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ORIGINAL ARTICLE

Allergen-Specific Immunotherapy and Biologics

An exhausted phenotype of T_H2 cells is primed by allergen exposure, but not reinforced by allergen-specific immunotherapy

Shu-Hung Wang[1,2,3] | Ulrich M. Zissler[1,2,3] | Maren Buettner[4] | Sonja Heine[1,2,3] | Alexander Heldner[1,2,3] | Sebastian Kotz[5] | Lisa Pechtold[5] | Josephine Kau[5] | Mirjam Plaschke[5] | Julia T. Ullmann[1,2,3] | Ferdinand Guerth[1,2,3] | Madlen Oelsner[1,2,3] | Francesca Alessandrini[1,2,3] | Simon Blank[1,2,3] | Adam M. Chaker[1,2,3,5] | Carsten B. Schmidt-Weber[1,2,3] | Constanze A. Jakwerth[1,2,3]

[1]Center of Allergy & Environment (ZAUM), German Research Center for Environmental Health, Technical University of Munich and Helmholtz Center Munich, Munich, Germany

[2]Members of the German Center of Lung Research (DZL), CPC-M, Munich, Germany

[3]Member of the Helmholtz I&I Initiative, Munich, Germany

[4]Institute of Computational Biology, Helmholtz Center Munich, German Research Center for Environmental Health, Neuherberg, Germany

[5]Department of Otorhinolaryngology and Head and Neck Surgery, Medical School, Technical University of Munich, Munich, Germany

Correspondence
Carsten B. Schmidt-Weber, Center of Allergy & Environment (ZAUM), Technical University and Helmholtz Center Munich, Biedersteiner Str. 29, 80802 Munich, Germany.
Email: csweber@tum.de

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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 was 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.

Abbreviations: AA, Allergic asthma; AAI, Allergic airway inflammation; AIT, Allergen-specific immunotherapy; AR, Allergic rhinitis; 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.

Shu-Hung Wang and Ulrich M. Zissler are equally contributed to the manuscript.

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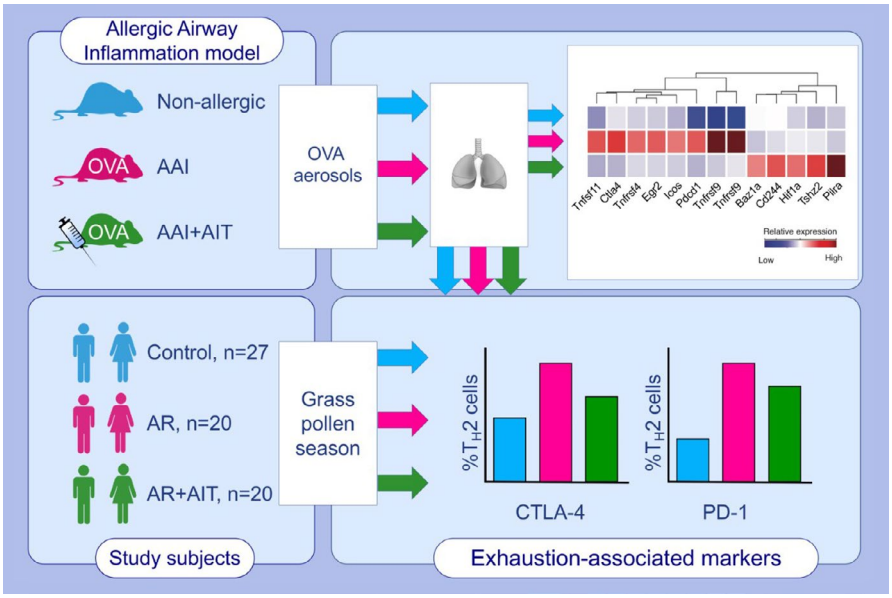
Conclusions: This study shows that allergen exposure promotes CTLA-4 and PD-1 expression on T_H2 cells and that the dynamic change in frequencies of exhausted T_H2 cells exhibits a differential pattern during the up-dosing versus the maintenance phases of AIT.

KEYWORDS
AIT, CTLA-4, PD-1, proallergic T_H2, T-cell exhaustion



GRAPHICAL ABSTRACT
This study shows that T_H2 cells upregulate the exhaustion-associated surface markers CTLA-4 and PD-1 upon in vivo allergen exposure in OVA-sensitized mice and grass pollen-allergic patients. This upregulation is reduced after AIT, in particular in T_H2 cells from local mouse lungs and AR patients with concurrent asthma. The transcriptome of allergic mouse lungs shows enhanced expression of both markers.

1 | 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, and 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), and 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 sensitization[25] 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.

2 | MATERIALS AND METHODS

2.1 | 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).

2.2 | Ovalbumin immunotherapy model

The model has been described earlier.[29] Mice were sensitized via intraperitoneal injections of 30 µg OVA (Grade V, Sigma-Aldrich, Merck, Darmstadt, Germany) in conjunction with 2 mg aluminum hydroxide (Imject™ Alum, Thermo Fisher Scientific, Waltham, MA, USA) in 200 µL phosphate-buffered saline (PBS) on days 0, 7, 14, and 28. Control mice received aluminum hydroxide (2 mg/200 µL PBS) only. After sensitization, the AIT group was treated on days 35, 39, 43, 47, 51, and 55 with subcutaneous injections of OVA (500 µg/200 µL 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.

2.3 | Patients and blood samples

Peripheral blood mononuclear cell (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) criteria[31]; a positive skin prick test wheal >3 mm in diameter; and a grass pollen specific IgE-level above 0.70 kU/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). PBMCs 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.

2.4 | 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 4 h 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).

2.5 | 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 normalization by inspecting the data distribution of each factor in a histogram. Then, we computed 15 nearest neighbors with the scanpy.pp.neighbors function and performed Louvain clustering with resolution 2.0.

Finally, we performed hierarchical clustering with the scanpy.tl.dendrogram function with default parameters (i.e. the Pearson

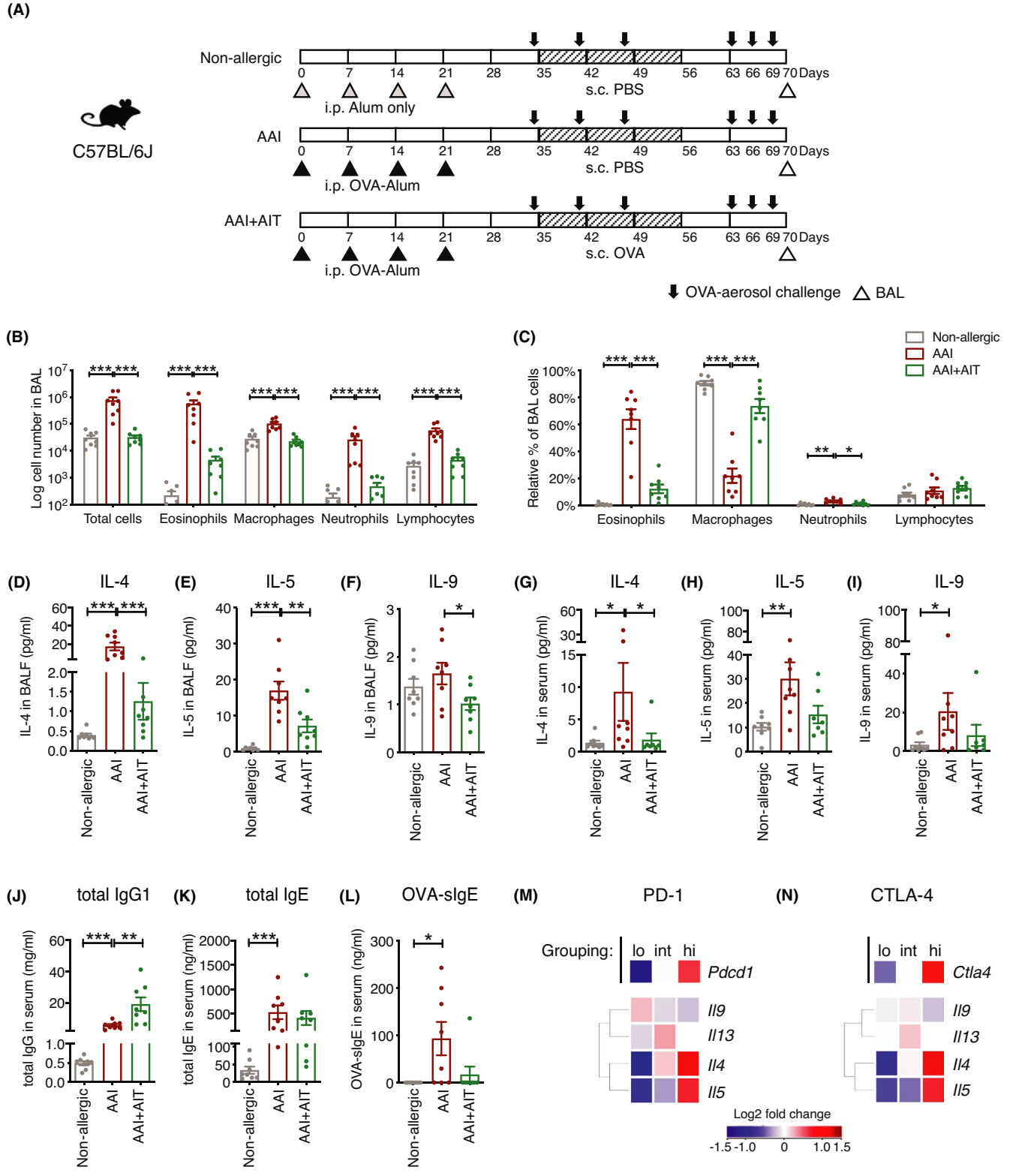


FIGURE 1 OVA-specific immunotherapy attenuated T_H2 response and intrapulmonary eosinophilia in mice. (A) Experimental scheme of OVA immunotherapy model. BAL fluid and sera from non-allergic, AAI and AAI + AIT mice were analyzed (n = 8 per group). (B) Total BAL cells, eosinophils, macrophages, neutrophils, 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. *$p \le .05$; **$p \le .01$; ***$p \le .001$; 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

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.

2.6 | 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.

3 | RESULTS

3.1 | OVA-specific immunotherapy attenuated allergic inflammation in mice

To examine the association of T-cell exhaustion with AIT, we implemented a murine immunotherapy model[29] 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 toward 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).

3.2 | 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 was 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 ≥ 1.5), but not with Foxp3 and TGF-β (data not shown). Next, the data were grouped per treatment condition and a total of 6500 regulated genes in AAI + AIT compared to AAI were identified, including 5243 upregulated genes and 1257 downregulated genes ($p \leq .05$; FC ≥ 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*, *and Cxcl5* (Figure S2A, B; Tables 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*), was 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.

3.3 | 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

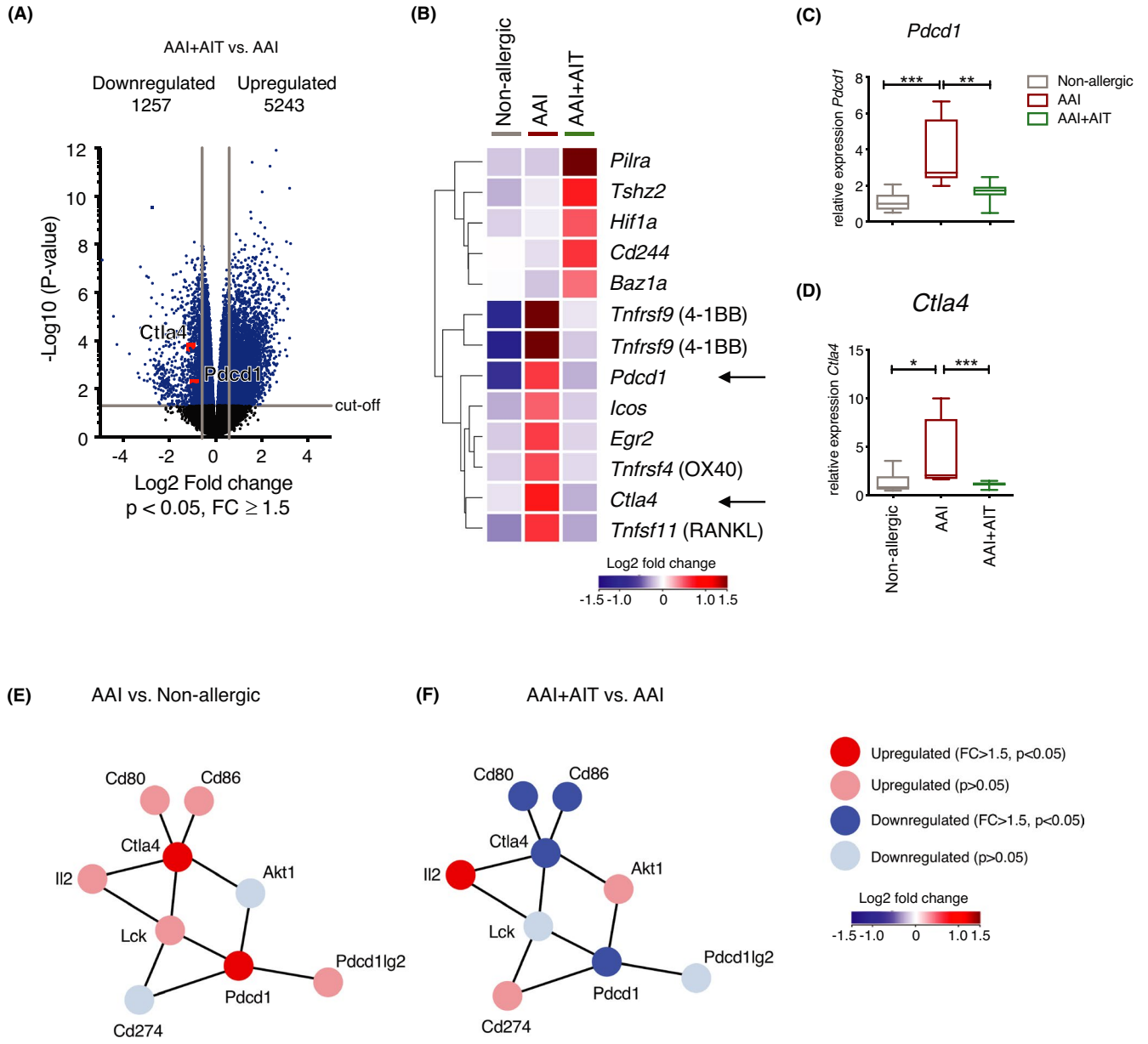


FIGURE 2 Transcriptional profiles of allergic mouse lungs reveal categories of genes associated with allergic inflammation and T-cell exhaustion. RNA whole transcriptome microarray analysis of mouse lung 24 h after the last OVA aerosol challenge. (A) Volcano plot illustrates differential gene expression 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 ≥ 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)

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 nonregulatory T_H2 cells was 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.

3.4 | 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 and 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.

3.5 | 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 toward 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.

4 | 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-1on 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 exhaustion[9] 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

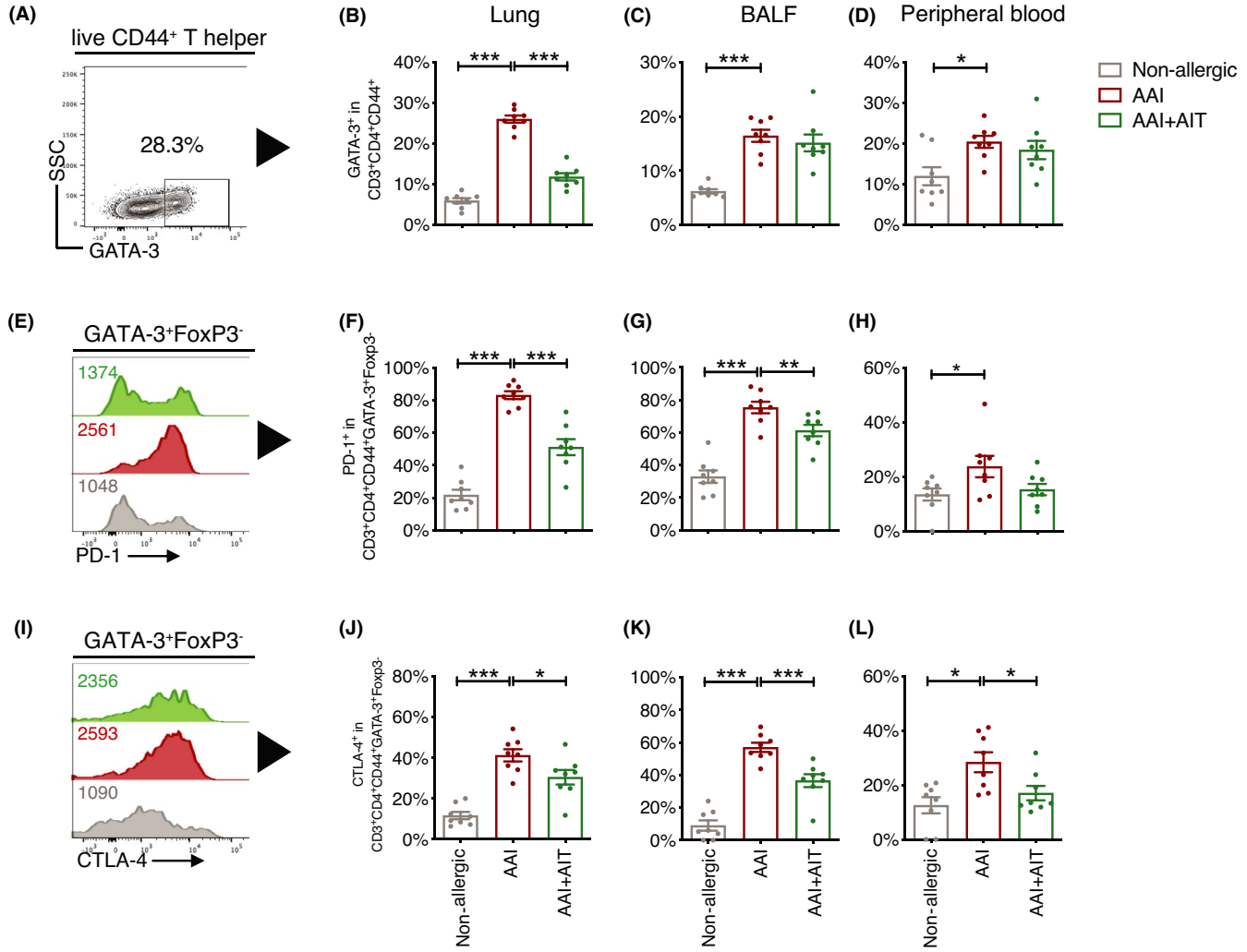


FIGURE 3 Recurrent allergen exposure drives upregulation of PD-1 and CTLA-4 on T_H2 cells. (A) A representative flow cytometric plot shows the gate of GATA-3$^+$ cells among live memory T helper cells (see complete gating strategy in Figure S4). Frequency of GATA3$^+$ T_H2 cells in the lungs (B), BAL 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. *$p \leq .05$; **$p \leq .01$; ***$p \leq .001$; Mann-Whitney *U* test

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

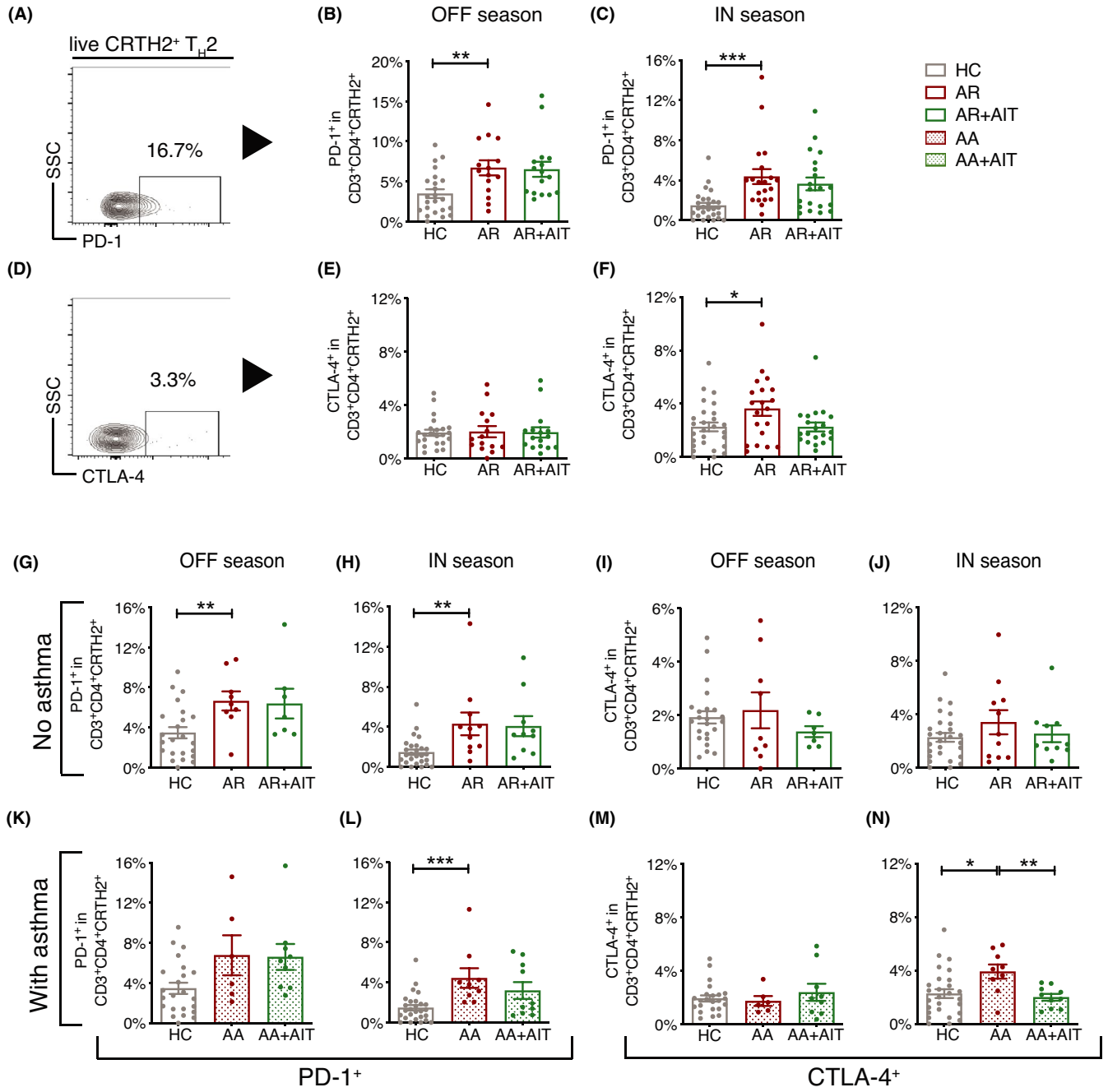


FIGURE 4 Exhaustion of circulating CRTH2$^+$ T_H2 cells is associated with allergen exposure and more profound in patients with allergic asthma. Flow cytometric analysis of CTLA-4 and PD-1 expression on circulating CRTH2$^+$ T_H2 cells from HC, AR, and AR + AIT groups. Representative flow cytometric 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

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

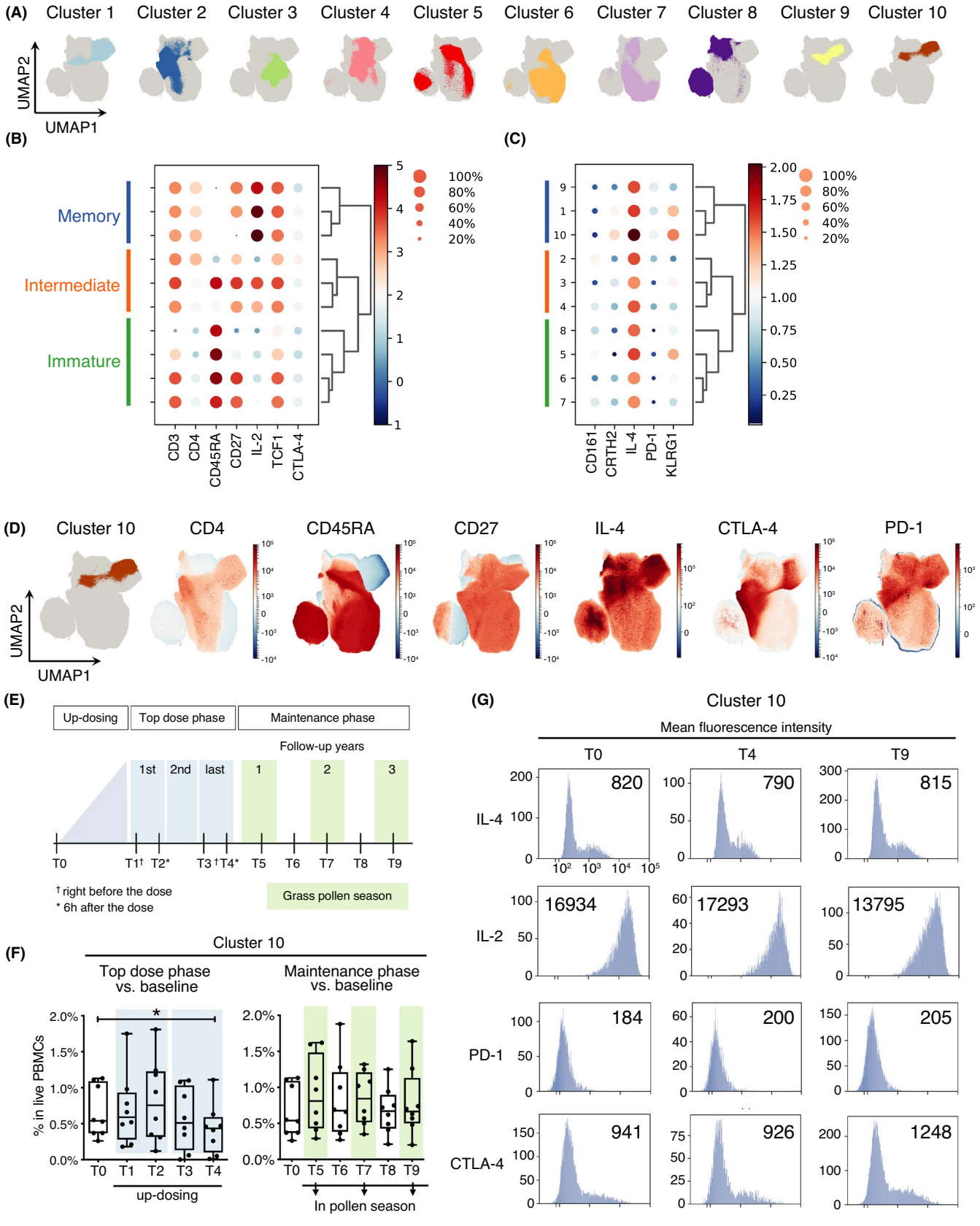


FIGURE 5 A late-differentiated T_H2 cells persisted throughout the course of AIT. (A) UMAP plots of ten lymphocyte subclusters. UMAP (Uniform Manifold Approximation and Projection) is a dimensional reduction algorithm. X-axis: UMAP-1; y-axis: UMAP-2. Dot plots depict the arcsinh-transformation values 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 scheme[5]: baseline (T0), right before (T1) and 6 h after the first (T2) and right before (T3) and 6 h 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). (F)Box plots show the dynamic change of cluster 10 ($CD45RA^-CD27^-CRTH2^+IL\text{-}4^+$) during up-dosing 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

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 decisions[49]: 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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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

A.M.C., C.A.J., C.S.W., S.B., S.H.W., and U.M.Z. designed the study. A.H., F.A., F.G., J.K., J.T.U., M.O., M.P., L.P., S.H., S.H.W., and S.K. involved in experiments execution. A.H., F.A., F.G., J.K., J.T.U., M.O., M.P., L.P., S.H., S.H.W., and S.K. collected the data. C.A.J., M.B., and S.H.W. analyzed the data. A.M.C., C.A.J., C.S.W., F.A., M.B., S.B., S.H.W., and U.M.Z. interpreted the data. A.M.C., C.A.J., C.S.W., S.H.W., U.M.Z. involved in literature search. All authors wrote the manuscript.

ORCID

Ulrich M. Zissler https://orcid.org/0000-0003-4826-3419
Francesca Alessandrini https://orcid.org/0000-0002-9854-8968
Simon Blank https://orcid.org/0000-0002-5649-1078
Carsten B. Schmidt-Weber https://orcid.org/0000-0002-3203-8084

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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