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Reduced CD4$^+$ terminally differentiated effector memory T cells in moderate-severe house dust mites sensitized allergic rhinitis patients

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Abstract

Background: Terminally differentiated effector memory (T_{EMRA}) T cells exert potent effector function after activation. The proportions of CD4$^+$ T cell subsets especially memory cells in allergic rhinitis (AR) patients sensitized to house dust mites (HDMs) have not been extensively studied.

Objective: This study aimed to compare the mean percentages and absolute counts of CD4$^+$ memory T cell subsets between: (i) non-allergic controls and AR patients; (ii) mild AR patients and moderate-severe AR patients.

Methods: Sensitization to *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* were determined in 33 non -allergic controls, 28 mild AR and 29 moderate-severe AR patients. Flow cytometry was used to determine the percentage of CD4$^+$ naïve (T_N; CD45RA$^+$CCR7$^+$), central memory (T_{CM}; CD45RA$^-$CCR7$^+$), effector memory (T_{EM}; CD45RA$^-$CCR7$^-$) and T_{EMRA} (CD45RA$^+$CCR7$^-$) T cells from the peripheral blood. The absolute counts of CD4$^+$ T cell subsets were obtained by dual platform method from flow cytometer and hematology analyzer.

Results: There were no significant differences in the mean percentages and absolute counts of CD4$^+$ T cell subsets between non-allergic controls and AR patients sensitized to HDMs. However, there were significant reduction in the mean percentage ($p = 0.0307$) and absolute count ($p = 0.0309$) of CD4$^+$ T_{EMRA} cells in moderate-severe AR patients compared to mild AR patients sensitized to HDMs and 13/24 (54.2%) moderate-severe AR patients sensitized to HDMs had persistent symptoms.

Conclusion: Reduction in the mean percentage and absolute count of CD4$^+$CD45RA$^+$CCR7$^-$ T_{EMRA} cells were observed in moderate-severe AR patients compared to mild AR patients in our population of AR patients sensitized to HDMs.

Key words: Allergic rhinitis, CD4$^+$ T cells, House dust mite, Memory T cells, T_{EMRA} cells

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Introduction

Allergic rhinitis (AR) is clinically defined as IgE-mediated inflammation of the nose after allergen exposure[1] associated with nasal symptoms including rhinorrhea, sneezing, nasal obstruction and nasal itchiness. The symptoms of AR usually bring impact to the quality of life by causing sleep disturbances, reduced work or school performance and abnormal daily activities. Comorbidities such as asthma, sinusitis, atopic dermatitis and otitis media are commonly associated with AR. AR is diagnosed mainly by allergic history and physical examination by the physician.[2] Skin prick test and specific IgE immunoassay from the blood samples are common and important allergic tests used to confirm the underlying allergic

sensitization that causes allergic rhinitis.

The severity of AR is classified into mild or moderate-severe based on the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines.[1] AR patients are classified as moderate-severe if they have any of these four impairments, i.e., sleep disturbance, impairment of daily activities including sport and leisure activities, impairment of work or school performance, and troublesome symptoms. AR patients are classified as having mild AR if they had none of the four impairments mentioned.[1]

The prevalence of AR is increasing in Malaysia and 35% of patients attending Otorhinolaryngology Clinics in Malaysia suffered from AR.[3] House dust mites (HDMs) are the most common allergens causing allergic sensitization among AR patients in Malaysia.[4,5] Previous study observed that 80% of AR patients in Malaysia were sensitized to HDMs based on skin prick test results[6] while another study found that *Dermatophagoides farinae* (*Der f*) was the common HDMs in 75.8% of adult AR patients.[7]

Memory T cells have been observed to play a role in AR.[8,9] Three subsets of $CD4^+$ memory T cells namely central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory (T_{EMRA}) cells can be distinguished by using CD45RA and CCR7 surface markers. A study in Sweden found that DNA methylation separated seasonal AR patients from non-allergic controls and suggested that this was due to the 41% reduction in the percentage of $CD4^+$ T_{CM} cells observed in AR patients compared to non-allergic controls.[10] The reduction of T_{CM} were also observed in another study in which the percentages of $CD4^+$ T_{CM} and $CD8^+$ T_{CM} were significant lowered in intermittent AR during birch pollen season.[11] However, another study on pollen-sensitive patients found a significant reduction in the percentage of $CD8^+$ T_{CM} but not in $CD4^+$ T_{CM} during pollen season, with an increase in total percentage of $CD4^+$ $CD45RA^+$ cells.[12]

There has been no study to date on memory T cell subsets in AR patients in the Malaysian population in which HDMs are the main allergens instead of pollens. This study aimed to compare the mean percentages and mean absolute counts of $CD4^+$ naïve (T_N), T_{CM}, T_{EM} and T_{EMRA} T cells between: (i) non-allergic controls and AR patients; (ii) mild AR patients and moderate-severe AR patients (in terms of AR severity).

Materials and Methods

Study Cohort and Data Collection

Adult AR patients (50 mild and 50 moderate-severe) and non-allergic controls (n = 50) aged between 18 years to 60 years old were recruited from April 2015 to May 2016. The severity of AR was diagnosed by physician based on the presence of clinical symptoms, nasal endoscopic examination and history of allergy. The classification of mild and moderate-severe AR was based on the ARIA guidelines.[1] AR patients were recruited from Otorhinolaryngology-Head and Neck Surgery (ORL-HNS) clinic in Hospital Universiti Sains Malaysia (HUSM) and non-allergic controls were recruited among staff and students from HUSM. Those who were pregnant, immunosuppressed, having autoimmune disorders, and/or taking oral steroids or cytotoxic drugs were not recruited. Verbal and written informed consent were given by all participants before enrolment. Clinical and demographic data including age, gender, comorbidities, smoking status and home location were collected. The protocol of this cross-sectional study was approved by the Human Research Ethics Committee of Universiti Sains Malaysia (USM/JEPeM/140390).

Blood Sample Processing

Ten ml of peripheral blood was collected from each AR patient and non-allergic controls into the vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson, Plymouth, UK). Two hundred µl of the blood was used for complete blood analysis using Sysmex XS-800i automated hematology analyzer (SYSMEX Corporation, Kobe, Japan). Then, plasma was isolated from the remaining blood and peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation method. Levels of total and specific IgE to *Der f* and *Dermatophagoides pteronyssinus* (*Der p*) in plasma were measured for each participant by using ImmunoCAP 100 (Phadia, Uppsala, Sweden). Specific IgE concentration level greater than or equal to 0.35 kUA/l was considered positive for sensitization to any allergen measured.

Determination of $CD4^+$ T cell Subsets by Flow Cytometer

Freshly isolated PBMCs were washed twice and suspended in phosphate-buffered saline (PBS) (SCIMEDX Corporation, Denville, NJ, USA). One hundred µl of cells with concentration of 5×10^6 cells/ml were taken from the suspension and stained with fluorochrome-conjugated antibodies: FITC anti-human CD4, APC anti-human CD45RA, PE anti-human CD197 (CCR7) and PE Mouse IgG2a, ĸ Isotype Control (FC) (all from BioLegend, San Diego, CA, USA). The cells were incubated on ice and in dark condition for 20 minutes. Next, cells were washed with PBS and resuspended in 400µl PBS. Then, the suspension was incubated on ice for 5 minutes in the dark and the cells were analyzed with BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA). Percentage of total $CD4^+$ T cells and its subsets were analyzed using FlowJo v10 (Ashland, OR, USA). The absolute count of total $CD4^+$ T cells were obtained by multiplying lymphocytes absolute count from hematology analyzer and percentage of total $CD4^+$ T cells from gated population.[13] Similarly, the absolute count of each $CD4^+$ T cell subset was obtained through multiplication of total $CD4^+$ T cells absolute count and percentage of $CD4^+$ T cell subsets from gated population.

Statistical Analysis

The categorical and numerical data for the demographic and clinical characteristics of non-allergic controls and AR patients were described using frequency (%) and mean (SD), respectively. The *p* values for the demographic and clinical characteristics data between non-allergic controls, mild AR patients and moderate-severe AR patients were obtained from one-way analysis of variance (ANOVA) with Tukey's multiple comparison test or Chi-square test. *P* values for the demographic and clinical characteristics data between mild AR patients and moderate-severe AR patients were obtained from independent t-test or Chi-square test. All statistical analyses of the demographic and clinical characteristics data were performed using IBM SPSS Statistics, version 22 (SPSS Inc, Chicago, Illinois, USA). The comparisons of the mean percentages and

absolute counts of total $CD4^+$ T cells and $CD4^+$ T cell subsets were done using independent t-test and were plotted in scatter plots, all in GraphPad Prism, version 6.03 (GraphPad Software Inc, San Diego, California, USA). *P* values of < 0.05 were considered to be statistically significant.

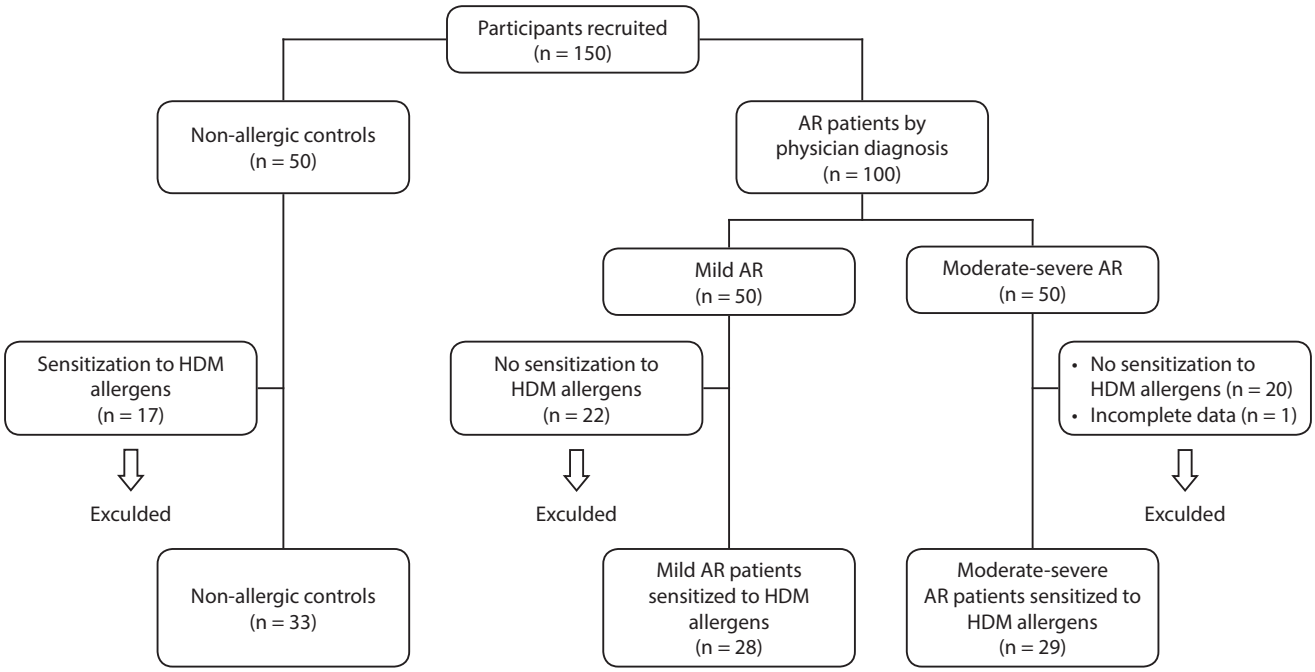
Results

Demographic and Clinical Data

Fifty non-allergic controls, 50 mild AR patients and 50 moderate-severe AR patients were recruited in this study. However, only 33 non-allergic controls were included in the analyses as others were excluded due to sensitization to the HDMs as measured by plasma specific IgE tests. 57% of our AR patients which consist of 28 mild AR patients and 29 moderate-severe AR patients, were sensitized to at least one of the HDM allergens tested. **Figure 1** illustrates the flowchart of the participants' recruitment.

The demographic and clinical characteristics of non-allergic controls and HDM sensitized AR patients are shown in **Table 1**. No significant difference in age was found between non-allergic controls, mild AR patients and moderate-severe AR patients. Non-allergic controls and AR patients were mostly female but there was no difference between gender, as well as body mass index, in non-allergic controls and AR patients. Asthma and atopic dermatitis comorbidities were reported in AR patients although no difference was observed in the incidence of these comorbidities between mild AR patients and moderate-severe AR patients.

There were no significant differences in specific IgE levels between mild and moderate-severe AR patients sensitized to HDM allergens measured. Total IgE level in plasma was significantly different between non-allergic controls, mild and moderate-severe AR patients ($p < 0.0001$). The significant difference in total IgE was found between non-allergic controls and mild



* House dust mite (HDM) allergens = *Dermatophagoides farinae, Dermatophagoides pteronyssinus*

Figure 1. Study flow chart of the selection of non-allergic controls and allergic rhinitis (AR) patients.

Table 1. Demographic and clinical characteristics of non-allergic controls and house dust mites sensitized allergic rhinitis patients

Characteristic	Non-allergic controls (n = 33)	Mild AR (n = 28)	Moderate-severe AR (n = 29)	*P-value*
Mean age (years) ± SD	28.0 ± 9.9	34.3 ± 13.4	28.6 ± 11.4	0.0804[a]
Gender				
Male	9 (27.3)	13 (46.4)	12 (41.4)	0.2720[c]
Female	24 (72.7)	15 (53.6)	17 (58.6)	
Mean BMI (kg/m^2) ± SD	22.7 ± 4.4	23.9 ± 4.8	23.8 ± 4.5	0.5109[a]

Abbreviations: AR, allergic rhinitis; BMI, body mass index; *Der f*, *Dermatophagoides farinae*; *Der p*, *Dermatophagoides pteronyssinus*; NA, not applicable; SD, standard deviation.
[a] One-way ANOVA; [b] Student's t-test; [c] Chi-square test

Table 1. Demographic and clinical characteristics of non-allergic controls and house dust mites sensitized allergic rhinitis patients

Characteristic	Non-allergic controls (n = 33)	Mild AR (n = 28)	Moderate-severe AR (n = 29)	*P-value*
Co-morbidities				
Asthma	NA	3 (10.7)	3 (10.3)	0.9640[c]
Atopic dermatitis	NA	3 (10.7)	3 (10.3)	0.9640[c]
Mean specific plasma IgE for house dust mites (kUA/l) for sensitized patients only, ± SD				
Der f	NA	34.3 ± 37.9 (n = 28)	37.7 ± 39.3 (n = 29)	0.7356[b]
Der p	NA	31.4 ± 33.5 (n = 27)	39.9 ± 39.6 (n = 28)	0.3973[b]
Mean total plasma IgE (kU/l) ± SD	119.0 ± 146.1	858.8 ± 746.9	597.8 ± 588.9	**< 0.0001**[a]
Currently smoking	1 (3.0)	2 (7.1)	0 (0.0)	0.3210[c]
Home location				
Urban	15 (45.5)	17 (60.7)	11 (37.9)	0.2150[c]
Rural	18 (54.5)	11 (39.3)	18 (62.1)	
Symptoms classification (%)				
Intermittent	NA	96.0 (n = 24)	45.8 (n = 11)	**< 0.0001**[c]
Persistent	NA	4.0 (n = 1)	54.2 (n = 13)	

Abbreviations: AR, allergic rhinitis; BMI, body mass index; *Der f*, *Dermatophagoides farinae*; *Der p*, *Dermatophagoides pteronyssinus*; NA, not applicable; SD, standard deviation.
[a] One-way ANOVA; [b] Student's t-test; [c] Chi-square test

AR patients ($p < 0.0001$), and between non-allergic controls and moderate-severe AR patients ($p = 0.0020$). The number of participants with reported current smoking was low in each group, with no significant difference between the groups. In addition, there was no significant difference in home location (urban or rural) between non-allergic controls and AR patients. However, there was a significant difference in symptom classification of intermittent or persistent between mild AR patients and moderate-severe AR patients sensitized to HDMs ($p < 0.0001$). The demographic and clinical characteristics of all AR patients recruited based on physician's diagnosis.

Populations of Total CD4⁺ T cells and CD4⁺ T cell Subsets

Figure 2 shows the gating strategy used to identify the populations of total CD4⁺ T cells and CD4⁺ T cell subsets. Firstly, the lymphocytes population was gated based on forward-scattered and side-scattered light (FSC/SSC) and the CD4⁺ T cells population was selected next with CD4 marker and FSC. Then, the CD4⁺ T cell subsets population was gated with CD45RA and CCR7 markers, and with the use of isotype control to identify the CCR7⁺ T cells population. The CD4⁺ T cell subsets were identified based on each subset characteristic markers: T_N (CD45RA⁺ CCR7⁺), T_{CM} (CD45RA⁻ CCR7⁺), T_{EM} (CD45RA⁻ CCR7⁻) and T_{EMRA} (CD45RA⁺ CCR7⁻). The percentage of total CD4⁺ T cells and CD4⁺ T cells subsets were obtained directly from the gated populations. Representative flow cytometric graphs of CD4⁺ T cell subsets in non-allergic controls, mild and moderate-severe AR patients are shown in **Figure 3A**. The mean percentages of total CD4⁺ T cells in non-allergic controls and AR patients sensitized to HDMs were 37% and 33% respectively. The mean absolute counts of total CD4⁺ T cells in non-allergic controls and AR patients sensitized to HDMs were 903 cells/µl and 869 cells/µl respectively. There was no significant difference in the percentage ($p = 0.0826$) and absolute count ($p = 0.6770$) of total CD4⁺ T cells between non-allergic controls and AR patients sensitized to HDMs as shown in **Figure 3B** and **Figure 3C**.

Analyses of CD4⁺ T cell Subsets

As depicted in **Figure 1**, AR patients have been defined based on: (i) physician's diagnosis only; and (ii) physician's diagnosis with positive sensitization to HDMs based on specific IgE test. Stratified analyses of the percentage and absolute count of CD4⁺ T cell subsets have been conducted to yield results for both definitions of AR patient, comparing between non-allergic controls and AR patients and subsequently in terms of AR severity. The results of CD4⁺ T cell subsets analyses are described in the next sections.

Percentage and Absolute Count of CD4⁺ T cell Subsets in AR Patients With Physician's Diagnosis Only

There was no significant difference in the percentages and absolute counts for all four CD4⁺ T cell subsets (T_N, T_{CM}, T_{EM} and T_{EMRA}) when compared between non-allergic controls to mild and moderate-severe AR patients diagnosed by physician.

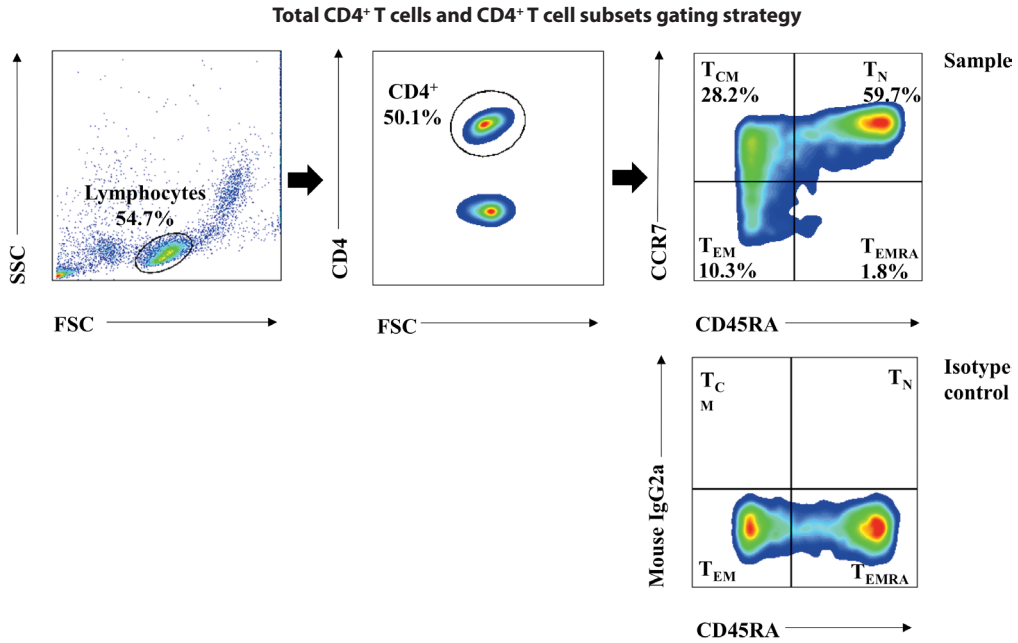


Figure 2. CD4⁺ T cell subsets gating strategy. FSC, forward-scattered; SSC, side-scattered.

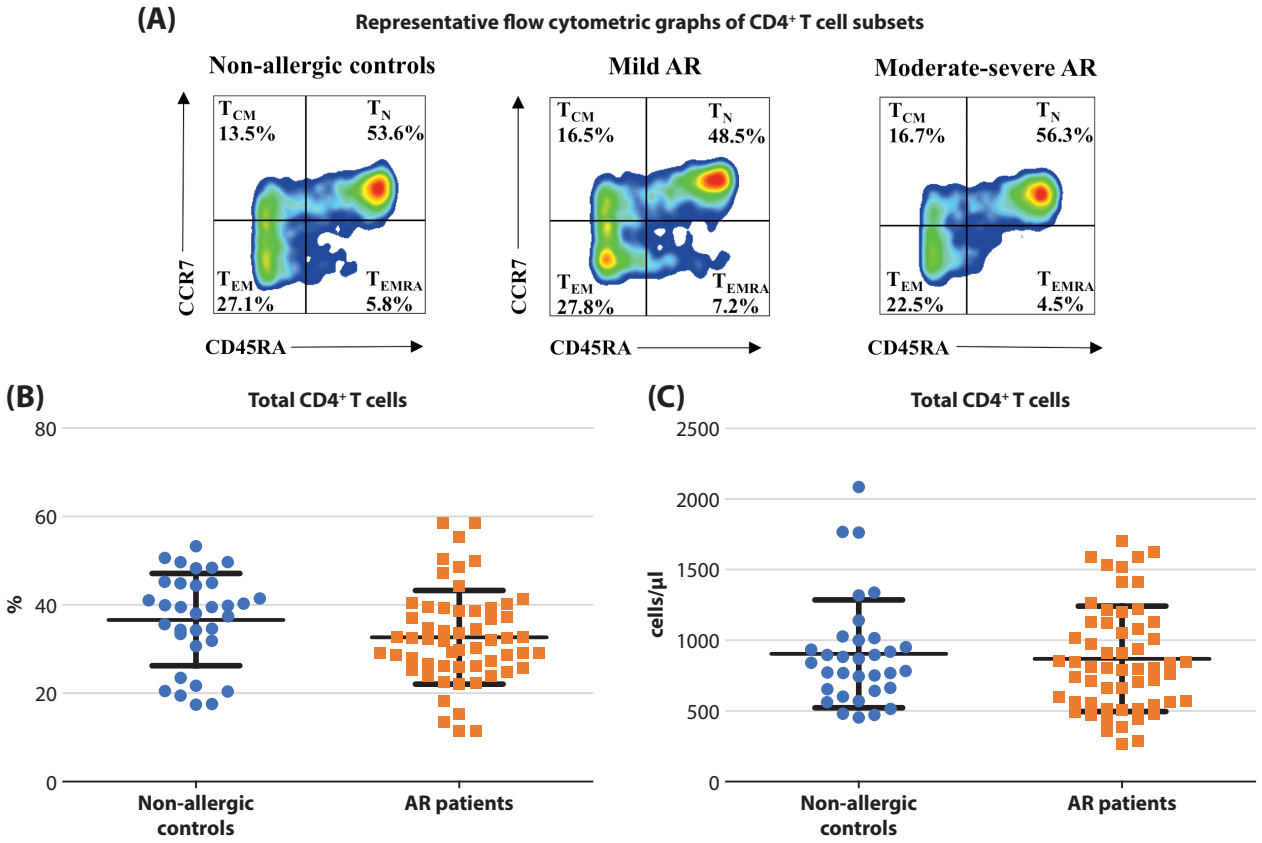


Figure 3. (A) CD4⁺ T cell subsets representative figures. (B) Mean percentage and (C) mean absolute count of total CD4⁺ T cells between non-allergic controls and allergic rhinitis (AR) patients sensitized to house dust mites.

Percentages and Absolute Counts of CD4⁺ T cell Subsets in AR patients Sensitized to HDM Allergens

The analyses on AR patients sensitized to HDMs showed no significant differences in the percentages of T_N, T_{CM} and T_{EM} subsets between mild and moderate-severe AR patients sensitized to HDMs (**Figure 4A–4C**). However, a significant reduction in the percentage of CD4⁺ CD45RA⁺ CCR7⁻ T_{EMRA} cells (p = 0.0307) was found in moderate-severe AR patients compared to mild AR patients (**Figure 4D**).

Similarly, there were no significant differences in the absolute counts of T_N, T_{CM} and T_{EM} subsets between mild and moderate-severe AR patients sensitized to HDMs (**Figure 5A-5C**),

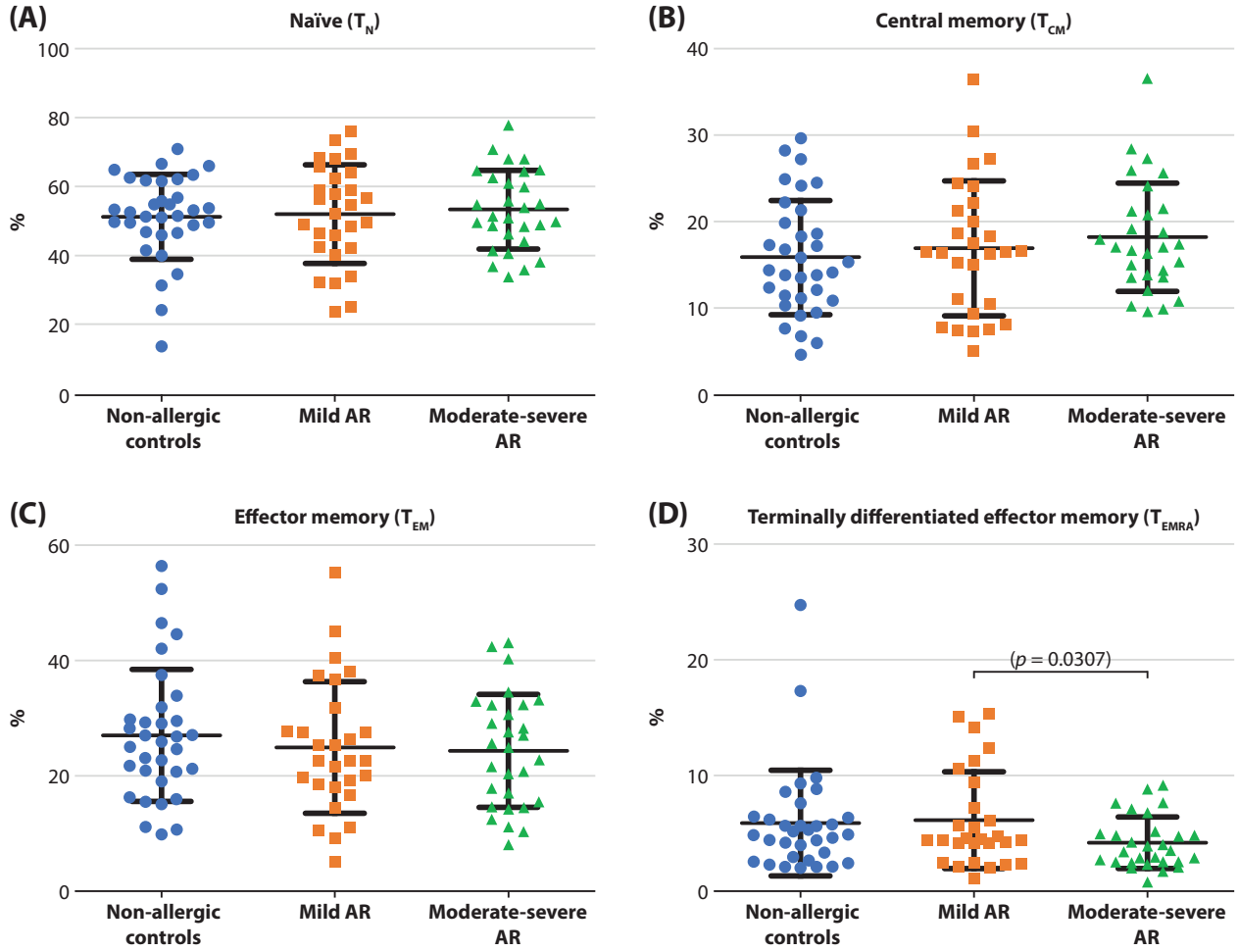


Figure 4. Mean percentages of (A) naïve (T_N), (B) central memory (T_{CM}), (C) effector memory (T_{EM}), (D) terminally differentiated effector memory (T_{EMRA}) $CD4^+$ T cells between non-allergic controls, mild allergic rhinitis (AR) patients and moderate -severe AR patients sensitized to house dust mites.

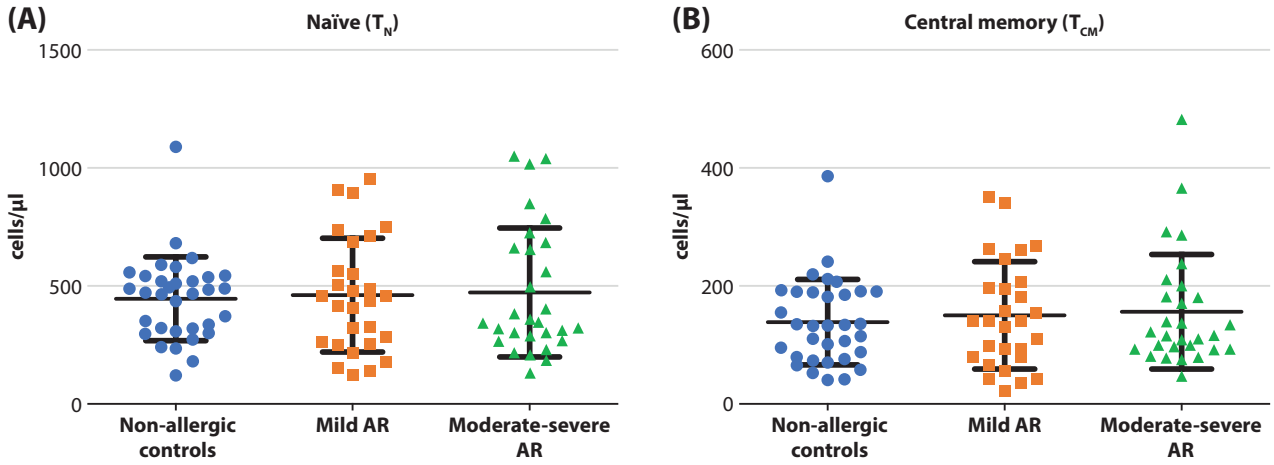


Figure 5. Mean absolute counts of (A) naïve (T_N), (B) central memory (T_{CM}), (C) effector memory (T_{EM}), (D) terminally differentiated effector memory (T_{EMRA}) $CD4^+$ T cells between non-allergic controls, mild allergic rhinitis (AR) patients and moderate-severe AR patients sensitized to house dust mites.

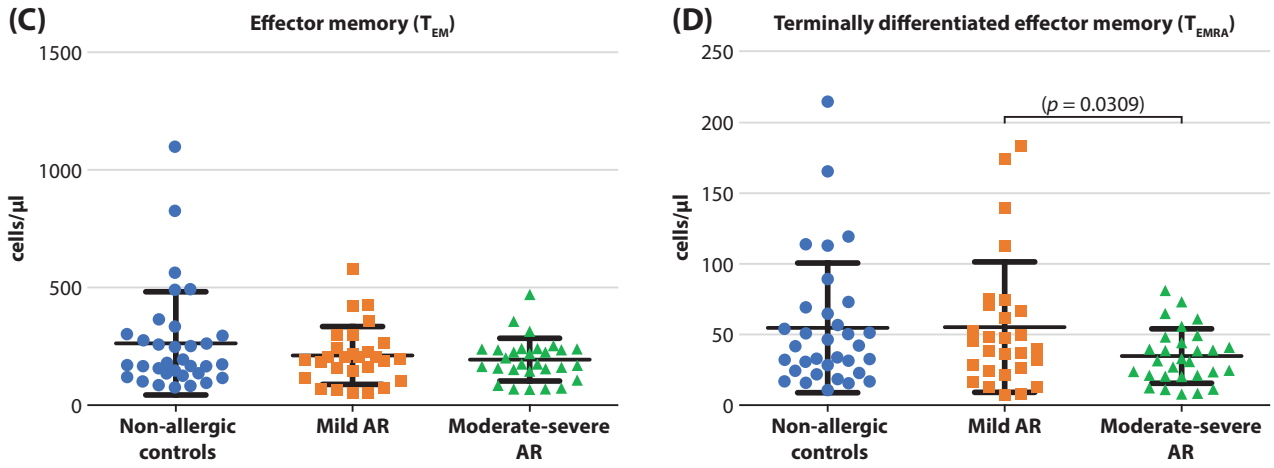


Figure 5. (Continued)

while the absolute count of $CD4^+$ $CD45RA^+$ $CCR7^-$ T_{EMRA} cells was significantly reduced ($p = 0.0309$) in moderate-severe AR patients with 34 cells/µl compared to mild AR patients with 55 cells/µl (**Figure 5D**).

The analysis of intermittent and persistent symptoms in mild AR patients and moderate-severe AR patients with HDMs sensitization showed significant difference ($p < 0.0001$), with the majority of mild AR patients [24/25 (96.0%)] had intermittent symptoms while most moderate-severe AR patients [13/24 (54.2%)] had persistent symptoms.

Discussion

An allergic response in AR starts with the deposition of allergens into the nasal mucous membrane.[14] These allergens are taken up and processed by antigen presenting cells and then presented to the T cells, which will be activated. Th2 cells are the main T cell subsets involved in allergic diseases, which release effector cytokines such as IL-4, IL-5 and IL-13 that will interact with B cells for the synthesis of allergen-specific IgE. These IgE will bind to the high affinity receptor on the surface of mast cells, leading to the release of mediators that results in nasal symptoms of rhinorrhea, sneezing, nasal itchiness and nasal blockage.[14]

Significant increase in IL-4 and IL-5 has been observed in children with AR compared to non-allergic controls.[15] The frequency of IL-4^+ $CD4^+$ T cells were significantly increased in the peripheral blood of seasonal AR patients sensitized to grass pollen after nasal allergen provocation which suggest that the local nasal provocation results in systemic activation of T_H2 cytokines in AR.[8]

In this study, there were no significant differences found in the percentage and absolute count of total $CD4^+$ T cells in peripheral blood between non-allergic controls and AR patients sensitized to HDMs. This is in accordance with previous studies using peripheral blood samples in seasonal AR patients that showed no significant difference in total $CD4^+$ T cells between AR patients and non-allergic controls.[10-12] The difference in these observations may reflect the local effect at nasal mucosa compared to systemic effects in peripheral blood of total amount of $CD4^+$ T cells, although both local and systemic events are important in the pathogenesis of allergic rhinitis.[16-18]

We observed that the mean percentages of total $CD4^+$ T cells in non-allergic controls and AR patients sensitized to HDMs were within the normal range in healthy adults of several populations including United States,[19] Iran[20] and Switzerland.[21] Accordingly, the mean absolute count of total $CD4^+$ T cells was also within the normal range compared to healthy adults from these populations and adults in Singapore.[22] These observations showed that the percentage and absolute count of total $CD4^+$ T cells in peripheral blood do not distinguish AR patients from non-allergic controls.

$CD4^+$ memory T cells responses have been observed in allergic airway inflammation during peptide immunotherapy,[23] and in a study on moderate-severe seasonal AR patients with grass pollen sensitization, in which systemic activation of $CD4^+$ memory T cells with increased $CD4^+$ memory T cells in the peripheral blood occured after nasal allergen provocation.[8] Furthermore, a study that utilized major histocompatibility complex class II peptide tetramers showed that $CD4^+$ memory T cells were detected against HDMs of *Der p* species and birch allergens in AR patients.[9] These findings indicated a role of $CD4^+$ memory T cells in AR and other allergic diseases.

The three subsets of $CD4^+$ memory T cells namely T_{CM}, T_{EM} and T_{EMRA} were distinguished by using CD45RA and CCR7 surface markers in this study. Both $CD4^+$ T_{CM} and T_{EM} cells which are $CD45RA^-$, are involved during the secondary exposure to the same pathogens. T_{CM} cells which express CCR7 efficiently stimulate dendritic cells and B-cells, and generate effector cells in the secondary lymphoid organs while T_{EM} cells that lack the expression of CCR7 mediate rapid inflammatory reactions or cytotoxicity in the peripheral tissues.[24] $CD4^+$ T_{EMRA} cells that re-expressed CD45RA which differentiate them from $CD4^+$ T_{CM} and T_{EM} cells are suggested to be generated in the bone marrow because they are found to be significantly higher in the bone marrow than blood and possess cytotoxic potential as they express high levels of granzyme B and perforin capable to exert potent effector functions.[25-30]

Previous studies have observed a significant reduction in the percentage of $CD4^+$ T_{CM} cells in AR patients compared to non-allergic controls.[10,11] The findings of the aforementioned studies were different to our results which showed no difference in the percentage and absolute count of $CD4^+$ T_{CM} cells between

non-allergic controls and our cohort of AR patients. We suggest that the difference in the allergens AR patients were sensitized to yielded differing results as more than half of our AR patients were sensitized to HDMs of *Dermatophagoides* species. These are perennial allergens present throughout the year while AR patients in the other populations are sensitized to birch and grass pollen which are seasonal allergens present during certain months annually. In addition, the discrepancy could be due to smaller sample sizes in these two studies, and there was no data on the absolute count of $CD4^+$ T_{CM} cells and the percentage of $CD4^+$ T_{CM} cells was also not compared according to the severity of AR.

In this study, we found that the percentage and absolute count of $CD4^+$ T_{EMRA} cells was significantly reduced in moderate-severe AR patients compared to mild AR patients sensitized to HDMs. Harari et al[31] suggested that $CD4^+$ T_{EMRA} population consists of terminally differentiated cells and is produced in condition of repetitive antigen exposure and low antigen load. These cells exerts potent effector function but are prone to death following antigen activation.[32] Therefore, our observation of reduced T_{EMRA} cells in moderate-severe AR patients may be due to cell death after frequent activation of these cells as the majority of the moderate-severe AR patients had persistent symptoms while only a few mild AR patients had persistent symptoms. The higher rate of persistent symptoms in moderate-severe AR patients could suggest constant antigen exposure and activation that exhausted the $CD4^+$ T_{EMRA} cells.

A study that characterized human $CD4^+$ T cells showed that $CCR7^+CD45RA^-CD28^+$ subset produced only IL-2 while $CCR7^-CD45RA^-CD28^+$ and $CCR7^-CD45RA^+CD28^+$ subsets produced IL-2, IFN-γ and TNF-α.[25] IL-2 is crucial for the differentiation of $CD4^+$ T cells into defined effector T cell subsets following antigen-mediated activation and also for the maintenance of regulatory T cells[33] while IFN-γ is important for the resolution of allergic-related immunopathologies.[34] Similarly, another study also showed that $CD4^+CCR7^+CD45RA^-CD27^+CD28^+$ T_{CM} cells produced only IL-2 cytokine whereas the production of IFN-γ and IL-4 were observed in $CD4^+CCR7^-CD45RA^-CD27^+CD28^+$ T_{EM} cells.[35] As for $CD4^+$ T_{EMRA} cells in both $CCR7^-CD45RA^+CD27^+CD28^+$ and $CCR7^-CD45RA^+CD27^-CD28^-$ subsets, they are found to produce IFN-γ only and a very low number of cells produce IL-2.[35]

Among the limitations of our study is the lack local edaphic T cell samples from the nasal mucosa for comparison with the data from peripheral blood, which could show differences between local and systemic effect. However, data from the peripheral blood may be useful for comparison in future studies and there is still a big literature gap in the difference of $CD4^+$ memory T cell subsets in different severity of HDM sensitized AR. Another limitation of our study is the lack of CD3 marker in the characterisation of the memory cells described as there may be contamination with monocytes which express intermediate levels of CD4, within the lymphocytes population. However, measures including strict gating of lymphocyte population and selection of high CD4 intensity cell population, excluding those with intermediate CD4 intensity were taken. We also did not measure levels of cytokines especially those related to memory T cells e.g. IL-2. Therefore, the findings need to be interpreted with care taking into consideration the limitations.

Conclusion

This study showed that the percentage and absolute count of $CD4^+$ T_{EMRA} cells in peripheral blood were significantly reduced in moderate-severe AR patients compared to mild AR patients sensitized to HDMs in the Malaysian population. This observation, together with the association of persistent symptoms in moderate-severe AR patients, could be due to cell death following frequent cellular activation. However, further studies to validate this finding and to compare it to nasal mucosa samples in order to confirm the role of T_{EMRA} in AR is warranted.

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