Balkan Med J 2024;41:387-95

Comparison of Immune Checkpoint Molecule Expression in Different Years of House Dust Mite Subcutaneous Immunotherapy on CD4+ T and Treg Cells in Children with Allergic Rhinitis

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Background: Allergen-specific immunotherapy, a unique inducer of tolerance, may result in T cell exhaution.

Aims: To investigate how the duration of house dust mite (HDM) subcutaneous immunotherapy (SCIT) affects the expression of major immune checkpoint (ICP) molecules on the surface of CD4+ T-helper and regulatory T (Treg) cells.

Study Design: Cross-sectional study.

Methods: We enrolled 28 children with HDM-induced allergic rhinitis (AR) and six controls. The study participants were divided into six groups: one group each of patients in their first, second, and third years of HDM-SCIT; one group each comprising those in the first year following HDM-SCIT and those on pharmacotherapy; and the control group. The expression of ICPs on CD4+ T and Treg cells was determined using flow cytometry, and plasma levels of soluble ICPs were estimated by ELISA.

Results: Our results revealed a significant increase in the expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and lymphocyte activation gene 3 (LAG-3) on CD4+ T cells during the second and third years of SCIT, respectively. Additionally, a strong correlation was observed between the expression of CTLA-4 and T cell immunoglobulin and mucin domain containing molecule-3 in CD4+ T cells. Furthermore, we observed a significant correlation between the expressions of programmed cell death protein-1, CTLA-4, T cell Immunoreceptor with Immunoglobulin and Immunoreceptor Tyrosine-Based Inhibitory Motif domain, and LAG-3 on both CD4+ T and Treg cells. A robust correlation was observed between the plasma levels of soluble ICPs.

Conclusion: HDM-SCIT induces CD4+ T cell exhaution, which may contribute to tolerance induction in children with AR.

INTRODUCTION

Currently, allergen-specific immunotherapy (AIT) is the sole treatment capable of modifying the course of allergic diseases.[1,2] The primary objective is to induce permanent unresponsiveness to natural allergens, defined as tolerance, which results from complex interactions between diverse immune cells and molecules.[3-5] Dermatophagoides pteronyssinus (Der p) and dermatophagoides farinae (Der f) are two species of house dust mites (HDMs) that frequently induce allergic sensitization worldwide.[6] For children with persistent and moderate-to-severe allergic rhinitis (AR) induced by HDM, subcutaneous immunotherapy (SCIT) is an effective treatment strategy.[1,7]

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Received: Jun 11, 2024 **Accepted:** Aug 19, 2024 **Available Online Date:** September 06 2024 • **DOI:** 10.4274/balkanmedj.galenos.2024.2024-6-19

Available at www.balkanmedicaljournal.org

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Cite this article as: Hızlı Demirkale Z, Alpkıray MF, Engin A, Sönmez AD, Yücel E, Tamay Z, Özdemir C, Deniz G, Çetin Aktaş E. Comparison of Immune Checkpoint Molecule Expression in Different Years of House Dust Mite Subcutaneous Immunotherapy on CD4+ T and Treg Cells in Children with Allergic Rhinitis. Balkan Med J.; 2024; 41(5):387-95.

***Preliminary data from this work were presented in an oral presentation session at the WAC Congress, held from October 13-15, 2022, in İstanbul, Türkiye.**

T cell exhaustion is linked to a progressive decline in cell numbers and function because of persistent antigenic stimulation and has been extensively investigated in certain chronic viral infections, autoimmune diseases, and immune cells in the tumor milieu.[8-11] Exhausted T cells are characterized by the elevated expression of various inhibitory surface receptors, known as immune checkpoints (ICP), including 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), T cell Immunoreceptor with Immunoglobulin and Immunoreceptor Tyrosine-based Inhibitory Motif (TIGIT) domain, and lymphocyte activation gene-3 (LAG-3).[9,12] Given that AIT involves continuous and progressively increasing administration of high-dose allergen over several years, it is reasonable to hypothesize that $CD4^+$ T cell alterations that lead to an exhausted state might contribute to the development of tolerance. The precise function of the exhausted T cell phenotype in this process remains unclear, despite the fact that several studies suggest that the expression of certain ICPs on $CD4^+$ T cells may contribute to allergen-specific tolerance.[3,13-17] Though SCIT, we aimed to determine the impact of allergen exposure on the surface expression of PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 on the surface of $CD4^+$ T and Treg cells in children with moderate-to-severe AR, with or without asthma. We postulated that the expression of these ICPs may be influenced by HDM-SCIT duration. Furthermore, we measured the soluble plasma levels of PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 and examined the correlation between the membrane-bound and soluble forms of these molecules, as well as between their expression on $CD4^+$ T and Treg cells.

MATERIALS AND METHODS

Study design

The Ethics Committee of İstanbul University, İstanbul Faculty of Medicine, approved this cross-sectional study (approval number: 2022/821, date: 13.05.2022). All children and their parents provided written informed consent to participate in the study.

To assess the influence of HDM-SCIT duration on targeted ICPs, patients who received HDM-SCIT were divided into three subgroups: Group 1 (n = 5) during their first year, Group 2 (n = 7) during their second year, and Group 3 (n = 5) during their third year of treatment. Additionally, to ascertain the long-term sustainability of ICP expression, Group 4 (n = 6) included patients who had completed their HDM-SCIT within the previous year. Given that the cumulative dose of SCIT is substantially greater than that of natural allergen exposure, sensitive patients continue to experience allergen exposure.[3] To evaluate the impact of natural allergen exposure on ICPs, patients who were potential candidates for HDM-SCIT but were currently undergoing pharmacotherapy were included in Group 5 (n = 5). Finally, Group 6 (n = 6) comprised healthy non-atopic and non-allergic participants as a control group. Figure 1 summarizes the study groups.

Study population

We recruited 34 participants, including 28 patients with moderate-to-severe persistent HDM-induced AR with or without asthma as well as six participants in the control group. AR and asthma were defined in accordance with the guidelines of the Allergic Rhinitis and Its Impact on Asthma and Global Initiative for Asthma, respectively.[7] All participants underwent skin prick test (SPT) using 29 standard aeroallergen solutions, which included Der p and Der f extracts (Alk-Abello, Round Rock, TX, USA). The included patients had a clinical history of typical symptoms characteristic of allergy to HDMs, as confirmed by a positive SPT against Der p, Der f, or both (wheal diameter ≥ 3 mm larger than those control who were administered saline), while controls exhibited negative SPT results for all the aeroallergen solutions used.

The patients were administered a conventional regimen of standardized Der p extract adsorbed on aluminum hydroxide (Alutard SQ 510®, ALK-Abello A/S, Hørsholm, Denmark) over a three-year period. The pediatric allergists, who were previously involved in the application of AIT, made the decision to initiate HDM-SCIT. The treatment had already been initiated prior to the study and was

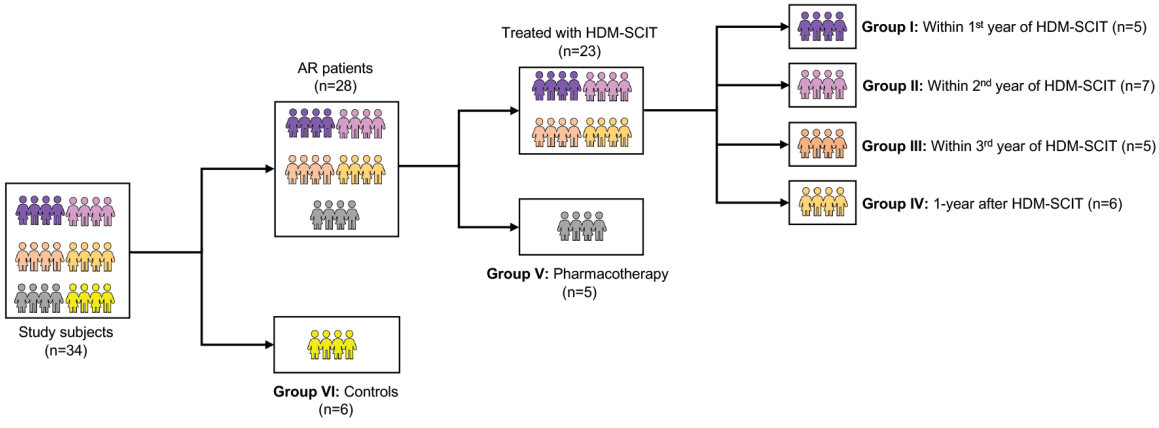

FIG. 1. Study groups.
AR, allergic rhinitis; HDM-SCIT, house dust mite subcutaneous immunotherapy; n, numbers of participants.

administered regardless of the patient's willingness to participate in the study. All HDM-SCIT extracts were made available through national health insurance and administered in accordance with the manufacturers' instructions.

We evaluated the severity of nasal symptoms using the visual analogue score (VAS). During the interview, patients who had undergone a minimum of six months of HDM-SCIT were requested to recall their symptoms prior to the initiation of treatment and provide a current total VAS. The VAS was represented by a 10-centimeter line, with the "worst symptom" (score: 10) at one end and "no symptom" (score: 0) at the other end. The VAS was employed for assessing the severity of each of the following symptoms: nasal itching, sneezing, nasal blockage, and nasal discharge. Additionally, the patients' medical records were compared to their recollected total VAS prior to initiating AIT to ensure that the score was consistent with their disease severity before the treatment.

Flow cytometric analyses of PD-1, CTLA-4, TIM-3, TIGIT and LAG-3 expressions on CD4⁺ T-helper and regulatory T cells

The peripheral blood samples were processed for surface staining employing the whole blood lysis method. The whole blood samples were labeled using anti-human CD45-Brilliant Violet 510™ (clone:2D1), anti-human CD3-Brilliant Violet 570™ (clone:UCHT1), anti-human CD4-PerCPCy5.5 (clone:OKT4), anti-human CD127-Brilliant Violet 421™ (clone:A019D5), anti-human CD25-PECy5 (clone:BC96), anti-human CD223 (LAG-3)-FITC (clone:11C3C65), anti-human TIGIT (VSTM3)-PE (clone:A15153G), anti-human CD152 (CTLA-4)-APC (clone:BNI3), anti-human CD279 (PD-1)-APC/cyanine7 (clone:EH12.2H7), and anti-human CD366 (TIM-3)-PECyanine7 (clone:F38-2E2) monoclonal antibodies (all from BioLegend, San Jose, CA, USA) for 20 minutes at room temperature in the dark. An autofluorescent tube was employed as an isotypic control for the analysis. Following incubation, red blood cells were lysed with FACS lysing solution (BD Biosciences, San Jose, CA, USA). In the forward scatter and side scatter (SSC) dot plot histograms, cells were gated by their size and granularity to identify lymphocytes. T-helper and Treg cells were gated within the SSC/CD45 and then $CD3^+CD4^+$ lymphocyte gate. Treg cells were defined as $CD3^+CD4^+CD25^+CD127^-$ cells in gated $CD3^+CD4^+$ T cells. The data analyses were conducted using the FlowJo™ 10.2 software (Tree Star Inc., USA).

Detection of soluble PD-1, CTLA-4, TIM-3, TIGIT and LAG-3 by ELISA

The ELISA was employed to analyze the plasma samples, which were stored at -80 °C Three of the 37 plasma samples subjected to ELISA were from additional patients (one in Group 3, one in Group 4, and one in Group 5). Human soluble PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 levels were detected using commercial ELISA kits according to the manufacturer's protocols for CTLA-4, PD-1, TIGIT, LAG-3 (Abbkine Inc., China), and TIM-3 (BT LAB, China). The ELx800 reader (BioTek Instruments) was employed to record the optical densities at 450 nm wavelengths.

Statistical analysis

Statistical analyses were conducted with the Python programming language version 3.8 (Python Software Foundation). Descriptive statistics are presented as means with standard deviations for variables with a normal distribution and as medians with minimum and maximum values in parentheses for variables with a non-normal distribution. Normality analysis was conducted using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Non-parametric tests (Kruskal-Wallis and Mann-Whitney U) were employed for further analyses. Correlation analyses were conducted using Spearman's rank correlation method. Heatmap correlation analysis was conducted using Python. The software GraphPad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, USA) was employed to generate the graphs. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of the subjects participating in the study

The study included 20 males and 14 females, with a mean age of 13.8 ± 2.2 years, and all groups were age-matched. Table 1 provides a summary of the demographic and clinical characteristics of the groups.

Expression of the immune checkpoints PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 on CD4⁺ T-helper cells

The expression of exhaustion-associated surface markers, such as PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3, on freshly isolated $CD4^+$ T cells from the study participants was analyzed using flow cytometry (Figure 2A). Our results indicated that Group 2 exhibited substantially higher levels of CTLA-4 expression on $CD4^+$ T cells than all other groups, except for Group 3 ($p = 0.048$, $p = 0.002$, $p = 0.018$, $p = 0.022$, respectively) (Figure 2B). Furthermore, Groups 2 and 3 exhibited significantly higher TIM-3 expression on $CD4^+$ T cells than Group 4, with p values of 0.035 and 0.03, respectively (Figure 2B). Additionally, $CD4^+$ T cell expression of LAG-3 reached statistical significance in the third year of treatment when compared to Group 4, Group 5, and Group 6 ($p = 0.03$, $p = 0.008$, $p = 0.017$, respectively).

Expression of the immune checkpoints PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 on regulatory T cells

The gating strategy for identifying $CD3^+CD4^+CD25^+CD127^-$ Treg cells is presented in Figure 3A. Representative dot plot expressions of PD-1 and TIGIT in a patient from Group 3 are illustrated in Figure 3B. Patients in Group 2 exhibited considerably greater expression of PD-1 on Treg cells compared to Group 4 ($p = 0.035$) (Figure 3C). However, TIGIT expression on Treg cells was significantly lower in Group 4 compared to Group 5 ($p = 0.03$). The study groups did not exhibit any substantial differences in the expression of CTLA-4, TIM-3, and LAG-3 on Treg cells (Figure 3C).

TABLE 1. Demographic and Clinical Characteristics of the Study Groups.

	Group 1, (n = 5)	Group 2, (n = 7)	Group 3, (n = 5)	Group 4, (n = 6)	Group 5, (n = 5)	Group 6, (n = 6)	p
Sex, M/F, (n)	2/3	5/2	3/2	3/3	3/2	4/2	N/A
Age (year), median (range)	13.4 (11.2-15.8)	13.0 (9.8-15.5)	14.2 (11.3-17.2)	15.2 (12.9-18.3)	13.6 (9.5-16.8)	14.4 (10.8-16.1)	0.404
AR duration (year), median (range)	7.2 (2.2-8.9)	6.9 (4.6-12.5)	8.8 (2.8-15.7)	11.0 (8.0-16.0)	4.6 (2.5-11.3)	-	0.072
HDM-SCIT duration (year), median (range)	0.8 (0.4-1.6)	2.2 (1.9-2.6)	2.9 (2.8-3.0)	0.6 (0.3-0.9)†	-	-	**0.001**
Diagnosis, AR/AR+asthma, (n)	3/2	3/4	2/3	3/3	3/2	-	N/A
Comorbid allergic diseases, (n)							
Atopic dermatitis	1	1	2	1	-	-	N/A
Chronic urticaria	1	1	-	-	-	-	
Food allergy	-	1	-	-	-	-	
Contact dermatitis	-	1	-	-	-	-	
SPT wheal size (mm), median (range)							
Der p	6.0 (4.5-12.5)	4.5 (4.5-10.0)	12.5 (6.0-17.5)	10.0 (6.0-12.5)	11.3 (3-17.5)	Negative	0.190
Der f	10.0 (6.0-12.5)	6.0 (4.5-12.5)	12.5 (7.5-17.5)	9.5 (4.5-10.0)	10.0 (7-12.5)	Negative	0.460
Co-sensitization pattern, (n)							
Grass	3	1	1	2	-	Negative	N/A
Furry animals	1	-	-	3	1	Negative	
Tree	1	-	-	-	1	Negative	
Mold	-	1	1	-	1	Negative	
Serum IgE (kU/l), median (range)	259.0 (64-676)	351.0 (10-2147)	599.5 (230-1738)	351.0 (196-2742)	309.0 (81-974)	59.5 (1-226)	**0.021**
	(n = 3)	**(n = 7)**	**(n = 5)**	**(n = 6)**			
Total VAS before HDM-SCIT	36 (36-37)	36 (34-38)	36 (33-40)	36 (33-39)	-	-	0.970
Total VAS after HDM-SCIT	12 (4-16)	8 (5-14)	10 (4-14)	13.5 (11-17)	-	-	0.119
VASΔ	25 (20-32)	29 (20-31)	26 (22-29)	23 (17-25)	-	-	0.128

AR, allergic rhinitis; Der f, dermatophagoides farina; Der p, dermatophagoides pteronyssinus; F, female; HDM, house dust mite; HDM-SCIT, house dust mite subcutaneous immunotherapy; IgE, immunoglobulin E; M, male; n, numbers of participants; N/A, not available; SPT, skin prick test; VAS, visual analog score; VASΔ, the changes in VAS between baseline and subsequent time points; †Represents the time elapsed after completion of a 3-year course of house dust mite subcutaneous immunotherapy. The bold *p* value indicates that statistically significant differences were determined using the Mann-Whitney U test.

Soluble PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 levels in plasma

We detected that all children exhibited detectable levels of the soluble ICPs, and the levels varied greatly among the study groups. Group 6 exhibited significantly higher plasma levels of sCTLA-4 than Group 2 ($p = 0.008$). Furthermore, the levels of sLAG-3 were significantly higher in Group 6 than in Group 1 and Group 2 ($p = 0.017$ and $p = 0.022$, respectively). Figure 4 depicts soluble ICP levels across all study groups.

Correlation analysis of membrane-bound and soluble forms of PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3

To gain a more comprehensive understanding of the relationship between the membrane-bound and soluble forms of PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3, we performed a heatmap analysis. Our analysis did not identify any correlation between the soluble levels of these ICPs and their expressions on both $CD4^+$ T cells and Treg cells (Figure 5A). However, we observed a robust relationship between the plasma levels of sPD-1, sCTLA-4, sTIM-3, sTIGIT, and sLAG-3. Additionally, a strong correlation was observed between the expressions of CTLA-4 and TIM-3 on $CD4^+$ T cells, while a weak correlation was also observed among other ICPs expressed on $CD4^+$ T cells (Figure 5A). Conversely, the ICP expressions on Treg cells, with the exception of a moderate correlation between PD-1 and TIM-3, did not exhibit substantial correlation patterns (Figure 5A). Interestingly, the expressions of PD-1, CTLA-4, TIGIT, and LAG-3 on $CD4^+$ T and Treg cells were moderately correlated with each other, except for TIM-3 (Figure 5B).

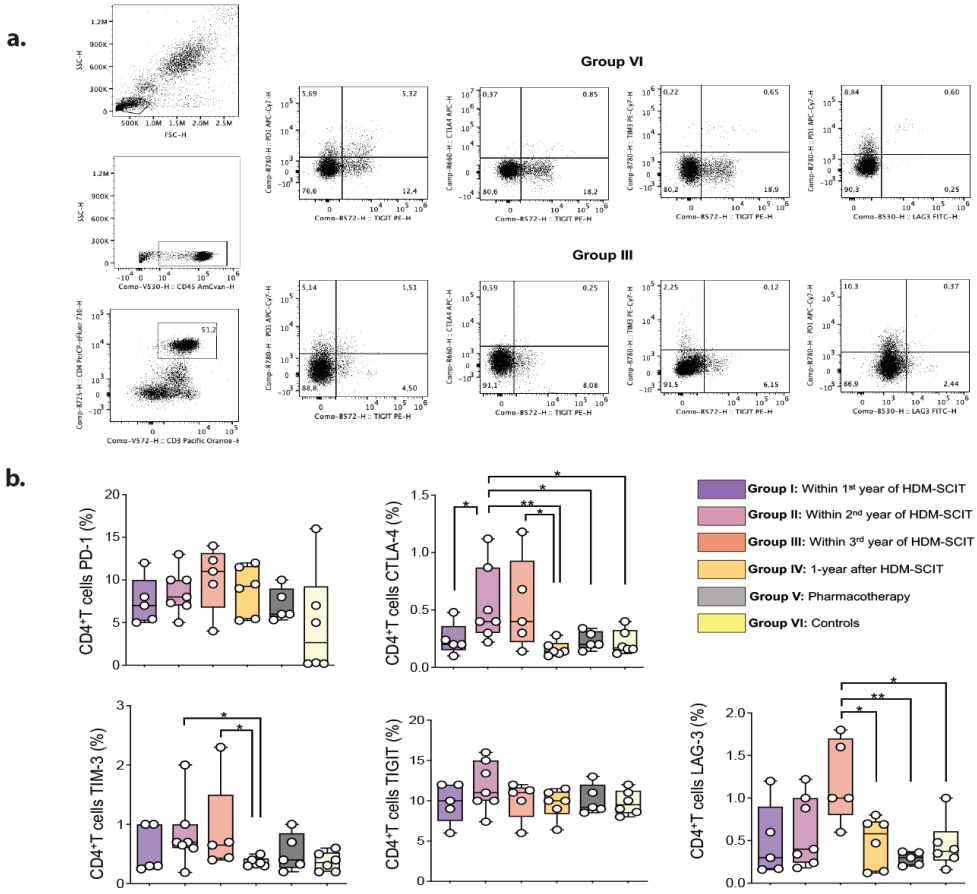

FIG. 2. Expression of PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 on peripheral blood $CD4^+$ T cells. (a) Representative dot plots demonstrating the gating strategy and expression of ICPs in a control participant (upper panel) and a patient treated with HDM-SCIT for three years (bottom panel). (b) Percentages of $CD4^+$ T cells expressing PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 in the study groups. Each data point represents a single donor among the study groups. Median values are indicated by a horizontal line; differences between the groups were analyzed using the Mann-Whitney U test ($^*p \leq 0.05$ and $^{**}p \leq 0.01$).

PD-1, programmed cell death protein-1; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; TIM-3, T cell immunoglobulin and mucin domain containing molecule-3; TIGIT, T cell Immunoreceptor with Immunoglobulin and Immunoreceptor Tyrosine-based Inhibitory Motif; LAG-3, lymphocyte activation gene-3; HDM-SCIT, house dust mite subcutaneous immunotherapy; ICP, immune checkpoint.

DISCUSSION

This study discovered a significant increase in CTLA-4 expression on $CD4^+$ T cells during the second year of HDM-SCIT. The expression remained elevated in the third year but decreased after treatment termination. Moreover, we noted a minor, though not statistically significant, increase in LAG-3 expression with each year of HDM-SCIT. This trend attained statistical significance in the third year of treatment. Similarly, we observed a consistent increase in PD-1 expression on $CD4^+$ T cells with each year of HDM-SCIT. However, statistical significance was not attained, possibly due to the limited sample size in each group of our dataset. These data indicate that an elevated expression of CTLA-4 and LAG-3 on $CD4^+$ T cells is observed on long-term and high-dose allergen administration through AIT for a minimum of two and three years (but not one year), respectively. This increased expression may cause $CD4^+$ T cells to transition to an exhausted state. Additionally, we observed a strong correlation between the expressions of CTLA-4 and TIM-3 on $CD4^+$ T cells, while other ICP receptor expressions showed a weak correlation. This result lends additional credence to the concept of $CD4^+$ T cell exhaustion. It is widely recognized that exhausted T cells co-express numerous ICPs in a variety of combinations. A greater number of co-expressed ICPs typically indicates a more severe state of exhaustion.[9] In the one-year post-treatment group, the expression of PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 was not upregulated on the surface of $CD4^+$ T cells. It is crucial to recognize that exhaustion does not represent a permanent terminal differentiation but rather an adaptive and reversible hyporesponsive state that is defined by persistent antigenic stimulation as opposed to terminated or intermittent exposure.[9,18] Therefore, it is conceivable that the exhaustion-associated molecules were reversed in the first-year post-treatment group. When assessed in conjunction with the pharmacotherapy group, it becomes evident that natural HDM exposure may not be sufficient for the expression of these ICPs on $CD4^+$ T cells.

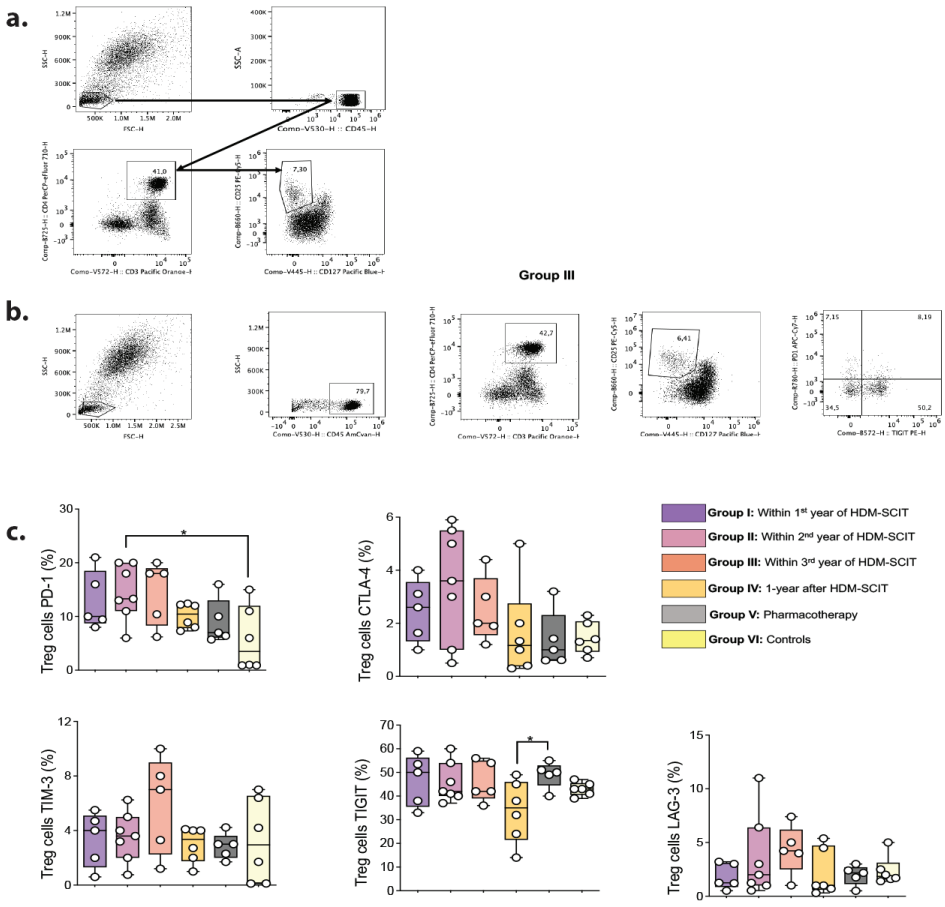

FIG. 3. Flow cytometric analyses of ICPs on Treg cells. (a) Gating strategy of $CD3^+CD4^+CD25^+CD127^-$ Treg cells are illustrated as dot plot histograms. (b) Representative dot plot depicting the expressions of PD-1 and TIGIT in a Group 3 patient. (c) Percentages of Treg cells expressing PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 in the study groups. Each data point represents a single donor among the study groups. Median values are indicated by a horizontal line; differences between the groups were analyzed using the Mann-Whitney U test ($^*p \leq 0.05$).

PD-1, programmed cell death protein-1; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; TIM-3, T cell immunoglobulin and mucin domain containing molecule-3; TIGIT, T-cell Immunoreceptor with Immunoglobulin and Immunoreceptor Tyrosine-based Inhibitory Motif; LAG-3, lymphocyte activation gene-3; HDM-SCIT, house dust mite subcutaneous immunotherapy; ICP, immune checkpoint.

In vitro stimulation of $CD4^+$ T cells with HDM extract resulted in a substantial increase in the expressions of PD-1, LAG-3, and CTLA-4, as demonstrated by Rosskopf et al.[17] in a previous study. Additionally, they discovered that inhibiting PD-1 boosts T cell proliferation and cytokine production. In another study, the expression of PD-1 and two other ICPs, B7-H1, and B and T lymphocyte attenuator (BTLA), was compared in patients allergic to Japanese cedar pollinosis between those who had received AIT and those who did not. The study found no significant differences in PD-1 and B7-H1 expressions between the two groups, but BTLA expression on $CD4^+$ T cells was enhanced in the AIT-treated group.[16] More recently, Wang et al.[14] reported that in a mouse model and two cohorts of pollen-allergic patients, the population of late-differentiated Th2 cells expressing CTLA-4 and PD-1 declined during the updosing phase of AIT but remained stable long-term during the maintenance phase. In a separate study, the transcriptional profile of $CD4^+$ T cells following AIT demonstrated the expression of MAF transcription factor, nuclear factor IL-3-regulated and costimulatory molecules, LAG-3, TIGIT, PD-1, and TIM-3 in a murine model.[19] Besides these studies, ICP molecules have also been examined in different populations.[20,21] One animal study demonstrated that CTLA-4 and TIM-3 might contribute to the maintenance of materno-fetal tolerance by regulating decidual $CD4^+$ T cell function.[20] Furthermore, increased expression of LAG-3 has been observed in β-thalassemia major patients, which may be linked to immune system abnormalities in this patient population.[21]

Treg cells are essential for preserving immune balance, and their role in controlling allergic inflammation is critical.[13] In the context

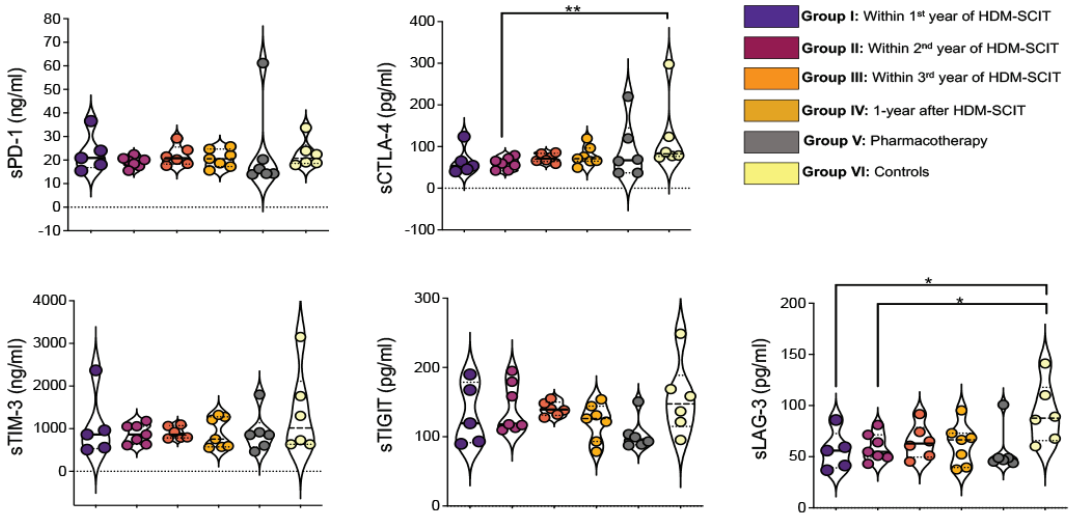

FIG. 4. Plasma levels of soluble PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3. Box and violin graphs illustrating the plasma levels of soluble PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3. Each data point represents the ELISA results of a single donor. Median values are indicated by a horizontal line; differences between the groups were analyzed using the Mann-Whitney U test ($^{*}p \leq 0.05$ and $^{**}p \leq 0.01$).

PD-1, programmed cell death protein-1; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; TIM-3, T cell immunoglobulin and mucin domain containing molecule-3; TIGIT, T cell Immunoreceptor with Immunoglobulin and Immunoreceptor Tyrosine-based Inhibitory Motif; LAG-3, lymphocyte activation gene-3; HDM-SCIT, house dust mite subcutaneous immunotherapy; ICP, immune checkpoint.

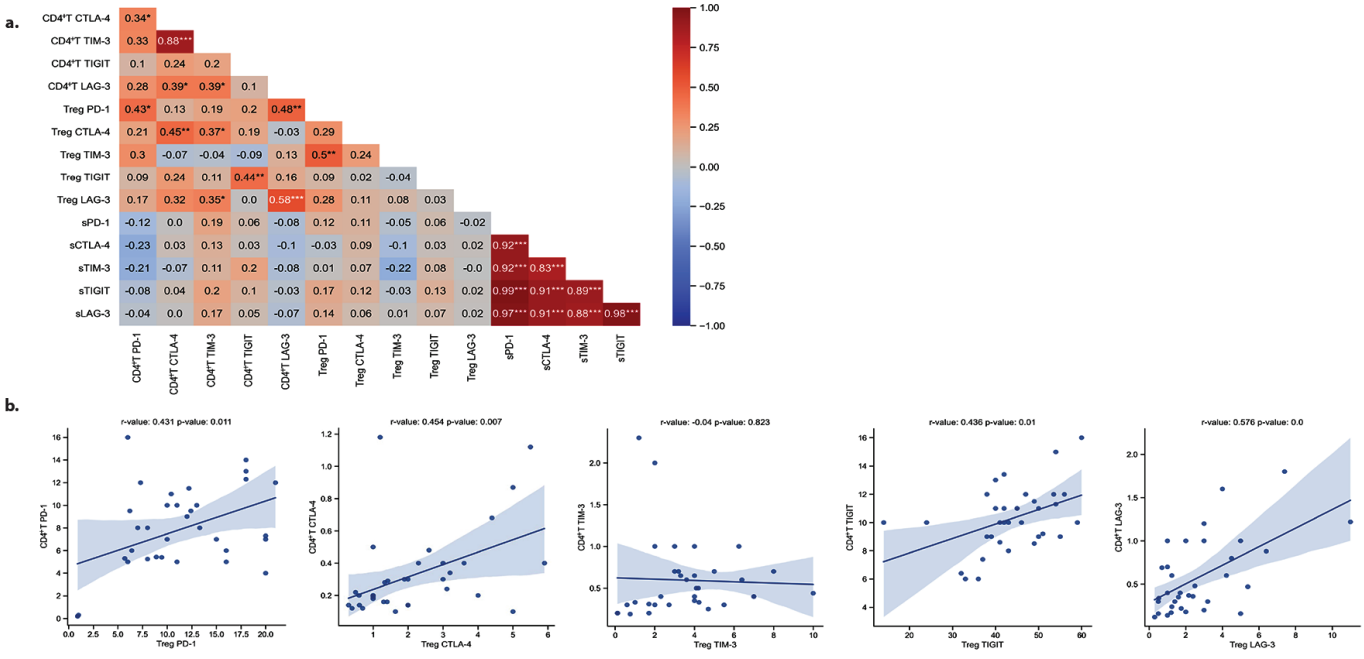

FIG. 5. Correlation analysis of membrane-bound and soluble forms of PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3, and their surface expressions on $CD4^{+}$ T and Treg cells. (a) The heatmap illustrates the analysis of the Spearman correlation coefficients between the membrane-bound and soluble forms of PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3. The color scale indicates the strength and direction of the correlation coefficient, with warmer colors suggesting stronger positive and cooler colors indicating stronger negative correlations. The Spearman correlation coefficients (*r* values) are denoted in each compartment, and significance levels (*p* values) are indicated as asterisks ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). (b) Correlation matrices for surface expressions of PD-1, CTLA-4, TIM-3, TIGIT, and LAG-3 between $CD4^{+}$ T and Treg cells. The Spearman correlation coefficients (*r* values) and significance levels (*p* values) are displayed above each graph. Each dot in the scatter plots represents a single study participant.

PD-1, programmed cell death protein-1; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; TIM-3, T cell immunoglobulin and mucin domain containing molecule-3; TIGIT, T cell Immunoreceptor with Immunoglobulin and Immunoreceptor Tyrosine-based Inhibitory Motif; LAG-3, lymphocyte activation gene-3.

of achieving tolerance through AIT, it remains uncertain whether AIT directly enhances the activity of Treg cells or merely boosts their overall number to restore balance between allergen-specific T cell subsets.[22,23] Our findings are significant in that they emphasize a correlation between PD-1, CTLA-4, TIGIT, and LAG-3-expressing $CD4^+$ T and Treg cells. Although continuous high-dose antigen stimulation plays a crucial role in the development of $CD4^+$ T cell exhaustion, the transition to the exhaustion phenotype is likely the result of a complex interaction between an altered immune microenvironment, inhibitory cytokines including interleukin-10 and transforming growth factor-beta, cell surface inhibitory and costimulatory receptors, and other cell populations such as Tregs.[9,13,22-24]

Soluble ICPs have been found to be upregulated in certain malignancies, and inhibiting them has been proven to enhance the anti-tumoral immune response.[25-29] However, in the field of pediatric allergy, there is limited research on soluble ICPs.[30] A previous study reported that serum sCTLA-4 levels reduced following administration of diverse Hymenoptera venom immunotherapy regimens.[30] The varying levels of these soluble ICPs in the plasma of healthy children made it challenging to interpret the impact of treatment duration on these molecules in our study. Previous studies have demonstrated that healthy adults have measurable levels of various soluble ICPs in their plasma, including sPD-1, sCTLA-4, sTIM-3, and sLAG-3.[26] Despite the absence of a significant correlation between the surface expression of ICPs on $CD4^+$ T and/or Treg cells and the soluble forms of them, we observed a strong correlation among their plasma levels. These results indicate that the regulation and function of soluble ICPs may vary from those of their $CD4^+$ T and/or Treg cell-bound counterparts. Therefore, additional studies are required to comprehend the origin, biological function, and baseline levels of soluble ICPs in healthy children and their impact on AIT.

While our study yielded promising results, it is crucial to acknowledge certain limitations when interpreting the results. First, this is a cross-sectional study that is devoid of longitudinal data on patients, which restricts our comprehensive understanding of the observed changes in checkpoint molecules in the cell populations during AIT. Furthermore, the sample size for each subgroup in our study was relatively small as a result of unforeseen obstacles that were encountered during the COVID-19 pandemic in accessing HDM-SCIT in Türkiye. Also, we did not conduct functional analyses for HDMs.

In conclusion, our cross-sectional study revealed that HDM-SCIT administration results in the upregulation of CTLA-4 and LAG-3 on $CD4^+$ T cells during the second and third years of treatment, respectively. Furthermore, we observed a strong correlation between the expression of CTLA-4 and TIM-3 on $CD4^+$ T cells. Our study also demonstrated correlations between the expression of PD-1, CTLA-4, TIGIT, and LAG-3 in both $CD4^+$ T and Treg cells. Nevertheless, larger-scale, comprehensive longitudinal studies are required to corroborate our findings and enhance our comprehension of the mechanisms related to exhaustion in the context of AIT.

Ethics Committee Approval: The Ethics Committee of İstanbul University, İstanbul Faculty of Medicine, approved this cross-sectional study (approval number: 2022/821, date: 13.05.2022).

Informed Consent: All children and their parents provided written informed consent to participate in the study.

Data Sharing Statement: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- Z.H.D., M.F.A., E.Ç.A.; Design- Z.H.D., E.Ç.A.; Supervision- Z.H.D., G.D., E.Ç.A.; Fundings- Z.H.D., E.Ç.A.; Materials- Z.H.D., M.F.A., A.E., A.D.S., Z.T., C.Ö.; Data Collection or Processing- Z.H.D., M.F.A., E.Y., Z.T., C.Ö.; Analysis or Interpretation- Z.H.D., A.E., A.D.S., E.Ç.A.; Literature Review- Z.H.D.; Writing- Z.H.D.; Critical Review- Z.H.D., E.Y., Z.T., C.Ö., G.D., E.Ç.A.

Conflict of Interest: The authors declare that they have no conflict of interest.

Funding: This work was supported by the Research Fund of Istanbul University (project number: TDP-2019-33335).

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