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Unconventional T cells in health and diseases

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1. INTRODUCTION

1.1. Immune system and its cellular components

The immune system consists of two parts that differ in the speed and specificity of their responses, known as innate and adaptive immunity. Innate immunity, as the name suggests, is inborn and natural. Its cellular components include neutrophils, monocytes, natural killer cells, dendritic cells, and soluble components such as complement proteins, cytokines, and acute phase proteins. Innate immunity responds rapidly to pathogen-associated molecular patterns and damage-associated molecular patterns. However, it lacks specificity and can sometimes damage healthy tissue. The cellular elements of adaptive immunity include T and B lymphocytes. B cells develop in the bone marrow and encounter antigens in lymphoid tissues, leading to a B cell response. T cells also develop in the bone marrow. Still, their maturation, including negative and positive selection and TCR rearrangement, occurs in the thymus through the processes of variable (V), diversity (D), and joining (J) recombination. Most T cells have a T cell receptor (TCR) composed of an α and β chain, while a minority of T cells also have γ and δ chains due to TCR rearrangement during development in the thymus.

Conventional T cells are known for recognizing peptide antigens presented on the major histocompatibility complex (MHC). Since MHC is highly polymorphic in humans, peptide antigens can be presented through either endogenous or exogenous pathways. However, this conventional T cell paradigm does not apply to unconventional T cells.

1.2. Unconventional T cells (Tu cells)

While our understanding of the thymic development of unconventional T cells (Tu cells) is limited, the role of the thymus is crucial in shaping their development. Tu cells recognize specific self and evolutionarily conserved molecules found in microbial agents through their invariant αβ and γδ T cell receptors (TCR). These receptors can identify non-peptide antigens like lipid antigens, non-peptide micro-bacterial ligands, and microbial riboflavin-derived antigens. They recognize these antigens when presented on non-polymorphic antigen presentation molecules like CD1d. In healthy individuals, Tu cells comprise a relatively small portion of the total T cell population in peripheral blood (PB).

Unconventional T cells with αβ TCRs can be classified based on their recognition of non-polymorphic antigen-presenting molecules such as CD1a, CD1b, CD1c, CD1d, HLA E, and MR1. Tu cells that recognize the CD1d molecule can be further divided into type I and type II, with type I also referred to as "invariant natural killer T cells." Mucosa-associated invariant T cells recognize riboflavin-derived antigens presented on MR1. Another population of unconventional T cells, γδ T cells, arises from somatic recombination during TCR rearrangement through V, D, and J segments. In humans, the cumulative frequency of Tu cells (iNKT, γδ T, and MAIT cells) varies by location: they represent 11% in the skin, 38% in the liver, 17% in the intestine, 6% in the lung, and 6% in the blood.

1.2.1. Mucosa Associated Invariant T (MAIT) cells

Mucosa-associated invariant T cells exhibit a unique ability to detect microbial antigens originating from riboflavin. These are presented via the non-polymorphic MHC class I-like protein MR1 molecule associated with β2-microglobulin. The T-cell receptor (TCR) repertoire of MAIT cells remains consistent across various species. Upon activation, these cells can initiate responses through TCR-dependent and TCR-independent pathways, ultimately leading to innate-effector responses. The TCR of MAIT cells consists of Vα7.2 (TRAV1-2) joined with Jα33 (TRAJ33). Within the riboflavin biosynthesis pathways, 5-A-RU (5-amino-6-D-ribitylaminouracil) acts as an intermediate molecule, facilitating the synthesis of lumazine- and pyrimidine-based antigens.

1.2.2. Invariant natural killer T cells (iNKT cells)

Invariant Natural Killer T cells, or iNKT cells, represent a distinctive subset of T cells renowned for their capacity to recognize lipid antigens via the non-polymorphic major histocompatibility complex class-I-like molecule, CD1d. The T cell receptor (TCR) of iNKT cells is defined by an invariant TCR α chain, formed by the unique Vα24-Jα18 gene arrangement, in conjunction with TCRβ chains Vβ11. These iNKT cells demonstrate a remarkable versatility in identifying lipid antigens, including glycolipids and diacylglycerol, sourced from diverse origins, encompassing commensal and pathogenic microorganisms and self-reactive lipid antigens. Despite their relatively low percentage among circulating T cells, the reduced frequency of iNKT cells in peripheral blood has been documented in several pathological conditions, underscoring their significance.

1.2.3. Gamma delta T cells (γδ T cells)

Gamma delta (γδ) T cells constitute a distinct and noteworthy subset of T lymphocytes, distinguished by their unique T cell receptor (TCR) composition. The γ gene for the human TCR chain is situated on the short arm of chromosome 7 (7p15), while the δ gene for the TCR chain is located within the chromosome 14 locus. These γδ T cells exhibit a diverse repertoire of TCR genes, encompassing eight variable (V) δ gene segments (Vδ1 - Vδ8) and seven variable (V) γ gene segments (Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9, and Vγ11) available for rearrangement. In human peripheral blood, up to 95% of γδ T cells comprise the TCR Vγ9Vδ2 gene segment, while Vδ1 and Vδ3 γδ T cells constitute only a tiny fraction of the T cell population. These distinctive T cells are prominently distributed in the skin and gastrointestinal tissues, underscoring their critical role as the frontline defense against infections. What distinguishes γδ T cells is their capacity to recognize cells exposed to non-peptide, low-molecular-weight micro bacterial ligands, often termed phospho-antigens.

1.3. Age-dependent descent of the immune system and gender bias

Immunosenescence significantly impacts the immune system, primarily driven by mammal thymus involution. Thymus involution begins early, with a reduction in thymic epithelial cells and an accumulation of adipose tissues starting in the first year of life. Sex hormones play a crucial role in thymus involution, with males experiencing a more rapid decline, mainly due to the impact of androgens. Additionally, thymic involution mediated by progesterone occurs during pregnancy, which is essential for successful pregnancies. Several factors contribute to the differences in the immune system between men and women, including reproductive status, sex chromosomal genes, sex hormones, and environmental factors.

1.4. MAIT cells, Vα7.2+/CD161- T cells and mucosal/skin barriers

Currently, human MAIT cells are typically identified using 5-OP-RU loaded MR-1 tetramers or by the co-expression of CD161++ along with TCR Vα7.2-Jα33. Within the Vα7.2+ T cell compartment, subsets with CD161- expression can also be distinguished. CD161, a C-type lectin-like protein, is a type-II transmembrane protein expressed on approximately 25% of human adult T cells, encompassing CD4 and CD8 subsets. CD161 expression is generally associated with memory cells. Within the

Vα7.2+ T cell compartment, the subsets of CD161- (Vα7.2+/CD161- T cells) and CD161+ (MAIT cells) cells have distinct gene expression profiles despite sharing Vα7.2 expression.

MAIT cells possess an array of tissue-homing chemokine receptors, indicating their capacity to migrate into tissues in response to local inflammation. These versatile cells can be activated through T cell receptor (TCR)-dependent and TCR-independent mechanisms. MAIT cells play an essential function in interacting with commensal bacteria via TCR-dependent pathways. They protect the mucosal barrier by generating key factors like IL-17, IL-22, VEGF, and TGFβ, aiding tissue repair. The precise function of Vα7.2+/CD161- T cells remains uncertain. However, there is a notable increase in their presence in the PB of chronic HCV and HIV-1 infected patients compared to healthy controls, and MAIT cells seem to be compromised in this context.

This burgeoning field of research delves into the intricate interplay between MAIT cells and various skin conditions, shedding light on their potential roles as defenders and instigators of cutaneous health. MAIT cells have attracted considerable scientific interest owing to their potential in addressing skin infections and influencing the progression of inflammatory skin disorders. More importantly, they are crucial in maintaining skin homeostasis, reassuring us about the body's natural defense mechanisms. Unraveling the involvement of MAIT cells in skin diseases offers fresh insights and suggests a potential avenue for developing innovative therapeutic approaches.

1.5. Atopic Dermatitis, MAIT cells and Vα7.2+/CD161- T cells

Atopic dermatitis (AD) is a chronic inflammatory skin condition with a complex and multifaceted etiology. While genetic and environmental risk factors have been identified, the precise cause of the disease remains unclear. It arises from a combination of factors, including compromised skin barrier function, microbiota dysbiosis leading to increased colonization by Staphylococcus aureus, IgE-mediated hypersensitivity, and dysregulated immune responses. The inflammatory milieu in AD involves a complex interplay of cytokines, implicating dendritic cells, Langerhans cells, mast cells, and various immune cell subsets such as T helper (Th) 2, Th22, Th17, Th1 cells, and innate lymphoid cells

(ILC2). During the acute phase, Th2/22 cells drive vigorous type 2 inflammation, while in the chronic phase, the prevailing inflammation shifts from Th2 to Th1.

MAIT cell subsets play an active role in the pathogenesis of skin disorders such as psoriasis and hidradenitis suppurativa, where they serve as an additional source of IL-17, exacerbating the condition. The influence of MAIT cells on AD in humans is still under investigation, and the role of Vα7.2+/CD161- T cells remains uncertain.

1.6. Problem Statement

The pro and anti-inflammatory roles of unconventional T (Tu) cells have been thoroughly investigated in various pathogenic conditions, encompassing infections, immune-mediated disorders, autoimmune diseases, and cancers. However, the impact of age and gender on the immunosenescence of invariant Natural Killer T (iNKT), γδ T, and Mucosa-Associated Invariant T (MAIT) cells collectively remains largely unexplored, and the establishment of reference ranges for these cell populations is an unresolved challenge. This knowledge gap hampers the widespread utilization of Tu cells in research and standard laboratory diagnostics. Our hypothesis posits that Tu cell populations undergo variations between genders and across different age groups, and these changes are indicative of an immunosenescent process that warrants in-depth investigation and understanding.

The growing body of evidence supports the significance of Mucosa-Associated Invariant T (MAIT) cells in various skin disorders. Atopic dermatitis (AD) is one of the most prevalent inflammatory skin conditions in developed countries, characterized by a multifactorial and intricate pathophysiological mechanism. Despite the intricacies of AD, there has been limited exploration into the role of peripheral blood MAIT cells in its progression. MAIT cells, a crucial link between the innate and adaptive arms of the immune system, are more abundant in peripheral blood than other unconventional T-cell subsets. Alongside CD161+ MAIT cells, CD161- T cells are also present within the Vα7.2+ T cell compartment. However, their specific role remains to be thoroughly investigated. Based on observations, we hypothesize that MAIT cells, Vα7.2+/CD161- T cells, and their subsets likely play a pivotal role in the

pathogenesis of AD. Understanding their involvement in this context is crucial for shedding light on the underlying mechanisms driving this prevalent and complex skin condition.

MAIT cells can be activated via TCR-dependent and cytokine-dependent mechanisms and can secrete a range of cytokines and cytotoxic effector molecules upon activation. The response of MAIT cells to different stimuli, the effects of immunomodulatory drugs on MAIT cells, and the comparison of MAIT cells with other Vα7.2+/CD161- T subsets under the same conditions are not well understood. Therefore, we aimed to investigate the response of MAIT cells to various stimuli, including PMA/Ionomycin, 5-OP-RU, and 5-OP-RU/IL-12/IL-33, and the effects of immunomodulatory drugs (cyclosporin A and Vitamin D3) on MAIT cells based on different stimulatory conditions *in vitro*. Additionally, the study aims to compare the response of MAIT cells with Vα7.2+/CD161- T cells under the same conditions. Understanding these factors can provide insights into the role of MAIT cells in the immune response and their potential as a therapeutic target.

2. AIMS

i. The reference range of unconventional T cells (iNKT, γδ T cells, and MAIT cells) in healthy populations was unknown, making it difficult to study them under clinical conditions. Our objective was to determine age—and gender-dependent reference ranges, including the absolute number and percentage of total T cells in peripheral blood.

ii. The decline of immune cells due to aging significantly impacts the immune system, primarily driven by thymus involution. The immunosenescence of unconventional T cells (iNKT, γδ T cells, and MAIT cells) has not been studied yet, and we aimed to investigate immunosenescence in these populations.

iii. Atopic dermatitis (AD) is one of developed nations' most prevalent inflammatory skin conditions. MAIT cells are a crucial link between the innate and adaptive arms of the immune system. However, their role is unknown in AD. Alongside CD161- T cells are also present within the Vα7.2+ T cell compartment with almost no information. We aim to investigate the involvement of human peripheral blood MAIT and $V\alpha7.2^{+}/CD161^{-}$ T cells in atopic dermatitis.

iv. Mucosa-associated invariant T cells can be activated via TCR-dependent and independent mechanisms. The response of MAIT cells to different stimuli is still under exploration. Cyclosporin A (CsA) is a calcineurin inhibitor frequently used to treat atopic dermatitis, and Vitamin D3 (VitD) immunomodulates the functional activities of innate and adaptive immune cells targeting the vitamin D receptor (VDR). We aimed to investigate the impact of immunomodulatory drugs (CsA and VitD) on MAIT and $V\alpha7.2^{+}/CD161^{-}$ T cells in different stimulatory conditions. Furthermore, we planned to compare MAIT and $V\alpha7.2^{+}/CD161^{-}$ T cells under the same conditions.

3. METHODS

3.1. Flow cytometry

3.1.1. Monoclonal antibodies (MAbs)

Multiparametric flow cytometry experiments were conducted using a pre-titrated mouse anti-human monoclonal antibody panel. The antibodies were sourced from various manufacturers, including Beckman Coulter (Brea, CA, USA), Dako (Glostrup, Denmark), Biolegend (San Diego, CA, USA), BD/Pharmingen (Franklin Lakes, NJ, USA), Exbio (Prague, Czech Republic), and MACS Miltenyi Biotec.

3.1.2. Lyse-wash protocol of flow cytometric staining

The stain, lyse, and wash method was utilized for cell surface staining. In a FACS tube, 100 µL of peripheral blood (PB) was mixed with pre-titrated monoclonal antibodies (mAbs) and incubated for 15 minutes in the dark at room temperature (20-22°C). Subsequently, 1 mL of 1x RBC lysis buffer (BD FACS lysing solution) was added and incubated for 10 minutes. After incubation, tubes were washed with 1 mL of phosphate-buffered saline and centrifuged at 1500 rpm for 5 minutes. The resulting pellet was then resuspended in 400 µL of 1% paraformaldehyde.

3.1.3. Sample acquisition on a flow cytometer

All data acquisitions were conducted on a BD FACS Canto II flow cytometer. To monitor equipment performance, routine internal quality control assessments were executed using a cytometer setup and tracking beads (CS&T).

3.2. Peripheral blood mononuclear cells stimulation and intracellular staining

3.2.1. Peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells were isolated from 6-8 mL of PB collected in a heparin tube using the density gradient centrifugation method. 3 mL of Ficoll-Hypaque was added to a 15 mL falcon tube, and heparinized blood was layered gently upon it. The tubes were centrifuged at 1500 RPM for 30 minutes at room temperature. Post-centrifugation, only a buffy layer was isolated in another tube and

dissolved in RPMI-1640 media containing 1% streptomycin/penicillin and 10% fetal bovine serum. Cells were rewashed with centrifugation at 1500 RPM for 5 minutes before performing cell count and were adjusted around $1x10^6 – 2x10^6$ cells/100 µl.

3.2.2. PBMC stimulation with phorbol 12-myristate 13-acetate (PMA)/Ionomycin

Peripheral blood mononuclear cells were stimulated with 30 ng/mL of phorbol 12-myristate 13-acetate (PMA) and 1 µg/mL of ionomycin. The total volume was adjusted to 1 mL by adding RPMI medium, and the FACS tube was then incubated for four hours at 37°C with a 5% CO2 incubator. Following the 40-minute incubation period with PMA/Ionomycin, 20 µg/mL of Brefeldin-A was added and gently vortexed. Incubation was continued for an additional 3 hours and 20 minutes.

3.2.3. Intracellular staining protocol

Cells were first washed with PBS, followed by adding fluorescently labeled cellular surface antibodies at the appropriate concentration. The mixture was vortexed and then incubated for 15 minutes in the dark at room temperature. Subsequently, 100 µL of Intraprep permeabilization buffer 1 (Beckman Coulter, Brea, CA, USA) was added, vortexed, and incubated for another 15 minutes. The tubes were then washed with 4 mL of PBS through centrifugation (1500 RPM for 5 minutes), and the supernatant was decanted. Next, 100 µL of Intraprep permeabilization buffer 2 was added and incubated for 5 minutes without vortexing. Following this, fluorescently labeled cytoplasmic antibodies were added and incubated for 30 minutes in the dark at room temperature. After incubation, cells were washed with 4 mL of PBS, centrifuged, and resuspended in 400 µL of paraformaldehyde. The acquisition was performed as described above in 3.1.3. section.

3.3. Ethics Clearance

The institutional review board of the Faculty of Medicine at the University of Debrecen approved the study (DE RKEB/IKEB 5404–2020).

3.4. Establishing the reference range of Tu

3.4.1. Blood sample collection and complete blood cell count

Three mL peripheral blood (PB) samples of healthy controls (HC) were collected through venipuncture in a BD vacutainer tube containing tri-potassium ethylene-diamine tetra acetic acid. A complete blood count (CBC) was performed using a six-part hematology analyzer Siemens ADVIA® 2120i.

3.4.2. Exclusion criteria

We meticulously defined strict exclusion criteria to evaluate HC samples, known as the SENIEUR protocol. This protocol, which was modified due to the COVID-19 pandemic situation, ensured that only the most reliable data was included in our study. Briefly, any infections (<6 weeks), inflammation (acute and chronic), autoimmune disorder, human immunodeficiency virus infection, hepatitis B virus infection, hepatitis C virus infection, SARS CoV-2 infection (within one year), diabetes mellitus, immunosuppressive drugs, alcoholism and drug abuse, current pregnancy or breastfeeding, malignancy, immunomodulatory therapy, vaccination (<6 weeks), and other conditions which influence immune system were excluded from the study.

3.4.3. Healthy control study population

To establish the reference range of Tu cells, we have collected homogeneous samples from one population. All the healthy adult samples collected for this prospective study belonged to the Caucasian population residing in central Europe, mainly from Hungary. A total of 295 samples were obtained, of which 93 were excluded based on the exclusion criteria defined above in section 3.5.2, and 202 samples were included in our study. Four different age groups were defined as 18-35, 36-50, 51-65, and 66-90 years, and samples were categorized within these age groups. Also, to establish the gender-based reference range, samples were classified based on gender. 93 samples were from males, and 109 samples belonged to females

3.4.4. Flow cytometric panel, phenotyping, and data analysis

A multicolor flow cytometric panel was employed to identify unconventional T (Tu) cells using the monoclonal antibodies, following the lyse-wash methodology outlined in sections 3.1.2 and 3.1.2. Data

was analyzed using FACSDiva version 6.1.3 software after sequentially gated flow cytometric plots. The T-cell receptor (TCR) of invariant natural killer T cells was stained using the Vα24-Jα18 clone 6B11 and characterized as CD45+/CD3+/Vα24+/Vα24-Jα18(6B11)+. The TCR of γδ T cells was stained with Vγ9 and Vδ2 and characterized as CD45+/CD3+/Vγ9+/Vδ2+. Mucosal-associated invariant T (MAIT) cells' TCR was identified as Vα7.2 and classified as CD45+/CD3+/Vα7.2+/CD161bright+. The absolute counts of total invariant natural killer T cells, γδ T cells, and mucosal-associated invariant T cells were calculated utilizing the absolute white blood cell (WBC) and lymphocyte counts (expressed as x10^9/L) obtained from the complete blood count (CBC) report through a dual-platform approach. These values were expressed as cells per microliter (cells/μL).

3.5. Characterization of MAIT and Vα7.2+/CD161- T cells in Atopic dermatitis

3.5.1. Sample collection and counting

PB samples from 14 untreated Atopic Dermatitis (AD) patients were obtained from the dermatology department, and ten gender and age-matched control samples were obtained from the Department of Laboratory Medicine volunteers. Three mL PB samples were collected in a BD vacutainer tube containing tri-potassium ethylene-diamine tetra acetic acid (K3-EDTA), 6 mL blood was collected in a BD vacutainer containing sodium heparin anticoagulant, and 3 mL of PB collected in BD clot activator, silicon coated tube from each donor.

3.5.2. Cell surface immunophenotyping

K3-EDTA anticoagulated blood was processed using the lyse wash protocol described above in section 3.1.2. A multicolor flow cytometric panel was designed to stain MAIT and Vα7.2+/CD161- T cells and their subsequent phenotypes with pre-titrated antibodies

3.5.3. Cytokine balance measurement

PBMC was isolated from the heparinized peripheral blood, and PMA/Ionomycin stimulation was performed as described in section 3.2.2. Surface and intracellular staining were performed to detect Th1, Th2, and Th17 cytokines using an intracellular antibody panel, following the protocol in section 3.2.3.

3.5.4. Flow cytometric data analysis

Multiparametric data were collected through four distinct tubes from each sample. The Flowjo v10.9.0 software was employed for the sequential gating T cells and subsequent subsets and for conducting multidimensional data analysis.

3.5.5. Sequential gating of T cells, MAIT cells, and Vα7.2+/CD161- T cells

In a standardized approach, data underwent initial cleaning using PeacoQC; then, doublets were removed by analyzing forward scatter (FSC) area vs. height and FSC area vs. width. White blood cells (WBCs) were subsequently identified as CD45+ events, from which lymphocytes were gated. Further refinement of the data involved the exclusion of antibody residues based on the CD45 vs. CD3 plot. Events positive for Vα7.2 and CD161 were classified as Mucosal-Associated Invariant T (MAIT) cells, while Vα7.2+/CD161- cells were designated as Vα7.2+/CD161- T cells. Subsets such as CD4, CD8, PD-1, CD69, CD38, cutaneous lymphocyte antigen (CLA), interferon-gamma (IFNγ), interleukin-4 (IL-4), IL-22, IL-17A, tumor necrosis factor-alpha (TNFα), granzyme B (GzB), TNFα/GzB, interleukin-1 beta (IL-1β), and interleukin-18 receptor alpha (IL-18Rα) were gated within T cells, MAIT cells, and Vα7.2+/CD161- T cells, respectively. The proportion of these subsets from the parent gate was used in future statistical analysis.

3.5.6. Multidimensional data analysis

The same universal gates were applied to isolate T cells for multidimensional data analysis. A down-sampling approach was employed, reducing the number of T cells to 20,000. Subsequently, gates based on CD3 expression were down-sampled and concatenated into single files, encompassing both atopic dermatitis (AD) and healthy control (HC) samples. Flowjo plugins were utilized to implement machine learning algorithms. A dimensionality reduction technique, Uniform Manifold Approximation and Projection (UMAP) was applied to visualize high-dimensional data. The Phenograph clustering algorithm, integrated into FlowJo as a plugin, was employed to refine and determine the number of clusters. Subsequently, FlowSOM was run to construct self-organizing maps and identify meta-clusters of T cells. The optimization of FlowSOM meta cluster numbers involved multiple clustering iterations

with varying numbers of meta clusters to prevent over- and under-clustering. A slight preference for over-clustering was adopted to avoid overlooking less expressive phenotypes.

3.6. Effects of immunomodulatory drugs on MAIT and Vα7.2+/CD161- T cells

3.6.1. Reagent preparation

MAIT cells are activated by microbial riboflavin-derived antigens, such as 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU). 5-OP-RU are potent activators of MAIT cells and are formed by non-enzymatic reactions between 5-amino-6-d-ribitylaminouracil (5-A-RU), an early intermediate in bacterial riboflavin synthesis and glyoxal or methylglyoxal. We prepared 5-OP-RU by mixing a 1:50 ratio of 5-A-RU and methylglyoxal. 5-A-RU was purchased commercially from Cayman Chemical, and 40% H2O of the methylglyoxal solution was purchased from Merck. 10 nM/mL of the final 5-OP-RU concentration stimulated MAIT cells.

MAIT cells were also activated with TCR-dependent and independent mechanisms, i.e., 5-OP-RU + IL-12 and IL-33 cytokines. 50 ng/mL (IL-12 and IL-33) final concentration was used in the experiment. PMA was diluted in RPMI media, and a final 20 ng/mL concentration was used. Ionomycin was used straight from stock solution (1.5 μg/mL). 10 ng/mL of Brefeldin-A was used diluted in RPMI.

Immunomodulatory drugs (Vitamin D3 and Cyclosporin A) were purchased from Merck. Vitamin D3 was diluted in ethanol; the final concentration was 0.1 nM/mL. Cyclosporin A was diluted in RPMI media, and the final concentration was 400 ng/mL.

3.6.2. Sample collection and PBMCs isolation

18 mL of PB was collected through venipuncture in a BD vacutainer containing sodium heparin anticoagulant from 7 healthy control donors. PBMCs were isolated, and cell concentration was adjusted following the protocol mentioned in section 3.2.1

3.6.3. Experiment design

The experiment was performed in a FACS tube containing $1x10^6 – 2x10^6$ PBMCs/100 μL, and the total volume was adjusted to 1 mL of RPMI. All the tubes were vortexed and incubated at 37^0C with 5% CO_2

for 18 hours, followed by Brefeldin A, and incubation was continued for six more hours. Finally, cells were washed, and cellular surface and intracellular staining were performed using monoclonal antibodies following the protocol mentioned above in section 3.2.3. Three multicolor panels of antibodies containing surface and intracellular markers were stained from each experimental tube to see the changes in MAIT cells.

3.6.4. Flow cytometric measurement

Post-staining cells were dissolved in 400 µL of paraformaldehyde, and the acquisition was performed as described above in section 3.1.3. Syto 16 nucleic acid dye was added before the acquisition, and 200,000 events were targeted and measured.

3.6.5. Data analysis

Flow cytometric data were analyzed using sequential gating of MAIT cells and Vα7.2+/CD161- T cells. Surface and intracellular expression were gated within MAIT cells using Flowjo v10.9.0 software. Percentage of MAIT and Vα7.2+/CD161- T cells among T cells and % of CD69, PD-1, CD69/PD-1, CCR6, CLA, CCR6/CLA, IFNγ, IL-4, IL-13, IFNγ/IL-4, IFNγ/IL-13, IL-17, IL-22, IL-17/IL-22, CD38, GzB, TNFα, GzB/TNFα among MAIT and Vα7.2+/CD161- T cells were exported in spreadsheet and data were plotted using GraphPad Prism.

3.7. Statistical analysis

Statistical analyses were conducted using GraphPad Prism versions 5.0 and 9.0. The Kolmogorov-Smirnov (SK) and Shapiro-Wilk tests were used to determine whether the data distribution was normal. Descriptive statistics such as median, minimum, maximum, 25th-75th percentile, and reference range (2.5-97.5%) were computed as needed. To compare continuous variables across many related and independent samples, a one-way analysis of variance (ANOVA) was used. The Kruskal-Wallis test with Dunn's post hoc test was used for non-normal and non-parametric data distribution. In contrast, parametric standard one-way ANOVA with Tukey's multiple comparison test tests was applied for normally distributed data. To compare continuous variables between two related and independent samples, normally distributed data were analyzed with parametric t-tests and the Mann-Whitney test for

non-parametric, non-normally distributed data. Correlation and linear regression analyses were carried out between two continuous variables. Furthermore, a two-way ANOVA was used to compare different stimulations and treatments, with statistical significance at a p-value of less than 0.05.

4. RESULTS

4.1. Immunosenescence in Tu cells

The correlation between the percentage and the absolute number of iNKT cells and age was non-significant ($p = 0.1456$, $r = 0.1027$ and $p = 0.01042$, $r = 0.1144$, respectively). Both the percentage and absolute numbers of γδ T cells exhibited a marked negative correlation with age ($p < 0.0001$, $r = 0.2719$, $r = 0.3027$), indicating an apparent age-related decline. Similarly, MAIT cells, in terms of percentage and absolute numbers, displayed a significant negative correlation with age ($p < 0.0001$, $r = 0.3761$, $r = 0.4023$), further supporting the age-dependent decline observed.

Data were grouped by gender to identify gender biases. In males, the percentage and absolute numbers of iNKT cells displayed non-significant correlations with age ($p = 0.6614$, $r = 0.04601$ and $p = 0.7740$, $r = 0.03018$ respectively). However, a noteworthy negative correlation emerged between γδ T cells and age ($p = 0.0003$, $r = 0.3647$and $p = 0.0002$, $r = 0.3802$ respectively). Moreover, MAIT cells exhibited a significant negative correlation with age ($p < 0.0001$, $r = 0.4861$ and $r = 0.5071$). Interestingly, in females, all data displayed substantial negative correlations with age. The p-values for iNKT cells (percentage and absolute numbers) were 0.0047, $r = 0.2689$, and $p = 0.0016$, $r = 0.2986$, respectively. Similarly, γδ T cells exhibited correlation p-values of 0.0007, $r = 3198$ and $p = 0.0003$, $r = 0.3407$, and MAIT cells showed correlation p-values <0.0001, $r = 0.4951$ and $r = 0.5527$.

4.1.1. Relationship between Tu cells

Our correlation analysis aimed to establish relationships between the percentages of iNKT, γδ T, and MAIT cells, revealing significant positive correlations between iNKT cells and both γδ T cells and MAIT cells. Similarly, γδ T cells positively correlated with MAIT cells, highlighting the interconnectedness of these Tu cell types.

4.1.2. Gender-dependent differences in Tu cells

The dataset analyzed quantitative differences in Tu cells (% and absolute numbers) across genders. Student's t-tests assessed significant differences, and corresponding p-values were reported.

Noteworthy disparities were found within each gender concerning iNKT and γδ T cells. Interestingly, MAIT cells did not exhibit any gender-specific differences. Specifically, iNKT cells (both in percentage and absolute count) were higher in females than males ($p = 0.0289$ and 0.0187, respectively). In comparison, γδ T cells were higher in males than females ($p = 0.0155$ and 0.0419, respectively). Surprisingly, T cells (% of lymphocytes) also showed a significant gender disparity, with females displaying a higher percentage of T cells than males ($p = 0.0044$).

4.1.3. Gender-dependent relationships between Tu cells

To explore gender-specific relationships between iNKT, γδ T cells, and MAIT cells, the data were stratified by gender, and correlation analyses were performed.

In the male group, a significant positive correlation was found between iNKT cells and MAIT cells ($p = 0.0027$), while no significant correlation was observed between iNKT cells and γδ T cells ($p = 0.4017$). Notably, a significant positive correlation was evident between γδ T cells and MAIT cells ($p = 0.0134$).

Conversely, within the female group, all correlations were significantly positive, including correlations between iNKT cells and γδ T cells ($p = 0.0074$), iNKT cells and MAIT cells ($p = 0.0017$), and γδ T cells and MAIT cells ($p = 0.0427$).

4.2. Age-dependent changes and reference ranges of Tu cells

The data were grouped based on the age groups outlined in the Material and Methods section 3.5. Descriptive statistics were also computed to establish the peripheral blood (PB) reference ranges for Tu cells. Percentages and absolute numbers (cells/μL) were derived from hematological parameters via the dual platform method.

4.2.1. Age-dependent changes of Tu cells

iNKT cells did not exhibit statistically significant differences in age groups. However, γδ and MAIT cells displayed substantial variations across different age brackets. For iNKT cells (expressed as a percentage and cells/μL), the p-values between various age groups were 0.1281 and 0.0757, respectively. An observable decrease in median values was noticed between age groups 18–35 (0.136)

and 36–50 (0.078); nevertheless, the subsequent age groups demonstrated a recovery in these differences.

In contrast, significant differences were noted within age groups for both γδ T cells (percentage and absolute number) ($p < 0.0001$). Further scrutiny using Dunn's multiple comparisons test revealed significance between the 18–35 group versus the 36–50, 51–65, and 66–90 age groups. This implies that γδ T cells do not substantially decline during the transition from middle to older age.

Additionally, MAIT cells exhibited significant differences between age groups ($p < 0.0001$). However, Dunn's multiple comparisons demonstrated that the younger age group (18-35) and the middle-aged group (36–50) exhibited significantly higher numbers of MAIT cells than the older group.

4.2.2. Descriptive statistics of total Tu cells

Descriptive statistics encompassed the median, minimum, and maximum values, the 25th to 75th percentiles, and the 2.5th to 97.5th percentiles (representing the reference range). The percentage of iNKT among T cells was calculated at 0.095%, ranging from 0.007% to 3.844%, with an absolute number of iNKT cells at 1.3 cells/µL, ranging from 0.1 to 63.5 cells/µL. γδ T cells comprised 2.17% of T cells, ranging from 0.078% to 16.09%, with an absolute number of 30 cells/µL, varying from 1 to 249 cells/µL. MAIT cells represented 2.99% of T cells, ranging from 0.11% to 18.3%, with an absolute number of MAIT cells at 42 cells/µL, varying from 2 to 261 cells/µL. Reference ranges for iNKT, γδ T, and MAIT cells among T cells were determined as 0.01–1.12%, 0.31–11.9%, and 0.27–10.7%, respectively.

4.2.3. Descriptive statistics of age-grouped Tu cells

The dataset was segmented into distinct age groups previously defined in the Material and Methods section (3.5). Four age brackets spanning 18–90 years (18-35; 36-50; 51-65; 66-90) were established, and descriptive statistics for Tu cells were compiled for each group.

18–35 years age group: In this cohort, iNKT cells comprised 0.13% (ranging from 0.007% to 3.84%), γδ T cells accounted for 3.78% (ranging from 0.685% to 16.09%), and MAIT cells constituted 5.02% (ranging from 0.67% to 11.9%). Absolute numbers were recorded as follows: iNKT cells ranged from 0.1 to 63.5 cells/μL (median of 2 cells/μL), γδ T cells ranged from 9 to 249 cells/μL (median of 56.5 cells/μL), and MAIT cells ranged from 10 to 183 cells/μL (median of 71 cells/μL). The reference ranges for iNKT, γδ T, and MAIT cells were determined as 0.0074–2.93%, 0.80–14.5%, and 0.79–11.3%, respectively.

36–50 years age group: Within this age bracket, the percentages were as follows: iNKT cells at 0.078% (ranging from 0.01% to 2.6%), γδ T cells at 1.98% (ranging from 0.11% to 11.9%), and MAIT cells at 3.42% (ranging from 0.3% to 18.3%). Absolute numbers revealed the following ranges: iNKT cells ranged from 0.1 to 44.1 cells/μL (median of 1.1 cells/μL), γδ T cells ranged from 1 to 203 cells/μL (median of 28 cells/μL), and MAIT cells ranged from 5 to 261 cells/μL (median of 45 cells/μL). The reference ranges for iNKT, γδ T, and MAIT cells were 0.011–1.98%, 0.26–10.5%, and 0.4–13.2%, respectively.

51–65 years age group: In this cohort, iNKT cells represented 0.108% (ranging from 0.011% to 1.15%), γδ T cells accounted for 1.66% (ranging from 0.078% to 7.23%), and MAIT cells constituted 1.85% (ranging from 0.11% to 10.59%). Absolute numbers were as follows: iNKT cells ranged from 0.1 to 13.2 cells/μL (median of 1.15 cells/μL), γδ T cells ranged from 1 to 122 cells/μL (median of 23.5 cells/μL), and MAIT cells ranged from 2 to 102 cells/μL (median of 23.5 cells/μL). The reference ranges for iNKT, γδ T, and MAIT cells were 0.012–0.994%, 0.153–6.98%, and 0.13–10.2%, respectively.

66–90 years age group: Within this age category, the percentages were recorded as iNKT cells at 0.098% (ranging from 0.011% to 0.816%), γδ T cells at 1.5% (ranging from 0.11% to 15.3%), and MAIT cells at 1.34% (ranging from 0.2% to 13.2%). Absolute numbers exhibited the following ranges: iNKT cells ranged from 0.1 to 16 cells/μL (median of 1 cells/μL), γδ T cells ranged from 1 to 160 cells/μL (median of 20 cells/μL), and MAIT cells ranged from 2 to 211 cells/μL (median of 16.5

cells/μL). The reference ranges for iNKT, γδ T, and MAIT cells were 0.011–0.81%, 0.11–15.3%, and 0.2–13.2%, respectively.

4.2.4. Descriptive statistics of gender-dependent Tu cells

The examination of gender-dependent percentages and absolute numbers for iNKT, γδ, and MAIT cells was conducted based on the total dataset.

Males: Among males, the percentage of iNKT cells was recorded at 0.076% (ranging from 0.007% to 0.816%), with an absolute number ranging from 0.1 to 16 cells/μL. γδ T cells exhibited a percentage of 2.52% (ranging from 0.31% to 16.09%) and an absolute number ranging from 3 to 249 cells/μL. MAIT cells showed a percentage of 3.04% (ranging from 0.11% to 10.75%), with an absolute number of MAIT cells ranging from 2 to 211 cells/μL. The reference ranges (2.5–97.5%) for iNKT, γδ T, and MAIT cells were 0.0087–0.56%, 0.43–14.2%, and 0.20–10.2%, respectively.

Females: Among females, the percentage of iNKT cells was recorded at 0.11% (ranging from 0.011% to 3.84%), with an absolute number ranging from 0.1 to 63.5 cells/μL. γδ T cells exhibited a percentage of 1.79% (ranging from 0.078% to 12.4%) and an absolute number ranging from 1 to 203 cells/μL. MAIT cells showed a percentage of 2.67% (ranging from 0.2% to 18.3%), with an absolute number of MAIT cells ranging from 2 to 261 cells/μL. The reference ranges for iNKT, γδ T, and MAIT cells were 0.015–2.1%, 0.11–11.6%, and 0.28–13%, respectively.

4.3. Involvement of peripheral blood MAIT and Vα7.2+/CD161- T Cells in atopic dermatitis (AD)

4.3.1. Cellular surface marker characterization of T cells, MAIT cells, and Vα7.2+/CD161- T cells in AD

We used two-dimensional sequential gating to analyze T cell properties and the Vα7.2+ compartment, encompassing mucosal-associated invariant T (MAIT) cells and Vα7.2+/CD161- T cells. Individuals with atopic dermatitis (AD) exhibited a significantly higher percentage of T cells (as a percentage of lymphocytes) compared to healthy controls (HC), particularly CD38+ activated T cell subsets. Conversely, in AD patients, the percentage of MAIT cells (as a percentage of T cells) decreased, while

CD38+ activated MAIT cell subsets increased. Additionally, in AD, the fraction of Vα7.2+/CD161- T cells declined, whereas CD38+ subsets increased. No significant differences were observed in surface markers such as CD69, PD-1, cutaneous lymphocyte antigen (CLA), CD4, and CD8 between T cells, MAIT cells, and Vα7.2+/CD161- T cells.

4.3.2. Type of T cell, MAIT cell and Vα7.2+/CD161- T cell response in AD

Our findings confirm the suggested Th2/Th22 dominance in atopic dermatitis (AD) characterized by a decreased percentage of IFNγ-producing T cells and a marginally elevated, non-significant IL-4 production. Moreover, a higher rate of IL-22-producing T cells was observed, with no significant difference in IL-17A-producing T cells. Notably, AD's polyfunctional double-positive fraction of Tumor Necrosis Factor α/Granzyme B (GzB)-generating MAIT cells was significantly enhanced, suggesting the potential involvement of peripheral blood (PB) MAIT cells in disease pathogenesis. However, no significant differences were observed in single-positive GzB, TNFα, or IFNγ production. Vα7.2+/CD161- T cells demonstrated similar responses to conventional T cells. Specifically, Th1 (IFNγ)-producing cells decreased, while IL-4 production showed a slight increase, albeit not statistically significant. Notably, Th22 (IL-22)-producing cells significantly increased, whereas no substantial change was observed in IL-17A-generating Vα7.2+/CD161- T cells. Additionally, there were no significant differences detected in intracellular markers such as IL-17A, IL-22, and IL-18Rα of MAIT cells, TNFα, GzB, TNFα/GzB, and IL-18Rα of T cells, or Vα7.2+/CD161- T cells.

4.3.3. Comparison of MAIT and Vα7.2+/CD161- T cells

In our comparison between MAIT cells and Vα7.2+/CD161- T cells in individuals with atopic dermatitis (AD) and healthy controls (HC), significant differences were observed in all datasets from HC individuals, except for IL-17A and the GzB/TNFα subsets of MAIT and Vα7.2+/CD161- T cells. Within the Vα7.2+ compartment, MAIT cells were found to be more abundant than Vα7.2+/CD161- T cells. MAIT cells exhibited a higher expression of CD69 compared to CD38, while Vα7.2+/CD161- T cells showed the opposite pattern. Additionally, MAIT cells, primarily composed of CD8+ cytotoxic T cells, displayed notably higher PD-1 expression than Vα7.2+/CD161- T cells. On the other hand, Vα7.2+/CD161- T cells were more likely to have a CD4+ helper phenotype and express cutaneous

lymphocyte antigen (CLA). Compared to Vα7.2+/CD161- T cells, MAIT cells exhibited increased expression of IFNγ, decreased expression of IL-4, and no significant difference in IL-17A. Furthermore, MAIT cells produced more IL-22 and less granzyme B (GzB) and tumor necrosis factor-alpha/granzyme B (TNFα/GzB) compared to Vα7.2+/CD161- T cells. Additionally, MAIT cells demonstrated substantially higher levels of interleukin-18 receptor alpha (IL-18Rα) and TNFα than Vα7.2+/CD161- T cells, indicating their distinct characteristics.

In the comparison related to atopic dermatitis (AD), alterations were observed only in groups affected by AD pathogenesis. The fraction of MAIT cells (as a percentage of T cells) that were downregulated in AD became non-significant. Similarly, IL-22-generating Vα7.2+/CD161- T cells increased in AD but became non-significant. AD's polyfunctional TNFα/GzB-generating MAIT cells significantly increased.

4.3.4. Multidimensional T cell analysis

The multidimensional analysis of T cells revealed multiple immunological clusters that exhibited significant differences between patients with atopic dermatitis (AD) and healthy controls (HC). The phenotypes of these immune clusters were evaluated using marker expression from the respective tubes. Notably, the following subsets demonstrated statistically significant differences: T cells, CD161+ T cells, Vα7.2+/CD161- T cells, mucosal-associated invariant T (MAIT) cells, CD69+ MAIT cells, CD38+ T cells, CD4+/CD161+ T cells, CD8+/Vα7.2+/CD161- T cells, CD38+/Vα7.2+/CD161- T cells, CD4+MAIT cells, CD8+MAIT cells, double-negative (DN) MAIT cells, IFNγ+ T cells, IL-22+ T cells, and IFNγ+ Vα7.2+/CD161- T cells.

In AD patients, CD3+ T cells were more prevalent than CD161+ T cells, MAIT cells, CD69+ MAIT cell subsets, and Vα7.2+/CD161- T cell subsets compared to healthy controls. Tube 2 provided additional insights into the subsets of these cells based on cellular surface expression. In atopic dermatitis (AD), the following observations were made: a reduction in CD4+/CD161+ T cells, an increase in CD38+ T cells, a decrease in MAIT cell subsets (CD4, CD8, double-negative), a reduction in CD8+/Vα7.2+/CD161- T cells, and an increase in CD38+ activated Vα7.2+/CD161- T cells. Data from Tube 3 indicated a Th2/Th22 response in T cells, with an increase in IL-22-producing CD3+ T

cells and a decrease in IFNγ-producing cells. Specifically, AD suppresses IFNγ-producing Vα7.2+/CD161- T cells.

4.4. Effect of immunomodulation on MAIT cells and Vα7.2+/CD161- T cells under different stimulatory conditions

4.4.1. Effects of various stimulants on MAIT and Vα7.2+/CD161- T Cells

Significant differences between different stimulants were determined using untreated data to mitigate treatment bias. After activation, a consecutive decrease in MAIT cell frequency was observed; however, the data were not statistically significant. In contrast, Vα7.2+/CD161- T cell frequency did not show any reduction. Under steady-state conditions, MAIT cells express CCR6, but a considerable decrease was observed under various stimulatory conditions, especially with PMA/Ionomycin. Minimal CCR6 expression was detected in Vα7.2+/CD161- T cells, and no changes were noted with the application of stimulants. Similar results were observed even after exposure to different stimulatory conditions, consistent with previous findings indicating the lack of CLA expression in PB MAIT cells. However, compared to other stimulants, PMA stimulation enhanced CLA expression on the surface of Vα7.2+/CD161- T cells. Interestingly, CLA/CCR6+ Vα7.2+/CD161- T cells exhibited a sharp increase in surface expression post-PMA stimulation, while MAIT cells remained relatively inexpressive.

CD69, an acute activation marker, demonstrated a sharp increase in expression across all stimulants compared to unstimulated MAIT cells, with the highest expression observed in response to 5-OP-RU and 5-OP-RU along with cytokines (IL-12 and IL-33). In the case of Vα7.2+/CD161- T cells, only PMA stimulation induced CD69 expression, while the remaining stimulants elicited a nonsignificant response compared to unstimulated cells. CD38, a chronic activation marker, exhibited higher expression variation across different stimulants in MAIT cells than unstimulated cells, whereas Vα7.2+/CD161- T cells showed no significant differences. A slight increase in PD-1 expression was observed in MAIT cells; however, the data were not statistically significant, and no significant results were noted for Vα7.2+/CD161- T cells. Interestingly, PD-1/CD69 expression significantly increased

with 5-OP-RU and 5-OP-RU/IL-12/IL-33 stimulation, whereas only PMA stimulation showed a significant increase in double-positive

4.4.2. Cytokine production fluctuates with varied stimuli

Under steady-state conditions, MAIT cells exhibited characteristics of Th2-type cells. They expressed higher levels of IL-13 and no IFNγ. However, after stimulation, there was a significant decrease in IL-13 and an increase in IFNγ. A substantial rise in IFNγ was observed following PMA stimulation, as expected. Polyfunctional MAIT cells (IFNγ/IL-13) were absent under steady-state conditions. However, interactions with MAIT cell ligands resulted in a significantly higher number of these polyfunctional cells, particularly in the presence of proinflammatory cytokines and MAIT cell ligands (5-OP-RU/IL-12/IL-33). Although there was a visible increase in IL-17A-producing MAIT cells, the data were not statistically significant. In contrast, PMA-stimulated Vα7.2+/CD161- T cells showed a different pattern. Only PMA-stimulated MAIT cells and Vα7.2+/CD161- T cells produced significantly higher levels of IL-22. The other stimulants did not reach statistical significance. Similar results were observed for IL-17A/IL-22+ MAIT cells and Vα7.2+/CD161- T cells, with statistically significant upregulation only observed with PMA.

This was expected for Vα7.2+/CD161- T cells since they were only stimulated by PMA/Ionomycin. However, when considering IL-17A, IL-22, and IL-17A/IL-22-producing MAIT cells, only PMA showed a significant difference compared to the ligand (5-OP-RU). IL-4-producing MAIT cells were not detected. But Vα7.2+/CD161- T cells expressed significantly higher levels of IL-4 and IFNγ/IL-4 post-PMA stimulation. No significant differences were noted in Granzyme B (GzB)-producing MAIT cells and Vα7.2+/CD161- T cells with stimulants. 5-OP-RU/IL-12/IL-33-stimulated TNFα-producing MAIT cells did not significantly differ from the unstimulated control. However, PMA and 5-OP-RU-stimulated cells did show significant differences. TNFα-producing Vα7.2+/CD161- T cells showed no significant difference with different stimulations compared to the control. Interestingly, 5-OP-RU with or without IL-12/IL-33 showed significantly higher levels of polyfunctional TNFα/GzB-producing MAIT cells. However, no significant difference was noted in Vα7.2+/CD161- T cells.

4.4.3. Cyclosporin A and Vitamin D_3 modulatory changes in the cellular surface of MAIT and Vα7.2+/CD161- T cells under different stimulation

No significant effect of immunomodulatory drugs was observed on MAIT cell numbers. However, when Vα7.2+/CD161- T cells were stimulated with 5-OP-RU and treated with CsA, a significantly higher number was observed than untreated cells. CsA downregulated CCR6 expression in MAIT cells when stimulated with 5-OP-RU ligand in the presence of proinflammatory cytokines (IL-12 and IL-33), while no significant difference was noted in Vα7.2+/CD161- T cells. CsA and VitD treatments did not affect CLA expression in MAIT and Vα7.2+/CD161- T cells. However, CsA downregulated CLA/CCR6 expression in MAIT cells in the presence of cytokines (IL-12 and IL-33), with no observed effects on Vα7.2+/CD161- T cells. VitD treatment upregulated CD69 expression in MAIT cells when stimulated with PMA, while CsA significantly upregulated CD69 expression with 5-OP-RU stimulation. CD69 expression in Vα7.2+/CD161- T cells remained stable compared to the untreated control. Specific changes in CD38 expression were noted with the 5-OP-RU ligand, with CsA showing downregulation and VitD showing upregulation in MAIT cells. No difference was noted in CD38 expression of Vα7.2+/CD161- T cells during treatment. PD-1 expression remained stable with immunomodulatory drug treatment in MAIT cells. However, VitD treatment showed significantly reduced PD-1 expression during PMA stimulation in Vα7.2+/CD161- T cells. PD-1/CD69 expression in MAIT cells was modulated by CsA and VitD in the presence of IL-12 and IL-33, with a significant decrease noted with both treatments. Nevertheless, there was no difference observed in Vα7.2+/CD161- T cell treatment.

4.4.4. Cyclosporin A and Vitamin D_3 modulatory changes in cytokine production of MAIT and Vα7.2+/CD161- T cells under varied stimulation

CsA was found to block IFNγ-producing MAIT cells post-stimulation, and VitD treatment showed blockade with 5-OP-RU ligand-specific stimulation. As expected, CsA treatment also showed IFNγ blockade in PMA-stimulated Vα7.2+/CD161- T cells. When stimulated with 5-OP-RU, VitD promoted IL-13-producing MAIT cells in a dependent manner, while no significant treatment-specific differences were noted in IL-13-producing Vα7.2+/CD161- T cells. During ligand-specific stimulation, a sharp

blockade was observed in polyfunctional IL-13/IFNγ-producing MAIT cells with CsA and VitD treatment. A similar observation was made during PMA stimulation of Vα7.2+/CD161- T cells. No significant changes were noted with IL-17A-producing MAIT cells due to immunomodulation; however, CsA-treated, IL-17A-producing Vα7.2+/CD161- T cells increased when stimulated with 5-OP-RU ligand. There were no treatment-based significant differences in IL-22-producing MAIT and Vα7.2+/CD161- T cells. Similarly, IL-17A/IL-22+ MAIT cells did not show any treatment-based changes. However, unstimulated Vα7.2+/CD161- T cells treated with VitD showed increased polyfunctional IL-17A/IL-22-producing cells. MAIT cells did not express IL-4; no treatment-based differences were found in IL-4 and IFNγ/IL-4-producing Vα7.2+/CD161- T cells. The treatments did not affect GzB-producing MAIT cells and Vα7.2+/CD161- T cells. CsA-dependent decline in TNFα-producing MAIT cells and Vα7.2+/CD161- T cells was noted with PMA-specific stimulation; however, CsA could not help with MAIT cell-specific stimulants. Conversely, GzB/TNFα-producing MAIT cells were blocked by CsA and VitD when stimulated with 5-OP-RU. Interestingly, no drug effect was noted in the 5-OP-RU ligand with IL-12/IL-33 stimulation. The treatment-based blockade was reported in GzB/TNFα-producing Vα7.2+/CD161- T cells.

5. DISCUSSION

Extensive research in the past two decades has highlighted the significance of non-traditional T cells, known as unconventional T cells (Tu). These cells play a crucial role in various health conditions and act as a bridge between the innate and adaptive immune response. They do not depend on MHC peptide complexes. They share tissue-resident phenotypes and can be activated through TCR-dependent and independent mechanisms (via cytokines). The interplay between aging, gender, and immune responses sheds light on the complex nature of the human immune system. Aging significantly influences the immune system, primarily regulated by thymus involution in mammals. Sex hormones, particularly androgens, play a crucial role in accelerating thymus involution in males, while in females, progesterone-mediated thymic involution during pregnancy is also essential for successful pregnancies. The age-related decline in unconventional T cells raises concerns about their potential impact on age-related pathological conditions. However, establishing a direct association requires more in-depth studies. In our initial research phase, we established age and gender-dependent immunosenescence and reference ranges for unconventional T cells and identified a relationship between them. This study lays the groundwork for future research and clinical studies comparing unconventional T cells in age-related conditions, disease progression, and follow-up assessments.

Our research findings show a strong association between aging and changes in unconventional T-cell subsets. We found a significant negative correlation with age for γδ T cells and mucosa-associated invariant T (MAIT) cells. At the same time, there was no significant relationship between age and the percentage or absolute count of invariant natural killer T (iNKT) cells. Our research revealed gender-specific associations with age. Male iNKT cells showed no significant correlation with age, while females displayed a strong negative correlation, suggesting a potential gender-dependent link to immune-mediated diseases. γδ T cells negatively correlated with age in males and females, indicating an increased risk of immune-related diseases as people age. Additionally, males and females showed decreased mucosa-associated invariant T (MAIT) cells with age.

No significant variation was found in the levels of invariant natural killer T (iNKT) cells across age groups, contrary to the expected concept of immunosenescence. Age-related changes in γδ T cells show

higher γδ T cells in younger individuals, and middle-older individuals showed a slight, statistically non-significant reduction. Age-related changes in MAIT cells were observed, with lower percentages in middle-aged and elderly individuals compared to the young.

Variations in immune cell composition are believed to be influenced by factors such as sex hormones, sex chromosomes, reproductive status, and environmental factors. Our study found significant gender differences in iNKT cells, where females had more iNKT cells than males. We also found that males had higher γδ T cells than females. However, MAIT cells didn't show gender-dependent differences.

The overall number of unconventional T cells showed a significant positive correlation, especially between iNKT and MAIT cells across genders. However, iNKT cells and γδ T cells in men did not correlate. MAIT and iNKT cells share transcriptional factors like T-bet, RORγt, and PLZF, along with genes involved in intra-thymic development, including members of the SLAM family receptor. This suggests potential similarities in their developmental pathways and functional characteristics. In CD1d knockout mice, there is an increase in mucosal-associated invariant T (MAIT) cells, while MR1-deficient mice show an increase in invariant natural killer T (iNKT) cells. This suggests a potential link between these cell types through shared antigen-presenting molecules and signaling pathways. Despite their frequency differences, iNKT and MAIT cells complement each other, highlighting the possible synergies between different unconventional T cell populations in the immune system.

We used descriptive statistics to establish the reference ranges for unconventional T (Tu) cells in peripheral blood. Our findings on invariant natural killer T (iNKT) cells align with those reported by several studies involving healthy adults. The majority of γδ T cells in peripheral blood use Vγ9 and Vδ2, commonly referred to as γδ T cells. Our data aligns with similar studies on healthy adult Japanese and Portuguese cohorts. The frequency of mucosa-associated invariant T (MAIT) cells in peripheral blood is noteworthy among unconventional T cells. Our results align with similar studies conducted on healthy Korean individuals and blood samples across a wide age range.

Limited research has been conducted on healthy individuals. Our study is the only attempt to collectively establish reference ranges for iNKT cells, γδ T cells, and MAIT cells. This study was

prospective, single-center, and focused on a homogeneous population (Caucasian) with gender-balanced samples, covering iNKT cells, γδ T cells, and MAIT cells across four age groups with rigorous exclusion criteria. We used 6B11 monoclonal antibody clone designed to bind to the CDR3 region of the canonical Vα24Jα18 TCR rearrangement of iNKT cells. This choice was made due to its ability to detect lower yet specific iNKT cells, mainly when used with Vα24 within CD3+ T cells.

In the second part of our study, we aimed to investigate the importance of MAIT cells and Vα7.2+/CD161- T cells in healthy individuals and those in the early stages of atopic dermatitis (AD). Our study found that individuals with AD had a higher percentage of T cells but fewer MAIT cells and Vα7.2+/CD161- T cells than healthy controls (HC). MAIT cells play a crucial role in immune responses by recognizing microbial vitamin B2 metabolites. Staphylococcus aureus exacerbates AD and possesses the riboflavin biosynthesis pathway, which generates MR1 antigens recognized by MAIT cells. This interaction suggests a potential mechanism for MAIT cells to contribute to immune responses against S. aureus in AD. Recent research suggests that MAIT cells aid in wound healing by being recruited to the wound site and contributing to tissue repair by producing factors such as amphiregulin. The reduced levels of MAIT and Vα7.2+/CD161- T cells in the blood of individuals with atopic dermatitis may be due to their migration to skin lesions. Further research on skin lesions is needed to understand the role of MAIT cells and Vα7.2+/CD161- T cells in AD.

In chronically activated AD patients, CD4+/CD38+ T cells were involved in a defective polyfunctional response to staphylococcal antigens. These findings highlight the importance of CD38 as a valuable marker for monitoring chronic activation status in AD. Our study found elevated CD38 across all subsets, including T cells, making it a more accurate activation marker in AD than CD69. Type 2 inflammation driven by Th2/22 cells aligns with the classic hallmarks of AD, and our results in AD patients exhibited Th2/22 response, characterized by an increase in IL-22-producing T cells and a decrease in Th1 (IFNγ)-producing T cells. MAIT cells are known for their polyfunctional response, and in our results, we found that AD patients have elevated levels of polyfunctional MAIT cell response, particularly those that produce GzB and TNFα. Granzyme B (GzB) and tumor necrosis factor-alpha (TNFα) may play a role in the immune response associated with AD, indicating the disease's complex

nature. Our study found a decrease in IFN-γ-producing Vα7.2+/CD161- T cells and an increase in IL-22-producing Vα7.2+/CD161- T cells, suggesting a role in Th2/22-mediated inflammation in AD.

Our study compared the immunophenotypes of Vα7.2+/CD161+ (MAIT cells) and Vα7.2+/CD161- T cells in healthy controls (HC) and individuals with AD. In HC, Vα7.2+/CD161- T cells demonstrated higher IL-4 and granzyme B (GzB) expression levels than MAIT cells. Additionally, these cells exhibited higher levels of the CD38 activation marker but lower levels of CD69 than MAIT cells. Moreover, Vα7.2+/CD161- T cells were predominantly CD4 (helper T) cells, whereas MAIT cells were CD8 (cytotoxic T) cells. Vα7.2+/CD161- T cells exhibited significantly lower levels of IL-18Rα than MAIT cells, suggesting their distinct nature from MAIT cells.

The multidimensional analysis method examines all fluorescence signals simultaneously, providing a more comprehensive view than traditional gating. It offers a nuanced understanding of the complex immunophenotypic landscape, revealing crucial insights into immunological changes. This approach was used to analyze downsampled T cells, uncovering unique cell types and interactions that traditional gating may miss. The study revealed differences in the following populations, including CD8+ subsets of Vα7.2+/CD161- T cells, CD69+ cells, CD4+ cells, CD8+ cells, double-negative (DN) MAIT cells, and CD4+/CD161+ T cells. Notably, CD161+ T cells and CD4-expressing subsets were observed. CD161 is highly functional and closely associated with a memory phenotype (central or effector). Our research aligns with previous studies showing fewer helper T cells in AD than healthy controls, emphasizing the need for multidimensional analysis to detect complex immunophenotypic changes.

MAIT cells have a comprehensive immunological response, with different roles in blood and mucosa. Stimulation settings and the tissue microenvironment can influence their responses and diverse functions in healthy and damaged tissues. MAIT cells express high levels of MDR1, which provides resistance to cytotoxic chemicals like daunorubicin but not to immunosuppressive drugs' effects. Our *in vitro* study focused on their activation, exhaustion, and various cytokine responses under commonly known stimulatory conditions for MAIT cells. We compared Vα7.2+ T cells (CD161-) and MAIT cells (CD161+) to discern differences.

After stimulation, we noticed a slight decrease in MAIT cell, likely due to TCR internalization from the

immunological synapse. This is necessary for sustained TCR signaling and downregulation, which enhances T-cell proliferation in response to stimulation. CsA treatment reduced CCR6 expression in the presence of 5-OP-RU/IL-12/33. Stimulation with 5-OP-RU, with or without IL-12/IL-33, increased CD69 and CD38 expression. CD69 expression rises in a CsA-dependent manner and is a marker of early T cell activation, while CD38 is associated with T cell activation and proliferation. CsA decreased CD38 expression, while Vitamin D increased it. A trial with 16 cystic fibrosis patients found a positive correlation between Vitamin D therapy and CD38 expression and a negative correlation with PD-1 expressing MAIT cells. The findings support the potent immunomodulatory effects of Vitamin D in clinical settings. PD-1/CD69 cells significantly increase when stimulated with 5-OP-RU alone or with IL-12/IL-33. CsA and Vitamin D effectively regulate CD69/PD-1 expression in IL-12/IL-33-stimulated cells.

MAIT cells fail to sustain IFNγ production during TCR-dependent activation, leading to increased expression of tissue-repair genes. Our results show both CsA and Vitamin D significantly inhibit IFNγ production in MAIT cells during PMA and 5-OP-RU activation. CsA mechanism of action targets to block TCR-mediated calcium signaling and effectively suppresses IFNγ production in MAIT cells even in the presence of proinflammatory cytokines. Our results showed stimulation-induced downregulation of IL-13-producing MAIT cells, particularly in the presence of IL-12/IL-33. However, proinflammatory cytokines notably increase the number of polyfunctional MAIT cells producing IL-13/IFNγ.

MAIT cells are highly flexible, integrating environmental signals to adapt and change their functions. This flexibility is influenced by tissue location, clonal identity, and activation state. Vitamin D balances the immune system by reducing IFNγ and increasing IL-13 from MAIT cells when exposed to MR-1 ligands without proinflammation cytokines. MAIT cells in the buccal mucosa produce more IL-17 than those in peripheral blood. Conversely, decidual MAIT cells stimulated with PMA release very little IL-17 and almost no IL-22, unlike MAIT cells in the female vaginal mucosa. Our findings align with previous studies, showing minimal IL-17A, IL-22, and polyfunctional IL-17A/IL-22 production by peripheral blood MAIT cells upon PMA stimulation.

MAIT cells are identified using flow cytometry by expressing Vα7.2 and the c-type lectin CD161. The discovery of the MR-1 ligand led to the development of a 5-OP-RU-loaded MR-1 tetramer to detect

TCR-specific MAIT cells. However, not all MAIT cells identified by Vα7.2/CD161+ binding to the MR-1 tetramer, and not all MR-1 binding tetramers express Vα7.2/CD161+. A recently discovered host-derived MR-1 ligand, cholic acid 7-sulfate (CA7S), is recognized by MAIT cells. Unlike 5-OP-RU, CA7S increases MAIT cell survival and upregulates a gene associated with wound healing and immunoregulation. Our in vitro study found apparent differences between Vα7.2+/CD161+ (MAIT) and Vα7.2+/CD161- T cells despite sharing the Vα7.2 compartment. Since this population was only exposed to the 5-OP-RU ligand and not the newly discovered CA7S ligand, it would be interesting to investigate the effects of both ligands on MAIT and Vα7.2+/CD161- T cells.

Our research suggests different ways of activating MAIT cells lead to diverse responses. MAIT cell activation through the proinflammatory cytokine-supported 5-OP-RU ligand resulted in the highest CD69 expression and IFNγ production. MAIT cells showed polyfunctional reactions to the same stimulus, with elevated levels of PD-1/CD69, IL-13/IFNγ, and TNFα/GzB. Cyclosporin A, a potent immunosuppressant, significantly reduced migration, activation, and cytokine responses to several MAIT cell stimuli. The 5-OP-RU ligand did not activate Vα7.2+/CD161- T cells, regardless of IL-12 or IL-33 levels, which confirms that MAIT cells differ from Vα7.2+/CD161- T cells and respond differently to Cyclosporin A and vitamin D. However, PMA stimulation increased the production of IL-17A, IL-4, IL-17A/IL-22, and IFNγ/IL-4, cytokines that MAIT cells typically produce rarely. We extensively studied the MAIT cell response to CsA and Vitamin D under various stimuli. Our analysis offers further insights into MAIT cell behavior and therapeutic potential.

6. NEW SCIENTIFIC FINDINGS

To summarize, the frequency of γδ and MAIT cells is higher in the younger age group, revealing a negative correlation with age and supporting the concept of immunosenescence. In contrast, iNKT cell frequency shows no statistically significant difference across age groups and no observable negative correlation with age. Gender-specific analysis uncovers higher levels of iNKT cells in females, exhibiting a negative correlation with age. In comparison, γδ T cells display higher levels in males, with both genders negatively correlating with age. MAIT cells, on the other hand, exhibit no gender-based differences and manifest a negative correlation with age in both males and females. The intriguing discovery of a positive correlation among uT cells lays a foundation for future studies exploring immune-mediated diseases and their relationship with uT cells.

In vitro study revealed that MAIT cells were producing IL-13 in steady state conditions and respond robustly to the 5-OP-RU ligand, specifically in the presence of proinflammatory cytokines (IL-12/33). Our in vitro experiments showed that MAIT cells regulate the surface expression of CD69 and CD38 and produce IFNγ and effector (TNFα/GzB) cytokines. We found a similar pattern of cell surface markers and cytokines in AD. This phenotype may correspond to an activated and fully differentiated effector cell type capable of producing cytokines and acting as a cytotoxic cell suggesting their involvement in the pathomechanism of the disease. Cyclosporin A is a potent immunosuppressing agent that significantly blocks the proinflammatory cytokines in MAIT cells, while vitamin D3 could support MAIT cell activation and IL-13 production to bring the steady state conditions.

Despite sharing the Vα7.2 compartment, MAIT cells and Vα7.2+/CD161- T cells show clear differences. Vα7.2+/CD161- T cells resemble conventional T cells and do not respond to 5- OP-RU, although PMA stimulation increases the production of certain cytokines.

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List of publications related to the dissertation

1. **Singh, P.**, Széles, M., Baráth, S., Hevessy, Z.: A Comprehensive Investigation of Stimulatory Agents on MAIT and Vα7.2+/CD161-T Cell Response and Effects of Immunomodulatory Drugs.
   Int. J. Mol. Sci. 25 (11), 1-16, 2024.
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3. **Singh, P.**, Széles, M., Mezei, Z. A., Baráth, S., Hevessy, Z.: Age-dependent frequency of unconventional T cells in a healthy adult Caucasian population: a combinational study of invariant natural killer T cells, [gamma][delta] T cells, and mucosa-associated invariant T cells.
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4. **Singh, P.**, Széles, M., Mezei, Z. A., Baráth, S., Hevessy, Z.: Gender-dependent frequency of unconventional T cells in a healthy adult Caucasian population: a combinational study of invariant NKT cells, [gamma] [delta] T cells, and mucosa-associated invariant T cells.
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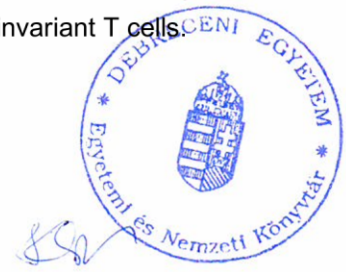

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List of other publications

5. Tóth, E. L., Orbán-Kálmándi, R. A., Bagoly, Z., Lóczi, L., Deli, T., Török, O., Deliné Molnár, S., Baráth, S., **Singh, P.**, Hevessy, Z., Katona, É., Fagyas, M., Szabó, A. Á., Molnár, S., Krasznai, Z. T.: Case report: Complex evaluation of coagulation, fibrinolysis and inflammatory cytokines in a SARS-CoV-2 infected pregnant woman with fetal loss.
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