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The Minor T Cell Subsets in Blood may not be Minor in Helping to Assess Human Health

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Abstract

The $CD4^+$ T cells referred to as helpers or Major Histocompatibility Complex Class II (MHCII)-responsive cells and $CD8^+$ referred to as cytolytic or MHCI-responsive T cells are the major subsets in human blood. However, minor subsets of T cells exist in blood and may have usefulness in assessing health and response to environmental stresses including exposures to pathogen, chemical, physical, or psychological stress. The minor subsets include phenotypically and functionally diverse populations of T cells not expressing CD4 or CD8 (double negative), expressing both CD4 and CD8 (double positive), and cells expressing natural killer markers (CD56 ± CD16). Some characteristics of these subsets are discussed and the levels in blood of females and males provided. Longitudinal changes of individuals were much narrower than ranges amongst all blood donors. As for the neutrophil to lymphocyte ratio, which may be diagnostic for a health problem, the ratio of subpopulations in the blood may aid diagnosis or prognosis. However, populations including double-positive, double-negative and natural killer T cells need to be more routinely reported to physicians so that results can be compared to variant conditions among patients.

Introduction

Peripheral blood populations provide a window into the immune system health of an individual and can be an important indicator of an infection, cancer, or environmental stress. Accurate measurement of lymphoid (T, B, NK, and NKT lymphocytes) as well as myeloid (granulocytes, monocytes, megakaryocytes, erythrocytes, and mast cell) lineages, including minor populations such as the Innate Lymphoid Cells (ILCs) and progenitors of lymphoid and myeloid cells by flow cytometry can aid evaluation of health and environmental influences. Our premise is the composite of all circulating cells highlights the ongoing response to the endogenous and exogenous environment, and even cell types usually at low numbers in blood can be diagnostically or prognostically informative. Blood is a relatively non-invasive isolate, yet it represents a picture of the whole organism, in that it traffics through all organs picking up or losing cells and modulators along its path. Leukocyte ratios, such as the Neutrophil to Lymphocyte Ratio (NLR) may increase because of a cancer [1-3], septicemia [4], or a physical/psychological stress on an organism [5]. An early rise in $CD3^+CD4^-CD8^-$ T cells referred to as Double Negative (DN) Ts may be more indicative of an infection than antibodies to the pathogen [6]. During certain viral infections, such as Human Immunodeficiency Virus (HIV), a dramatic drop in Single Positive (SP) $CD4^+$ T cells may be observed [7,8], but they may rise again with Highly Active Antiretroviral Therapy (HAART). Additional markers will assist quantification of lymphoid and myeloid subsets, which may aid further diagnostics and prognostic evaluations. Blood mast cell progenitors (Lin^- $CD34^{hi}CD117^{int/hi}$ $Fc\varepsilon RI^+$) may suggest allergic asthma with greater lung pathophysiology [9-11]. Increased amounts of $\gamma\delta$ T cells are more frequently observed in HIV patients (29%) compared with renal transplant patients (11%) and healthy donors (3%), and $\gamma\delta$ T cells differ based on CD8 dimers, which may be α/α, α/β, or β/β [12].The number of intermediate ($CD14^{hi}CD16^+$) monocytes in blood has been related to cardiovascular risk with chronic kidney disease [13,14]. Dendritic Cell (DC) number in blood and expression of IL-12 or IL-10 may assist evaluation of Hepatitis-C Virus (HCV) effects on the liver [15]. An abbreviated list of cell types that can be quantified in the blood includes naïve, central memory, and effector memory $CD4^+$and $CD8^+$ T cells; T helper subsets(Th1, Th2, Th9, Th17, and Th22); regulatory suppressor T cells (Treg); antibody-producing (IgM, IgA, or IgG) B cells and regulatory $CD24^{hi}CD38^{hi}$ and $CD24^{hi}CD27^+$ B cells, which both produce IL-10 [16]; Innate Lymphoid Cells (ILCs), which include Nature Killer (NK) cells, and progenitor and mature myeloid subsets, such as monocyte and DC subsets, eosinophils, basophils, neutrophils, and mast cells.

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In addition to the major lymphocyte populations of T, B, and NK cells, flow cytometric analyses have revealed the presence of several minor T cell subsets, which can be identified and quantified during routine clinical immunophenotyping procedures. These minor subsets are the DNTs mentioned earlier, double-positive T cells (DPTs or $CD3^+CD4^+CD8^+$cells), and NKTs ($CD56^+CD16^+CD3^+$). Changes in the cell counts for these minor subsets have now been associated with various disease states. Besides an increase with some early responses to pathogens, an increase in DNTs has been observed in patients with systemic lupus erythematosus [17]. Increases in DPTs have been associated with active chronic hepatitis [18] and HIV [19]. Perturbations in the numbers of NKTs have been observed in patients with allergies and asthma [20]. During recent years much more has been learned about these minor T cell subsets and their association with a few diseases. Although not routinely assayed, other elevated amounts of minor populations in the blood such as $\gamma\delta$ T cells, Innate ILCs, and progenitor subsets may assist in diagnoses. Herein, our discussion relates mainly to the analysis of the DPTs, DNTs, and NKTs and their association with a variety of disease states and presence in blood of females and males.

Thymus-derived cells (T cells) are heterogeneous and plastic due to epigenetic modulations within the thymus and thereafter. Although the T cells in the blood are grouped as helpers (Th cells), cytolytic (Tc cells), natural killer (NK) T cells, and suppressors (Treg cells), each of these populations have subpopulations with different immunophenotypes and functions. The $CD4^+$ Th subsets are closely mirrored by ILCs, which include NK cells (part of the ICL1 subset), ILC2 and ILC3 cells. ILCs are like the $CD4^+$ Th subsets regarding signaling molecules and cytokines, e.g., T-bet and interferon-gamma (IFNγ) for Th1 and NK (ILC1) cells, GATA-3 and IL-4 for Th2 and ILC2 cells, and RORγT and IL-17 for Th17 and some ILC3 cells. However, ILCs lack the antigen-specific T Cell Receptor (TCR)-CD3 complex, and thus, lack adaptive antigen-specificity, which defines them as innate.

Ontogenic development of T, B, and NK cells and ILCs begins as Common Lymphoid Progenitors (CLPs) that develop first in yolk sac followed by fetal liver and finally bone marrow. The expression of biomarkers that identify lymphocytes as T cells occurs within the thymus, the primary lymphoid organ for T cells. Progenitor T cells go through several stages as they develop within the thymus before they exit mainly as $TCR\alpha/\beta^+CD3^+CD4^+CD8^-$ or $TCR\alpha/\beta^+CD3^+CD4^-CD8^+$ cells, which are often referred to as Single Positive (SP) T cells. NK cells and other ILCs reach maturity without need for thymus, whereas NKTs, invariant (i) NKTs, and $TCR\gamma/\delta^+$ T cells initiate their development in the thymus, but they are generated at much lower numbers. T cell progenitors begin their differentiation within the thymic subcapsular area with expression of pan T cell biomarkers, such as CD5 and CD7, but they lack the surface expression of the pan T cell marker CD3 and surface receptors CD8 and CD4 used to define Major Histocompatibility Complex Class I (MHCI) and Class II (MHCII) responsive T cells, respectively. Four stages of DNTs have been differentiated. The DN1, DN2, DN3, and DN4 T cells progress in their differentiation toward the DPT cells in the cortex of the thymus (Figure 1) [21-23]. At the DN3 stage, the expression of TCR, which is either $TCR\alpha/\beta$ or $TCR\gamma/\delta$, begins with intracellular expression of the pan T cell CD3 complex. The majority of the DN4 T cells continue their development with co-expression of CD4 and CD8, the DPTs. As the cells begin to express TCR, a large proportion of the DPTs die due to "neglect" or negative selection [24,25]. To

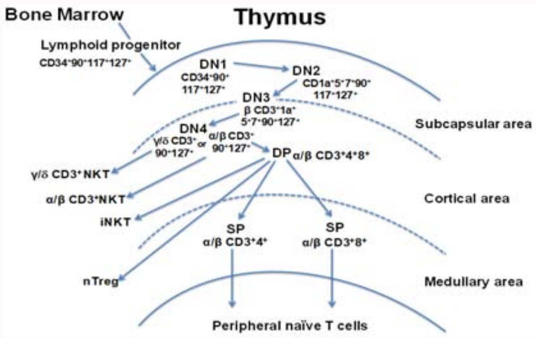

Figure 1: Ontology of T cells in the thymus. The stages in development and the immunophenotypes are presented, in part, from the descriptions in references [21-23].

enhance negative selection to self-peptides, medullary Thymic Epithelial Cells (mTECs), express autoimmune regulator (Aire), a gene that enables expression of Peripheral Tissue Antigens (PTAs) with enhancement of central tolerance to self-antigens [26]. Surviving negative selection, the DPTs receive signals from MHCI or MHCII expressing myeloid cells, the developing DPTs receive positive stimulation (thymic education) for further maturation. Dependent on their TCR specificity and affinity for peptide-altered MHCI or MHCII, the DPTs lose expression of either CD4 or CD8 based on transcription control by ThPOK and Runx3, respectively. Mature immunocompetent SP $TCR^+CD3^+CD4^+CD8^-$ and $CD4^-CD8^+$ T cells emigrate from the thymus. However, due to thymic escape and/or peripheral modulations, DNTs and DPTs exist in the periphery. Under most circumstances even considering some pathology, the numbers of non-malignant DNT and DPTs in the blood are low in comparison to the SP T cells or NK cells. With normal conditions, DPTs plus DNTs usually total <5% of the T cells in the blood. Like DNTs and DPTs, the numbers of NKTs in blood are usually low. Although the DNTs, DPTs and NKTs naturally exist in the peripheral blood and their numbers are usually low, their presence in the blood can help to define the immune status of an individual and can be indicative of a disorder. Quantification of DNTs, DPTs, and NKTs has been diagnostic for certain immunopathologies.

Although DNTs, DPTs, and NKTs have been implicated in various pathologies, they often have not been included in routine clinical laboratory analyses of T cell subsets. This is especially troublesome for DPTs, in that their percentages are occasionally added into both the SP CD4 and CD8 subsets. Lab reports designating the number of $CD3^+CD4^+$ cells should indicate whether this value represents $CD3^+CD4^+CD8^-$ cells or $CD3^+CD4^+CD8^+$ plus $CD3^+CD4^+CD8^-$ cells. With flow cytometry and no additional antibody reagents using ≥4-color analyses, DNTs and DPTs can be quantified by expression of $CD3^+$, $CD45^{hi}$, and Side-Light Scatter $(SSC)^{low}$ with or without CD4 and CD8 and NKTs by expression of CD3 and CD56 ± CD16. A difficulty in quantitative analysis of DNTs, DPTs, and NKTs for clinical purposes has been that some clinical software programs had not included DPTs, DNTs, and/or NKTs and have not excluded analysis of non-viable cells and cell aggregates. Without clear differentiation of DPTs from SP T cells and inclusion of DNTs and NKTs in cellular immunology analysis, normal reference ranges for the T cell subsets are lacking and thus, clinical usefulness continues to be delayed. Although as mentioned, the DPT percentages are usually low (<2% of $CD3^+$ cells), higher percentages of DPTs or DNTs have been reported. A recent report indicated that a HIV-infected patient had 40% of the T cells expressing CD4 and CD8 along with 7% and 47 % of the $CD3^+$ cells being $CD4^+CD8^-$ and $CD4^-CD8^+$, respectively [27]. If reported as 87% $CD3^+CD8^+$ and 47% $CD3^+CD4^+$, this would lead to obvious inappropriate conclusions. Different vendor immunophenotyping kits have also reported NK cells based on the expression of CD56 + CD16 or just CD56 alone, which would affect the numbers of NK and NKT cells. In this report, we list some of the characteristics of these T cell subsets. Physicians need to get reports with subsets clearly designated, which should minimally include DNTs, DPTs, NKTs, SP T cells, B cells, and NK cells. In the early 1980s, some oncologists did not see the need for immunophenotyping for therapeutic interventions, but as more immunophenotyping was provided on patient reports and related to patient responses to therapies, the usefulness of knowing more about subtypes was realized. "Bench to bed" results cannot improve patient care if certain existent data is not provided.

The DNTs, DPTs and NKTs can be further subdivided based on TCR and additional CD antigens; however, for this review, we limit consideration of additional biochemical and CD variances. However, TCR differences are discussed since differentiating T cells as TCRα/β or TCRγ/δ may be important for better defining some immunopathologies [28]. Additionally, peripheral blood TCRα/β^+ DNTs were reported to have a higher marker percentage of activation (HLA-DR^+), memory (CD45RO) and cytotoxic function (CD56, CD57, CD11b) than TCRα/β^+ SP T cells [29]; however, it has been reported that they are mostly naïve (CD45RA) T cells [30]. TCRα/β^+ DNTs from patients with Chagas' disease had inflammatory activities, whereas TCRγ/δ^+ DNTs had immunosuppressive activity [31]. Conflicting results may reflect methodology, gating strategies, or patient differences.

Double Negative T cells (DNTs)

One of the first reports demonstrating an increased percentage of TCRα/β^+ DNTs in the blood of patients with Systemic Lupus Erythematous (SLE) noted their ability to enhance autoantibody production [32]. As mentioned earlier, peripheral T cells, including DNTs, can express TCRα/β or TCRγ/δ, and TCRγ/δ DNTs also can promote autoantibody production [33,34]. Steroid treatment of SLE patients induced concomitant decline of autoimmunity and numbers of TCRγ/δ DNTs in SLE patients [34]. Although the role of DNTs in autoimmune disease remains to be more fully characterized and defined, their enhanced presence is indicative of a health concern [35]. It has been suggested that cAMP Responsive Element Modulator alpha (CREMα) induces epigenetic down modulation of CD8 generating the DNTs [36,37]. Thus, peripheral blood DNTs could be due to premature emigration from the thymus and/or peripheral down-modulation of CD8 or CD4 from SP T cells. The presence of DNTs in HIV-1 infected persons has been suggested to result from internalization of CD4 and its lysosomal degradation due to the Nef protein, encoded by HIV [38,39]. Intracellular CD4 expression was detectable in HIV-1p24(+) DNTs [40], and inflammatory IL-17 expressing DNTs have been implicated in the persistence of HIV during Anti Retroviral Therapy (ART)[41]. The positive and negative aspects of DNT's inflammatory activities have been reviewed [35]. Although DNTs can enhance inflammation [42-44], there also have been reports of immunosuppressive DNTs after transplantation or with an autoimmunity trying to regulate immunity [45-47]. The diverse activities of DNTs indicate the need for more thorough delineation of this T cell population.

An elevated presence of DNTs has been reported for patients with autoimmune diseases, inflections [48,49], Graft Vs Host Disease (GVHD) [50], and a novel lymphoproliferative/autoimmune syndrome resembling murine lpr/gld disease [51], which may have related to Autoimmune Lympho Proliferative Syndrome (ALPS). Increased numbers of DNTs also occur after organ transplants [47]. SLE and ALPS are the two autoimmune diseases most often cited to have patients with increased amounts of DNTs, but elevated numbers of DNTs also have been reported for autoimmune thyroid disease [52], Sjögren's syndrome [42], Behcet's disease [53], and pediatric autoimmunity [54]. DNTs associated with ALPS have been identified with additional biomarkers, e.g., expression of the common γ subunit of the activating Fc receptors (FcRγ), which possess immunosuppressive activity [55]. Quantifications of IL-10 and DNTs have been cited as reliable biomarkers of ALPS [56]. Unique spectra type histograms of the DNTs from patients with ALPS have suggested that the DNTs are not derived from SP CD4 or CD8 T cells [57]; however, in contrast, it has been suggested that their Vβ TCR expression indicates a clonal relationship to SP CD8 T cells [58]. The immunophenotypic profiles and functional variances associated with ALPS are likely due to presence of DNT subpopulations expressing either TCRα/β or TCRγ/δ, which relates to the auto aggressive or immunosuppressive activities that can accompany ALPS [59]. Functionally distinct and polar forms of TCR$\alpha\beta$ and TCR$\gamma\delta$ DNTs, aggressive and suppressive, respectively, have also been described in individuals with Chagas' disease [31]. The numbers of DNTs are expanded in thymus, bone marrow, lymph nodes, spleen and liver of many patients with ALPS and they express CD45RA; DNTs in the blood have been reported to express CD45RA, HLA-DR, and CD57 [60]. ALPS are usually considered a pediatric pathology; however, adults with elevated DNTs have been diagnosed with ALPS, which is also referred to as Canale-Smith syndrome [61]. ALPS was first reported by Canale and Smith [62]. Like ALPS, elevated presence of DNTs has been reported for a mutation of the LPS-Responsive and Beige-Like Anchor (LRBA) gene. Patients with the LRBA mutation have immunodeficiency, enteropathy and autoimmune cytopenias [63].

Although the total numbers of DNTs were suggested not to significantly increase in some patients with SLE, the percentage of activated TCRα/β DNTs has been reported to be increased [64-66] and to possess the Th2 phenotype with IL-4 expression [67]. Patients with SLE having anti-phospholipids syndrome had DNTs with increased mitochondrial mass [68]. As discussed for ALPS, TCR expression can define DNTs from patients with SLE who have suppressive or autogressive promotor activity [69]. DNTs from patients with SLE [70] and Sjogren's syndrome [42,71] were reported to demonstrate the Th17 phenotype, which coincides with the inflammatory nature of these autoimmune diseases. A substantial proportion of Mucosal-Associated Invariant T (MAIT) cells are DNTs, and their presence has been implicated in a few diseases, and they usually possess Th1- and Th17-associated features [72-74]. Interestingly, MAIT cells are deficient in SLE patients [75].

TCRγ/δ DNTs have been implicated in the pathogenesis of graft coronary disease; however, these DNTs were expanded *in vitro* with IL-2 [76]. Therefore, the DNT phenotype may not have been causing the pathogenesis but trying to control it since IL-2 is known to aid Treg development. DNTs expressing high levels of CD69, CD28 and CD40L (CD154) are also present in the kidney of patients with Acute Kidney Injury (AKI) [77]. AKI is a complex disorder with

Table 1: Lymphoid subpopulations in blood of female and males.

Subpopulation	Females (n=104)	Males (n=43)	Significance (pvalue)
B cells	263.7 ± 5.6 (54-921)*	227.2 ±11.7 (58-2149)	<0.001
NK cells	221.5 ± 4.8 (48-1069)	236.2 ± 7.9 (43-726)	NS
T cells	1372.9 ± 17.2 (369-3006)	1228.8 ± 25.4 (522-2693)	<0.001
SP CD4+ T cells	900.8 ± 12.8 (287-2621)	833.2± 18.2 (301-1884)	<0.001
SP CD8+ T cells	403.7 ± 6.9 (76-1064)	337.2 ±12.0 (100-1245)	<0.001
DPTs	22.8 ± 1.4 (0-253)	9.5 ± 0.6 (1-94)	<0.001
DNTs	41.4 ± 1.5 (0-236)	41.7± 2.5 (6-247)	NS
NKTs	88.1 ± 3.4 (0-826)	74.2± 4.5 (13-326)	<0.001

*Mean±SEM (range) of all samples from females (717) and males (310); female vs. male difference by Mann-Whitney Rank Sum Test with inclusion of all analyses. Analyses for individuals varied from one to a maximum of 29 analyses over 12 years and 23 analyses over 10 years for females and males, respectively.

Table 2: Female and male ranges of DPTs, DNTs and NKTs.

Subset	Cell Number/μL (Range)	Females (n=104)	Males (n=42)
DPT	≤20	70.20%	90.50%
	>20-<70	22.10%	7.10%
	>70	7.70%	0.20%
DNT	≤20	23.10%	23.80%
	>20-<70	71.10%	64.30%
	>70	5.8%	11.90%
NKT	≤20	12.50%	11.90%
	>20-<70	43.30%	33.30%
	>70	44.20%	54.80%

multiple causative factors including sepsis and nephrotoxins and ischemia [78]. Responding DNTs in the injured kidney produce the anti-inflammatory cytokines IL-27 and IL-10; DNTs possess immunosuppressive activity associated with hematopoietic stem cell transplantation with allogeneic cells [79]. Like the classical Treg cells ($CD3^+CD4^+CD25^{hi}CD127^{low}FoxP3^+$), DNTs have suppressor activity with production of TGF-β and IL-10, which aids the control of autoimmune diseases and allograft survival [80]. Chronic GVHD occurred in patients after allogeneic bone marrow transplants in those with decreased numbers of TCRγ/δ DNTs [81]. Presence of DNTs with Treg activity has been shown to limit severe manifestations of GVHD in the HLA mismatched recipients, but they often have not been assessed for TCR subtype [82]. During pregnancy, estrogen signaling of mast cells in the thymus has been suggested to initiate release of DNTs through efferent lymphatic vessels [83]. This DN T cell pathway is suggested to give rise to three subsets of TCRγ/δDN T-cells and one subset of TCRα/β DN T-cells, and the TCRγ/δDN T-cells are suggested to be essential for the maintenance of pregnancy. At birth, the number of DN T cells may be predictors for Late-Onset Sepsis (LOS) and necrotizing enterocolitis in preterm infants [84].

Double Positive T cells (DPTs)

It should not be surprising that DPTs, like DNTs, can be expressed at different levels and with differential functions dependent on their immunophenotype. In fact, there has been reported to be increased numbers of DNTs, DPTs and NKTs in the blood of children with β-Thalassemia Major (BTM) [85]. Since there are variant immune aberrances associated with immunopathologies, it should be expected that immune subsets with differential functions exist. Over thirty years ago, DPTs were reported in patients with chronic active hepatitis [18], idiopathic thrombocytopenic purpura [86], myasthenia gravis [87], and SLE [88]. Like the peripheral presence of DNTs, which could be due to loss of CD4 or CD8 from peripheral SP T cells or to premature migration of developing T cells from the thymus, peripheral DPTs could be due to early escape from the thymus or induction of previously suppressed CD4 or CD8. IL-4 has been reported to induce CD8 expression by SP CD4 T cells [89]; the IL-4 may be an autocrine effect since DPTs have been reported to express high levels of IL-4, at least, in patients with systemic sclerosis [90]. However, the DP expression may only be a transient phenotype [91], and CD8 is expressed as α/α and not α/β [92]. It also has been suggested that DPTs are rare and may be due to artifacts with non-specific staining and/or analysis of aggregates of SP T cells [93]. DPTs are rare in healthy individuals; however, more recent studies have confirmed the existence of DPTs. Early flow cytometric analyses may have thought DPTs to be artifacts, because earlier studies did not use means, such as filtration or gating by FSC-H *vs.* FSC-A, to remove aggregates from the analysis.

Table 3: Longitudinal variance of DPTs.

Subject	Age (range)	Number of analyses	DPT/μL (mean ± SEM)	DPT/μL (range)
Female	50-59	16	9.2 ± 0.9	4-20
Female	29-38	21	9.6 ± 0.7	3-17
Female	53-67	12	13.3-1.1	8-19
Female	60-66	17	36.8 ± 1.8	26-51
Female	49-56	18	10.9 ± 0.8	3-17
Female	43-53	24	26.0 ± 0.9	21-38
Female	42-54	29	12.1 ±1.1	0-23
Female	37-49	27	8.1 ± 0.5	3-14
Female*	55-60	14	146.7 ± 12.6	77-253
Female	42-48	15	71.1 ± 2.5	55-93
Female	50-54	15	35.7 ± 2.3	22-58
Male	40-45	11	10.4 ± 0.5	7-14
Male	41-44	10	1.9 ± 0.2	1-3
Male	47-58	23	12.7 ± 1.2	3-20
Male	61-72	26	9.3 ± 0.6	2-11
Male	51-58	29	7.1 ± 0.6	2-14

*Female had type II diabetes and heart disease.

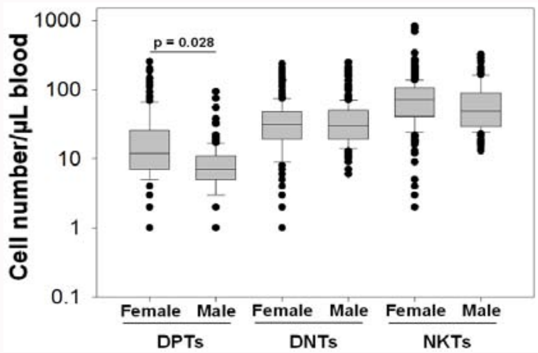

Figure 2: The average values of the DPT, DNT and NKT subsets for an individual were used in plotting the median and percentiles of females (n=104) and males (n=43).

As for DNTs, the DPTs appear to be heterogeneous regarding function [94], which indicates that they also have multiple subsets [95], which may carry transcription factors for CD4 and CD8 SP T cells and have functions that bridge both subsets as described in RA patients [96]. DPTs have ant-viral activity [19].

Natural Killer T Cells (NKTs)

As discussed, lymphoid subsets have diverse phenotypes and functions, and the lower presence of some such as the NKTs makes their presence in blood seem less useful especially when seemingly conflicting results are reported. NKTs are even harder to place into a single category for being involved in pathology than DPTs and DNTs, partly, because they are more variant in their phenotypes and functions than DPTs and DNTs, and their presence in blood only represents a small proportion of their presence and activity at an organ site. Like the Th and ILC subsets, there are NKT1, NKT2, NKT17, and NKT10 subsets [97-99].

There are two major types of NKTs. The type I NKTs (also called classical NKTs or invariant (i) NKTs) have a semi-invariant TCR with expression of Vα24-Ja18, which usually pairs with a Vβ11 chain. The TCR of NKTs does not recognize peptides in association with MHC class I or II molecules; their TCRs recognize lipids or glycolipids presented by CD1d, a non-polymorphic non-classical MHC class I molecule [100]. The iNKTs are heterogeneous regarding expression of CD4 and CD8 [100] as well as recognition by monoclonal antibody to Vα24-Ja18 (6B11) and α-GalCer-loaded dextramer [101]. The $CD4^+$iNKT cells have functions in common with Th1, Th2 or Treg cells; whereas the $CD4^-$iNKT cells ($CD8^+$ or DN) have greater cytolytic activity and are more Th1-like. The blood levels of iNKT cells range from 0.01-1%. Presences of peripheral $CD4^+$iNKTs are suggested to be due to thymic output, whereas $CD4^-$iNKT can be expanded in the periphery [102]. Thymic NKTs are $CD4^+$ $CD161^{-/low}$, whereas most NKTs in the blood are $CD4^-CD161^+$ [103].

The types II NKTs have a relatively diverse TCR repertoire. In humans, there is more type II NKTs than type I NKTs. Like type I NKTs, the TCRs of type II NKTs recognize lipid and CD1d; however, other MHC class I-like forms (CD1a, CD1b, and CD1c) also bind lipids and are recognized by the TCR of type II NKTs [104]. As for all T cell subsets, a current routine analysis is limited so that with only CD3 and CD56 or CD56 + CD16 no further assessment of NKT subsets is delineated. However, knowing general levels in blood may be useful, which requires reference ranges.

Quantification of DPTs, DNTs, and NKTs

Immunophenotyping methodology has undergone marked improvements during the past decades. Flow cytometry has replaced microscopy and there have been advances in the use of multi-color techniques and analysis with automated software. These improvements have greatly decreased the assay to assay variation within the context of one sample.

As part of a New York State proficiency testing program, we established a reference range for some lymphocyte subpopulations in human blood of employees of the Department of Health. Donor consent was approved by the department's IRB (Protocol 98-108). Blood was collected into K2 EDTA vacutainers (BD Bioscience) and processed and analyzed within 24 hr. The blood (50 μL) was placed into Trucount™ tubes that were preloaded with fluorochrome conjugated antibodies. The blood was lysed with 500 μL of FacsLyse and assayed with Canto flow cytometer without washing. The lymphocyte, monocyte, and granulocyte populations were gated from a CD45 *vs*. SSC plot. As described earlier, prior gating by FSC *vs*. SSC and FSC-H *vs*. FSC-A for elimination of debris and aggregated leukocytes is needed. All analyses of total T cells, SP $CD4^+$ and $CD8^+$ T cells, B cells, NK cells, DPTs, DNTs, and NKTs were used to determine the mean ± SEM differences between females and males (Table 1). The DPT proportions seemed to differ the most between females and males when only the subset average for a person was used (Figure 2). The DPTs, DNTs, and NKTs were arbitrarily divided into groups based on ranges (<20, >20 to <70, and >70 cells/ul) to further evaluate female and male differences. A greater percentage of females had more DPTs/μl (29.8%) than males (7.3%). Even though the DPT range is broad especially for females (0-253 DPTs/uL), an individual's range over time was much lower, which was the basis for the groups shown (Table 2). Some examples of the longitudinal variances for DPTs are reported (Table 3) since DPTs differed more than DNTs and NKTs between females and males. Interestingly, the female who had a relatively high consistent level of DPTs (Table 3), and a consistent low level of NKTs (5.0±0 NKTs/μL) had type II diabetes and heart disease. The DPT: NKT ratio for this person was 29.34; whereas, the average ratio for females and males was 0.505±0.218 and 0.18±0.022 (mean±SEM), respectively. Some NKTs are known to suppress autoimmune disease such as type I diabetes [103,105].

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