Changes in Immunological Parameters by Aging in Rural Healthy Indian Adults and their Associations with Sex and Lifestyle

Dhiraj Agarwal
King Edward Memorial Hospital Research Centre
Sourav Paul
National Centre for Cell Science
Pallavi Lele
King Edward Memorial Hospital Research Centre
Vikrant Piprode
National Centre for Cell Science
Anand Kawade
King Edward Memorial Hospital Research Centre
Neerja Hajela
Yakult Danone India Pvt. Ltd.
Ashish Bavdekar
King Edward Memorial Hospital Research Centre
Varsha Parulekar
DiagnoSearch Life Sciences Pvt. Ltd.
Manisha Ginde
DiagnoSearch Life Sciences Pvt. Ltd.
Gandhali Paranjape
DiagnoSearch Life Sciences Pvt. Ltd.
Tetsuji Hori
Yakult Central Institute
Kazunori Matsuda
Yakult Central Institute
Sanjay Juvekar
King Edward Memorial Hospital Research Centre
Girdhari Lal (glal@nccs.res.in)
National Centre for Cell Science

Research Article

Keywords: Aging, Natural killer cells, T cells, B cells, Dietary pattern, Serum cotinine

Posted Date: January 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1216293/v1

Abstract

Several factors including sex and lifestyle have been reported to contribute to age-related alteration of immune functions. The study was undertaken to determine age-related differences in the proportion of peripheral blood mononuclear lymphocytes in the Indian population using blood samples from 67 healthy adults (33 females and 34 males) aged between 20 and 80 years old. In the linear regression analysis to estimate the relationship with age categories, there was a significant increase in the frequency of natural killer cells with aging, while their cytolytic activity significantly declined. The frequency of $CD4^+$ T cells increased with age whereas that of $CD8^+$ T cells decreased, resulting in age-associated increase of CD4/CD8 ratio. The subsets of B cells did not show any significant relationship with age. Although there were variations between the male and female subgroups in effect size of aging, the trends were in the same direction in all the parameters. Reduced fat intake was associated with the lower frequency of $CD4^+$ T cells, and higher serum cotinine level was associated with higher CD4/CD8 ratio. The results indicate that cellular immunity in the Indian population is affected with aging, while the humoral immunity is less susceptible to aging.

1. Introduction

Humans are regularly exposed to a plethora of infectious agents like bacteria, viruses, fungi, and parasites, resulting in an infection that can be asymptomatic or that can lead to a spectrum of diseases. The cellular and humoral immune responses play a critical role in detecting and clearing pathogens [1]. The intensity of the immune response is highly variable between individuals and is influenced by several genetic and non-genetic factors, including age, gender, metabolic, and nutritional factors [1]. Among these, age is one of the most well-known factors and a decline in immune function with age is known as ‘immunosenescence’ which is characterised by an impairment of different immunological parameters, including adaptive and innate immune cells [2, 3].

Low levels of natural killer (NK) cell activity have been observed in the elderly and those with autoimmune diseases and cancer. In contrast, the elderly, where the NK cells are well preserved both in numbers and function, help them lead healthier and longer lives [4]. Animal and some human studies have shown that there is an alteration in cell numbers and activity of T cell sub populations ($CD4^+$ and $CD8^+$ T cells) with age. The level of interferon (IFN)-γ generally tends to decrease with age [5]. The decline in both qualitative and quantitative levels of immune response with aging results in increased susceptibility to infection compromises the ability to fight infection and increases mortality, malignancies and autoimmune diseases [6].

Besides age, sex-specific differences in immune response have also been previously observed for many bacterial and viral pathogens. It has been shown that geographical variation can influence immune response, as observed in studies conducted in Austria, France, Spain, Italy, Japan, and India [7–11]. However, multiple immune parameters which include both acquired and innate immune cells at rural location in different age groups in Western India has not been well studied.

This study was aimed to obtain the baseline information about immune parameters, including NK cells and subsets of T cells and B cells in healthy rural Indian adults aged between 20 and 80 years old, and to see age-associated changes in the composition. Further, we assessed their variations by sex and lifestyle factors including dietary pattern and nicotine intake. The study will provide key new findings that will help evaluate dietary intervention such as functional foods to improve immune functions in India.

2. Materials And Methods

2.1. Study Site and Participants

The study was conducted in 22 villages in Pune district covered by Vadu Health and Demographic Surveillance System (HDSS) of KEM Hospital Research Centre (Pune, Maharashtra, India) from September 2017 to January 2018 as an observational study. The study area was primarily rural but also included a few villages that had undergone semi-urbanization. Participants were randomly selected through stratification according to sex and age categories (21–30, 31–40, 41–50, 51–60, 61–70, and 71–80 years) to achieve a minimum of 60 eligible ones. The participants were excluded if they had any known/reported allergy or hypersensitivity to specific medicines and foods, acute or chronic illness, or had a high fever of 38°C at blood sampling. Out of the total 70 participants enrolled in the study, 67 participants were eligible for analysis, and the age-stratified number was as follow: 11 participants (5 females, 6 males) were in the age group of 21–30 years, 13 in 31–40 years (7 females, 6 males), 11 in 41–50 years (6 females, 5 males), 12 in 51–60 years (5 females, 7 males), 10 in 61–70 years (5 females, 5 males), and 10 in 71–80 years (5 females, 5 males). The participants were asked to fill up the questionnaire that asked about taking diet low in salt or fat (Yes/No) and taking fermented foods (Yes/No).

2.2. Blood Collection

Standard phlebotomy protocol was followed for blood sample collection, ensuring the safety of study participants as well as the team member. Venous blood (25–30 ml) was collected in heparin-containing sterile tubes (BD Biosciences; San Jose, CA, USA). The whole blood sample was immediately shipped to the National Centre for Cell Science, Pune, India at 2–8°C in an insulated box for processing and analysis.

2.3. Isolation of PBMCs and NK cells

PBMCs were isolated using Lymphoprep and SepMate PBMC isolation tubes (Stem Cell Technology; Vancouver, Canada) as per the manufacturer’s guidelines. NK cells from the blood were purified using the RosetteSep antibody cocktail (Stem Cell Technologies) as per manufacturer’s guidelines. Purified NK cells were used for the assays testing NK cell activity.

2.4. Analysis of NK Cell Activity

NK cell activity was measured in terms of the cytolytic function and degranulation potential. NK cell cytolityc activity on K562 target cells was measured by the CFSE-based assay (direct assay) [12]. The

degranulation potential of NK cells after co-culturing with K562 target cells was measured by the CD107-based degranulation assay (indirect assay)[12, 13]. In a comparison of the results between the two assays, there was a significant positive linear correlation (Figure S1; ρ = 0.523, $p < 0.001$).

2.4.1. Direct Assay

Human leukemia K562 cell line was used as a target for NK cell lytic activity. K562 cell line was cultured in RPMI-1640 medium (HiMedia; Mumbai, India) supplemented with 10% FBS and antibiotics (Penicillin 100U/ml, Streptomycin 100 mg/ml) in humidified 5% CO_2 incubator at 37°C. K562 cells were washed with PBS and stained with 5 μM CFSE (Thermo Fisher Scientific; Waltham, MA, USA). Freshly- isolated NK cells (5×10^4 cells/well) as effector cells and CFSE-labelled K562 cells as target cells were co-incubated at effector/target (E/T) ratio of 10:1 at 37°C in U-bottomed 96 well plates. After 5 hours of incubation at 37°C, cells were harvested and stained with APC-conjugated anti-human CD56 antibody (clone NCAM; BioLegend; San Diago, CA, USA), eFluor 450-conjugated anti-human CD3ε (clone UCHT1; BioLegend) and 7AAD (Sigma Aldrich; St. Louis, MO, USA) for 30 minutes in the dark. Cells were washed, and apoptosis of CFSE-labelled K562 cells was analysed using Flow cytometry (FACS Canto II; BD Biosciences). Increased frequency of $7\text{-AAD}^+\text{CFSE}^+$ cells represent apoptosis of the cells. Data were analysed after gating on $\text{CFSE}^+\text{CD56}^-\text{CD3}\varepsilon^-$ cells using FlowJo software (Tree Star; Ashland, OR, USA).

2.4.2. Indirect Assay

Freshly isolated NK cells were used as effector cells and K562 cells as target cells. Effector and target cells were co-incubated at E:T ratio of 10:1 for 4 hours at 37°C. PE-conjugated anti-human CD107a antibody (10 μg/ml; clone H4A3; BioLegend) was added in the culture. Phorbol-12-myristate-13-acetate (50 ng/ml; Sigma Aldrich) and ionomycin (250 ng/ml; Sigma Aldrich) were used as a positive control whereas medium alone was used as unstimulated control. After stimulation, cells were stained with APC-conjugated anti-human CD56 antibody, Alexa Fluor 488-conjugated anti-human CD3ε antibody. Cells were washed with cold medium and acquired using flow cytometry (FACS Canto II, BD Bioscience), and analysed after gating of $\text{CD56}^+\text{CD3}\varepsilon^-$ cells using FlowJo software. Higher $\text{CD107}^+\text{CD56}^+$ cells indicate increased degranulating NK cells.

2.5. Analysis of T Cells and Intracellular IFN-γ Staining

PBMCs (1×10^6 cells) were stimulated in 1 ml complete RPMI 1640 medium in the presence of cell stimulation cocktail containing protein transport inhibitor cocktail (BioLegend) in 24 well plates. Plates were incubated in 5% CO_2 incubator at 37°C for 6 hours. Cells were harvested, surface stained for Alexa Fluor 488 anti-human CD3ε, BV421 anti-human CD4 (clone OKT4; BioLegend), and PE/Cy7 anti-human CD8 (clone SK1; BioLegend) on ice for 30 minutes. After washing with PBS, cells were fixed and permeabilised using Fix/Perm Buffer (BioLegend) as per manufacturer's guidelines and stained with PE anti-human IFN-γ (clone 4S.B3; BioLegend). Cells were acquired using FACS Canto II and analysed using FlowJo software.

2.6. Analysis of B Cells

PBMCs (1×10^6 cells) were surface stained with APC-conjugated anti-human CD138 (clone MI15; BioLegend), APC-Cy7-conjugated anti-human CD19 (clone HIB19; BioLegend) BV510-conjugated anti-human CD27 (clone O323, BioLegend), BV421-conjugated anti-human CD38 (clone HB-7; BioLegend), PE-conjugated anti-human IgG (clone M1310G05; BioLegend), and Dylight 488-conjugated anti-human IgA antibody (Abcam; Cambridge, UK). Cells were washed, fixed with 1% paraformaldehyde solution and acquired using FACS Canto II. Total $CD19^+CD27^+CD38^{hi}CD138^+$ plasma cells, $CD19^+CD27^+CD38^+CD138^-$ plasmablast cells, $CD19^+IgA^+$ B cells, and $CD19^+IgG^+$ B cells were analysed.

2.7. Measurement of Serum Cotinine Level

Serum cotinine level was measured using Cotinine ELISA Kit (cat. no. KA0930; Abnova; Taipei, Taiwan) according to manufacturer's guidelines.

2.8. Statistical Analysis

The statistical analysis was performed in R v. 3.4.1 software (R Foundation for Statistical Computing; Vienna, Austria). There was neither formal sample size calculation nor hypothesis testing for the study. All the study parameters, including immunological parameters are presented as descriptive statistics (Table 1). It includes mean, standard error of the mean, median, minimum, maximum, and 95% confidence interval (CI) for the parameters. Further stratification by different age groups was also performed for the parameters. R package glm was used for linear regression analysis. The linear regression analysis used the age groups (categories were converted to numbers from 1 to 6 in age order) as a sole explanatory variable; and each of the immunological parameters as a response variable. The fixed effect size of explanatory variable, and its 95% CI and *p*-value: Pr(>|t|) were estimated in total 67 subjects and in sex subgroups. All statistical tests were performed at the 0.050 level of significance. All *p*-values were rounded to three decimal places and are presented as "$p < 0.001$" if they were below 0.001 after rounding. Owing to observational character of the study, no correction for multiplicity testing was applied.

Table 1
Immunological parameters of the study population.

Item	Statistics	Female (*n* = 33)	Male (*n* = 34)
$CD3^{-}CD56^{+}$ NK cells (%)	Mean ± SD	13.03 ± 7.20	13.83 ± 8.19
	95% CI	[10.48, 15.58]	[10.98, 16.69]
	Median (min, max)	11.3 (2.4, 32.9)	12.6 (0.5, 33.7)
$CD3^{-}CD56^{+}$ NK cells	Mean ± SD	6820.2 ± 4687.9	6568.8 ± 4561.3
(counts/10^5 PBMCs)	95% CI	[5158.0, 8482.5]	[4977.3, 8160.3]
	Median (min, max)	5481 (467, 18540)	5481 (480, 19264)
$CFSE^{+}7AAD^{+}$ target cells (%)	Mean ± SD	7.38 ± 4.45	5.89 ± 3.16
	95% CI	[5.80, 8.96]	[4.77, 7.02]
	Median (min, max)	5.7 (2.0, 21.9)	5.1 (1.6, 16.6)
$CD107a^{+}CD56^{+}$ NK cells (%)	Mean ± SD	8.89 ± 5.48	9.66 ± 6.01
	95% CI	[6.94, 10.83]	[7.56, 11.76]
	Median (min, max)	6.9 (2.4, 27.1)	8.25 (1.1, 26.8)
$CD3^{+}CD4^{+}$ T cells (%)	Mean ± SD	51.95 ± 11.37	50.26 ± 9.47
	95% CI	[47.92, 55.98]	[46.95, 53.56]
	Median (min, max)	52.6 (27.4, 75.2)	50.35 (33.1, 71.9)
$CD3^{+}CD8^{+}$ T cells (%)	Mean ± SD	27.61 ± 9.46	28.98 ± 10.29
	95% CI	[24.26, 30.97]	[25.39, 32.57]
	Median (min, max)	26.9 (10.0, 51.8)	30.7 (8.6, 46.2)
CD4/CD8 ratio	Mean ± SD	2.28 ± 1.51	2.17 ± 1.50
	95% CI	[1.74, 2.82]	[1.65, 2.70]
	Median (min, max)	1.88 (0.59, 7.37)	1.70 (0.72, 8.12)
IFN-$\gamma^{+}CD4^{+}$ T cells (%)	Mean ± SD	13.27 ± 5.66	13.01 ± 5.64

Item	Statistics	Female (*n* = 33)	Male (*n* = 34)
	95% CI	[11.26, 15.28]	[11.04, 14.98]
	Median (min, max)	11.9 (5.1, 30.5)	12.4 (4.9, 28.7)
IFN-γ^{+}CD8^{+} T cells (%)	Mean ± SD	41.38 ± 16.49	41.67 ± 15.97
	95% CI	[35.54, 47.23]	[36.10, 47.25]
	Median (min, max)	38.9 (11.0, 72.6)	42.9 (13.2, 76.5)
CD19^{+} B cells (%)	Mean ± SD	16.01 ± 5.72	13.68 ± 6.14
	95% CI	[13.98, 18.04]	[11.53, 15.82]
	Median (min, max)	15.3 (7.2, 33.6)	11.6 (2.4, 26.5)
CD38^{+}CD138^{+} Plasma cells (%)	Mean ± SD	59.24 ± 10.32	60.50 ± 10.40
	95% CI	[55.45, 63.03]	[56.75, 64.25]
	Median (min, max)	59.9 (42.3, 78.8)	60.0 (41, 82)
CD38^{+}CD138^{-} Plasmablast cells (%)	Mean ± SD	40.75 ± 10.32	39.49 ± 10.40
	95% CI	[36.96, 44.53]	[35.74, 43.24]
	Median (min, max)	40.1 (21.2, 57.7)	40.0 (18.0, 59.0)
IgG^{+}CD19^{+} B cells (%)	Mean ± SD	7.17 ± 3.60	6.33 ± 3.15
	95% CI	[5.85, 8.49]	[5.19, 7.47]
	Median (min, max)	6.1 (2.0, 15.0)	5.8 (2.6, 17.2)
IgA^{+}CD19^{+} B cells (%)	Mean ± SD	7.47 ± 4.55	6.97 ± 3.33
	95% CI	[5.80, 9.14]	[5.77, 8.18]
	Median (min, max)	6.5 (2.8, 24.8)	6.5 (1.7, 15.2)

3. Results

3.1. Age-associated Changes in NK Cells

Among 70 participants initially enrolled in the study, 67 participants (33 females and 34 males) were eligible for the analysis. Blood sample was collected from each participant and analysed for specific immune parameters. The descriptive statistics of entire baseline data of the immune parameters are represented in Table 1. We first analysed impact of aging on NK cells by linear regression analysis. There were significant age-related increases of $CD3^{-}CD56^{+}$ NK cells both in percentage (estimate, 1.86; $p < 0.001$; Figure 1a) and absolute number in the peripheral blood mononuclear cells (PBMCs) (estimate, 972.8; $p = 0.003$; Figure 1b). As summarised in **Table S1**, these trends were stronger in the male subgroup (estimate, 2.73 and 1279.4, respectively) than the female subgroup (estimate, 0.94 and 652.5, respectively). The cytolytic function and degranulation potential of NK cells were measured by the carboxy-fluorescein succinimidyl ester (CFSE)-based assay (direct assay) [12] and the CD107-based degranulation assay (indirect assay) [12, 13], respectively. In the direct assay, there was no significant age-associated change in the NK cytolytic activity against K562 cells (Figure 1c). In the indirect assay, it was observed that degranulation potential represented by the percentage of $CD107a^{+}CD56^{+}$ NK cells was significantly reduced with age (estimate, −0.88; $p = 0.034$; Figure 1d), although the trend diminished in both male and female subgroups **(Table S1)**.

3.2. Age-associated Changes in T Cell Subsets

The linear regression analysis showed a significant increase in the percentage of $CD3^{+}CD4^{+}$ T cells with age (estimate, 2.27; $p = 0.002$; Figure 2a), while that of $CD3^{+}CD8^{+}$ T cells significantly declined (estimate, −3.04; $p < 0.001$; Figure 2b), resulting in an increase in CD4/CD8 ratio (estimate, 0.43; $p < 0.001$; Figure 2c). This trend was common to both male and female subgroups (**Table S1**). IFN-γ is a mediator of the cell-mediated immune response of $CD4^{+}$ T and $CD8^{+}$ T cells. The mean percentage of IFN-$\gamma^{+}CD4^{+}$ T cells was stable with age (Figure 2d), while that of IFN-$\gamma^{+}CD8^{+}$ T cells significantly increased with age (estimate, 5.05; $p < 0.001$; Figure 2e), which was common in both male and female subgroups (**Table S1**).

3.3. Age-associated Changes in B Cell Subsets

With respect to the frequency of total B cells, plasma cells, plasmablast cells, $IgG^{+}CD19^{+}$ B cells, and $IgA^{+}CD19^{+}$ B cells, no significant age-associated change was observed in either of total subjects and the sex subgroups (Figure 3 and **Table S1**).

3.4. Effect of Lifestyle Factors on Immune Parameters

We evaluated lifestyle factors affecting immune parameters. The questionnaire data that asked the subjects about taking diet low in salt or fat (Yes/No), and taking fermented foods (Yes/No) was used for the analysis. We observed a significant relationship only between fat intake and $CD3^{+}CD4^{+}$ T cells (estimate, −7.17; $p = 0.010$; Figure 4a), showing that individuals having diet low in fat had significant reduced frequency of $CD3^{+}CD4^{+}$ T cells in the PBMCs. The impact of smoking and chewing tobacco was also evaluated. We measured the metabolite of nicotine, cotinine levels in the serum of individuals, and

the data of < 20 ng/ml was regarded as 'low' and that of ≥ 20 ng/ml was as 'high', respectively. Our results showed that high serum cotinine level is significantly associated with increased frequency of $CD3^+CD4^+$ T cells (estimate, 5.42; $p = 0.034$; Figure 4b), reduced frequency of $CD3^+CD8^+$ T cells (estimate, −5.57; $p = 0.021$; Figure 4c), and higher CD4/CD8 ratio (estimate, 0.75; $p = 0.042$; Figure 4d).

4. Discussion

In this study, we assessed age-associated immune changes in the rural population in Maharashtra state of India. Several parameters of cellular immunity were found to be affected by aging, especially in frequency and activity of NK cell and T cell sub populations (Figure 1 and 2). We could not observe any significant age-associated changes in any parameters of humoral immunity (Figure 3).

The $CD3^-CD56^+$ NK cells represent 5–10% in the PBMCs [14]. Saxena et al. [9] previously reported the value of the healthy Indian populations ($n = 501$) was 12.44 ± 7.55% (Mean ± SD) in the PBMCs, and our results were consistent with those observations (Table 1). We found an increased number but reduced functional capacity of NK cells with aging (Figure 1b and 1d). Similar results have been observed in the studies done in multiple countries where the number of NK cells significantly increased with age in the Spanish, French, and Australian populations, but not in the Austrian population [10, 15]. There were few studies focusing on functional capacities of NK cells, and Hazeldine et al. reported higher NK cell activity in the healthy young subjects (20–35 years) than the old ones (61–91 years) [16]. Healthy lifestyles such as no-smoking, doing physical exercise, and ingestion of probiotics as a dietary supplement showed significantly higher NK cell activity [17, 18]. These lifestyle factors plus geographical and genetic factors may play an important role in determining immunosenescence patterns between populations.

There was a significant increase in the proportion of $CD4^+$ cells and decrease in the proportion of $CD8^+$ cells (Figure 2a and 2b). Although we did not analyse the feature of decreased $CD8^+$ T cells in the study, it has been reported that the decline in $CD8^+$ T cells is more prominent in TCRαβ than in the TCRγδ subsets [15]. Previous studies have also shown that there is a decrease in the percentage of naïve T cells and an increase in the percentage of effector memory T cells with aging [19]. We also found a decreased number but augmented functional ability of $CD8^+$ cells (Figure 2b and 2e). The age-associated changes in NK cells and T cells might be compensated by number and function to maintain homeostasis of the cellular immunity.

This study showed a significant age-associated increase of CD4/CD8 ratio in healthy Indian rural population (Figure 2c). Hirokawa et al., also reported CD4/CD8 ratio showed a distinct age-related increase in the Japanese population [5]. These results indicates that the increase of CD4/CD8 ratio could be a potential marker of immunosenescence. It has been reported that T cell subsets such as $CD4^+$ and $CD8^+$ T cells show a wide range of frequency distribution in the different states of India at different geographical locations across the country[9]. The populations living in the Northern (Uttar Pradesh, Punjab, and Delhi) and Western (Maharashtra) states showed higher mean values of CD4/CD8 ratio (1.24 and above) as compared to the Southern states (0.91 in Tamil Nadu and 0.99 in Kerala). This observation

was supported by another study in Rajasthan, a Northern state of India, reporting that CD4/CD8 ratio was 1.43 ± 0.56 in males and 1.78 ± 0.76 in females [20]. The study area was located in Maharashtra, the Western state of India, and the mean value of CD4/CD8 ratio was higher than those previous studies, indicating that the results might reflect the geographic or genetic variation in India.

Lifestyle variations between individuals have impacts on immune system and immune defence mechanism. The two important concepts have been evolved through several epidemiological surveys: contributions of environment (hygiene hypothesis) and of diet (dietary hypothesis) on immune parameters of individuals. It has been shown that dietary salt intake has an impact on adaptive $CD4^+$ T cells such as inflammatory Th17 [21, 22], regulatory $CD4^+$ T cells [23], and innate immune cells including macrophages [24, 25] and neutrophils [26], and excessive salt intake is now recognised as a potential modulator of inflammatory and autoimmune diseases [27]. Dietary lipid administration also has been reported to modulate immune parameters [28]. Our results showed that the subgroup reducing fat in diet showed a lower mean frequency of circulating $CD4^+$ T cells in the PBMCs (Figure 4a). Dietary fat intake is directly correlated with gut microbiota and alteration in type and amount of the fat intake may lead to dysbiosis and inflammatory response [29–32]. Given that $CD4^+$ T cells increased with aging (Figure 2a), excessive fat intake can be a factor to accelerate this age-associated change. Smoking and chewing tobacco alters the innate and adaptive immune systems, and affect immune defence mechanism [33, 34]. High nicotine intake in the form of smoking or chewing tobacco is known to have an impact on development of several neurodegenerative diseases and cancers [34–36]. We observed that the level of serum cotinine, a metabolite of nicotine, related with the altered immune parameters with respect to $CD4^+$ and $CD8^+$ T cells (Figure 2a and 2b), which was concordant with the previous results in the Indian population [9]. The role of $CD8^+$ T cells has been well documented in infectious diseases including viral infection and cancer[37], and nicotine is known to ablate the function of $CD8^+$ T cells to fight cancer [38]. Considering that $CD8^+$ T cells reduced with aging (Figure 2b), tobacco consumption can be regarded as a factor to the age-related change of $CD8^+$ T cells.

Although there were some variations between the male and female subgroups in the effect size of aging, the trends were in the same direction in all the immune parameters analysed **(Table S1)**. Steroid hormones affect both humoral and cellular immune responses, and estrogen is an important factor for different immunological response between male and female [39]. In general, while estrogen action increases immune response [40], it has been shown that mitochondrial oxidative stress is higher in male than in female and that higher levels of estrogens in females protects them against age-related decline of the immune response by up-regulating the expression of antioxidants and longevity-related genes [41]. Yan et al. reported the ratio of activated B cells ($CD20^+CD69^+$ B cells) in the Australian populations significantly decreased with age only in the male subgroup but not in the female one [15]. Our study did not show any significant variations in the B cell subsets with age and sex (Figure 3), and further investigations are necessary to obtain consistent results on sex differences in immune status.

The study had several limitations in its study design. We enrolled 67 subjects, but the sample size became much smaller when stratified according to their age (around 10 subjects in each age category). Another limitation lies in the fact that the participants reside in nearby area, and the results might contain regional characteristics of the study area. We used the qualitative questionnaire to collect the participants' dietary information, but quantitative assessment would be necessary to more specifically determine effects of dietary factors. Further investigations, involving a larger sample size across multiple regions, would provide more concrete information on immunosenescence and related factors (sex and lifestyle) in the Indian population.

5. Conclusions

The study provided evidences for immunosenescence in the Indian population. The cellular immunity was found to be affected by aging and some lifestyle factors of fat intake and tobacco consumptions, while the humoral immunity was not significantly affected by either of age, sex, and lifestyle factors.

Declarations

Institutional Review Board Statement: The study was conducted according to ICH-GCP and Indian ethical guidelines, and approved by Ethics Committees of the KEM Hospital Research Centre, Pune, Maharashtra, India (Reference No. 1720) and the National Centre for Cell Science, Pune, Maharashtra, India (Reference No. NCCS/IEC/2017-II/1).

Informed Consent Statement: Written informed consent was obtained from all the study participants involved in the study.

Acknowledgments: We gratefully acknowledge all the subjects for their participation in the study. We also acknowledge the field research assistants and the laboratory technicians of Vadu HDSS of the KEM Hospital Research Centre for their work on data collection and blood sample collection, respectively.

Funding statement: This study received funding from Yakult Honsha Co., Ltd. (Minato-ku, Tokyo, Japan).

Conflicts of Interest: G.L. received an honorarium from the National Centre for Cell Science for giving the technical services to the KEM Hospital Research Centre. K.M. and T.H. are employees of the Yakult Central Institute of Yakult Honsha Co., Ltd. N.H. is an employee of Yakult Danone India Pvt. Ltd. Va.P., M.G. and G.P. are employee of DiagnoSearch Life Science Pvt. Ltd. All authors declare no other competing interest.

Author Contributions: S.J., T.H., N.H., K.M., and G.L. led to the development of the study. D.A., P.L., A.K., A.B., S.J. recruited the participants and collected the blood from the participants. S.P., V.Pi., and G.L. performed all the laboratory tests and collected the primary data. D.A., K.M., T.H., and V.Pa. performed the statistical tests. M.G. and G.P. provided medical review. D.A., K.M., T.H., N.H., and G.L. wrote the manuscript. All authors have reviewed and approved the present version of the manuscript.

References

[1] K. Shida, K. Nomoto, Probiotics as efficient immunopotentiators: translational role in cancer prevention, Indian J Med Res, 138 (2013) 808-814.

[2] R. Solana, G. Pawelec, R. Tarazona, Aging and innate immunity, Immunity, 24 (2006) 491-494.

[3] T. Fulop, A. Larbi, K. Hirokawa, A.A. Cohen, J.M. Witkowski, Immunosenescence is both functional/adaptive and dysfunctional/maladaptive, Seminars in immunopathology, (2020).

[4] L. Kaszubowska, A. Dettlaff-Pokora, L. Hak, M. Szarynska, M. Ryba, J. Mysliwska, A. Mysliwski, Successful ageing of nonagenarians is related to the sensitivity of NK cells to activation, J Physiol Pharmacol, 59 Suppl 9 (2008) 187-199.

[5] K. Hirokawa, M. Utsuyama, Y. Hayashi, M. Kitagawa, T. Makinodan, T. Fulop, Slower immune system aging in women versus men in the Japanese population, Immun Ageing, 10 (2013) 19.

[6] T. Fulop, G. Dupuis, J.M. Witkowski, A. Larbi, The Role of Immunosenescence in the Development of Age-Related Diseases, Rev Invest Clin, 68 (2016) 84-91.

[7] A. Santagostino, G. Garbaccio, A. Pistorio, V. Bolis, G. Camisasca, P. Pagliaro, M. Girotto, An Italian national multicenter study for the definition of reference ranges for normal values of peripheral blood lymphocyte subsets in healthy adults, Haematologica, 84 (1999) 499-504.

[8] S.S. Uppal, S. Verma, P.S. Dhot, Normal values of CD4 and CD8 lymphocyte subsets in healthy indian adults and the effects of sex, age, ethnicity, and smoking, Cytometry B Clin Cytom, 52 (2003) 32-36.

[9] R.K. Saxena, V. Choudhary, I. Nath, S.N. Das, R.S. Paranjape, G. Babu, S. Ramlingam, D. Mohanty, H. Vohra , M. Thomas, Q.B. Saxena, N.K. Ganguly, Normal ranges of some select lymphocyte sub-populations in peripheral blood of normalhealthy Indians, Current Science, 86 (2004) 969-975.

[10] M.P. Vasson, M.C. Farges, N. Goncalves-Mendes, J. Talvas, J. Ribalta, B. Winklhofer-Roob, E. Rock, A. Rossary, Does aging affect the immune status? A comparative analysis in 300 healthy volunteers from France, Austria and Spain, Immun Ageing, 10 (2013) 38.

[11] P. Kanodia, G. Kaur, P. Coshic, K. Chatterjee, T. Neeman, A. George, S. Rath, V. Bal, S.B. Prabhu, Characterization of biological variation of peripheral blood immune cytome in an Indian cohort, Scientific reports, 9 (2019) 14735.

[12] E. Aktas, U.C. Kucuksezer, S. Bilgic, G. Erten, G. Deniz, Relationship between CD107a expression and cytotoxic activity, Cell Immunol, 254 (2009) 149-154.

[13] G. Alter, J.M. Malenfant, M. Altfeld, CD107a as a functional marker for the identification of natural killer cell activity, Journal of immunological methods, 294 (2004) 15-22.

[14] S. Paul, G. Lal, The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy, Frontiers in immunology, 8 (2017) 1124.

[15] J. Yan, J.M. Greer, R. Hull, J.D. O'Sullivan, R.D. Henderson, S.J. Read, P.A. McCombe, The effect of ageing on human lymphocyte subsets: comparison of males and females, Immun Ageing, 7 (2010) 4.

[16] J. Hazeldine, P. Hampson, J.M. Lord, Reduced release and binding of perforin at the immunological synapse underlies the age-related decline in natural killer cell cytotoxicity, Aging Cell, 11 (2012) 751-759.

[17] Y. Kusaka, H. Kondou, K. Morimoto, Healthy lifestyles are associated with higher natural killer cell activity, Prev Med, 21 (1992) 602-615.

[18] M. Reale, P. Boscolo, V. Bellante, C. Tarantelli, M. Di Nicola, L. Forcella, Q. Li, K. Morimoto, R. Muraro, Daily intake of Lactobacillus casei Shirota increases natural killer cell activity in smokers, Br J Nutr, 108 (2012) 308-314.

[19] J. Nikolich-Zugich, The twilight of immunity: emerging concepts in aging of the immune system, Nat Immunol, 19 (2018) 10-19.

[20] A. Rungta, S. Hooja, N. Vyas, S. Rishi, A. Rao, A. Gupta, Enumeration of CD 4 and CD 8 T lymphocytes in healthy HIV seronegative adults of northwest India: A preliminary study, Indian J Pathol Microbiol 51 (2008) 127-129.

[21] M. Kleinewietfeld, A. Manzel, J. Titze, H. Kvakan, N. Yosef, R.A. Linker, D.N. Muller, D.A. Hafler, Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells, Nature, 496 (2013) 518-522.

[22] C. Wu, N. Yosef, T. Thalhamer, C. Zhu, S. Xiao, Y. Kishi, A. Regev, V.K. Kuchroo, Induction of pathogenic TH17 cells by inducible salt-sensing kinase SGK1, Nature, 496 (2013) 513-517.

[23] A.L. Hernandez, A. Kitz, C. Wu, D.E. Lowther, D.M. Rodriguez, N. Vudattu, S. Deng, K.C. Herold, V.K. Kuchroo, M. Kleinewietfeld, D.A. Hafler, Sodium chloride inhibits the suppressive function of FOXP3+ regulatory T cells, J Clin Invest, 125 (2015) 4212-4222.

[24] W.C. Zhang, X.J. Zheng, L.J. Du, J.Y. Sun, Z.X. Shen, C. Shi, S. Sun, Z. Zhang, X.Q. Chen, M. Qin, X. Liu, J. Tao, L. Jia, H.Y. Fan, B. Zhou, Y. Yu, H. Ying, L. Hui, X. Liu, X. Yi, X. Liu, L. Zhang, S.Z. Duan, High salt primes a specific activation state of macrophages, M(Na), Cell Res, 25 (2015) 893-910.

[25] K.J. Binger, M. Gebhardt, M. Heinig, C. Rintisch, A. Schroeder, W. Neuhofer, K. Hilgers, A. Manzel, C. Schwartz, M. Kleinewietfeld, J. Voelkl, V. Schatz, R.A. Linker, F. Lang, D. Voehringer, M.D. Wright, N. Hubner, R. Dechend, J. Jantsch, J. Titze, D.N. Muller, High salt reduces the activation of IL-4- and IL-13-stimulated macrophages, J Clin Invest, 125 (2015) 4223-4238.

[26] K. Jobin, N.E. Stumpf, S. Schwab, M. Eichler, P. Neubert, M. Rauh, M. Adamowski, O. Babyak, D. Hinze, S. Sivalingam, C. Weisheit, K. Hochheiser, S.V. Schmidt, M. Meissner, N. Garbi, Z. Abdullah, U. Wenzel, M. Holzel, J. Jantsch, C. Kurts, A high-salt diet compromises antibacterial neutrophil responses through hormonal perturbation, Sci Transl Med, 12 (2020).

[27] N. Wilck, M.G. Matus, S.M. Kearney, S.W. Olesen, K. Forslund, H. Bartolomaeus, S. Haase, A. Mahler, A. Balogh, L. Marko, O. Vvedenskaya, F.H. Kleiner, D. Tsvetkov, L. Klug, P.I. Costea, S. Sunagawa, L. Maier, N. Rakova, V. Schatz, P. Neubert, C. Fratzer, A. Krannich, M. Gollasch, D.A. Grohme, B.F. Corte-Real, R.G. Gerlach, M. Basic, A. Typas, C. Wu, J.M. Titze, J. Jantsch, M. Boschmann, R. Dechend, M. Kleinewietfeld, S. Kempa, P. Bork, R.A. Linker, E.J. Alm, D.N. Muller, Salt-responsive gut commensal modulates TH17 axis and disease, Nature, 551 (2017) 585-589.

[28] M.A. de Pablo, M. Angeles Puertollano, G. Alvarez de Cienfuegos, Immune cell functions, lipids and host natural resistance, FEMS Immunol Med Microbiol, 29 (2000) 323-328.

[29] P.D. Cani, R. Bibiloni, C. Knauf, A. Waget, A.M. Neyrinck, N.M. Delzenne, R. Burcelin, Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice, Diabetes, 57 (2008) 1470-1481.

[30] P.J. Turnbaugh, F. Backhed, L. Fulton, J.I. Gordon, Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome, Cell host & microbe, 3 (2008) 213-223.

[31] M.D. Trottier, R. Irwin, Y. Li, L.R. McCabe, P.J. Fraker, Enhanced production of early lineages of monocytic and granulocytic cells in mice with colitis, Proceedings of the National Academy of Sciences, 109 (2012) 16594-16599.

[32] G. Jakobsdottir, J. Xu, G. Molin, S. Ahrne, M. Nyman, High-fat diet reduces the formation of butyrate, but increases succinate, inflammation, liver fat and cholesterol in rats, while dietary fibre counteracts these effects, PLoS ONE, 8 (2013) e80476.

[33] W.H. Piao, D. Campagnolo, C. Dayao, R.J. Lukas, J. Wu, F.D. Shi, Nicotine and inflammatory neurological disorders, Acta pharmacologica Sinica, 30 (2009) 715-722.

[34] F. Qiu, C.L. Liang, H. Liu, Y.Q. Zeng, S. Hou, S. Huang, X. Lai, Z. Dai, Impacts of cigarette smoking on immune responsiveness: Up and down or upside down?, Oncotarget, 8 (2017) 268-284.

[35] J.D. Johnson, D.P. Houchens, W.M. Kluwe, D.K. Craig, G.L. Fisher, Effects of mainstream and environmental tobacco smoke on the immune system in animals and humans: a review, Crit Rev Toxicol, 20 (1990) 369-395.

[36] Y. Geng, S.M. Savage, S. Razani-Boroujerdi, M.L. Sopori, Effects of nicotine on the immune response. II. Chronic nicotine treatment induces T cell anergy, J Immunol, 156 (1996) 2384-2390.

[37] A.M. van der Leun, D.S. Thommen, T.N. Schumacher, CD8(+) T cell states in human cancer: insights from single-cell analysis, Nature reviews, 20 (2020) 218-232.

[38] C.C. Cheng, H.C. Lin, Y.W. Chiang, J. Chang, Z.L. Sie, B.L. Yang, K.H. Lim, C.L. Peng, A.S. Ho, Y.F. Chang, Nicotine exhausts CD8(+) T cells against tumor cells through increasing miR-629-5p to repress IL2RB-mediated granzyme B expression, Cancer Immunol Immunother, 70 (2021) 1351-1364.

[39] D. Caruso, A.M. Barron, M.A. Brown, F. Abbiati, P. Carrero, C.J. Pike, L.M. Garcia-Segura, R.C. Melcangi, Age-related changes in neuroactive steroid levels in 3xTg-AD mice, Neurobiol Aging, 34 (2013) 1080-1089.

[40] S. Oertelt-Prigione, The influence of sex and gender on the immune response, Autoimmunity reviews, 11 (2012) A479-485.

[41] J. Vina, J. Gambini, R. Lopez-Grueso, K.M. Abdelaziz, M. Jove, C. Borras, Females live longer than males: role of oxidative stress, Current pharmaceutical design, 17 (2011) 3959-3965.

Figures

Figure 1

Age-associated changes in NK cells. Linear regression analysis to evaluate effect of aging on the percentage of $CD3^{-}CD56^{+}$ NK cells gated to total lymphocyte population (**a**), the number of $CD3^{-}CD56^{+}$ NK cells in 10^5 PBMCs. (**b**), cytolytic activity of NK cells by direct assay (**c**), and degranulation potential of NK cells by indirect assay (**d**). The linear regression curve is plotted for the data of 67 subjects (33 females: open circle, 34 males: closed triangle), and its equation and *p*-value (* $p < 0.050$, ** $p < 0.010$) for the effect size of aging are shown. The statistics of analysis are summarised in Table S1.

Figure 2

Age-associated changes in T cell subsets. Linear regression analysis to evaluate effect of aging on the frequencies of $CD4^{+}$ T cells (**a**; $CD3^{+}CD4^{+}$ T cells), $CD8^{+}$ T cells (**b**; $CD3^{+}CD8^{+}$ T cells), IFN-$\gamma^{+}CD4^{+}$ T cells (**d**) and IFN-$\gamma^{+}CD8^{+}$ T cells (**e**), and CD4/CD8 ratio (**c**). The linear regression curve is plotted for the data of 67 subjects (33 females: open circle, 34 males: closed triangle), and its equation and *p*-value (** $p < 0.010$) for the effect size of aging are shown. The statistics of analysis are summarised in Table S1.

Figure 3

Age-associated changes in B cell subsets. Linear regression analysis to evaluate effect of aging on the frequencies of total B cells (**a**; $CD19^+$ B cells), plasma cells (**b**; $CD38^+CD138^+$ Plasma cells), plasmablast cells (**c**; $CD38^+CD138^-$ Plasma cells), IgG^+CD19^+ B cells (**d**), and IgA^+CD19^+ B cells (**e**). The linear regression curve was plotted for the data of 67 subjects (33 females: open circle, 34 males: closed triangle), and its equation and *p*-value for the effect size of aging are shown. The statistics of analysis are summarised in Table S1.

Figure 4

Effect of dietary fat intake and serum cotinine on $CD4^+$ and $CD8^+$ T cells. Linear regression analysis to evaluate effect of reduce dietary fat intake on CD4 T cells (**a**), and serum cotinine levels on $CD4^+$ T cells (**b**), $CD8^+$ T cells (**c**) and ratio of CD4/CD8 T cells (**d**) in the PBMCs. The linear regression curve was plotted for the data of 67 subjects (33 females: open circle, 34 males: closed triangle), and its equation and *p*-value (* $p < 0.050$) for the effect size of the explanatory valuable are shown.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.docx
- TableS1.docx