

DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Unconventional T cells in health and diseases

by Parvind Singh

UNIVERSITY OF DEBRECEN
KÁLMÁN LAKI DOCTORAL SCHOOL

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List of abbreviations

AD	Atopic dermatitis	PB	Peripheral blood
BCL-6	B-cell lymphoma 6	PMA	Phorbol 12-myristate 13-acetate
CLA	Cutaneous lymphocyte antigen	PLZF	Promyelocytic leukemia zinc-finger
FlowSOM	Self-Organizing Maps	PLZF	Promyelocytic leukemia zinc-finger
GzB	Granzyme B	PBMC	Peripheral blood mononuclear cells
$\gamma\delta$ T cells	Gamma delta T cells	ROR γ	RAR-related orphan receptor gamma
HLA	Human leukocyte antigen	SLAM	Signalling lymphocytic activation molecule
HCV	Hepatitis C virus	TCR	T cell receptor
HIV	Human immunodeficiency Virus	TNF α	Tumor necrosis factor-alpha
HC	Healthy control	t-SNE	t-distributed stochastic neighbor embedding
ICOS	Inducible T cell costimulatory molecule	TGF β	Transforming growth factor-beta
IL	Interleukin	uT cells	Unconventional T cells
ILC2	Innate lymphoid cells	UMAP	Uniform manifold approximation and projection
IgE	Immunoglobulin E	VEGF	Vascular endothelial growth factor
iNKT cells	Invariant natural killer T cells	Vit D	Vitamin D ₃
IFN γ	Interferon gamma	WBCs	White blood cells
K3-EDTA	Tri-potassium ethylene-diamine tetra acetic acid	5-A-RU	5-amino-6-D-ribitylaminouracil
MICB	MHC class I polypeptide-related sequence B	5-OP-RU	5-(2 oxopropylideneamino)-6-D-ribitylaminouraci
MICA	MHC class I polypeptide-related sequence A	6-FP	6-formylpterin
MR-1	Major histocompatibility complex, class I-related		
mAbs	Monoclonal antibodies		
MAIT cells	Mucosa-associated invariant T cells		
MHC	Major histocompatibility complex		
NKG2D	Natural killer group 2 D		
PD-1	Programmed death receptor		

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 (Figure 1).

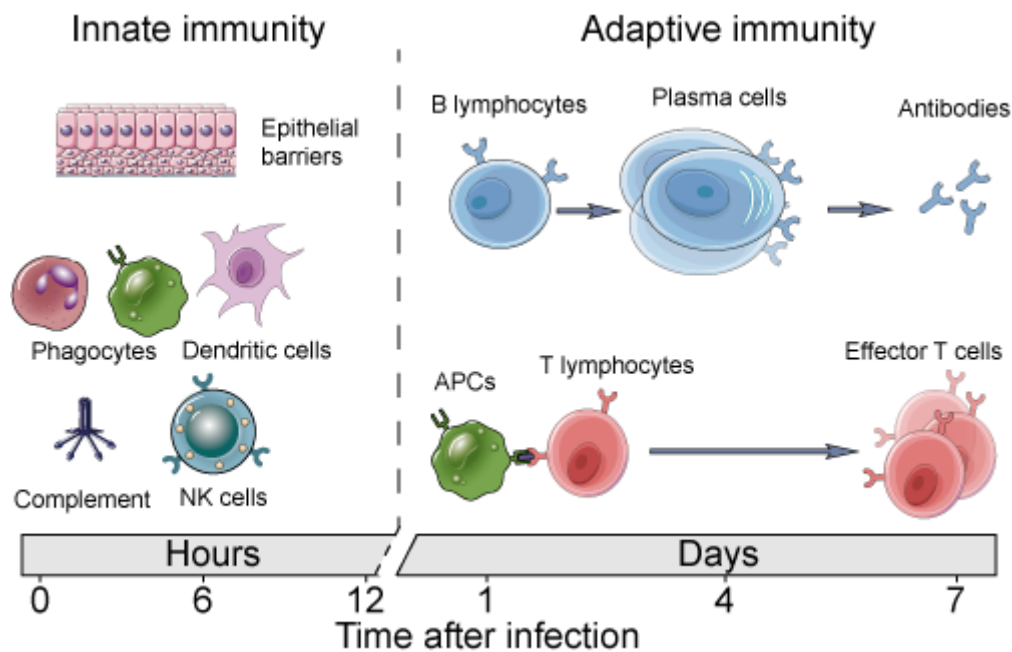


Figure 1: Kinetics of innate and adaptive immune responses. Adapted from Creative Diagnostic [1].

On the other hand, adaptive immunity is specific to particular antigens and only targets those. Its response is slower, taking several days to a week, as it involves antigen recognition through the major histocompatibility complex (MHC), proliferation, and response. Additionally, adaptive immunity has a memory response, which is rapid and robust upon subsequent

exposure to the same antigen. 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. This response can be either Th2-dependent or independent, resulting in the generation of plasma cells and the secretion of immunoglobulins. Plasma cells can be short-lived or long-lived, with long-lived plasma cells residing in the bone marrow and providing lifelong immunity against a specific antigen.

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. Following these processes, naïve CD4 or CD8 T cells bearing CD3⁺ appear. Once primed by antigen-presenting cells through the MHC peptide complex, they undergo antigen-specific proliferation and differentiation and eventually become effector cells. 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. CD8⁺ T cells are cytotoxic cells with specific responses through MHC class I peptide complexes, while CD4⁺ cells depend on MHC class II peptide complexes. Their activation leads to the production of various cytokines, depending on the stimulus through the MHC peptide complex, and, in turn, they facilitate communication among immune cells [2] [3].

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 (uT cells)

While our understanding of the thymic development of unconventional T cells (uT cells) is limited, the role of the thymus is crucial in shaping their development. However, some of these

lineages may develop outside of the thymus. Research has shown that a transcription factor called promyelocytic leukemia zinc-finger (PLZF) is a master regulator in their development and function. uT cells recognize specific self and evolutionarily conserved molecules found in microbial agents through their invariant $\alpha\beta$ and $\gamma\delta$ T cell receptors (TCR) [4]. 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 (Figure 2). In healthy individuals, uT cells comprise a relatively small portion of the total T cell population in peripheral blood (PB) [5].

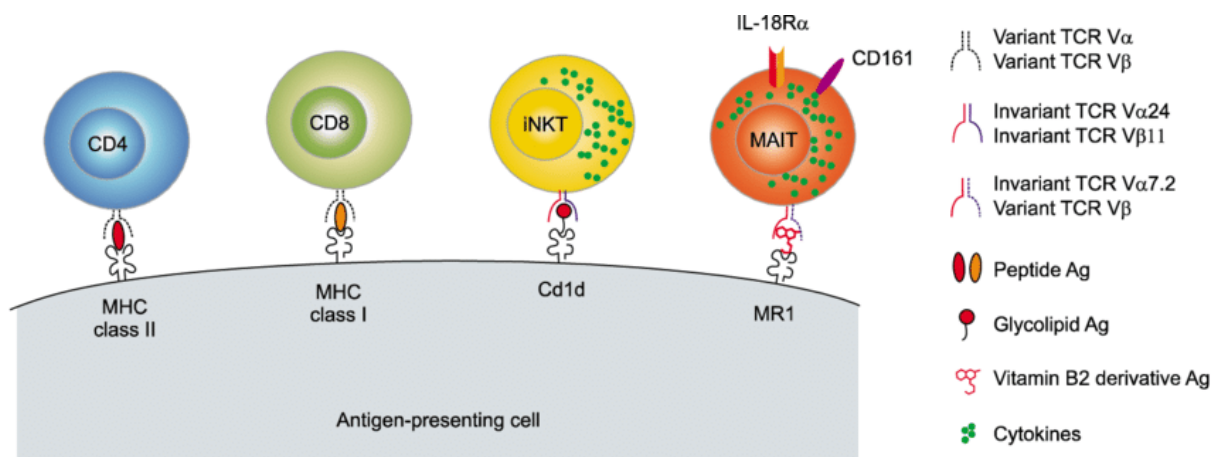


Figure 2: Antigen presentation differences among conventional T cells, invariant natural killer T (iNKT) cells, and mucosal-associated invariant T (MAIT) cells in humans. Adapted from Park et al.[6].

uT cells with $\alpha\beta$ TCRs can be classified based on their recognition of non-polymorphic antigen-presenting molecules such as CD1a, CD1b, CD1c, CD1d, HLA E, and MR1. uT 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". Invariant natural killer T (iNKT) cells express joining region 18 (TRAJ18) and the invariant TCR chain variable region 10 (TRAV10). Additionally, mucosa-associated invariant T cells recognize riboflavin-derived antigens presented on MR1. Mucosa-associated invariant T (MAIT) cells have TCR chains that express

a semi-invariant chain, V α 7.2-J α 33, and β chain. Another population of uT cells, $\gamma\delta$ T cells, arises from somatic recombination during TCR rearrangement through V, D, and J segments [5].

During a microbial infection, some antigen-specific $\alpha\beta$ T cells differentiate into specialized effector cells known as tissue-resident memory T cells. These T cells migrate into nonlymphoid tissues, where they take up residence for extended periods to combat the microbial infection and respond to re-exposure. uT cells share characteristics with tissue-resident T cells. They are believed to be a bridge between innate and adaptive immunity because they can be activated through TCR-dependent and -independent mechanisms [7]. In humans, the cumulative frequency of uT cells (iNKT, $\gamma\delta$ 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. MAIT cells play a role in maintaining tissue homeostasis and are found in immune tissue epithelial surfaces, particularly in symbiotic microbial environments such as the respiratory tract, oral cavity, skin, intestine, and female genital mucosa [8].

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, which are presented via the non-polymorphic MHC class I-like protein MR1 (depicted in Figure 2), a molecule associated with β 2-microglobulin [9]. 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 V α 7.2 (TRAV1-2) joined with J α 33 (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. Pyrimidine-based antigens are produced synthetically from the condensation of 5-A-RU with methylglyoxal, resulting in the formation

of 5-OP-RU (5-(2-oxopropylideneamino)-6-D-ribitylaminouracil). Researchers employ 5-OP-RU, either in its pure form or loaded onto MR-1 tetramers, for the in vitro and in vivo investigation of MAIT cells [10]–[14]. MAIT cells express abundant IL-12 and IL-18 receptors and can respond to cytokines independently of TCR stimulation. Studies have demonstrated that TCR-dependent stimulation leads to rapid polyfunctional activation involving proinflammatory cytokines such as $\text{TNF}\alpha$, $\text{IFN}\gamma$, IL-17, and IL-22. However, IL-12 + IL-18-mediated stimulation results in a slower and more restricted response primarily mediated by $\text{IFN}\gamma$ [15].

MAIT cells defend against viral and bacterial infections [16]. Within healthy individuals, the expression levels of resident and recirculating MAIT cells vary across different anatomical sites. The majority of epidermal MAIT cells express CD103 and cutaneous lymphocyte antigen (CLA), whereas only a tiny proportion in the dermal layer express CD103 [17]. Numerous studies have established a link between MAIT cells, riboflavin-producing bacteria, and a smaller subset of infections caused by non-riboflavin-producing bacteria and fungi. Additionally, MAIT cells have been implicated in viral infections [18]–[21]. Furthermore, these cells have been associated with either a direct or indirect impact on various autoimmune and immune-mediated diseases [22]–[24]. Several types of solid tumors have shown a negative correlation with the frequency of circulating MAIT cells [25]–[29]. Recently, the pro-tissue repair function of MAIT cells was investigated during skin injury in a mouse model. The migration of MAIT cells to inflamed tissues is mediated by CXCR6/CXCL16 interaction, independent of MR-1. Upon recruitment, they produce several tissue repair signals, with amphiregulin being among the most prominent, resulting in accelerated wound healing [30].

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 [31]. The T cell receptor (TCR) of iNKT cells is defined by an invariant TCR α chain, formed by the unique V α 24-J α 18 gene arrangement, in conjunction with TCR β chains V β 11. These iNKT cells demonstrate a remarkable versatility in identifying lipid antigens, including glycolipids and diacylglycerol, sourced from a diverse array of origins, encompassing commensal and pathogenic microorganisms, as well as self-reactive lipid antigens (Figure 2) [32] [33]. Their distinctive maturation process within the thymus sets iNKT cells apart from conventional T cells, where they undergo positive selection alongside thymocytes. This process results in their specificity, which remains remarkably conserved across mammalian species. Moreover, iNKT cells are characterized as tissue-resident T cells, and their functionality is intricately linked to their specific localization within tissues [34]. Notably, cancerous cells frequently exhibit an elevated presence of the CD1d molecule, rendering them a prime target for activation by iNKT cells [35] [36]. Hematopoietic stem cell-engineered iNKT cells possess unique attributes and offer multiple avenues for engagement with the cancer microenvironment, as demonstrated by several clinical trials showcasing the measurable effectiveness of modified iNKT cells in combatting malignancies [37]–[39]. In addition to their pivotal role in cancer immunotherapy, iNKT cells have garnered attention due to their connection with various immune-mediated diseases. Despite their relatively low percentage among circulating T cells, the reduced frequency of iNKT cells in PB has been documented in several pathological conditions, underscoring their significance [40]–[45].

1.2.3. Gamma delta T cells ($\gamma\delta$ T cells)

Gamma delta ($\gamma\delta$) 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 $\gamma\delta$ T cells exhibit a diverse repertoire of TCR genes, encompassing eight variable (V) δ gene segments (V δ 1 - V δ 8) and seven

variable (V) γ gene segments ($V\gamma 2$, $V\gamma 3$, $V\gamma 4$, $V\gamma 5$, $V\gamma 8$, $V\gamma 9$, and $V\gamma 11$) available for rearrangement [46] [47].

Notably, $V\delta 1$, $V\delta 2$, and $V\delta 3$ rearrangements are the most prevalent among the δ gene segments, with the remaining segments being less frequent. In human PB, up to 95% of $\gamma\delta$ T cells comprise the TCR $V\gamma 9V\delta 2$ gene segment, while $V\delta 1$ and $V\delta 3$ $\gamma\delta$ 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 $\gamma\delta$ T cells is their capacity to recognize cells exposed to non-peptide, low-molecular-weight microbacterial ligands, often termed phospho-antigens (Figure 3) [48] [49]. These phospho-antigens are generated through a unique isoprenoid biosynthesis pathway exclusive to bacteria, and synthetic phosphate analogues, known as artificial phospho-antigens, also exist [50]–[52].

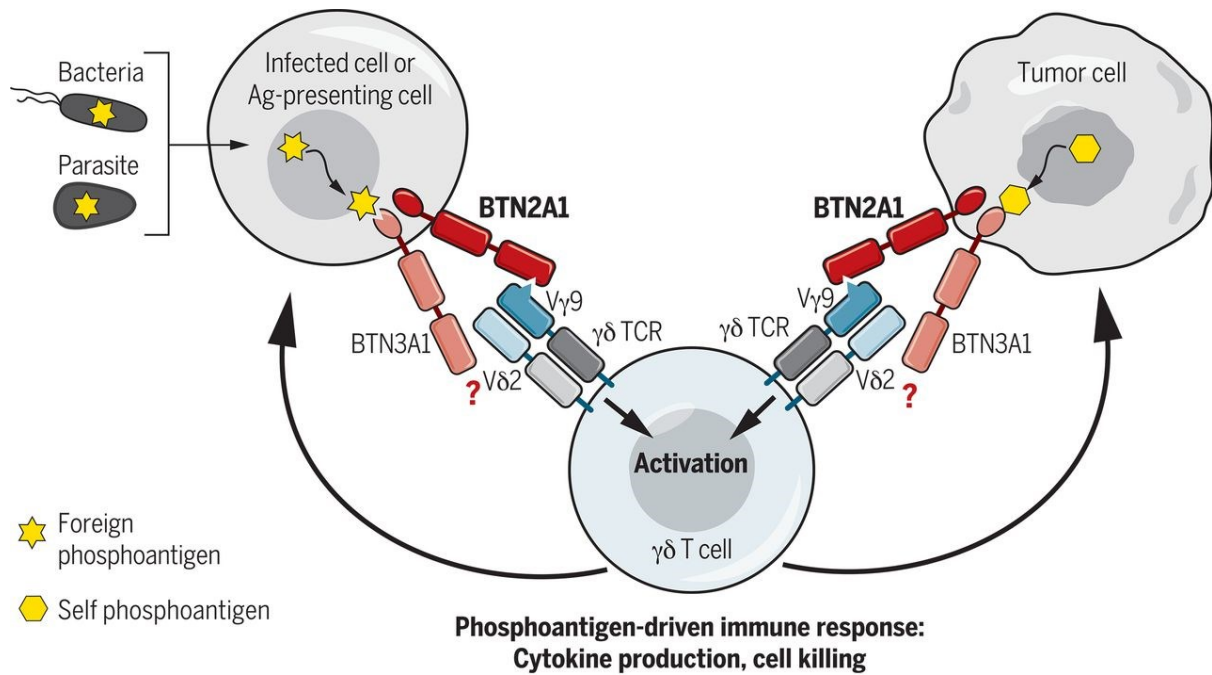


Figure 3: $\gamma\delta$ T cells get activated in response to microbial and cancer-derived phosphoantigens. The molecular mechanism for phosphoantigen recognition remained unclear. Adapted from Rigau et al.[53]

Importantly, unlike conventional T cells, $\gamma\delta$ T cells do not rely on major histocompatibility complex (MHC) molecules for antigen recognition. Non-peptide ligands, including phosphorylated mevalonate metabolites, are present in mammalian and microbial cells and they often upregulated in damaged or stressed cells [50] [45]. This unique attribute enables $\gamma\delta$ T cells to function as a primary defense against various infections, including *Mycobacterium tuberculosis* and tetanus toxoid, and they are implicated in a spectrum of immune-mediated pathological conditions [48],[54],[63],[55]–[62]. Furthermore, $\gamma\delta$ T cells play a crucial role in immune surveillance against the onset of malignancies. They possess the distinct capability to detect stress-induced MICA or MICB-expressing cells through the natural killer group 2 member D (NKG2D) receptor, further emphasizing their importance in immune surveillance and cancer immunity [52],[64],[65]. These intriguing characteristics and roles render $\gamma\delta$ T cells an indispensable and versatile component of the immune system, with the potential to influence a broad range of health and disease conditions [49],[66]–[68].

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

The intricate interplay of aging, gender, and immune responses underscores the multifaceted nature of the human immune system. The aging process significantly impacts the immune system, primarily driven by thymus involution in mammals. Thymus involution begins early, with a reduction in thymic epithelial cells and an accumulation of adipose tissues starting in the first year of life. This reduction in thymic cellularity is most pronounced in the first few decades of life, with an annual decline of 3 to 5% until middle age, after which it slows down to around 1%. 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. Metabolic regulation and adipogenesis also contribute to thymic involution [69],[70].

Immunosenescence, which is primarily a consequence of aging, renders elderly individuals more susceptible to infectious diseases, autoimmune conditions, and cancer [71]. Gender plays a significant role in shaping the immune response, leading to gender bias in both innate and adaptive immunological responses [72] [73]. Males tend to have more natural killer (NK) cells in their PB, while females have more B cells. When peripheral blood mononuclear cells (PBMC) are stimulated with phytohemagglutinin, in females, a significant increase is noted in B and T cells, whereas males show a rise in NK cells [74]. 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. These immune system disparities result in males being more susceptible to non-reproductive tumors, while females are at a higher risk of autoimmune diseases [75],[76].

Hormonal influences significantly contribute to gender-based variations in immune responses to different bacterial and viral vaccinations. Generally, females demonstrate a more robust response compared to males. For instance, estradiol stimulates the production of interferon

(IFN) and pro-inflammatory cytokines like IL-1, IL-6, and TNF, thereby bolstering both cell-mediated and humoral immunity. Progesterone receptors on diverse immune cells, including NK cells, macrophages, dendritic cells, and T cells, exert extensive anti-inflammatory effects [73].

1.4. MAIT cells, V α 7.2+/CD161- T cells and mucosal/skin barriers

MAIT cells, known for their rapid and adaptable responses to diverse stimuli, play pivotal roles in preserving the integrity of mucosal barriers and safeguarding against commensal and pathogenic challenges. They are instrumental in immune surveillance and protection across multiple mucosal obstacles, such as the oral mucosa, respiratory tract, female genital tracts, and the skin [77]–[80]. Despite their abundance in the liver, constituting up to 45% of liver T cells, MAIT cells also represent approximately 10% of T cells in PB [81]–[83].

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. Upon activation, MAIT cells display a diverse cytokine profile, generating various cytokines, including Th1 (TNF- α , IFN- γ), Th2 (IL-2, IL-4, IL-10), and Th17 (IL-17, IL-22) cytokines. The specific cytokine profile exhibited by MAIT cells is influenced by the tissue of origin and the nature of antigenic stimulation they encounter [84].

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 V α 7.2-J α 33⁺ [13]. Within the V α 7.2⁺ 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 [85]. CD161 expression is generally associated with memory cells. In the CD4 compartment, both CD161⁺ and CD161⁻ subsets can be identified. While, in the CD8 compartment CD161 can be further categorized into

CD161⁻, CD161⁺, and CD161⁺⁺ subsets [86][87]. Within the V α 7.2⁺ T cell compartment, the subsets of CD161⁻ (V α 7.2⁺/CD161⁻ T cells) and CD161⁺ (MAIT cells) cells have distinct gene expression profiles despite sharing V α 7.2 expression. However, they exhibit some common upregulated and downregulated genes that differ from conventional T cells, offering insights into the relationship between these distinct cell types [88].

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 TGF β , aiding tissue repair. MAIT cells respond to pathogens when infected or dysbiotic. In addition to direct TCR-dependent interactions, they can be activated in a TCR-independent way by inflammatory cytokines such as IL-7, IL-12, IL-15, IL-18, and type I interferon. The dual activation mode triggers the production of proinflammatory cytokines and cytotoxic mediators, including IL-17, TNF α , IFN γ , granzyme, and perforins, which aid in pathogen clearance. Nevertheless, excessive activation of MAIT cells can precipitate chronic mucosal inflammatory disorders [84]. The precise function of V α 7.2⁺/CD161⁻ T cells remains uncertain. However, there is a notable increase in their presence in the PB of chronic HCV patients compared to healthy controls, and MAIT cells seem to be compromised in this context [89]. Chronic HIV-1 infection results in an elevation of V α 7.2⁺/CD161⁻ T cells and a reduction in MAIT cells relative to healthy controls, with no significant alteration in total T cell count [90].

A recent study has identified populations of MAIT cells in tonsils that express conventional follicular helper T cell markers, including B-cell lymphoma 6 (BCL-6), and exhibit the CXCR5⁺ phenotype. Furthermore, compared to PB MAIT cells, follicular MAIT cells demonstrate upregulation of inducible T cell costimulatory molecule (ICOS) and programmed death receptor 1 (PD-1). Notably, these CXCR5⁺ follicular MAIT cells produce higher levels of IL-21, suggesting a role in activating T-dependent B cells in germinal centers [91]. 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, alongside their role in maintaining skin homeostasis. Unraveling the involvement of MAIT cells in skin diseases offers fresh insights into the body's defense mechanisms and suggests a potential avenue for developing innovative therapeutic approaches.

1.5. Atopic dermatitis, MAIT cells and V α 7.2+/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). Disruption of the epidermal barrier triggers keratinocytes to recruit ILC2 and Th2 cells through chemokines, producing key cytokines such as IL-4, IL-5, and IL-13. Allergen-activated dendritic cells trigger the release of both IL-4 and IL-13 from naive T cells. These type 2 cytokines play crucial roles in inducing IgE class switching, recruiting eosinophils, and promoting Th22 differentiation, all of which contribute to heightened skin barrier disruption and a pro-inflammatory environment. 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 [92]–[95].

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 [96],[97]. The influence of MAIT cells on AD in humans is still under

investigation, and the role of $V\alpha 7.2+/CD161-$ T cells remains uncertain. Naidoo et al. conducted a study utilizing a mouse model to shed light on the potential involvement of MAIT cells in AD. Their findings revealed that deleting MR1 in mice or functionally blocking MAIT cell activation using an antagonist called 6-formylpterin (6-FP) significantly reduced AD progression. Eosinophils and basophils play a significant role in type 2 inflammation in AD. Functional blockade of MAIT cells resulted in reduced skin-infiltrating eosinophils, and IL-4-producing basophils failed to activate, leading to a substantial reduction in inflammation [98].

1.6. Multicolor flow cytometry

Multicolor flow cytometry provides a robust tool for examining and quantifying the characteristics of individual cells within heterogeneous populations. Flow cytometry's heart is the ability to rapidly and accurately measure a myriad of cellular properties, including size, granularity, phenotype, and functionality, all in a high-throughput manner. Flow cytometry operates on the fundamental principle of analyzing individual cells or particles as they flow in a single file through a focused laser beam.

Flow cytometry measures both light scattering and fluorescence. When a cell passes through the laser beam, it scatters light, providing information about cell size (forward scatter, FSC) and granularity (side scatter, SSC). Additionally, if the cell is labeled with antibodies conjugated with fluorescent dyes, it emits fluorescence when excited by the laser. The emitted fluorescence is captured and can be used to determine various characteristics of the cell, such as its phenotype, viability, or intracellular molecules. Flow cytometry can simultaneously measure multiple parameters (8-13 fluorescent signals plus scatter characteristics) in a single experiment by combining different fluorochromes with distinct emission spectra. This enables the characterization of complex cell populations and the investigation of various cellular features simultaneously. Flow cytometry can also sort cells based on their properties. It allows the isolation of pure cell populations for further research [99].

1.7.Multidimensional data analysis

Flow cytometry is a powerful technique for simultaneously measuring multiple parameters on single cells, producing high-dimensional data. Analyzing these complex datasets effectively has been challenging, but traditional methods and recent advancements have significantly improved our ability to interpret and visualize the information they contain.

Traditional flow cytometry data analysis often begins with manual gating. Researchers define regions, or "gates," on bivariate plots to isolate specific cell populations. While adequate for simple analyses, it can be subjective and time-consuming. Histograms and density plots are graphical representations providing an overview of marker expression distributions. Histograms show the frequency of marker expression levels, while density plots display the overlap of multiple markers' distributions. Heatmaps visualize the expression of various markers across different cell populations. They help to identify marker co-expression patterns but may not capture the full complexity of high-dimensional data.

The recent advancements of machine learning algorithms such as t-SNE (t-Distributed Stochastic Neighbour Embedding), FlowSOM (Self-Organizing Maps), and Phenograph, along with traditional methods, have revolutionized the analysis of high-dimensional flow cytometric data. They offer more objective, comprehensive, and sophisticated insights into cell populations, marker relationships, and biological processes, facilitating groundbreaking discoveries in immunology, oncology, and many other fields. t-SNE is a dimensionality reduction technique that maps high-dimensional data to a lower-dimensional space while preserving local similarities. t-SNE helps uncover previously unnoticed cell populations and relationships among markers [100]. FlowSOM is an unsupervised clustering algorithm that groups cells based on marker expression patterns. It generates self-organizing maps and hierarchically organizes the data into meta clusters, revealing complex cell population structures. FlowSOM allows for deeper insight into data organization and cell subsets [101].

1.8. Problem Statement

The pro and anti-inflammatory roles of uT 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 iNKT, $\gamma\delta$ T, and 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 uT cells in research and standard laboratory diagnostics. Our hypothesis posits that uT 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 MAIT cells in various skin disorders. 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 PB MAIT cells in its progression. MAIT cells, a crucial link between the innate and adaptive arms of the immune system, are more abundant in PB than other uT cell subsets. Alongside CD161⁺ MAIT cells, CD161⁻ T cells are also present within the V α 7.2⁺ T cell compartment. However, their specific role remains to be thoroughly investigated. Based on observations, we hypothesize that MAIT cells, V α 7.2⁺/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 V α 7.2⁺/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 V α 7.2+/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, $\gamma\delta$ 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 PB.
- 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, $\gamma\delta$ T cells, and MAIT cells) has not been studied yet, and we aimed to investigate immunosenescence in these populations.
- iii. 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^{negative} T cells are also present within the V α 7.2⁺ T cell compartment with almost no information. We aim to investigate the involvement of human PB MAIT and V α 7.2⁺/CD161⁻ T cells in AD.
- iv. MAIT 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 AD, 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 α 7.2⁺/CD161⁻ T cells in different stimulatory conditions. Furthermore, we planned to compare MAIT and V α 7.2⁺/CD161⁻ T cells under the same conditions.

3. MATERIAL AND 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 (Table 1). 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.

mAb Name	Clone	Fluorescence	Manufacturer
anti-human CD16	3G8	Fluorescein isothiocyanate (FITC)	BC/IOT
anti-human IL-1 β	JK1B-1		Biolegend
anti-human Cutaneous lymphocyte antigen (CLA)	HECA-452		Biolegend
anti-human TCR V γ 9	B3		Biolegend
anti-Human IFN- γ	25723.11		Biolegend
anti-Human IL-13	3010.211		Biolegend
anti-Human IL-4	JES10-5A2	Phycoerythrin (PE)	Biolegend
anti-human TNF- α	REA656		MACS
anti-human CD38	HB-7		BD
anti-human CD56	MOC-1		Dako
anti-human CD7	8H8.1		BC/IOT
anti-human TCR V α 24-J α 18	6B11	Peridinin chlorophyll protein/Cynine 5.5 (PerCP/Cy5.5)	Biolegend
anti-human TCR V δ 2	B6		Biolegend
anti-human TCR V α 7.2	3C10		Biolegend
anti-human TCR V α 24	C15	PE-Cynine 7 (PC7)	BC/IOT
anti-human CD161	191B8		BC/IOT
anti-human CD3	SK7	Allophycocyanin (APC)	BD
anti-human IL-18R α	H44		Biolegend
anti-human IL-22	2G12A41		Biolegend
anti-human CD279 (PD-1)	REAL383		MACS
anti-human CD8	SK1		BD

anti-human CD3	SK7	Allophycocyanin cyanine 7 (APC-H7)	BD
anti-human CD4	RPA-T4	Pacific blue (PB)	BD
anti-human IL-17A	BL168		Biolegend
anti-human granzyme B (GzB)	QA16A02		Biolegend
anti-human CD69	FN50		Biolegend
anti-human CD45	HI30	Pacific orange (PO)	Exbio

Table 1: Monoclonal antibodies used in various experiments, their clones, and different fluorescent materials tagged along with manufacturer details are given. BC/IOT: Beckman Coulter (Brea, CA, USA), Dako (Glostrup, Denmark), Biolegend (San Diego, CA, USA), BD/Pharmingen (Franklin Lakes, NJ, USA), Exbio (Prague, Czech Republic), MACS Miltenyi Biotec (Bergisch Gladbach, Germany).

3.1.2. Antibody titration and stain index

Cell surface and intracellular markers were titrated to determine the optimal flow cytometric staining concentration. Surface markers; anti-human TCR V γ 9, anti-human TCR V α 24-J α 18 (clone 6B11), anti-human TCR V δ 2, anti-human TCR V α 7.2, anti-human TCR V α 24, anti-human CD161, anti-human CD69, anti-human Cutaneous lymphocyte antigen (CLA), anti-human CD279 (PD1), and anti-human CD3. Separately, intracellular staining markers were also titrated, such as anti-human IFN- γ , anti-human IL-22, anti-human IL-4, anti-human IL-17A, anti-human IL-1 β , anti-human TNF- α , anti-human granzyme B, and anti-human IL-18R α . The remaining antibodies were previously titrated.

Cellular surface staining was performed using stain, lyse, and wash protocol as described below. Briefly, recommended concentrations of antibodies were used as stock and then diluted further (2x, 4x, 8x, 16x, and 32x) in PBS. These dilutions were then incubated with PB. Later, cells were subjected to RBC lysis and washed using centrifugation before acquisition. Pre-titrated anti-human CD3-APC was used as a backbone marker to gate T cells.

For intracellular staining, isolated PBMCs were stimulated with Phorbol 12-myristate 13-acetate (PMA)/Ionomycin in the presence of Brefeldin. A detailed protocol is described below in section 3.3.2. Median fluorescence intensity (MFI) was recorded in the respective channel,

and the stain index was calculated as follows:

$$(Median\ of\ Positive - Median\ of\ Negative) \div (SD\ of\ Negative \times 2).$$

3.1.3. Fluorescence minus one control and gating

A fluorescence minus one (FMO) control was run to identify the fluorescence spread of antibodies used to stain the population of interest. Staining was performed using a lyse-wash protocol on the PB collected in EDTA anticoagulant, as described below. Four tubes were stained for each of the uT cells according to the FMO matrix (Table 2).

Tube	FITC	PE	PerCP/Cy5.5	PC7	APC	APC-H7	PB	PO
Unstained	-	-	-	-	-	-	-	-
iNKT	CD16	CD56	6B11	Vα24	CD3	-	-	CD45
iNKT	CD16	CD56		Vα24	CD3	-	-	CD45
iNKT	CD16	CD56	6B11		CD3	-	-	CD45
Unstained	-	-	-	-	-	-	-	-
γδ T	Vγ9	CD7	Vδ2	-	CD3	CD8	CD4	CD45
γδ T		CD7	Vδ2	-	CD3	CD8	CD4	CD45
γδ T	Vγ9	CD7		-	CD3	CD8	CD4	CD45
Unstained	-	-	-	-	-	-	-	-
MAIT	-	CD56	Vα7.2	CD161	CD3	CD8	CD4	CD45
MAIT	-	CD56		CD161	CD3	CD8	CD4	CD45
MAIT	-	CD56	Vα7.2		CD3	CD8	CD4	CD45

Table 2: A fluorescence minus one (FMO) matrix of antibodies was used to stain invariant Natural killer T cells, Gamma delta T cells, and Mucosa-associated invariant T cells. Three tubes were used to stain TCRs for each uT cell. Each tube lacked one antibody, and the remaining antibodies in the panel remained the same to see the fluorescence spread. Unstained tubes were used as controls.

3.1.4. Lyse-wash protocol of flow cytometric staining

The stain, lyse, and wash method was utilized for cell surface staining. In a FACS tube, 100 μ L of 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 μ L of 1% paraformaldehyde.

3.1.5. Sample acquisition on a flow cytometer

All data acquisitions were conducted on a BD FACS Canto II flow cytometer (Franklin Lakes, NJ, USA). To monitor equipment performance, routine internal quality control assessments were executed using a cytometer setup and tracking beads (CS&T). Additionally, participation in the UK-NEQAS Leukaemia Immunophenotyping Programme ensured external quality control for equipment and procedures. Tubes were processed manually or using a carousel setup, with acquisition rates set at medium to high speed and a stop gate established at 300,000 events per tube.

3.2. Sample stability

To establish the stability of the samples, we performed experiments where uT cells were measured from ten samples repeatedly every 24 hours for four days. The stain-lyse wash protocol was performed as described above (section 3.1.4.) by adding SYTO dye to discriminate between live and dead cells. uT cells were gated as described in section 3.5.4. and data from all measurements were collected. Statistical calculations were performed as described in 3.8. One-way ANOVA test revealed no significant difference across the measured period.

3.3. PBMCs stimulation and intracellular staining

3.3.1. PBMCs isolation

PBMCs 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 (Merck KGaA, Darmstadt, Germany). Cells were rewashed with centrifugation at 1500 RPM for 5 minutes before performing cell count and were adjusted around 1×10^6 – 2×10^6 cells/100 μ L.

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

PBMCs were stimulated with 30 ng/mL of phorbol 12-myristate 13-acetate (PMA) and 1 μ g/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 μ g/mL of Brefeldin-A was added and gently vortexed. Incubation was continued for an additional 3 hours and 20 minutes.

3.3.3. 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 μ L 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 μ L 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 μ L of paraformaldehyde. The acquisition was performed as described above at 3.1.5. section.

3.4. Ethics Clearance

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

3.5. Establishing reference range of uT

3.5.1. Blood sample collection and complete blood cell count

Three mL PB samples of healthy controls (HC) were collected through venipuncture in a BD vacutainer tube containing tri-potassium ethylene-diamine tetra acetic acid (Becton Dickinson, San Jose, CA, USA). A complete blood count (CBC) was performed using a six-part hematology analyzer Siemens ADVIA® 2120i (Siemens Healthcare GmbH, Erlangen, Germany).

3.5.2. Exclusion criteria

We defined strict exclusion criteria to evaluate HC samples, known as the SENIEUR protocol. This protocol was modified due to the COVID-19 pandemic situation [102]. 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 1 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.

3.5.3. Healthy control study population

To establish the reference range of uT 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—Clinical Laboratory Standards Institute 2008 guidelines defined the method [48] to establish reference ranges. 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. Ninety-three samples were from males, and one hundred and nine samples belonged to females (Table 3).

	Age groups (years)				Gender	
	18-35	36-50	51-65	66-90	Male	Female
Total samples included (n)	56	77	52	18	93	109
Mean age $\pm$ SD (years)	29 $\pm$ 5	44 $\pm$ 4	58 $\pm$ 4	73 $\pm$ 4	44 $\pm$ 14	45 $\pm$ 14

Table 3: Description of samples used to establish reference range and immunosenescence of uT cells. Age-wise and gender-wise distribution of samples with different age groups, mean age, and standard deviation are given.

3.5.4. Flow cytometric panel, phenotyping, and data analysis

A multicolor flow cytometric panel was employed to identify uT cells using the monoclonal antibodies detailed in Table 4, following the lyse-wash methodology outlined in sections 3.1.4 and 3.1.5. Data analysis was conducted using FACSDiva version 6.1.3 software (BD Biosciences, San Jose, CA, USA) after sequentially gated flow cytometric plots. The T-cell receptor (TCR) of iNKT cells was stained using the V α 24-J α 18 clone 6B11 and characterized as CD45⁺/CD3⁺/V α 24⁺/V α 24-J α 18(6B11)⁺. The TCR of $\gamma\delta$ T cells was stained with V γ 9 and V δ 2 and characterized as CD45⁺/CD3⁺/V γ 9⁺/V δ 2⁺. Mucosal-associated invariant T (MAIT)

cells' TCR was identified as V α 7.2 and classified as CD45+/CD3+/V α 7.2+/CD161bright+ (Figure 4). The percentages of lymphocytes were estimated among white blood cells, with T cells gated among lymphocytes and iNKT cells and MAIT cells gated among total T cells and $\gamma\delta$ T cells were categorized into subsets including V δ 2, V γ 9, and conventional T cells. The absolute counts of total iNKT cells, $\gamma\delta$ T cells, and mucosal-associated invariant T cells were calculated utilizing the absolute white blood cell (WBC) and lymphocyte counts (expressed as $\times 10^9/L$) obtained from the complete blood count (CBC) report through a dual-platform approach. These values were expressed as cells per microliter (cells/ μ L).

Tube	FITC	PE	PerCP/Cy5.5	PC7	APC	APC-H7	PB	PO
iNKT	CD16	CD56	6B11	V α 24	CD3	-	-	CD45
$\gamma\delta$ T cells	V γ 9	CD7	V δ 2	-	CD3	CD8	CD4	CD45
MAIT	-	CD56	V α 7.2	CD161	CD3	CD8	CD4	CD45

Table 4: Monoclonal antibody panel to stain uT cells for the healthy control study to establish reference ranges and immunosenescence.

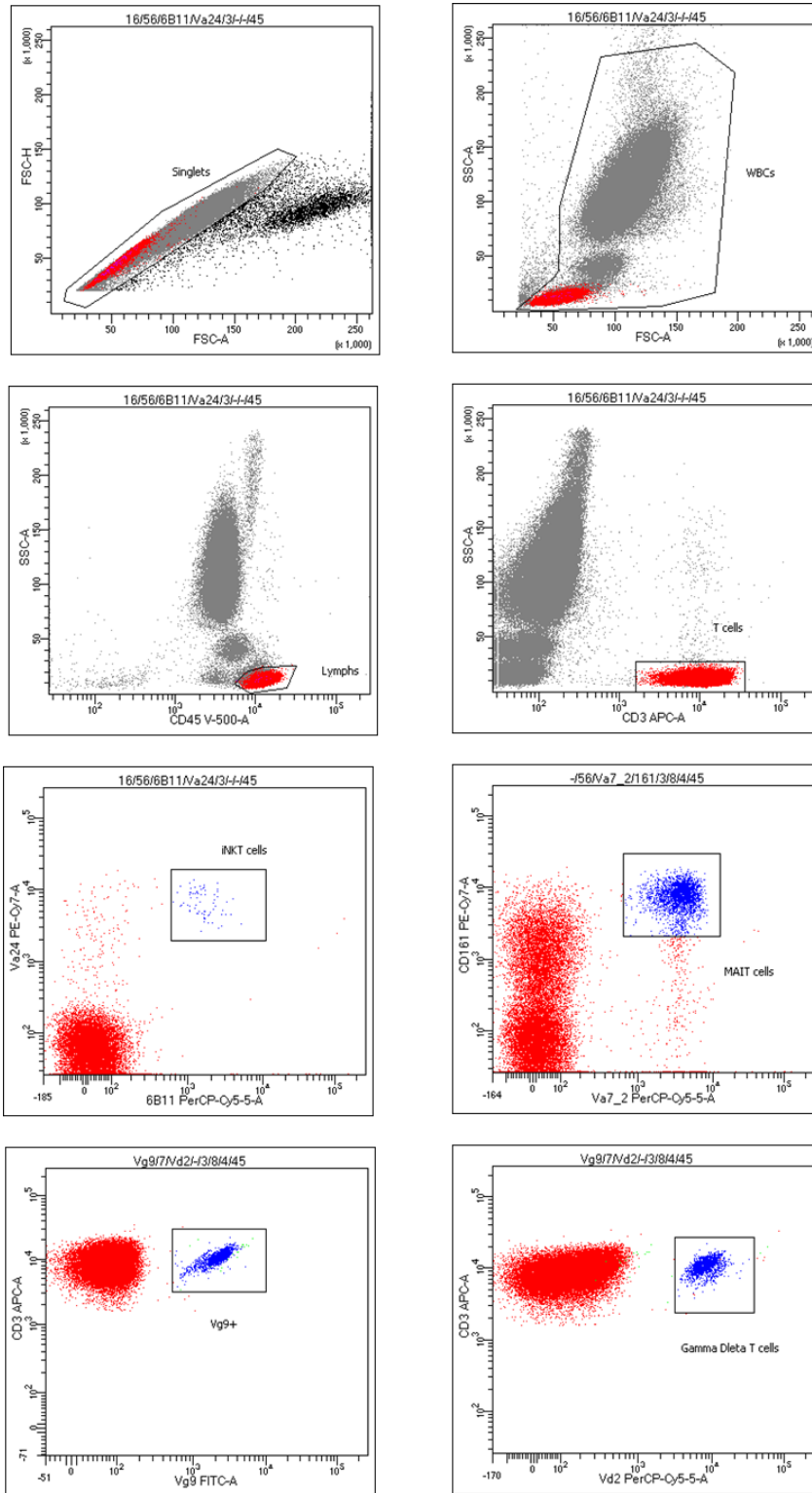


Figure 4: Sequential gating strategy utilized in the analysis, beginning with isolating universally singlet cells from the total events. Subsequently, white blood cells (WBCs) were gated among singlets, followed by the isolation of lymphocytes among the WBC population. Further gating was performed to extract T cells among the identified lymphocytes. Within the T cell subset, gating was applied to isolate invariant natural killer T (iNKT) cells, Mucosal-associated invariant T (MAIT) cells, and V γ 9 cells, followed by gating for V δ 2 cells within the V γ 9 subset, identified as Gamma Delta T cells.

3.6. Characterization of MAIT and V α 7.2+/CD161- T cells in AD

3.6.1. Sample collection and counting

PB samples from 14 untreated AD patients were obtained from the Department of Dermatology, 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 (Becton Dickinson, San Jose, CA, USA) from each donor. CBC was performed using a six-part hematology analyzer Siemens ADVIA® 2120i (Siemens Healthcare GmbH, Erlangen, Germany).

3.6.2. Cell surface immunophenotyping

K3-EDTA anticoagulated blood was processed using the lyse wash protocol described above in section 3.1.4. A multicolor flow cytometric panel was designed to stain MAIT and V α 7.2+/CD161- T cells and their subsequent phenotypes with pre-titrated antibodies (Table 5).

	FITC	PE	PerCP/Cy5.5	PC7	APC	APC-H7	PB	PO
Tube 1	CLA	CD38	V α 7.2	CD161	CD3	CD8	CD4	CD45
Tube 2	-	-	V α 7.2	CD161	PD-1	CD3	CD69	CD45
Tube 3*	cyIFN- γ	cyIL-4	V α 7.2	CD161	cyIL-22	CD3	cyIL-17A	CD45
Tube 4*	cyIL-1 β	cyTNF- α	V α 7.2	CD161	IL-18R α	CD3	cyGzB	CD45

*Table 5: Monoclonal antibody panel to stain cellular surface and intracellular markers of mucosa-associated invariant T cells and V α 7.2+/CD161- T cells for atopic dermatitis and healthy control samples. Heparinized peripheral blood was used to stain Tubes 1 and 2 using a lyse wash protocol. *For Tubes 3 and 4, PBMCs were isolated and stimulated with PMA/Ionomycin, and intracellular staining was performed to detect cytokines post-brefeldin A treatment.*

3.6.3. Cytokine balance measurement

PBMC was isolated from the heparinized PB, and PMA/Ionomycin stimulation was performed as described in section 3.3.2. Surface and intracellular staining were performed to detect Th1, Th2, and Th17 cytokines using an intracellular antibody panel (Table 5), following the intracellular staining protocol mentioned in section 3.3.3.

3.6.4. Flow cytometric data analysis

Multiparametric data were collected through four distinct tubes from each sample. Tubes A and B were designated for surface marker analysis, while tubes C and D focused on assessing intracellular cytokines following PMA stimulation. The Flowjo v10.9.0 software (TreeStar Inc., Ashland, OR, USA) was employed for the sequential gating of T cells and their subsequent subsets and for conducting multidimensional data analysis.

3.6.5. Sequential gating of T cells, MAIT cells, and V α 7.2+/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. As depicted in Figure 5, events positive for V α 7.2 and CD161 were classified as Mucosal-Associated Invariant T (MAIT) cells, while V α 7.2+/CD161- cells were designated as V α 7.2+/CD161- T cells. Subsets such as CD4, CD8, PD-1, CD69, CD38, cutaneous lymphocyte antigen (CLA), interferon-gamma (IFN γ), interleukin-4 (IL-4), IL-22, IL-17A, tumor necrosis factor-alpha (TNF α), granzyme B (GzB), TNF α /GzB, interleukin-1 beta (IL-1 β), and interleukin-18 receptor alpha (IL-18R α) were gated within T cells, MAIT cells, and V α 7.2+/CD161- T cells, respectively. The proportion of these subsets from the parent gate was used in future statistical analysis.

UNIVERSAL GATING

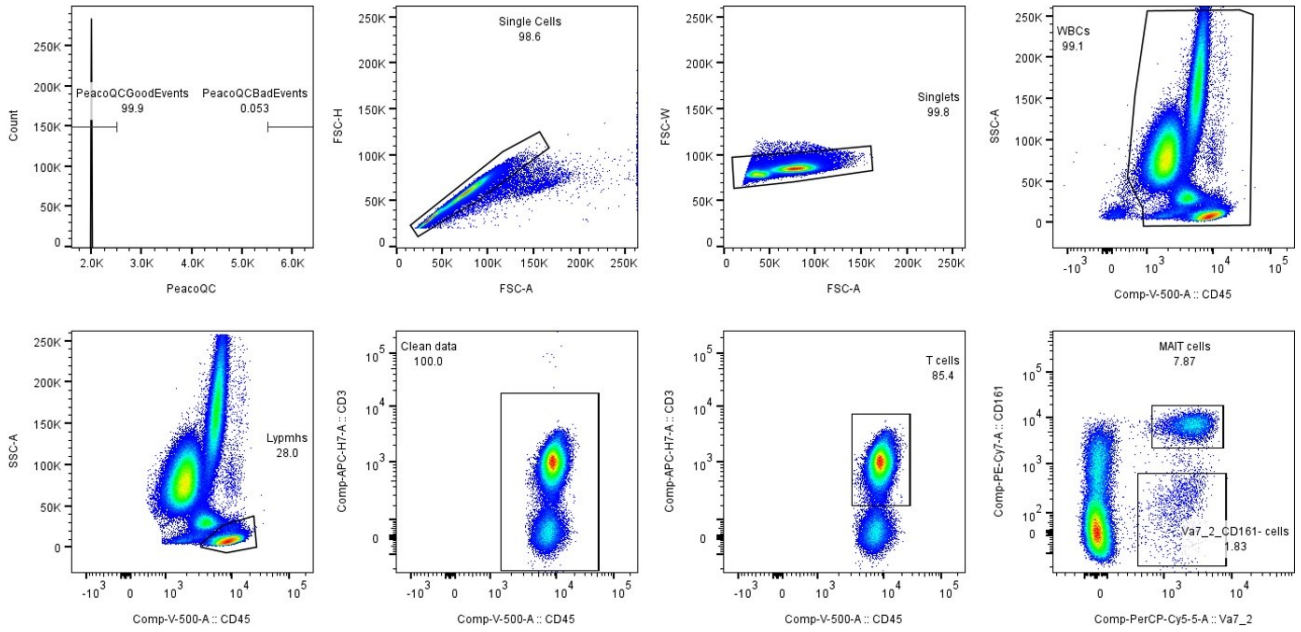


Figure 5: Universal gating of T cells. Sequential gating of cells from left to right and top to bottom. Events are gated on PeacoQC good events; doublets were removed from FSC area vs. height and area vs. width. WBCs were gated in CD45 vs SSC, and lymphocytes were gated within the WBC gate. A clean gate on CD45 vs CD3 was applied to remove antibody precipitates, and T cells were gated among clean lymphocyte gates. $V\alpha 7.2^+$ subsets were gated as CD161⁺ (MAIT cells) and CD161⁻ T cells.

3.6.6. Multidimensional data analysis

The same universal gates were applied to isolate T cells for multidimensional data analysis, as illustrated in Figure 5. 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 for each of the four tubes (1, 2, 3, and 4) individually, encompassing both AD and 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 [103]. 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 [101]. The optimization of FlowSOM

meta cluster numbers involved multiple clustering iterations with varying numbers of meta clusters in preventing over and under-clustering, resulting in the identification of ten clusters in tube 1, eighteen in tube 2, fourteen in tube 3, and sixteen in tube 4 (Figures 6 and 7). A slight preference for over-clustering was adopted to avoid overlooking less expressive phenotypes.

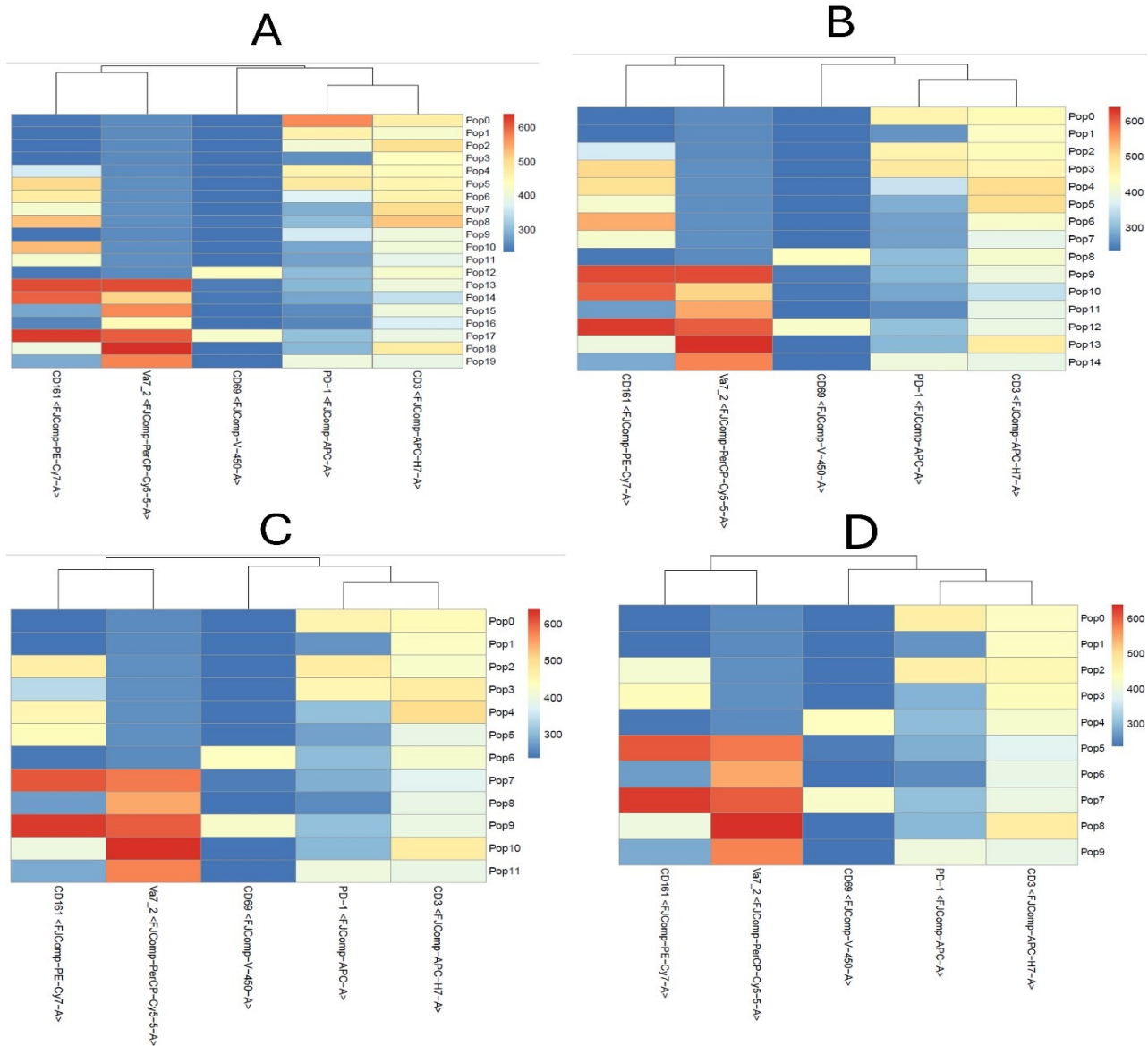


Figure 6: FlowSOM clustering was repeatedly performed on datasets to optimize meta clusters, decreasing clusters in each iteration. Heatmaps representing fluorescence expression in different channels from different clusters are shown from Tube 1. 20 meta-clusters (A) show over-clustering, and multiple clusters show similar expressions. (B) meta clusters are reduced to 15, but similar expression was identified between several clusters. (D) Ten meta-clusters show that biologically significant clusters are getting merged. Twelve meta-clusters were optimal since they are slightly over-clustered and not under-clustered (C).

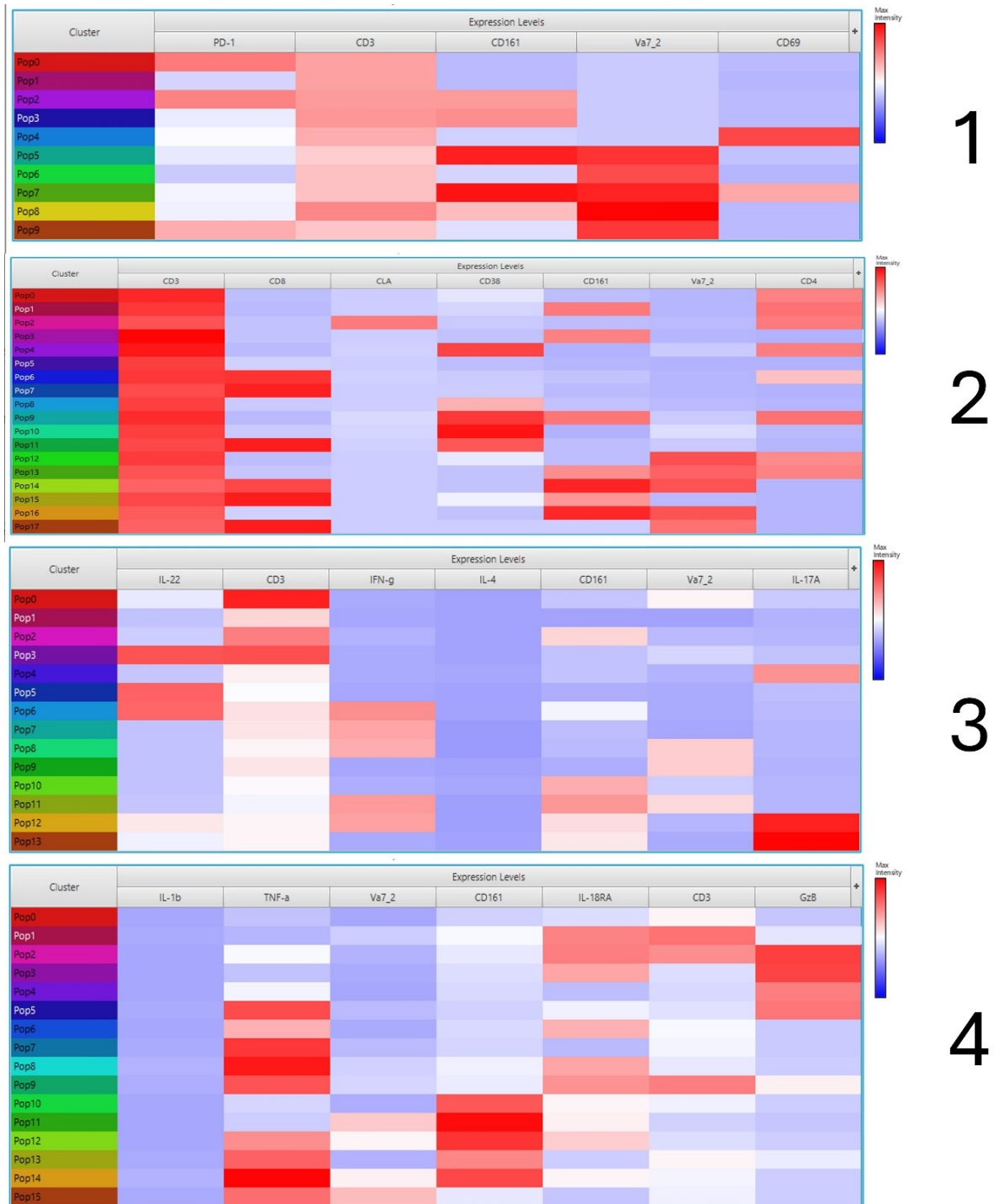


Figure 7: FlowSOM biologically relevant immune clusters identified within different tubes. (1) Ten immune clusters were identified within this tube expressing different subsets of T cells. (2) Eighteen immune clusters were identified within this tube expressing distinct T cell subsets. (3) Fourteen immune clusters were identified within this tube expressing distinct T cell subsets, and (4) Sixteen immune clusters were identified within this tube expressing distinct T cell subsets.

3.7. Effects of immunomodulatory drugs on MAIT and V α 7.2+/CD161- T cells

3.7.1. 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 (Michigan, USA), and 40% H₂O of the methylglyoxal solution was purchased from Merck (KGaA, Darmstadt, Germany). 5-OP-RU were made freshly before the experiment and diluted in dimethyl sulfoxide (DMSO), where 5-A-RU and methylglyoxal were incubated for 2 hours at room temperature. 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. Both cytokines were purchased from Biolegend (San Diego, CA, USA) and diluted in RPMI media, and 50 ng/mL 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 μ g/mL). Ten ng/mL of Brefeldin-A was used diluted in RPMI.

Immunomodulatory drugs (Vitamin D₃ and Cyclosporin A) were purchased from Merck (KGaA, Darmstadt, Germany). 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.

3.7.2. Sample collection and PBMCs isolation

18 mL of PB was collected through venipuncture in a BD vacutainer containing sodium heparin anticoagulant (Becton Dickinson, San Jose, CA, USA) from 7 HC donors. PBMCs were isolated, and cell concentration was adjusted following the protocol mentioned in section 3.3.1.

3.7.3. Experiment design

The experiment was performed in a FACS tube containing $1 \times 10^6 - 2 \times 10^6$ PBMCs/100 μ L, and the total volume was adjusted to 1 mL of RPMI. The complete experiment design and setup are described in Table 6. All the tubes were vortexed and incubated at 37°C with 5% CO₂ 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 3.3.3. Three multicolor panels of antibodies containing surface and intracellular markers (Table 7) were stained from each experimental tube to see the changes in MAIT cells.

Tube no	Stimulation	Cyclosporin A	Vitamin D3
1	Unstimulated	-	-
2	PMA/Ionomycin	-	-
3	5-OP-RU	-	-
4	5-OP-RU+IL-12 and IL-33	-	-
5	Unstimulated	+	-
6	PMA/Ionomycin	+	-
7	5-OP-RU	+	-
8	5-OP-RU+IL-12 and IL-33	+	-
9	Unstimulated	-	+
10	PMA/Ionomycin	-	+
11	5-OP-RU	-	+
12	5-OP-RU+IL-12 and IL-33	-	+

Table 6: Experimental design to test the effect of immunomodulatory drugs on MAIT cells in different stimulatory conditions. Three stimulatory conditions (PMA/Ionomycin, 5-OP-RU, and 5-OP-RU+IL12+IL33) and unstimulated (control). All tubes were incubated with or without immunomodulatory drugs (Cyclosporin A or Vitamin D3) for 24 hours; for the last 6 hours, Brefeldin-A was added.

Tube	FITC	PE	PerCP/Cy5.5	PC7	APC	APC-H7	PB	PO
Tube 1	CLA	CCR6	V α 7.2	CD161	PD-1	CD3	CD69	CD45
Tube 2	cyIFN- γ	cyIL-4/IL-13	V α 7.2	CD161	cyIL-22	CD3	cyIL-17A	CD45
Tube 3	Syto	cyTNF- α	V α 7.2	CD161	CD38	CD3	cyGzB	CD45

Table 7: Monoclonal antibody panel to stain MAIT cells after the immunomodulatory drug experiment, including surface and cytoplasmic antibodies. Tube 1 contains only surface markers, while tubes 2 and 3 contain surface and intracellular targets.

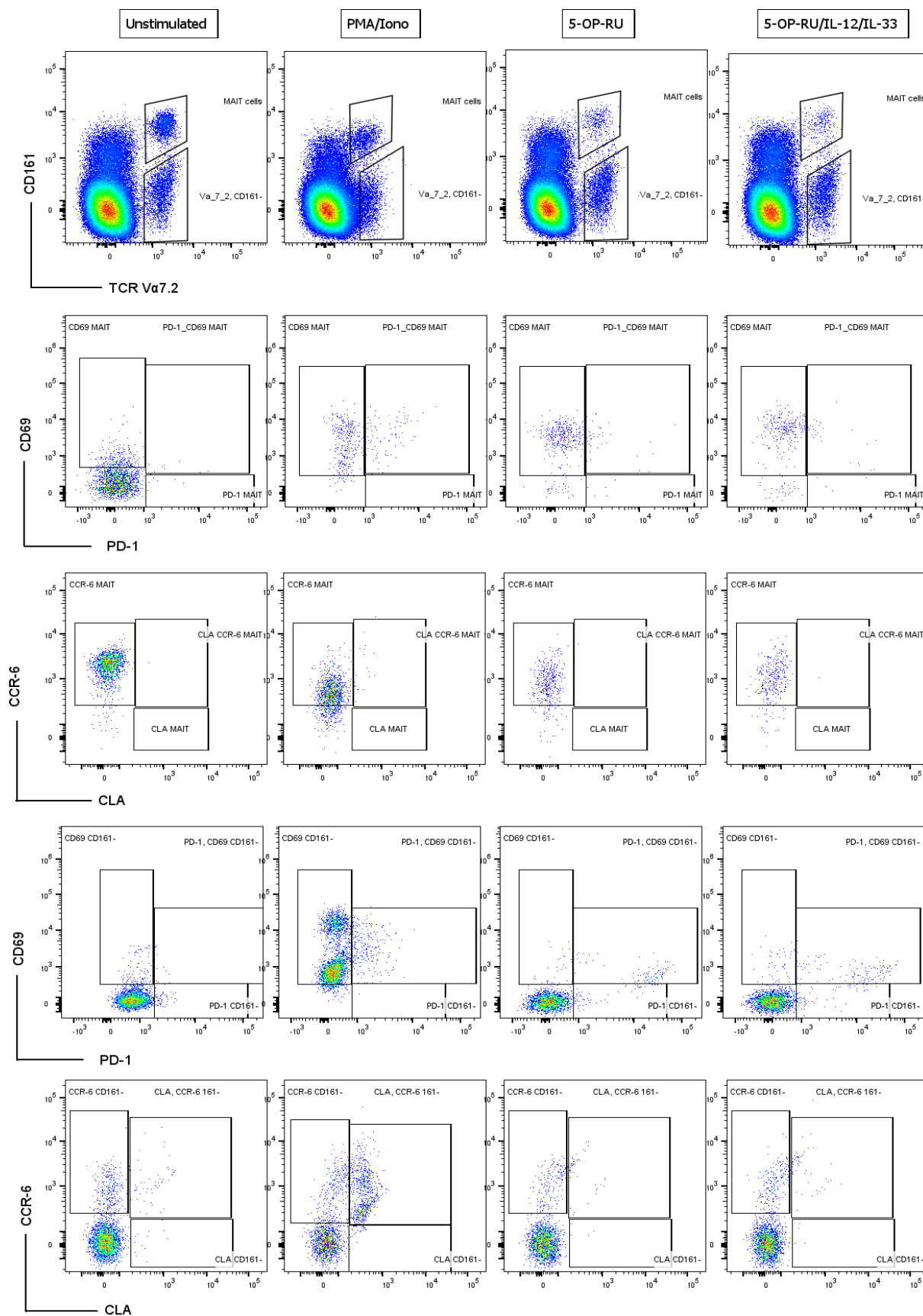
3.7.4. Flow cytometric measurement

Post-staining cells were dissolved in 400 μ L of paraformaldehyde, and the acquisition was performed as described above in section 3.1.5. Syto 16 nucleic acid dye was added before the acquisition, and 200,000 events were targeted and measured.

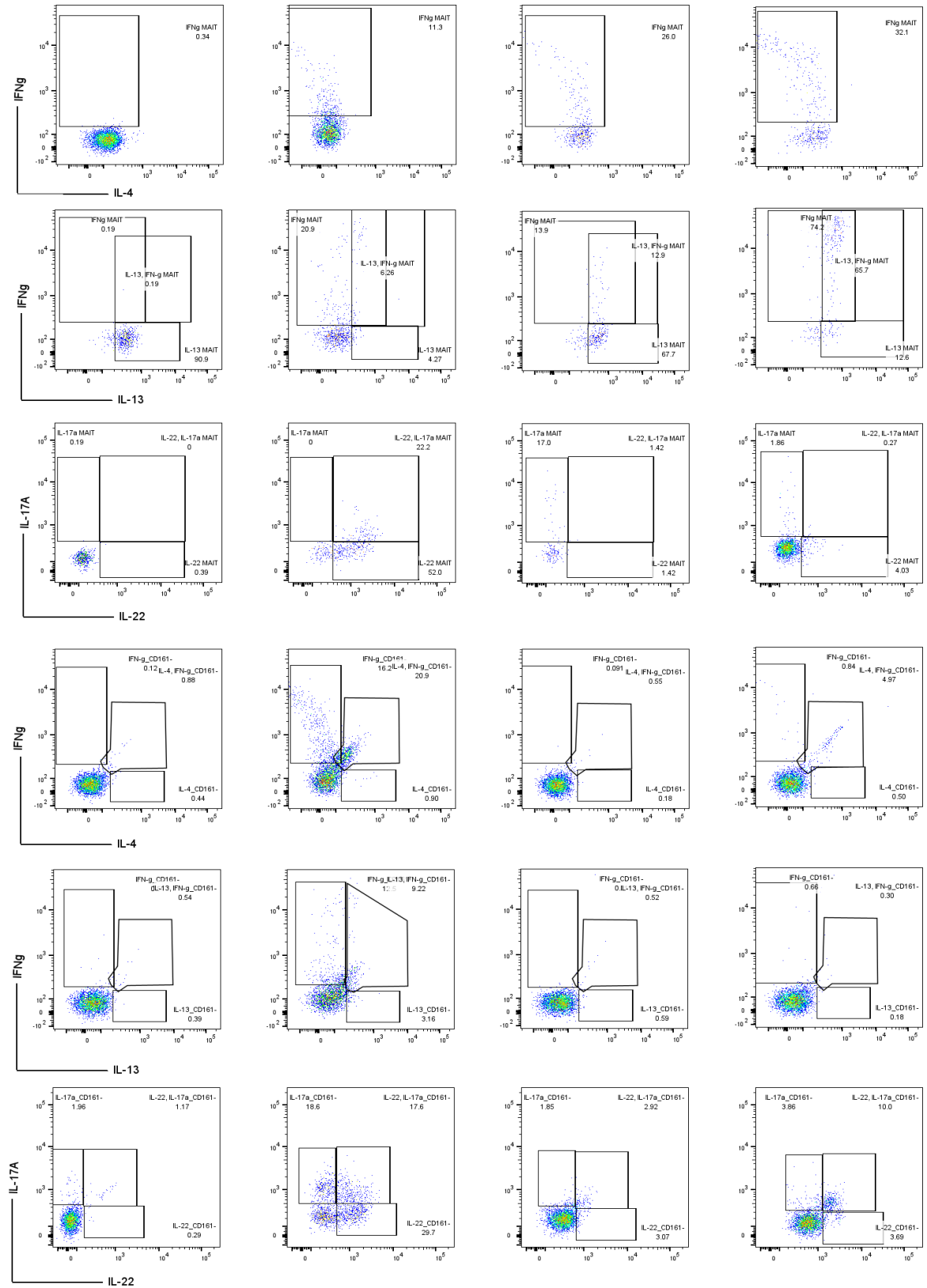
3.7.5. Data analysis

Flow cytometric data were analyzed using sequential gating of MAIT cells and V α 7.2+/CD161- T cells. Surface and intracellular expression were gated within MAIT cells using Flowjo v10.9.0 software (Figure 8A/B/C). Percentage of MAIT and V α 7.2+/CD161- T cells among T cells and % of CD69, PD-1, CD69/PD-1, CCR6, CLA, CCR6/CLA, IFN γ , IL-4, IL-13, IFN γ /IL-4, IFN γ /IL-13, IL-17, IL-22, IL-17/IL-22, CD38, GzB, TNF α , GzB/TNF α among MAIT and V α 7.2+/CD161- T cells were exported in spreadsheet and data were plotted using GraphPad Prism.

A



B



C

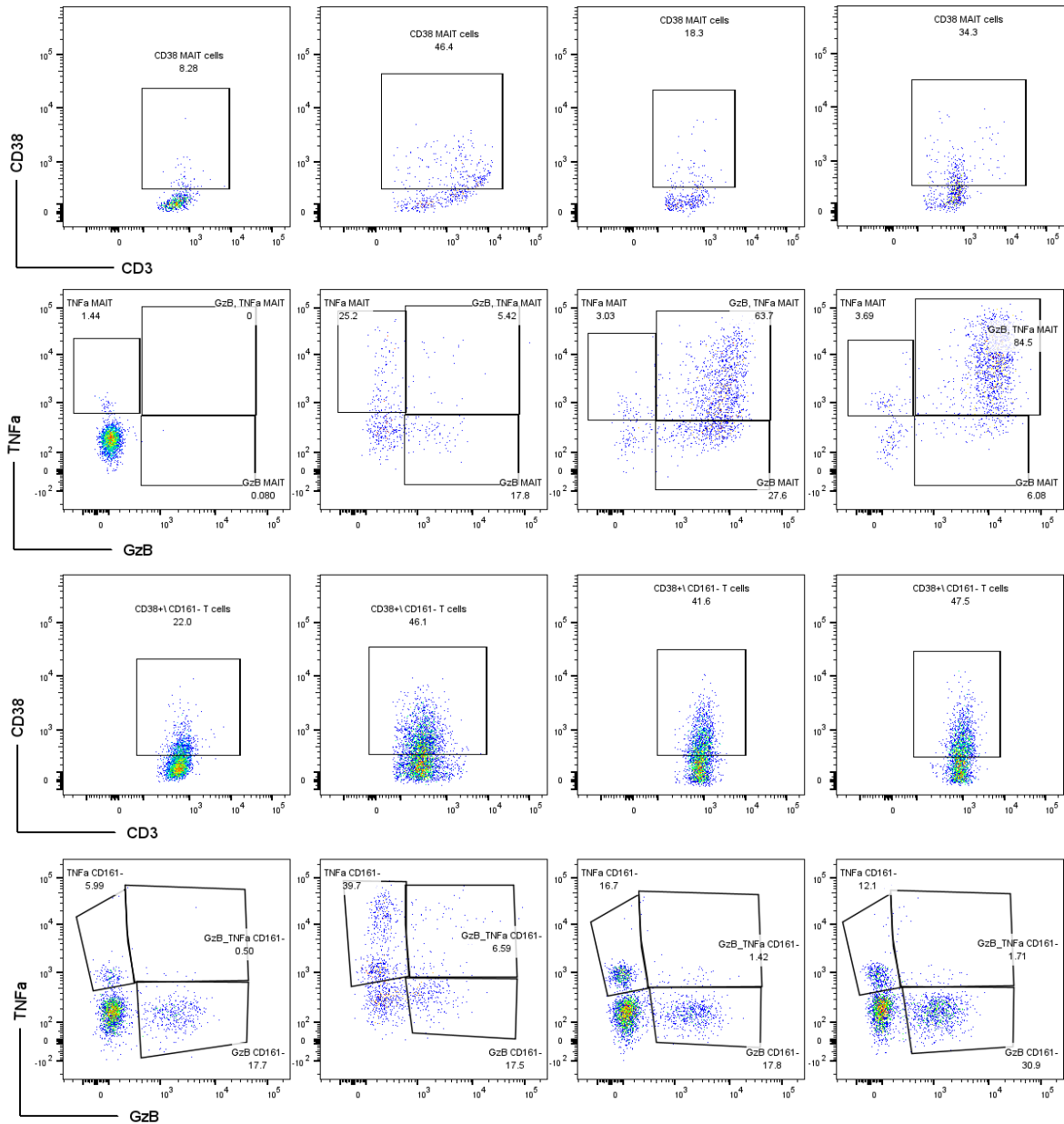


Figure 8: Mucosal-associated invariant T (MAIT) and Va7.2+/CD161- T cells and subsequent subsets were gated using Flowjo. Data was pre-processed as described for universal T cell gating in Figure 5—changes in phenotypic character within these populations based on different stimulatory conditions. Panel left belongs to the unstimulated control group, Phorbol 12-myristate 13-acetate (PMA)/Ionomycin stimulated PBMCs showed next, followed by 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU) and lastly extreme right panel belongs to 5-OP-RU+IL12+IL33 stimulation. Top to bottom (A) represents cellular surface staining tubes containing CD69, PD-1, CCR6, and CLA markers. MAIT and Va7.2+/CD161- T cells subsets representation for this marker are shown. (B) represents intracellular staining tube containing IFN- γ , IL-4, IL-13, IL-17A and IL-22 markers. MAIT and Va7.2+/CD161- T cells subsets representation for these markers are given. Tube (C) contains CD38, TNF α , and GzB markers. Subsets of MAIT and Va7.2+/CD161- T cells representation for this marker are shown.

3.8. Statistical analysis

Statistical analyses were conducted using GraphPad Prism versions 5.0 and 9.0 (GraphPad Software, San Diego, USA). 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 analysed 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 defined at a p-value of less than 0.05.

4. RESULTS

4.1. Immunosenescence in uT cells

Immunosenescence in uT cells was investigated by analyzing data correlating donor age (in years) with uT cell percentage among T cells and absolute cell numbers (cells/ μ L). The correlation analysis revealed intriguing patterns.

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 $\gamma\delta$ 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, both 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 (Figure 9).

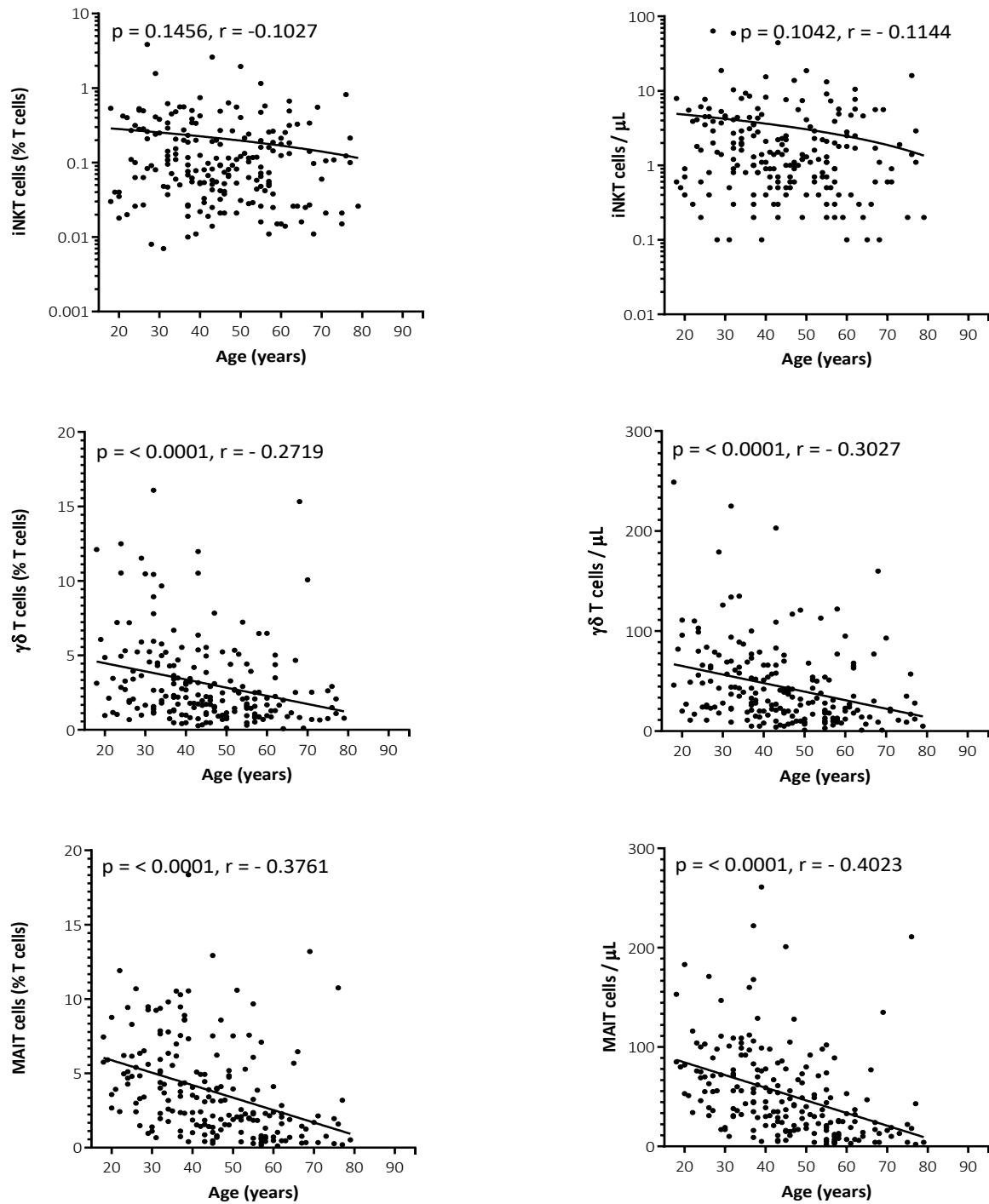


Figure 9: Analysis of immunosenescence in unconventional T cells. The X-axis denotes age, while the Y-axis represents the individual counts of uT cells. The correlation between age and the total data from percentages and absolute numbers (cells/ μL) of iNKT, $\gamma\delta$ T, and MAIT cells was examined. For each correlation, P values and regression lines were calculated and plotted.

To identify gender biases data were grouped by gender. 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 $\gamma\delta$ 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 $r = 0.5071$) (Figure 10). 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, $\gamma\delta$ T cells exhibited correlation p-values of 0.0007 , $r = 0.3198$ and $p = 0.0003$, $r = 0.3407$, and MAIT cells showed correlation p-values <0.0001 , $r = 0.4951$ and $r = 0.5527$ (Figure 11).

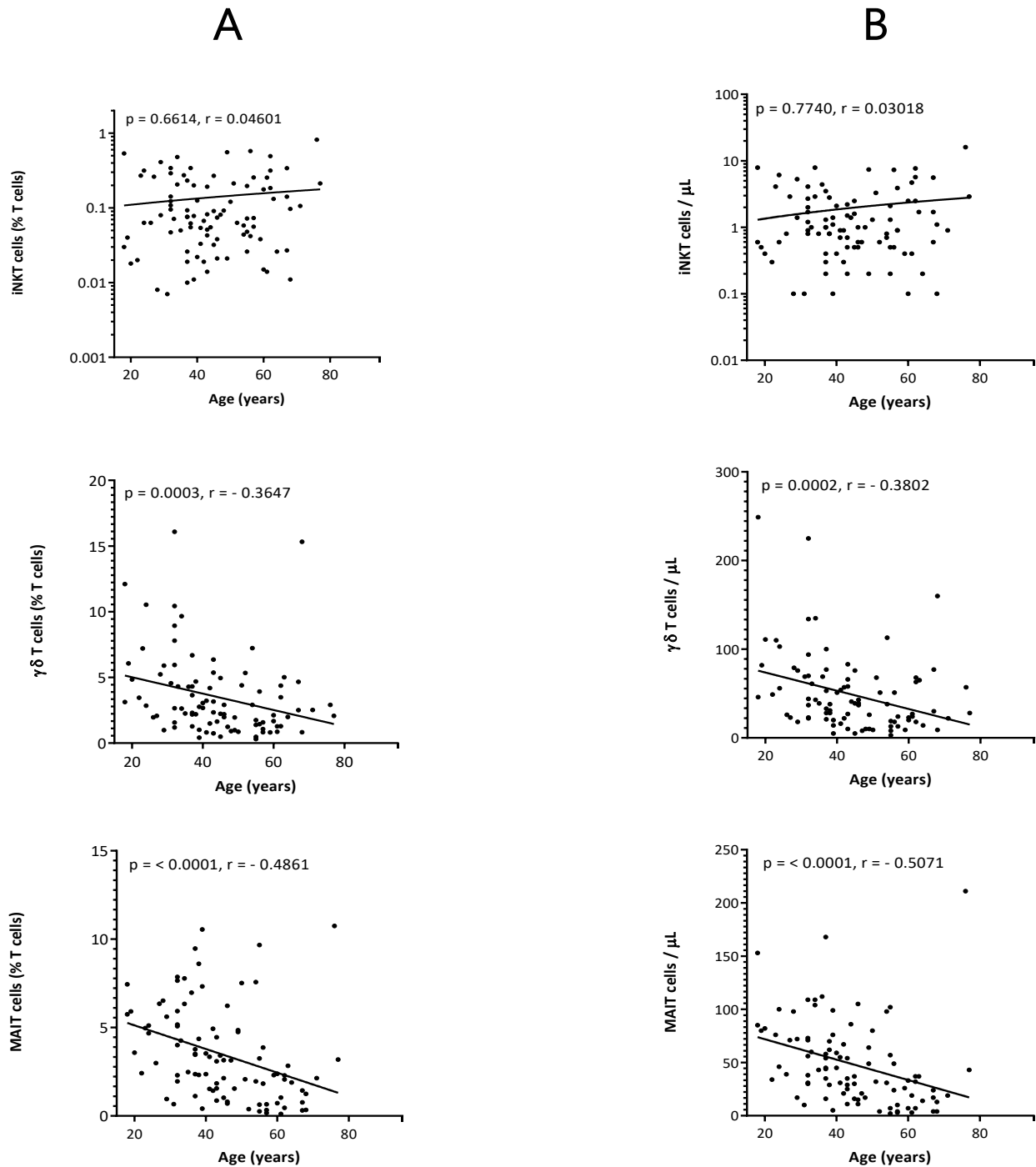


Figure 10: Assessment of immunosenescence in age-dependent male-specific unconventional T cells. The X-axis illustrates age, while the Y-axis displays the individual counts of uT cells. Panel A represents the percentage of uT cells, and Panel B exhibits absolute numbers (cells/ μ L). Data obtained from peripheral blood samples of males concerning iNKT, $\gamma\delta$ T, and MAIT cells were correlated with age. Each correlation analysis includes plotted P values and regression lines.

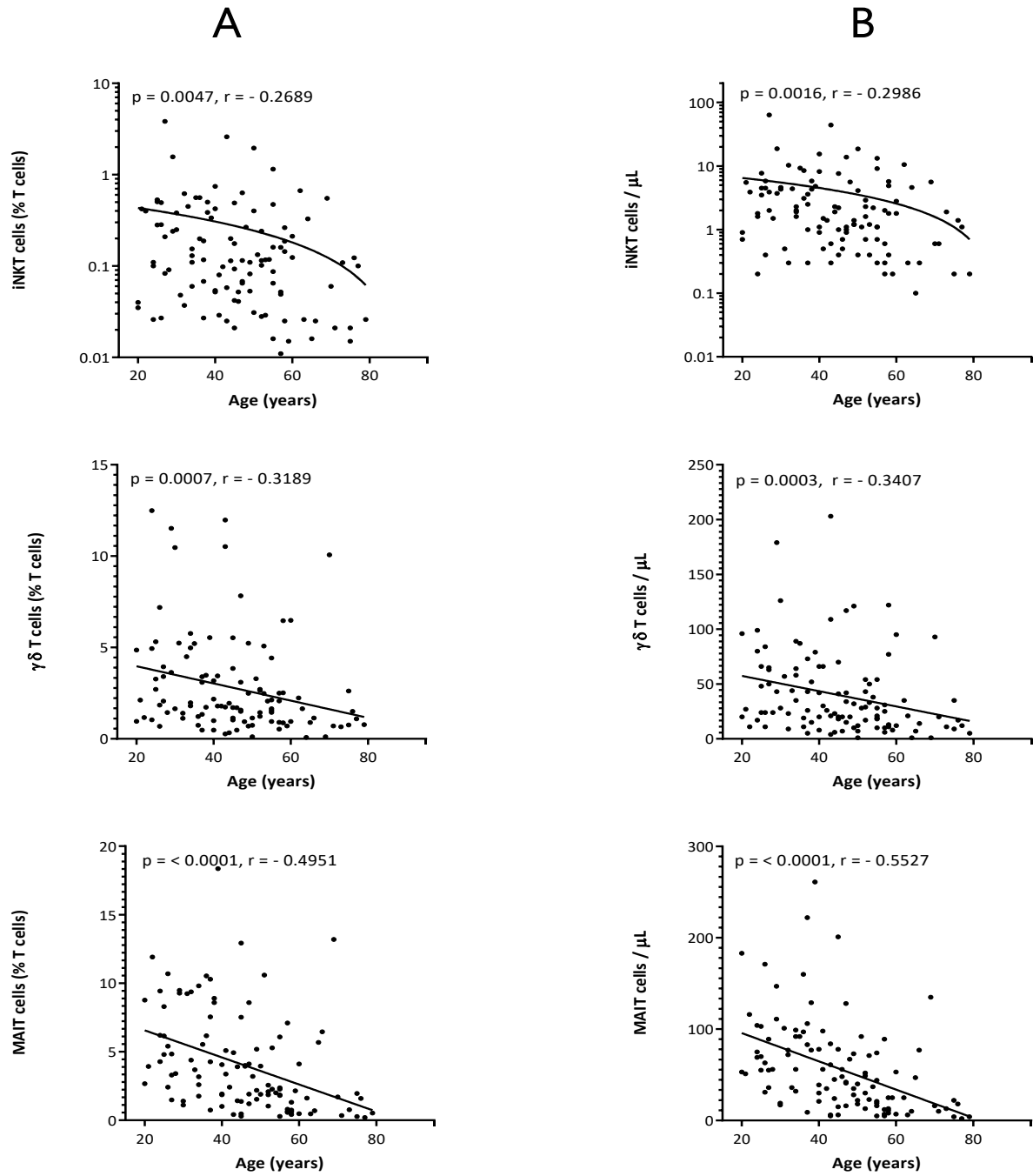


Figure 11: Examination of immunosenescence in age-dependent female-specific unconventional T cells. The X-axis represents age, while the Y-axis illustrates the individual counts of uT cells. Panel A showcases the percentage of uT cells, while Panel B demonstrates absolute numbers (cells/ μ L). Data obtained from peripheral blood samples of females concerning iNKT, $\gamma\delta$ T, and MAIT cells were analyzed for correlation with age. Each correlation analysis includes plotted P values and regression lines.

4.1.1. Relationship between uT cells

To establish relationships between the percentages of iNKT, $\gamma\delta$ T, and MAIT cells, a correlation analysis was conducted to investigate potential connections among these uT cell types. Significant positive correlations were observed between iNKT cells and both $\gamma\delta$ T cells ($p = 0.0262$) and MAIT cells ($p < 0.0001$). Similarly, $\gamma\delta$ T cells positively correlated with MAIT cells ($p = 0.0020$) (Figure 12).

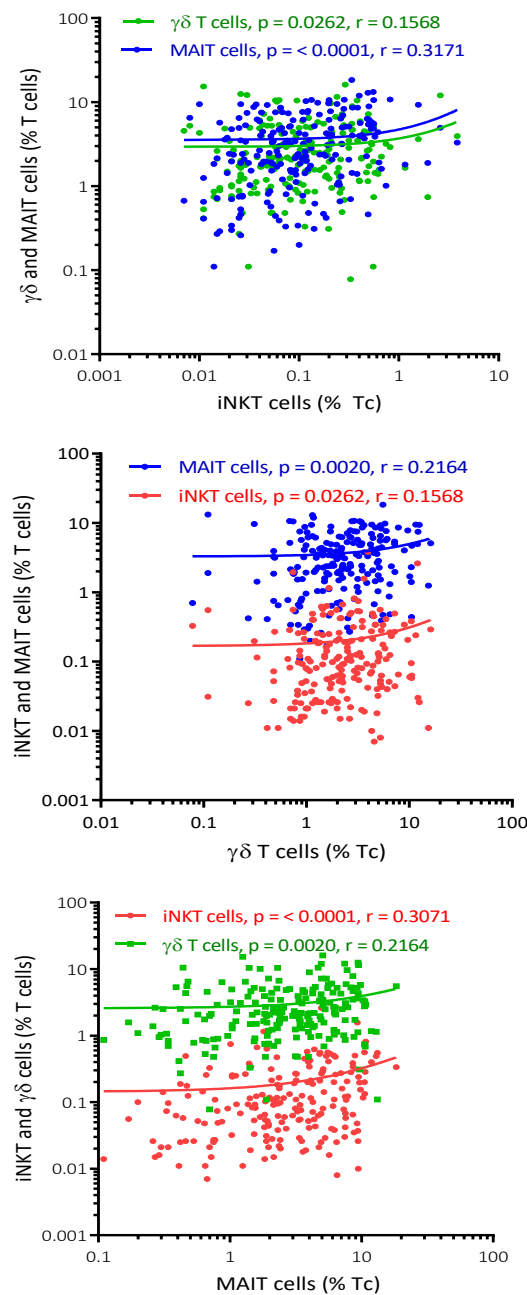


Figure 12: Examination of the relationship between different types of unconventional T cells. Correlation analysis was conducted between the percentages of individual unconventional T cells. The X-axis denotes subtype of unconventional T cells, while the Y-axis represents the percentage of each unconventional T cell subset among the total T cell population. The corresponding p values and Spearman's rank correlation coefficients for each correlation are separately indicated within the graphics.

4.1.2. Gender-dependent differences in uT cells

The dataset analyzed quantitative differences in uT 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 genders concerning iNKT and $\gamma\delta$ 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, $\gamma\delta$ 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 ($p = 0.0044$) (Figure 13).

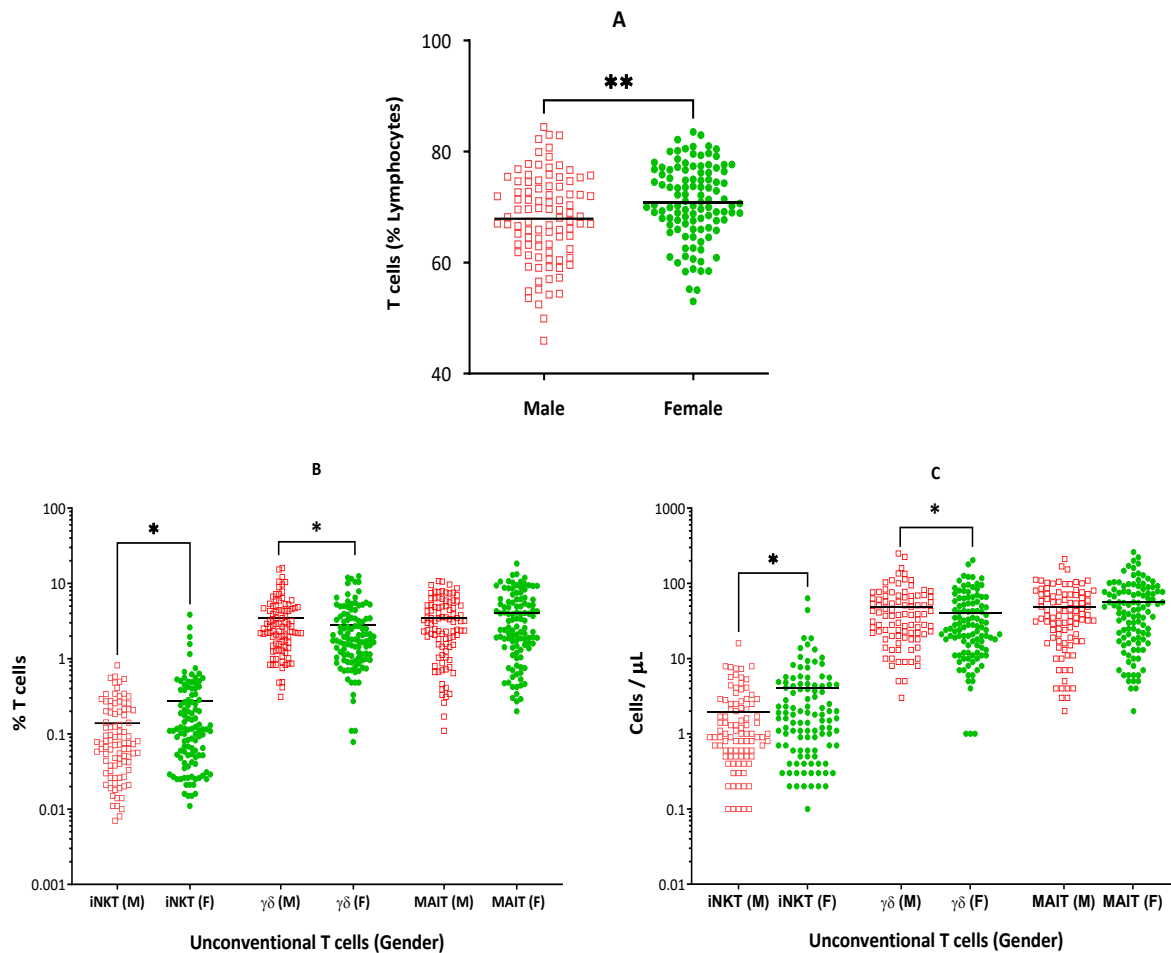


Figure 13: Evaluation of gender-specific differences in unconventional T cells (uT cells). The analysis includes T cell percentages among lymphocytes in males and females (Panel A), percentages of iNKT cells, $\gamma\delta$ T cells, and MAIT cells (Panel B), and absolute numbers of iNKT cells, $\gamma\delta$ T cells, and MAIT cells (Panel C) within the peripheral blood of healthy adult males compared to healthy adult females. The X-axis represents gender and different subsets of uT cells, while the Y-axis depicts the percentage of uT cells or their absolute numbers individually. Student's t-tests were performed to identify significant differences between genders, with the corresponding p values displayed within the graphics using asterisks to indicate significance levels ($p \leq 0.05$; ** $p \leq 0.01$).*

4.1.3. Gender-dependent relationships between uT cells

To explore gender-specific relationships between iNKT, $\gamma\delta$ 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 $\gamma\delta$ T

cells ($p = 0.4017$). Notably, a significant positive correlation was evident between $\gamma\delta$ T cells and MAIT cells ($p = 0.0134$).

Conversely, within the female group, all correlations were significantly positive, including correlations between iNKT cells and $\gamma\delta$ T cells ($p = 0.0074$), iNKT cells and MAIT cells ($p = 0.0017$), and $\gamma\delta$ T cells and MAIT cells ($p = 0.0427$) (Figure 14).

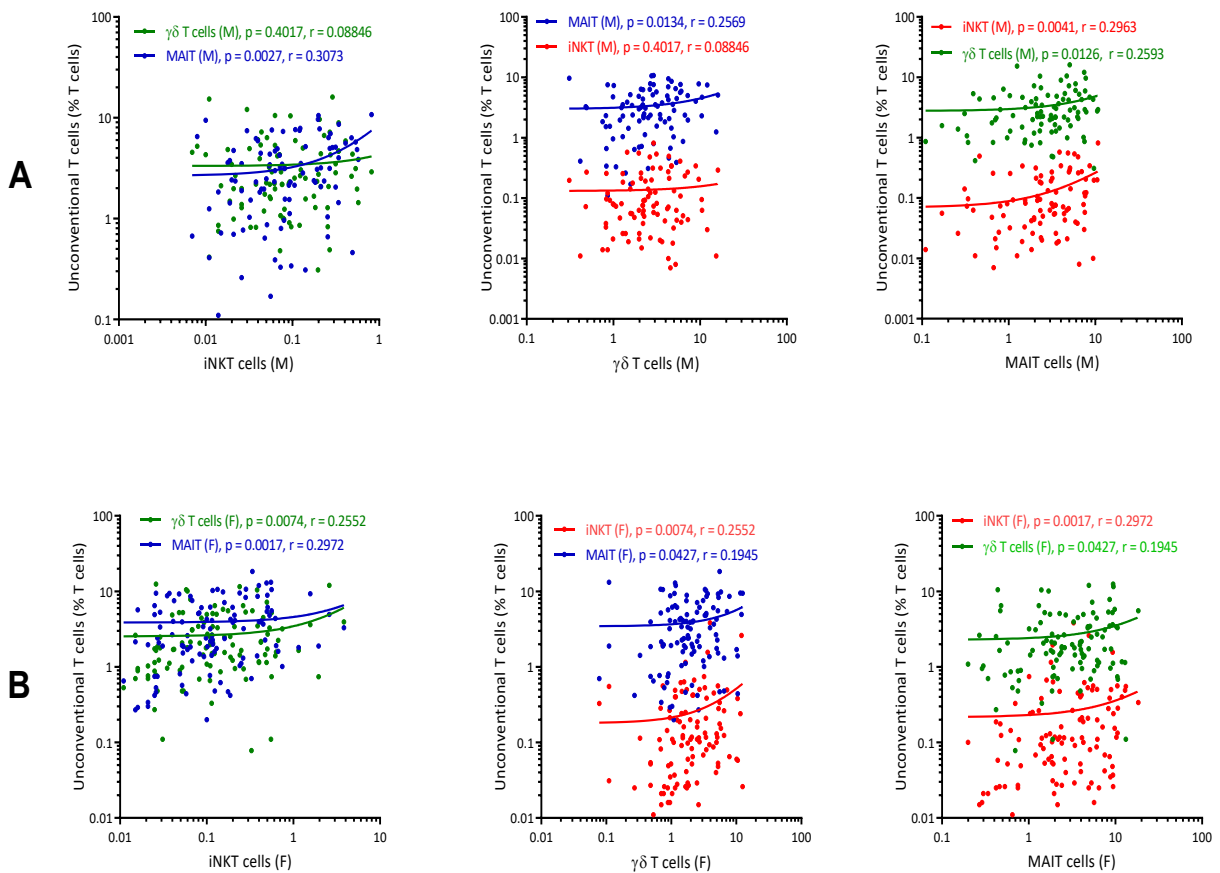


Figure 14: Examination of gender-dependent relationships between various subsets of unconventional T cells (uT cells). Panels A and B display the percentages of iNKT cells, $\gamma\delta$ T cells, and MAIT cells in the peripheral blood of healthy adult males and females, respectively. Correlation analyses were conducted between the percentages of individual uT cell subsets. The X-axis represents different unconventional T cell subsets, while the Y-axis indicates the percentage of each unconventional T cell subset among the total T cell population. The corresponding p values and Spearman's rank correlation coefficients for each correlation are separately indicated within the graphics.

4.2. Age-dependent changes and reference ranges of uT 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 PB reference ranges for uT cells. Percentages and absolute numbers (cells/ μ L) were derived from hematological parameters via the dual platform method described in the Material and Methods section.

4.2.1. Age-dependent changes of uT cells

iNKT cells did not exhibit statistically significant differences in age groups. However, $\gamma\delta$ and MAIT cells displayed substantial variations across different age brackets. For iNKT cells (expressed as a percentage and cells/ μ L), 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 (Figure 15).

In contrast, significant differences were noted within age groups for both $\gamma\delta$ 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 during the transition from middle to older age, $\gamma\delta$ T cells do not exhibit a substantial decline (Figure 15). Additionally, MAIT cells exhibited significant differences between age groups ($p < 0.0001$). However, Dunn's multiple comparisons demonstrated that not only the younger age group (18–35) but also the middle-aged group (36–50) exhibited significantly higher numbers of MAIT cells compared to the older group (Figure 15).

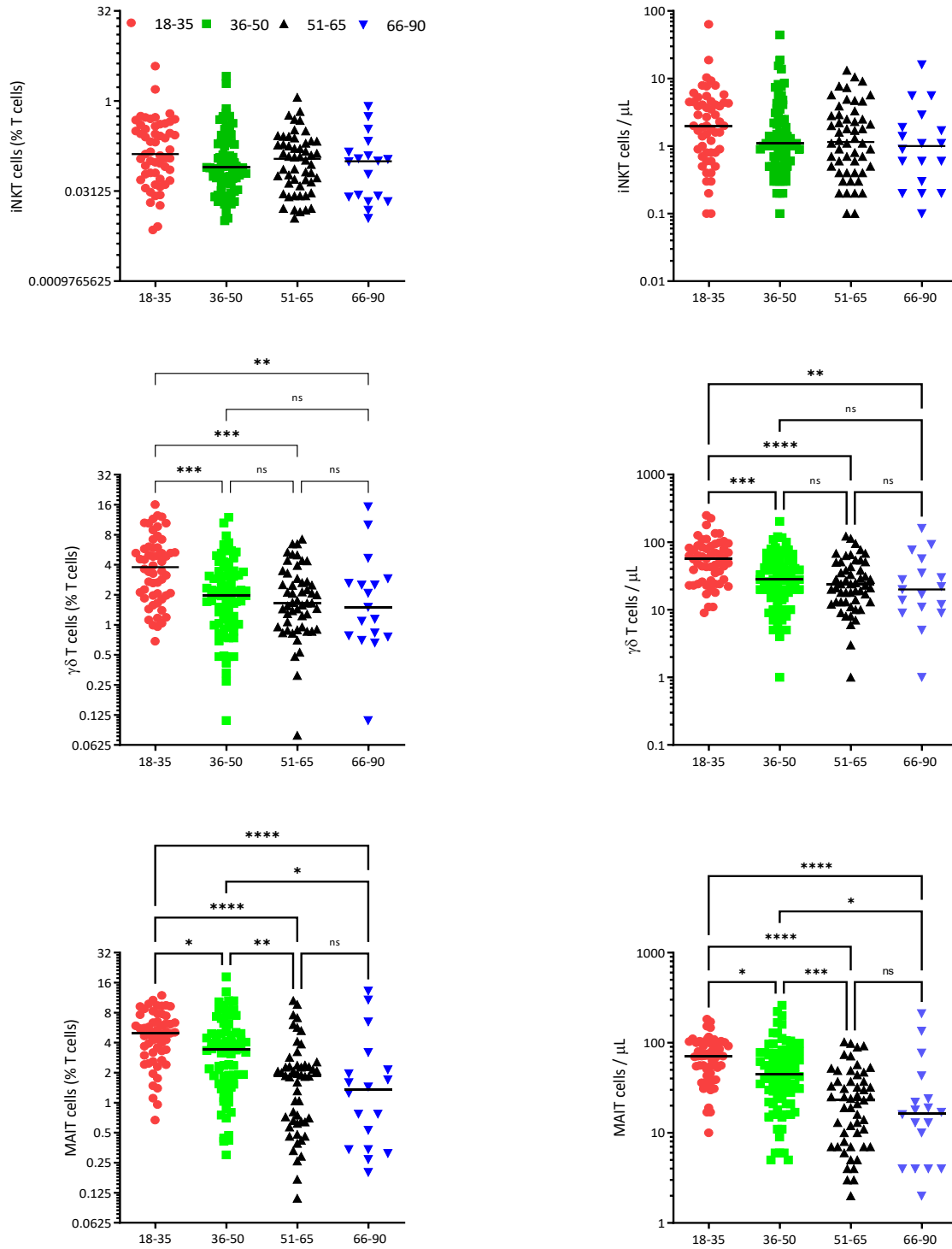


Figure 15: Assessment of age group-dependent decline in unconventional T cells (uT cells). The X-axis delineates different age groups, while the Y-axis represents individual counts of uT cells. The left panel illustrates the percentage of uT cells, and the right panel displays absolute numbers (cells/ μL). One-way ANOVA was performed to identify significant differences between the age groups. The corresponding P values are depicted within the graphics using asterisks to denote significance levels ($*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$, $****p \leq 0.0001$).

4.2.2. Descriptive statistics of total uT 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/ μ L, ranging from 0.1 to 63.5 cells/ μ L. $\gamma\delta$ T cells comprised 2.17% of T cells, ranging from 0.078% to 16.09%, with an absolute number of 30 cells/ μ L, varying from 1 to 249 cells/ μ L. 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/ μ L, varying from 2 to 261 cells/ μ L. Reference ranges for iNKT, $\gamma\delta$ T, and MAIT cells among T cells were determined as 0.01–1.12%, 0.31–11.9%, and 0.27–10.7%, respectively (Table 8).

Method (Equipment)	Parameter	Median (Min-Max)	25-75% percentile	2.5-97.5% percentile
Hematology analyzer	WBC (x10 ⁹ /L)	6.66 (3.76-12.35)	5.86-7.77	4.59-10.62
	Lymphocytes (x10 ⁹ /L)	2.04 (0.97-4.91)	1.71-2.42	1.08-3.39
Flow cytometer	T cell (% Lymphocytes)	69.78 (45.92-84.42)	64.67-75.2	53.65-82.84
	iNKT cells (% T cell)	0.095 (0.007-3.84)	0.042-0.254	0.01-1.12
	$\gamma\delta$ T cell (% T cell)	2.17 (0.078-16.09)	1.163-4.31	0.31-11.9
	MAIT cells (% T cell)	2.99 (0.11-18.3)	1.43-5.4	0.27-10.7
Dual platform	iNKT (cells/ μ L)	1.3 (0.1-63.5)	0.6-3.6	0.1-18.34
	$\gamma\delta$ T cell (cells/ μ L)	30 (1-249)	17.75-63	4.07-158
	MAIT (cells/ μ L)	42 (2-261)	18-76	4-181

Table 8: Reference ranges of uT cells (2.5-97.5%) obtained from total healthy adult PB samples of the Caucasian population. Descriptive statistics of iNKT, $\gamma\delta$ T, and MAIT cells (% and absolute numbers), WBCs, and lymphocytes are given.

4.2.3. Descriptive statistics of age-grouped uT cells

The dataset was segmented into distinct age groups previously defined in the Material and Methods section (3.5), and descriptive statistics for uT 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%), $\gamma\delta$ 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/ μ L (median of 2 cells/ μ L), $\gamma\delta$ T cells ranged from 9 to 249 cells/ μ L (median of 56.5 cells/ μ L), and MAIT cells ranged from 10 to 183 cells/ μ L (median of 71 cells/ μ L). The reference ranges for iNKT, $\gamma\delta$ T, and MAIT cells were determined as 0.0074–2.93%, 0.80–14.5%, and 0.79–11.3%, respectively (Table 9).

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%), $\gamma\delta$ 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/ μ L (median of 1.1 cells/ μ L), $\gamma\delta$ T cells ranged from 1 to 203 cells/ μ L (median of 28 cells/ μ L), and MAIT cells ranged from 5 to 261 cells/ μ L (median of 45 cells/ μ L). The reference ranges for iNKT, $\gamma\delta$ T, and MAIT cells were 0.011–1.98%, 0.26–10.5%, and 0.4–13.2%, respectively (Table 9).

51–65 years age group: In this cohort, iNKT cells represented 0.108% (ranging from 0.011% to 1.15%), $\gamma\delta$ 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/ μ L (median of 1.15 cells/ μ L), $\gamma\delta$ T cells ranged from 1 to 122 cells/ μ L (median of 23.5 cells/ μ L), and MAIT cells ranged from 2 to 102 cells/ μ L (median of 23.5 cells/ μ L). The reference ranges for iNKT, $\gamma\delta$ T, and MAIT cells were 0.012–0.994%, 0.153–6.98%, and 0.13–10.2%, respectively (Table 9).

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%), $\gamma\delta$ 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/ μ L (median of 1 cells/ μ L), $\gamma\delta$ T cells ranged from 1 to 160 cells/ μ L (median of 20 cells/ μ L), and MAIT cells ranged from 2 to 211 cells/ μ L (median of 16.5 cells/ μ L). The reference ranges for iNKT, $\gamma\delta$ T, and MAIT cells were 0.011–0.81%, 0.11–15.3%, and 0.2–13.2%, respectively (Table 9).

Age groups	Method (Equipment)	Parameter	Median (Min-Max)	25-75% percentile	2.5-97.5% percentile
18-35	Hematology analyzer	WBC ($\times 10^9$ /L)	6.79 (3.76-12.24)	5.88-7.83	3.91-11.49
		Lymphocytes ($\times 10^9$ /L)	2.07 (1.02-4.91)	1.778-2.465	1.04-4.24
	Flow cytometer	T cell (% Lymphocytes)	69.61 (49.93-83.53)	66.19-72.25	53.86-82.94
		iNKT cells (% T cell)	0.13 (0.007-3.84)	0.052-0.395	0.0074-2.93
		$\gamma\delta$ T cell (% T cell)	3.78 (0.685-16.09)	1.97-5.938	0.80-14.5
		MAIT cells (% T cell)	5.02 (0.67-11.9)	3.038-7.218	0.79-11.3
	Dual platform	iNKT (cells/ μ L)	2.0 (0.1-63.5)	0.8-4.5	0.1-61.76
		$\gamma\delta$ T cell (cells/ μ L)	56.5 (9-249)	26.25-86.25	9.85-238
		MAIT (cells/ μ L)	71 (10-183)	43.75-98.75	12.98-177
36-50	Hematology analyzer	WBC ($\times 10^9$ /L)	6.66 (4.71-10.63)	5.91-7.62	4.79-10.13
		Lymphocytes ($\times 10^9$ /L)	2.13 (1.05-3.05)	1.71-2.49	1.17-3.03
	Flow cytometer	T cell (% Lymphocytes)	70.86 (53.59-83.01)	65.82-75.83	54.2-82.95
		iNKT cells (% T cell)	0.078 (0.01-2.6)	0.041-0.199	0.011-1.98
		$\gamma\delta$ T cell (% T cell)	1.98 (0.11-11.9)	1.02-3.315	0.26-10.5
		MAIT cells (% T cell)	3.42 (0.3-18.3)	1.555-5.02	0.4-13.2
	Dual platform	iNKT (cells/ μ L)	1.1 (0.1-44.1)	0.55-2.5	0.195-19.88
		$\gamma\delta$ T cell (cells/ μ L)	28 (1-203)	15-52.5	3.85-125
		MAIT (cells/ μ L)	45 (5-261)	23-77.5	5-224
	Hematology analyzer	WBC ($\times 10^9$ /L)	6.77 (4.59-12.35)	5.91-7.89	4.74-11.93
		Lymphocytes ($\times 10^9$ /L)	2.04 (0.97-4)	1.715-2.313	0.976-3.93

51-65	Flow cytometer	T cell (% Lymphocytes)	67.79 (45.92-84.42)	61.4-76.17	48.22-83.71
		iNKT cells (% T cell)	0.108 (0.011-1.15)	0.039-0.208	0.012-0.994
		$\gamma\delta$ T cell (% T cell)	1.66 (0.078-7.23)	0.95-2.86	0.153-6.98
		MAIT cells (% T cell)	1.85 (0.11-10.59)	0.642-2.378	0.13-10.2
	Dual platform	iNKT (cells/ μ L)	1.15 (0.1-13.2)	0.425-2.875	0.1-12.32
		$\gamma\delta$ T cell (cells/ μ L)	23.5 (1-122)	13-48.25	1.65-119
		MAIT (cells/ μ L)	23.5 (2-102)	7.25-42.25	2.32-100
66-90	Hematology analyzer	WBC ($\times 10^9$ /L)	6.31 (4.54-9.24)	4.90-7.37	4.54-9.24
		Lymphocytes ($\times 10^9$ /L)	1.72 (1.2-3.56)	1.47-2.258	1.2-3.56
	Flow cytometer	T cell (% Lymphocytes)	69.81 (52.45-81)	60.65-76.32	52.45-81
		iNKT cells (% T cell)	0.098 (0.011-0.816)	0.024-0.159	0.011-0.81
		$\gamma\delta$ T cell (% T cell)	1.5 (0.11-15.3)	0.77-2.765	0.11-15.3
		MAIT cells (% T cell)	1.34 (0.2-13.2)	0.34-2.403	0.2-13.2
	Dual platform	iNKT (cells/ μ L)	1 (0.1-16)	0.275-2.15	0.1-16
		$\gamma\delta$ T cell (cells/ μ L)	20 (1-160)	10.0-46.0	1-160
		MAIT (cells/ μ L)	16.5 (2-211)	4-28.75	2-211

Table 9: Age-dependent reference ranges of uT cells (2.5-97.5%) obtained from total healthy adult PB samples of the Caucasian population. Descriptive statistics of iNKT, $\gamma\delta$ T, and MAIT cells (% and absolute numbers) along with WBCs and lymphocytes are given, and data were categorized based on age group distribution.

4.2.4. Descriptive statistics of gender-dependent uT cells

The examination of gender-dependent percentages and absolute numbers for iNKT, $\gamma\delta$, 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/ μ L. $\gamma\delta$ 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/ μ L. 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/ μ L. The

reference ranges (2.5–97.5%) for iNKT, $\gamma\delta$ T, and MAIT cells were 0.0087–0.56%, 0.43–14.2%, and 0.20–10.2%, respectively (Table 10).

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/ μ L. $\gamma\delta$ 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/ μ L. 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/ μ L. The reference ranges for iNKT, $\gamma\delta$ T, and MAIT cells were 0.015–2.1%, 0.11–11.6%, and 0.28–13%, respectively (Table 10).

Gender	Method (Equipment)	Parameter	Median (Min-Max)	25-75%	Mean $\pm$ SD	2.5-97.5%
Male	Hematology analyzer	WBC ($\times 10^9$ /L)	6.6 (3.7-11)	5.8-7.5	6.7 $\pm$ 1.3	4.5-9.8
		Lymphocytes ($\times 10^9$ /L)	2 (1-3.7)	1.7-2.4	2 $\pm$ 0.49	1.1-3.2
	Flow cytometer	T cell (% Lymphocytes)	68 (45.9-84.4)	62.2-73.9	67.8 $\pm$ 7.9	50.8-82.9
		iNKT (% T cell)	0.076 (0.007-0.816)	0.038-0.203	0.18 $\pm$ 0.51	0.0087-0.56
		$\gamma\delta$ T cell (% T cell)	2.52 (0.31-16.09)	1.4-4.6	3.5 $\pm$ 3	0.43-14.2
		MAIT (% T cell)	3.04 (0.11-10.75)	1.4-5	3.4 $\pm$ 2.6	0.20-10.2
	Dual platform	iNKT (cells/ μ L)	1 (0.1-16)	0.5-2.5	2.5 $\pm$ 6.4	0.1-7.9
		$\gamma\delta$ T cell (cells/ μ L)	38 (3 -249)	20-67	48.9 $\pm$ 42	5-202
		MAIT (cells/ μ L)	37.5 (2-211)	18-71	48.5 $\pm$ 38	3-162
Female	Hematology analyzer	WBC ($\times 10^9$ /L)	6.7 (4.1-12.3)	5.8-7.8	7 $\pm$ 1.6	4.6-11
		Lymphocytes ($\times 10^9$ /L)	2 (0.97-4.91)	1.6-2.4	2 $\pm$ 0.62	1-3.6
		T cell (% Lymphocytes)	70.6 (53-83.5)	66.8-76.5	70.8 $\pm$ 6.7	55.1-82.3
		iNKT (% T cell)	0.11 (0.011-3.84)	0.048-0.306	0.27 $\pm$ 0.5	0.015-2.1

	Flow cytometer	$\gamma\delta$ T cell (% T cell)	1.79 (0.078-12.4)	1-3.4	2.7 ± 2.6	0.11-11.6
		MAIT (% T cell)	2.67 (0.2-18.3)	1.4-5.8	4 ± 3.5	0.28-13
	Dual platform	iNKT (cells/ μ L)	1.6 (0.1-63.5)	0.6-4.4	4 ± 7.9	0.2-25
		$\gamma\delta$ T cell (cells/ μ L)	26 (1-203)	13-55	39.7 ± 37	1-139
		MAIT (cells/ μ L)	45 (2-261)	18-78	56.5 ± 49.8	4-206

Table 6: Gender-dependent reference ranges of uT cells (2.5-97.5%) obtained from total healthy adult PB samples of the Caucasian population. Descriptive statistics of iNKT, $\gamma\delta$ T, and MAIT cells (% and absolute numbers) along with WBCs and lymphocytes are given, and data were categorized based on age and gender distribution.

4.3. Involvement of PB MAIT and V α 7.2+/CD161- T Cells in AD

4.3.1. Cellular surface marker characterization of T cells, MAIT cells, and V α 7.2+/CD161- T cells in AD

We used two-dimensional sequential gating to analyze T cell properties and the V α 7.2+ compartment, encompassing mucosal-associated invariant T (MAIT) cells and V α 7.2+/CD161- T cells. Individuals with AD exhibited a significantly higher percentage of T cells (as a percentage of lymphocytes) compared to HC, particularly CD38+ activated T cell subsets (Figure 16A). Conversely, in AD patients, the percentage of MAIT cells (as a percentage of T cells) decreased, while CD38+ activated MAIT cell subsets increased (Figure 16B). Additionally, in AD, the fraction of V α 7.2+/CD161- T cells declined, whereas CD38+ subsets increased (Figure 16C). 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 V α 7.2+/CD161- T cells.

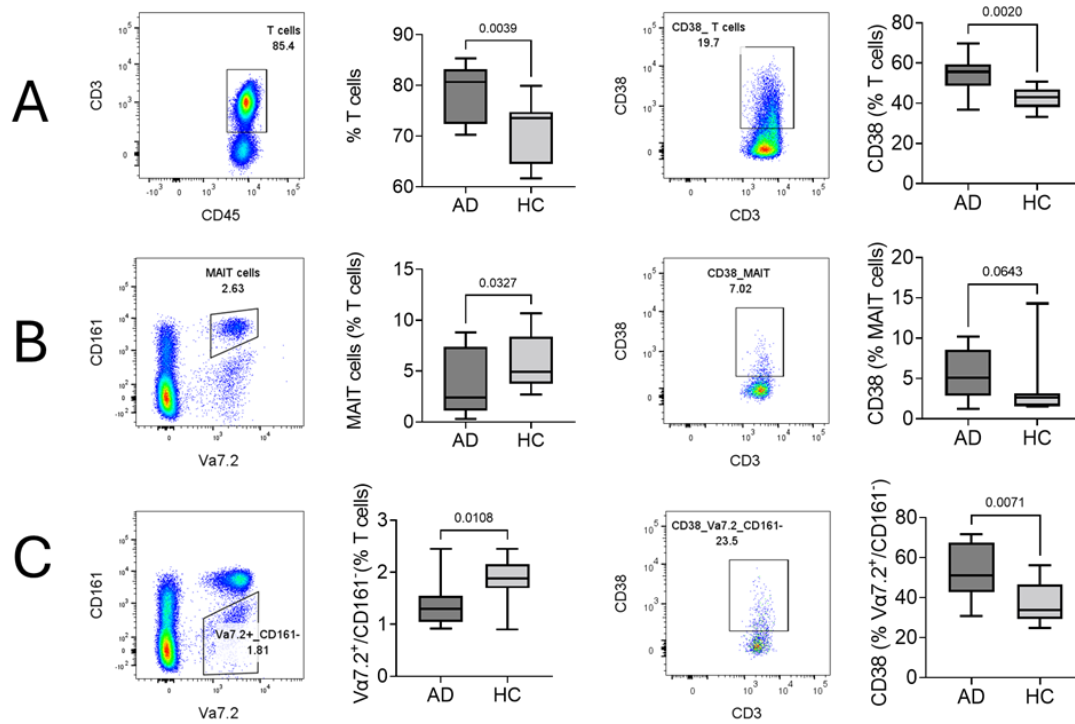


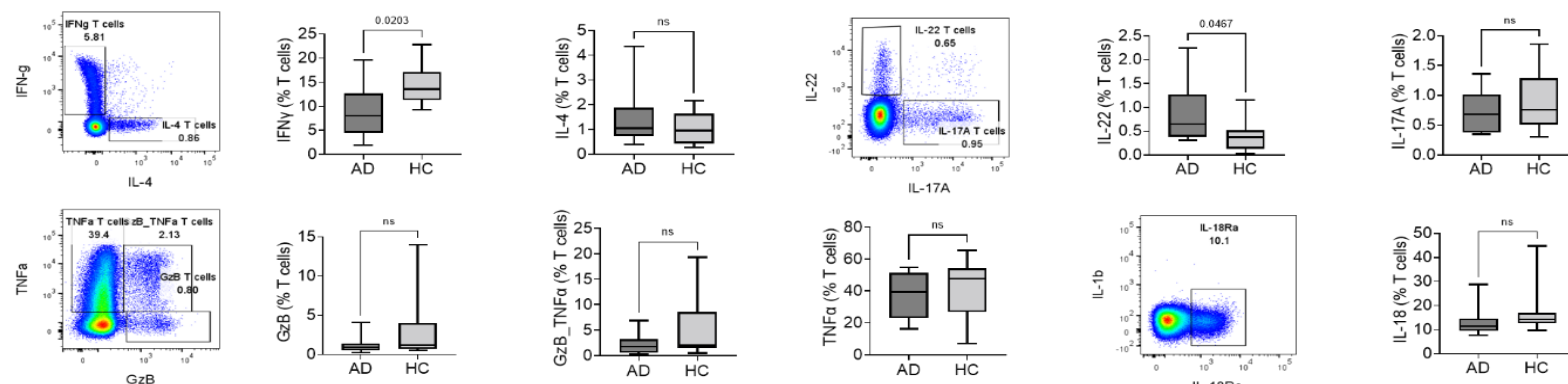
Figure 16 illustrates the significant differences in the expression of cellular surface markers among T cells, MAIT cells, and Va7.2+/CD161- T cells between subjects with atopic dermatitis (AD) and healthy controls (HC). Representative flow cytometric dot plots and their respective statistical analyses are provided. Panel (A) pertains to T cells, panel (B) represents MAIT cells, and panel (C) corresponds to Va7.2+/CD161- T cells. The statistical significance between AD and HC was determined using the Student's t-test. The X-axis indicates the sample category (AD and HC), while the Y-axis represents the percentage from T cells, MAIT cells, and Va7.2+/CD161- cells, respectively. P-values indicating significance levels are displayed within the plots.

4.3.2. Type of T cell, MAIT cell and Va7.2+/CD161- T cell response in AD

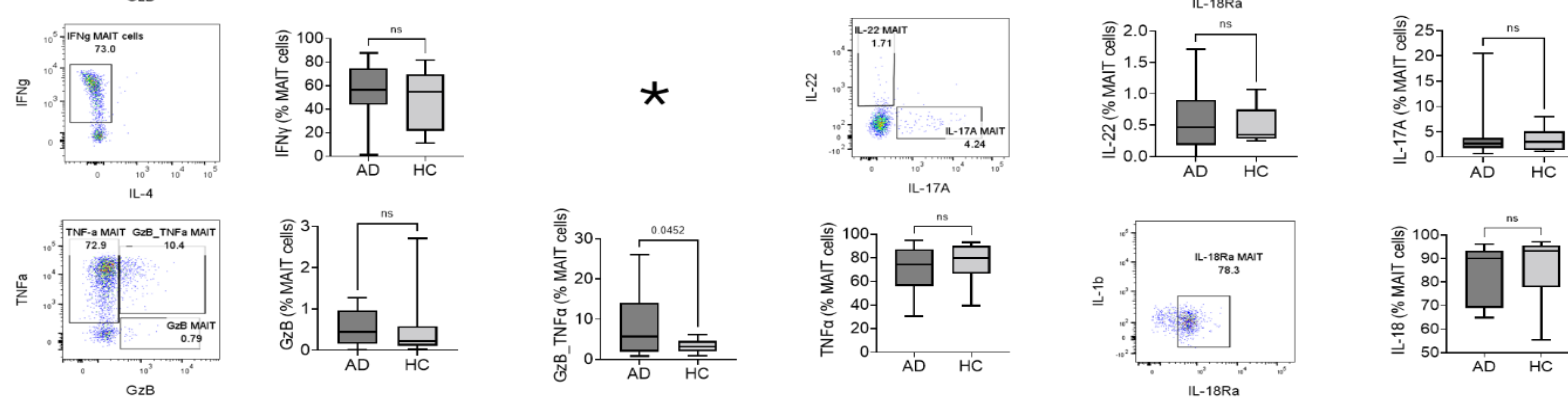
Our findings confirm the suggested Th2/Th22 dominance in AD characterized by a decreased percentage of IFN γ -producing T cells and a marginally elevated, non-significant IL-4 production. Moreover, a higher rate of IL-22-producing T cells was observed, with no significant difference in IL-17A-producing T cells (Figure 17A). Notably, the polyfunctional double-positive fraction of Tumor Necrosis Factor α /Granzyme B (GzB)-generating MAIT cells was significantly enhanced in AD (Figure 17B), suggesting the potential involvement of PB MAIT cells in disease pathogenesis. However, no significant differences were observed in single-positive GzB, TNF α , or IFN γ production. Va7.2+/CD161- T cells demonstrated similar

responses to conventional T cells. Specifically, Th1 (IFN γ)-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 V α 7.2⁺/CD161⁻ T cells (Figure 17C). Additionally, there were no significant differences detected in intracellular markers such as IL-17A, IL-22, and IL-18R α of MAIT cells, TNF α , GzB, TNF α /GzB, and IL-18R α of T cells, or V α 7.2⁺/CD161⁻ T cells.

A



B



C

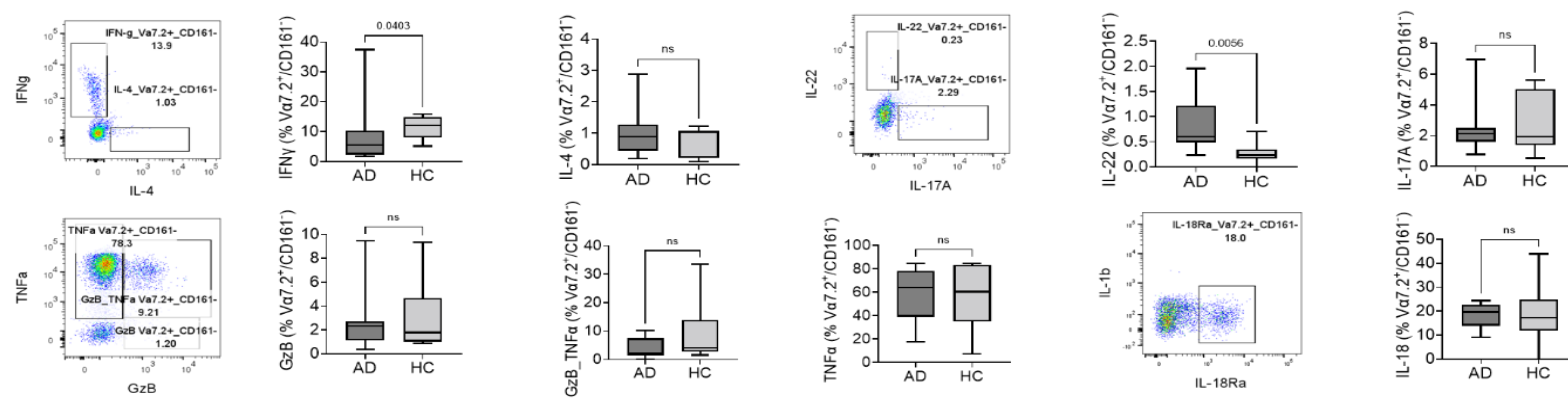


*Figure 17: Illustrates the significant differences in the expression of intracellular cytokine-producing cells following phorbol 12-myristate 13-acetate stimulation between subjects with atopic dermatitis (AD) and healthy controls (HC). Representative flow cytometric dot plots and their respective statistical analyses are provided. Panel (A) pertains to T cells, panel (B) represents MAIT cells, and panel (C) corresponds to Vα7.2+/CD161- T cells. The statistical significance between AD and HC was determined using the Student's t-test. The X-axis indicates the sample category (AD and HC), while the Y-axis represents the percentage from T cells, MAIT cells, and Vα7.2+/CD161- cells, respectively. P-values indicating significance levels are displayed within the plots, where p-values > 0.05 are denoted as ns (non-significant). Additionally, * denotes that the event number of IL-4-producing MAIT cells was below the detection limit. Hence, statistical calculation was not performed for this parameter.*

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 AD and 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 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 V α 7.2⁺/CD161⁺ T cells increased in AD but became non-significant. AD's polyfunctional TNF α /GzB-generating MAIT cells significantly increased (Figure 18).

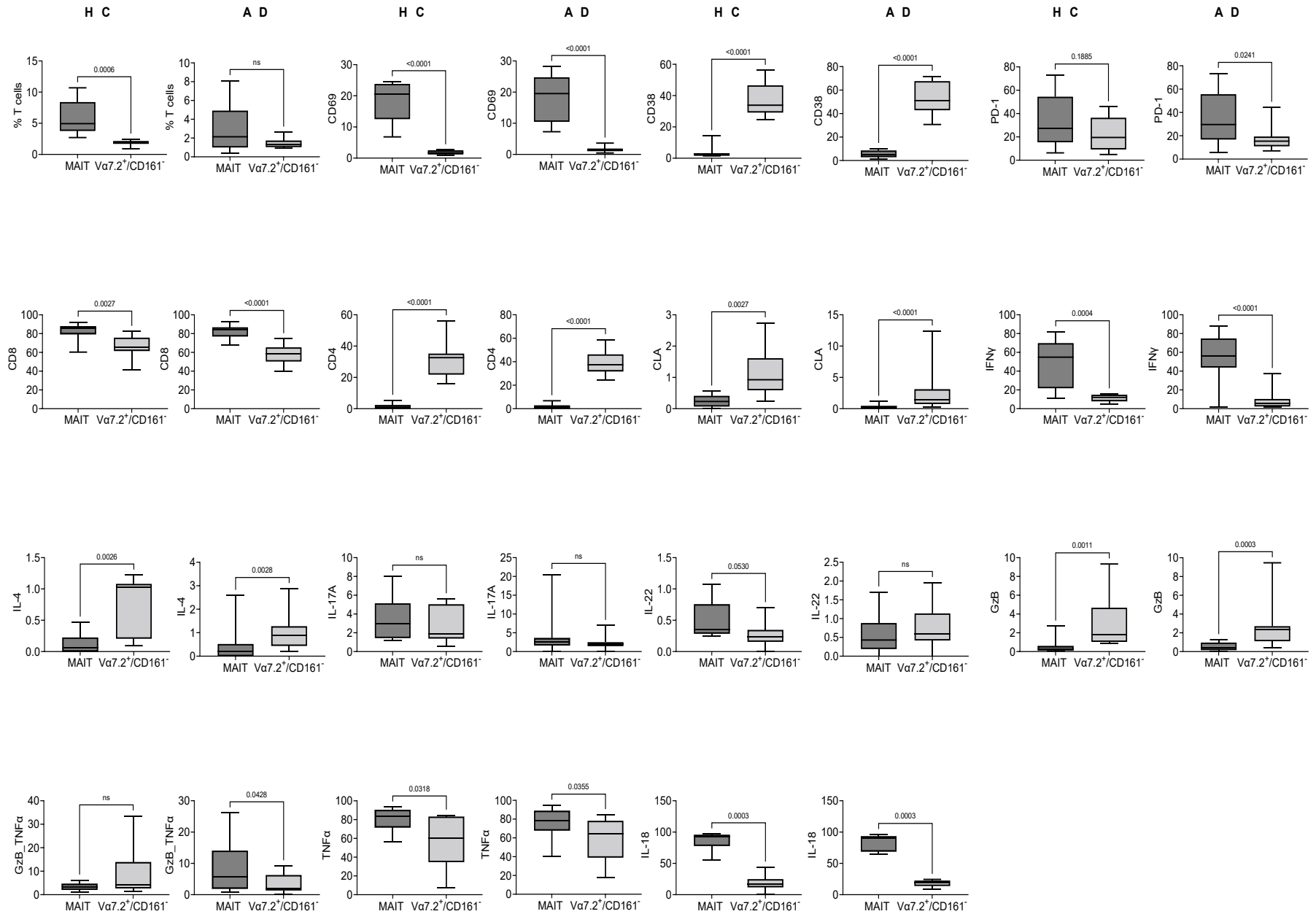


Figure 18 compares MAIT cells and Vα7.2+/CD161- T cells and their subsets in healthy control (HC) samples and individuals with atopic dermatitis (AD). The statistical significance between MAIT and Vα7.2+/CD161- T cells was determined using the Student's t-test. The X-axis represents the cells of MAIT and Vα7.2+/CD161- T cells, while the Y-axis represents different subsets among MAIT and Vα7.2+/CD161- T cells. P-values indicating significance levels are displayed within the plots, where p-values > 0.05 are denoted as ns (non-significant). Each comparison between HC and AD columns is provided in parallel from top to bottom.

4.3.4. Multidimensional T cell analysis

The multidimensional analysis of T cells from tubes 1, 2, and 3 revealed multiple immunological clusters that exhibited significant differences between patients with AD and 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, total 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 (Figure 19).

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 HC (Figure 20A). Tube 2 provided additional insights into the subsets of these cells based on cellular surface expression. In 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 (Figure 20B). 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, Figure 20C illustrates that AD suppresses IFNγ-producing Vα7.2+/CD161- T cells. Multidimensional analysis revealed no significant differences from Tube 4.

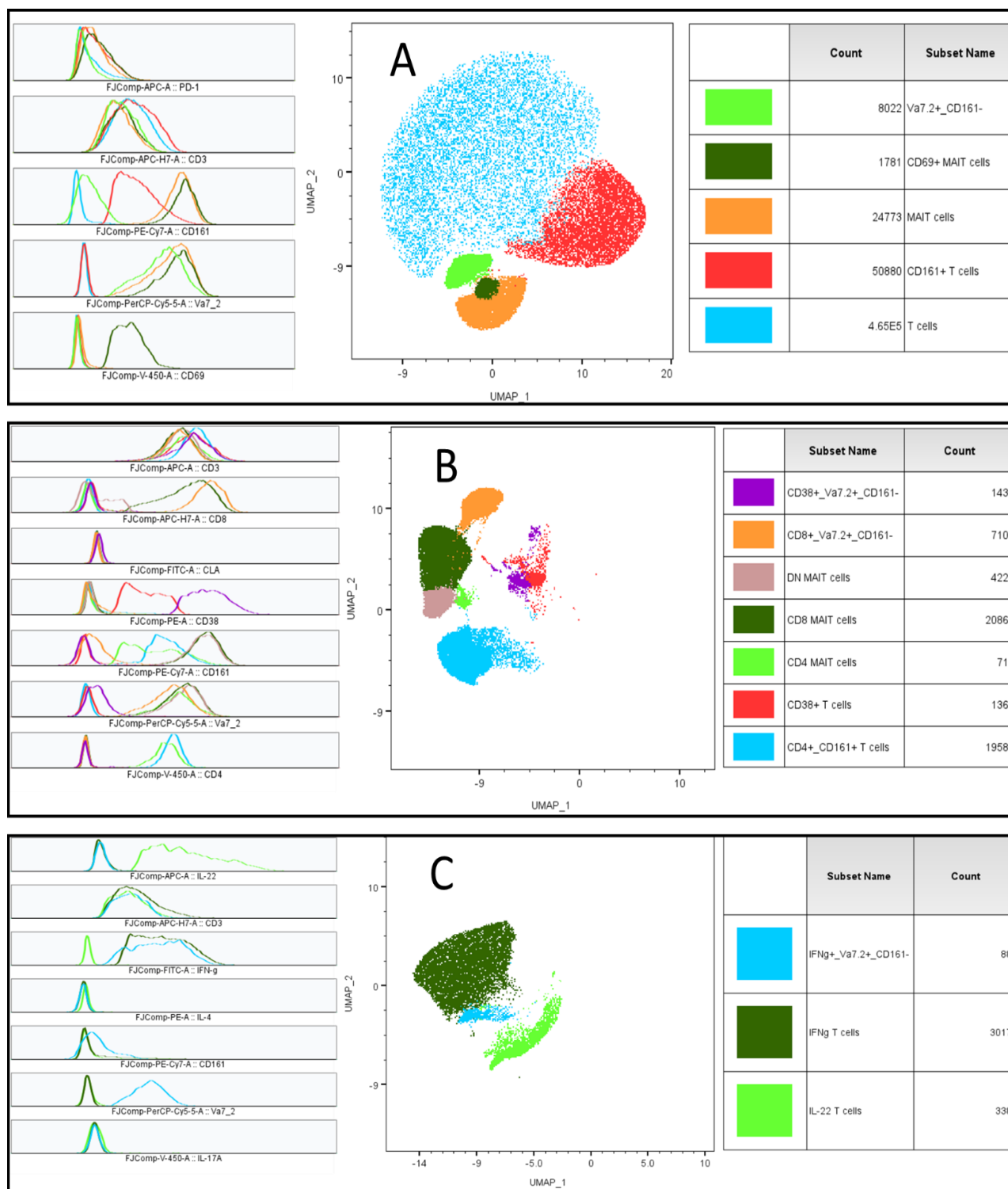


Figure 19 depicts dimensionality reduction (UMAP) and clustering (FlowSOM) of flow cytometric data. T cell subsets that significantly differ between atopic dermatitis (AD) and healthy controls (HC) within tubes 1, 2, and 3 are represented as A, B, and C, respectively. The middle plots show UMAP_1 and UMAP_2 plots, with their corresponding phenotypes displayed in multiple overlay plots on the left side. Counts from the total T cells and the names of subsets are provided in the right-side panel.

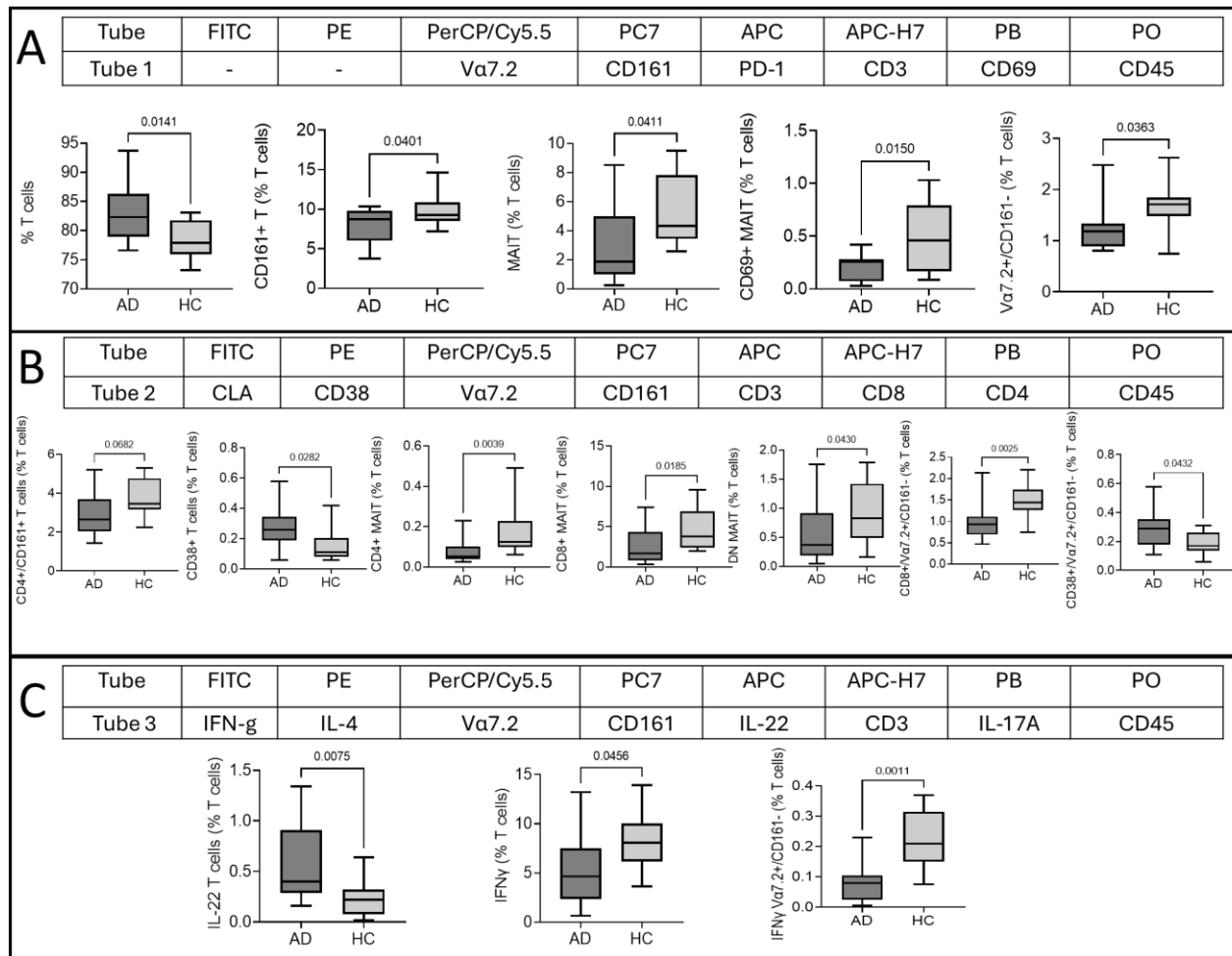


Figure 20 illustrates the significantly different subsets of T cells within healthy controls (HC) and individuals with atopic dermatitis (AD), analyzed using multidimensional data analysis. Figures A, B, and C show the significantly different subsets within tubes 1, 2, and 3, respectively. No statistically significant results were found between tube 4 using multidimensional analysis. Monoclonal antibodies used in each tube are indicated. Subsets of T cells that are significantly different between AD and HC are compared using the Student's *t*-test and represented with box plots. The X-axis represents cells of AD and HC, while the Y-axis represents different subsets among T cells. P-values indicating significance levels are displayed within the plots.

4.4. Effect of immunomodulation on MAIT cells and V α 7.2+/CD161- T cells under different stimulatory conditions

4.4.1. Effects of various stimulants on MAIT and V α 7.2+/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, V α 7.2+/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 V α 7.2+/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 V α 7.2+/CD161- T cells. Interestingly, CLA/CCR6+ V α 7.2+/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 V α 7.2+/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 V α 7.2+/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 V α 7.2+/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 cells (PD-1/CD69) in V α 7.2+/CD161- T cells (Figure 21).

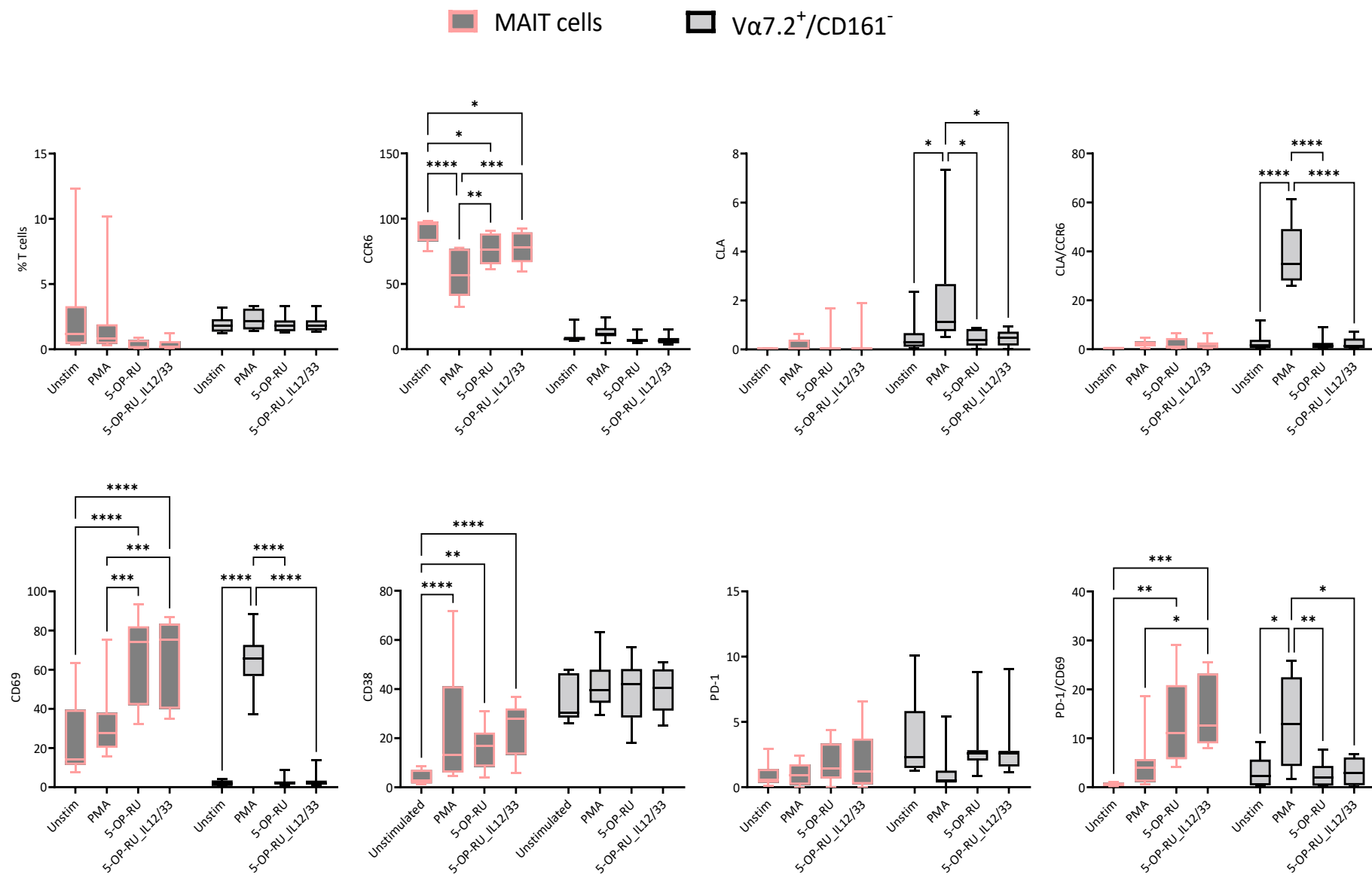


Figure 21: Cellular surface expression of MAIT and Vα7.2+/CD161- T cells were compared across different stimulatory conditions. MAIT cells are depicted in a grey color gradient with a pink border, while Vα7.2+/CD161- cells are represented in a grey color gradient with black borders. The x-axis illustrates various stimulatory conditions (unstimulated, phorbol myristate acetate (PMA)/ionomycin, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU), 5-OP-RU/IL-12/IL-33), and the y-axis represents marker expression as a percentage of MAIT cells or Vα7.2+/CD161- T cells. Statistical analysis was conducted using Two-way ANOVA. "" denotes $P \leq 0.05$, "**" signifies $P \leq 0.01$, "***" represents $P \leq 0.001$, and "****" indicates $P \leq 0.0001$.*

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 IFN γ . However, after stimulation, there was significant decrease in IL-13 and increase in IFN γ . Significant increase in IFN γ was observed following PMA stimulation, as expected. Polyfunctional MAIT cells (IFN γ /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 together, only PMA-answer showed a significant difference compared to the ligand

(5-OP-RU). IL-4-producing MAIT cells were not detected. But V α 7.2+/CD161- T cells expressed significantly higher levels of IL-4 and IFN γ /IL-4 post-PMA stimulation. No significant differences were noted in Granzyme B (GzB)-producing MAIT cells and V α 7.2+/CD161- T cells with stimulants. 5-OP-RU/IL-12/IL-33-stimulated TNF α -producing MAIT cells did not show a significant difference compared to the unstimulated control. However, PMA and 5-OP-RU-stimulated cells did show significant differences. TNF α -producing V α 7.2+/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 TNF α /GzB-producing MAIT cells. However, no significant difference was noted in V α 7.2+/CD161- T cells (Figure 22).

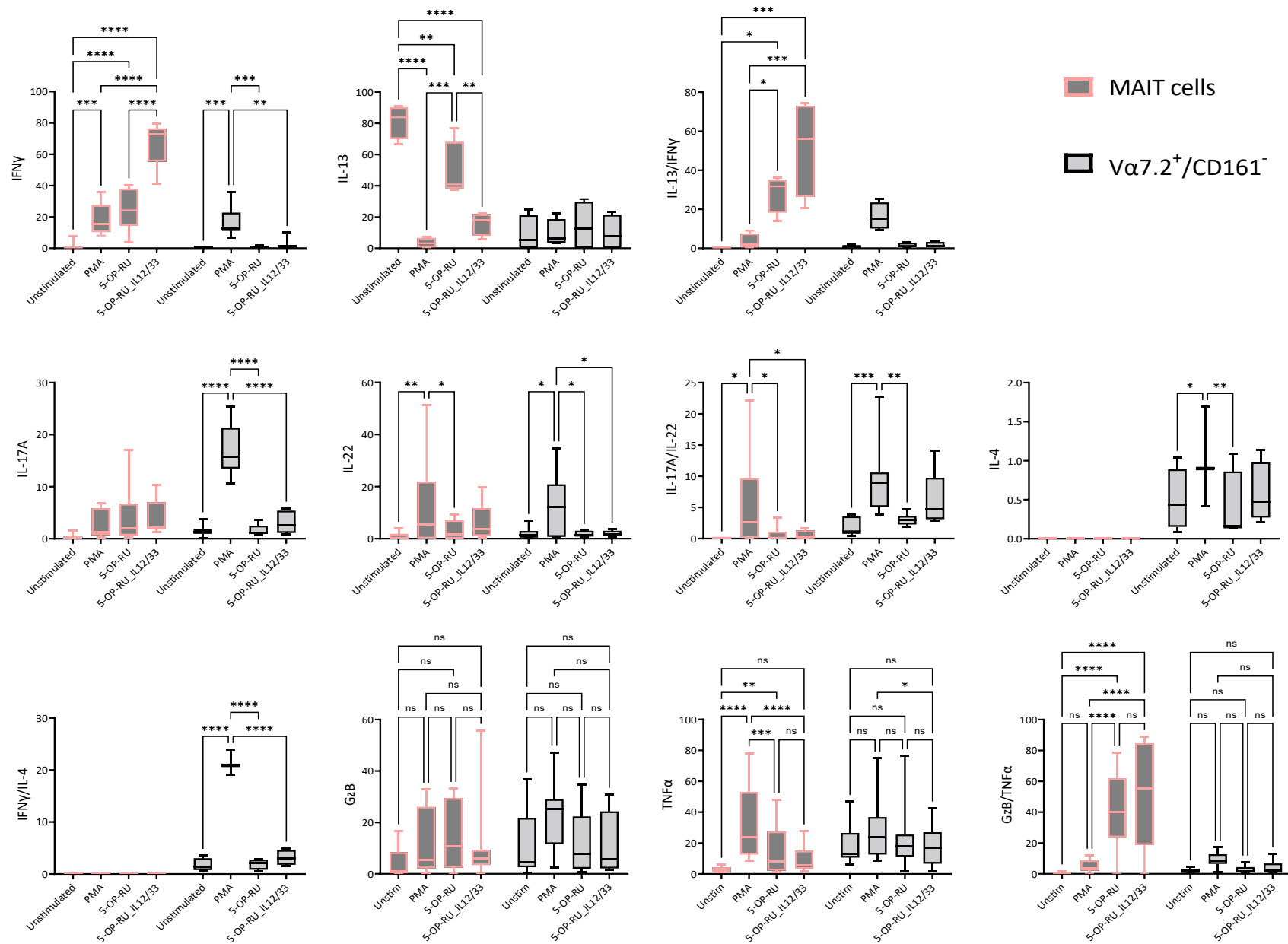


*Figure 22: Intracellular cytokine production by MAIT and Vα7.2+/CD161- T cells were compared across different stimulatory conditions. MAIT cells are depicted in a grey color gradient with a pink border, while Vα7.2+/CD161- cells are represented in a grey color gradient with black borders. The x-axis illustrates various stimulatory conditions (unstimulated, Phorbol myristate acetate (PMA)/ionomycin, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU), 5-OP-RU/IL-12/IL-33), and the y-axis represents marker expression as a percentage of MAIT cells or Vα7.2+/CD161- T cells. Statistical analysis was conducted using Two-way ANOVA. ** denotes $P \leq 0.05$, " ** signifies $P \leq 0.01$, "*** represents $P \leq 0.001$, and "*****" indicates $P \leq 0.0001$.*

4.4.3. Cyclosporin A and Vitamin D₃ 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 V α 7.2+/CD161- T cell treatment (Figure 23).

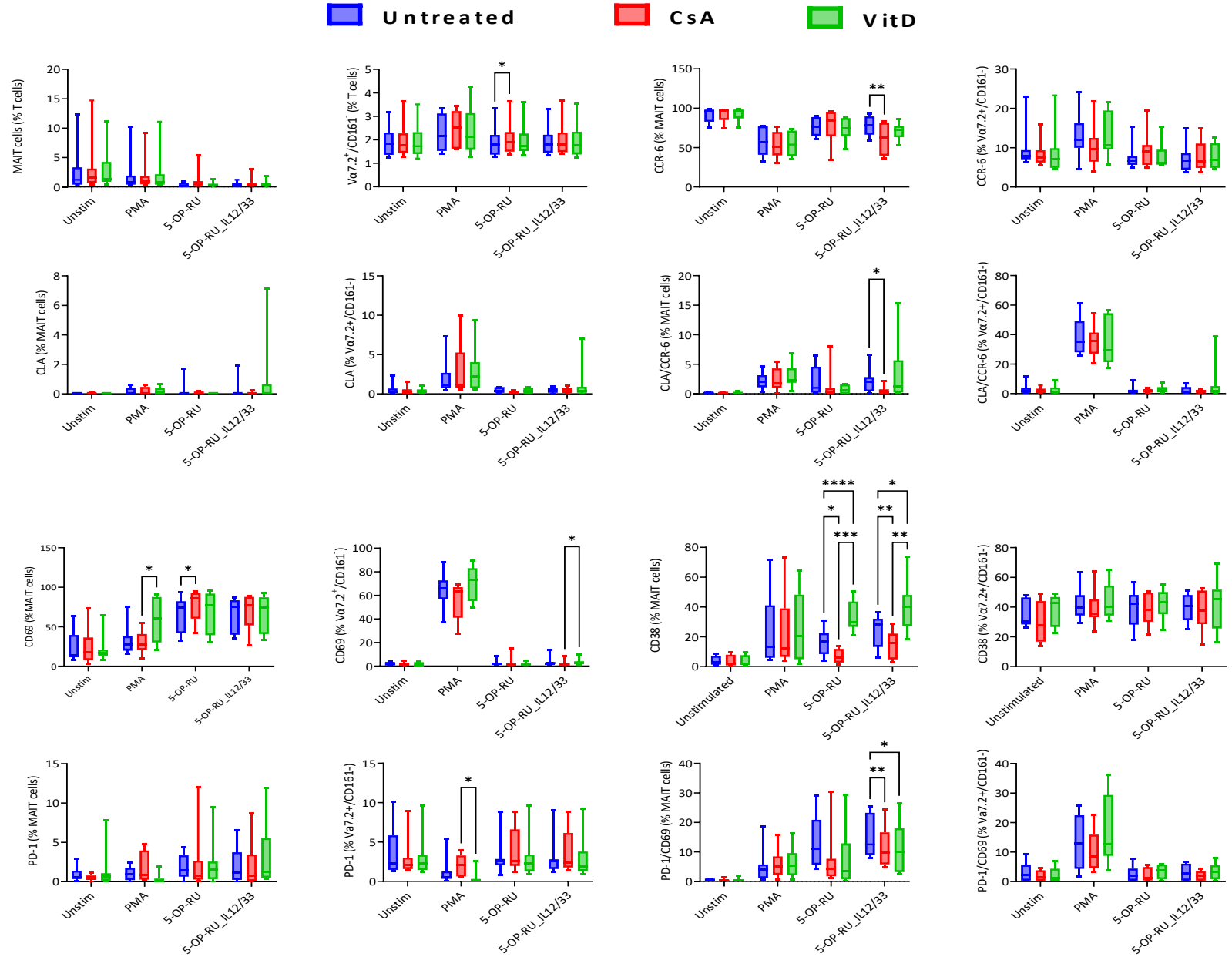


Figure 23: Treatment-based changes in cellular surface markers of MAIT and Vα7.2+/CD161- T Cells under different stimulatory conditions. The blue color gradient represents untreated cells, the red color gradient represents Cyclosporin A (CsA) treated cells, and the green color gradient represents Vitamin D3 (VitD) treated cells. The x-axis illustrates different stimulatory conditions (unstimulated, Phorbol myristate acetate (PMA)/ionomycin, 5-(2-oxopropylideneamino)-6-d-ribitylamouracil (5-OP-RU), 5-OP-RU/IL-12/IL-33), while the y-axis represents marker expression as a percentage of MAIT cells or Vα7.2+/CD161- T cells. Statistical analysis was performed using Two-way ANOVA to compare individual treatment groups between untreated, CsA-treated, and VitD-treated cells. "" denotes $P \leq 0.05$, "**" signifies $P \leq 0.01$, "***" represents $P \leq 0.001$, and "****" indicates $P \leq 0.0001$.*

4.4.4. Cyclosporin A and Vitamin D₃ modulatory changes in cytokine production of MAIT and Vα7.2+/CD161- T cells under varied stimulation

CsA was found to block IFN γ -producing MAIT cells post-stimulation, and VitD treatment showed blockade with 5-OP-RU ligand-specific stimulation. As expected, CsA treatment also showed IFN γ 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/IFN γ -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, and no treatment-based differences were found in IL-4 and IFN γ /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 TNF α -producing MAIT cells and V α 7.2+/CD161- T cells was noted with PMA-specific stimulation; however, CsA could not help with MAIT cell-specific stimulants. Conversely, GzB/TNF α -producing MAIT cells were blocked by CsA and VitD when stimulated with 5-OP-RU. Interestingly, no effect of drugs was noted in the case of 5-OP-RU ligand with IL-12/IL-33 stimulation. The treatment-based blockade was noted in GzB/TNF α -producing V α 7.2+/CD161- T cells (Figure 24).

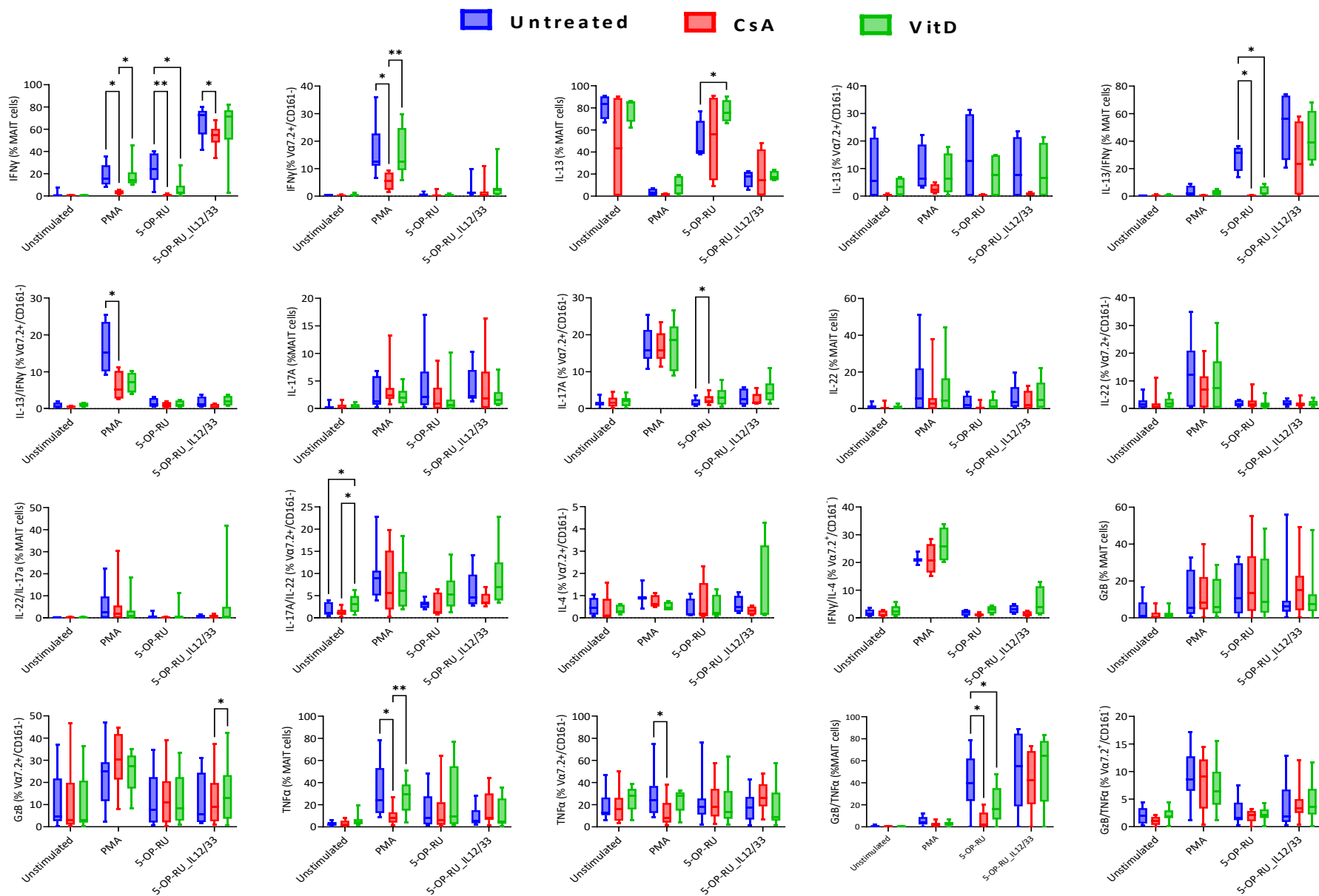


Figure 24: Treatment-based changes in intracellular cytokines of MAIT and $V\alpha 7.2^{+}/CD161^{-}$ T cells under different stimulatory conditions. The blue color gradient represents untreated cells, the red color gradient represents Cyclosporin A (CsA) treated cells, and the green color gradient represents Vitamin D3 (VitD) treated cells. The x-axis represents different stimulatory conditions (unstimulated, Phorbol myristate acetate (PMA)/ionomycin, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU), 5-OP-RU/IL-12/IL-33), while the y-axis represents marker expression as a percentage of MAIT cells or $V\alpha 7.2^{+}/CD161^{-}$ T cells. Statistical analysis was performed using Two-way ANOVA to compare individual treatment groups between untreated, CsA-treated, and VitD-treated cells. "" denotes $P \leq 0.05$, "**" signifies $P \leq 0.01$, "***" represents $P \leq 0.001$, and "*****" indicates $P \leq 0.0001$.*

5. DISCUSSION

Extensive research in the past two decades has highlighted the significance of non-traditional T cells, known as uT. These cells play a crucial role in a wide range of 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 uT 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 uT cells and identified a relationship between them. This study lays the groundwork for future research and clinical studies comparing uT cells in age-related conditions, disease progression, and follow-up assessments.

Our research findings show a strong association between aging and changes in uT-cell subsets. We found a significant negative correlation with age for $\gamma\delta$ T cells and MAIT cells, while there was no significant relationship between age and the percentage or absolute count of iNKT cells. Other studies have also identified negative correlations between different age groups in $\gamma\delta$ T cells and MAIT cells [104]–[109]. 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. [110],[111]. This contrasts the findings of Jing et al., who reported a negative correlation of

iNKT cells with age in both males and females [112]. $\gamma\delta$ T cells negatively correlated with age in males and females, indicating an increased risk of immune-related diseases as people age. Fonseca et al. found similar results, with females showing a steeper decline than males [104]. Additionally, both males and females showed decreased MAIT cells with age, consistent with Chen et al.'s findings [109].

No significant variation was found in the levels of iNKT cells across age groups, contrary to the expected concept of immunosenescence. Although there was a slight decline in median values between young and middle-aged groups, these values returned to normal in other age groups. Our findings differ from earlier studies, such as by Jing et al. involving 49 young and 101 senior individuals, which reported a significant difference between the two groups [112]. Peralbo et al. observed significantly lower $V\alpha 24^+$ $V\beta 11^+$ cells among lymphocytes in elderly subjects than in young individuals [113]. Similarly, Delarosa et al. reported a decreased percentage of $V\alpha 24^+$ NKT cells in older people compared to the young [114]. These disparities may stem from variations in study design, sample sizes, and methodology. Our study found age-related changes in $\gamma\delta$ T cells, with younger individuals having higher percentages and absolute numbers of these cells. Middle-aged and older individuals showed a slight, statistically non-significant reduction. This aligns with previous studies suggesting a gradual decline in $\gamma\delta$ T cells after the age of 20–30 [105]–[107]. Age-related changes in MAIT cells were observed, with lower percentages in middle-aged and elderly individuals compared to the young. Lee et al. and Chen et al. demonstrated a progressive increase in MAIT cell frequency from cord blood to young age, followed by a decline in older age groups [115], [109]. These findings highlight the dynamic nature of MAIT cells with aging.

Gender bias in immune cell type frequency is crucial for accurate assessments. Our study found that females have a significantly higher percentage of $CD3^+$ T cells than males. Other studies have supported this, indicating a greater fraction of T cells in females [116]. These variations in

immune cell composition are believed to be influenced by factors such as sex hormones, sex chromosomes, reproductive status, and environmental factors [73]. Our study found significant gender differences in the percentage and absolute number of iNKT cells. Females had more iNKT cells than males, which contradicts previous findings [117]. Montoya et al. found a slightly more significant frequency trend in iNKT cells in females compared to males, although the results did not reach statistical significance [118]. Further research into these gender-specific variations is important, especially in immunological diseases where these cells may have a significant role. We found males have higher percentages and absolute numbers of $\gamma\delta$ T cells than females, as reported by several other studies [105],[119],[120]. However, one study found no significant difference between genders [104]. Our study found gender differences in $\gamma\delta$ T cells, but not in MAIT cells. These results align with those of Novak et al., who also found no statistically significant differences between males and females in MAIT cells [108]. Gender differences in uT cell populations highlight the complexity of immune system dynamics, emphasizing the need for further research to understand the underlying mechanisms and potential implications for immunological responses.

The overall number of uT cells showed a significant positive correlation, especially between iNKT and MAIT cells across genders. However, iNKT cells and $\gamma\delta$ T cells in men did not correlate. Gherardin et al. reported a favorable association between the percentages of iNKT cells and MAIT cells, as well as $\gamma\delta$ T cells and MAIT cells, in a study involving 27 healthy adult PB samples [83]. MAIT and iNKT cells share transcriptional factors like T-bet, ROR γ t, 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 [121] [122]. In CD1d knockout mice, there is an increase in mucosal-associated invariant T (MAIT) cells, while MR1-deficient mice show an increase in iNKT cells. This suggests a potential link between these cell types through shared antigen-presenting molecules

and signaling pathways [123]. Despite their frequency differences, iNKT and MAIT cells complement each other [124], highlighting the potential synergies between different uT cell populations in the immune system.

We used descriptive statistics to establish the reference ranges for uT cells in PB. Our findings on iNKT cells align with those reported by several studies involving healthy adults [118],[125]–[127]. Fereidouni et al. observed a higher percentage of 6B11-iNKT cells among T lymphocytes in their study of 40 healthy adults from Iran [117] (Table 11). The difference may be due to the younger age of their subjects and the smaller sample size compared to our study. The majority of $\gamma\delta$ T cells in PB use V γ 9 and V δ 2, commonly referred to as $\gamma\delta$ T cells [128]. Our data aligns with similar studies on healthy adult Japanese and Portuguese cohorts [105] [104] (Table 12). However, conflicting results arise when considering the pan anti-TCR $\gamma\delta$ T cell marker [104],[105],[129] (Table 13). The frequency of MAIT cells in PB is noteworthy among uT cells. Our results align with similar studies conducted on healthy Korean individuals and blood samples across a wide age range [10] [111] (Table 14).

iNKT cells					
References	Sample size	% T cells (range)	% Mean $\pm$ SD	Cells/ μ L (range)	Mean $\pm$ SD
Our results	203	0.095 (0.007-4.94)	0.2348 $\pm$ 0.5125	1.3 (0.1-63.5)	3.334 $\pm$ 7.27
Montoya et. al., 2006	90	0.01 - 0.92	0.17 $\pm$ 0.19	NA	NA
Peter et.al., 2002	10	0.01 - 0.1 of PBMC	NA	NA	NA
Lucas, et.al, 2002	11	0.01 - 0.14 of PBMC	NA	NA	NA
Sandberg, et.al., 2003	75	0.05 (0.01 - 0.19) of PBMC	NA	NA	NA
Fereidouni et al., 2009	40	0.54 (0.14 - 1.78)	NA	NA	NA

Table 11: Comparison of our results of invariant natural killer T cells with previously published studies by different groups

$\gamma\delta$ T cells					
References	Sample size	% T cells (range)	% Mean $\pm$ SD	Cells/ μ L (range)	Mean $\pm$ SD
Our results	203	2.175 (0.078 - 16.9)	3.119 $\pm$ 2.86	30 (1 to 249)	44 $\pm$ 40.06
Michishita et. al., 2011	120	NA	NA	1 - 153	43 $\pm$ 36
Sonia Fonseca et. al., 2020	30	NA	NA	39 (6 - 243)	63 $\pm$ 62

Table 12: Comparison of our results of $\gamma\delta$ T cells identified with $V\gamma 9V\delta 2$ marker with previously published studies which identified subsets of $\gamma\delta$ T cells with $V\gamma 9V\delta 2$ marker positivity

$\gamma\delta$ T cells					
References	Sample size	% T cells (range)	% Mean $\pm$ SD	Cells/ μ L (range)	Mean $\pm$ SD
Our results	203	2.175 (0.078 - 16.9)	3.119 $\pm$ 2.86	30 (1 to 249)	44 $\pm$ 40.06
Michishita et. al., 2011	120	NA	NA	4 - 207	68 $\pm$ 44
Sonia Fonseca et. al., 2020	30	4.3 (1.2 - 15.4)	5.0 $\pm$ 3.6	63 (9 - 253)	NA
Andreu-Ballester et. al., 2012	157	3.13 (0.19 - 14.03)	NA	69.8 (4.5 - 318.7)	NA

Table 13: Comparison of our results of $\gamma\delta$ T cells identified with $V\gamma 9V\delta 2$ marker with previously published studies which identified $\gamma\delta$ T cells with a pan anti-TCR $\gamma\delta$ T cell marker

MAIT cells					
References	Sample size	% T cells (range)	% Mean $\pm$ SD	Cells/ μ L (range)	Mean $\pm$ SD
Our results	203	2.99 (0.11 - 18.36)	3.801 $\pm$ 3.175	42 (2 - 261)	52.88 $\pm$ 45.1
Lee. et al, 2013	133	2.12 (0.19 - 21.7)	-	-	-
Novak et al, 2014	100	1.195	-	19	-

Table 14: Comparison of our results of mucosa-associated invariant T cells with previously published studies for MAIT cells

Limited research has been conducted on healthy individuals. Our study is the only attempt to collectively establish reference ranges for iNKT cells, $\gamma\delta$ T cells, and MAIT cells. Discrepancies with previous studies can be attributed to factors such as different antibody clones and earlier study periods. Our study used the 6B11 monoclonal antibody clone designed to bind to the CDR3 region of the canonical V α 24J α 18 TCR rearrangement of iNKT cells. This choice was made due to its ability to detect lower yet specific iNKT cells, particularly when used with V α 24 within CD3+ T cells [118],[130]. In Jing et al.'s study, an imbalance in male and female representation may have affected the results. Additionally, the difference in results compared to Fereidouni et al. can be attributed to the different sample sizes. Our study was prospective, single-center, and focused on a homogeneous population (Caucasian) with gender-balanced samples, covering iNKT cells, $\gamma\delta$ T cells, and MAIT cells across four age groups with rigorous exclusion criteria.

In the second part of our study, we aimed to investigate the importance of MAIT cells and V α 7.2+/CD161- T cells in healthy individuals and those in the early stages of AD. Our study found that individuals with AD had a higher percentage of T cells but fewer MAIT cells and V α 7.2+/CD161- T cells than in PB of HC. MAIT cells play a crucial role in immune responses by recognizing microbial vitamin B2 metabolites [131]. *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 [132] [133]. As shown by Stanberg et al., after a *Staphylococcal* enterotoxin-triggered cytokine storm, MAIT cells may become unresponsive, indicating possible manipulation by *Staphylococcus aureus* and functional impairment [134]. 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 [30]. The reduced levels of MAIT and V α 7.2+/CD161- T cells in the blood of individuals with AD may be due to

their migration to skin lesions. Further research on skin lesions is needed to understand the role of MAIT cells and V α 7.2+/CD161- T cells in AD.

Orfali *et al.* found that 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 [135]. In our study, we found elevated CD38 across all subsets, including T cells, making it a more accurate activation marker in AD compared to CD69 [136], [137]. 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 (IFN γ)-producing T cells [92]–[95][138]. Keratinocytes, broadly expressing the IL-22 receptor, play an essential role in skin barrier defence and AD pathogenesis via IL-22-keratinocyte crosstalk [139]. Real-time PCR data support this, indicating strong gene activation of Th22 immunological pathways in skin samples during the acute phase, which gradually increases during the chronic phase of the disease [140].

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 TNF α . These cells are lower in the female vaginal tract after *E. coli* stimulation compared to blood [77]. Granzyme B (GzB) and tumor necrosis factor-alpha (TNF α) may play a role in the immune response associated with AD, indicating the disease's complex nature [141]–[143]. Our study found a decrease in IFN- γ -producing V α 7.2+/CD161- T cells and an increase in IL-22-producing V α 7.2+/CD161- T cells suggesting a role in Th2/22-mediated inflammation in AD. Further research is needed to understand their specific activities and contributions to AD's inflammatory processes. In contrast to AD, previous investigations found that chronic Hepatitis C Virus (HCV) and Human Immunodeficiency Virus type 1 (HIV-1) patients had significantly higher levels of V α 7.2+/CD161- T cells in their PB compared to HC, but MAIT cells were

decreased [89] [90]. Although both cell groups express the V α 7.2, they appear to be two completely different cell populations. In our study, we compared the immunophenotypes of V α 7.2+/CD161+ (MAIT cells) and V α 7.2+/CD161- T cells in HC and individuals with AD. In HC, V α 7.2+/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, V α 7.2+/CD161- T cells were predominantly CD4 (helper T) cells, whereas MAIT cells were CD8 (cytotoxic T) cells. V α 7.2+/CD161- T cells exhibited significantly lower levels of IL-18R α than MAIT cells, suggesting their distinct nature from MAIT cells. Dusseaux et al. described that IL-18R α was a surrogate marker for MAIT cells regulated by PLZF and RAR-related orphan receptor gamma transcription factors [144]. Park et al. found that TCR V α 7.2+CD161- T cells differ from MAIT cells but resemble conventional T cells. The overall gene expression profile indicates distinct characteristics between these cell types [88] [145]. In patients with AD, MAIT and V α 7.2+/CD161- T cell subsets showed similar results to those in HC, with a few exceptions in cell frequencies and subsets. AD is characterized by a decrease in total MAIT cells, an increase in GzB/TNF α -producing MAIT cells, and an increase in IL-22-producing V α 7.2+/CD161- T 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 [146]–[149]. The study revealed differences in the following populations, including CD8+ subsets of V α 7.2+/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) [150]. Our research aligns with previous studies showing fewer helper T cells in AD compared to HC [151] [152], emphasizing the need for multidimensional analysis to detect complex immunophenotypic changes.

Research on the role of MAIT cells in human AD is limited. One study in a mouse model found that MAIT cells promote AD by activating eosinophils and recruiting IL-4-producing cells [98]. Another study reported no significant change in MAIT cell frequency in the PB of human AD patients compared to HC [153]. The variation in study results could be due to sample size, patient demographics, and methodology. Limitations include reliance on PB samples and lack of skin biopsy analysis. Future research with larger sample sizes and comprehensive analyses across various disease stages would better understand MAIT cells and V α 7.2+/CD161- T cells in AD pathogenesis [154]. It's crucial to consider these factors for accurately interpreting research findings. This approach can help us to understand the complex interplay between immune cells and skin tissues in AD and potentially lead to the development of more targeted therapeutic interventions.

MAIT cells have a wide immunological response, with different roles in blood and mucosa. Their responses can be influenced by stimulation settings and the tissue microenvironment [84]. Summarizing MAIT cell signals, Konecny et al. emphasized their diverse functions in healthy and damaged tissues, highlighting the computational complexities of analyzing cell-cell communication using single-cell data [155]. Fergusson et al. found that MAIT cells express high levels of MDR1, which provides resistance to cytotoxic chemicals like daunorubicin but not to immunosuppressive drugs' effects [156]. CD161, a C-type lectin-like receptor, is an identifier for MAIT cells alongside the V α 7.2 TCR α chain. V α 7.2+/CD161- T cells constitute a distinct population separate from MAIT cells despite sharing the V α 7.2+ compartment. These cells exhibit unique gene expression patterns compared to MAIT cells [157]. Recent research has

found that cells respond differently in cases of AD than MAIT cells. V α 7.2+/CD161- T cells demonstrate a conventional Th2/22 response, while MAIT cells show an upregulation of polyfunctional TNF α /GzB-producing cells [158]. Our *in vitro* study focused on their activation, fatigue, and various cytokine responses under commonly known stimulatory conditions for MAIT cells. We compared V α 7.2+ T cells (CD161-) and MAIT cells (CD161+) to discern differences.

After stimulation, we noticed a slight decrease in MAIT cell counts, 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 [159]. Figure 25 summarizes different outcomes from treatments with specific stimulants. CsA treatment reduced CCR6 expression in the presence of 5-OP-RU/IL-12/33. Juno et al. observed that in renal patients, reduced CXCR3 expression was compensated by increased expression of CCR6 and CXCR6 [160]. Exploring the potential compensation of CCR6 with other migratory chemokines, such as CCR9, CCR5, and CXCR6, by examining known MAIT cell migratory chemokine receptors under similar conditions would have been beneficial [84],[144],[160]. 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 [161],[162]. CsA decreased CD38 expression, while Vitamin D increased it. In a trial with 16 cystic fibrosis patients, Pincikova et al. found a positive correlation between Vitamin D therapy and CD38 expression, and a negative correlation with PD-1 expressing MAIT cells [163]. 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 IFN γ production during TCR-dependent activation, leading to

increased expression of tissue-repair genes [164],[165]. Our results show both CsA, and Vitamin D significantly inhibit IFN γ production in MAIT cells during PMA, and 5-OP-RU activation. CsA mechanism of action targets to block TCR-mediated calcium signaling [166] and effectively suppresses IFN γ 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/IFN γ .

MAIT cells are highly flexible, integrating environmental signals to adapt and change their functions. This flexibility is influenced by factors such as tissue location, clonal identity, and activation state [33],[131],[167]. Vitamin D balances the immune system by reducing IFN γ and increasing IL-13 from MAIT cells when exposed to MR-1 ligands without proinflammation cytokines. Hinks et al. demonstrated that TCR-stimulated MAIT cells increase Vitamin D receptor expression in vitro and in mice [168]. In response to *E. coli* stimulation in organs like the liver and female reproductive tract, significant numbers of IL-17A and IL-22-producing MAIT cells are detected [10] [77]. MAIT cells in the buccal mucosa produce more IL-17 than those in PB. 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 [77],[78],[169]. Our findings align with previous studies, showing minimal IL-17A, IL-22, and polyfunctional IL-17A/IL-22 production by PB MAIT cells upon PMA stimulation.

MAIT cells are identified using flow cytometry by expressing V α 7.2 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 V α 7.2/CD161⁺ binding to the MR-1 tetramer, and not all MR-1 binding tetramers express V α 7.2/CD161⁺ [83]. 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 [170]. In our *in vitro* study, we found clear differences between V α 7.2+/CD161+ (MAIT) and V α 7.2+/CD161- T cells despite sharing the V α 7.2 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 V α 7.2+/CD161- T cells. Park et al. noted that TCR V α 7.2+CD161- T cells differ from V α 7.2+/CD161+ cells and closely resemble conventional T cells based on RNA sequencing. MAIT and V α 7.2+/CD161- T cells exhibit similar patterns of activated and downregulated genes [88]. Kathrin et al. studied the CDR3 repertoire of V α 7.2+ chains and found that both canonical and noncanonical clones were prevalent within MAIT cells. In contrast, V α 7.2+/CD161- subsets were polyclonal [145]. Our research suggests that 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 IFN γ production. MAIT cells showed polyfunctional reactions to the same stimulus, with elevated levels of PD-1/CD69, IL-13/IFN γ , and TNF α /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 V α 7.2+/CD161- T cells, regardless of IL-12 or IL-33 levels, which confirms that MAIT cells differ from V α 7.2+/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 IFN γ /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 did not cover the CA7S ligand, which has been shown to enhance survival and upregulate genes associated with wound healing and immunoregulation. Including this ligand in future studies could offer further insights into MAIT cell behaviour and therapeutic potential.

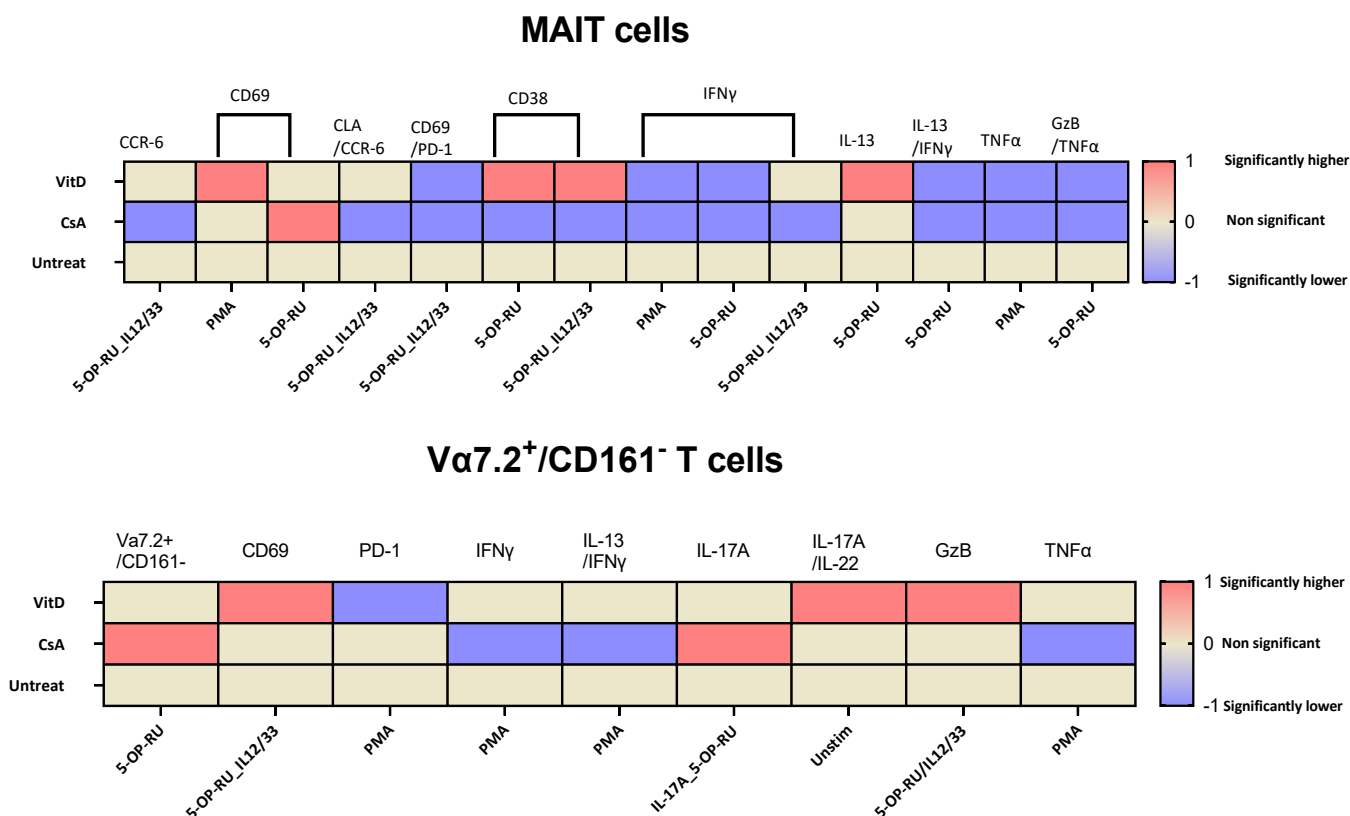


Figure 25: Summarizing the data found to be statistically significant between the treatment group comparison of MAIT and Va7.2⁺/CD161⁻ T cells. Only the statistically significant data are summarised here; non-significant data are not mentioned here. The heatmap gradient shows 0, which means nonsignificant (Brown color), -1, which shows significantly lower (Blue color), and 1, which indicates considerably higher (Red color). On the top, the different markers are given; on the left corners different treatment are mentioned (Untreated, Cyclosporin A and Vitamin D3) and the stimulant (Phorbol myristate acetate (PMA)/ionomycin, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU), 5-OP-RU/IL-12/IL-33) are given in lower part of the figure.

6. NEW SCIENTIFIC FINDINGS

To summarize, the frequency of $\gamma\delta$ 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, $\gamma\delta$ 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 IFN γ and effector (TNF α /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 V α 7.2 compartment, MAIT cells and V α 7.2+/CD161- T cells show clear differences. V α 7.2+/CD161- T cells resemble conventional T cells and do not respond to 5-OP-RU, although PMA stimulation increases the production of certain cytokines.

7. REFERENCES

1. Anon. Innate and adaptive immunity time line. *Creative Daignostics*. Available at: <https://www.creative-diagnostics.com/innate-and-adaptive-immunity.htm> [Accessed November 5, 2023].
2. Parkin J, Cohen B. An overview of the immune system. *The Lancet*. 2001; 357:1777–1789. Available at: <https://www.sciencedirect.com/science/article/pii/S0140673600049047>. DOI: [https://doi.org/10.1016/S0140-6736\(00\)04904-7](https://doi.org/10.1016/S0140-6736(00)04904-7).
3. Weiskopf D, Weinberger B, Grubeck-Loebenstien B. The aging of the immune system. *Transplant International*. 2009; 22:1041–1050. DOI: 10.1111/j.1432-2277.2009.00927.x.
4. Pellicci DG, Koay H-F, Berzins SP. Thymic development of unconventional T cells: how NKT cells, MAIT cells and $\gamma\delta$ T cells emerge. *Nature Reviews Immunology*. 2020; 20:756–770. Available at: <https://doi.org/10.1038/s41577-020-0345-y>. DOI: 10.1038/s41577-020-0345-y.
5. Godfrey DI, Uldrich AP, Mccluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nature Immunology*. 2015; 16:1114–1124. DOI: 10.1038/ni.3298.1114.
6. Park Y-W, Kee S-J. Mucosal-associated Invariant T cells: A New Player in Innate Immunity. *jrd*. 2015; 22:337–345. Available at: <http://dx.doi.org/10.4078/jrd.2015.22.6.337>. DOI: 10.4078/jrd.2015.22.6.337.
7. Legoux F, Salou M, Lantz O. Unconventional or preset $\alpha\beta$ T cells: Evolutionarily conserved tissue-resident T cells recognizing nonpeptidic ligands. *Annual Review of Cell and Developmental Biology*. 2017; 33:511–535. DOI: 10.1146/annurev-cellbio-100616-060725.
8. Amini A, Pang D, Hackstein CP, Klenerman P. MAIT Cells in Barrier Tissues: Lessons from Immediate Neighbors. *Frontiers in Immunology*. 2020; 11:1–20. DOI: 10.3389/fimmu.2020.584521.
9. Treiner E, Duban L, Bahram S, Radosavljevic M, Wanner V, Tilloy F, Affaticati P, *et al*. Erratum: Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1

(Nature (2003) 422 (164-169)). *Nature*. 2003; 423:1018.DOI: 10.1038/nature01700.

10. Godfrey DI, Koay H-FF, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nature Immunology*. 2019; 20:1110–1128. Available at: <http://dx.doi.org/10.1038/s41590-019-0444-8>.DOI: 10.1038/s41590-019-0444-8.

11. Godfrey DI, Koay HF, McCluskey J, Gherardin NA. The biology and functional importance of MAIT cells. *Nature Immunology*. 2019; 20:1110–1128. Available at: <http://dx.doi.org/10.1038/s41590-019-0444-8>.DOI: 10.1038/s41590-019-0444-8.

12. Corbett AJ, Eckle SBG, Birkinshaw RW, Liu L, Patel O, Mahony J, Chen Z, *et al*. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature*. 2014; 509:361–365.DOI: 10.1038/nature13160.

13. Reantragoon R, Corbett AJ, Sakala IG, Gherardin NA, Furness JB, Chen Z, Eckle SBG, *et al*. Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells. *Journal of Experimental Medicine*. 2013; 210:2305–2320.DOI: 10.1084/jem.20130958.

14. Rahimpour A, Koay HF, Enders A, Clanchy R, Eckle SBG, Meehan B, Chen Z, *et al*. Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *Journal of Experimental Medicine*. 2015; 212:1095–1108.DOI: 10.1084/jem.20142110.

15. Lamichhane R, Schneider M, de la Harpe SM, Harrop TWR, Hannaway RF, Dearden PK, Kirman JR, *et al*. TCR- or Cytokine-Activated CD8⁺ Mucosal-Associated Invariant T Cells Are Rapid Polyfunctional Effectors That Can Coordinate Immune Responses. *Cell Reports*. 2019; 28:3061-3076.e5. Available at: <https://www.sciencedirect.com/science/article/pii/S2211124719311027>.DOI: <https://doi.org/10.1016/j.celrep.2019.08.054>.

16. Toubal A, Nel I, Lotersztajn S, Lehuen A. Mucosal-associated invariant T cells and disease.

- Nature Reviews Immunology*. 2019; 19:643–657. Available at: <http://dx.doi.org/10.1038/s41577-019-0191-y>.DOI: 10.1038/s41577-019-0191-y.
17. Li J, Reantragoon R, Kostenko L, Corbett AJ, Varigos G, Carbone FR. The frequency of mucosal-associated invariant T cells is selectively increased in dermatitis herpetiformis. *Australasian Journal of Dermatology*. 2017; 58:200–204.DOI: 10.1111/ajd.12456.
18. Salou M, Franciszkievicz K, Lantz O. MAIT cells in infectious diseases. *Current Opinion in Immunology*. 2017; 48:7–14. Available at: <http://dx.doi.org/10.1016/j.coi.2017.07.009>.DOI: 10.1016/j.coi.2017.07.009.
19. Meermeier EW, Harrieff MJ, Karamooz E, Lewinsohn DM. MAIT cells and microbial immunity. *Immunology and Cell Biology*. 2018; 96:607–617.DOI: 10.1111/imcb.12022.
20. Ussher JE, Willberg CB, Klenerman P. MAIT cells and viruses. *Immunology and Cell Biology*. 2018; 96:630–641.DOI: 10.1111/imcb.12008.
21. Parrot T, Gorin JB, Ponzetta A, Maleki KT, Kammann T, Emgård J, Perez-Potti A, *et al*. MAIT cell activation and dynamics associated with COVID-19 disease severity. *Science Immunology*. 2020; 5:1–14.DOI: 10.1126/SCIIMMUNOL.ABE1670.
22. Rouxel O, Lehuen A. Mucosal-associated invariant T cells in autoimmune and immune-mediated diseases. *Immunology and Cell Biology*. 2018; 96:618–629.DOI: 10.1111/imcb.12011.
23. Kurioka A, Walker LJ, Klenerman P, Willberg CB. MAIT cells: new guardians of the liver. *Clinical & Translational Immunology*. 2016; 5:e98.DOI: 10.1038/cti.2016.51.
24. Magalhaes I, Kiaf B, Lehuen A. iNKT and MAIT cell alterations in diabetes. *Frontiers in Immunology*. 2015; 6:6–12.DOI: 10.3389/fimmu.2015.00341.
25. Ling L, Lin Y, Zheng W, Hong S, Tang X, Zhao P, Li M, *et al*. Circulating and tumor-infiltrating mucosal associated invariant T (MAIT) cells in colorectal cancer patients. *Scientific Reports*. 2016; 6:1–10.DOI: 10.1038/srep20358.
26. Zabijak L, Attencourt C, Guignant C, Chatelain D, Marcelo P, Marolleau JP, Treiner E.

Increased tumor infiltration by mucosal-associated invariant T cells correlates with poor survival in colorectal cancer patients. *Cancer Immunology, Immunotherapy*. 2015; 64:1601–1608.DOI: 10.1007/s00262-015-1764-7.

27. Won EJ, Ju JK, Cho YN, Jin HM, Park KJ, Kim TJ, Kwon YS, *et al*. Clinical relevance of circulating mucosal-associated invariant T cell levels and their anti-cancer activity in patients with mucosal-associated cancer. *Oncotarget*. 2016; 7:76274–76290.DOI: 10.18632/oncotarget.11187.

28. Duan M, Goswami S, Shi JY, Wu LJ, Wang XY, Ma JQ, Zhang Z, *et al*. Activated and exhausted MAIT cells foster disease progression and indicate poor outcome in hepatocellular carcinoma. *Clinical Cancer Research*. 2019; 25:3304–3316.DOI: 10.1158/1078-0432.CCR-18-3040.

29. Mérédis F, Koen V, Sylvia F, Ken M, Kim De V, Elke De B, Xavier L, *et al*. Both mucosal-associated invariant and natural killer T-cell deficiency in multiple myeloma can be countered by PD-1 inhibition. *Haematologica*. 2016; 23:471–473.

30. du Halgouet A, Darbois A, Alkobtawi M, Mestdagh M, Alphonse A, Premel V, Yvorra T, *et al*. Role of MR1-driven signals and amphiregulin on the recruitment and repair function of MAIT cells during skin wound healing. *Immunity*. 2023; 56:78-92.e6. Available at: <https://www.sciencedirect.com/science/article/pii/S1074761322006409>.DOI: <https://doi.org/10.1016/j.immuni.2022.12.004>.

31. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, *et al*. CD1d-restricted and TCR-mediated activation of V(α)14 NKT cells by glycosylceramides. *Science*. 1997; 278:1626–1629.DOI: 10.1126/science.278.5343.1626.

32. Mattner J, DeBord KL, Ismail N, Goff RD, Cantu C, Zhou D, Saint-Mezard P, *et al*. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature*. 2005; 434:525–529.DOI: 10.1038/nature03408.

33. Kinjo Y, Tupin E, Wu D, Fujio M, Garcia-Navarro R, Benhnia MREI, Zajonc DM, *et al.* Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nature Immunology*. 2006; 7:978–986.DOI: 10.1038/ni1380.
34. Crosby CM, Kronenberg M. Tissue-specific functions of invariant natural killer T cells. *Nature Reviews Immunology*. 2018; 18:559–574. Available at: <https://doi.org/10.1038/s41577-018-0034-2>.DOI: 10.1038/s41577-018-0034-2.
35. Metelitsa LS, Weinberg KI, Emanuel PD, Seeger RC. Expression of CD1d by myelomonocytic leukemias provides a target for cytotoxic NKT cells. *Leukemia*. 2003; 17:1068–1077.DOI: 10.1038/sj.leu.2402943.
36. Fais F, Tenca C, Cimino G, Coletti V, Zanardi S, Bagnara D, Saverino D, *et al.* CD1d expression on B-precursor acute lymphoblastic leukemia subsets with poor prognosis. *Leukemia*. 2005; 19:551–556.DOI: 10.1038/sj.leu.2403671.
37. Zhu Y, Smith DJ, Zhou Y, Li YR, Yu J, Lee D, Wang YC, *et al.* Development of Hematopoietic Stem Cell-Engineered Invariant Natural Killer T Cell Therapy for Cancer. *Cell Stem Cell*. 2019; 25:542-557.e9. Available at: <https://doi.org/10.1016/j.stem.2019.08.004>.DOI: 10.1016/j.stem.2019.08.004.
38. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue S, Sasada T, Zeng W, *et al.* HHS Public Access. 2018; 23:3510–3519.DOI: 10.1158/1078-0432.CCR-16-0600.Adoptive.
39. Heczey A, Courtney AN, Montalbano A, Robinson S, Liu K, Li M, Ghatwai N, *et al.* Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis. *Nature Medicine*. 2020; 26:1686–1690. Available at: <http://dx.doi.org/10.1038/s41591-020-1074-2>.DOI: 10.1038/s41591-020-1074-2.
40. Beristain-Covarrubias N, Canche-Pool E, Gomez-Diaz R, Sanchez-Torres LE, Ortiz-Navarrete V. Reduced iNKT cells numbers in type 1 diabetes patients and their first-degree relatives. *Immunity Inflammation and Disease*. 2015; 3:411–419.DOI: 10.1002/iid3.79.

41. Zhou C, Zhou X, He D, Li Z, Xie X, Ren Y. Reduction of Peripheral Blood iNKT and $\gamma\delta$ T Cells in Patients With Parkinson's Disease: An Observational Study. *Frontiers in Immunology*. 2020; 11:1–7.DOI: 10.3389/fimmu.2020.01329.
42. Berzins SP, Smyth MJ, Baxter AG. Presumed guilty: Natural killer T cell defects and human disease. *Nature Reviews Immunology*. 2011; 11:131–142. Available at: <http://dx.doi.org/10.1038/nri2904>.DOI: 10.1038/nri2904.
43. Lam PY, Nissen MD, Mattarollo SR. Invariant natural killer T cells in immune regulation of blood cancers: Harnessing their potential in immunotherapies. *Frontiers in Immunology*. 2017; 8:1–12.DOI: 10.3389/fimmu.2017.01355.
44. Sbihi Z, Dossier A, Boutboul D, Galicier L, Parizot C, Emarre A, Hoareau B, *et al.* iNKT and memory B-cell alterations in HHV-8 multicentric Castleman disease. *Blood*. 2017; 129:855–865.DOI: 10.1182/blood-2016-06-719716.
45. King LA, Lameris R, de Gruijl TD, van der Vliet HJ. CD1d-Invariant Natural Killer T Cell-Based Cancer Immunotherapy: α -Galactosylceramide and Beyond. *Frontiers in Immunology*. 2018; 9.DOI: 10.3389/fimmu.2018.01519.
46. Erin J. Adams, Gu S, Luoma AM. Human gamma delta T cells: evolution and ligand recognition. *Cell Immunol*. 2015; 296:31–40.DOI: 10.1016/j.cellimm.2015.04.008.Human.
47. Murre C, Waldmann RA, Morton CC, Bongiovanni KF, Waldmann TA, Shows TB, Seidman JG. Human γ -chain genes are rearranged in leukaemic T cells and map to the short arm of chromosome 7. *Nature*. 1985; 316:549–552.DOI: 10.1038/316549a0.
48. Bröker B, Lydyard PM, Emmrich F. The role of $\gamma\delta$ T cells in the normal and disordered immune system. *Klinische Wochenschrift*. 1990; 68:489–495.DOI: 10.1007/BF01648239.
49. Hayday AC. $\gamma\delta$ Cells: A right time and a right place for a conserved third way of protection. *Annual Review of Immunology*. 2000; 18:975–1026.DOI: 10.1146/annurev.immunol.18.1.975.
50. Constant P, Davodeau F, Peyrat MA, Poquet Y, Puzo G, Bonneville M, Fournié JJ.

Stimulation of human $\gamma\delta$ T cells by nonpeptidic mycobacterial ligands. *Science*. 1994; 264:267–270.DOI: 10.1126/science.8146660.

51. Belmant C, Espinosa E, Halary F, Tang Y, Peyrat M-A, Sicard H, Kozikowski A, *et al.* A chemical basis for recognition of nonpeptide antigens by human $\gamma\delta$ T cells*. *The FASEB Journal*. 2000; 14:1669–1670.DOI: 10.1096/fj.99-0909fje.

52. Ferrarini M, Ferrero E, Dagna L, Poggi A, Zocchi MR. Human $\gamma\delta$ T cells: A nonredundant system in the immune-surveillance against cancer. *Trends in Immunology*. 2002; 23:14–18.DOI: 10.1016/S1471-4906(01)02110-X.

53. Rigau M, Ostrouska S, Fulford TS, Johnson DN, Woods K, Ruan Z, McWilliam HEG, *et al.* Butyrophilin 2A1 is essential for phosphoantigen reactivity by $\gamma\delta$ T cells. *Science*. 2020; 367:eaay5516. Available at: <https://doi.org/10.1126/science.aay5516>.DOI: 10.1126/science.aay5516.

54. Kozbor D, Trinchieri G, Monos DS, Isobe M, Russo G, Haney JA, Zmijewski C, *et al.* Human TCR- γ +/ δ +, CD8+ T lymphocytes recognize tetanus toxoid in an MHC-restricted fashion. *Journal of Experimental Medicine*. 1989; 169:1847–1851.DOI: 10.1084/jem.169.5.1847.

55. Joseph Holoshitz, Frits Koning, John E. Coligan JDB& SS. Isolation of CD4- CD8-mycobacteria-reactive T lymphocyte clones from rheumatoid arthritis synovial fluid. *Nature*. 1989; 339:226–229.

56. Haregewoin A, Soman G, Hom RC, Finberg RW. Human $\gamma\delta$ + T cells respond to mycobacterial heat-shock protein. *Nature*. 1989; 340:309–312.DOI: 10.1038/340309a0.

57. Granel B, Camoin L, Serratrice J, De Roux-Serratrice C, Brunet C, Pache X, Swiader L, *et al.* Étude rétrospective de 55 patients présentant une lymphocytose T γ / δ dans le sang circulant. *Revue de Medecine Interne*. 2002; 23:137–143.DOI: 10.1016/S0248-8663(01)00529-X.

58. Meraviglia S, El Daker S, Dieli F, Martini F, Martino A. T cells cross-link innate and adaptive immunity in mycobacterium tuberculosis infection. *Clinical and Developmental Immunology*.

2011; 2011.DOI: 10.1155/2011/587315.

59. Chen ZW, Letvin NL. V γ 2V δ 2+ T cells and anti-microbial immune responses. *Microbes and Infection*. 2003; 5:491–498.DOI: 10.1016/S1286-4579(03)00074-1.

60. Zheng J, Liu Y, Lau YL, Tu W. $\gamma\delta$ -T cells: An unpolished sword in human anti-infection immunity. *Cellular and Molecular Immunology*. 2013; 10:50–57.DOI: 10.1038/cmi.2012.43.

61. Terrazzini N, Kern F. Cell-mediated immunity to human CMV infection: A brief overview. *F1000Prime Reports*. 2014; 6:1–9.DOI: 10.12703/P6-28.

62. Rajoriya N, Fergusson J, Leithead JA, Klenerman P. Gamma delta T-lymphocytes in hepatitis C and chronic liver disease. *Frontiers in Immunology*. 2014; 5:1–9.DOI: 10.3389/fimmu.2014.00400.

63. Chen ZW. Immune biology of Ag-specific $\gamma\delta$ T cells in infections. *Cellular and Molecular Life Sciences*. 2011; 68:2409–2417.DOI: 10.1007/s00018-011-0703-9.

64. Silva-Santos B, Serre K, Norell H. $\gamma\delta$ T cells in cancer. *Nature Reviews Immunology*. 2015; 15:683–691.DOI: 10.1038/nri3904.

65. Maria Raffaella Zocchi and Alessandro P. Role of GammaDelta T lymphocytes in tumor defense. *Frontiers in Bioscience* 9, 2588-2604. 2004; 85:A7.DOI: 10.1016/s0003-9993(04)00507-6.

66. Fisch P, Moris A, Rammensee H, Handgretinger R. Cells in Tumour Immunity and Autoimmunity. *Immunology today*. 2000:187–191.

67. Veronika Groh, Alexander Steinle, Stefan Bauer TS. Recognition of Stress-Induced MHC Molecules by Intestinal Epithelial $\gamma\delta$ T Cells. *SCIENCE*. 1998; 279:1737–1740.

68. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived $\gamma\delta$ T cells of MICA and MICB. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:6879–6884.DOI: 10.1073/pnas.96.12.6879.

69. Liang Z, Dong X, Zhang Z, Zhang Q, Zhao Y. Age-related thymic involution: Mechanisms and functional impact. *Aging cell*. 2022; 21:e13671.DOI: 10.1111/accel.13671.
70. Bodey B, Bodey BJ, Siegel SE, Kaiser HE. Involution of the mammalian thymus, one of the leading regulators of aging. *In vivo (Athens, Greece)*. 1997; 11:421–440.
71. Mousset CM, Hobo W, Woestenenk R, Preijers F, Dolstra H, van der Waart AB. Comprehensive Phenotyping of T Cells Using Flow Cytometry. *Cytometry Part A*. 2019; 95:647–654.DOI: 10.1002/cyto.a.23724.
72. Spitzer JA. Gender differences in some host defense mechanisms. *Lupus*. 1999; 8:380–383.DOI: 10.1177/096120339900800510.
73. Klein SL, Flanagan KL. Sex differences in immune responses. *Nature Reviews Immunology*. 2016; 16:626–638.DOI: 10.1038/nri.2016.90.
74. Abdullah M, Chai PS, Chong MY, Tohit ERM, Ramasamy R, Pei CP, Vidyadaran S. Gender effect on in vitro lymphocyte subset levels of healthy individuals. *Cellular Immunology*. 2012; 272:214–219. Available at: <http://dx.doi.org/10.1016/j.cellimm.2011.10.009>.DOI: 10.1016/j.cellimm.2011.10.009.
75. Jacobson DL, Gange SJ, Rose NR, Graham NMH. Epidemiology and estimated population burden of selected autoimmune diseases in the United States. *Clinical Immunology and Immunopathology*. 1997; 84:223–243.DOI: 10.1006/clin.1997.4412.
76. Cook MB, Dawsey SM, Freedman ND, Inskip PD, Wichner SM, Quraishi SM, Devesa SS, *et al*. Sex disparities in cancer incidence by period and age. *Cancer Epidemiology Biomarkers and Prevention*. 2009; 18:1174–1182.DOI: 10.1158/1055-9965.EPI-08-1118.
77. Gibbs A, Leeansyah E, Introini A, Paquin-Proulx D, Hasselrot K, Andersson E, Broliden K, *et al*. MAIT cells reside in the female genital mucosa and are biased towards IL-17 and IL-22 production in response to bacterial stimulation. *Mucosal Immunology*. 2017; 10:35–45. Available at: <https://doi.org/10.1038/mi.2016.30>.DOI: 10.1038/mi.2016.30.

78. Sobkowiak MJ, Davanian H, Heymann R, Gibbs A, Emgård J, Dias J, Aleman S, *et al.* Tissue-resident MAIT cell populations in human oral mucosa exhibit an activated profile and produce IL-17. *European Journal of Immunology*. 2019; 49:133–143.DOI: 10.1002/eji.201847759.
79. Wong EB, Ndung'u T, Kasprowicz VO. The role of mucosal-associated invariant T cells in infectious diseases. *Immunology*. 2017; 150:45–54.DOI: 10.1111/imm.12673.
80. Constantinides MG, Link VM, Tamoutounour S, Wong AC, Perez-Chaparro PJ, Han SJ, Chen YE, *et al.* MAIT cells are imprinted by the microbiota in early life and promote tissue repair. *Science*. 2019; 366.DOI: 10.1126/science.aax6624.
81. Dusseaux M, Martin E, Serriari N, Péguillet I, Premel V, Louis D, Milder M, *et al.* Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161 hi IL-17-secreting T cells. *Blood*. 2011; 117:1250–1259.DOI: 10.1182/blood-2010-08-303339.
82. Le Bourhis L, Martin E, Péguillet I, Guihot A, Froux N, Coré M, Lévy E, *et al.* Antimicrobial activity of mucosal-associated invariant T cells. *Nature Immunology*. 2010; 11:701–708.DOI: 10.1038/ni.1890.
83. Gherardin NA, Souter MNT, Koay HF, Mangas KM, Seemann T, Stinear TP, Eckle SBG, *et al.* Human blood MAIT cell subsets defined using MR1 tetramers. *Immunology and Cell Biology*. 2018; 96:507–525.DOI: 10.1111/imcb.12021.
84. Nel I, Bertrand L, Toubal A, Lehuen A. MAIT cells, guardians of skin and mucosa? *Mucosal Immunology*. 2021; 14:803–814. Available at: <http://dx.doi.org/10.1038/s41385-021-00391-w>.DOI: 10.1038/s41385-021-00391-w.
85. Lanier LL, Chang C, Phillips JH. Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *The Journal of Immunology*. 1994; 153:2417–2428. Available at: <https://doi.org/10.4049/jimmunol.153.6.2417>.DOI: 10.4049/jimmunol.153.6.2417.

86. Takahashi T, Dejbakhsh-Jones S, Strober S. Expression of CD161 (NKR-P1A) Defines Subsets of Human CD4 and CD8 T Cells with Different Functional Activities¹. *The Journal of Immunology*. 2006; 176:211–216. Available at: <https://doi.org/10.4049/jimmunol.176.1.211>. DOI: 10.4049/jimmunol.176.1.211.
87. Wyrożemski Ł, Qiao S-W. Immunobiology and conflicting roles of the human CD161 receptor in T cells. *Scandinavian Journal of Immunology*. 2021; 94:e13090. Available at: <https://doi.org/10.1111/sji.13090>. DOI: <https://doi.org/10.1111/sji.13090>.
88. Park D, Kim HG, Kim M, Park T, Ha H-H, Lee DH, Park K-S, *et al.* Differences in the molecular signatures of mucosal-associated invariant T cells and conventional T cells. *Scientific Reports*. 2019; 9:7094. Available at: <https://doi.org/10.1038/s41598-019-43578-9>. DOI: 10.1038/s41598-019-43578-9.
89. Barathan M, Mohamed R, Vadivelu J, Chang LY, Saeidi A, Yong YK, Ravishankar Ram M, *et al.* Peripheral loss of CD8(+) CD161(++) TCRV α 7·2(+) mucosal-associated invariant T cells in chronic hepatitis C virus-infected patients. *European journal of clinical investigation*. 2016; 46:170–180. DOI: 10.1111/eci.12581.
90. Leeansyah E, Ganesh A, Quigley MF, Sönnernborg A, Andersson J, Hunt PW, Somsouk M, *et al.* Activation, exhaustion, and persistent decline of the antimicrobial MR1-restricted MAIT-cell population in chronic HIV-1 infection. *Blood*. 2013; 121:1124–1135. DOI: 10.1182/blood-2012-07-445429.
91. Jensen O, Trivedi S, Meier JD, Fairfax KC, Hale JS, Leung DT. A subset of follicular helper-like MAIT cells can provide B cell help and support antibody production in the mucosa. *Science Immunology*. 2022; 7:1–28. DOI: 10.1126/sciimmunol.abe8931.
92. Schuler CF, Billi AC, Maverakis E, Tsoi LC, Gudjonsson JE. Novel insights into atopic dermatitis. *Journal of Allergy and Clinical Immunology*. 2023; 151:1145–1154. Available at: <https://www.sciencedirect.com/science/article/pii/S0091674922014804>. DOI:

<https://doi.org/10.1016/j.jaci.2022.10.023>.

93. Weidinger S, Beck LA, Bieber T, Kabashima K, Irvine AD. Atopic dermatitis. *Nature reviews. Disease primers*. 2018; 4:1.DOI: 10.1038/s41572-018-0001-z.

94. Humeau M, Boniface K, Bodet C. Cytokine-Mediated Crosstalk Between Keratinocytes and T Cells in Atopic Dermatitis. *Frontiers in immunology*. 2022; 13:801579.DOI: 10.3389/fimmu.2022.801579.

95. Bantz SK, Zhu Z, Zheng T. The Atopic March: Progression from Atopic Dermatitis to Allergic Rhinitis and Asthma. *Journal of clinical & cellular immunology*. 2014; 5.DOI: 10.4172/2155-9899.1000202.

96. Gallagher C, Mahon J Mac, O'Neill C, Cassidy FC, Dunbar H, Barra C De, Cadden C, *et al*. Mucosal associated invariant T cells are altered in patients with Hidradenitis Suppurativa and contribute to the inflammatory milieu. *bioRxiv*. 2022:2022.01.17.476587. Available at: <http://biorxiv.org/content/early/2022/01/18/2022.01.17.476587.abstract>.DOI: 10.1101/2022.01.17.476587.

97. Teunissen MBM, Yeremenko NG, Baeten DLP, Chielie S, Spuls PI, de Rie MA, Lantz O, *et al*. The IL-17A-Producing CD8⁺ T-Cell Population in Psoriatic Lesional Skin Comprises Mucosa-Associated Invariant T Cells and Conventional T Cells. *Journal of Investigative Dermatology*. 2014; 134:2898–2907. Available at: <https://www.sciencedirect.com/science/article/pii/S0022202X1536557X>.DOI: <https://doi.org/10.1038/jid.2014.261>.

98. Naidoo K, Woods K, Pellefigues C, Cait A, O'Sullivan D, Gell K, Marshall AJ, *et al*. MR1-dependent immune surveillance of the skin contributes to pathogenesis and is a photobiological target of UV light therapy in a mouse model of atopic dermatitis. *Allergy: European Journal of Allergy and Clinical Immunology*. 2021; 76:3155–3170.DOI: 10.1111/all.14994.

99. Shapiro HM. *Practical Flow Cytometry*. Wiley; 2005. Available at:

<https://books.google.hu/books?id=JhSyimPKuJwC>.

100. Belkina AC, Ciccolella CO, Anno R, Halpert R, Spidlen J, Snyder-Cappione JE. Automated optimized parameters for T-distributed stochastic neighbor embedding improve visualization and analysis of large datasets. *Nature Communications*. 2019; 10:5415. Available at: <https://doi.org/10.1038/s41467-019-13055-y>.DOI: 10.1038/s41467-019-13055-y.

101. Van Gassen S, Callebaut B, Van Helden MJ, Lambrecht BN, Demeester P, Dhaene T, Saeys Y. FlowSOM: Using self-organizing maps for visualization and interpretation of cytometry data. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*. 2015; 87:636–645.DOI: 10.1002/cyto.a.22625.

102. Ligthart GJ, Corberand JX, Fournier C, Galanaud P, Hijmans W, Kennes B, Müller-Hermelink HK, *et al.* Admission criteria for immunogerontological studies in man: The senieur protocol. *Mechanisms of Ageing and Development*. 1984; 28:47–55.DOI: 10.1016/0047-6374(84)90152-0.

103. McInnes L, Healy J. UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. *ArXiv*. 2018; abs/1802.0. Available at: <https://api.semanticscholar.org/CorpusID:3641284>.

104. Fonseca S, Pereira V, Lau C, Teixeira M dos A, Bini-Antunes M, Lima M. Human Peripheral Blood Gamma Delta T Cells: Report on a Series of Healthy Caucasian Portuguese Adults and Comprehensive Review of the Literature. *Cells*. 2020; 9:729.DOI: 10.3390/cells9030729.

105. Michishita Y, Hirokawa M, Guo YM, Abe Y, Liu J, Ubukawa K, Fujishima N, *et al.* Age-associated alteration of $\gamma\delta$ T-cell repertoire and different profiles of activation-induced death of V δ 1 and V δ 2 T cells. *International Journal of Hematology*. 2011; 94:230–240.DOI: 10.1007/s12185-011-0907-7.

106. Argentati K, Re F, Donnini A, Tucci MG, Franceschi C, Bartozzi B, Bernardini G, *et al.*

Numerical and functional alterations of circulating $\gamma\delta$ T lymphocytes in aged people and centenarians. *Journal of Leukocyte Biology*. 2002; 72:65–71.DOI: 10.1189/jlb.72.1.65.

107. Caccamo N. Sex-specific phenotypical and functional differences in peripheral human V α 9/V β 2 T cells. *Journal of Leukocyte Biology*. 2006; 79:663–666.DOI: 10.1189/jlb.1105640.

108. Novak J, Dobrovolny J, Novakova L, Kozak T. The Decrease in Number and Change in Phenotype of Mucosal-Associated Invariant T cells in the Elderly and Differences in Men and Women of Reproductive Age. *Scandinavian Journal of Immunology*. 2014; 80:271–275.DOI: 10.1111/sji.12193.

109. Chen P, Deng W, Li D, Zeng T, Huang L, Wang Q, Wang J, *et al.* Circulating mucosal-associated invariant T Cells in a large cohort of chinese individuals from newborn to elderly. *Frontiers in Immunology*. 2019; 10:1–12.DOI: 10.3389/fimmu.2019.00260.

110. Molling JW, Kölgen W, Van Der Vliet HJJ, Boomsma MF, Kruizenga H, Smorenburg CH, Molenkamp BG, *et al.* Peripheral blood IFN- γ -secreting V α 24+V β 11 + NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *International Journal of Cancer*. 2005; 116:87–93.DOI: 10.1002/ijc.20998.

111. Wang Y, Sedimbi S, Löfbom L, Singh AK, Porcelli SA, Cardell SL. Unique invariant natural killer T cells promote intestinal polyps by suppressing TH1 immunity and promoting regulatory T cells. *Mucosal Immunology*. 2018; 11:131–143.DOI: 10.1038/mi.2017.34.

112. Jing Y, Gravenstein S, Rao Chaganty N, Chen N, Lysterly KH, Joyce S, Deng Y. Aging is associated with a rapid decline in frequency, alterations in subset composition, and enhanced Th2 response in CD1d-restricted NKT cells from human peripheral blood. *Experimental Gerontology*. 2007; 42:719–732.DOI: 10.1016/j.exger.2007.01.009.

113. Peralbo E, DelaRosa O, Gayoso I, Pita ML, Tarazona R, Solana R. Decreased frequency and proliferative response of invariant V α 24V β 11 natural killer T (iNKT) cells in healthy elderly. *Biogerontology*. 2006; 7:483–492.DOI: 10.1007/s10522-006-9063-5.

114. DelaRosa O, Tarazona R, Casado JG, Alonso C, Ostos B, Peña J, Solana R. V α 24+ NKT cells are decreased in elderly humans. *Experimental Gerontology*. 2002; 37:213–217.DOI: 10.1016/S0531-5565(01)00186-3.
115. Lee OJ, Cho YN, Kee SJ, Kim MJ, Jin HM, Lee SJ, Park KJ, *et al.* Circulating mucosal-associated invariant T cell levels and their cytokine levels in healthy adults. *Experimental Gerontology*. 2014; 49:47–54. Available at: <http://dx.doi.org/10.1016/j.exger.2013.11.003>.DOI: 10.1016/j.exger.2013.11.003.
116. Ahnstedt H, McCullough LD. The impact of sex and age on T cell immunity and ischemic stroke outcomes. *Cellular Immunology*. 2019; 345.DOI: 10.1016/j.cellimm.2019.103960.
117. Fereidouni M, Hosseini RF, Azad FJ, Schenkel J, Varasteh A, Mahmoudi M. Frequency of circulating iNKT cells among Iranian healthy adults. *Cytometry Part B - Clinical Cytometry*. 2010; 78:65–69.DOI: 10.1002/cyto.b.20489.
118. Montoya CJ, Pollard D, Martinson J, Kumari K, Wasserfall C, Mulder CB, Rugeles MT, *et al.* Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. *Immunology*. 2007; 122:1–14.DOI: 10.1111/j.1365-2567.2007.02647.x.
119. Schultze-Florey CR, Chukhno E, Goudeva L, Blasczyk R, Ganser A, Prinz I, Förster R, *et al.* Distribution of major lymphocyte subsets and memory T-cell subpopulations in healthy adults employing GLP-conforming multicolor flow cytometry. *Leukemia*. 2021; 35:3021–3025. Available at: <http://dx.doi.org/10.1038/s41375-021-01348-5>.DOI: 10.1038/s41375-021-01348-5.
120. Caccamo N, Dieli F, Wesch D, Jomaa H, Eber M. Sex-specific phenotypical and functional differences in peripheral human V 9/V 2 T cells. *Journal of Leukocyte Biology*. 2006; 79:663–666.DOI: 10.1189/jlb.1105640.
121. Kim EY, Lynch L, Brennan PJ, Cohen NR, Michael B, States U, States U, *et al.* The

- transcriptional programs of iNKT cells. 2019; 27:26–32.DOI: 10.1016/j.smim.2015.02.005.The.
122. Koay HF, Su S, Amann-Zalcenstein D, Daley SR, Comerford I, Miosge L, Whyte CE, *et al.* A divergent transcriptional landscape underpins the development and functional branching of MAIT cells. *Science Immunology*. 2019; 4:1–17.DOI: 10.1126/sciimmunol.aay6039.
123. Koay HF, Gherardin NA, Enders A, Loh L, Mackay LK, Almeida CF, Russ BE, *et al.* A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage. *Nature Immunology*. 2016; 17:1300–1311.DOI: 10.1038/ni.3565.
124. Lukasik Z, Elewaut D, Venken K. Mait cells come to the rescue in cancer immunotherapy? *Cancers*. 2020; 12:1–18.DOI: 10.3390/cancers12020413.
125. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V α 24 natural killer T cells. *Journal of Experimental Medicine*. 2002; 195:637–641.DOI: 10.1084/jem.20011908.
126. Lucas M, Gadola S, Meier U, Young NT, Harcourt G, Karadimitris A, Coumi N, *et al.* Frequency and Phenotype of Circulating V α 24/V β 11 Double-Positive Natural Killer T Cells during Hepatitis C Virus Infection. *Journal of Virology*. 2003; 77:2251–2257.DOI: 10.1128/jvi.77.3.2251-2257.2003.
127. Sandberg JK, Bhardwaj N, Nixon DF. Dominant effector memory characteristics, capacity for dynamic adaptive expansion, and sex bias in the innate V α 24 NKT cell compartment. *European Journal of Immunology*. 2003; 33:588–596.DOI: 10.1002/eji.200323707.
128. Schondelmaier S, Wesch D, Pechhold K, Kabelitz D. V γ gene usage in peripheral blood $\gamma\delta$ T cells. *Immunology Letters*. 1993; 38:121–126.DOI: 10.1016/0165-2478(93)90176-3.
129. Andreu-Ballester JC, García-Ballesteros C, Benet-Campos C, Amigó V, Almela-Quilis A, Mayans J, Ballester F. Values for $\alpha\beta$ and $\gamma\delta$ T-lymphocytes and CD4⁺, CD8⁺, and CD56⁺ subsets in healthy adult subjects: Assessment by age and gender. *Cytometry Part B - Clinical Cytometry*. 2012; 82 B:238–244.DOI: 10.1002/cyto.b.21020.

130. Boeck CL, Amberger DC, Doraneh-Gard F, Sutanto W, Guenther T, Schmohl J, Schuster F, *et al.* Significance of Frequencies, Compositions, and/or Antileukemic Activity of (DC-stimulated) Invariant NKT, NK and CIK Cells on the Outcome of Patients with AML, ALL and CLL. *Journal of Immunotherapy*. 2017; 40:224–248.DOI: 10.1097/CJI.0000000000000171.
131. Kjer-Nielsen L, Patel O, Corbett AJ, Le Nours J, Meehan B, Liu L, Bhati M, *et al.* MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature*. 2012; 491:717–723. Available at: <https://doi.org/10.1038/nature11605>.DOI: 10.1038/nature11605.
132. Patrycja O, Klaudia S, Joanna E, Małgorzata U, Agata W-P, Wioletta B-R, Dariusz Ś, *et al.* Staphylococcus aureus from Atopic Dermatitis Patients: Its Genetic Structure and Susceptibility to Phototreatment. *Microbiology Spectrum*. 2023; 11:e04598-22. Available at: <https://doi.org/10.1128/spectrum.04598-22>.DOI: 10.1128/spectrum.04598-22.
133. Le Bourhis L, Martin E, Péguillet I, Guihot A, Froux N, Coré M, Lévy E, *et al.* Antimicrobial activity of mucosal-associated invariant T cells. *Nature Immunology*. 2010; 11:701–708. Available at: <https://doi.org/10.1038/ni.1890>.DOI: 10.1038/ni.1890.
134. Sandberg JK, Norrby-Teglund A, Leeansyah E. Bacterial deception of MAIT cells in a cloud of superantigen and cytokines. *PLoS biology*. 2017; 15:e2003167.DOI: 10.1371/journal.pbio.2003167.
135. Orfali RL, Yoshikawa FSY, Oliveira LM da S, Pereira NZ, de Lima JF, Ramos YÁL, Duarte AJ da S, *et al.* Staphylococcal enterotoxins modulate the effector CD4⁺ T cell response by reshaping the gene expression profile in adults with atopic dermatitis. *Scientific Reports*. 2019; 9:13082. Available at: <https://doi.org/10.1038/s41598-019-49421-5>.DOI: 10.1038/s41598-019-49421-5.
136. Malavasi F, Deaglio S, Funaro A, Ferrero E, Horenstein AL, Ortolan E, Vaisitti T, *et al.* Evolution and Function of the ADP Ribosyl Cyclase/CD38 Gene Family in Physiology and Pathology. *Physiological Reviews*. 2008; 88:841–886. Available at:

<https://doi.org/10.1152/physrev.00035.2007>.DOI: 10.1152/physrev.00035.2007.

137. Seneviratne SL, Black AP, Jones L, Bailey AS, Ogg GS. The role of skin-homing T cells in extrinsic atopic dermatitis. *QJM: An International Journal of Medicine*. 2007; 100:19–27.

Available at: <https://doi.org/10.1093/qjmed/hcl132>.DOI: 10.1093/qjmed/hcl132.

138. Nograles KE, Zaba LC, Shemer A, Fuentes-Duculan J, Cardinale I, Kikuchi T, Ramon M, *et al*. IL-22-producing ‘T22’ T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. *The Journal of allergy and clinical immunology*. 2009; 123:1244–52.e2.DOI: 10.1016/j.jaci.2009.03.041.

139. Pan Y, Du D, Wang L, Wang X, He G, Jiang X. The Role of T Helper 22 Cells in Dermatological Disorders. *Frontiers in immunology*. 2022; 13:911546.DOI: 10.3389/fimmu.2022.911546.

140. Gittler JK, Shemer A, Suárez-Fariñas M, Fuentes-Duculan J, Gulewicz KJ, Wang CQF, Mitsui H, *et al*. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. *The Journal of allergy and clinical immunology*. 2012; 130:1344–1354.DOI: 10.1016/j.jaci.2012.07.012.

141. Zhang Q, Wang Q, Zhang L-X. Granzyme B: A novel therapeutic target for treatment of atopic dermatitis. *Indian journal of dermatology, venereology and leprology*. 2023; 89:166–169.DOI: 10.25259/IJDVL_260_2022.

142. Turner CT, Zeglinski MR, Richardson KC, Santacruz S, Hiroyasu S, Wang C, Zhao H, *et al*. Granzyme B Contributes to Barrier Dysfunction in Oxazolone-Induced Skin Inflammation through E-Cadherin and FLG Cleavage. *Journal of Investigative Dermatology*. 2021; 141:36–47. Available at: <https://www.sciencedirect.com/science/article/pii/S0022202X20316602>.DOI: <https://doi.org/10.1016/j.jid.2020.05.095>.

143. Lopatnikova JA, Alshevskaya AA, Krugleeva OL, Nepomnyschih VM, Gladkikh VS, Lukinov VL, Karaulov A V, *et al*. Expression of TNF α Receptors on Immunocompetent Cells

Is Increased in Atopic Dermatitis. *International archives of allergy and immunology*. 2017; 174:151–160.DOI: 10.1159/000481135.

144. Dusseaux M, Martin E, Serriari N, Péguillet I, Premel V, Louis D, Milder M, *et al*. Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17–secreting T cells. *Blood*. 2011; 117:1250–1259. Available at: <https://www.sciencedirect.com/science/article/pii/S0006497120586011>.DOI: <https://doi.org/10.1182/blood-2010-08-303339>.

145. Held K, Bhonsle-Deeng L, Siewert K, Sato W, Beltrán E, Schmidt S, Rühl G, *et al*. $\alpha\beta$ T-cell receptors from multiple sclerosis brain lesions show MAIT cell–related features. *Neurology - Neuroimmunology Neuroinflammation*. 2015; 2:e107. Available at: <http://nn.neurology.org/content/2/4/e107.abstract>.DOI: 10.1212/NXI.0000000000000107.

146. Ferrer-Font L, Mayer JU, Old S, Hermans IF, Irish J, Price KM. High-Dimensional Data Analysis Algorithms Yield Comparable Results for Mass Cytometry and Spectral Flow Cytometry Data. *Cytometry. Part A: the journal of the International Society for Analytical Cytology*. 2020; 97:824–831.DOI: 10.1002/cyto.a.24016.

147. Rahim A, Meskas J, Drissler S, Yue A, Lorenc A, Laing A, Saran N, *et al*. High throughput automated analysis of big flow cytometry data. *Methods*. 2018; 134–135:164–176. Available at: <https://www.sciencedirect.com/science/article/pii/S1046202317301640>.DOI: <https://doi.org/10.1016/j.ymeth.2017.12.015>.

148. Liu P, Liu S, Fang Y, Xue X, Zou J, Tseng G, Konnikova L. Recent Advances in Computer-Assisted Algorithms for Cell Subtype Identification of Cytometry Data . *Frontiers in Cell and Developmental Biology* . 2020; 8. Available at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.00234>.

149. Baumgaertner P, Sankar M, Herrera F, Benedetti F, Barras D, Thierry A-C, Dangaj D, *et al*. Unsupervised Analysis of Flow Cytometry Data in a Clinical Setting Captures Cell Diversity

and Allows Population Discovery . *Frontiers in Immunology* . 2021; 12. Available at: <https://www.frontiersin.org/articles/10.3389/fimmu.2021.633910>.

150. Fergusson JR, Fleming VM, Klenerman P. CD161-expressing human T cells. *Frontiers in immunology*. 2011; 2:36.DOI: 10.3389/fimmu.2011.00036.

151. Roesner LM, Floess S, Witte T, Olek S, Huehn J, Werfel T. Foxp3(+) regulatory T cells are expanded in severe atopic dermatitis patients. *Allergy*. 2015; 70:1656–1660.DOI: 10.1111/all.12712.

152. Samochocki Z, Alifier M, Boder P, Jeziorkowska R, Rosiak E, Jurkiewicz B, Glińska O, *et al.* T-regulatory cells in severe atopic dermatitis: alterations related to cytokines and other lymphocyte subpopulations. *Archives of dermatological research*. 2012; 304:795–801.DOI: 10.1007/s00403-012-1290-9.

153. Cassius C, Branchtein M, Battistella M, Amode R, Lepelletier C, Jachiet M, de Masson A, *et al.* Persistent deficiency of mucosal-associated invariant T cells during dermatomyositis. *Rheumatology*. 2020; 59:2282–2286. Available at: <https://doi.org/10.1093/rheumatology/kez564>.DOI: 10.1093/rheumatology/kez564.

154. Hijnen DJ. Shifting paradigms in the immunology of atopic dermatitis. *The Journal of allergy and clinical immunology*. 2020; 145:1360–1362.DOI: 10.1016/j.jaci.2020.02.030.

155. Konecny AJ, Huang Y, Setty M, Prlic M. Signals that control MAIT cell function in healthy and inflamed human tissues. *Immunological reviews*. 2024.DOI: 10.1111/imr.13325.

156. Fergusson JR, Ussher JE, Kurioka A, Klenerman P, Walker LJ. High MDR-1 expression by MAIT cells confers resistance to cytotoxic but not immunosuppressive MDR-1 substrates. *Clinical and experimental immunology*. 2018; 194:180–191.DOI: 10.1111/cei.13165.

157. Fergusson JR, Smith KE, Fleming VM, Rajoriya N, Newell EW, Simmons R, Marchi E, *et al.* CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. *Cell reports*. 2014; 9:1075–1088.DOI: 10.1016/j.celrep.2014.09.045.

158. Singh P, Gaspar K, Szegedi A, Sajtos L, Barath S, Hevessy Z. Investigating V α 7.2+/CD161⁺ T Cell and MAIT Cell Profiles Using Flow Cytometry in Healthy Subjects and Subjects with Atopic Dermatitis. *International Journal of Molecular Sciences*. 2024; 25.DOI: 10.3390/ijms25063486.
159. Martínez-Martín N, Fernández-Arenas E, Cemerski S, Delgado P, Turner M, Heuser J, Irvine DJ, *et al.* T cell receptor internalization from the immunological synapse is mediated by TC21 and RhoG GTPase-dependent phagocytosis. *Immunity*. 2011; 35:208–222.DOI: 10.1016/j.immuni.2011.06.003.
160. Yu H, Yang A, Liu L, Mak JYW, Fairlie DP, Cowley S. CXCL16 Stimulates Antigen-Induced MAIT Cell Accumulation but Trafficking During Lung Infection Is CXCR6-Independent. *Frontiers in immunology*. 2020; 11:1773.DOI: 10.3389/fimmu.2020.01773.
161. Paquin-Proulx D, Greenspun BC, Costa EASS, Segurado AC, Kallas EG, Nixon DF, Leal FE. MAIT cells are reduced in frequency and functionally impaired in human T lymphotropic virus type 1 infection: Potential clinical implications. *PLoS ONE*. 2017; 12:1–17.DOI: 10.1371/journal.pone.0175345.
162. Li S, Simoni Y, Becht E, Loh CY, Li N, Lachance D, Koo S-L, *et al.* Human Tumor-Infiltrating MAIT Cells Display Hallmarks of Bacterial Antigen Recognition in Colorectal Cancer. *Cell reports. Medicine*. 2020; 1:100039.DOI: 10.1016/j.xcrm.2020.100039.
163. Pincikova T, Paquin-Proulx D, Sandberg JK, Flodström-Tullberg M, Hjelte L. Vitamin D treatment modulates immune activation in cystic fibrosis. *Clinical and experimental immunology*. 2017; 189:359–371.DOI: 10.1111/cei.12984.
164. Slichter CK, McDavid A, Miller HW, Finak G, Seymour BJ, McNevin JP, Diaz G, *et al.* Distinct activation thresholds of human conventional and innate-like memory T cells. *JCI insight*. 2016; 1.DOI: 10.1172/jci.insight.86292.
165. Leng T, Akther HD, Hackstein C-P, Powell K, King T, Friedrich M, Christoforidou Z, *et*

- al.* TCR and Inflammatory Signals Tune Human MAIT Cells to Exert Specific Tissue Repair and Effector Functions. *Cell Reports*. 2019; 28:3077-3091.e5. Available at: <https://www.sciencedirect.com/science/article/pii/S2211124719310988>.DOI: <https://doi.org/10.1016/j.celrep.2019.08.050>.
166. Sharma M, Zhang S, Niu L, Lewinsohn DM, Zhang X, Huang S. Mucosal-Associated Invariant T Cells Develop an Innate-Like Transcriptomic Program in Anti-mycobacterial Responses. *Frontiers in immunology*. 2020; 11:1136.DOI: 10.3389/fimmu.2020.01136.
167. Corbett AJ, Eckle SBGG, Birkinshaw RW, Liu L, Patel O, Mahony J, Chen Z, *et al.* T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature*. 2014; 509:361–365. Available at: <https://doi.org/10.1038/nature13160>.DOI: 10.1038/nature13160.
168. Hinks TSC, Marchi E, Jabeen M, Olshansky M, Kurioka A, Pediongco TJ, Meehan BS, *et al.* Activation and In Vivo Evolution of the MAIT Cell Transcriptome in Mice and Humans Reveals Tissue Repair Functionality. *Cell Reports*. 2019; 28:3249-3262.e5. Available at: <https://www.sciencedirect.com/science/article/pii/S2211124719309362>.DOI: <https://doi.org/10.1016/j.celrep.2019.07.039>.
169. Vazquez J, Chavarria M, Chasman DA, Welch Schwartz R, Tyler CT, Lopez G, Fisher RC, *et al.* Multiomic analysis reveals decidual-specific transcriptional programming of MAIT cells. *American journal of reproductive immunology (New York, N.Y. : 1989)*. 2021; 86:e13495.DOI: 10.1111/aji.13495.
170. Ito E, Inuki S, Izumi Y, Takahashi M, Dambayashi Y, Ciacchi L, Awad W, *et al.* Sulfated bile acid is a host-derived ligand for MAIT cells. *Science Immunology*. 2024; 9:eade6924. Available at: <https://doi.org/10.1126/sciimmunol.ade6924>.DOI: 10.1126/sciimmunol.ade6924.

8. KEYWORDS

Unconventional T cells; iNKT cells; $\gamma\delta$ T cells; MAIT cells; V α 7.2+/CD161- T cells; Atopic dermatitis; Immunosenescence; Reference range; Cyclosporin A; Vitamin D; t-SNE; FlowSOM; Multidimensional data analysis; Age-dependent; Frequency; Gender dependency; Flow cytometry; Dimensionality reduction; Unsupervised clustering; Stimulation; Immunomodulation

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11. LIST OF PUBLICATIONS RELATED TO DISSERTATION



Registry number:
Subject:

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PhD Publication List

Candidate: Parvind Singh

Doctoral School: Kálmán Laki Doctoral School

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 V α 7.2+/CD161-T Cell Response and Effects of Immunomodulatory Drugs.
Int. J. Mol. Sci. 25 (11), 1-16, 2024.
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IF: 5.6 (2022)
2. **Singh, P.**, Gáspár, K., Szegedi, A., Sajtos, L., Baráth, S., Hevessy, Z.: Investigating V α 7.2+/CD161- T Cell and MAIT Cell Profiles Using Flow Cytometry in Healthy Subjects and Subjects with Atopic Dermatitis.
Int. J. Mol. Sci. 25 (6), 1-15, 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.
GeroScience. 44 (4), 2047-2060, 2022.
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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.
J. Leukoc. Biol. 112 (5), 1155-1165, 2022.
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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.
Front. Immunol. 15, 1-10, 2024.
DOI: <http://dx.doi.org/10.3389/fimmu.2024.1329236>
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6. Baráth, S., **Singh, P.**, Hevessy, Z., Ujfalusi, A., Mezei, Z. A., Balogh, M., Széles, M., Kappelmayer, J.: Enhancing HLA-B27 antigen detection: Leveraging machine learning algorithms for flow cytometric analysis.
Cytom. Part B-Clin. Cytom. [Epub ahead of print], 2024.
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