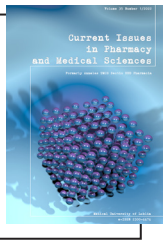
DOI: 10.2478/cipms-2024-0018 Curr. Issues Pharm. Med. Sci., Vol. 37, No. 2, Pages 110-113

Current Issues in Pharmacy and Medical Sciences
Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA

journal homepage: http://www.curipms.umlub.pl/



Seasonal changes and sex differences in peripheral blood γδ T and iNKT cells in healthy Polish adults

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ARTICLE INFO

Received 25 January 2024
Accepted 27 May 2024

Keywords:
vitamin D,
particulate matter,
seasonal changes,
iNKT,
γδ T.

ABSTRACT

Vitamin D regulates not only bone metabolism but also many other processes, including the functioning of lymphocytes. Human T cells have a nuclear receptor for vitamin D (VDR). Studies to date have shown significant seasonal variations in conventional T cell populations in humans living in temperate climates.

Objectives. The aim of the current paper was an assessment of seasonal changes of γδ T and iNKT cells in healthy individuals.

Material and methods. Peripheral blood was drawn from healthy volunteers – approx. 20 a month – and an additional cohort of 20 volunteers donated blood four times, once every four months. Percentages of γδ T and iNKT cells was assessed with flow cytometry.

Results. A pronounced accumulation of iNKT cells was noted in spring, the differences in γδ T cells were less notable. Vitamin D significantly hampers γδ T proliferation *in vitro*. Conclusions. In the presented article, we show seasonal variability within two populations of unconventional T lymphocytes – γδ T and iNKT.

INTRODUCTION

Human T cells can be divided into two subsets – conventional and unconventional. The latter comprises less than 10% and consists of γδ T, iNKT and MAIT cells [1]. Unconventional T cells share functional characteristics of conventional T cells and innate immunity, e.g. NK cells. They have fully functional TCR that can be built of either γ and δ chains for γδ T or α and β chains for iNKT and MAIT, but they remain MHC unrestricted [2-4]. With innate cells, mostly NK, unconventional T cells share the capacity for rapid response to stimuli and a plethora of activating and cytotoxicity-related receptors, e.g. NKG2D, CD226 and NKp30 [5].

Human γδ T are classically divided based on the variable fragments of the δ chain into Vδ1-Vδ5 [6]. The majority of studies have focused so far on the phosphoantigen-reactive Vδ2 population that dominates in the peripheral blood [7]. That subset can be easily and selectively expanded *in vitro* with phosphoantigen stimulation, either directly (HMBPP, BrHPP or IPP) or indirectly with N-aminobisphosphonates, most commonly zoledronate [8]. Vδ2 cells usually dominate among total γδ T lymphocytes in the peripheral blood in adults, while Vδ1 is the major subset in newborns and children. The Vδ1/Vδ2 balance switches with age towards Vδ2 domination [9].

Khoo *et al*. discovered that seasonal changes in serum vitamin D are reflected by conventional αβT CD4+ and CD8+, the numbers of which generally rise in summer [10]. It has been also previously demonstrated by Bernicke *et al*. that human γδ T cells vary significantly between seasons and that vitamin D hampers their proliferation response to phosphoantigens [11]. Moreover, phosphoantigen-dependent activation of human γδ T cells seems to significantly up-regulate the expression of the vitamin D receptor (VDR) [12]. Thus, we decided to re-evaluate the seasonal changes in γδ T cells and additionally of iNKT – another unconventional T cell subset.

AIM

We aimed to assess the seasonal variations among peripheral γδ T and iNKT cells in healthy individuals in Poland.

MATERIAL AND METHODS

Participants

Healthy volunteers of both sexes aged between 20 and 30 were recruited for the study. After signing written informed

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consent, 5 ml of peripheral blood was drawn into an EDTA-containing tube. Approx. 20 individuals were recruited each month for a whole year. Additionally, a separate 20 people had their blood drawn once every three months for a total of four blood collections. Participants were also weighed and had their height measured and were asked about whether they had COVID-19 infection and how many times they have had any symptoms of minor infection in the past 12 months. The study was approved by the Bioethical Committee of the Medical University of Lublin.

Cell isolation

Immediately following the collection, peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation. Briefly, blood was layered on top of LSM-1077 (PromoCell) and centrifuged at 600× g for 20 minutes. Next, the inter-phase was collected and washed twice in PBS.

Cell staining and cytometry

Approx. 1 million PBMC were stained with an antibody cocktail: anti-human CD3 PE-Cy5 (BioLegend, #300410, clone: UCHT1), anti-human CD3 beFluor 647 (BioEastern, Lublin, Poland, #433164, clone: UCHT1), FITC anti-human TCRγδ (BioEastern, #433721, clone: B1) and anti-human iNKT PE (BioLegend, #342904, clone: 6B11). After 20 minutes of incubation at room temperature in darkness, the cells were washed with PBS. Immediately afterwards, samples were acquired with BD FACS Canto II. Data were analyzed via FlowJo (BD Biosciences). Gating is presented in Figure 1A.

Cultures with vitamin D

Approximately 1 million PBMC was suspended in RPMI-1640 + 10% FBS + penicillin/streptomycin and placed in 24-well plates. Samples were stimulated with zoledronate (2.5 μM) + IL-2 (50 IU/ml) +/- vitamin D (10 mM) for γδ T (Vδ2) proliferation. Fresh IL-2 was added every 2 days. When necessary cells were split or had their medium exchanged. After 2 weeks, cultures were collected. Cells were labeled with anti-CD3 APC (BioLegend, #300439, clone: UCHT1) and anti-TCRγδ FITC. Counting beads (Spherotech, #ACBP-150-10) were also added. Samples were then acquired with BD FACS Canto II. Data were analyzed utilizing FlowJo.

Particulate matter (PM)

Daily concentrations of PM2.5 and PM10 measured in Lublin by the Regional Inspectorate for Environmental Protection from the time of the study were accessed via The World Air Quality Project [https://aqicn.org]. Mean values for each month were calculated.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9 (GraphPad Software) and JASP 0.17.2 (University of Amsterdam). Data distribution was assessed with the Shapiro-Wilk test. Kruskal-Wallis and U Mann-Whitney tests were used to compare the groups and to calculate p values. The Spearman test was applied to assess correlations between iNKT, γδ T, PM2.5, PM10 and biomedical parameters.

RESULTS

γδ T percentage did not differ between seasons

At first, we assessed the month-to-month and seasonal differences in γδ T percentage. As presented in Figure 1B, there were inconsistent but significant differences in month-to-month comparison, but no real differences between seasons. Similar results were obtained for the individuals who had their blood collected every three months (Figure 1D). In the latter case, some tendency for a higher percentage in spring and summer can be noted.

iNKT percentage seems to rise significantly in spring

We then assessed the iNKT cells. No differences were observed for month-to-month comparisons or for seasonal observations in the whole group (Figure 1C). Noteworthy, however, a significant expansion of iNKT cells was observed in our seasonal cohort (blood drawn every three months) (Figure 1E).

The percentage of γδ T and iNKT did not differ between biological sexes

Our group contained roughly equal numbers of men (N=99) and women (N=108). Thus, we decided to compare the γδ T and iNKT percentages between them. No significant difference was noted (Figure 1F, 1G).

Particulate matter concentration did not correlate with iNKT and γδ T percentage

No significant correlation was noted for either PM2.5 or PM10 mean monthly concentration on one hand and the percentage of either iNKT or γδ T on the other (Figure 1H, 1I).

Vitamin D significantly hampered γδ T proliferation *in-vitro*

Addition of vitamin D to zoledronate-stimulated cultures of γδ T cells led to a significant decrease in proliferation (Figure 1J).

DISCUSSION

The percentage of iNKT and γδ T varied between months, but, more importantly, significant differences were noted when those same donors were analysed 4 times a year (Figure 1D-E). Such seasonal variation in unconventional T cells in healthy donors has important potential implications e.g. study and control groups for immunological examinations should be recruited at the same time of year to prevent seasonal interference. Recruiting a study group in winter and control in summer or vice versa could potentially lead to significant differences unrelated to the disease or state studied.

Similarly to our observation of the peak in iNKT cells, Afoke *et al*., described a significant peak of T helper cells in spring and a similar, yet weaker peak in total T cells [13]. Macrophages followed closely with the lowest value for spring, but a high peak in summer [13]. Higher percentages of T helper and T cytotoxic cells in winter were also noted by Maes *et al*. [14]. Ter Horst *et al*. compared seasonal and non-seasonal factors and their influence on peripheral blood lymphocytes; even after correcting for non-seasonal factors,

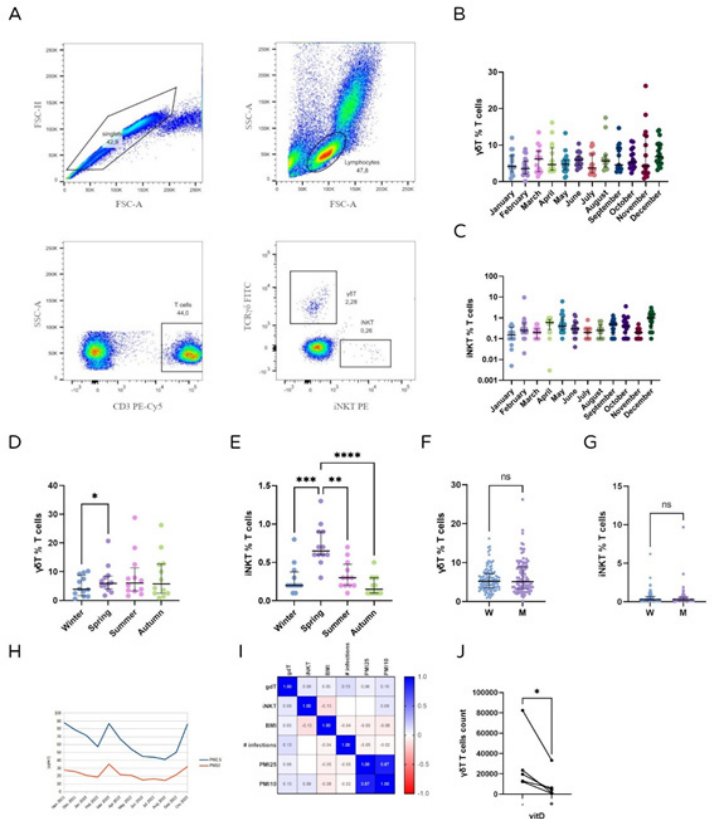
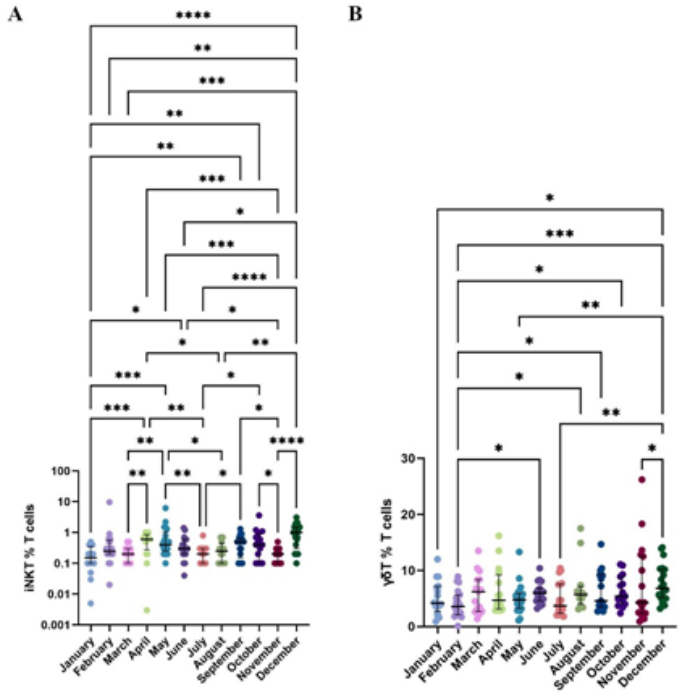


Figure 1. Panel A shows the gating strategy: first single cells were gated on FSC-H vs FSC-A, then lymphocytes were selected n FSC-A vs SSC-A. T cells were gated based on the CD3 expression, finally iNKT and γδT cells were gated among total T cells. Monthly differences are summarized in panels B and C; the majority of comparisons in both panels B and C yielded $p<0.05$ and for clarity are not summarized in the figure (full data available in Supp. Figure 1). Panels D and E present the differences in the seasonal cohort. Biological sex-related differences are presented in F and G. Panels H, I present the PM2.5 and PM10 concentration in Lublin, as well as their correlations with immunological parameters. Impact of vitamin D on γδ T proliferation is presented in panel J



Supp. Figure 1. Monthly comparisons with full spectrum of comparisons. Panel A shows differences in iNKT, while panel B in γδT cells

significant differences in T and B cells remained [15]. They noted a significant impact on the number of total T and B cells, but not on total T helper and cytotoxic T cells.

Mann *et al*. described significant seasonal differences in cytokine output in Rhesus monkeys. Furthermore, they saw a significant difference in the proliferation response of T cells (with a much better answer in Winter) [16]. This is also something we have noted in response to phosphoantigens by human γδ T cells – a somewhat better response was noted in winter [data unpublished]. Vitamin D seems to lower the output of IFN-γ and TNF in T cells in response to C. albicans [17]. In general, higher vitamin D concentration seems to favor Th2-response over Th1 and Th17 [18]. Altogether this suggests that γδ T cells for immunotherapy should in the best-case scenario be expanded from donations done in winter. This should provide us with material more suited for cellular immunotherapy – higher IFN-γ and TNF output and better *in vitro* proliferation. Importantly, serum concentration of vitamin D is usually at its lowest in early spring (March/April) [19]. Virtually all T cells express vitamin D receptor (VDR) even at the early thymocyte stage. VDR seems to be especially important for iNKT generation [20]. Indeed, children born in summer had notably lower counts of all major T cell subsets, including iNKT and γδ T [21].

As recently pointed out by Gałuszka-Bulaga *et al*., part of the seasonal variation may be related to different concentrations of particulate matter in air during winter and summer [22]. As was previously described by Williams *et al*., particulate matter concentration significantly impacts T cell proliferation in response to polyclonal stimulation [23]. Interestingly, this effect can be partially mitigated by N-acetylcysteine [24]. The exposure to particulate matter was not assessed directly in the current study, which should be considered an important limitation of the study. Nevertheless, we have noted no correlation between particulate matter concentration in Lublin and either T subset analyzed. Nevertheless, the impact of other forms of air pollution was not assessed and may be of importance.

The dose-dependent inhibition of T cell proliferation by calcitriol and other active forms of vitamin D is relatively well known [25-27]. Only recently was it first tested on unconventional T cells – Bernicke *et al*. demonstrated similar inhibition of γδ T proliferation in zoledronate-stimulated PBMC culture [11]. As demonstrated in Figure 1, we have obtained similar results.

Study limitations

The current study is based on a limited cohort of young people living in one city. Thus, some local factors might have influenced the results. Additionally, only young donors were recruited for the

study, thus it remains to be assessed whether similar trends are observed in older people. Finally, no subsets of γδ T cells were assessed.

CONCLUSIONS

Some important differences in both γδ T and iNKT cells were observed in the current study. Interestingly, γδ T cells seems to be at their lowest in winter, while iNKT cells are at their highest in spring. Those differences may be a result of numerous external factors – air pollution, sun exposure, vitamin D deficiency and supplementation, and finally infectious factors.

FUNDING

The study was funded by the National Science Center in Poland, Preludium grant number 2019/35/N/NZ6/02973.

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