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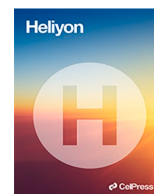

Research article

A comprehensive analysis of the immune system in healthy Vietnamese people

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ABSTRACT

Lifestyle, diet, socioeconomic status and genetics all contribute to heterogeneity in immune responses. Vietnam is plagued with a variety of health problems, but there are no available data on immune system values in the Vietnamese population. This study aimed to establish reference intervals for immune cell parameters specific to the healthy Vietnamese population by utilizing multi-color flow cytometry (MCFC). We provide a comprehensive analysis of total leukocyte count, quantitative and qualitative shifts within lymphocyte subsets, serum and cytokine and chemokine levels and functional attributes of key immune cells including B cells, T cells, natural killer (NK) cells and their respective subpopulations. By establishing these reference values for the Vietnamese population, these data contribute significantly to our understanding of the human immune system variations across diverse populations. These data will be of substantial comparative value and be instrumental in developing personalized medical approaches and optimizing diagnostic strategies for individuals based on their unique immune profiles.

1. Introduction

The human immune system serves as a sophisticated defense mechanism, playing a pivotal role in safeguarding the body against both infectious diseases as well as cancer. Innate and adaptive immunity provide a coordinated repetoire of diverse cells and molecules. The intricate interplay between these compartments is essential for effective immune function. Key players within the immune system are myeloid and lymphoid cells, notably B and T cells, which act as central mediators of immune responses. Both these linages are further categorized into distinct subsets, as identified by specific cluster of differentiation (CD) markers. The extensive cellular diversity within these subsets encodes the remarkable complexity of the immune system [1].

Functioning of the immune system is influenced by a multitude of factors, both intrinsic and extrinsic, ranging from physiological sex, age, nutritional status, stress, chemical exposure and genetic constitution [2–4]. Any significant alterations in the immune system caused by these factors serve as indicators of potential dysfunctions within the immune system. While the immune system is highly

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individualistic, comprised of various cell phenotypes and components, it is possible to measure specific markers to assess its performance [5,6]. However, interpreting these results necessitates the development of reference values that reflect diversity of the human population [7–10].

In Europe, Melzer et al. (2015) performed a comprehensive investigation using 10-color flow cytometry phenotyping of 608 German adults. They set accurate reference intervals for different types of adult leukocyte subpopulations [7]. Their work illuminates not only the general characteristics of the German population, but also significant trends related to gender and age. Furthermore, Claus et al. (2016) proposed a practical approach for detecting immune function alterations [11]. This method provides detailed quantitative and qualitative data encompassing total leukocyte counts, functional properties of T cells, NK cells and monocytes, changes in lymphocyte subpopulation composition and serum cytokine concentrations.

Surprisingly, establishment of reference intervals for immune cell parameters specific to the Vietnamese population has remained uncharted territory. This study aims to fill this gap by presenting a comprehensive dataset derived from healthy Vietnamese adults. It encompasses data on total leukocyte counts, quantitative and qualitative shifts in lymphocyte subsets, serum cytokine and chemokine levels and the functional attributes of T, NK, B cells, monocytes and their respective subpopulations. These data introduce the first preliminary set of reference ranges tailored to the Vietnamese population, ultimately enabling the identification of outliers in immune function of individuals.

2. Materials and methods

1. Subjects

Forty healthy individuals, including 25 female (15 being ≤45 and 10 being >45) and 15 male (13 being ≤45 and 2 being >45), ranging in age from 22 to 67 were recruited between September 25, 2021, and June 8, 2022. They all had middle to high income levels and completed at least a high school education. All participants voluntarily consented to participate and were not employees of our medical institution. The study was conducted with approval of the medical ethics committee at Vinmec International Hospital and adhered to the principles outlined in the Helsinki Declaration.

2. Plasma Storage and Isolation of Peripheral Mononuclear Cells

Peripheral venous blood was collected in heparinized and EDTA vacutainer tubes from Becton-Dickinson (BD) and Company, Germany. The buffy coat was collected, washed twice with PBS at 420×*g* for 5 min and 200×*g* for 10 min, respectively. Peripheral blood mononuclear cells (PBMCs) were were obtained via density-gradient centrifugation using Ficoll-Paque (density of 1.077 ± 0.001 g/mL, GE Healthcare, USA) at 760×*g* for 20 min. This was followed by a deceleration without braking at room temperature.

To collect plasma, peripheral blood from heparinized tubes was centrifuged for 5 min at 1710×*g* at 20 °C. The resulting plasma supernatant was harvested and stored at −80 °C for cytokine level assessments.

3. Surface Staining

Whole blood samples were collected in EDTA blood collection tubes at room temperature (20 °C–25 °C). The EDTA-*anti*-coagulated blood was stained within 24 h and analyzed within 1 h after staining.

All reagents are listed in Table Appendix-1. For tube No.01, reagents were added to 50 μl of whole blood, which was then stained for 15 min at room temperature in the dark. For tube No.02-06, 100 μl of whole blood and washed blood cells (in the case of tube No.03) were stained with marker antigens/reagents as detailed in Table Appendix-1 for 30 min at room temperature in the dark.

To lyse red blood cells, 450 aμl of BD FACS lysing solution was added to tube No.01 and 2 mL was used for the remaining tubes. Erythrocytes were eliminated by lysing in the dark at room temperature for 15 min.

Tube No.01 was analyzed via flow cytometery within 1 h after lysing. The unstained tube and tube No.02 were subjected to centrifugation at 500×*g* for 5 min. Pellets were washed twice with 2 mL of PBS and centrifuged at 500×*g* for 5 min. During each washing step, the supernatant was carefully aspirated to avoid cell loss. Following the final wash, pellets were resuspended in 300 μl of PBS 1 and analyzed by flow cytometry within 1 h.

4. Flow Cytometry Analysis

The absolute count and percentage of T cell subtypes, B cells and NK cells (tube No.01) were analyzed using the IVD BD FACSuite Clinical application.

The absolute number of lymphocytes was determined using the BD Multitest™ 6-Color TBNK with True Count tubes Kit (Catalog No. 337166). Lymphocytes were gated in all events on side scatter (SSC-A) and CD45. Based on the number of events of labeled lymphocytes and the volume of sample, we calculated the absolute number of lymphocytes as follows:

$$\text{Absolute Number of Cells} = \frac{\text{Number of events in cell population}}{\text{Number of absolute events''}} \text{x} \frac{\text{Number of beads/test}}{\text{test volume}}$$

For analyzing B and NK cells subpopulations, gating conditions were set at 300 s or 5000 events for B cells, B cells subpopulations, NK cells and NK cell subpopulations. For all other cells, gates were set at 300 s or 300.000 events.

The BD FACSuite application was used to analyze the dataset for lymphocyte and monocyte subpopulations (tube No.02), B cell subpopulations (tube No.03), T cell subpopulations (tube No.04), NK-T activation and maturation population (tube No.05) and activating NK cell receptors (tube No.06).

Cultured T cells were stained with Hu CD3 BV510 UCHT1 and Hu CD4 APC-H7 RPA-T4 at room temperature for 20 min in the dark. Subsequently, stained cells were washed with 2 mL PBS at 500×*g* for 5 min. The resulting pellet was suspended in 300 μl PBS and analyzed using the BD FACSuite application to assess CSFE dilution in the $CD3^+$ and $CD3^+CD4^+$ populations.

5. T Cell Proliferation

To monitor T cell proliferation, PBMCs (or cultured T cells) were loaded with 0.5 μM of green, fluorescent carboxyfluorescein succinimidyl ester (CFSE) from Thermo Scientific, Eugene, OR, USA. Cells were then washed twice to remove any unbound CFSE and resuspended in RPMI containing 10 % FCS and 1 % penicillin/streptomycin (Fresh medium). Labeled cells were plated at a density of 1×10^6 cells per well in 24-well flat-bottom plates, which were either coated with mAb anti-CD3 and mAb anti-CD28 at 0.5 μM and 2.5 μM (BD Biosciences), or no additional stimulation in RPMI that served as the control. Approximately half of the culture medium was replaced with fresh medium every three days.

Cells were cultured for six days, and CSFE dilution in $CD3^+$ and $CD3^+CD4^+$ events were analyzed to assess T cell proliferation using the FlowJo Proliferation analysis platform.

6. Cytotoxicity Analysis

The human MHC-I deficient erythroleukemic cell line, K562, was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin. Cells were maintained at 37 °C in a humidified 5 % CO2 incubator and used as target cells in cytotoxicity assays once they reached the mid-log phase of congruence. These K562 cells were employed as target cells (T) and were labeled by incubating with Calcein-AM (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) for 30 min at 37 °C. Labeled cells were then washed with PBS and seeded into a 96-well flat-bottomed plate at a density of 1×10^4 cells per well in RPMI containing 10 % FBS. PBMCs were then added as effector cells (E) to the target cells at four different E:T ratios: 40:1, 20:1, 10:1, and 5:1. To ensure that all cells settled to the bottom of the plate, plates were gently centrifuged for 2 min at 50×*g*, after which initial fluorescence intensity (FI) was measuring using a Terascan VPC2 counter. The alkaline reagent DCN90 was subsequently added to control wells to facilitate maximum dye-release in the control cells which was fllowed by incubation for 2 h. After incubation, plates were once again centrifuged for 2 min at 50×*g*. Following this, 80 μL of supernatant was removed followed by addition of 80 μL of fresh RPMI to each well. The plate underwent another 2 min centrifugation at 50×*g* and a second measurement was taken using the Terascan VPC2 counter.

To calculate cytotoxicity (%), the following formula was used: experimental value is the combined fluorescence of E + T, the spontaneous value is the FI of T + medium and the maximum (positive control) value is the FI of T + DCN 90.

$$\% \text{ cytotoxicity} = \frac{\textbf{experiment} - \textbf{negative control}}{\textbf{positive control} - \textbf{negative control}} \times 100$$

7. Detection of Growth Factors and Interleukins (Cytokines)

To quantify interleukins and growth factors (shown in Table Appendix-2), frozen plasma stored at −80 °C was thawed and subjected to the manufacturer's instructions using the Human ProcartaPlex Mix&Match 26-plex kit and ProcartaPlex Human Basic kit from ThermoFisher Scientific, US, as follows: Serial dilutions were made to prepare a standard curve by making 4-fold diluted concentrations of each interleukin and growth factor. Plasma samples, standards and backgrounds were mixed with capture beads in the corresponding well in a 96-well plate. Plates were sealed and incubated with gentle shaking at room temperature for 2 h. Cells then underwent two washes with wash buffer and were subsequently incubated with 1x detection antibody mix for 30 min at RT with shaking. After another two washes with wash buffer, Streptavidin-PE was added and incubated the cells with shaking for 30 min at room temperature. Plates were washed two additional times with wash buffoer before adding reading buffer and subsequently shaking for 5 min at room temperature to detect luminescent signals using a LuminexTM200TM (Invitrogen, US). Data analysis was carried out using the ProcartaPlex Analysis App (ThermoFisher Scientific, US) and results were calculated from two technical replicates.

8. Data Analysis

Descriptive statistics for quantitative variables were summarized using means, standard deviations and 95 % confidence intervals. The Shapiro-Wilk test implemented in RStudio was used to assess normality of distribution for every immunological biomarker. The results indicated a statistically significant departure from normality for all biomarkers, with p-values consistently below 0.001.

2.1. Age and gender stratification

The dataset included the categorical variables of gender (male/female) and age (less than 45 and greater than or equal to 45 years). Selection of 45 years as the age threshold for distinguishing younger and older individuals aligns with the established frameworks for

classifying middle age [[12,13]]. This classification provides a reasonable justification for the chosen cutoff point, allowing for a systematic evaluation of potential disparities between younger and older individuals.

2.2. Statistical tests

Given the heterogeneity of varianes of the biomarkers, the Wilcoxon rank-sum test was employed as the appropriate non-parametric test to assess statistically significant differences between groups. For each biomarker, pairwise comparisons were conducted between the two age groups and between genders. A p-value less than 0.05 was considered statistically significant, indicating a demonstrable difference between the groups.

2.3. Software and significance level

All statistical analyses were conducted using RStudio software. A significance level of $\alpha = 0.05$ was adopted throughout the study. This approach ensures a rigorous statistical framework by accounting for the non-normal distribution of the biomarkers and employing appropriate non-parametric tests to accurately identify statistically significant variation.

3. Results

1. Absolute Lymphocyte Population in Peripheral Blood

The absolute number of peripheral lymphocyte subsets was determined in healthy Vietnamese subjects These data revealed significant expected variability in both the absolute number and proportion of peripheral lymphocyte subsets, with coefficients of variation ranging from 12 % (percentage $CD3^+$ T cells) to 80 % (absolute CD56+NK cells) (see Table 1).

Following stringent gating criteria, we analyzed the proportional composition of distinct subsets within these cells. Subsequent comparative assessments were conducted by age and gender, yielding the following results (Fig. 1).

2. Lymphocyte Subsets

We devised six panels comprising 8 to 10 markers each (as detailed in Table Appendix-1) to evaluate three distinct leukocyte groups: B cells, T cells and NK cells. We also measured their activation status as well as proliferation and maturation markers.

3.1. B cells

Total B cells were isolated and their subpopulations were evaluated. The most notable variation as assessed by the coefficient of variation was observed in the immature B cell (125 %) and CD21low (112 %) subpopulations (Table 2).

3.1.1. B cell subpopulations as assessed by age and gender

Significant disparities were detected in the proportions of naive (53.87 ± 13.00 vs 36.53 ± 12.81; $p < 0.001$), memory (29.15 ± 13.89 vs 7.26 ± 10.64; $p < 0.001$) and CD21low (10.92 ± 17.69 vs 52.16 ± 19.15; $p < 0.001$) B cells, as depicted in Fig. 2A. B-cell subsets did not exhibit gender-based variations within our cohort (Fig. 2B).

Analysis of B cell subpopulations revealed significant variations associated with age, but not gender. Specifically, subjects aged ≤ 45 years demonstrated distinct B cell profiles compared to those >45 years old (panel A, circled data). Interestingly, no statistically significant differences were observed between male and female individuals in terms of B cell subpopulations (panel B).

Table 1

Lymphocyte Populations in Peripheral Blood of Healthy Vietnamese Subjects.

This table presents the total counts of various lymphocyte and monocyte populations measured in the peripheral blood of healthy Vietnamese subjects.

Populations/Subpopulations		Mean (±SD)	Min - Max	95 % CI
Lymphocyte	Abs (n/mm^3)	2615.20 (±1008.31)	1228–7386	2293 - 2938
T Cells (CD3$^+$)	Abs (n/mm^3)	1710.33 (±541.26)	896–3809	1537 - 1883
	%	66.71 (±8.48)	46.45–80.01	64 - 69
Helper T Cells (CD4$^+$)	Abs (n/mm^3)	920.90 (±319.45)	433–2352	819 - 1023
	%	35.92 (±7.25)	21.53–53.62	34 - 38
Cytotoxic T Cells (CD8$^+$)	Abs (n/mm^3)	681.20 (±279.30)	174–1329	592 - 771
	%	26.35 (±7.62)	12.66–41.86	24 - 29
B Cells (CD19$^+$)	Abs (n/mm^3)	343.00 (±170.88)	140–1044	288 - 398
	%	13.04 (±3.45)	8.08–27.58	12 - 14
NK Cells (CD56$^+$)	Abs (n/mm^3)	539.70 (±430.13)	135–2477	402 - 677
	%	19.44 (±8.44)	5.09–38.12	17 - 22
CD4/CD8 ratio		1.54 (±0.74)	0.66–4.04	1.3 - 1.8

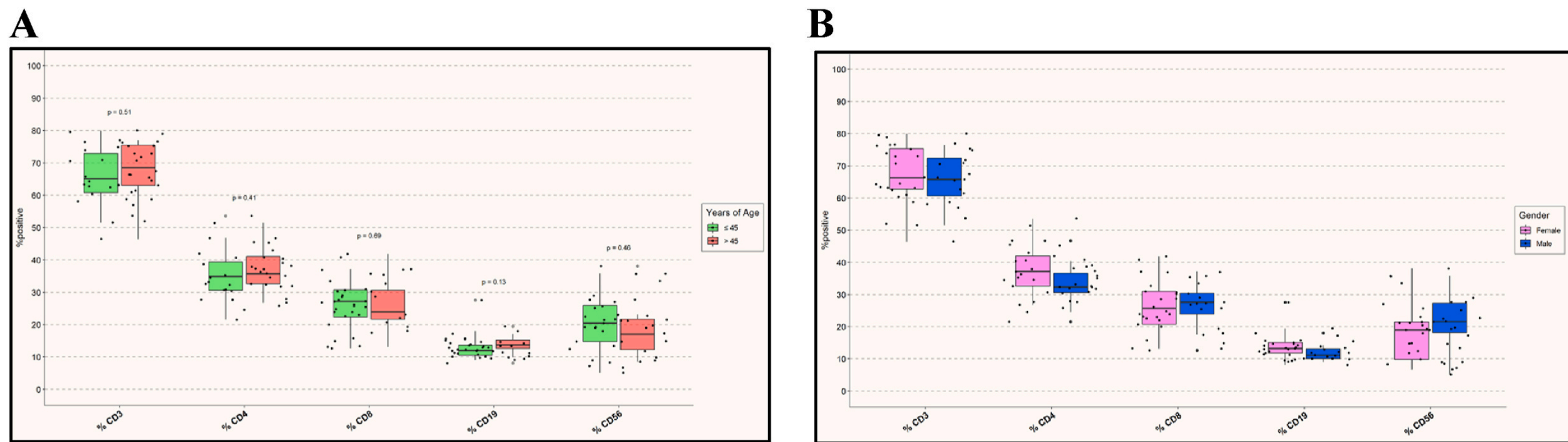


Fig. 1. No Age- or Gender-Related Differences in Major T Cell Populations.There were no statistically significant differences associated with either age or gender. Within the defined $CD3^{+}$ lymphocyte gate, no discernible variations were observed in the relative proportions of $CD4^{+}$ helper T cells, $CD8^{+}$ cytotoxic T cells or CD45RA + naive T cells between individuals aged ≤45 and > 45 years (panel A). Similarly, no statistically significant differences were detected in these major T cell subpopulations between male and female subjects (panel B).

Table 2
Gated B Cell Population and Subpopulations in Peripheral Blood of Healthy Vietnamese Subjects.
This table presents the relative abundance (percentages) of the gated B cell population and their constituent subpopulations within the peripheral blood of healthy Vietnamese subjects.

Populations/Subpopulations	Mean (±SD)%	Min – Max%	95 % CI
B cells	7.68 (±2.52)	3.44–15.74	6.9 - 8.5
CD21lowCD38low	3.64 (±2.53)	0.41–11.73	2.8 - 4.4
Transitional	1.96 (±2.20)	0.2–13.09	1.3 - 2.7
Plasmablasts	0.62 (±0.56)	0.04–3.19	0.45 - 0.80
Plasma cells	17.24 (±14.59)	0–66.67	13 - 22
Class-switched	53.84 (±15.42)	15.67–81.81	49 - 59
Non-switched	21.41 (±11.26)	2.48–51.05	18 - 25
B naive	48.66 (±15.10)	17.26–75.66	44 - 53
B memory	22.59 (±16.39)	1.01–48.51	17 - 28
B immature	5.45 (±6.82)*	1.78–44.35	3.3 - 7.6
B CD21 low	23.30 (±26.20)**	2.1–74.36	15 - 32

Coefficient of variation: * 125 %; **112 %.

3.2. T and NK cells

Subcategories of helper T cells (Th) and cytotoxic T cells (Tc) are shown in Figure Appendix-3.

3.2.1. T and NK cell subpopulations

Our investigation aimed to assess the presence of various T cell subpopulations within the peripheral blood of healthy Vietnamese individuals. This included Treg cells, double negative ($CD4^-CD8^-$) T cells, CD4 $CD28^-$ T cells, $CD4^+$ naive T cells, $CD4^+$ effector T cells, and $CD4^+$ memory T cells, along with their $CD8^+$ counterparts. Additionally, we evaluated the presence of natural killer (NK) cell subsets distinguished by CD62L expression (intermediate state of NK maturation) and CD197 expression (associated with lymph node homing). The data presented in Table 3 represent characterization of these cell types in a healthy Vietnamese population within the context of this study. While the sample size is currently limited, it offers a preliminary picture of their distribution.

3.2.2. T cell subpopulations in age and gender comparison

There was a marked distinction between individuals aged ≤45 and those over 45 years (as depicted in Fig. 3, A and C). Notably, within the older population (>45 years old), there was a pronounced reduction in naive $CD4^+$ T cells (CD45RA + CD62L+ and CD45RA + CD197+), coupled with a substantial increase in memory $CD4^+$ T cells (CD45RA- CD62L+ and CD45RA-CD197+) as compared to the younger (≤45 years old) cohort (Fig. 3A). This trend was mirrored in the CD8 T cell subpopulations within the older group (>45 years old), with reductions in naive CD8 T cells (CD45RA + CD62L+ and CD45RA + CD197+) coupled with an increase in one subset of memory cells (CD8, CD45RA-CD197+). Interestingly, the older group also exhibited an increase in other subsets, namely, age-related TCD8 cells (CD8, $CD28^-$), effector memory T cells (CD8, $CD45^+$CD62L-), and central memory T cells (CD8, $CD45^+$CD197-) (Fig. 3C). There were no significant differences in any subpopulation based on gender (as evident in Fig. 3, B and D).

Analysis of T cell subpopulations revealed age-dependent alterations, with significant changes observed in those subjects greater than 45 years of age (circled data in panels A and C). Notably, no statistically significant differences were detected between male and female subjects (panels B and D).

3.2.3. NK cell subpopulations as assessed by age and gender

Analysis of Table 4 revealed no statistically significant differences in the NK cell subpopulations (NK, CD62L+ and NK, CD197+) across age groups (≤45 and > 45 years) or gender (male and female) within this specific sample. However, to draw more definitive conclusions about potential age- and gender-related variations in NK cell dynamics, further investigations with larger sample sizes and a broader analysis of the immune cell landscape are warranted.

3.2.4. T and NK cell activation and maturation

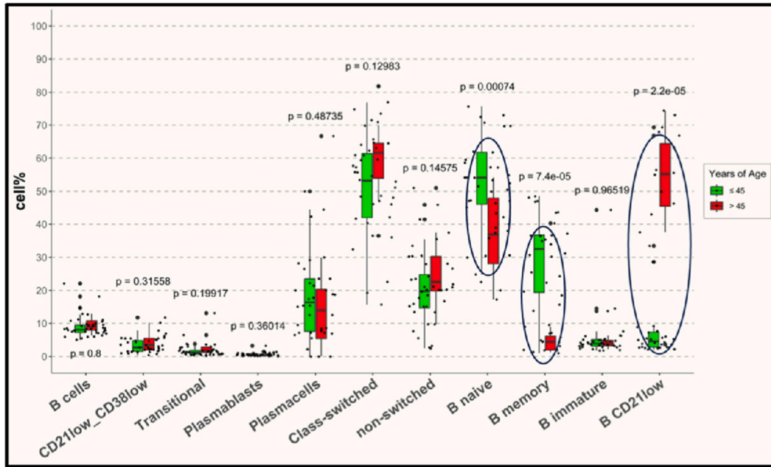
Modifications in immune function often entail alterations in the activation status of immune cells or may lead to changes in maturation [14]. In this comprehensive investigation focusing on lymphocytes, specifically T and NK cell activation and maturation, we employed monoclonal antibodies tailored to track expression of markers associated with these processes (Figure Appendix-4). A detailed breakdown of gating statistics can be found in Table 6.

Examination of T and NK cell activation and maturation parameters revealed significant age-related changes, with a threshold observed at 45 years old (circled data in panel A). Interestingly, no statistically significant differences were identified between male and female subjects in terms of these parameters (panel B).

Increasing age was associated with an elevation in some markers associated with maturation and proliferation within the age group exceeding 45 years. However, no discernible differences were identified between men and women (Fig. 4).

3.2.5. T cell and NK cell activating receptors

Motivated by the well-established principle that cell activation often triggers modulation of receptor expression [15], we sought to



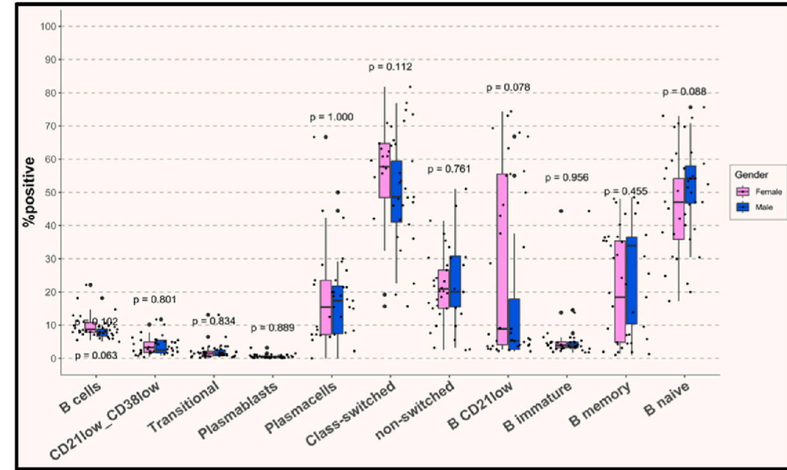


Fig. 2. Age-dependent alterations in B cell subpopulations.

Table 3
Characterization of T and NK Cell Subpopulations in Healthy Human Peripheral Blood.
This table presents a detailed analysis of T and NK cell subpopulations in the peripheral blood of healthy individuals. The data are further categorized by specific T cell subsets:
(A) CD4$^+$ T Cells: This section presents the mean ± standard deviation (SD, Min-Max %)and 95 % CI values for various subpopulations within the CD4$^+$ T cell compartment.
(B) CD8$^+$ T Cells: Similarly, this section focuses on the CD8$^+$ T cell population, providing mean ± SD, Min-Max %)and 95 % CI data for its constituent subpopulations.
(C) NK Cells: Finally, this section presents the mean ± SD, Min-Max %)and 95 % CI values for various subpopulations within the NK cell compartment.

Populations/Subpopulations	Mean (±SD)%	Min – Max%	95 % CI
Treg cells (CD25$^+$CD127-/low)	13.07 (±18.68)	2.53–98.92	7.1 – 19
CD4$^-$CD8$^-$ T cells	7.60 (±3.13)	2.76–16.84	6.6 – 8.6
(A) Mean ± SD, Min-Max %)and 95 % CI data of the subpopulations of CD4 T cells			
CD4, CD28$^-$	4.92 (±4.87)	2.53–98.92	3.4 – 6.5
CD4, CD45RA + CD62L+ (naive)	35.77 (±11.45)	11.79–60.37	32 - 39
CD4, CD45RA + CD197+ (naive)	36.08 (±12.07)	10.7–61.34	32 - 40
CD4, CD45RA-CD62L- (effector)	22.49 (±6.39)	12.05–42.94	20 - 25
CD4, CD45RA-CD197- (effector)	19.33 (±7.62)	8.7–42.19	17 - 22
CD4, CD45RA + CD62L- (naïve)	1.53 (±2.83)	0.11–16.28	0.62 – 2.4
CD4, CD45RA + CD197- (naïve)	1.66 (±3.00)	0.14–15.18	0.71 – 2.6
CD4, CD45RA-CD62L+ (memory)	40.22 (±8.11)	25.98–61.43	38 - 43
CD4, CD45RA-CD197+ (memory)	42.93 (±12.63)	15.99–75.01	39 - 47
(B) Mean ± SD, Min-Max %)and 95 % CI data of the subpopulations of CD8 T cells			
CD8, CD28$^-$	32.98 (±14.44)	10.41–66.19	28 - 38
CD8, CD45RA + CD62L+ (naive)	40.42 (±17.17)	9.85–76.02	35 - 46
CD8, CD45RA + CD197+ (naive)	34.93 (±17.63)	6.34–73.47	29 - 41
CD8, CD45RA-CD62L- (effector)	28.25 (±11.65)	7.86–54.59	25 - 32
CD8, CD45RA-CD197- (effector)	30.85 (±11.46)	12.15–55.27	27 - 35
CD8, CD45RA + CD62L- (ILC)	18.81 (±11.10)	3.84–52.07	15 - 22
CD8, CD45RA + CD197- (ILC)	22.72 (±10.82)	4.25–55.64	19 - 26
CD8, CD45RA-CD62L+ (memory)	12.51 (±6.11)	5.23–26.19	11 - 14
CD8, CD45RA-CD197+ (memory)	11.51 (±7.99)	0.91–32.85	8.9–14
(C) Mean ± SD, Min-Max %)and 95 % CI data of the subpopulations of NK cells			
NK, CD62L+	53.21 (±33.09)	8.23–99.96	43 - 64
NK, CD197+ (CCR7+)	1.56 (±1.24)	0–5.28	1.2 – 2.0

quantify the dynamic landscape of activating receptors on both T and NK cells. Surface expression of key T cell activating receptors, including 2B4, DNAM-1, and NKG2D, exhibited heterogeneity across the T cell population, as evidenced by variable percentages of positively stained cells for each receptor (Table 6). A similar pattern of diverse expression, albeit quantified through mean fluorescence intensity (MFI), was observed for activating receptors (2B4, DNAM, NKG2D, NKp30, and NKp46) on NK cells (Table 6). Interestingly, NKp44 expression stood as a notable exception. Unlike the other activating receptors we assessed, NKp44 exhibited a robust upregulation upon activation, remaining virtually undetectable on the resting cells analyzed in this study (Figure Appendix-5C).

Analysis of activating receptor expression revealedage-related variations but no differences associated with gender. Specifically, individuals exceeding 45 years of age demonstrated significantly higher expression of two T cell activating receptors, 2B4 and DNAM. Conversely, one NK cell receptor, NKP44, exhibited lower expression in subjects younger than 45 years.

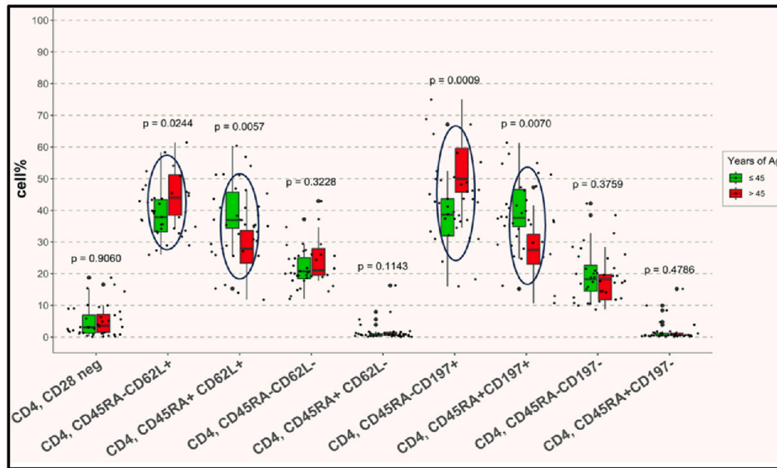
We conducted a comparative analysis of expression of activating receptors on T cells and NK cells based on age and gender. Our findings revealed age-related differences in the expression of 2B4+ and DNAM + receptors on T cells (Fig. 5A) and the NKp44 receptor on NK cells (Fig. 5B). However, we observed no gender-based differences for any of the receptors (Fig. 5C and D).

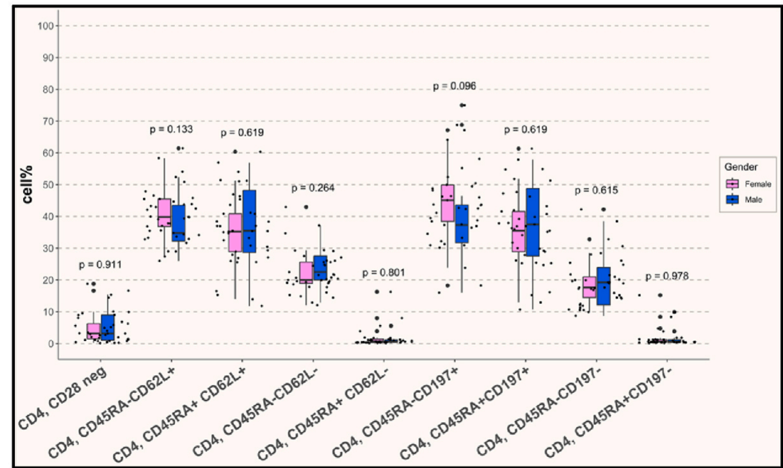
3.2.6. T cell proliferation

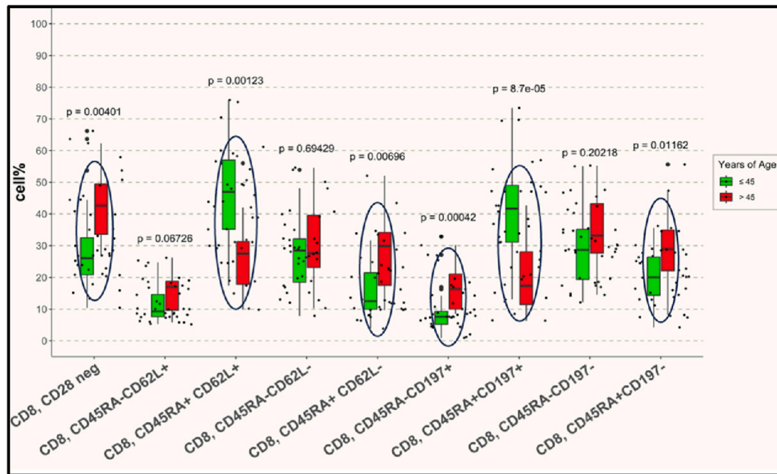
Evaluating immune cells is crucial, not only in terms of quantity but also concerning their functional quality. In this study, we place our focus on the functional quality of two vital cell types with implications for cancer resistance and sepsis: T cells and NK cells. These cell types are susceptible to a multitude of intrinsic and extrinsic influences that can potentially modify their functionality [16,17]. T-cell proliferation was gauged through co-stimulation using anti-CD3 and anti-CD28 over six days, followed by an analysis of carboxyfluorescein succinimidyl ester (CFSE) dilution among CFSE-labeled T cells. This process allowed us, as detailed in the Research Methods section, to determine the number of proliferative generations within each stimulated T cell sample (as depicted in Fig. 6A). Additionally, to affirm the proliferative capacity of specific T cell subsets, we also assessed the proliferative potential of helper T cells (CD4$^+$ T) (Fig. 6B). Remarkably, this capacity paralleled the broader T-cell proliferation (CD3$^+$).

The results of T-cell proliferation were calculated using the FlowJo software proliferation analysis platform. Typically, data are presented as a percentage of precursor cells that have undergone division. The cell division index provides an average count of cell divisions executed by cells within the initial population, even accounting for those cells that do not undergo division. In contrast, the proliferation index exclusively considers cells that have undergone division, representing the quotient of the total number of divisions relative to the number of cells that have participated in the division process (Fig. 6C and D; Table 7).

Analysis of all three indices of both T cells (panel A) and CD4$^+$ T cells (panel B) populations revealed no statistically significant







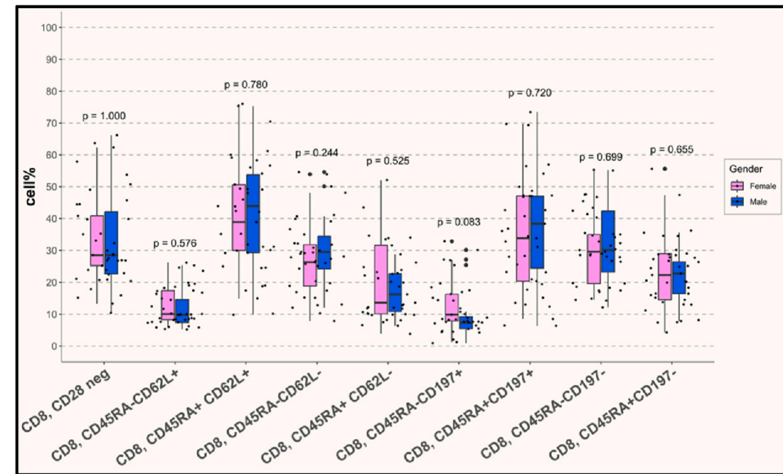


Fig. 3. Impact of age and sex on T cell subpopulations.

Table 4
Similarity of NK Cell Subpopulations in Healthy Vietnamese Adults with No Age or GenderDifferences.
This table presents the mean ± standard deviation (SD) data for various NK cell subpopulations measured in the peripheral blood of healthy Vietnamese adults. Notably, the analysis revealed no statistically significant differences in any of the NK cell subpopulations between subjects categorized by age (≤45 years and >45 years) or gender (male and female).

Characteristic	≤45	>45	*p-value*[b]	Male	Female	*p-value*[b]
NK, CD62L+	56.93 (±33.41)[a]	44.54 (±32.01)[a]	*0.11*	47.03 (±30.59)	63.52 (±35.55)	*0.3*
NK, CD197+	1.32 (±1.02)	2.12 (±1.54)	*0.072*	1.80 (±1.41)	1.16 (±0.77)	*0.15*

[a] Mean (±SD).
[b] Wilcoxon rank sum exact test; Wilcoxon rank sum test.

Table 5
Characterization of T and NK Cell Activation and Maturation in Healthy Human Peripheral Blood.
This table presents the mean ± standard deviation (SD, Min-Max %)and 95 % CI data for key markers associated with activation and maturation of T and NK cells in the peripheral blood (PB) of healthy individuals.

Populations/Subpopulations	Mean (±SD)%	Min –Max%	95 % CI
T, $CD25^+$	18.97 (±5.37)	10.23–37.82	17 - 21
T, $CD57^+$	14.03 (±7.66)	3.49–34.43	12 - 16
T, $CD69^+$	2.13 (±1.48)	0.32–7.42	1.7 – 2.6
T, GITR+	10.07 (±7.20)	1.37–31.94	7.8 – 12
T, KLRG1+	33.87 (±12.26)	11.29–62.26	30 - 38
T, NKG2C+	2.07 (±4.70)	0.33–30.43	0.57 – 3.6
NK, $CD25^+$	11.05 (±11.86)	0.98–43.72	7.3 – 15
NK, $CD57^+$	68.60 (±13.85)	34.23–88.88	64 - 73
NK, $CD69^+$	4.32 (±2.87)	0.57–15.35	3.4 – 5.2
NK, GITR+	8.01 (±5.33)	0.35–20.4	6.3 – 9.7
NK, KLRG1+	47.94 (±22.74)	4.21–87.15	41 - 55
NK, NKG2C+	35.90 (±23.60)	0.9–80.81	28 - 43

Table 6
T and NK Cell Activating Receptors in Healthy Vietnamese Adults.
This table presents the mean ± standard deviation (SD), Min-Max % and 95 % CI data for a panel of activating receptors expressed by T and NK cells in 40 healthy Vietnamese individuals.

Receptors	Mean (±SD)	Min - Max	95 % CI
T, 2B4 (%)	28.38 (±10.82)	6.35–52.66	25 - 32
T, DNAM-1 (%)	51.25 (±12.48)	26.57–76.57	47 - 55
T, NKG2D (%)	43.25 (±9.48)	22.42–58.6	40 - 46
NK, 2B4 (MFI)	787.78 (±202.77)	449–1220	723 – 853
NK, DNAM (MFI)	820.60 (±168.38)	446–1233	767 – 874
NK, NKG2D (MFI)	635.42 (±121.05)	457–958	597 – 674
NK, NKp30 (MFI)	205.62 (±229.70)	4–1199	132 – 279
NK, NKp44 (MFI)	16.90 (±12.18)	1–41	13 - 21
NK, NKp46 (MFI)	440.68 (±393.67)	100–2278	315 – 567

differences associated with either age or gender.

When comparing differences between two age groups, specifically those aged ≤45 and those over 45, as well as between men and women, our analysis revealed no statistically significant distinctions in general T cell populations and $CD4^+$ T cells (Fig. 7).

3.2.7. NK cell cytotoxicity

Natural Killer (NK) cells play a pivotal role in identifying and eliminating cells that are either virally infected or have undergone transformation. When activated, NK cells establish direct contact with target cells, directing their cytolytic granules towards the immune synapse and releasing cytolytic proteins aimed at the target cells [18]. In this study, we assessed NK cell cytotoxicity against K562 tumor cells target cells using PBMC as the effector cells (Table 8). There were no differences in NK cell cytotoxicity in the two age groups ≤45 and > 45 as well as between men and women (Fig. 8).

3. Cytokines

Cytokines, which are non-antibody proteins or glycoproteins, are produced and released by inflammatory leukocytes and other non-leukemic cells. These proteins serve as vital regulatory mediators facilitating communication between cells throughout the body and they play an indispensable role in the proper functioning of the immune system. Utilizing the Human ProcartaPlex Mix & Match 26-plex kit and ProcartaPlex Human Basic kit (ThermoFisher Scientific, US), we were able to quantitatively assess 27 cytokines

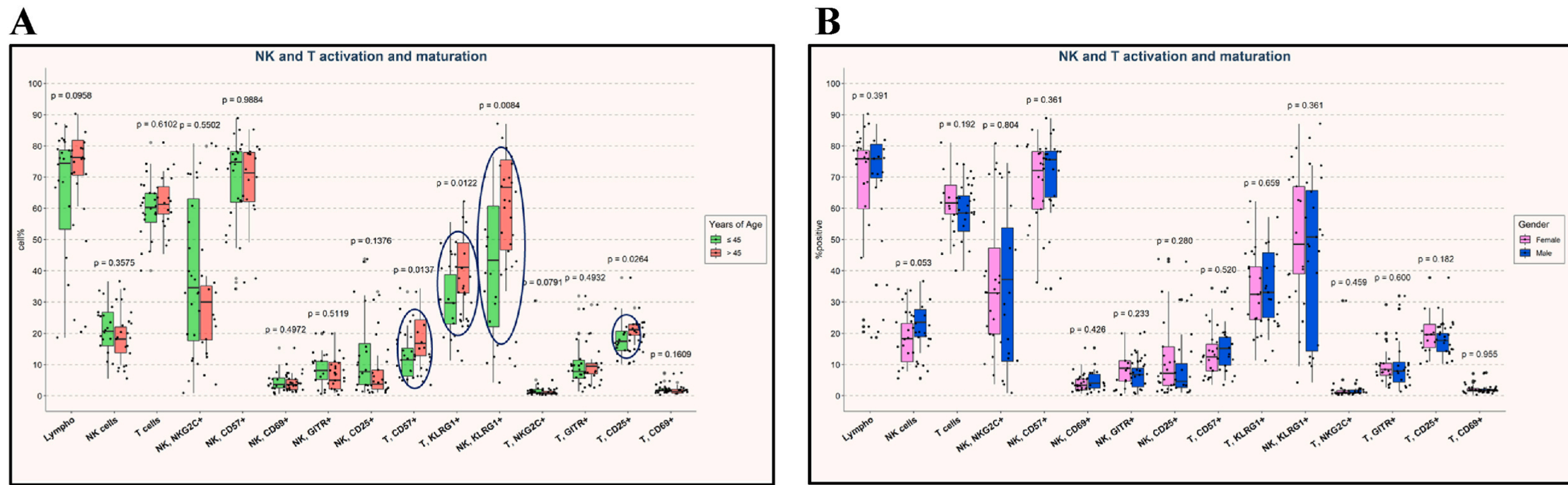


Fig. 4. Age-dependent alterations in T and natural killer cell activation and maturation.

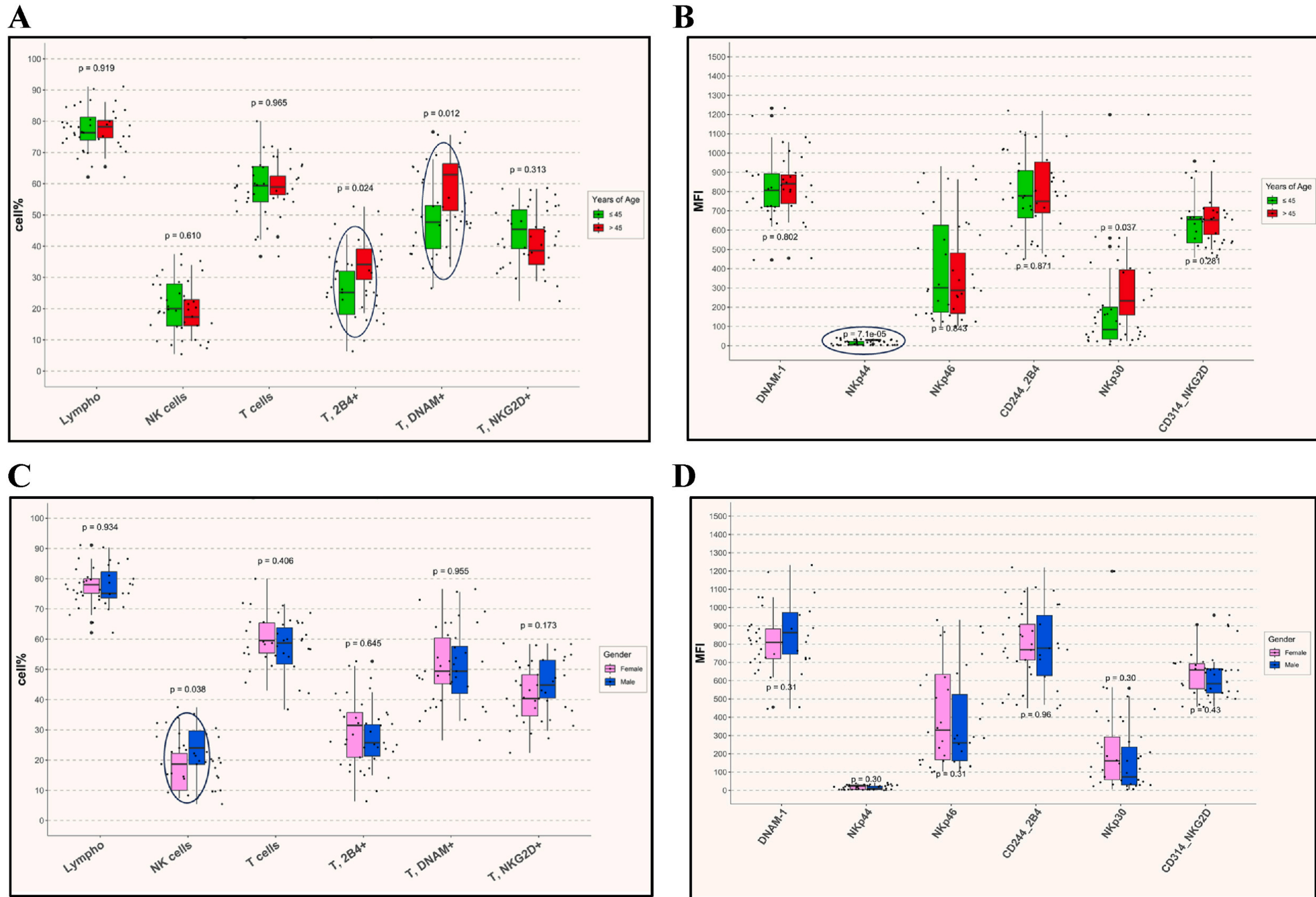


Fig. 5. Age-related variations and lack of gender differences in activating receptor expression on T and NK cells.

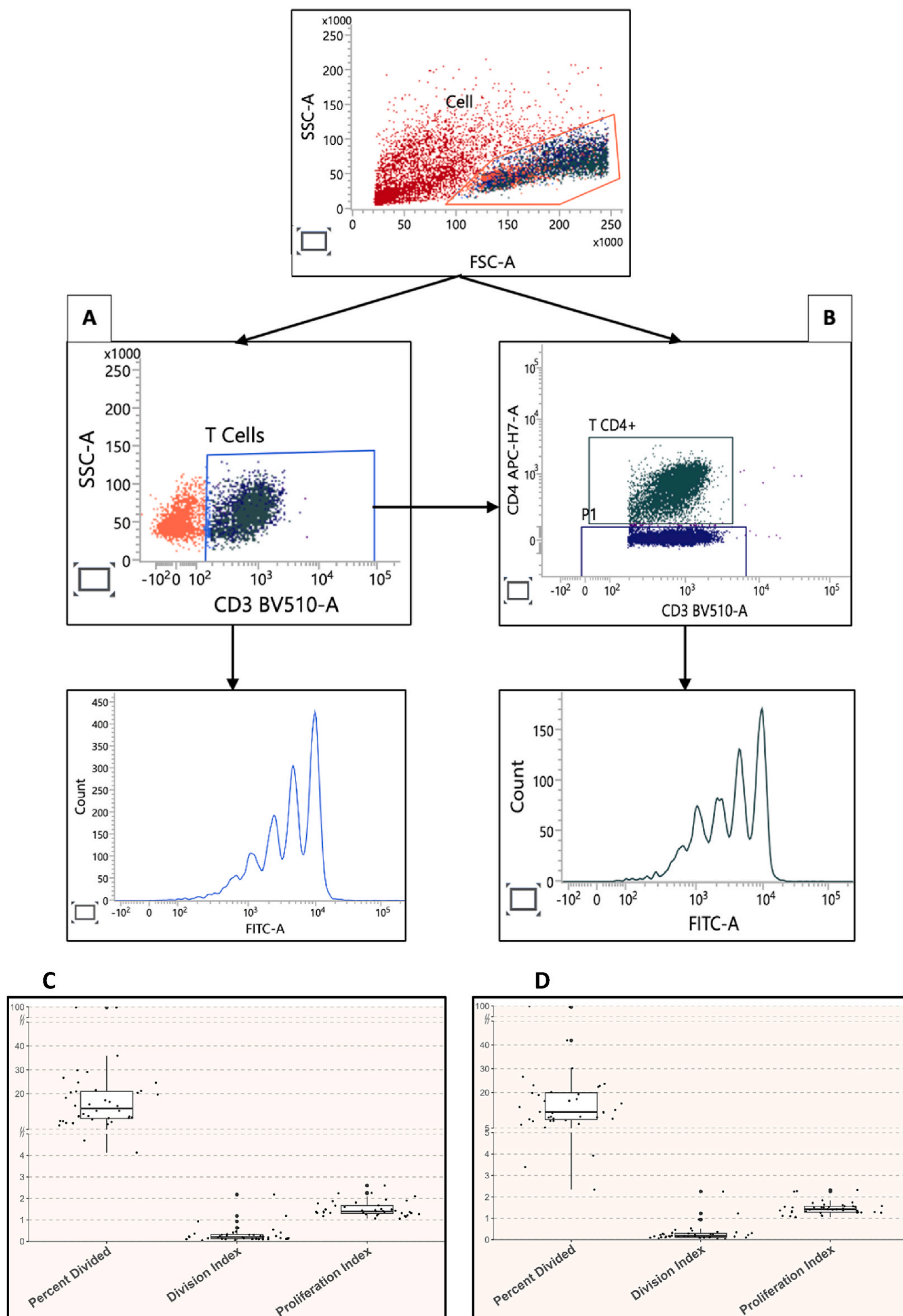


Fig. 6. Characterization of T Cell Proliferation.

This figure depicts the analysis of T cell proliferation following antigenic stimulation. Peripheral blood mononuclear cells (PBMCs) were labeled with carboxyfluorescein succinimidyl ester (CFSE) and subsequently stimulated with anti-CD3 and anti-CD28 antibodies for 6 days. T cell responses were assessed based on forward scatter (FSC) and side scatter (SSC) characteristics after gating on the CD3 marker.

(A and C): The gating strategy employed for identifying T cells is illustrated. Panel A shows a representative example from one experiment, demonstrating the gating approach based on CD3 expression and SSC. Panel C summarizes the data from 40 healthy donors, visualizing the typical

gating strategy used for T cell identification.
(B and D): Further analysis focused on $CD4^+$ T cells within the gated T cell population. Panel B displays the gating strategy for $CD4^+$ T cells from one representative experiment, utilizing the additional CD4 marker. Panel D presents the summarized gating approach for $CD4^+$ T cell identification across 40 healthy donors.

Table 7
T Cell and T $CD4^+$ Cell Proliferation Analysis.
This table presents the data from experiments analyzing the proliferative capacity of T cells and T $CD4^+$ cells in a specific cell population isolated from human peripheral blood based on the provided image. The table displays mean values (mean ± SD, Min-Max % and 95 % CI) for various proliferation indices measured in these cell subpopulations.

Indexes	T cell			T $CD4^+$ cell		
	Mean (±SD) %	Min – Max (%)	95 % CI	Mean (±SD) %	Min – Max (%)	95 % CI
Percent divided	19.22 (±19.65)	4.13–97.50	[13.1–25.3]	18.2 (±20.0)	2.34–99.2	11.9 – 24.3
Division index	0.31 (±0.38)	0.05–2.18	[0.19–0.42]	0.29 (±0.39)	0.03–2.25	0.17 – 0.41
Proliferation index	1.51 (±0.35)	1.06–2.6	[1.39–1.61]	1.47 (±0.3)	1.05–2.32	1.38 – 1.56

(Table 9).

Among the cohort of healthy individuals in our study, four cytokines in this population were non-detectable: Fibroblast Growth Factor −2 (FGF-2), Interleukin (IL)-4, IL-5, and IL-13. These cytokines play a crucial role in immune regulation, particularly in the coordination of interactions between T and B cells. The remaining cytokines (23 in total) exhibited a wide range of concentrations in healthy subjects, with notable variability, especially in the case of IL-1 Receptor Antagonist (IL-1RA), Monocyte Chemoattractant Protein-1 (MCP-1) (CCL2), Macrophage Inflammatory Protein-1β (MIP-1 β) (CCL4) and Vascular Endothelial Growth Factor A (VEGF-A).

In terms of the group of anti-inflammatory interleukins, including IL-1RA, IL-4, IL-6, IL-10 and IL-13, only IL-1RA displayed a considerably high value that was quite variable with a coefficient of variation of 248 % (91.49 ± 227.70 ng/mL). This suggests that among these healthy individuals, there may be an elevated risk of certain autoimmune diseases, such as diabetes mellitus [19] or obesity [20]. Additionally, these levels may also be influenced by immunomodulation following the administration of mRNA-based COVID-19 vaccines [21], as most of these healthy individuals had received such vaccinations.

Notably, in our cohort of healthy subjects, pro-inflammatory cytokines such as IL-1beta, IL-6, and tumor necrosis factor (TNF) alpha displayed limited variation. This underscores the fact that the subjects were healthy and unaffected by ongoing inflammatory reactions. These findings are further substantiated by the quantification results for chemokine ligands (CCL), growth-promoting cytokines G-CSF (Granulocyte-Colony Stimulating Factor), GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor), eotaxin, PDGF (Platelet-Derived Growth Factor), and VEGF which exhibited concentrations well within the expected range for healthy individuals.

3.3. *Exploring age and gender-related variation in cytokines*

In our investigation into the potential impact of age and gender on cytokine levels, we stratified the data into two distinct age groups: those aged ≤45 and those over 45. As depicted in Fig. 9, the results revealed a lack of significant differences among both age groups and across both genders, with a solitary exception. Notably, there was statistically significant elevation in IL-12 and MIP-1β (CCL4) levels in the older population (>45).

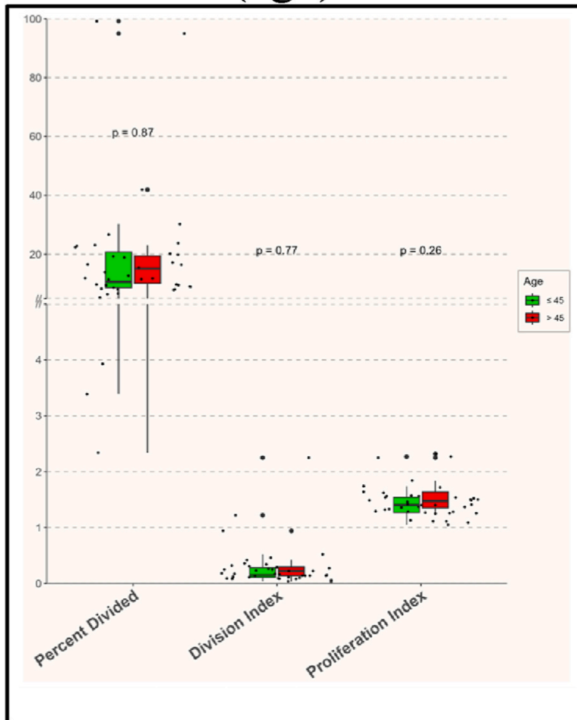
This figure depicts the analysis of plasma cytokine levels in relation to both age and gender. Panels (A) and (B) focus on age comparisons, presenting the concentrations of specific cytokines/interleukins measured in two distinct age groups. Similarly, panels (C) and (D) explore gender differences by showing the concentrations of the same cytokines/interleukins in male and female subjects.

4. Discussion

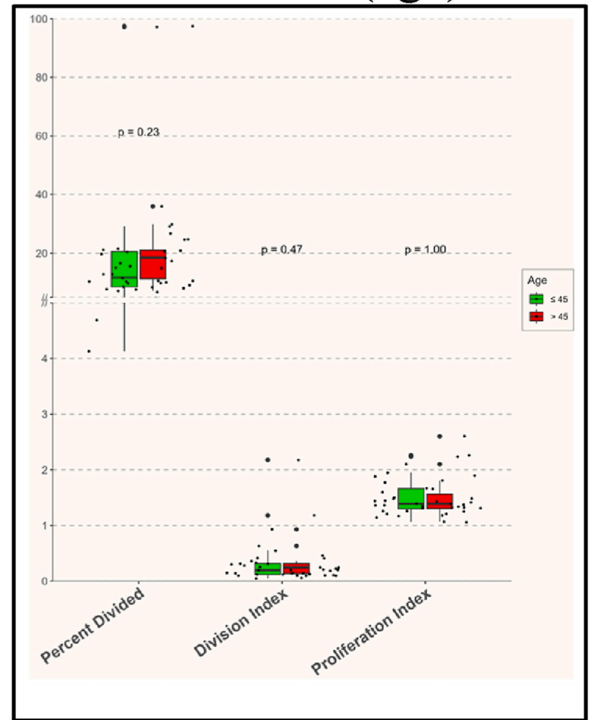
Multi-Color Flow Cytometry. The use MCFC, particularly in clinical settings,has rapidly evolved, rendering the identification and categorization of functional immune cell subpopulations more convenient and useful for diagnoses. We used the same techniques and antibodies recommended by Claus et al. [11] with 10 color MCFC to phenotype all of the major human leukocyte subsets in healthy Vientamese subjects. A comprehensive assessment of the immune system equips clinicians with the ability to make more precise and timely treatment recommendations. Furthermore, a sophisticated and comprehensive analysis of immune cell subpopulations will assist in establishing a detailed immunological reference standard for the Vietnamese population.

MCFC is a sophisticated method for studying different types of immune cell subpopulations that have different roles. These include naïve cell subpopulations, memory cells, efficient cells, and markers for maturation and proliferation, among others. Although the standardization of flow cytometric methods has historically posed a significant challenge in accurately characterizing the immune systems of healthy and afflicted individuals [22], meticulous adherence to the manufacturer's instructions and the use of specific reagents can yield reliable data. To facilitate comparisons with other existing studies, we adopted the gate selection method utilized in a prior German study [11], with minor modifications tailored to our laboratory. As such, data generated from this study will contribute to the establishment of a permanent reference dataset for immune cell populations among the Vietnamese population.

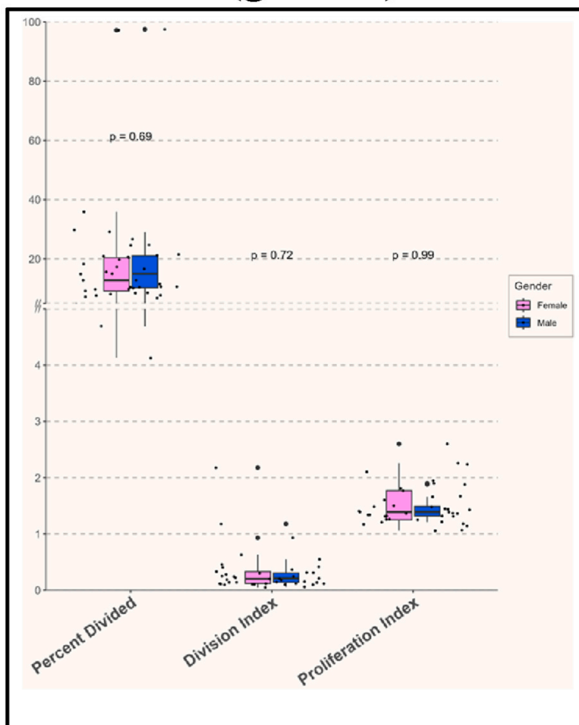
A:T cell (age)



B: CD4+ T cell (age)



C: T cell (gender)



D: CD4+ T cell (gender)

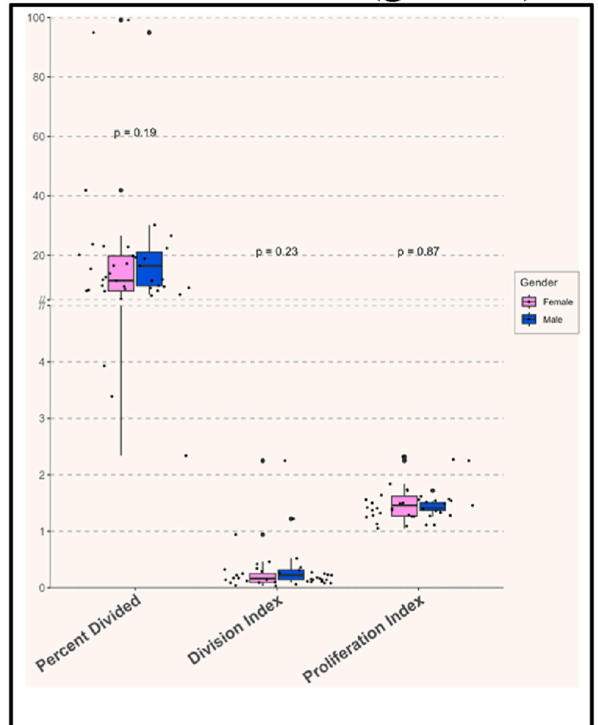


Fig. 7. Comparison of proliferation indices in T and CD4+T cell populations.

Table 8
NK cell cytotoxicity.
The cytotoxicity percentage was computed using the formula outlined in the methods section, demonstrating the capacity of the NK cells within PBMCs to kill K562 cells.

PBMC:K562 cells	Specific lysis %	Min-Max%	95 % CI
40:1	65.17 (±19.25)	14–92	59 - 71
20:1	48.23 (±19.01)	10–82	42 - 54
10:1	29.30 (±16.68)	4–66	24 - 35
5:1	18.40 (±11.44)	1–48	15 - 22

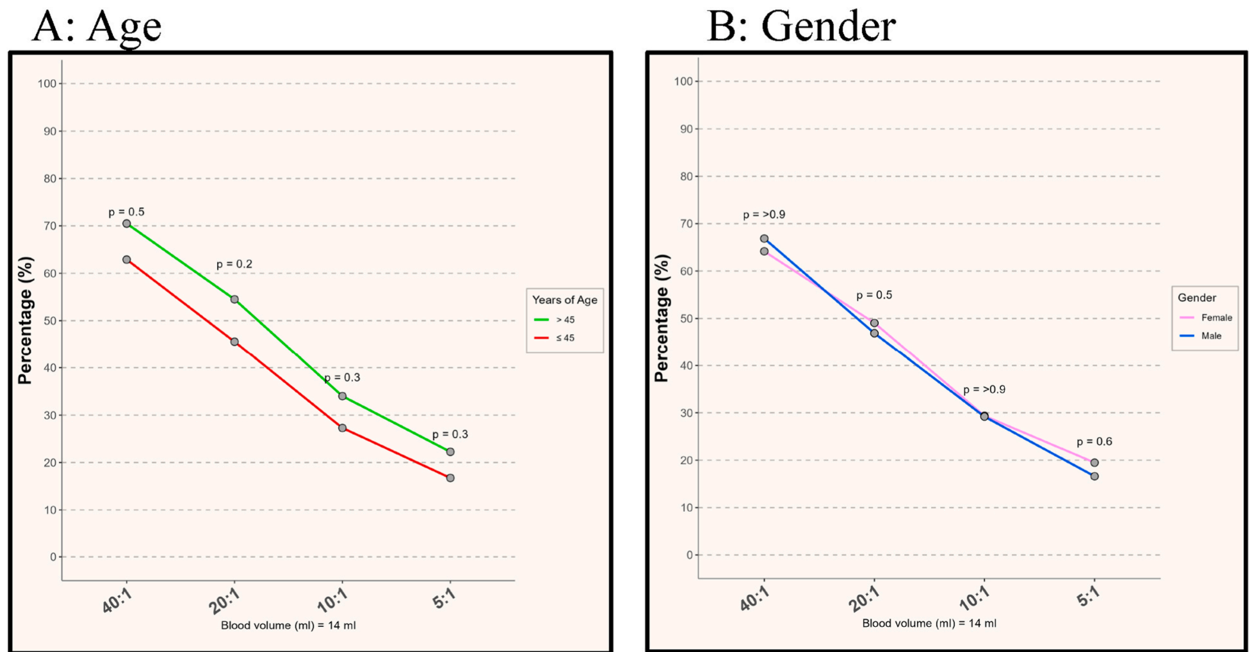


Fig. 8. NK Cell Cytotoxic Activity Analysis.
Examination of NK cell cytotoxicity revealed no statistically significant variations linked to either age or gender.
(A): The age comparison chart demonstrates no discernible differences in NK cell cytotoxic activity between individuals aged ≤45 and > 45 years.
(B): Similarly, the gender comparison figure indicates no statistically significant difference in NK cell cytotoxicity between male and female subjects.

Immune Changes After 45 Years of Age. One of our major findings is the variation of several functional immune cell populations between ages ≤45 and > 45 years, especially those of B cells and T cells but not for the NK cells. We were surprised to detect changes generally associated with a much older population in a population defined as only >45 years of age, which may be a function of the Vietnamese population These findings align well with established effects in older indviduals, characterized by a decline in naive $CD4^+$ and $CD8^+$ T cells and an increase in memory T cell subpopulations [23–26]. This shift is believed to be a result of the progressive reduction in thymic naïve T cells with thymic aging and degeneration, as well as increased immunological memory due to exposure to environmental antigens and pathogens [27]. Perhaps these changes occur in younger healthy Vietnamese than in Western populations.

The impact of aging on T cell frequency has been documented across various subsets. Notably, the increased occurrence of Treg cell populations ($CD4^+CD25^+$ and CD4+FOXP3+) has been well-documented [28,29]. Our study, however, did not identify any significant changes in low Treg frequency with advancing age. This discrepancy in the overall increase in Treg frequency with age compared to previous studies may be attributed to our relatively limited sample size.

B cells in Healthy Vietamese Subjects. B cells play a pivotal role in adaptive immunity, contributing to pathogen defense through their diverse subsets and functional states. Understanding the distribution and proportions of B cell subpopulations in healthy individuals establishes a baseline for assessing immune health and aids in identifying potential deviations associated with various diseases. In this study, we conducted a comprehensive analysis of B cell subpopulations in a cohort of healthy Vietnamese individuals and compared our findings with existing data reported in the literature. Our results align with previous studies [30,31] that reported the predominance of naive B cells and a significant proportion of class-switched memory B cells in healthy individuals. The percentages of transitional B cells and plasmablasts in our cohort are consistent with the dynamic nature of B cell differentiation and activation.

The decline in naive and memory B cell levels beyond the age of 45 may likely result from some reasons such as thymus involution [32], bone marrow alterations [33] or intrinsic defects in class switch with age [34]. However, the increase in CD21low in individuals

Table 9
Plasma Cytokine Profiles in Healthy Individuals.
This table presents the mean ± standard deviation (SD, Min – Max pg/mL and 95 % CI) values of various cytokines measured in the plasma of 40 healthy donors. The analysis employed the Human ProcartaPlex Mix & Match 26-plex kit and ProcartaPlex Human Basic kit (ThermoFisher Scientific, USA) to comprehensively assess a broad spectrum of immune molecules.

Cytokines	Mean (±SD) pg/mL	Min – Max pg/mL	95 % CI
Eotaxin (CCL11)	30.08 (±18.75)	2.25–107.58	24 - 36
FGF-2	0[a]	0	N/A – N/A
G-CSF (CSF-3)	7.29 (±10.42)	0–43.76	4.0 – 11
GM-CSF	0.47 (±2.96)	0–18.47	-0.48 - 1.4
IFN gamma	0.19 (±1.20)	0–7.62	-0.19 - 0.58
IL-1 beta	0.15 (±0.98)	0–6.19	-0.16 - 0.47
IL-1RA	91.49 (±227.70)	0–880.36	19 – 164
IL-2	0.44 (±1.96)	0–8.93	-0.18 - 1.1
IL-4	0[a]	0	N/A – N/A
IL-5	0[a]	0	N/A – N/A
IL-6	1.04 (±4.68)	0–24.88	-0.46 - 2.5
IL-7	0.25 (±0.28)	0–1.18	0.16 – 0.34
IL-8 (CXCL8)	0.15 (±0.94)	0–5.93	-0.15 - 0.45
IL-9	0.44 (±2.11)	0–12.36	-0.23 - 1.1
IL-10	0.41 (±0.78)	0–3.5	0.17 – 0.66
IL-12/IL-23p40	2.44 (±2.11)	0.07–10.28	1.8 – 3.1
IL-13	0[a]	0	N/A – N/A
IL-15	4.77 (±9.01)	0–49.89	1.9 – 7.7
IL-17A (CTLA-8)	0.05 (±0.29)	0–1.86	-0.05 - 0.14
IP-10 (CXCL10)	8.21 (±6.32)	1.27–29.39	6.2 – 10
MCP-1 (CCL2)	94.28 (±47.11)	8.65–194.36	79 – 109
MIP-1 alpha (CCL3)	5.94 (±6.15)	0–31.16	4.0 – 7.9
MIP-1 beta (CCL4)	67.16 (±117.37)	12.61–773.13	30 – 105
PDGF-BB	6.12 (±2.31)	2.73–12.89	5.4 – 6.9
TNF alpha	2.75 (±9.20)	0–57.85	-0.19 - 5.7
VEGF-A	176.97 (±312.76)	56.65–2079.73	77–277
RANTES (CCL5)	34.26 (±9.78)	16.65–65.03	31 - 37

[a] *LLOD: FGF-2 = 12.79 pg/mL; IL-13 = 3.47 pg/mL; IL4 = 13.38 pg/mL; IL-5 = 9.25 pg/mL.*

over 45 years of age may signify a potential autoimmune disorder [35], warranting further investigation.

Comparing our results with the existing literature, we observed that CD21 low-expressing B cells in healthy individuals consist of a mixture of naïve and memory B cells [36], and their variability is higher in our cohort [37]. This observation may arise from individual differences or technical variations in the methods used for cell isolation and analysis. Future studies should investigate the factors contributing to this variability. Additionally, the percentage of plasma cells in our study appears lower than some reported values [38], suggesting potential variations in age, ethnicity or environmental factors influencing plasma cell differentiation and maintenance.

4.1. T lymphocytes, natural killer cells and their receptors

T lymphocytes (T cells) and natural killer (NK) cells are fundamental components of the adaptive and innate immune systems, respectively. Understanding the distribution and phenotypic characteristics of these cell subsets in healthy individuals is crucial for deciphering immune system dynamics across diverse populations. This study presents a very detailed analysis of T cell subsets and NK cells in a cohort of healthy Vietnamese individuals aged 20 to 70.

To identify T and NK cells within the lymphocyte population, we analyzed expression of CD3 and CD56. The CD25 glycoprotein is a component of the IL-2 receptor and plays a pivotal role in T cell proliferation, activation-induced cell death and the functions of both regulatory (Treg) and effector (Teff) T cells [39]. It is expressed on activated T and NK cells, as well as Treg and memory T cells [40,41] (see Table 5). The CD57 antigen serves as a reliable marker for identifying terminally differentiated 'senescent' cells characterized by reduced proliferative capacity and altered functional attributes. While CD57 expression on human lymphocytes signifies an inability to proliferate, these cells simultaneously exhibit heightened cytotoxic potential. CD57-positive NK cells demonstrate memory-like characteristics and potent effector functions. Consequently, the frequencies of $CD57^+$ cells in blood and tissues correlate with clinical prognoses in cases of chronic infections, various cancers and human aging. Modulating the functionality of senescent $CD57^+$ T cells and mature $CD57^+$ NK cells presents innovative avenues for assessing human immunological aging and addressing various chronic diseases. CD57 (14.03 ± 7.66 %) and KLRG1 (33.87 ± 12.26 %) (Table 5) are associated with the advanced stages of NK cell maturation and are also present on senescent T cells [42,43]. As a final selected marker for activating receptors on T and NK cells, NKG2C has been linked to CMV reactivation and NK cell memory [44]. The glucocorticoid-induced TNFR-related protein (GITR), also recognized as TNFRSF18, belongs to the TNFR superfamily and is expressed on regulatory T cells (Tregs) along with select activated immune cells, including effector T lymphocytes, NK) cells and neutrophils [45–48]. Both CD69 and GITR are upregulated on activated cells [49,50], and they are lowly expressed on resting cells (Table 5).

Our findings revealed a relatively high percentage of double negative T cells ($CD4^-CD8^-$, 7.60 % of total T cells) compared to data from Western populations (Table 3A) [51,52]. Additionally, the proportion of regulatory T cells (Tregs, $CD25^+$CD127-/low) was

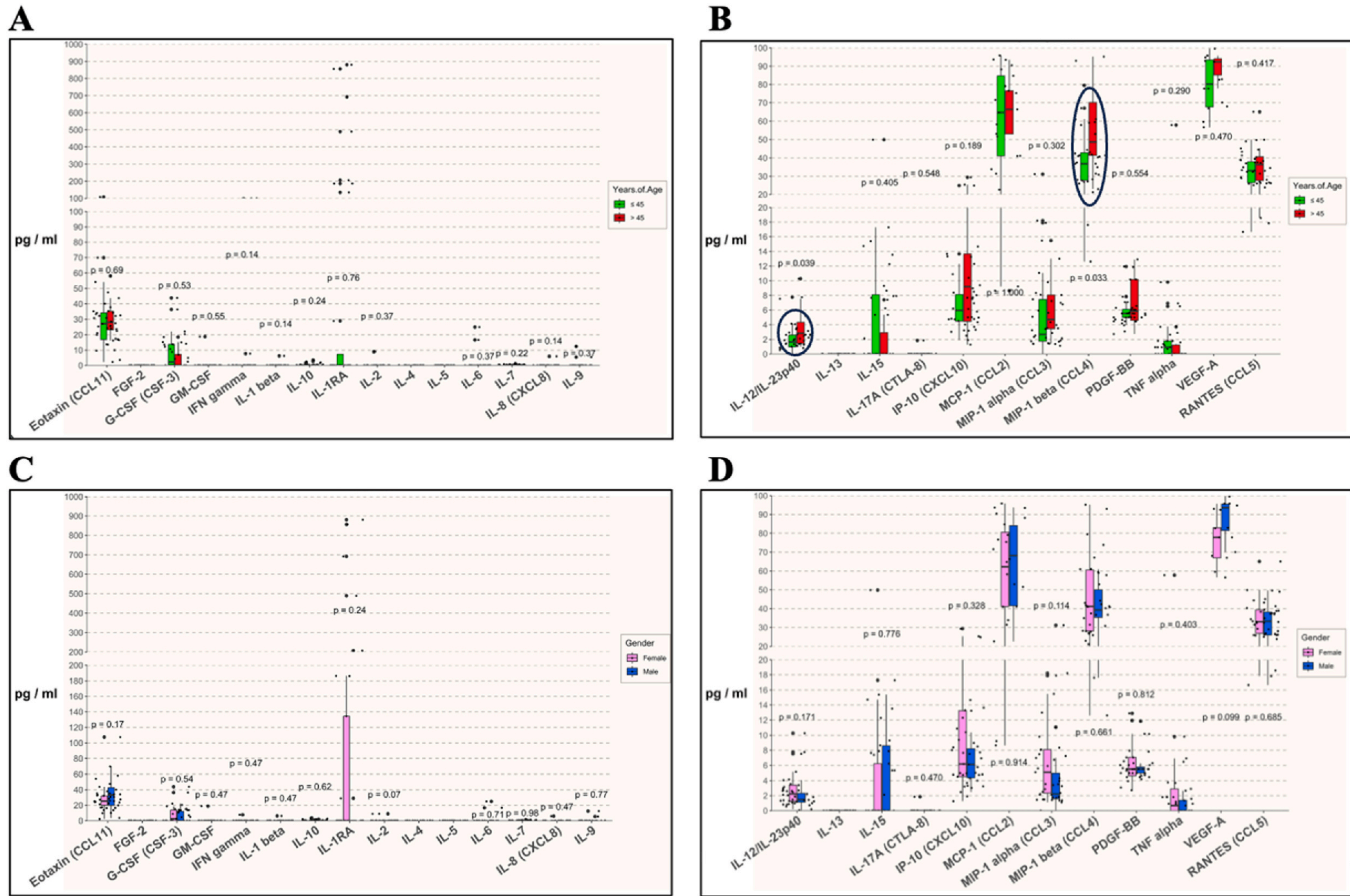


Fig. 9. Plasma cytokine concentrations - age and gender comparisons.

elevated at 13.07 %, suggesting a potentially robust regulatory capacity within the Vietnamese immune system.

CD28 is a co-stimulatory molecule essential for T cell activation. Activated T cells may downregulate CD28 expression, reflecting increased differentiation potential and potential antigen experience [53]. We assessed CD28 expression on $CD4^+$ and $CD8^+$ T cells (Table 4, B & C). The observed ratios of CD4/CD28- T cells (4.92 ± 4.87 %) and CD8/CD28- T cells (32.98 ± 14.44 %) provide temporary reference values for Vietnamese individuals within this age range.

We characterized naive, effector, and memory T cell populations using established markers (CD45RA, CD62L, and CD197). Similar patterns were observed across these subsets in both $CD4^+$ and $CD8^+$ T cells (Table 3, B & C). These findings suggest a balanced distribution of naive, effector and memory T cells in the Vietnamese population. The presence of a higher proportion of Tregs and CD4/CD28- T cells highlights the potential contribution of unconventional T cell subsets to immune regulation and immune surveillance in the Vietnamese population.Our data also revealed some differences in the percentages of specific T cell subsets compared to existing literature [54,55]. These variations are likely attributable to factors such as geographic location, demographics, sample size and flow cytometry gating strategies. Notably, the observed variability in CD8/CD28- T cells aligns with previous reports [56].

NK cell cytotoxicity is a critical mechanism of innate defense against infections and malignancies. NK cells are cytotoxic lymphocytes that can directly kill target cells without prior sensitization. In the cytotoxicity assay, the percentage of specific lysis of K562 cells by PBMCs increases with increasing effector-to-target (E:T) ratios. In this study, the percentage of specific lysis at an E:T ratio of 10:1 is 29.30 % (±16.68 %); at 20:1 is 48.23 % (±19.1 %), and at 40:1 is 65.17 % (±19.25 %). A comparison of these data with other authors' data [57] shows that the level of NK cell cytotoxicity in our study is slightly higher than that of healthy individuals. The increase in specific lysis with increasing E:T ratios is also a well-established phenomenon in NK cell biology [58].

We further investigated NK cell subsets based on CD62L expression. CD62L + NK cells represent an intermediate stage in NK cell maturation. Interestingly, our study revealed that CD62L + NK cells comprised over 50 % of the total NK cell population, exceeding values reported in a German study (43.38 ± 11.64 %) [11]. This suggests a potential delay in NK cell maturation within the Vietnamese population. In contrast, the proportion of CD197+ (CCR7+) NK cells, known to migrate to lymph nodes for T cell interactions [59–61], was relatively low at 1.56 % of total NK cells, suggesting a potential bias towards tissue-resident NK cells.

Our data on the proliferation and activation markers ($CD25^+$, $CD57^+$, $CD69^+$, GITR+, KLRG1+) of T and NK cells in the >45-year-old group are consistent with published reports for healthy adults [62–64]. This suggests normal age-related changes in these immune cell subsets.

Overall, this study provides a detailed characterization of T cell subsets and NK cells in a Vietnamese population. The observed variations compared to Western populations may reflect geographic and demographic differences. Further research is needed to explore the functional implications of these findings in health and disease.

Pro-and Anti-Inflammatory Cytokines. With regard to cytokine profiles, the present findings highlight the significant impact of age and gender on cytokine expression. Notably, our observation of elevated IL-12 and MIP-1β levels in individuals exceeding 45 years of age (Fig. 9B) prompts the hypothesis that this age-related increase might be intricately intertwined with the physiological processes of aging, chronic inflammation and the intricate interplay between the immune and nervous systems. IL-12 is widely recognized as a critical regulator of Th1 responses, promoting the expansion and survival of activated T-cells and NK cells while modulating the cytotoxic activity of CTLs and NK cells. Similarly, MIP-1β, a key member of the chemokine family, plays a pivotal role in orchestrating the recruitment of immune cells to inflammatory sites and regulating the subsequent inflammatory response.

Limitations of this study. The eligibility criteria for inclusion were confined to individuals who exhibited no ongoing medical symptoms, maintained regular work activities, and did not express any health-related complaints. Regrettably, we could not definitively ascertain whether these individuals harbor latent diseases. Moreover, the constrained availability of volunteers restricted the sample size. Consequently, our sample may not be entirely representative of the broader healthy population that may have underlying but undectable health issues.

Our study participants were volunteers recruited from the greater Hanoi area and they all belonged to middle-to-high income brackets. This limits the generalizability of our immunity data to only this population and excludes lower-income Vietnamese subjects. Many studies use a limit of 60–70 years in studies on aging. However, we used an accepted cut-off limit of >45 years (12 subjects) because we were interested in potential changes that occur after middle age. The small number of participants in this study, shown by the limitation of men >45 years old, is also another limitation of the study.

We conducted a comparative analysis of cell viability between fresh whole blood and cryopreserved PBMC and found no discernible differences. However, the published medical literature suggests a potential for minimal distinctions in the phenotypic markers utilized in our study [65–67]. Furthermore, as frequently encountered in flow cytometric studies, cross-referencing our data with other investigations poses challenges due to variations in antibodies used, measurement techniques and characteristics of the patient populations [68,69]. As a result, we did not place excessive emphasis on directly comparing our immunological indices with numerous other studies derived from patients in other populations. Our primary focus was to construct reference ranges for immune indices pertinent to the Vietnamese population and underscore the significance of acknowledging these variations among different racial groups.

5. Conclusion

This study constitutes a seminal contribution to clinical immunology by establishing the first comprehensive dataset of immune parameters in healthy Vietnamese individuals. It reveals profound discrepancies in immune signatures compared to established reference ranges and patterns observed in Western populations. These findings significantly expand our understanding of immune variation across diverse populations and hold immense potential for informing tailored clinical approaches in the Vietnamese context.

These data serve as a critical temporary immunological reference values for healthy Vietnamese subjects. This empowers clinical practitioners, when confronted with serious diseases like systemic infections or cancer, to confidently utilize these immune parameters for patient assessment. Such informed decisions will minimize further immune system compromise and pave the way for the most effective treatment strategies. Furthermore, this comprehensive dataset represents a valuable tool for evaluating the impact of diverse interventions on the human immune system. Its applications extend to assessing vaccination efficacy, monitoring treatment responses in infectious and autoimmune diseases and guiding immune interventions employed in cancer therapy.

Ethical approval

All procedures performed in this study were approved by the Vinmec-VinUni Ethical Review Committee (ethics approval number: 64/202/QD-VMEC) and were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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6. Data availability

The datasets used and analyzed during the current study available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Dinh Chien Huynh: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Minh Phuong Nguyen:** Resources, Methodology. **Dinh Trung Ngo:** Resources, Methodology, Conceptualization. **Nguyen Xuan Hung:** Writing – review & editing, Resources, Methodology, Conceptualization. **Dac Tu Nguyen:** Writing – review & editing, Resources, Methodology. **Thi Hien Mai:** Writing – review & editing, Resources, Methodology. **Thi Huyen Le:** Writing – review & editing, Resources, Methodology. **Duy Hoang Mai:** Writing – review & editing, Data curation, Conceptualization. **Khac Linh Le:** Writing – review & editing. **Khoi Quan Nguyen:** Writing – review & editing. **Viet Hoang Nguyen:** Writing – review & editing, Data curation. **Keith W. Kelley:** Writing – review & editing, Validation, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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APPENDIX 1

Table Appendix-1
Marker antigens, reagents with coupled fluorochromes, distributors, and Ab volume used to stain whole blood cells were listed.

Panel No.	Label	Tube	Marker antigens/Reagent	Company	Volume
01	Tube No.01	BD Tru-count™ Tube	BD Multitest™ 6-Color TBNK	BD Biosciences	20 μl
02	Tube No.02	Sample Tube	Hu CD19 BV421 HIB19	BD Biosciences	5 μl
			Hu CD3 BV510 UCHT1	BD Biosciences	5 μl
			Hu CD16 FITC 3G8	BD Biosciences	20 μl
			Hu CD14 PE MphiP9	BD Biosciences	5 μl
			Hu HLA-DR PerCP-Cy5.5 L243 (G46-6)	BD Biosciences	5 μl
			Hu CD64 PE-Cy7 10.1	BD Biosciences	5 μl
			Hu CD56 APC B159	BD Biosciences	20 μl
			Hu CD45 Alexa 700 HI30	BD Biosciences	5 μl
03	Tube No.03	Sample Tube	Hu CD19 BV421 HIB19	BD Biosciences	5 μl

(*continued on next page*)

Table Appendix-1 (*continued*)

Panel No.	Label	Tube	Marker antigens/Reagent	Company	Volume
			Hu CD138 BV510 MI15	BD Biosciences	5 µl
			Hu IgD FITC IA6-2	BD Biosciences	20 µl
			Hu/NHP CD21 PE B-ly4	BD Biosciences	5 µl
			Hu CD27 PerCP-Cy5.5 M-T271	BD Biosciences	5 µl
			Hu CD38 PE-Cy7 HIT2	BD Biosciences	5 µl
			Hu IgM APC G20-127	BD Biosciences	20 µl
			Hu CD45 Alexa 700 HI30	BD Biosciences	5 µl
04	Tube No.04	Sample Tube	Hu CD127 BV421 HIL-7R-M21	BD Biosciences	5 µl
			Hu CD45RA BV510 HI100 50Tst	BD Biosciences	5 µl
			Hu CD62L BV605 DREG-56	BD Biosciences	5 µl
			Hu CD8 FITC RPA-T8	BD Biosciences	20 µl
			Hu CCR7 (CD197) PE 2-L1-A	BD Biosciences	5 µl
			Hu/NHP CD28 PerCP-Cy5.5 CD28.2	BD Biosciences	5 µl
			Hu CD25 PE-Cy7 M-A251	BD Biosciences	5 µl
			Hu CD56 APC B159	BD Biosciences	20 µl
			Hu CD3 Alexa 700 UCHT1 100ug	BD Biosciences	5 µl
			Hu CD4 APC-H7 RPA-T4	BD Biosciences	5 µl
05	Tube No.05	Sample Tube	KLRG1 100 test	Miltenyi Biotec	2 µl
			BD OptiBuild™ BV510 Mouse Anti-Human GITR (CD357)	BD Biosciences	5 µl
			BD OptiBuild™ BV605 Mouse Anti-Human CD159c (NKG2C)	BD Biosciences	5 µl
			Hu CD57 PE NK-1 100Tst	BD Biosciences	5 µl
			Hu CD56 PerCP-Cy5.5 B159	BD Biosciences	5 µl
			Hu CD25 PE-Cy7 M-A251	BD Biosciences	5 µl
			Hu CD3 Alexa 700 UCHT1	BD Biosciences	5 µl
			Hu/NHP CD69 APC-H7 FN50	BD Biosciences	5 µl
06	Tube No.06	Sample Tube	Hu CD56 Horizon V450 B159	BD Biosciences	5 µl
			Hu CD314 (NKG2D) BV510 1D11	BD Biosciences	5 µl
			Hu CD337 (NKp30) BV605 p30-15	BD Biosciences	5 µl
			Hu CD226 FITC DX11 100Tst	BD Biosciences	20 µl
			HU NKP44 (CD336) PE P44-8	BD Biosciences	20 µl
			Hu CD335 (NKP46) PE-Cy7 9E2/NKP46	BD Biosciences	5 µl
			Hu CD244 APC 2-69	BD Biosciences	5 µl
			Hu CD3 Alexa 700 UCHT1 100ug	BD Biosciences	5 µl
07	Unstain-ed Tube	Sample Tube	No marker antigens/Reagent		

Table Appendix-2
The bead number used in measuring the growth factors and interleukins.

No	Name of interleukin/growth factor	Bead number
1.	Eotaxin (CCL11)	33
2.	FGF-2	75
3.	G-CSF (CSF-3)	42
4.	GM-CSF	44
5.	IFN gamma	43
6.	IL-1Beta	18
7.	IL-10	28
8.	IL-12/IL-23p40	64
9.	IL-13	35
10.	IL-15	65
11.	IL-17A (CTLA-8)	36
12.	IL-1 RA	38
13.	IL-2	19
14.	IL-4	20
15.	IL-5	21
16.	IL-6	25
17.	IL-7	26
18.	IL-8 (CXCL10)	27
19.	IL-9	52
20.	IP-10 (CXCL10)	22
21.	MCP-1 (CCL2)	51
22.	MIP-1 alpha (CCL3)	12
23.	MIP-1 beta (CCL4)	47
24.	PDGF-BB	77
25.	TNF alpha	45
26.	VEGF-A	78
27.	Rantes (CCL5)	42

APPENDIX II

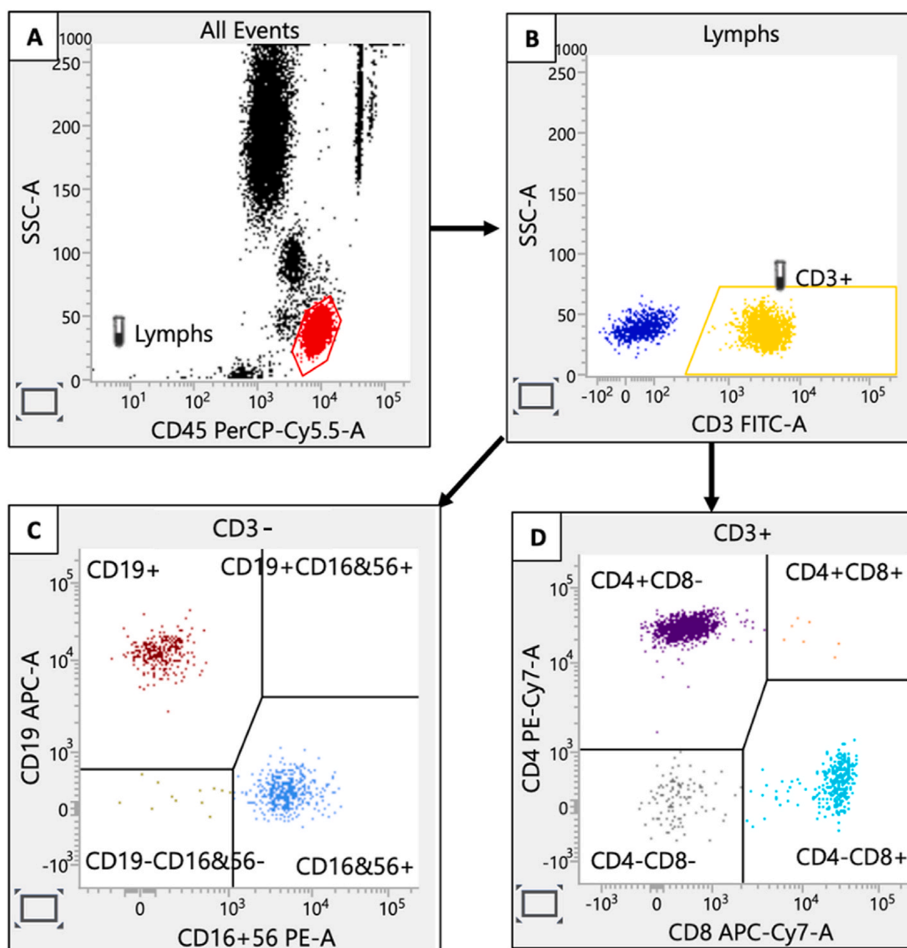

Fig. ure Appendix-1. Identification of Lymphocyte Subpopulations and Antibody-Coated Beads.
Lymphocyte populations and antibody-coated beads were distinguished based on their combined fluorescence intensity for CD45 and side scatter (SSC) properties. A subsequent gating strategy in a second fluorescence channel eliminated background events for accurate bead quantification. Within the defined lymphocyte gate, T cells ($CD3^+$), helper T cells ($CD4^+$), cytotoxic T cells ($CD8^+$), B cells ($CD19^+$), and natural killer (NK) cells ($CD56^+$) were further classified.

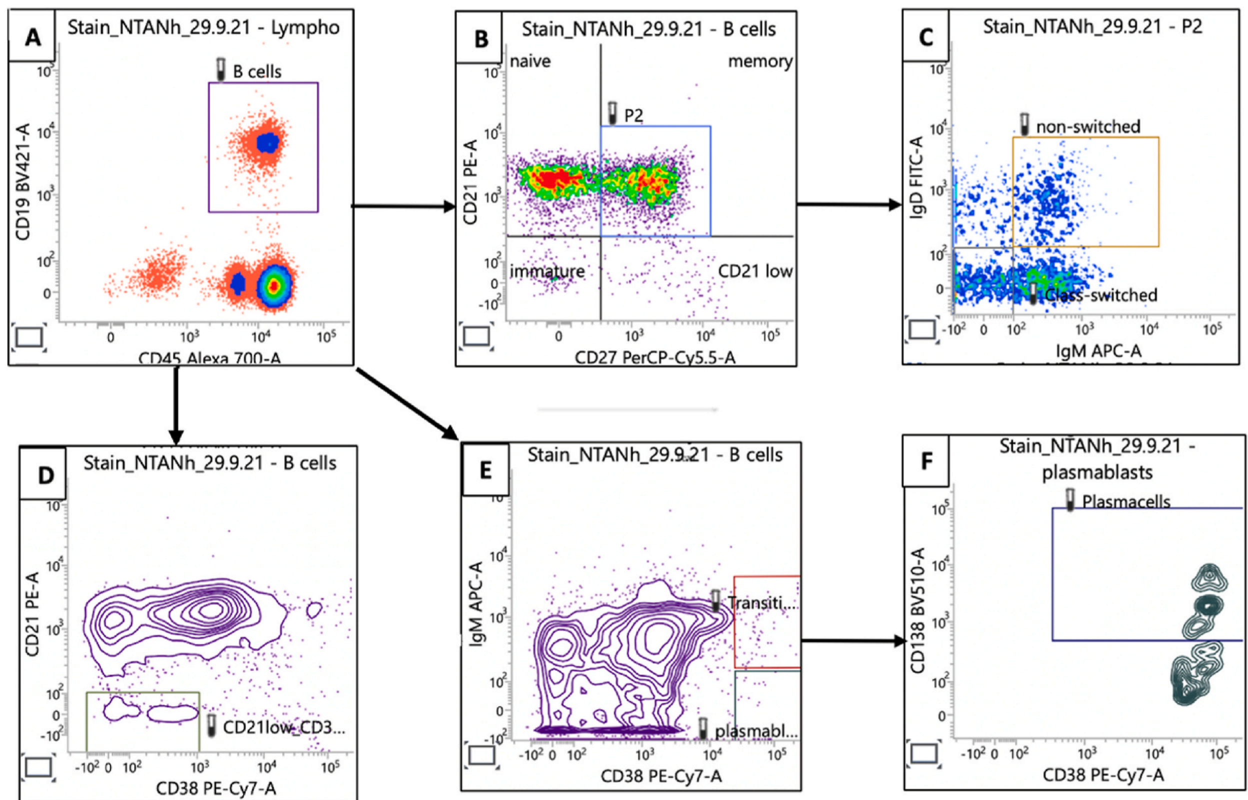

Fig. ure Appendix-2. B cell subpopulations. (A) B cells were identified by CD45 and CD19 expression. (B) Immature, naive, memory and CD21low B cells were identified by CD21/CD27 subgating. Class-switched versus non-switched memory B cells were separated by IgM and IgD expression based on the memory B cell gate (C). (D) Activated CD21low CD38low B cells were identified by CD21 and CD38 staining from the B cell gate. (E) $CD38^{+}$ transitional B cells and plasmablasts in the B cell population were discriminated by expression of IgM and plasma cells (CD138+IgM-) were identified by further subgating from plasmablasts (F).

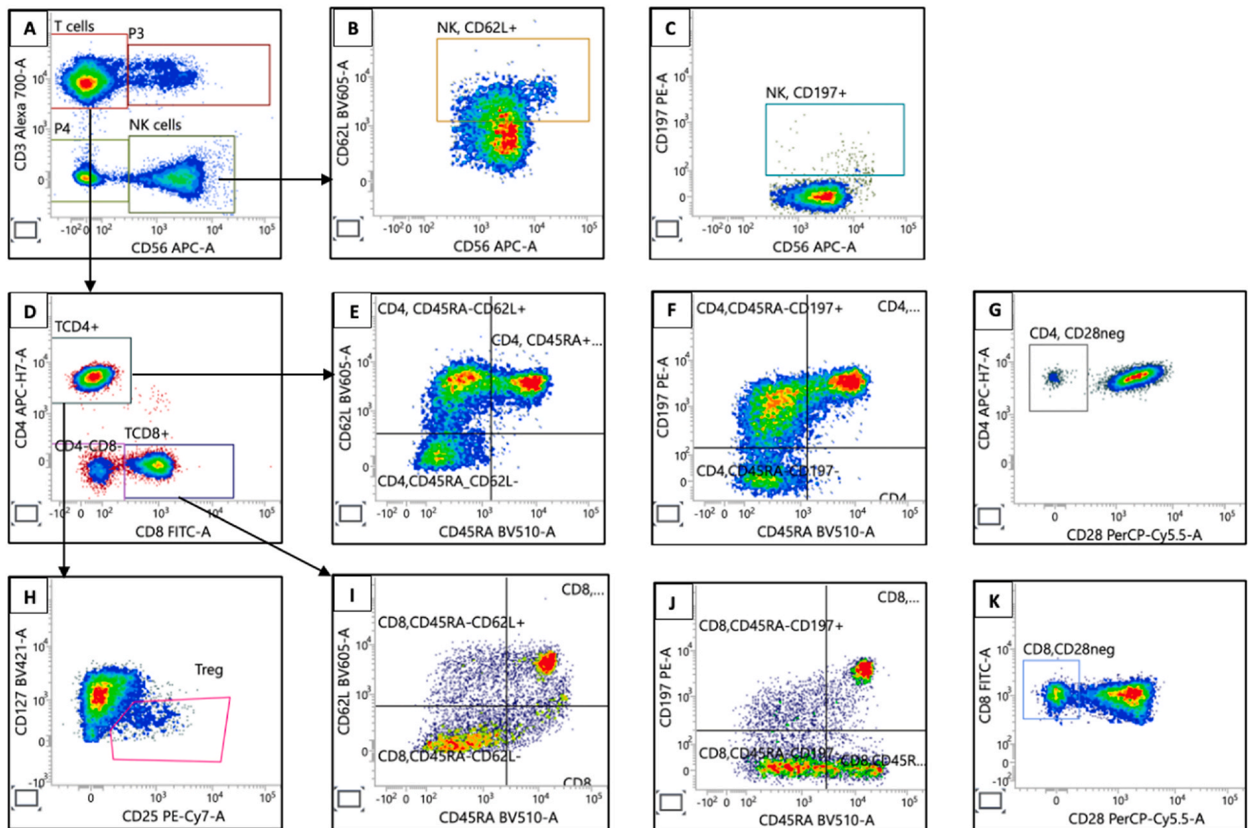

Fig. ure Appendix-3. Characterization of T Cell Subpopulations
(A): Separation of T and KK cells employed fluorescently labeled anti-CD3 and anti-CD56 antibodies.
(B & C): Homing marker expression (CD62L and CD197) on NK cells was assessed.
(D): Helper T cells and cytotoxic T cells were identified using specific anti-CD4 and anti-CD8 antibodies. Helper T cell subpopulations were further categorized as regulatory T ($CD25^{+}$CD127low), naive (CD45RA + CD62L + or CD45RA + CD197+), memory (CD45RA-CD62L + or CD45RA-CD197+), and aged-related ($CD28^{-}$) cells (panels E, F, G, and H, respectively). Cytotoxic T cell subpopulations included naive (CD45RA + CD62L + or CD45RA + CD197+), memory (CD45RA-CD62L + or CD45RA-CD197+), and aged-related ($CD28^{-}$) cells (panels I, J, and K, respectively).

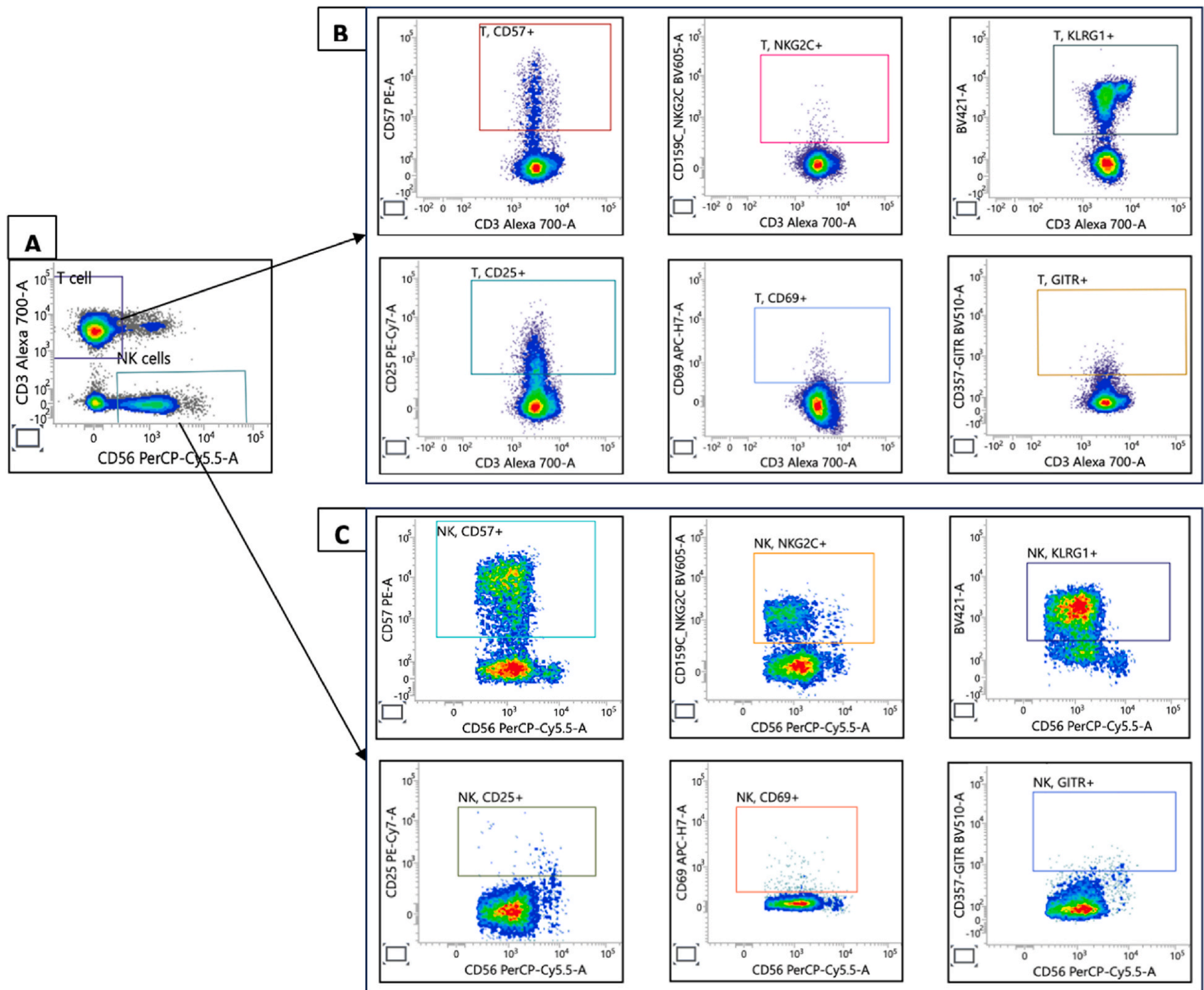

Fig. ure Appendix-4. Characterization of T and NK Cell Maturation and Activation Profiles
(A) Initial separation of T and NK cell populations was achieved using fluorescently labeled anti-CD3 and anti-CD56 antibodies. Subsequently, the identified T (panel B) and NK cell populations (panel C) were further analyzed to assess the expression of specific surface markers associated with cellular maturation (CD57, NKG2C, KLRG1) and activation status (CD25, CD69, GITR). The mean percentage ± standard deviation of each gated marker within these populations, based on data from 40 healthy subjects, is presented in detail in Table 5.

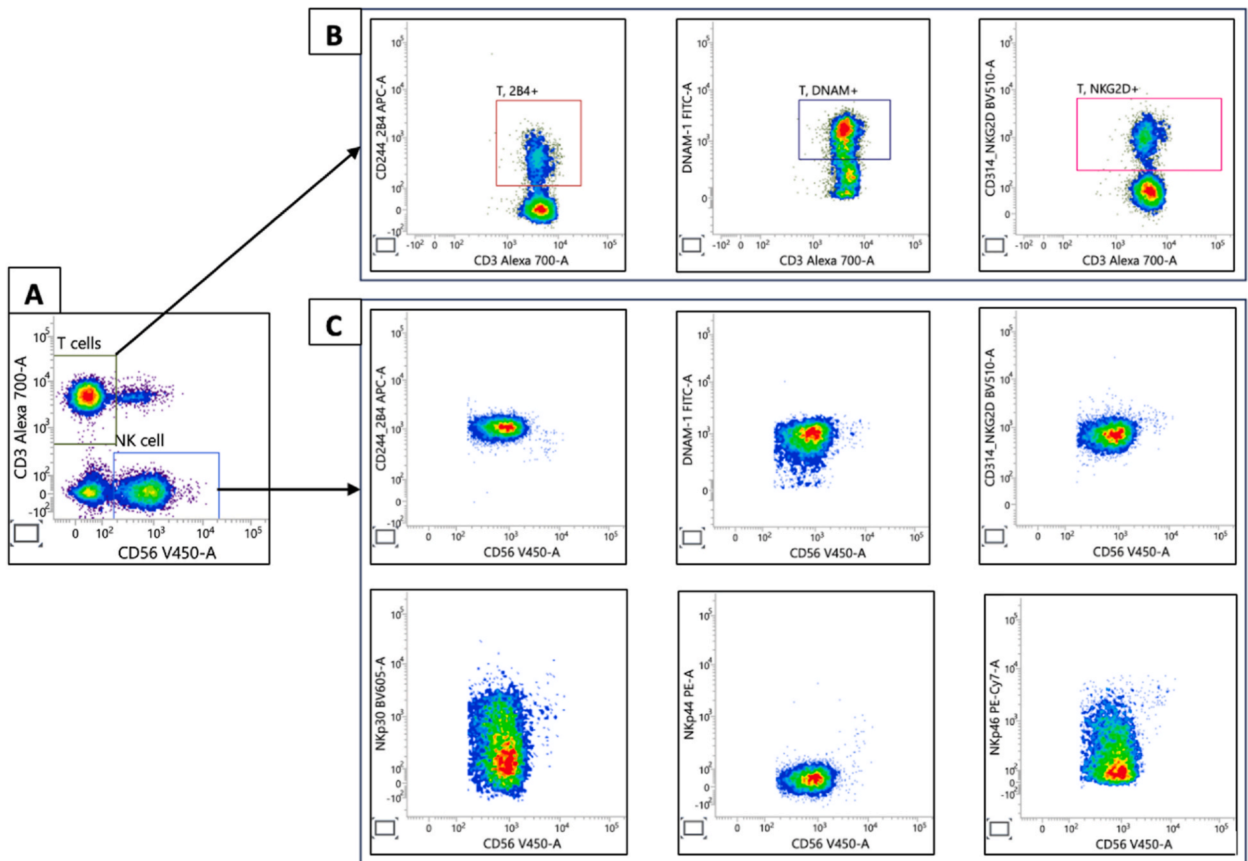

Fig. ure Appendix-5. Characterization of Activating Receptors in T and NK Cell Populations.
(A) Initial separation of NK and T cell populations was achieved through differential expression of surface markers CD3 and CD56.
(B) Within the identified T cell population, the percentage of cells expressing the activating receptors 2B4, DNAM, and NKG2D was determined and presented as the percent positive cells.
(C) For NK cells, expression of the activating receptors 2B4, DNAM, NKG2D, NKP30, NKP44, and NKP46 was assessed and quantified using the median fluorescence intensity (MFI) parameter.

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