (RIN < 7.5). RNA-Seq analysis was performed in CXCR5^{lo} samples collected from 5 allergic and 4 AIT-derived samples and CXCR5^{hi} samples from 5 allergic and 3 AIT-derived samples, leaving two CXCR5^{lo} samples and one CXCR5^{hi} sample without corresponding match.

Microscaled RNA sequencing

Total RNA was purified as described previously³⁰ and is described in detail in the supplemental material.

RNA sequencing analysis

RNA sequence analysis was performed as described previously³⁰ and is described in detail in the supplemental material.

Tfh/Tfr marker expression following TCR stimulation/rIL-2 culture

CD4+ T cells were isolated from previously frozen PBMC by negative selection using CD4+ T cell isolation kit II per the manufacturers instructions (Miltenyi, San Diego, CA). The isolated CD4 cell population was stained as described above. Tfh cells (CD4+CD3+CD45RO+CXCR5+) were isolated using a BD FACS Aria II cell sorter. After sorting, cells were plated in a round-bottom 96-well plate at 2 x 10⁵ /well in 200 µl serum-free AIM-V medium (Life Technologies) with human rIL-7 (4 ng/ml). Tfh cells were incubated in medium alone, with TCR stimulation

(Dynabeads, human Tactivator CD3/28, Life technologies) or with human rIL-2 (125 µg/ml), all in the presence or absence of anti-human IL-2 (50 µg/ml). After 5 days of incubation, cells were harvested, washed and surface stained with antibodies for CD4 (eBioscience, San Diego, CA, clone RPA-T4), CD3 (BD, Franklin Lakes, NJ, clone UCHT1), CD45RO (eBioscience, clone UCHL1), CXCR5 (Biolegend, clone J252D4). For exclusion, stains for CD19 (BD, clone HIB19), CD14 (BD, clone M5E2), CD8 (BD, clone RPA-T8) were also performed. Intracellular stains for FOXP3 and Helios were performed as described above.

Statistical analysis

A one- or two-tailed Mann–Whitney U test was used for statistical analysis as indicated in the figure legends. For paired sample comparison, Wilcoxon signed-rank test was used. Differences with a p value <0.05 were considered significant.

Online repository figure legends:

Supplemental figure E1: Automated gating analysis reveals reduced CXCR5 expression in AIT-treated patients compared to untreated allergic and non-allergic controls. a: Representative dotplots showing Tfh cells in an allergic, AIT-treated and non-allergic patient. b: Percentage Tfh in allergics (n=24), AIT-treated (n=32) and non-allergic (n=13) patients. Statistical analysis was performed using the Mann-Whitney test, one-tailed. **-p<0.01.

Supplemental figure E2: Cytokine production after PMA/Ionomycin stimulation in Tfr cells ex vivo. Intracellular cytokine staining for IL-10, IFN γ , IL-4 and IL-13 after 6 hours of stimulation with PMA/Ionomycin was performed in Tfr cells from 4 healthy donors. Percentage of cytokine producing cells out of the total CD4 subset is shown.

111 Supplemental table E1: Demographic and clinical information of donors recruited
 112 for this study.

Donor	Cohort	Gender	Birth year	Race	Donation Date	Wheal size*	AIT report**
D00013	Non-allergic	M	1979	White	2008-07-15	0	n/a
D00014	Non-allergic	F	1982	White	2008-07-15	0	n/a
D00030	Non-allergic	F	1954	White	2008-08-13	0	n/a
D00032	Non-allergic	M	1982	White	2008-10-13	0	n/a
D00034	Non-allergic	F	1971	White	2008-10-16	0	n/a
D00036	Non-allergic	M	1987	White	2008-10-21	0	n/a
D00038	Non-allergic	F	1980	White	2008-10-22	0	n/a
D00049	Non-allergic	F	1957	Hispanic	2008-11-21	0	n/a
D00055	Non-allergic	F	1948	White	2009-01-05	0	n/a
D00082	Non-allergic	M	1976	Black	2009-12-17	0	n/a
D00086	Non-allergic	F	1974	White	2010-03-09	0	n/a
D00091	Non-allergic	M	1971	Hispanic	2010-04-22	0	n/a
D00118	Non-allergic	F	1951	White	2008-12-12	0	n/a
D00008	Allergic	M	1980	White	2008-07-08	11	n/a
D00052	Allergic	F	1968	White	2008-12-12	10	n/a
D00056	Allergic	M	1967	Hispanic	2009-01-08	5	n/a
D00073	Allergic	F	1965	Black	2009-10-26	12	n/a
D00084	Allergic	M	1979	White	2009-01-20	17	n/a
D00090	Allergic	M	1967	Hispanic	2009-01-08	10	n/a
D00117	Allergic	F	1958	White	2011-01-24	36	n/a
D00122	Allergic	F	1972	White	2011-03-01	20	n/a
D00137	Allergic	F	1985	White	2012-05-22	6	n/a
D00139	Allergic	F	1961	White	2012-06-07	48	n/a
D00140	Allergic	F	1977	White	2012-06-12	10	n/a

D00141	Allergic	M	1991	White	2012-06-14	14	n/a
D00145	Allergic	F	1961	White	2012-07-05	8	n/a
D00149	Allergic	F	1992	White	2012-08-01	5	n/a
D00156	Allergic	F	1960	Black	2012-08-22	17	n/a
D00158	Allergic	M	1982	White	2012-09-04	31	n/a
D00159	Allergic	F	1976	Black	2012-09-04	10	n/a
D00160	Allergic	F	1964	Black	2012-09-11	31	n/a
D00161	Allergic	F	1981	Hispanic	2012-09-11	17	n/a
D00163	Allergic	M	1984	Black	2012-09-18	22	n/a
D00166	Allergic	M	1982	White	2012-09-21	28	n/a
D00167	Allergic	M	1949	White	2012-09-21	13	n/a
D00168	Allergic	M	1969	White	2012-09-24	21	n/a
D00170	Allergic	M	1966	White	2012-09-26	37	n/a
D00172	Allergic	F	1953	White	2012-09-26	12	n/a
D00031	AIT	F	1969	Hispanic	2008-08-25	7	yes
				Asian/Pacific			
D00063	AIT	M	1970	Islander	2009-03-25	15	yes
D00072	AIT	F	1954	White	2009-10-14	8	yes
D00080	AIT	M	1973	White	2009-12-02	25	yes
D00087	AIT	M	1973	White	2010-03-23	18	yes
D00106	AIT	F	1971	White	2010-09-16	9	yes
D00101	AIT	M	1964	White	2009-11-12	17	yes
D00109	AIT	F	1980	Black	2010-09-27	10	yes
D00111	AIT	M	1963	White	2010-09-29	11	yes
D00114	AIT	F	1961	White	2010-10-21	11	uncertain
D00126	AIT	M	1969	White	2011-03-14	17	yes
D00127	AIT	M	1973	White	2010-03-23	14	yes
D00129	AIT	F	1990	White	2012-01-23	17	uncertain
D00130	AIT	M	1973	White	2012-01-24	15	yes
D00132	AIT	M	1969	White	2012-02-14	11	yes
D00133	AIT	M	1973	White	2012-02-14	11	yes
D00134	AIT	F	1954	White	2012-02-16	15	yes
D00135	AIT	M	1962	White	2012-03-15	13	yes
D00136	AIT	F	1946	White	2012-03-16	10	yes
D00142	AIT	F	1984	White	2012-06-28	5	no
D00143	AIT	F	1960	White	2012-06-29	7	yes
D00144	AIT	M	1978	White	2012-07-02	9	yes
D00146	AIT	M	1982	Black	2012-07-09	7	yes
D00147	AIT	M	1981	White	2012-07-25	6	yes
D00148	AIT	F	1967	Black	2012-07-25	10	no
D00150	AIT	F	1966	White	2012-08-02	6	yes
D00151	AIT	F	1961	White	2012-08-02	9	no
D00152	AIT	F	1974	White	2012-08-03	10	yes

D00153	AIT	M	1967	White	2012-08-03	5	yes
D00155	AIT	M	1985	Black	2012-08-14	9	yes
D00157	AIT	F	1987	White	2012-08-27	10	yes
D00162	AIT	F	1956	White	2012-09-14	7	yes

* wheal size of skin prick test with Timothy grass

** self report on whether patient felt that AIT treatment helped to improve allergic symptoms

113

114 Supplemental table E2: A list of genes significantly differentially expressed by the

115 CXCR5^{lo} vs CXCR5^{hi} subset. *IKZF2* and *CXCR5* shown for reference.

	Gene	Protein	Fold change	p adjusted	Median CXCR5 ^{hi}	Median CXCR5 ^{lo}
1	CCR8	CCR8	>100	0.008	0	10
2	LRRC32	Garp	33.3	0.001	2	54
		Zinc finger and BTB domain-containing				
3	ZBTB32	protein 32	11.6	0.046	2	19
4	ENC1	CD93	11.5	0.005	9	107
		Hydroxyprostaglandin dehydrogenase 15-				
5	HPGD	(NAD)	9.7	0.028	5	49
6	SEMA5A	Semaphorin 5A	8.6	0.000	5	41
7	CHN1	Chimerin 1	8.3	0.009	28	234
8	FOXP3	Fox p 3	7.5	0.000	12	91
9	LAG3	Lag3	6.0	0.000	13	77
10	GZMA	Granzyme A	6.0	0.000	44	264
		coiled-coil domain containing 141				
11	CCDC141		5.5	0.004	16	90
12	MKI67	Ki67	5.5	0.018	6	35
13	RTKN2	Rhotekin 2	5.3	0.008	36	191
14	CLECL1	C-type lectin like 1	4.5	0.000	13	59
		Natural killer cell granule protein 7				
15	NKG7		4.0	0.023	52	210

16	LGALS3	Galectin 3	4.0	0.004	75	302
17	CD70	CD70	3.6	0.020	3	11
18	CCL5	Rantes	3.6	0.000	350	1254
19	LOC541471	n.d.	3.2	0.004	39	123
20	ZEB2	Zinc finger E-box-binding homeobox 2	2.9	0.041	37	107
21	CD200	CD200	2.9	0.001	49	144
22	MLF1IP	Centromere protein U	2.8	0.028	26	74
23	CST7	Cystatin-F	2.6	0.008	242	621
24	LYAR	LYAR	2.5	0.008	148	366
25	SMC4	SMC-4	1.9	0.018	233	441
26	FLJ22184	n.d.	0.0	0.028	8	0
27	IKZF2	Helios	11.9	0.160	9	104
28	CXCR5	CXCR5	0.4	-	2321	968

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