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An Exhausted Phenotype of T_H2-Cells Is Primed by Allergen Exposure, But Not Reinforced by Allergen-specific Immunotherapy

Short running title:

Allergen exposure primes T_H2 cell exhaustion

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Abstract:

Background: Studies show that proallergic T_H2 -cells decrease after successful allergen-specific immunotherapy (AIT). It is likely that iatrogenic administration of allergens drives these cells to exhaustion due to chronic T cell receptor stimulation. This study aimed to investigate the exhaustion of T cells in connection with allergen exposure during AIT in mice and two independent patient cohorts.

Methods: OVA-sensitized C57BL/6J mice were challenged and treated with OVA, and the development of exhaustion in local and systemic T_H2 -cells was analyzed. In patients, the expression of exhaustion-associated surface markers on T_H2 -cells was evaluated using flow cytometry in a cross-sectional grass pollen allergy cohort with and without AIT. The treatment effect was further studied in PBMC collected from a prospective long-term AIT cohort.

Results: The exhaustion-associated surface markers CTLA-4 and PD-1 were significantly upregulated on T_H2-cells upon OVA aerosol exposure in OVA-allergic compared to non-allergic mice. CTLA-4 and PD-1 decreased after AIT, in particular on the surface of local lung T_H2-cells. Similarly, CTLA-4 and PD-1 expression were enhanced on T_H2-cells from patients with allergic rhinitis with an even stronger effect in those with concomitant asthma. Using an unbiased Louvain clustering analysis, we discovered a late-differentiated T_H2 population expressing both markers that decreased during up-dosing but persisted long-term during the maintenance phase.

Conclusions: This study shows that allergen exposure promotes CTLA-4 and PD-1 expression on T_H2-cells, and that the dynamic change in frequencies of exhausted T_H2-cells exhibits a differential pattern during the up-dosing versus the maintenance phases of AIT.

Key words: AIT; CTLA-4; PD-1; proallergic T_H2; T cell exhaustion

Abbreviations:

AAI	Allergic airway inflammation
AIT	Allergen-specific immunotherapy
AR	Allergic rhinitis
AA	Allergic asthma
BAL	Bronchoalveolar lavage
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
DEG	Differentially expressed genes
IL	Interleukin
LAG-3	lymphocyte activation gene-3
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cell
PD-1	Program death protein-1

TCF-1	T-cell factor 1
TCR	T-cell receptor
TIM-3	T-cell immunoglobulin- and mucin-domain-containing molecule-3
UMAP	Uniform Manifold Approximation and Projection

Total word counts: 3485

Introduction

Successful allergen-specific immunotherapy (AIT) in allergic airway disease effectively reduces symptom and the need for anti-allergic rescue medication such as antihistamines or corticosteroids while improving the quality of life in allergic patients.¹⁻⁴ The induction of clinical tolerance to allergen is associated with multiple anti-inflammatory effects. In parallel to clinical improvement, proallergic T_H2-cells have been shown to decrease after several years of AIT.⁵⁻⁷ Given this, during AIT, proallergic T_H2-cells are chronically exposed to allergens administered iatrogenically, T cell exhaustion should be considered as a potential mechanism leading to tolerance induction.⁸

T cell exhaustion is characterized by diminished effector function and sustained expression of inhibitory receptors, such as programmed cell death protein-1 (PD-1), cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), T-cell immunoglobulin- and mucin-domain-containing molecule-3 (TIM-3), as well as lymphocyte activation gene-3 (LAG-3).⁹⁻¹² Engagement of these inhibitory receptors downmodulates T-cell receptor (TCR) signaling cascades and restricts the downstream PI3K/AKT pathway in a nonredundant manner.¹³⁻¹⁶ While functional exhaustion can be partially reversed by blocking these receptors using immune-checkpoint inhibitors (ICI),^{11,16,17} epigenetic reprogramming turns the exhausted state into a stable phenotype.^{18,19} Notably, some exhausted T cells retain the expression of the transcription factor TCF-1 and thus their self-renewal potential, whereas some do not and are therefore prone to apoptosis and resistance to ICI.²⁰⁻²²

While CD4⁺ T cell exhaustion has been addressed in variable chronic infections and autoimmune diseases,^{11,23,24} its role in the context of allergy and AIT is not fully understood. Using murine models of asthma, several studies exhibited the role of PD-1 and CTLA-4 in limiting allergen sensitization²⁵ and allergic airway inflammation.^{26,27} In addition, it has been reported that allergen-specific CD4⁺ T cells are terminally differentiated and preferentially deleted during

AIT.^{6,7} However, the contribution of T cell exhaustion to these observations is uncertain. Upon *in vitro* stimulation with common inhalant allergens, CD4⁺ T cells upregulated PD-1 and CTLA-4, while PD-1 but not CTLA-4 blockade increased their proliferation.²⁸ Still, it remains largely unknown whether proallergic T_H2-cells would reach a state of exhaustion upon AIT.

We hypothesized that allergen exposure during AIT could render T_H2-cells functionally exhausted. In this study, we therefore sought to determine the development of the exhausted phenotype of T_H2-cells in an AIT mouse model and further to validate the findings in grass-pollen allergic patient cohorts.

Materials and Methods

Animals

Female specific pathogen-free C57BL/6J mice, six weeks of age, were purchased from Charles River (Sulzfeld, Germany) and provided with food and water *ad libitum*. The experiment was conducted following German federal guidelines for the use and cares of laboratory animals. The study was approved by the Government of the District of Upper Bavaria and the Animal Care and Use Committee of the Helmholtz Center Munich (55.2-1-54-2532-50-2017).

Ovalbuminimmunotherapy model

The model has been described earlier.²⁹ Mice were sensitized via intraperitoneal injections of 30µg OVA (Grade V, Sigma-Aldrich, Merck, Darmstadt, Germany) in conjunction with 2mg aluminum hydroxide (Imject™ Alum, Thermo Fisher Scientific, Waltham, MA, USA) in 200µl phosphate-buffered saline (PBS) on days 0, 7, 14 and 28. Control mice received aluminum hydroxide (2mg/200µl PBS) only. After sensitization, the AIT group was treated on days 35, 39, 43, 47, 51 and 55 with subcutaneous injections of OVA (500µg/200µl PBS), while the rest received sham treatment with PBS. All mice were challenged with 1% nebulized OVA for 15 min on days 34, 41 and 48 during the immunotherapy phase and on days 63, 66 and 69 toward the end of the experiment. All mice were euthanized on day 70. After bleeding, bronchoalveolar lavage fluid (BALF) was collected and cell counts were reported as one of us (AF) and colleagues have previously described.³⁰ The online repository contains a detailed description of the analysis of immunoglobulins and cytokines in serum and BALF and other methods.

Patients and blood samples

PBMC samples collected from a cross-sectional patient cohort were used for *ex vivo* phenotyping of T_H2-cells. Forty grass-pollen allergic patients were recruited who met the following criteria: at least a two-year history of moderate-severe and chronic persistent allergic rhinitis during the grass-pollen season, as defined by ARIA (Allergic Rhinitis and its Impact on Asthma) criteria³¹; a positive skin prick test wheal >3 mm in diameter; and a grass-pollen specific IgE-level above 0.70kU/l (**Table S1**). Twenty of them received AIT treatment before recruitment. Twenty-seven non-allergic individuals without a clinical history of chronic rhinosinusitis were recruited as controls. The study was approved by the ethics commission of the Technical University of Munich (5534/12). After written and informed patient consent and in accordance with the Helsinki Declaration, peripheral blood was obtained from patients twice, once in (May–July) and once out of grass pollen season (October–January). Peripheral blood mononuclear cells (PBMC) were isolated using density-gradient centrifugation and cryopreserved until analysis.

To identify exhausted T_H2-cell clusters during AIT, we used PBMC samples collected in the course of the longitudinal Prospective Allergy and Clinical Immune Function Cohort study (PACIFIC, EudraCT 2015–003545-25).⁵ Patient characteristics are summarized in **Table S2**. At

each time point, eight samples were included for analysis. Due to patient attrition, samples could not be linked timewise, rendering the analysis cross-sectional.

Flow cytometry

Human PBMCs and murine lymphocytes isolated from lungs, BALF, and blood were analyzed by flow cytometry, and 100,000 cells were acquired per sample. Antibodies used are listed in **Table S3**. Cells were incubated with fixable viability dye to exclude dead cells and labeled with antibodies against surface markers. For intracellular staining, surface-labeled cells were fixed/permeabilized and then stained with antibodies against intracellular molecules. Cells were stained with isotype antibodies to determine the nonspecific binding. For cytokine assay, cells were incubated for 4h in medium containing PMA, ionomycin and brefeldin-A (all from Sigma-Aldrich, Merck, Darmstadt, Germany) before intracellular staining. The samples were analyzed using a BD LSRII Fortessa Flow Cytometer and FlowJo software (Ashland, OR, USA).

Louvain cluster analysis and differential abundance test

The flow cytometric data (pre-gated in live populations) were analyzed with scanpy (version 1.5.1) in Python version 3.8. We used the FlowCytometryTools package (<https://github.com/eyurtsev/FlowCytometryTools>, github version tag 0.5.0) to read the data and subsequently converted them into the anndata (version 0.7.4) format. We compensated the data matrix using the numpy (version 1.19.1) matrix multiplication function.

We normalized the compensated flow cytometric data using the arcsinh-transformation (with cofactor 150) and checked the feasibility of the normalisation by inspecting the data distribution of each factor in a histogram. Then, we computed 15 nearest neighbors with the scanpypp.neighbors function and performed Louvain clustering with resolution 2.0.

Finally, we performed hierarchical clustering with the scanpytl.dendrogram function with default parameters (i.e. the Pearson correlation and the 'complete' linkage method as implemented in the scipy cluster module (scipy version 1.5.2)).

We performed Differential Abundance tests for all Louvain clusters and merged clusters using the Dirichlet Regression model (implemented in the DirichletReg R package;

<https://epub.wu.ac.at/4077/>) for predefined pairwise comparisons in R (version 3.5.2). All p-values were corrected for multiple testing using the Benjamini-Hochberg method.

Statistics

GraphPad Prism6 (GraphPad Software, La Jolla, CA, USA) was used to perform the statistical analysis. Specifically, Kruskal-Wallis test and two-tailed Mann-Whitney *U* test were used to evaluate statistical significance ($P \leq .05$), where appropriate.

Results

OVA-specific immunotherapy attenuated allergic inflammation in mice

To examine the association of T cell exhaustion with AIT, we implemented a murine immunotherapy model²⁹ where OVA-sensitized mice were subjected to sham treatment or OVA-specific immunotherapy, denoted as allergic airway inflammation (AAI) and AAI+AIT groups, respectively (**Figure 1A**). After OVA aerosol challenge, the total cell number in the BAL fluid

(BALF) increased roughly 40-fold in AAI over than in non-allergic mice, while the number decreased 25-fold after AIT (**Figure 1B**). While non-allergic mice had a typical BAL composition dominated by macrophages, AAI mice displayed a typical lung inflammatory pattern, with eosinophils predominating(**Figure 1C**). An extensive decrease of BAL inflammatory infiltrate was observed in AAI+AIT, in particular of eosinophils, returning to a macrophage-predominant BAL composition.

Furthermore, BALF concentrations of IL-4 and IL-5 significantly increased in AAI compared to non-allergic mice, while AIT significantly reduced the levels of IL-4, IL-5, and IL-9 (**Figure 1D–F**). Similarly, serum levels of IL-4 were significantly lower in AAI+AIT compared to AAI, while there was a tendency towards reductions in IL-5 and IL-9(**Figure 1G–I**). In addition to the type-2 cytokines, secretion of IL-6, TNF- α , and IL-17A into BALF, and serum levels of IL-6 were also significantly decreased in AAI+AIT compared to AAI (**Figure S1**). In contrast, the amount of IFN- γ in BALF and serum was comparable across groups, while IL-10 was mostly below detection limits (data not shown).

Moreover, AAI showed a 12-fold increase of total IgG1 and nearly a 30-fold increase of total IgE compared to non-allergic mice (**Figure 1J,K**). AIT resulted in a further 3-fold increase of total IgG1 but did not alter the levels of total IgE compared to AAI. OVA-specific IgE was significantly higher in AAI than in non-allergic mice and returned to control levels after AIT (**Figure 1L**).

Transcriptional profiles of allergic mouse lungs reveal categories of genes associated with allergic inflammation and T cell exhaustion

In addition, the transcriptome of murine lung homogenates was analyzed using a whole-genome microarray. Considering the regulatory role of CTLA-4 and PD-1 on T cell signaling and their association with T cell exhaustion, the data were first grouped according to their expression levels and the dose-dependent effect of CTLA-4 and PD-1 expression on other genes were tested(**Figure 1M,N**). CTLA-4 and PD-1 showed an expression-level correlation with IL-4 and IL-5 (high vs. low: $P \leq .05$; $FC \geq 1.5$), but not with Foxp3 and TGF- β (data not shown). Next, the data were grouped per treatment condition and a total of 6,500 regulated genes in AAI+AIT compared to AAI were identified, including 5,243 upregulated genes and 1,257 downregulated genes ($P \leq .05$; $FC \geq 1.5$; **Figure 2A**). AIT augmented expression of *Gpr35*, *Sema5a*, and IL-17 cytokine family

members, but downregulated genes associated with allergic inflammation, such as *Saa1/3*, *Ccl11*, *Ccl20*, *Cxcl5* (Figure S2A,B; Table S5 and S6). The microarray data were further probed using a curated gene expression profile of exhausted CD4⁺ T cells to see whether the cells mirrored the exhausted phenotype (Table S4). AAI mouse lung cells enhanced the expression of *Ctla4*, *Pdcd1* and several co-stimulatory molecules. In contrast, *Pilra* and *Cd244*, molecules associated with inhibition of TCR signaling, were upregulated after AIT (Figure 2B; Table S7). The expression of *Havcr2* (TIM-3), *Lag3*, and exhaustion-associated transcription factors, *Eomes* or *Prdm1*, was comparable across groups (data not shown). Consistent with the microarray data, quantitative PCR confirmed diminished expression of *Ctla4* and *Pdcd1* in AAI+AIT compared to AAI (Figure 2C,D). On the other hand, gene set enrichment analysis (GSEA) revealed that AAI mouse lungs expressed a terminally differentiated signature comparable to exhausted CD8⁺ T cells (Figure S3A,B). The co-expressed genes associated with *Ctla4* and *Pdcd1* were selected from a database of protein-protein interactions,³² based on experimentally determined co-expression, physical interactions, functional associations, or knowledge extracted from other databases. Comparing AAI and non-allergic mice, it showed significant upregulation of CTLA-4 and PD-1 but not their ligands or associated signaling molecules (Figure 2E). While CTLA-4 was significantly downregulated in the lungs of AAI+AIT compared to AAI, the expression of its ligands CD80/86 was also downregulated (Figure 2F). Conversely, the expression of PD-1 ligands, PD-L1 (*Cd274*) and PD-L2 (*Pdcd1lg2*), were not significantly altered after AIT. Although the expression of the signaling molecules Lck and Akt1 was not significantly modulated, IL-2 was significantly upregulated comparing AAI+AIT with AAI.

Recurrent allergen exposure drives an exhausted phenotype of T_H2-cells

Given the mixed cell types in the microarray data of the murine lung homogenates and the central role of T_H2-cells in orchestrating type-2 immune response to allergens, we performed flow cytometry to look specifically on activated T_H2-cells (Figure 3A; Figure S4). Activated T_H2-cells were overrepresented in the lungs, BALF, and peripheral blood (PB) of AAI mice (Figure 3B–D). The frequency of intrapulmonary T_H2-cells was significantly lower in AAI+AIT compared to AAI (12% ± 0.9% vs. 26% ± 0.9%), whereas T_H2-cells in the BALF and PB remained comparable between the two groups. Additionally, PD-1 and CTLA-4 expression on non-regulatory T_H2-cells were higher in AAI (Figure 3E,I). While >75% of local lung and BALF T_H2-

cells expressed PD-1 in AAI, <33% of T_H2-cells expressed PD-1 in non-allergic mice (**Figure 3F,G**). There were also significantly more CTLA-4⁺ cells in the lungs and BALF of AAI compared to non-allergic mice, with differences up to 50% (**Figure 3J,K**). After AIT, 10-30% fewer local lung and BALF T_H2-cells expressed PD-1 and CTLA-4 compared to AAI. Overall, less than one-third of the circulating T_H2-cells expressed these markers, with approximately 10% differences between AAI and each of the other two groups (**Figure 3H,L**).

Collectively, these data demonstrate that T_H2-cells in the lungs of AAI mice responded strongly to OVA aerosol challenge and exhibited increased effector functions, yet an exhausted phenotype. After AIT, however, the frequency of intrapulmonary T_H2-cells decreased significantly.

Exhaustion of circulating CRTH2⁺T_H2-cells is more pronounced in patients with allergic asthma in pollen season

To verify these findings from the mouse model to grass pollen-allergic patients, PBMCs from healthy controls (HC; n = 27), allergic rhinitis patients with grass-pollen allergy (AR; n = 20) and AIT-treated AR patients (AR+AIT; n = 20; **Table S1A&B**) were isolated. The *ex vivo* expression of exhaustion-associated surface markers on CRTH2⁺T_H2-cells was determined (**Figure S5**). The frequency of PD-1⁺T_H2-cells was significantly higher in AR compared to HC (**Figure 4B,C**). In addition, while the expression of CTLA-4 was enhanced on T_H2-cells from AR in season compared to HC (3.6% ± 0.5% vs. 2.2% ± 0.3%), this expression was revoked by trend upon AIT (P = .07; **Figure 4E,F**). Further, no significant difference was detected in the expression of LAG-3 or TIM-3 on T_H2-cells (**Figure S6**). In the subsequent subgroup analysis, T_H2-cells from allergic asthma patients (AA; n = 9) upregulated CTLA-4 significantly compared to HC during pollen flight (**Figure 4N**), while in AIT-treated individuals (AA+AIT; n = 10) the CTLA-4 expression on T_H2-cells was significantly lower than in AA patients (2.0% ± 0.3% vs. 3.9% ± 0.5%). This latter effect, however, was restricted to patients with concomitant asthma (compare **Figure 4I,J**). On the other hand, PD-1 was upregulated in AR without asthma patients in and out of grass pollen season (**Figure 4G,H**) as well as in AA patients during pollen flight (**Figure 4L**). PD-1 expression, however, was unchanged by AIT across patient groups and overall the levels appeared higher out of season than in season. Taken together, in AR patients, upregulation of CTLA-4 on T_H2-cells was associated with allergen exposure and concomitant asthma, while upregulation of PD-1 was perennial, albeit with seasonal variations.

The frequency of late-differentiated T_H2-cells reached its base at top dose of AIT

Finally, to examine the treatment effect on exhaustion, flow cytometric analysis was performed using PBMC collected during a longitudinal AIT-treated patient cohort study (**Figure 5E; Table S2**). An unbiased graph-based Louvain-cluster analysis of flow cytometric cross-sectional data from each time point was performed, resulting in 42 clusters (see methods; **Figure S7**). The cluster distribution per sample corresponded to the cell-type composition per sample. The 42 clusters were merged into ten clusters based on the MFI pattern and the relationships in hierarchical clustering (**Figure 5A; Figure S8**). Based on the differential CD45RA expression in each of the ten clusters, three mother clusters were identified and assigned to “memory”, “intermediate”, and “immature” cells (**Figure 5B,C**).

Due to the lack of expression of CD45RA and CD27, cluster 10 was identified as late-differentiated T_H2-cells (CD45RA⁻CD27⁻CRTH2⁺IL-4⁺) with low-intermediate CD161 expression (**Figure 5B–D**), thus resembling a previously reported proallergic T_H2 population known as T_H2A.^{6,7} Expressing intermediate-level TCF-1 and KLRG-1 and high-level IL-2, this proallergic T_H2 population was also positive for exhaustion-associated markers CTLA-4 and PD-1, denoted as T_H2A_{EX} henceforth. Their percentage among live PBMCs decreased significantly six hours after the last top dose during up-dosing (T4) compared to baseline (T0; **Figure 5F**). Their frequency increased again in the following pollen season (T5), rendering it comparable to baseline and remained at this level towards the end of the follow-up phase (T9). While the MFI of IL-4 and PD-1 inside T_H2A_{EX} remained relatively stable over AIT, we observed a lower MFI of IL-2 in correspondence to an increase of CTLA-4 at T9 (**Figure 5G**). Overall, while T_H2A_{EX} decreased during up-dosing, it maintained its exhausted phenotype and persisted long-term during AIT.

Discussion

In the current study, we show that T_H2 -cells upregulate the exhaustion-associated surface markers CTLA-4 and PD-1 upon allergen exposure in allergic mice and AA patients, and that this upregulation is reduced after AIT. In an unbiased cluster analysis, we demonstrated that late-differentiated T_H2 -cells expressing both exhaustion markers decreased during up-dosing but were not eliminated by AIT.

The expression of CTLA-4 and PD-1 on T_H2 -cells was enhanced in AAI compared to their AIT-treated counterparts, implying that the exhausted phenotype of T_H2 -cells was associated with allergen exposure, whereas AIT did not render T_H2 -cells more exhausted. In comparison, T_H2 -cells from AR patients upregulated PD-1 expression perennially. Such sustained upregulation is a hallmark of T cell exhaustion⁹ and associated with epigenetic imprinting.^{18,19,33} By contrast, the upregulation of CTLA-4 was seasonal, indicating a more dynamic regulation of its expression and its reactivity to allergens. However, in AAI mice and allergic individuals, upregulation of these markers on T_H2 -cells is not synonymous with development of functional deficits. In fact, it has been shown that exhausted $CD4^+$ T cells were not functionally inert but could undergo functional adaption.^{9,34-37} Indeed, in contrast to the well-characterized role of PD-1 in exhaustion, some

previous studies show that PD-1 engagement could enhance T_H2 response and even exacerbate allergic airway inflammation.³⁸⁻⁴⁰ PD-1 ligation augmented GATA-3 expression and IL-4 production in murine T cells, while PD-1 deficiency resulted in lower serum levels of IL-4 and IL-13.³⁸ The administration of PD-L2-Fc increased serum IgE levels and BAL cell infiltrations in asthmatic mice,³⁹ whereas greater airway hyperreactivity and inflammation was observed in sensitized PD-L2^{-/-} mice compared with sensitized wild-type mice.⁴⁰

Although the expression of CTLA-4 and PD-1 was amplified on a transcriptional level in AAI mice and although GSEA provided ancillary evidence for T cell exhaustion, the mixed cell types in mouse lungs limit the possibility to delve into expression of exhaustion-associated genes specifically in T_H2 -cells. Meanwhile, the expression intensity of CTLA-4 and PD-1 showed a dose-dependent relation to type-2 cytokines, revealing no attenuation of effector functions in association with these markers.

Given that CTLA-4 is constitutively expressed on regulatory T cells (Tregs) and mediates their immunosuppressive functions,^{41,42} we also examine its expression on Tregs and found comparable expression levels across mouse groups, both locally and systemically (data not shown). Thus, upregulation of CTLA-4 on Foxp3-negative T_H2 -cells in AAI mice in this study was less likely associated with its regulatory role on Tregs. Additionally, since we have previously studied the dynamic changes of Treg population in AIT-treated grass pollen-allergic patient cohort,⁵ their role in the AIT-treated allergic mice was not investigated in more detail.

Upregulation of CTLA-4 and PD-1 on circulating T_H2 -cells was even more profound in AA patients as opposed to AR patients in pollen season, suggesting that, apart from allergen exposure, concurrent asthma could also promote the exhausted phenotype. Indeed, previous studies also showed that PD-1 expression was significantly higher in AA patients, whereas there was no consistent correlation with serum levels of IgE or disease severity.^{43,44} Association between CTLA-4 polymorphisms and serum IgE levels, asthma, and lower pulmonary function has been reported.⁴⁵

In the unbiased cluster analysis, T_H2A_{EX} decreased markedly during up-dosing, but were not eliminated by AIT, suggesting that the effect of AIT was different between the initial and the late stage, and that these exhausted T_H2 -cells could persist long-term in AIT-treated patients. Specifically, T_H2A_{EX} fell into the group of mature “memory T cells” and were of the effector memory T cell (T_{EM}) phenotype. Previous studies^{46,47} showed that T_{EM} cells recirculate between

blood and nonlymphoid tissue, such as lung and skin. Therefore, it is likely that the redistribution of these late-differentiated T_H2-cells contributes to their persistence in AIT-treated patients. By contrast, reduced frequency of T_H2-cells after AIT has been reported by several studies.⁵⁻⁷ However, as exemplified in our mouse study, AIT significantly reduced the frequency of intrapulmonary but not circulating T_H2-cells, implying that AIT affects local and systemic T_H2-cells differentially. In fact, fate tracing revealed that T_H2-cells expanded transiently after AIT and persisted in the lungs until subsequent allergen challenge.⁴⁸ This study implicates that AIT had a greater impact on intrapulmonary T_H2-cells, and that AIT did not necessarily drive T_H2-cells to exhaustion over time due to chronic antigen exposure but rather conditioned them for deletion upon secondary exposure. Future studies are warranted to address mechanisms that can accelerate this process and thereby shorten AIT treatment duration, while maintaining long-term efficacy.

Furthermore, we found that T_H2A_{EX} expressed high-level IL-2, despite its exhausted phenotype. However, there was a reciprocal decrease of IL-2 as CTLA-4 increased in the third follow-up year, implying that IL-2 production attenuated late in the course of AIT and, if AIT extends longer, declining viability might ensue. Conversely, while T_H2A_{EX} expressed a late-differentiation marker, KLRG1, their TCF-1 expression was not significantly reduced as observed in terminally differentiated T cells.^{20,21} Since TCF-1 is associated with self-renewal potential, this may partly account for their long-term persistence in AIT-treated patients. Notably, TCF-1 expression in CD4⁺ T cells was associated not only with the progenitor potential, but also with the fate decisions⁴⁹: for instance, T follicular helper cells express a high level of TCF-1 and sustain greater plasticity,⁵⁰ while TCF-1 and β -catenin can jointly promote GATA-3 expression and inhibit IFN- γ expression.⁵¹ Hence, T_H2-cells in general appear to have higher stemness by default, rendering them also more resistant to apoptosis.

In conclusion, this study demonstrates in a mouse model of AIT and in two independent patient cohorts that chronic allergen exposure results in higher expression of CTLA-4 and PD-1 on T_H2-cells, and that the proallergic T_H2-cells with an exhausted phenotype persist long-term during AIT, albeit with a reduction during up-dosing. The persistence of exhausted T_H2-cells may explain the long duration of AIT treatment required to effectively reduce symptom burden and improve the quality of life for allergic patients.

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Figure legends

Figure 1 OVA-specific immunotherapy attenuated T_H2 response and intrapulmonary eosinophilia in mice.(A) Experimental scheme of OVA immunotherapy model. BAL fluid and sera from non-allergic, AAI and AAI+AIT mice were analyzed (n = 8 per group). (B) Total BAL cells, eosinophils, macrophages, neutrophils and lymphocytes on a logarithmic scale. (C) Percentages of eosinophils, macrophages, neutrophils and lymphocytes within the total BAL cells. Levels of IL-4, IL-5, and IL-9 in BAL fluid (D–F) and mouse serum (G–I). Serum levels of total IgG1 (J), total IgE (K), and OVA-specific IgE (L). Graphs show means ± SEM. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$; Mann-Whitney *U* test. Transcriptional analysis of *Il4*, *Il5*, *Il9*, and *Il13* in relation to the expression levels of *Pdcd1* (M) and *Ctla4* (N), categorized as high, intermediate, and low groups. AAI, allergic airway inflammation.

Figure 2 Transcriptional profiles of allergic mouse lungs reveal categories of genes associated with allergic inflammation and T cell exhaustion. RNA whole transcriptome microarray analysis of mouse lung 24 h after the last OVA aerosol challenge. (A) Volcano plot illustrates differential gene expression comparing AAI+AIT and AAI mice. (B) Differentially expressed genes of AAI+AIT versus AAI mice were filtered using an entity list curated from exhausted CD4⁺ T cells (Table S7). Statistically significant entities ($P \leq .05$; $FC \geq 1.5$) are displayed. Real-time qPCR analysis of the indicated genes, *Pdcd1* (C) and *Ctla4* (D). * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$; Mann-Whitney *U* test. Co-expressed genes in relation to *Pdcd1* and *Ctla4* were selected from protein-protein association networks,³² based on experimentally determined interactions or computational prediction. The upregulation or downregulation of genes was color-coded according to the microarray analysis, comparing AAI versus non-allergic (E) and AAI+AIT versus AAI (F).

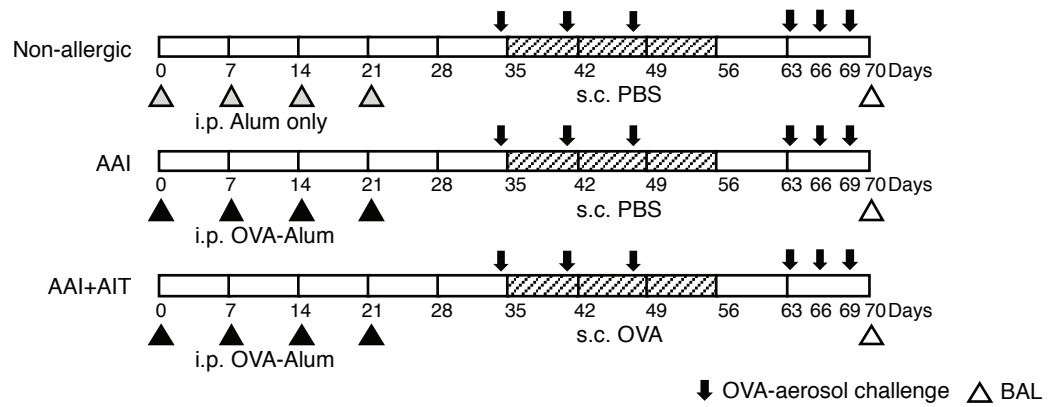
Figure 3 Recurrent allergen exposure drives upregulation of PD-1 and CTLA-4 on T_H2-cells.(A)A representative flow cytometric plot shows the gate of GATA-3⁺ cells among live memory T helper cells (see complete gating strategy in **Figure S4**). Frequency of GATA3⁺T_H2-cells in the lungs (B), BAL fluid (C) and peripheral blood (D) comparing different mouse groups. Mean fluorescence intensity (MFI) of PD-1 (E) and frequency of PD-1⁺ cells among nonregulatory T_H2-cells in the lungs (F), BAL fluid (G) and peripheral blood (H). MFI of CTLA-4 (I) and frequency of CTLA-4⁺ cells among nonregulatory T_H2-cells in the lungs (J), BAL fluid (K) and peripheral blood (L). Numbers within representative overlaying histograms denote MFI values. Graphs show means ± SEM. **P* ≤ .05; ***P* ≤ .01; ****P* ≤ .001; Mann-Whitney *U* test.

Figure 4 Exhaustion of circulating CRTH2⁺T_H2-cells is associated with allergen exposure and more profound in patients with allergic asthma. Flow cytometric analysis of CTLA-4 and PD-1 expression on circulating CRTH2⁺T_H2-cells from HC, AR and AR+AIT groups. Representative flow cytometric plots show the gates of PD-1⁺ (A) and CTLA-4⁺ cells (D) among live CRTH2⁺T_H2-cells (see complete gating strategy in **Figure S5**). Frequency of PD-1⁺ among CRTH2⁺T_H2-cells out of (=off) pollen season (B) and in pollen season (C). Frequency of CTLA-4⁺ among CRTH2⁺T_H2-cells off (E) and in season (F). In subgroup analysis based on concomitant asthma: (G-J) Patients without concomitant asthma. Percentage of PD-1⁺ cells off (G) and in season (H) and CTLA-4⁺ cells off (I) and in season (J). (K-N) Patients with concomitant asthma. Percentage of PD-1⁺ cells off (K) and in season (L) and CTLA-4⁺ cells off (M) and in season (N). Graphs show means ± SEM. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$; Mann-Whitney U test.

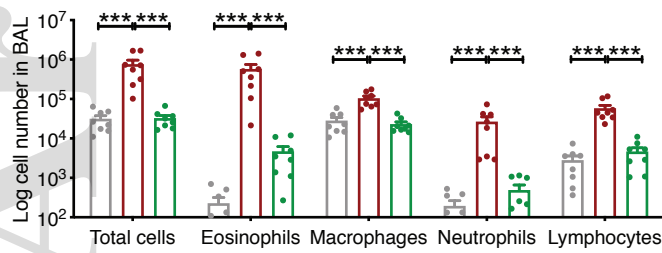
Figure 5 A late-differentiated T_H2-cells persisted throughout the course of AIT. (A) UMAP plots of ten lymphocyte subclusters. UMAP (Uniform Manifold Approximation and Projection) is a dimensional reduction algorithm. X-axis: UMAP-1; y-axis: UMAP-2. Dot plots depict the arcsinh-transformation values of MFI for higher-intensity markers in ten clusters (B) with cutoff at 1 and lower-intensity markers (C) with cutoff at 0.5. Based on differential CD45RA expression, the clusters were grouped into “memory,” “intermediate,” and “immature.” (D) UMAP plots of selected fluorescence markers: CD4, CD45RA, CD27, IL-4, CTLA-4, and PD-1, comparing to the UMAP of cluster 10 (left). X-axis: UMAP-1; y-axis: UMAP-2. (E) Study design scheme⁵: baseline (T0), right before (T1) and 6h after the first (T2) and right before (T3) and 6h after the last pre-seasonal top dose injection (T4) in year one of AIT, in (T5) and out of grass pollen season (T6) year 1 during maintenance phase, in (T7) and out of season (T8) year 2, and in season year 3 (T9). Box plots show the dynamic change of cluster 10 (CD45RA⁺CD27⁺CRTH2⁺IL-4⁺) during up-dosing (F) and maintenance phase. Comparison to the baseline by differential abundance tests using the Dirichlet Regression model: * $P \leq .05$. (G) MFI of IL-4, IL-2, PD-1, and CTLA-4 inside cluster 10 at different time points. Numbers within histograms denote MFI values.

FIGURE 1

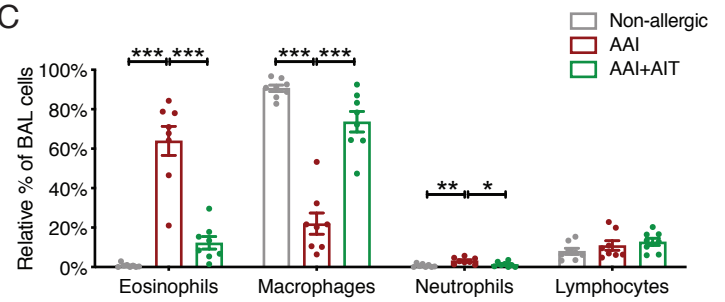
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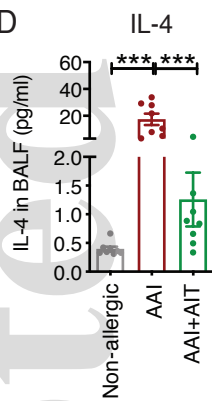
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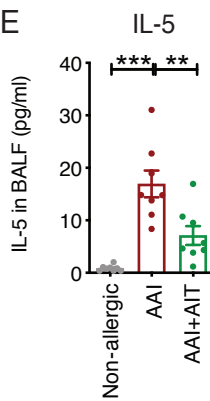
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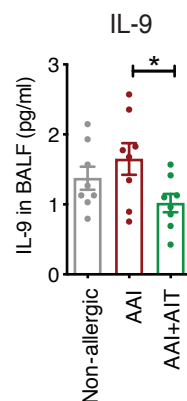
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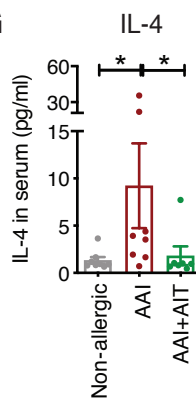
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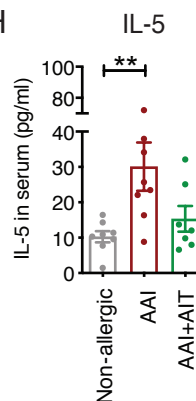
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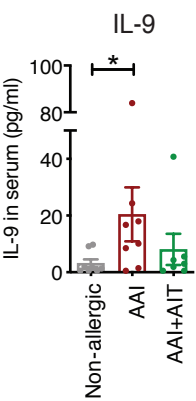
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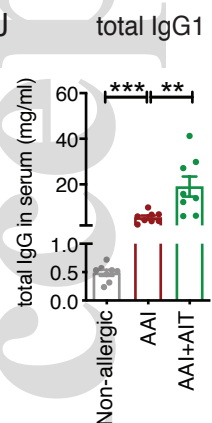
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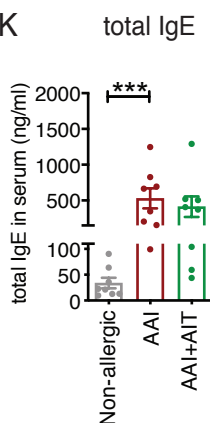
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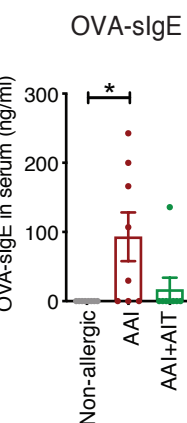
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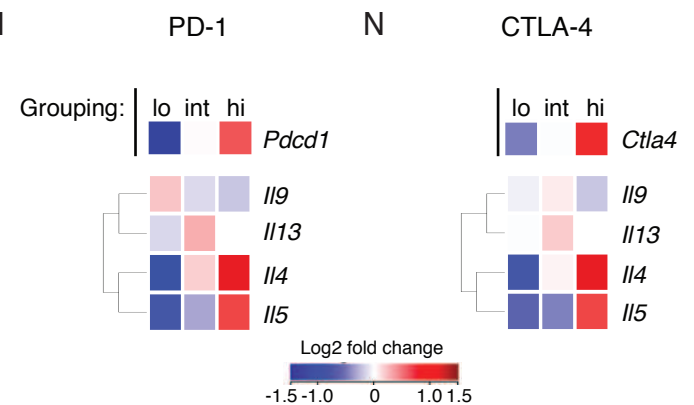


FIGURE 2

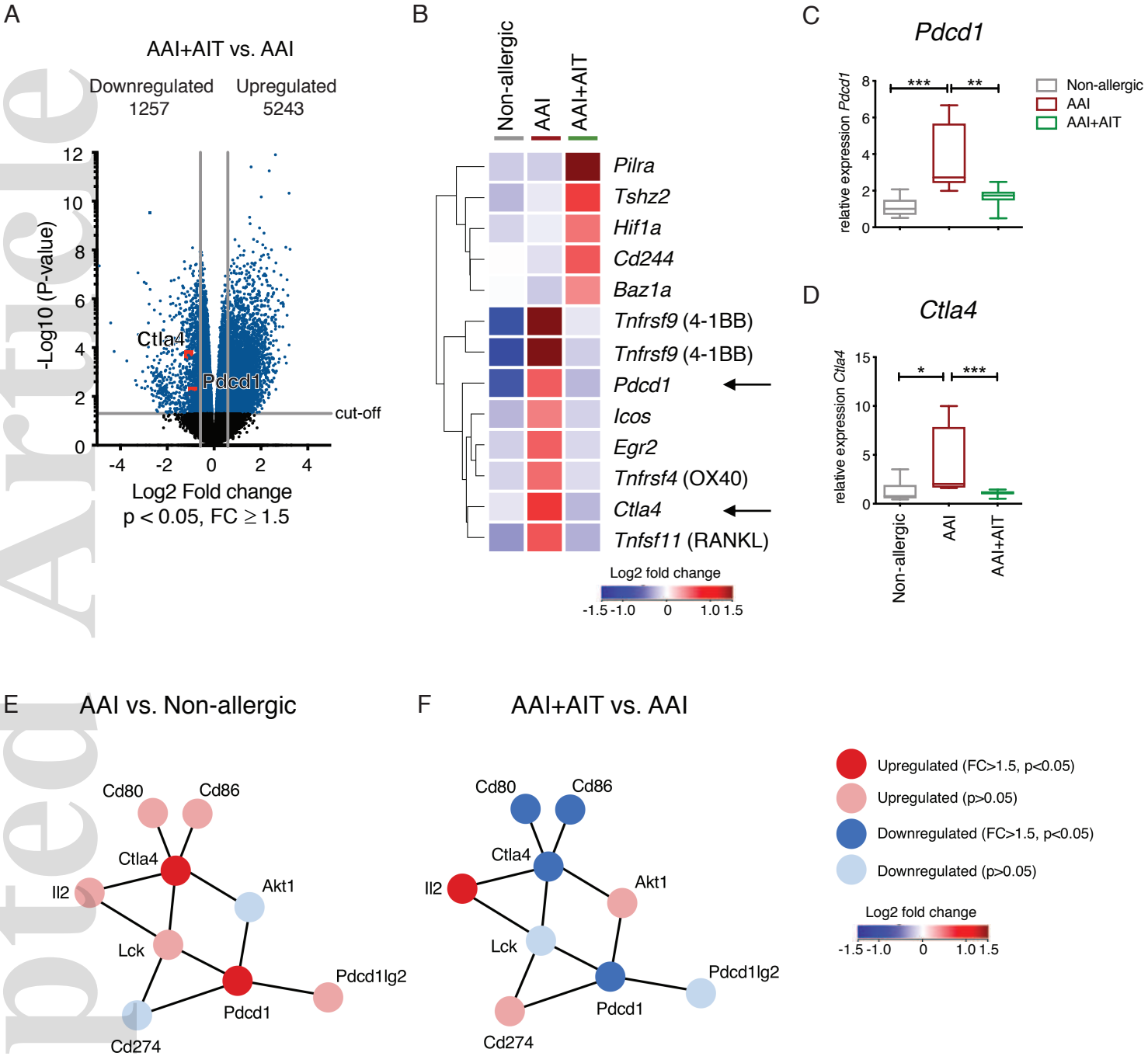


FIGURE 3

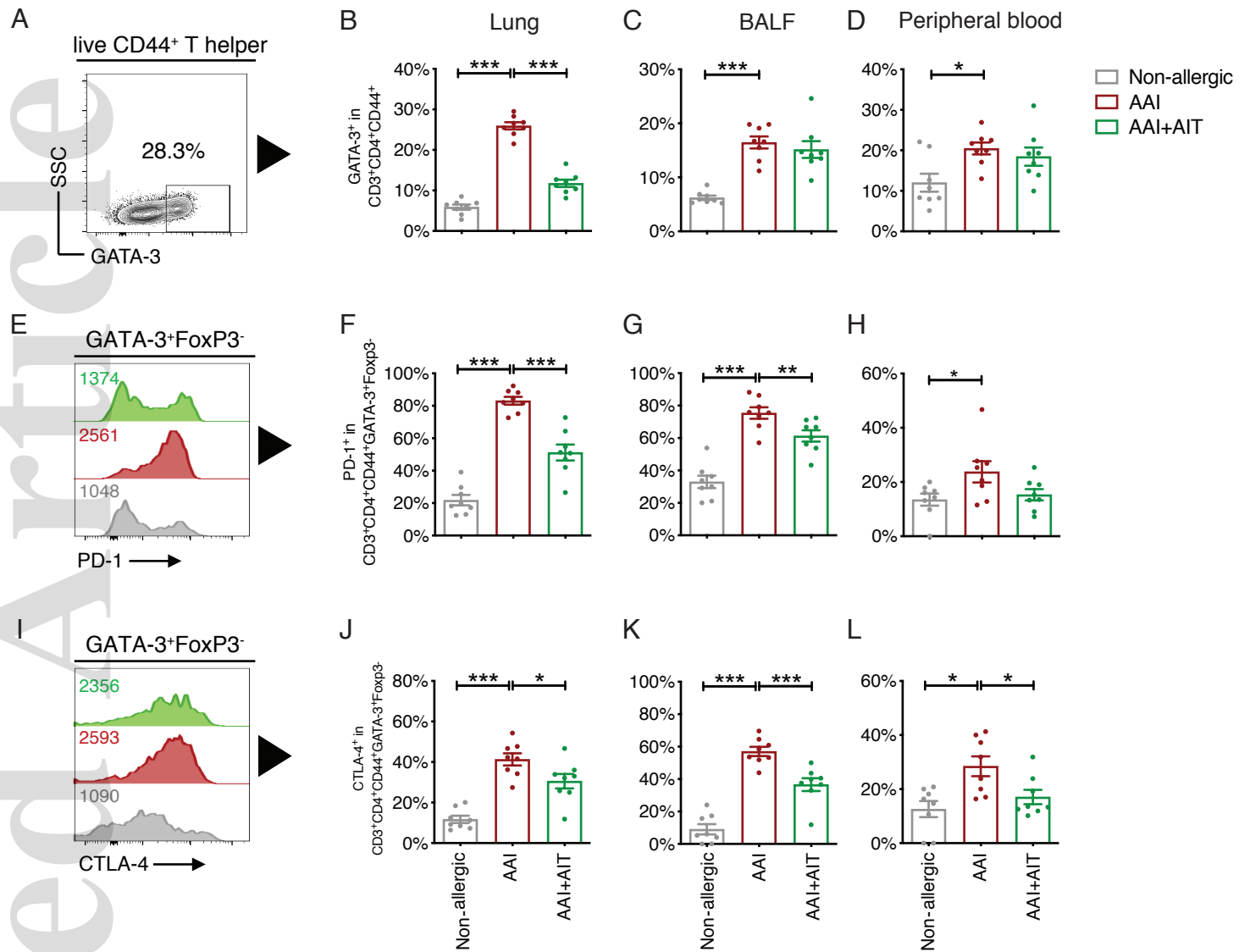


FIGURE 4 

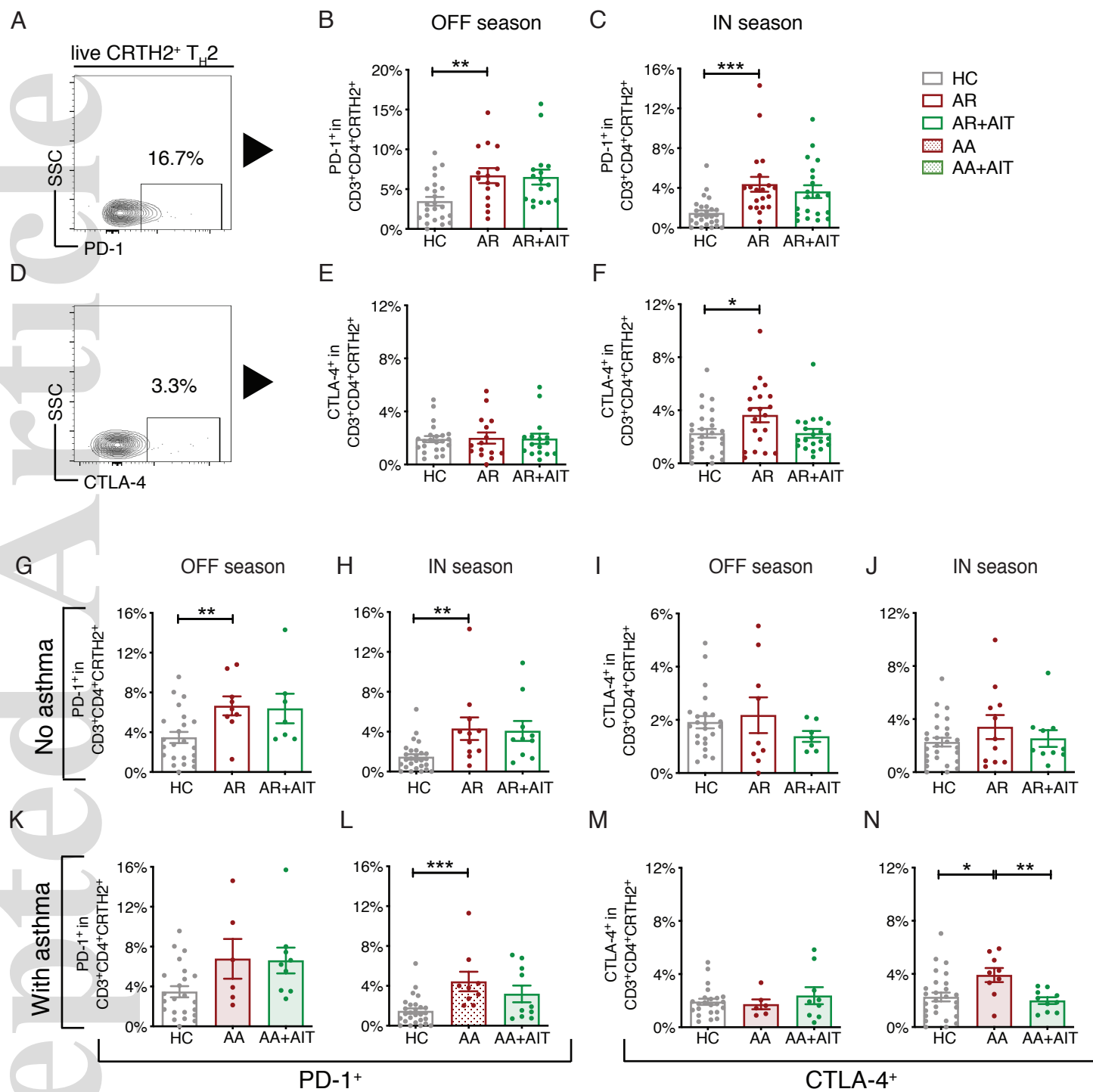
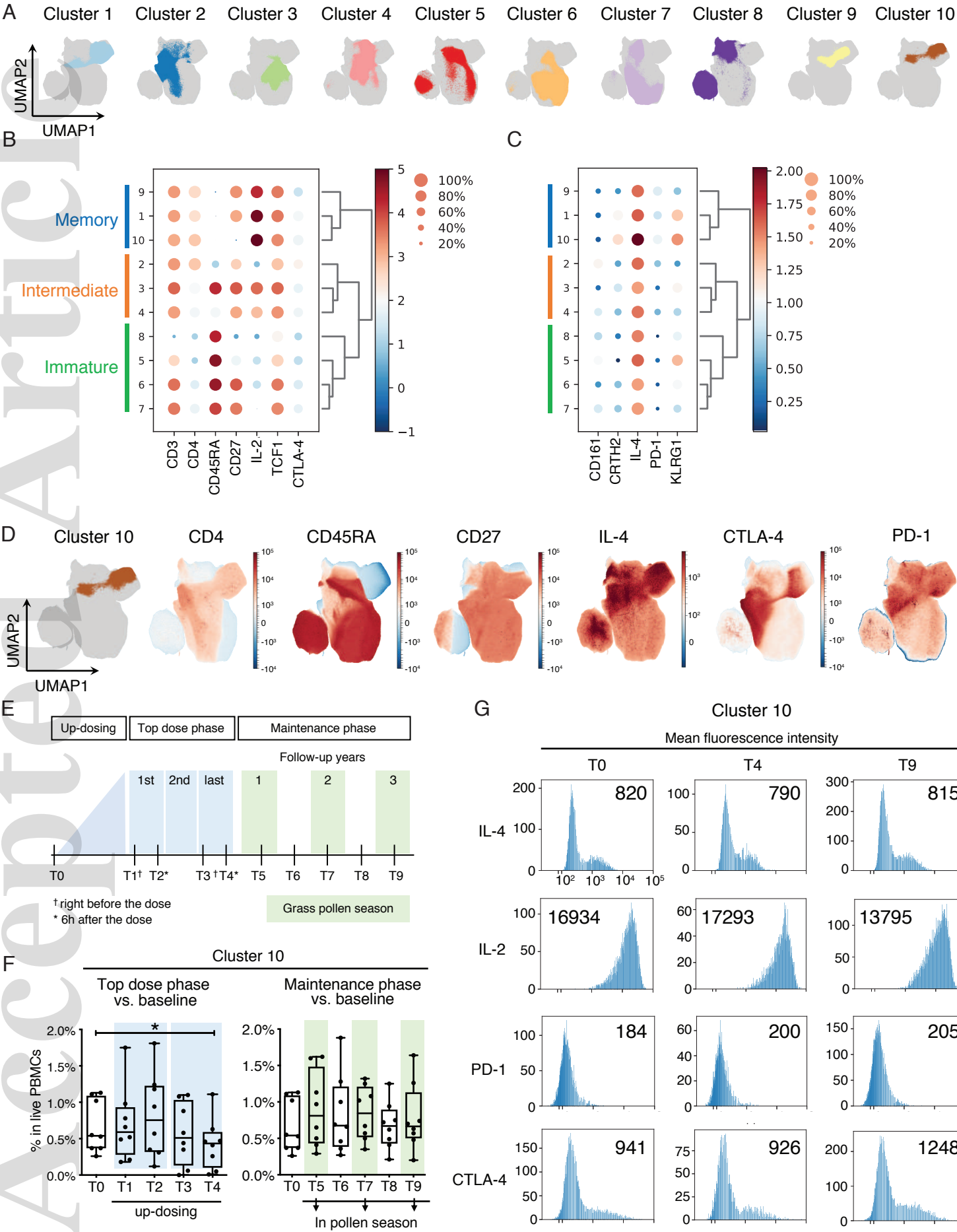


FIGURE 5 



Supplementary information (online repository)

Supplementary methods

Preparation of mouse blood and lung tissue samples

The mice were bled on day 70, 24 hours after the last allergen challenge. After centrifugation, plasma was collected and stored at -80°C for subsequent analysis. Cell pellets were treated with ACK lysing buffer (house-made) to remove erythrocytes and cryopreserved until analysis by flow cytometry.

The mouse lung tissues were incubated for 30 min in RPMI containing 1-mg/ml collagenase A and 100-µg/ml DNase I (both from Sigma-Aldrich, Merck, Darmstadt, Germany) and made into single-cell suspension after digestion. The intrapulmonary lymphocytes were isolated using density-gradient centrifugation and used for flow cytometric analysis.

Measurement of T helper cytokine, total IgG1, IgE, and OVA-specific IgE

LEGENDplex assays (BioLegend, San Diego, CA, USA) were used to determine the levels of cytokines in BAL fluid and mouse serum and total IgG1 and IgE in mouse serum. The samples were prepared according to the manufacturer's instructions and analyzed using a BD LSRII Fortessa™ Flow Cytometer. The data were analyzed using a cloud-based LEGENDplex™ Data Analysis Software. Measurement of OVA-specific IgE was performed using ELISA as described previously.¹

RNA extraction from mouse lung cells

The mouse lungs were stored in RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) at -80°C until RNA was extracted. For tissue disruption and homogenization, the mouse lung pieces were transferred to a 2-ml microcentrifuge tube containing ceramic beads (Precellys lysing kit for tissue homogenizer) and lysis buffer (Buffer RLT supplemented with 1% β -mercaptoethanol). The microcentrifuge tubes were placed in the Precellys 24 Tissue Homogenizer (Bertin Technologies SAS, Montigny Le Bretonneux, France) with the recommended setup per the manufacturer's manual. After centrifugation to precipitate the debris, the clear lysate was used for RNA extraction using RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The extracted RNA was quantified using ultraviolet-visible spectrophotometry (NanoDrop Technologies Inc., Wilmington, DE, USA). RNA integrity was determined using RNA 6000 Pico Chip Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Whole genome microarray analysis of mouse lungs

Extracted RNA samples from mouse lung tissue were processed and hybridized with SurePrint G3 Mouse Gene Expression v2 Microarrays (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. Quality-control checks and microarray data analysis were performed with GeneSpring GX 14.X (Agilent Technologies). Data import using a standard baseline transformation to the median of all values was performed, including log transformation and computation of fold changes. Subsequently, a principle component analysis (PCA) was conducted and revealed a homogenous component distribution. Compromised array signals (array spot is non-uniform if pixel noise of feature exceeds threshold or above saturation threshold) were excluded from further analysis. Differentially expressed genes were considered statistically

significant as an absolute log₂ fold change greater than 1.5 and a p-value less than 0.05 by using the Moderated T-Test (GeneSpring). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSEXXX (number will be substituted after acceptance).

To gain better insight into the differentially expressed genes (DEGs) between AAI+AIT and AAI mouse lungs, a gene ontology (GO) enrichment analysis of the DEGs was performed using an R package called clusterProfiler.² Gene set enrichment analysis (GSEA) was performed using software developed by Broad Institute (<http://www.broadinstitute.org/gsea/index.jsp>) and gene sets from the published genomic database (<https://www.ncbi.nlm.nih.gov/geo/>). Normalized enrichment score and q value were calculated by permutation testing.

Quantitative real-time PCR

Template cDNA, primers specific for each target region, and SYBR Green were mixed for a real-time PCR reaction using the ViiA 7 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Using the comparative C_T method,³ the target gene expression relative to control genes was compared between different groups and presented as fold change in expression.

67 **References (Online repository)**

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Supplementary Figure Legends

Figure S1 Significantly lower levels of IL-6 in BAL fluid and mouse serum after AIT. Levels of IL-6 (A), IL-17A (B), TNF- α (C), and IFN- γ (D) in BAL fluid. Levels of IL-6 (E), IL-17A (F), TNF- α (G), and IFN- γ (H) in mouse serum. Graphs show means $\pm$ SEM. $*P \leq .05$; $**P \leq .01$; $***P \leq .001$; Mann-Whitney U test.

Figure S2 Reduced expression of multiple chemoattractant and type-2 cytokine genes in mouse lungs following AIT. Because GO terms for biological processes were most enriched in association with chemotaxis and cytokine-mediated signaling pathway in AAI, the microarray data were subsequently filtered using the relevant GO terms (0072676, 0048247, and 0005125). (A) lymphocyte chemotaxis (GO term: 0072676 and 0048247; **Table S5**) and (B) cytokine activity (GO term: 0005125; **Table S6**).

Figure S3 Gene set enrichment analysis of the data reveals that AAI mouse lung cells expressed a terminally differentiated signature. GSEA plots comparing gene expression in lung cells of AAI+AIT versus AAI mice using publicly available gene sets of (A) antigen-specific TIM-3⁺ (GSE84105) and (B) KLRG1^{hi} exhausted CD8⁺ T cells (GSE10239) from GEO database. Data are from one experiment (n = 8 mice per group). NES, normalized enrichment scores; FDR, false discovery rate.

Figure S4 Gating strategy.

Expression of CTLA-4 and PD-1 on non-regulatory T_H2-cells (GATA-3⁺Foxp3⁻).

169 **Figure S5 Gating strategy.**

170 Expression of PD-1, CTLA-4, TIM-3, and LAG-3 on peripheral CRTH2⁺ T_H2-cells.

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Figure S6 Expression of LAG-3 and TIM-3 on circulating T_H2-cells was comparable across groups. Flow cytometric analysis of LAG-3 and TIM-3 expression on circulating CRTH2⁺T_H2-cells from HC, AR and AR+AIT groups. Representative flow cytometric plots show the gate of LAG-3⁺ (A) and TIM-3⁺ cells (D) among live CRTH2⁺T_H2-cells. Frequency of LAG-3⁺ cells among CRTH2⁺T_H2-cells out of (B) and in pollen season (C). Frequency of TIM-3⁺ cells among CRTH2⁺T_H2-cells out of (E) and in season (F).

Figure S7 A matrix plot depicts the 42 clusters generated by Louvain clustering analysis based on MFI. The compensated data were subjected to a Graph-based Louvain clustering analysis, resulting in 42 clusters. Each row corresponds to the mean fluorescence intensity (MFI) for different markers and each column represents one cluster, grouped in hierarchical clustering (shown as dendrogram above).

Figure S8 The dynamic change of different clusters throughout the course of AIT. (A) A line plot depicts the dynamic change in frequencies of ten clusters. (B–J) Box plots illustrate the percentage of clusters 1–9 among live PBMCs at different time points. Symbols represent individuals (n=8). Comparison to the baseline by differential abundance tests using the Dirichlet Regression model. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$.

Supplementary Tables

Supplementary Table S1. Demographic data of patient cohorts

Supplementary Table S2. Characteristics of grass pollen-allergic patients with immunotherapy (PACIFIC study)

Supplementary Table S3. Monoclonal antibodies used in flow cytometry analyses

Supplementary Table S4. List of DEGs between exhausted and memory T cells

Supplementary Table S5. Significant entities of genes filtered by GO term: 0072676 and 0048247 (AAI+AIT vs. AAI)

Supplementary Table S6. Significant entities of genes filtered by GO term: 0005125 (AAI+AIT vs. AAI)

Supplementary Table S7. Significant entities of genes filtered by curated gene list

251 **Table S1**

A. Demographic data of patient cohorts

Characteristics	HC (n = 27)	AR (n = 20)	AR+AIT (n = 20)
Age (years)*	25.5 ± 3.8	25.7 ± 4.8	28.1 ± 7.4
Male sex (%)	15 (56%)	9 (45%)	7 (35%)
BMI (kg/m ²)	21.0 ± 2.0	22.6 ± 3.3	21.5 ± 2.0
Total IgE (IU/L)	n.d.	147.1±48.9	40.8±14.4
Allergic asthma (%)	0 (0%)	9 (45%)	10 (50%)

HC, healthy control; AR, allergic rhinitis; AIT, allergen-specific immunotherapy; BMI, body mass index; n.d., not determined

Plus-minus values indicate SEM

*at informed consent procedure and inclusion into study

B. RQLQ (in season) of recruited patients.

	HC	AR	AR+AIT	AA	AA+AIT
RQLQ Score	1.2±0.2	2.5±0.3	1.3±0.4	1.1±0.4	0.8±0.4

HC, healthy control; AR, allergic rhinitis; AIT, allergen-specific immunotherapy AA, Allergic asthma
Data are presented as mean±SEM.

Table S2 Characteristics of grass pollen-allergic patients with immunotherapy (PACIFIC study)

Characteristics	Immunotherapy group n = 32
Age (years)*	25.6 ± 6.3
Male Sex (%)	16 (50%)
Total IgE (IU/L)	147.1 ± 167.1
Grass pollen allergy:	
Specific IgE (IU/L)	32.7 ± 27.9
Skin prick test (positive %)	100%
Allergic asthma (%)	14 (44%)

Plus-minus values indicate SD

*at informed consent procedure and inclusion into study

Table S3 Monoclonal antibodies used in flow cytometry analyses

A. Fluorochrome labelled anti-mouse antibodies.

Antigen	Fluorochrome	Company	Clone	Dilution
CD3	FITC	BD	145-2C11	1:400
CD4	AF700	BD	RM4-5	1:400
CD45	APC-eFluor780	Thermo Fisher	30-F11	1:400
CD44	BV605	BioLegend	IM7	1:200
PD-1	PE-Cy7	Thermo Fisher	J43	1:100
CTLA-4	PE-eFluor610	Thermo Fisher	UC10-4B9	1:100
GATA-3	eFluor660	Thermo Fisher	TWAJ	1:20

B. Fluorochrome labelled anti-human antibodies

Antigen	Fluorochrome	Company	Clone	Dilution
CD3	APC-Cy7	BioLegend	HIT3a	1:200
CD4	BV421	BioLegend	RPA-T4	1:200
CD45RA	PerCP-Cy5.5	BioLegend	HI100	1:200
CD27	AF700	BioLegend	O323	1:200
CD161	BV605	BioLegend	HP-3G10	1:100
CRTH2	APC	BioLegend	BM16	1:50
PD-1	BV650	BioLegend	EH12.2H7	1:100
CTLA-4	PE-Cy7	BioLegend	L3D10	1:100
LAG-3	BV650	BioLegend	11C3C65	1:100
TIM-3	PE-Dazzle	BioLegend	F38-2E2	1:100
IL-2	PE-Dazzle	BioLegend	MQ1-17H12	1:100
IL-4	AF488	BD	8D4-8	1:100
TCF-1	PE	BD	S33-966	1:50

Table S4 List of DEGs between exhausted and memory T cells⁴

<i>Ctla4</i>	<i>Tnfrsf4</i>	<i>Mcm6</i>	<i>Jun</i>
<i>Lilrb4</i>	<i>Tnfrsf11</i>	<i>Tshz2</i>	<i>Arntl</i>
<i>Pdcd1</i>	<i>Treml2</i>	<i>Sap30</i>	<i>Zfp1</i>
<i>Lag3</i>	<i>Tnfrsf9</i>	<i>Baz1a</i>	<i>Zfp81</i>
<i>Cd274</i>	<i>Cd40ig</i>	<i>Nfat5</i>	<i>Mef2d</i>
<i>Cd200</i>	<i>CD28</i>	<i>Mdfic</i>	<i>Zbtb20</i>
<i>Havcr2</i>	<i>Ikzf2</i>	<i>Pbx3</i>	<i>Hif1a</i>
<i>Cd244</i>	<i>Eomes</i>	<i>Plag1</i>	<i>Etv6</i>
<i>Pilra</i>	<i>Maf</i>	<i>Taf1a</i>	<i>Fosb</i>
<i>Sirpb1</i>	<i>Prdm1</i>	<i>Arid3b</i>	<i>Cebpa</i>
<i>Cd160</i>	<i>Pawr</i>	<i>Txnip</i>	<i>Maf</i>
<i>Btla</i>	<i>Jazf1</i>	<i>Zfp652</i>	<i>Fosl2</i>
<i>Lair1</i>	<i>Nab1</i>	<i>Spic</i>	<i>Stat4</i>
<i>Tnfrsf4</i>	<i>Nfatc1</i>	<i>Klf6</i>	<i>Egr2</i>
<i>Icos</i>	<i>Zbtb32</i>	<i>Vgll4</i>	<i>Bcl6</i>
<i>Tnfrsf9</i>	<i>Klf4</i>	<i>Junb</i>	

Table S5 Significant entities of genes filtered by GO term: 0072676 and 0048247 (AAI+AIT vs. AAI)

ProbeName	GeneSymbol	Description	p	FC (abs)	Regulation
A_55_P2739607	Bcar1	Mus musculus breast cancer anti-estrogen resistance 1 (Bcar1), transcript variant 2, mRNA [NM_009954]	7.50E-04	1.6917508	up
A_55_P2039681	Gpr35	Mus musculus G protein-coupled receptor 35 (Gpr35), transcript variant 1, mRNA [NM_022320]	4.26E-05	2.8183925	up
A_51_P458584	Fpr-rs3	Mus musculus formyl peptide receptor, related sequence 3 (Fpr-rs3), mRNA [NM_008040]	1.19E-04	4.5865965	up
A_51_P428372	Ppbbp	Mus musculus pro-platelet basic protein (Ppbbp), mRNA [NM_023785]	0.001356008	1.8507907	up
A_51_P288138	Fpr2	Mus musculus formyl peptide receptor 2 (Fpr2), mRNA [NM_008039]	0.0237685	1.5507616	up
A_55_P2717129	Bin2	bridging integrator 2 [Source:MGI Symbol;Acc:MGI:3611448] [ENSMUST00000183193]	0.001128301	1.8719321	up
A_51_P312485	Fpr1	Mus musculus formyl peptide receptor 1 (Fpr1), mRNA [NM_013521]	1.37E-04	2.5840476	up
A_55_P2738827	Itga1		1.66E-05	1.6260186	up
A_66_P140937	Hoxb9	Mus musculus homeobox B9 (Hoxb9), mRNA [NM_008270]	0.035362974	1.9729214	up
A_66_P125784	Plg	Mus musculus plasminogen (Plg), mRNA [NM_008877]	0.00264109	1.8488367	up
A_55_P2729378	Sema5a	Q6P1C9_MOUSE (Q6P1C9) Sema5a protein, partial (95%) [TC1599505]	0.015485268	2.4898396	up
A_55_P2171116	Lgals3	Mus musculus lectin, galactose binding, soluble 3 (Lgals3), transcript variant 1, mRNA [NM_001145953]	1.26E-05	1.6047273	down
A_51_P166886	Saa2	Mus musculus serum amyloid A 2 (Saa2), mRNA [NM_011314]	0.022200095	1.6082252	down
A_51_P101929	Lgals3	Mus musculus lectin, galactose binding, soluble 3 (Lgals3), transcript variant 2, mRNA [NM_010705]	4.49E-06	1.5282675	down
A_51_P327996	Ccl22	Mus musculus chemokine (C-C motif) ligand 22 (Ccl22), mRNA [NM_009137]	0.002465375	1.6434481	down
A_51_P185660	Ccl9	Mus musculus chemokine (C-C motif) ligand 9 (Ccl9), mRNA [NM_011338]	5.23E-06	2.554627	down
A_51_P400543	Aif1	Mus musculus allograft inflammatory factor 1 (Aif1), mRNA [NM_019467]	1.06E-04	1.6994497	down
A_51_P509573	Ccl4	Mus musculus chemokine (C-C motif) ligand 4 (Ccl4), mRNA [NM_013652]	0.005759234	1.9741256	down
A_52_P318673	Saa1	Mus musculus serum amyloid A 1 (Saa1), mRNA [NM_009117]	7.56E-05	3.286053	down
A_51_P460954	Ccl6	Mus musculus chemokine (C-C motif) ligand 6 (Ccl6), mRNA [NM_009139]	3.73E-07	1.9679912	down
A_55_P2105878	Ccr10	Mus musculus chemokine (C-C motif) receptor 10 (Ccr10), mRNA [NM_007721]	0.014573993	1.6671506	down
A_51_P140710	Ccl3	Mus musculus chemokine (C-C motif) ligand 3 (Ccl3), mRNA [NM_011337]	3.64E-05	2.7900608	down
A_51_P436652	Ccl7	Mus musculus chemokine (C-C motif) ligand 7 (Ccl7), mRNA [NM_013654]	1.24E-04	2.1809807	down
A_55_P2025765	Adam8	Mus musculus a disintegrin and metallopeptidase domain 8 (Adam8), transcript variant 1, mRNA [NM_007403]	5.12E-05	1.9376212	down
A_55_P2869822	Ccl11	Mus musculus chemokine (C-C motif) ligand 11 (Ccl11), mRNA [NM_011330]	3.55E-06	2.7997506	down

A_55_P2736435	Adam8	Mus musculus a disintegrin and metallopeptidase domain 8 (Adam8), transcript variant 1, mRNA [NM_007403]	1.52E-04	1.5322936	down
A_55_P2811679	Cxcl1	Mus musculus chemokine (C-X-C motif) ligand 1 (Cxcl1), mRNA [NM_008176]	1.20E-06	4.9006886	down
A_55_P2744077	Ccl4	Mus musculus chemokine (C-C motif) ligand 4 (Ccl4), mRNA [NM_013652]	0.007921795	1.9034137	down
A_55_P1966204	Cxcl12	Mus musculus chemokine (C-X-C motif) ligand 12 (Cxcl12), transcript variant 1, mRNA [NM_021704]	2.80E-05	1.6991965	down
A_51_P114462	Ccl17	Mus musculus chemokine (C-C motif) ligand 17 (Ccl17), mRNA [NM_011332]	2.36E-07	7.441073	down
A_51_P217463	Cxcl2	Mus musculus chemokine (C-X-C motif) ligand 2 (Cxcl2), mRNA [NM_009140]	1.52E-06	3.3652542	down
A_52_P249514	Ccl12	Mus musculus chemokine (C-C motif) ligand 12 (Ccl12), mRNA [NM_011331]	8.82E-06	2.3267226	down
A_52_P18116	Ccl24	Mus musculus chemokine (C-C motif) ligand 24 (Ccl24), mRNA [NM_019577]	1.57E-04	4.420853	down
A_51_P286737	Ccl2	Mus musculus chemokine (C-C motif) ligand 2 (Ccl2), mRNA [NM_011333]	1.88E-05	2.4999163	down
A_51_P408595	Ccl20	Mus musculus chemokine (C-C motif) ligand 20 (Ccl20), transcript variant 1, mRNA [NM_016960]	2.91E-10	6.7121267	down
A_52_P110052	Ackr1	Mus musculus atypical chemokine receptor 1 (Duffy blood group) (Ackr1), mRNA [NM_010045]	1.88E-04	1.9644057	down
A_55_P2904117	Ackr1	Mus musculus atypical chemokine receptor 1 (Duffy blood group) (Ackr1), mRNA [NM_010045]	3.29E-05	2.2581291	down
A_51_P237865	Il4	Mus musculus interleukin 4 (Il4), transcript variant 1, mRNA [NM_021283]	3.36E-04	2.2242913	down
A_66_P106131	Saa3	Mus musculus serum amyloid A 3 (Saa3), mRNA [NM_011315]	9.62E-06	6.951669	down
A_55_P1960386	Ccl1	Mus musculus chemokine (C-C motif) ligand 1 (Ccl1), mRNA [NM_011329]	1.67E-07	3.2391655	down
A_55_P1990032	Cxcl5	Mus musculus chemokine (C-X-C motif) ligand 5 (Cxcl5), mRNA [NM_009141]	4.47E-08	29.790846	down
A_51_P378789	Cxcl13	Mus musculus chemokine (C-X-C motif) ligand 13 (Cxcl13), mRNA [NM_018866]	1.07E-04	4.406385	down
A_51_P464703	Ccl8	Mus musculus chemokine (C-C motif) ligand 8 (Ccl8), mRNA [NM_021443]	3.81E-05	2.1218212	down

Table S6 Significant entities of genes filtered by GO term: 0005125 (AAI+AIT vs. AAI)

ProbeName	GeneSymbol	Description	p	FC (abs)	Regulation
A_55_P2741233	Il12b	Mus musculus interleukin 12b (Il12b), mRNA [NM_001303244]	1.73E-04	1.8477591	up
A_52_P656790	Ctf2	Mus musculus cardiotrophin 2 (Ctf2), mRNA [NM_198858]	0.005114046	2.241319	up
A_55_P2935935	Il2	Mus musculus interleukin 2 (Il2), mRNA [NM_008366]	1.84E-05	3.7589598	up
A_51_P112796	Il1f5	Mus musculus interleukin 1 family, member 5 (delta) (Il1f5), transcript variant 2, mRNA [NM_019451]	1.74E-04	2.7156081	up
A_55_P2744827	Il21	Mus musculus interleukin 21 (Il21), transcript variant 1, mRNA [NM_001291041]	3.37E-04	2.5235662	up
A_52_P87757	Il24	Mus musculus interleukin 24 (Il24), mRNA [NM_053095]	0.013938826	1.9922079	up
A_55_P2720030	Il1f6	Mus musculus interleukin 1 family, member 6 (Il1f6), mRNA [NM_019450]	0.002836923	2.5477848	up
A_55_P2166020	Ifna13	Mus musculus interferon alpha 13 (Ifna13), mRNA [NM_177347]	0.014385053	1.778753	up
A_51_P519301	Il17f	Mus musculus interleukin 17F (Il17f), mRNA [NM_145856]	0.011406475	1.6541293	up
A_55_P2732528	Il18	Mus musculus interleukin 18 (Il18), mRNA [NM_008360]	9.58E-05	1.8074615	up
A_55_P1985985	Il3	Mus musculus interleukin 3 (Il3), mRNA [NM_010556]	0.017051853	1.561119	up
A_55_P2199202	Il22	Mus musculus interleukin 22 (Il22), mRNA [NM_016971]	0.002910094	1.7656146	up
A_52_P482280	Ifna2	interferon alpha 2 [Source:MGI Symbol;Acc:MGI:107666] [ENSMUST00000105147]	0.023194369	2.0098205	up
A_55_P1996365	Ifna14	Mus musculus interferon alpha 14 (Ifna14), mRNA [NM_206975]	0.002628428	2.120864	up
A_55_P2030171	Ifna2	Mus musculus interferon alpha 2 (Ifna2), mRNA [NM_010503]	5.03E-06	2.3674192	up
A_51_P387681	Ifnab	GB	0.012984594	1.9567547	up
A_51_P317887	Sectm1b	Mus musculus secreted and transmembrane 1B (Sectm1b), mRNA [NM_026907]	6.69E-05	1.968251	up
A_51_P350005	Il17c	Mus musculus interleukin 17C (Il17c), mRNA [NM_145834]	7.56E-04	2.139632	up
A_52_P576049	Inhbc	Mus musculus inhibin beta-C (Inhbc), mRNA [NM_010565]	0.00268494	2.2109673	up
A_66_P136102	Lefty2	Mus musculus left-right determination factor 2 (Lefty2), mRNA [NM_177099]	0.002167371	1.6284444	up
A_66_P127574	Ifna12	Mus musculus interferon alpha 12 (Ifna12), mRNA [NM_177361]	0.01615672	2.1017344	up
A_55_P1994117	Ifnl3	Mus musculus interferon lambda 3 (Ifnl3), mRNA [NM_177396]	0.005939508	1.8560853	up
A_51_P429903	Ndp	Mus musculus Norrie disease (pseudoglioma) (human) (Ndp), mRNA [NM_010883]	1.52E-05	1.9270904	up
A_55_P2156697	Il17a	Mus musculus interleukin 17A (Il17a), mRNA [NM_010552]	8.47E-05	2.995631	up
A_52_P413646	Bmp6	Mus musculus bone morphogenetic protein 6 (Bmp6), mRNA [NM_007556]	4.05E-05	1.5316795	up
A_52_P633560	Nodal	Mus musculus nodal (Nodal), mRNA [NM_013611]	1.85E-04	1.6147039	up

A_55_P2001489	Il19	Mus musculus interleukin 19 (Il19), mRNA [NM_001009940]	4.06E-05	4.0247793	up
A_55_P1964960	Il33	Mus musculus interleukin 33 (Il33), transcript variant 1, mRNA [NM_001164724]	7.17E-05	1.656724	down
A_55_P2131483	Hmgb1	Mus musculus high mobility group box 1 (Hmgb1), transcript variant 1, mRNA [NM_001313894]	7.29E-08	1.5352703	down
A_51_P171075	Csf2	Mus musculus colony stimulating factor 2 (granulocyte-macrophage) (Csf2), mRNA [NM_009969]	4.23E-06	2.047887	down
A_51_P327996	Ccl22	Mus musculus chemokine (C-C motif) ligand 22 (Ccl22), mRNA [NM_009137]	0.002465375	1.6434481	down
A_51_P185660	Ccl9	Mus musculus chemokine (C-C motif) ligand 9 (Ccl9), mRNA [NM_011338]	5.23E-06	2.554627	down
A_51_P509573	Ccl4	Mus musculus chemokine (C-C motif) ligand 4 (Ccl4), mRNA [NM_013652]	0.005759234	1.9741256	down
A_66_P107818	Gdf3	Mus musculus growth differentiation factor 3 (Gdf3), mRNA [NM_008108]	0.007402576	1.9400156	down
A_52_P431159	Il1rn	Mus musculus interleukin 1 receptor antagonist (Il1rn), transcript variant 2, mRNA [NM_001039701]	9.78E-04	1.868729	down
A_51_P484371	Tnfsf8	Mus musculus tumor necrosis factor (ligand) superfamily, member 8 (Tnfsf8), mRNA [NM_009403]	1.92E-04	2.0129375	down
A_51_P460954	Ccl6	Mus musculus chemokine (C-C motif) ligand 6 (Ccl6), mRNA [NM_009139]	3.73E-07	1.9679912	down
A_52_P87713	Timp1	Mus musculus tissue inhibitor of metalloproteinase 1 (Timp1), transcript variant 1, mRNA [NM_001044384]	1.09E-05	2.511524	down
A_51_P140710	Ccl3	Mus musculus chemokine (C-C motif) ligand 3 (Ccl3), mRNA [NM_011337]	3.64E-05	2.7900608	down
A_51_P436652	Ccl7	Mus musculus chemokine (C-C motif) ligand 7 (Ccl7), mRNA [NM_013654]	1.24E-04	2.1809807	down
A_51_P303791	Inhba	Mus musculus inhibin beta-A (Inhba), mRNA [NM_008380]	6.95E-04	2.5073574	down
A_55_P2869822	Ccl11	Mus musculus chemokine (C-C motif) ligand 11 (Ccl11), mRNA [NM_011330]	3.55E-06	2.7997506	down
A_55_P2039359	Tnfsf11	Mus musculus tumor necrosis factor (ligand) superfamily, member 11 (Tnfsf11), mRNA [NM_011613]	1.69E-04	1.9583496	down
A_55_P2811679	Cxcl1	Mus musculus chemokine (C-X-C motif) ligand 1 (Cxcl1), mRNA [NM_008176]	1.20E-06	4.9006886	down
A_51_P430766	Il10	Mus musculus interleukin 10 (Il10), mRNA [NM_010548]	0.007507978	2.119456	down
A_55_P2614900	Il33	Mus musculus interleukin 33 (Il33), transcript variant 1, mRNA [NM_001164724]	3.69E-05	1.7525749	down
A_55_P2744077	Ccl4	Mus musculus chemokine (C-C motif) ligand 4 (Ccl4), mRNA [NM_013652]	0.007921795	1.9034137	down
A_55_P1966204	Cxcl12	Mus musculus chemokine (C-X-C motif) ligand 12 (Cxcl12), transcript variant 1, mRNA [NM_021704]	2.80E-05	1.6991965	down
A_51_P114462	Ccl17	Mus musculus chemokine (C-C motif) ligand 17 (Ccl17), mRNA [NM_011332]	2.36E-07	7.441073	down
A_51_P217463	Cxcl2	Mus musculus chemokine (C-X-C motif) ligand 2 (Cxcl2), mRNA [NM_009140]	1.52E-06	3.3652542	down
A_51_P239750	Inhba	Mus musculus inhibin beta-A (Inhba), mRNA [NM_008380]	2.82E-05	1.9964522	down
A_52_P249514	Ccl12	Mus musculus chemokine (C-C motif) ligand 12 (Ccl12), mRNA [NM_011331]	8.82E-06	2.3267226	down

A_52_P18116	Ccl24	Mus musculus chemokine (C-C motif) ligand 24 (Ccl24), mRNA [NM_019577]	1.57E-04	4.420853	down
A_55_P2029319	Cd70	Mus musculus CD70 antigen (Cd70), mRNA [NM_011617]	9.04E-04	2.096882	down
A_51_P286737	Ccl2	Mus musculus chemokine (C-C motif) ligand 2 (Ccl2), mRNA [NM_011333]	1.88E-05	2.4999163	down
A_51_P408595	Ccl20	Mus musculus chemokine (C-C motif) ligand 20 (Ccl20), transcript variant 1, mRNA [NM_016960]	2.91E-10	6.7121267	down
A_55_P1997756	Il6	Mus musculus interleukin 6 (Il6), transcript variant 1, mRNA [NM_031168]	9.88E-04	2.1384084	down
A_51_P237865	Il4	Mus musculus interleukin 4 (Il4), transcript variant 1, mRNA [NM_021283]	3.36E-04	2.2242913	down
A_52_P153179	Il31	Mus musculus interleukin 31 (Il31), mRNA [NM_029594]	0.01264545	1.8996928	down
A_55_P2106121	Il21	Mus musculus interleukin 21 (Il21), transcript variant 1, mRNA [NM_001291041]	0.002453658	2.772329	down
A_55_P1960386	Ccl1	Mus musculus chemokine (C-C motif) ligand 1 (Ccl1), mRNA [NM_011329]	1.67E-07	3.2391655	down
A_51_P185415	Il5	Mus musculus interleukin 5 (Il5), mRNA [NM_010558]	0.001098734	2.364928	down
A_55_P1990032	Cxcl5	Mus musculus chemokine (C-X-C motif) ligand 5 (Cxcl5), mRNA [NM_009141]	4.47E-08	29.790846	down
A_51_P378789	Cxcl13	Mus musculus chemokine (C-X-C motif) ligand 13 (Cxcl13), mRNA [NM_018866]	1.07E-04	4.406385	down
A_51_P464703	Ccl8	Mus musculus chemokine (C-C motif) ligand 8 (Ccl8), mRNA [NM_021443]	3.81E-05	2.1218212	down

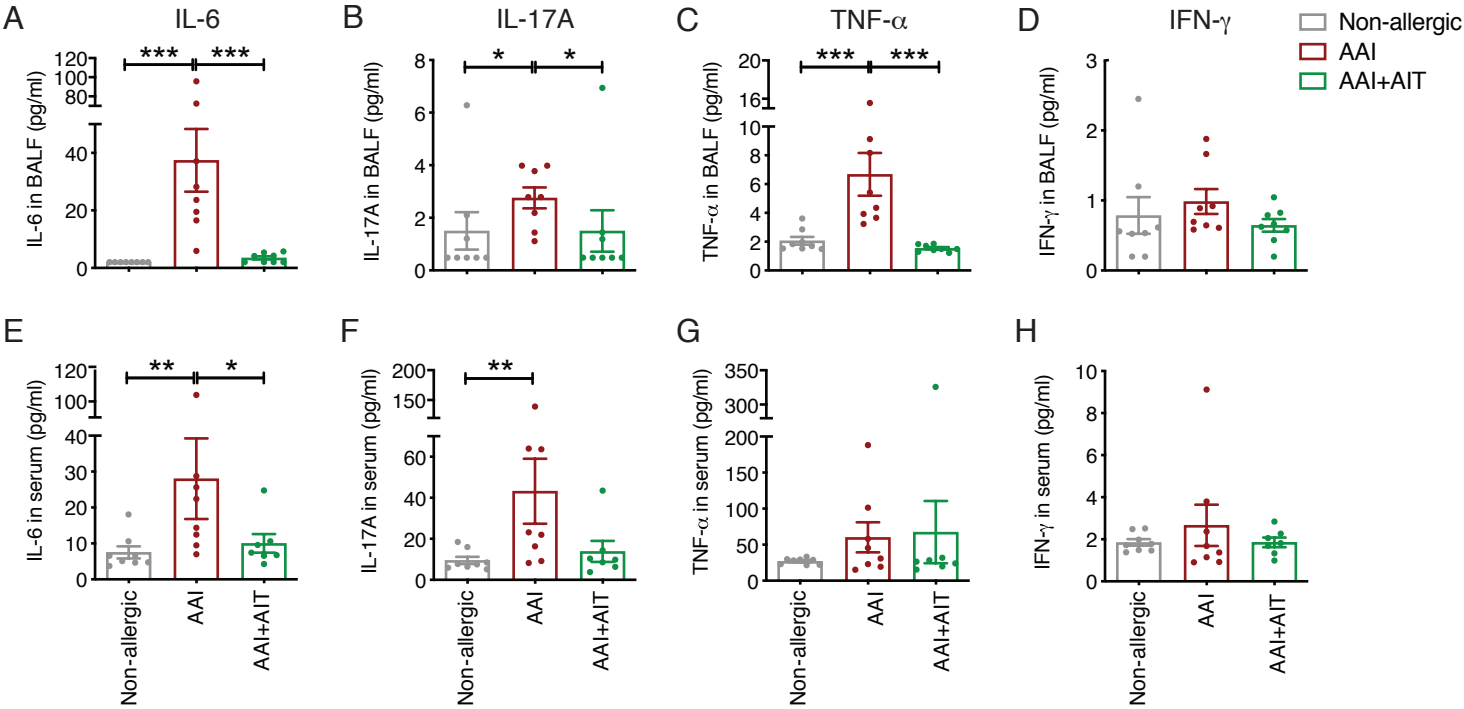
Table S7 Significant entities of genes filtered by curated gene list

ProbeName	GeneSymbol	Description	p	FC (abs)	Regulation
A_55_P2149011	Hif1a	hypoxia inducible factor 1, alpha subunit [Source:MGI Symbol;Acc:MGI:106918] [ENSMUST00000110464]	0.00300952	1.5157217	up
A_55_P2090612	Pilra	paired immunoglobulin-like type 2 receptor alpha [Source:MGI Symbol;Acc:MGI:2450529] [ENSMUST00000110980]	1.35E-04	3.4829297	up
A_55_P1977812	Cd244	Mus musculus CD244 natural killer cell receptor 2B4 (Cd244), mRNA [NM_018729]	1.93E-06	1.7166915	up
A_55_P2883248	Baz1a	bromodomain adjacent to zinc finger domain 1A [Source:MGI Symbol;Acc:MGI:1309478] [ENSMUST00000173176]	0.0301197	1.5790795	up
A_55_P2770047	Tshz2	PREDICTED: Mus musculus teashirt zinc finger family member 2 (Tshz2), transcript variant X2, mRNA [XM_006499424]	4.82E-07	1.8169348	up
A_55_P1988844	Klrg1	Mus musculus killer cell lectin-like receptor subfamily G, member 1 (Klrg1), mRNA [NM_016970]	4.10E-05	1.6108124	down
A_52_P682768	Icos	Mus musculus inducible T cell co-stimulator (Icos), mRNA [NM_017480]	8.70E-05	1.5901304	down
A_55_P2147712	Ctla4	Mus musculus cytotoxic T-lymphocyte-associated protein 4 (Ctla4), transcript variant 1, mRNA [NM_009843]	1.94E-04	2.0983176	down
A_55_P2039359	Tnfsf11	Mus musculus tumor necrosis factor (ligand) superfamily, member 11 (Tnfsf11), mRNA [NM_011613]	1.69E-04	1.9583496	down
A_51_P173043	Egr2	Mus musculus early growth response 2 (Egr2), transcript variant 1, mRNA [NM_010118]	2.89E-07	1.7080414	down
A_55_P2026903	Tigit	Mus musculus T cell immunoreceptor with Ig and ITIM domains (Tigit), mRNA [NM_001146325]	1.83E-05	2.227425	down
A_55_P2082180	Tnfrsf9	Mus musculus tumor necrosis factor receptor superfamily, member 9 (Tnfrsf9), transcript variant 1, mRNA [NM_011612]	5.07E-06	3.6775577	down
A_51_P256983	Klrg1	Mus musculus killer cell lectin-like receptor subfamily G, member 1 (Klrg1), mRNA [NM_016970]	2.39E-04	1.5172349	down
A_55_P2831060	Tnfrsf9	Mus musculus tumor necrosis factor receptor superfamily, member 9 (Tnfrsf9), transcript variant 1, mRNA [NM_011612]	4.12E-06	4.8933034	down
A_55_P2125491	Tnfrsf4	Mus musculus tumor necrosis factor receptor superfamily, member 4 (Tnfrsf4), mRNA [NM_011659]	2.82E-05	1.6231472	down
A_51_P285736	Pdcd1	Mus musculus programmed cell death 1 (Pdcd1), mRNA [NM_008798]	0.006098429	1.8720162	down

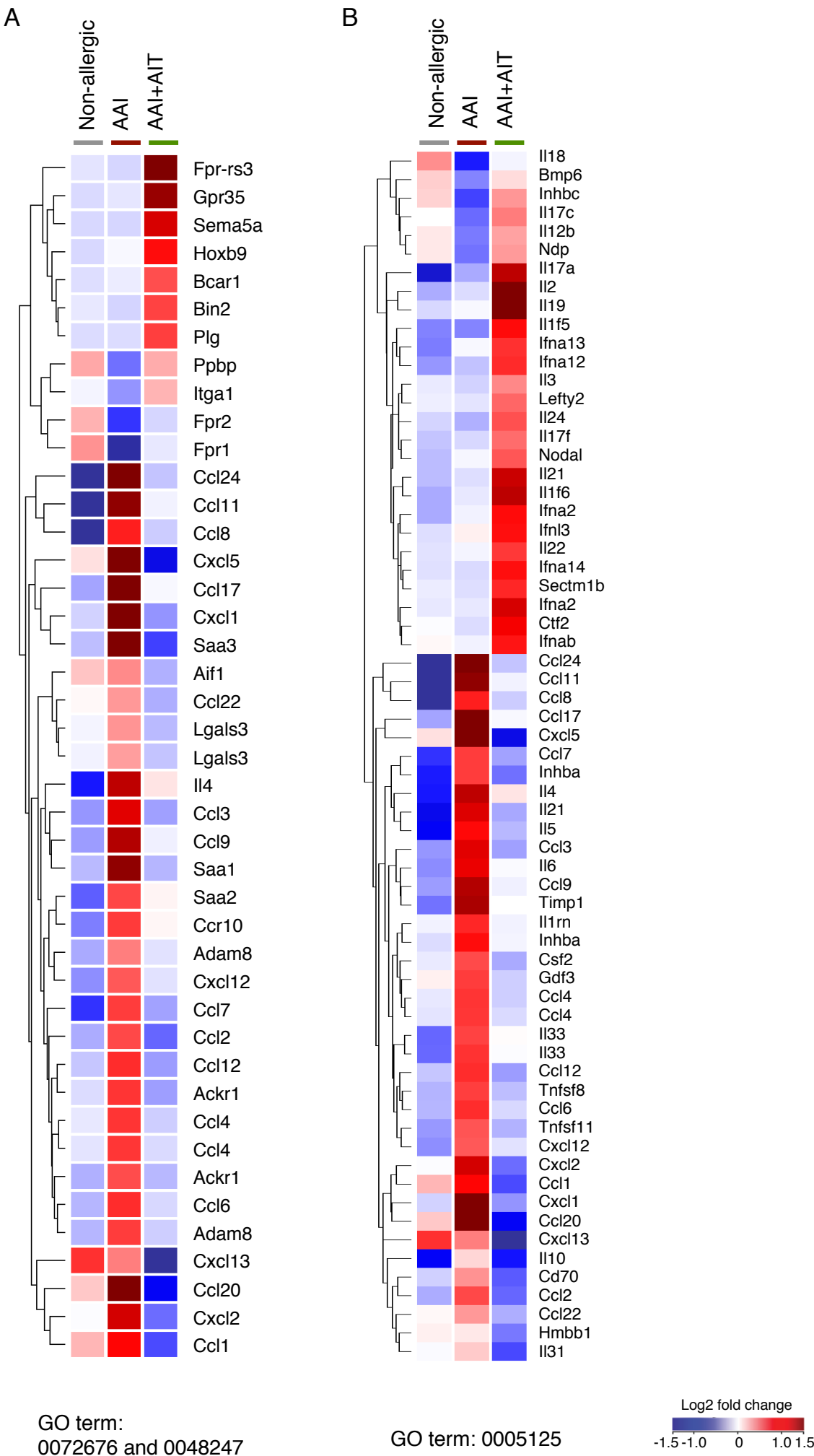
263

264

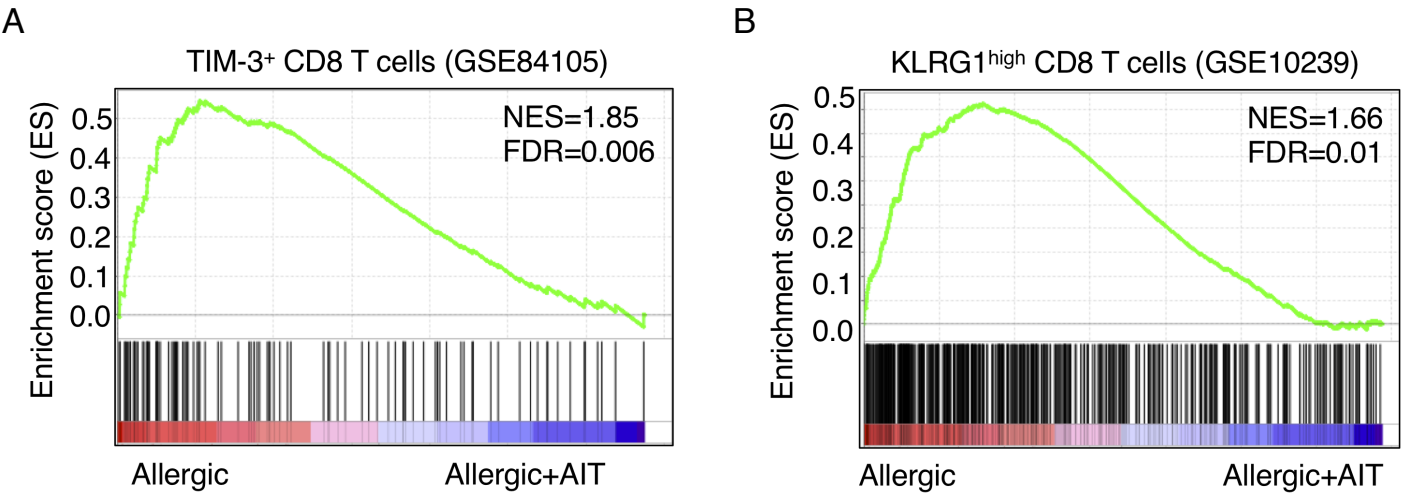
SUPPLEMENTARY FIGURE S1



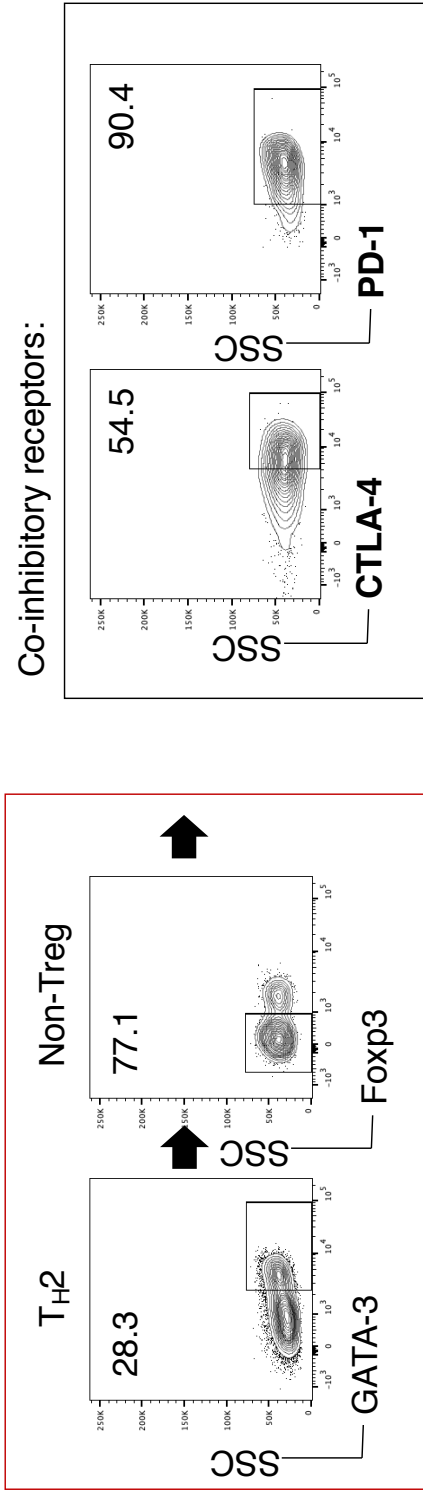
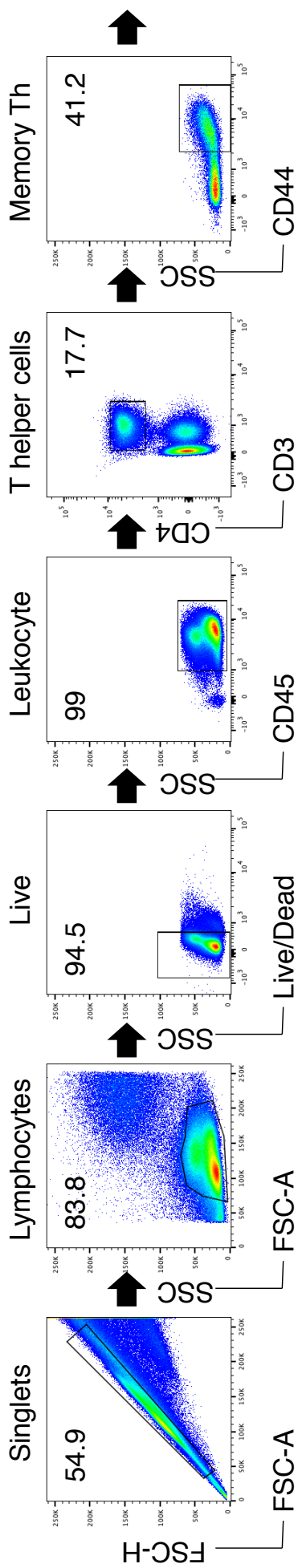
SUPPLEMENTARY FIGURE S2



SUPPLEMENTARY FIGURE S3

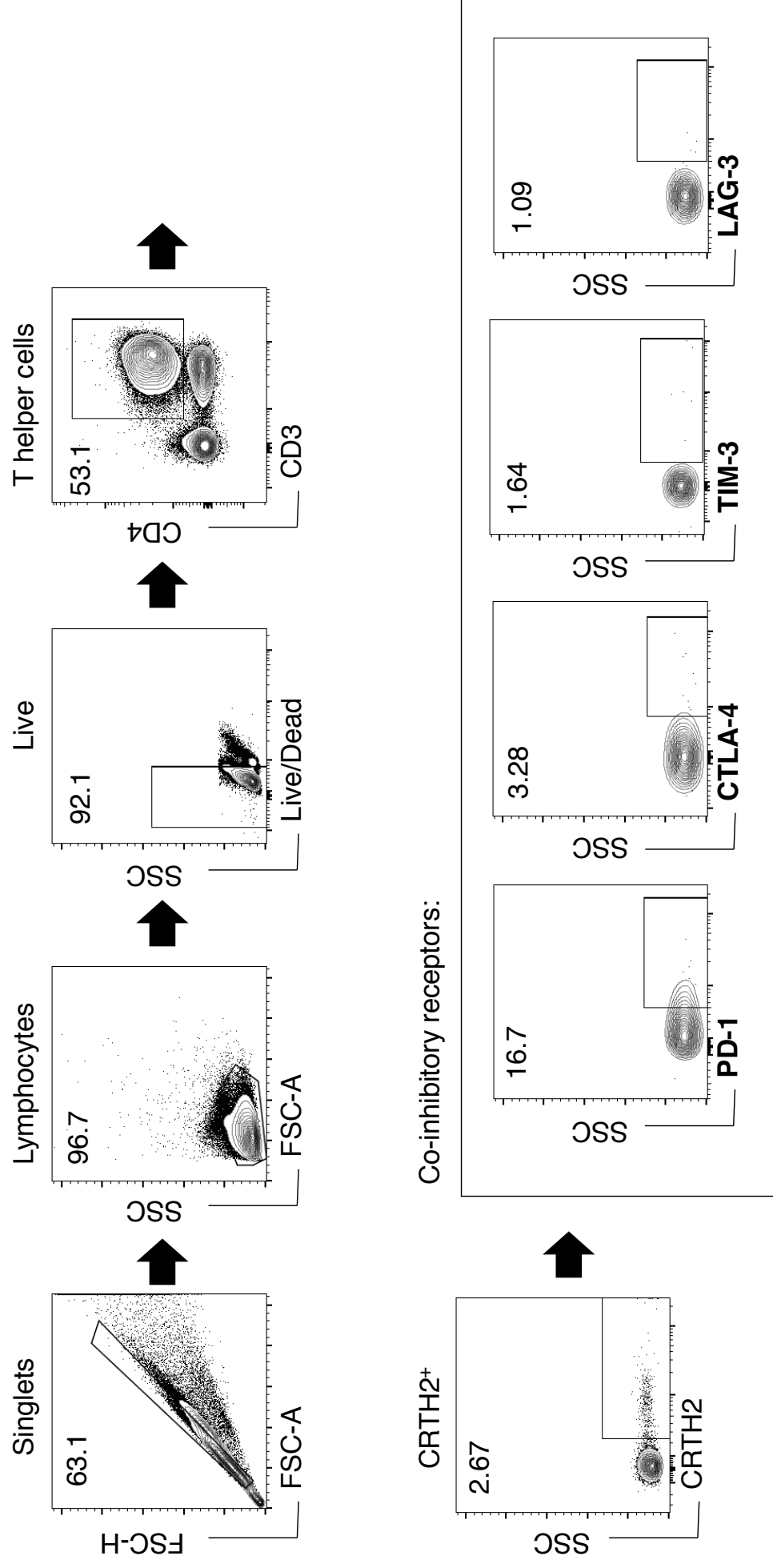


SUPPLEMENTARY FIGURE S4

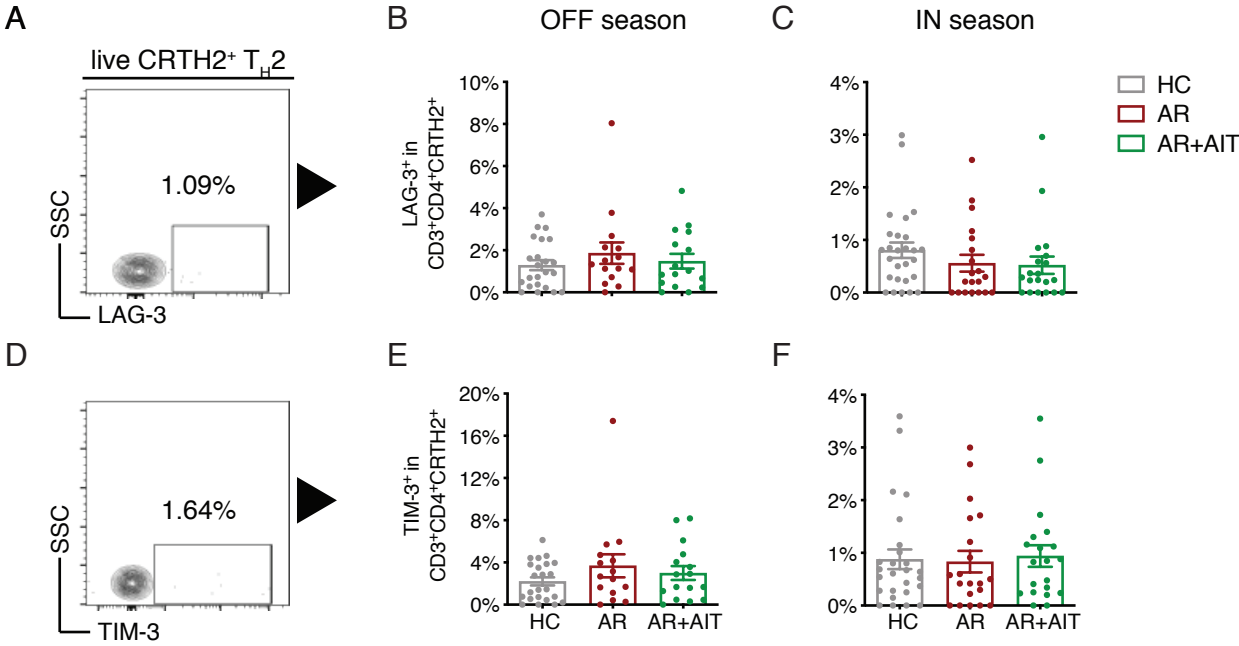


Proallergic T_H2 cells
GATA-3⁺Foxp3⁻

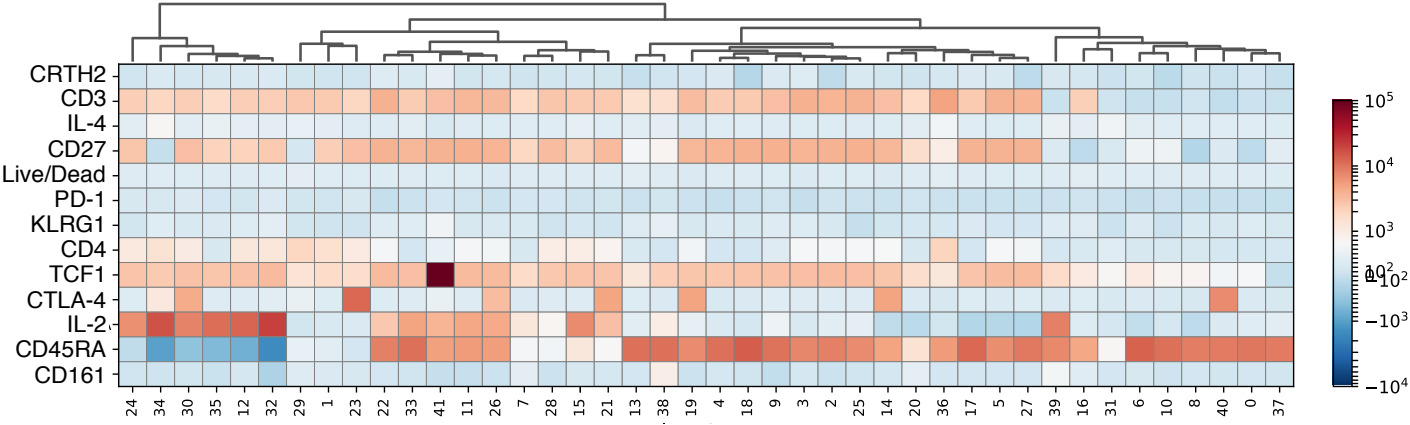
SUPPLEMENTARY FIGURE S5



SUPPLEMENTARY FIGURE S6

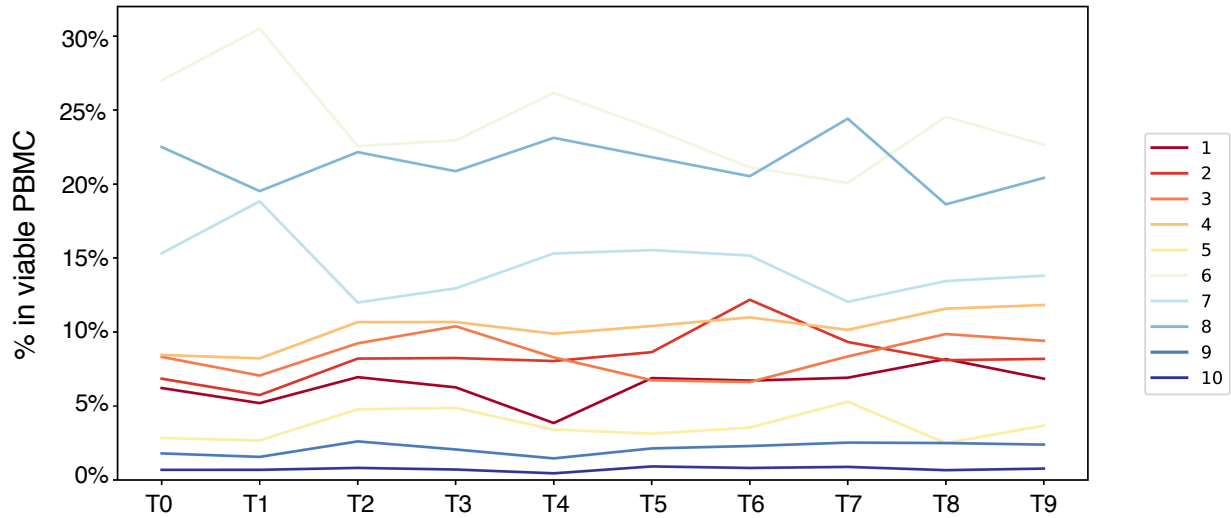


SUPPLEMENTARY FIGURE S7



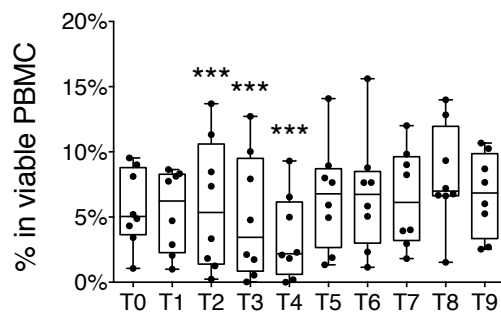
SUPPLEMENTARY FIGURE S8

A



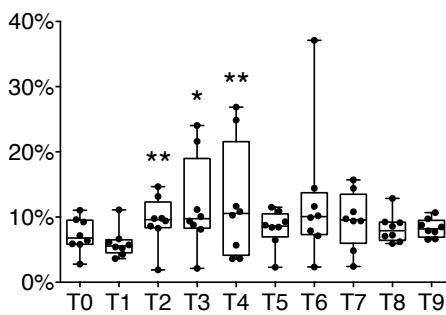
B

Cluster 1



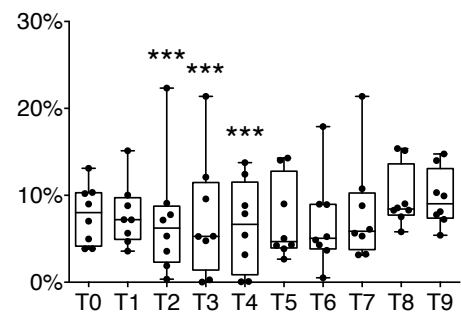
C

Cluster 2



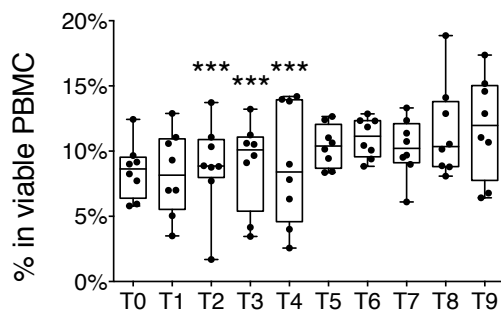
D

Cluster 3



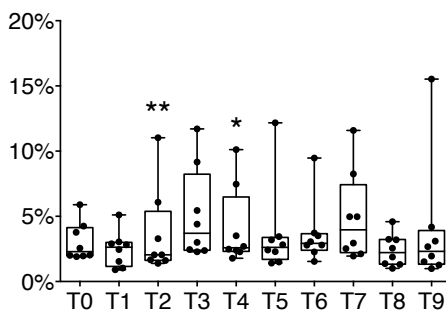
E

Cluster 4



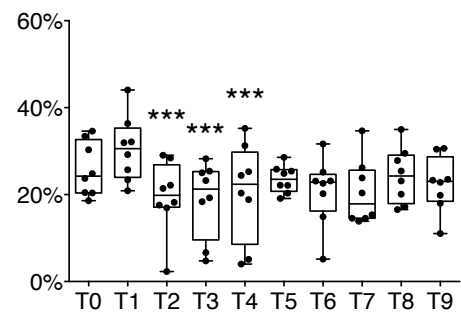
F

Cluster 5



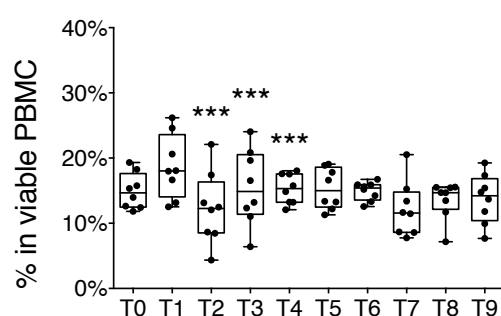
G

Cluster 6



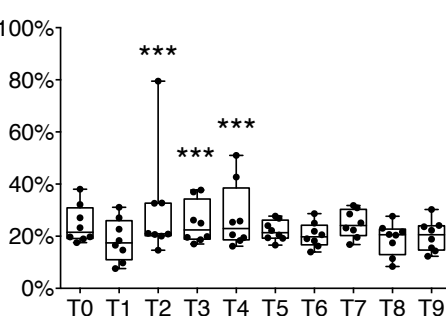
H

Cluster 7



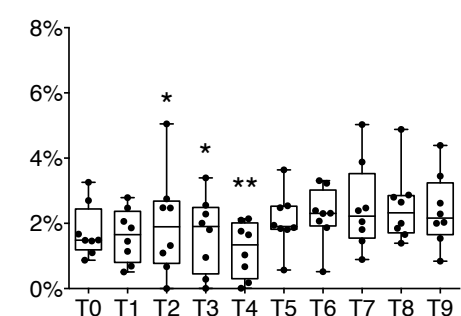
I

Cluster 8

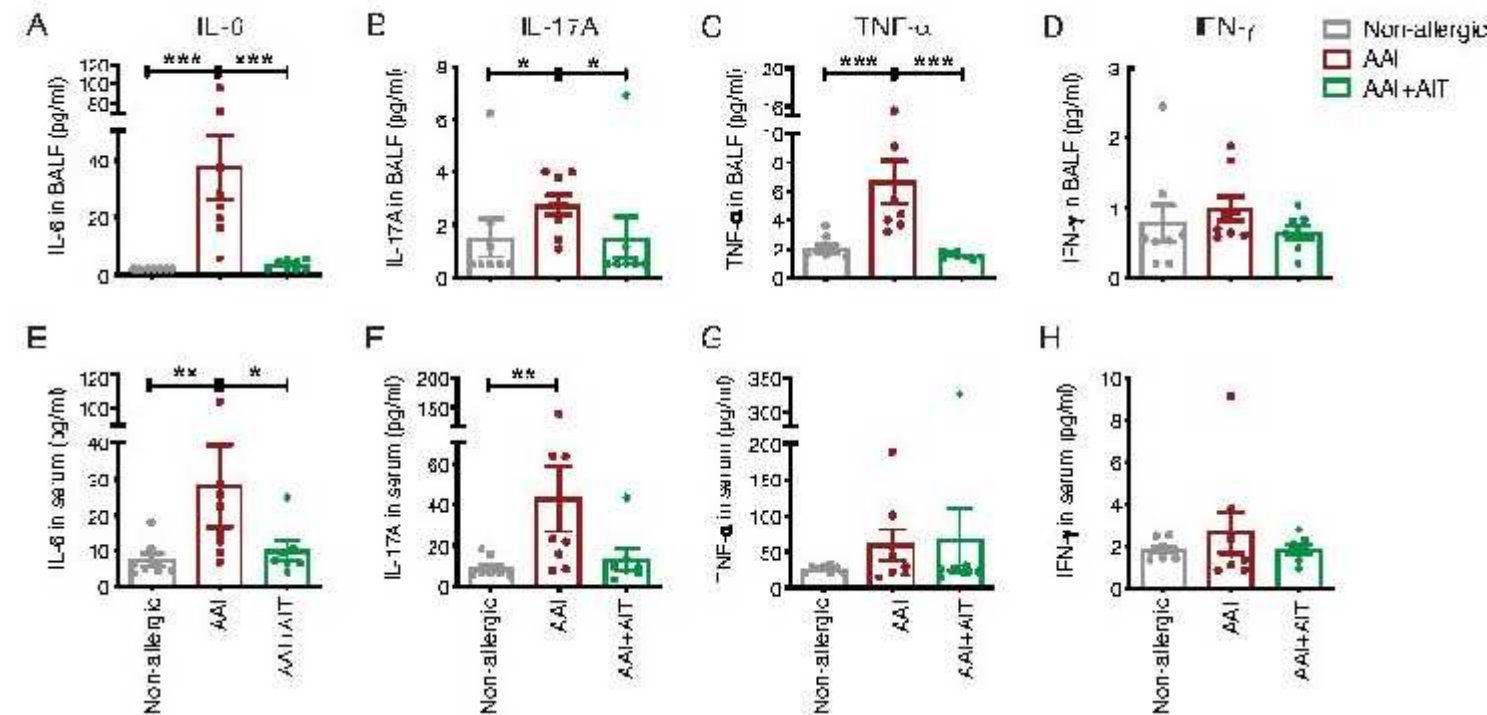


J

Cluster 9

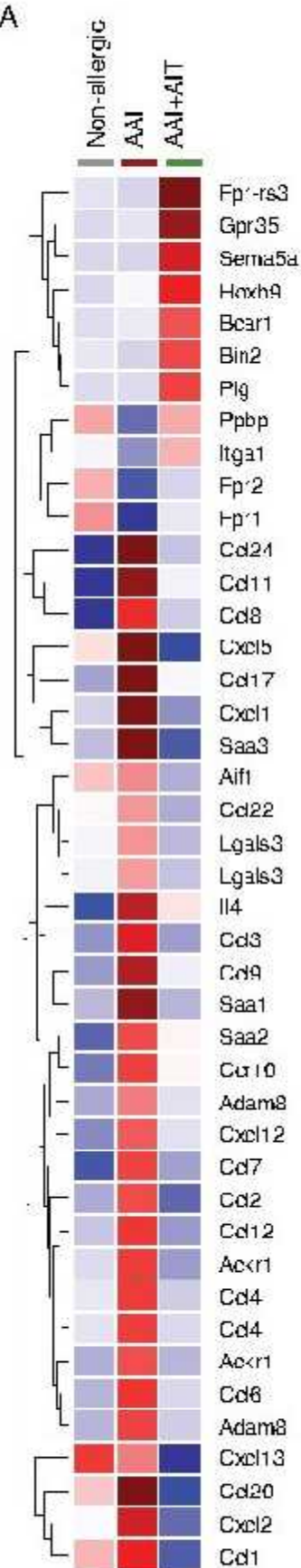


SUPPLEMENTARY FIGURE S1



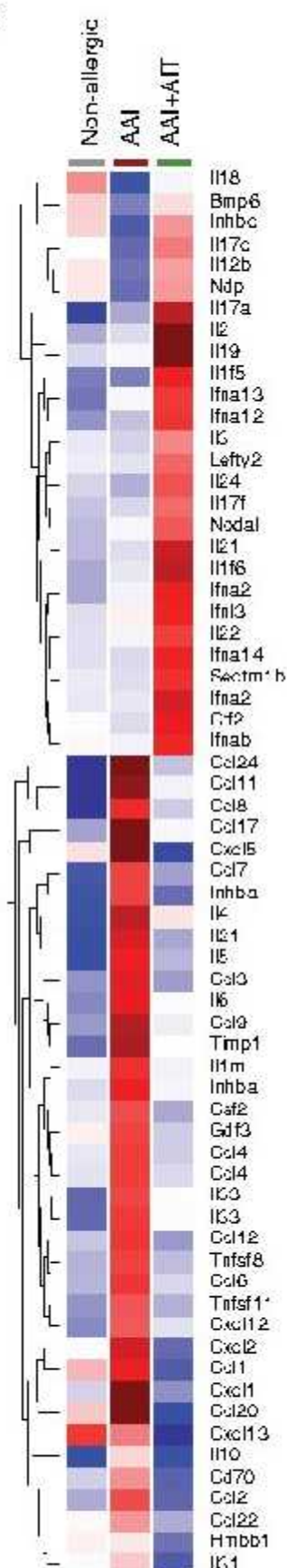
SUPPLEMENTARY FIGURE S2

A

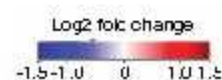


GO term:
0072676 and 0048247

B

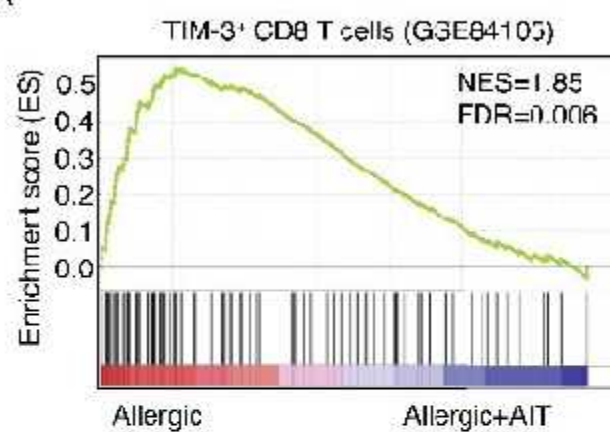


GO term: 0005125

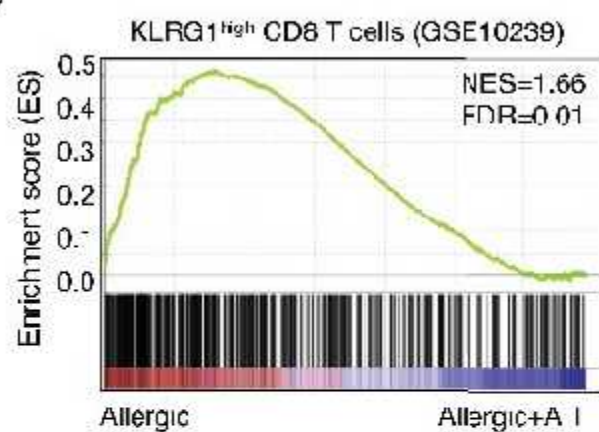


SUPPLEMENTARY FIGURE S3

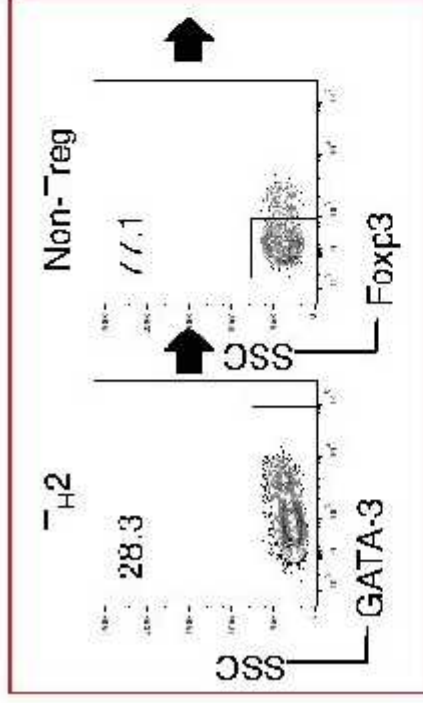
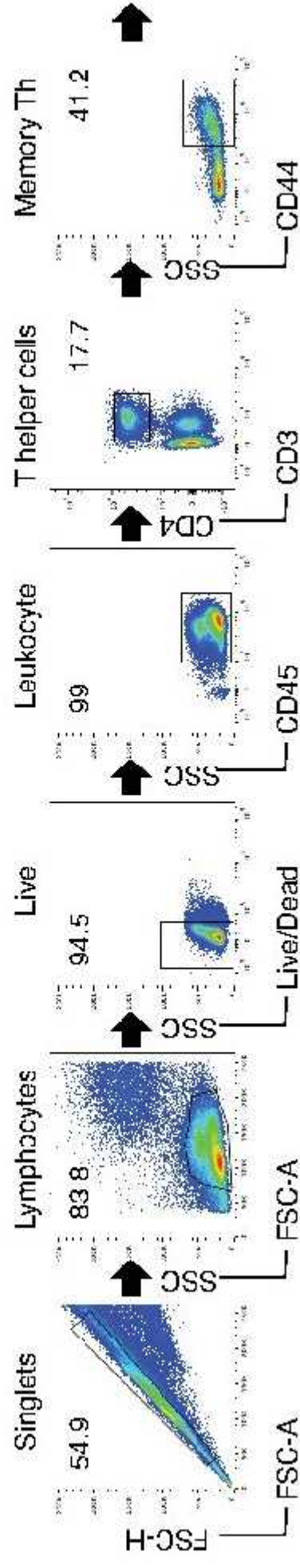
A



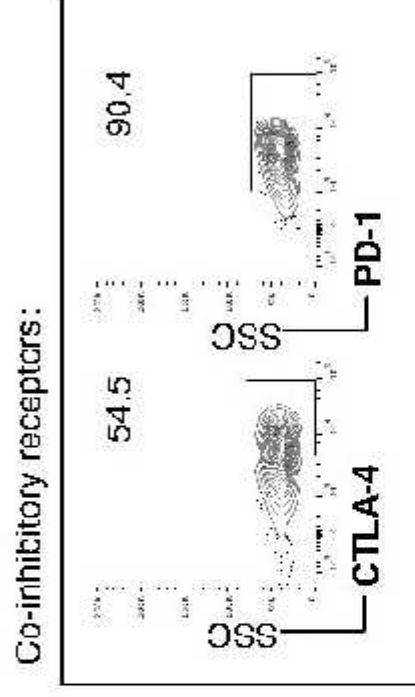
B



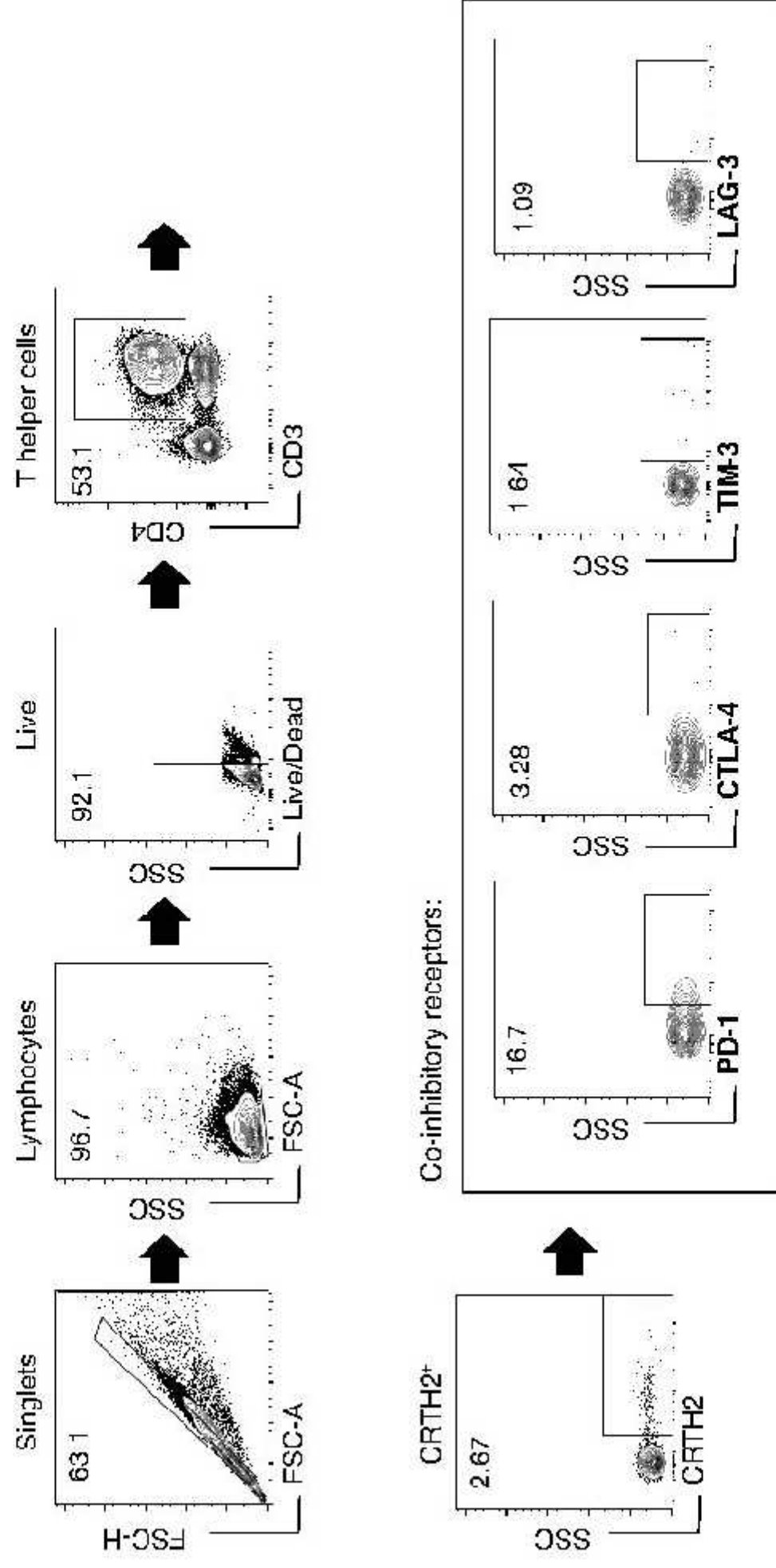
SUPPLEMENTARY FIGURE S4



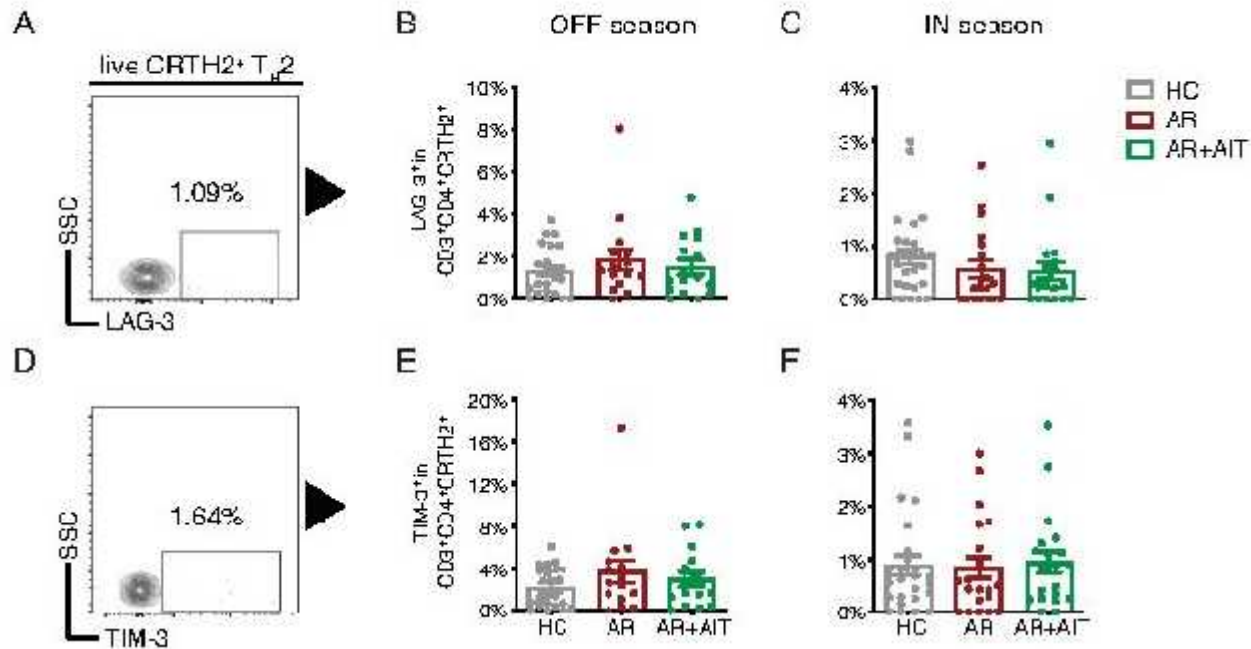
Proallergic TH2 cells
GATA-3⁺Foxp3⁻



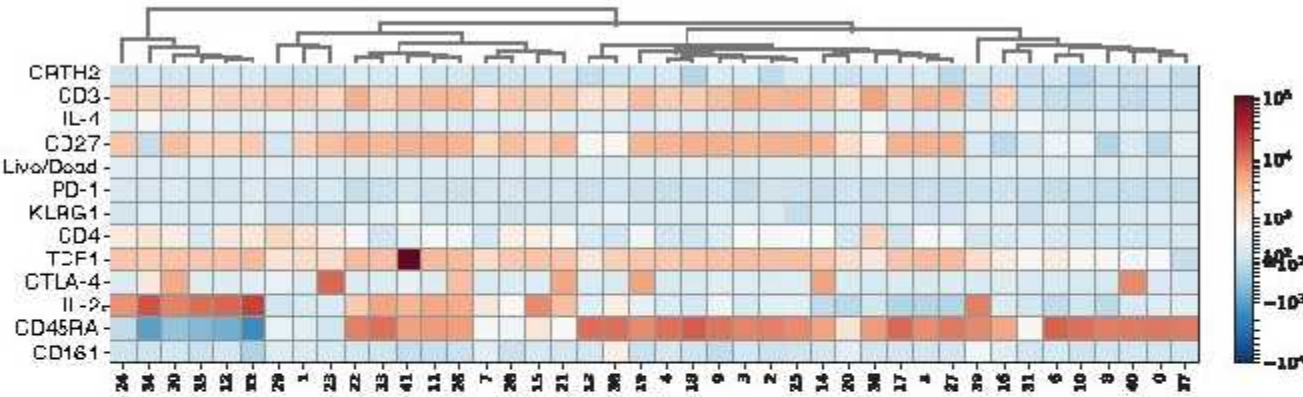
SUPPLEMENTARY FIGURE S5



SUPPLEMENTARY FIGURE S6

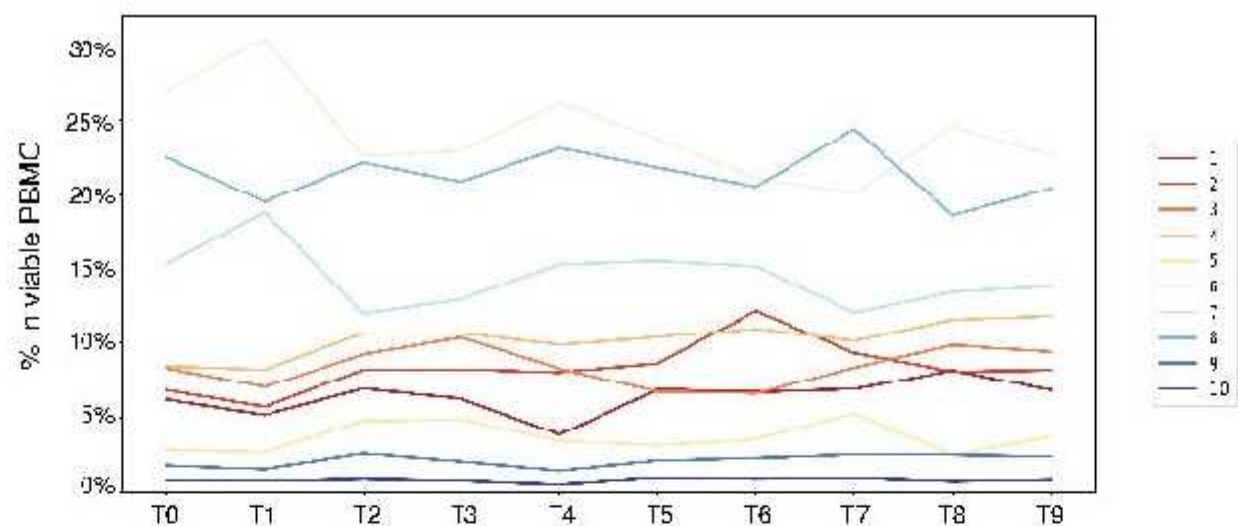


SUPPLEMENTARY FIGURE S7



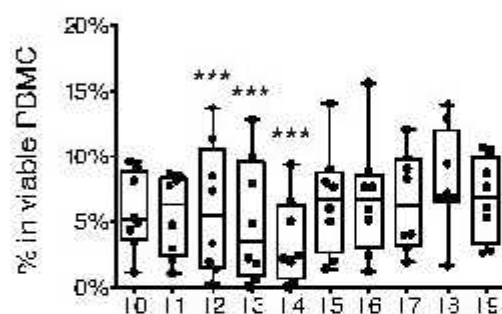
SUPPLEMENTARY FIGURE S8

A



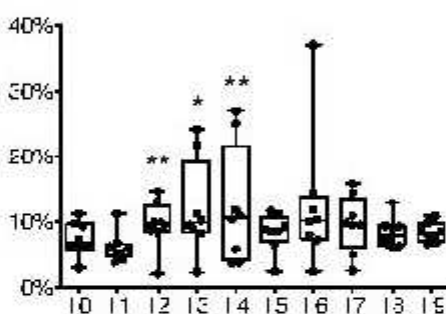
B

Cluster 1



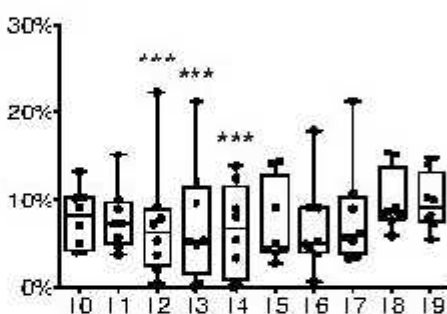
C

Cluster 2



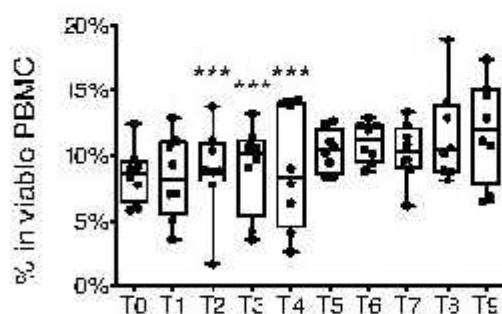
D

Cluster 3



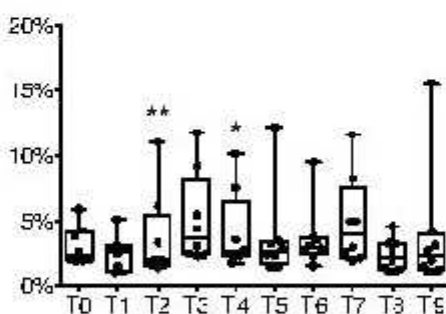
E

Cluster 4



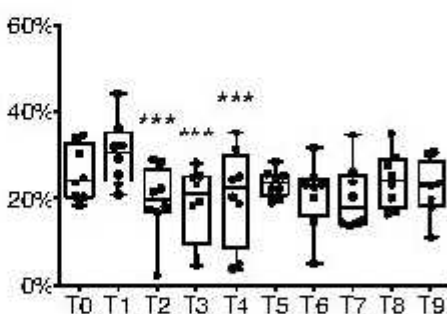
F

Cluster 5



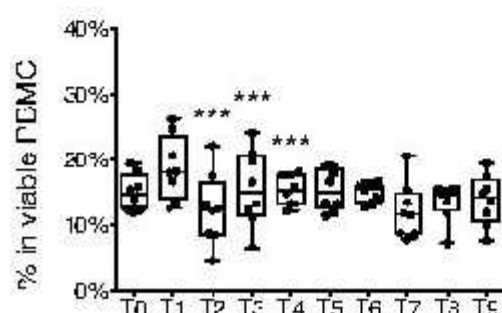
G

Cluster 6



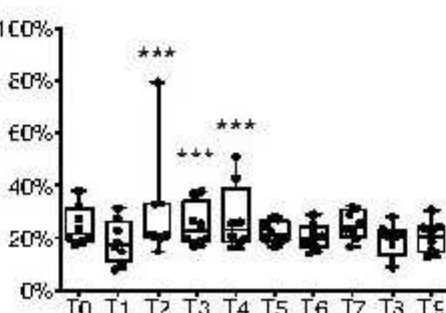
H

Cluster 7



I

Cluster 8



J

Cluster 9

