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CD3CD4CD8 (double negative) T cells: saviours or villains of the immune response?

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2 **CD3⁺CD4⁻CD8⁻ (double negative) T cells: saviours or villains of the immune**
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4 **response?**
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10 Fulvio D'Acquisto¹ and Tessa Crompton²
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29 **Running title:** Pathophysiological role CD4⁻CD8⁻ double negative T cells
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36 **Key words:** Thymocyte positive and negative selection; CD4⁻CD8⁻ DN T cells; unconventional T
37 cells; inflammation; autoimmune and infectious diseases.
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Abstract

Recent studies have shown that T cells are not just the latecomers in inflammation but might also play a key role in the early phase of this response. In this context, a number of T cell subsets including NKT cells, mucosal-associated invariant T cells and γ/δ T cells have been shown, together with classical innate immune cells, to contribute significantly to the development and establishment of acute and chronic inflammatory diseases. In this commentary we will focus our attention on a somewhat neglected class of T cells called $CD3^+CD4^-CD8^-$ double negative T cells and on their role in inflammation and autoimmunity. We will summarize the most recent views on their origin at the thymic and peripheral levels as well as their tissue localization in immune and non-lymphoid organs. We will then outline their potential pathogenic role in autoimmunity as well as their homeostatic role in suppressing excessive immune responses deleterious to the host. Finally, we will discuss the potential therapeutic benefits or disadvantages of targeting $CD3^+CD4^-CD8^-$ double negative T cells for the treatment of autoimmune disease. We hope that this overview will shed some light on the function of these immune cells and attract the interest of investigators aiming at the design of novel therapeutic approaches for the treatment of autoimmune and inflammatory conditions.

Introduction

One of the most important hallmarks of the inflammatory response is the migration and infiltration of leukocytes to the site of injury or infection [1, 2]. The identification of unique markers for the continuously growing number of cell types of the immune system has provided scientists with a wealth of information regarding the specific contribution of different subtypes of leukocytes to a variety of immune and inflammatory reactions. It is in fact possible these days to dissect out, isolate and characterize in great detail the types of leukocytes infiltrated in inflamed tissues based on the pattern of expression of their cell-surface markers.

However, the more we know about these cells the more we realize that these simple classifications are imperfect and that leukocytes can disguise themselves in many different ways. For instance, T cells have been typically identified based on the expression of the alpha and beta chains of the TCR ($\alpha\beta$ TCR) and further classified into helper or cytotoxic T cell populations based on the expression of CD4 or CD8, respectively. Interestingly, recent studies have shown that lymphocytes can also “adsorb” (or steal) classical antigen-presenting cell surface molecules through a process called “trogocytosis” (from the ancient Greek trogo, meaning “gnaw”). In the case of CD8⁺ T cells, acquisition of cognate MHC class I ligands induces cytotoxic T lymphocytes to “fratricide” antigen-specific cytolysis, thereby contributing to the clearance of CD8 [3, 4]. If the readers think that T cells are the only wolves in sheep’s’ clothing, they had better think twice. Recent evidence has shown that a subpopulation of neutrophils (about 5%) in both mouse and human possess the same key elements as T lymphocytes, in expressing the TCR-based immunoreceptors [5]. Similarly it has been shown that synovial neutrophils from patients suffering rheumatoid arthritis can transdifferentiate into dendritic cells and hence express key antigen presenting molecules such as MHC II and CD40, CD80, CD83 and CD86 [6].

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2 Thus it seems that immune cells, like human beings, like to transgress from their role and change
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4 their function or phenotype in specific settings. We have also learned that these T cells differ
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6 from their “conventional” siblings not just in their make-up of cell surface markers, but also in
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8 their function (Figure 1) since they appear to behave more like innate rather than adaptive
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10 immune cells.
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16 In this commentary we will be focusing our attention on $CD3^+CD4^-CD8^-$ double negative (DN) T
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18 cells, and on their pathophysiological functions. In particular, we will provide an overview of
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20 their origin and development as well as of their role as suppressor (DN regulatory T cells; DN
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22 Tregs) or effector (DN T cells) of the immune and inflammatory response.
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26 27 28 **A very brief overview of T cell development** 29

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31 Most T cells develop in the thymus, which provides a specialized environment that supports the
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33 maturation of lymphocyte precursors into functional T-cells [7]. The thymus is seeded by blood-
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35 borne progenitor cells from the foetal liver or adult bone-marrow which develop along a well-
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37 characterised programme, dependent on bidirectional signals between the developing thymocytes
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39 and the thymic epithelial cells (TEC) [8]. During $\alpha\beta$ T-cell development, thymic $CD4^-CD8^-$ DN
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41 cells give rise to the $CD4^+CD8^+$ double positive (DP) population, which differentiate to mature
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43 $CD8^+CD4^-$ or $CD8^-CD4^+$ single positive (SP) cells. The DN population can be subdivided by cell
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45 surface expression of CD25 and CD44. $CD44^+CD25^-$ (DN1) cells differentiate to become
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47 $CD44^+CD25^+$ (DN2) cells, which then differentiate to become $CD44^-CD25^+$ (DN3). The DN3
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49 population gives rise to the $CD44^-CD25^-$ (DN4) subset, which undergo a phase of rapid
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51 proliferation before differentiation into the DP population, in general *via* a cycling immature
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53 $CD8^+$ intermediate single positive (ISP) cell (Figure 1).
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2 Maturation from the DP population to the mature SP T-cell populations involves positive
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4 selection of the $\alpha\beta$ TCR repertoire to ensure appropriate MHC restriction and negative selection
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6 of potentially self-reactive clones. Many models have been proposed to describe how DP
7
8 thymocytes commit to the CD4 and CD8 lineages, and to explain how positive selection ensures
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10 that selected CD4 and CD8 SP populations express TCR appropriately restricted by MHC Class
11
12 II and Class I respectively. TCR repertoire selection is dependent on interactions between the
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14 TCR on the developing thymocyte and its MHC/peptide ligand on TEC [9-11]. Strength and
15
16 duration of TCR signal are thought to broadly determine the DP cell's fate with the strongest
17
18 signals leading to negative selection and apoptosis, (i.e. in the case of TCR recognizing self
19
20 antigens), intermediate signals leading to positive selection, and, weaker signals or lack of TCR
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22 signaling leading to DP cell death by neglect [12]. For DP thymocytes that are undergoing
23
24 positive selection, TCR signal strength and duration have been proposed to influence CD4 and
25
26 CD8 lineage commitment, with those cells receiving stronger TCR signals tending to be biased
27
28 towards the CD4 fate, and weaker/more transient signals tending to favor differentiation to CD8
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30 SP T cells [10]. Transcription factors, such as Th-POK, play essential roles in lineage
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32 commitment, and the ways in which the transcriptional regulation of lineage commitment relate
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34 to TCR signaling require further study [11]. In addition, other secreted factors from TEC, such
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36 as the morphogen Sonic hedgehog influence TCR repertoire selection and differentiation from
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38 DP to SP cell [13, 14].
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50 **Thymic and extrathymic origin of DN T cells**

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53 DN T cells have been proposed to originate in the thymus by escaping negative selection
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55 followed by migration in the periphery where they expand upon experiencing antigen [15].
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58 Similar to T helper cell lineage commitment, the strength of TCR signalling seems to be an
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60 important factor for the development of DN T cells. According to this hypothesis, lower intensity
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1 TCR signals would lead typically to the generation of mature CD4⁺ and CD8⁺ T cells while high
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4 TCR strength that borders deletion would promote the survival and escaping of DN T cells from
5
6 the thymus [16]. Using a model of thymocyte reaggregated culture Wang *et al.* demonstrated that
7
8 stimulation of cells with high concentrations of high affinity antigens leads to the conversion of
9
10 DP thymocytes into DN T cells *via* down-modulation of both CD4 and CD8 coreceptors
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12 expression [17]. It has alternatively been proposed that some DN T cells arise from the pre-TCR+
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14 DN population (late DN3 population). Under this model, strong signal transduction through the
15
16 pre-TCR/CD3 complex of $\gamma\delta$ TCR complex favours differentiation to DN T cell, whereas
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18 “normal/average” pre-TCR/CD3 complex signal transduction promotes differentiation to DP cell
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20 and commitment to the $\alpha\beta$ T cell lineage [18].
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29 Several line of evidences also support the theory that DN T cells are generated in the periphery
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31 rather than in the thymus. Using CD8-deficient mice and sublethally irradiated thymectomized
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33 mice reconstituted with T cell-depleted bone marrow cells Ford *et al.* demonstrated that
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35 functional regulatory DN T cells exist in CD8-deficient mice, hence they are not derived from
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37 antigen-stimulated CD8⁺ T cells. In contrast, a reduction in DN T cell number was observed in
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39 reconstituted thymectomized mice compared with nonthymectomized recipients. Interestingly,
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41 DN T cells that develop in thymectomized mice showed an increased suppressing activity
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43 compared to those that develop in sham-thymectomized mice [19].
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50 Mice lacking the expression of Fas/APO-1/CD95 (*lpr*) or Fas-ligand (*gld*) have been widely used
51
52 to investigate the developmental origin of DN T cells. These mice show a peculiar accumulation
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54 of DN T cells in the lymph nodes and other organs [20, 21] as a result of an impaired induction of
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56 apoptosis leading to lack of deletion of autoreactive T cells that is seen in normal mice [22]. *In*
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58 *vivo* antigenic stimulation of TCR transgenic/*lpr* mice resulted in peripheral deletion of T cells
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1 and in a nine fold increase in the percentage of DN T cells in the lymph nodes of the CD95-
2 deficient but not the CD95-intact mice [23] suggesting a peripheral origin of these cells during
3 immune stimulation. The clinical relevance of these observations has been confirmed in human.
4 Patients suffering from autoimmune lymphoproliferative syndrome (ALPS) have mutations in the
5 Fas apoptotic pathway that are associated with impaired apoptosis after TCR ligation of single
6 positive (CD4 or CD8) activated mature T cells [24]. Interestingly, these patients share similar
7 clinical features with *lpr* and *gld* mice and a selective accumulation of DN T cells in peripheral
8 blood [25-29].

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23 An extra-thymic origin for DN T cells has been also observed in inflammatory conditions. A
24 selective accumulation of DN T cells has been described in the peritoneal cavity of mice infected
25 with the intracellular pathogen *Listeria monocytogenes* and the appearance of these cells was still
26 observed when adult-thymectomized lethally irradiated bone marrow chimeras mice were tested
27 [30].
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38 **DN T cells as the villains of the immune system**

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40 Most of what we know about the pathogenic functions of DN T cells derives from the study of
41 mice expressing a transgenic TCR or from autoimmune-prone genetically modified mice. The
42 artificial expression of the transgenic $\alpha\beta$ TCR at the double negative stage of T cell development
43 has been proposed to mimic signals normally conveyed by the $\gamma\delta$ TCR and thus lead to the
44 generation of an artificial subpopulation of T cells with a unique profile of surface markers and
45 cytokine production. One example of these mice is the HY-TCR transgenic mice. HY mice
46 express a transgenic TCR that recognizes the male HY antigen presented by MHC class I
47 molecule. In female mice the HY transgenic T cells are positively selected to the CD8 lineage.
48 Conversely, male TCR transgenic mice show a strong reduction of the DP thymocytes and CD8⁺
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1 transgenic T cells [31, 32]. Interestingly, in male mice a relatively small number of DN T cells
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4 escape thymic selection and can be recovered in the periphery or as intraepithelial gut
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6 lymphocytes [31, 33].
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10 Analysis of the gene fingerprint of these cells by microarray demonstrated that, similar to
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12 $\gamma\delta$ TCR or NK T cells, HY-TCR⁺ DN T cells showed a “hair-trigger” reactivity that place them in
13
14 an intermediate position between innate and adaptive cells [34, 35]. In fact these cells showed an
15
16 unexpected upregulation of genes associated with the innate immune system and an increased
17
18 production of IL-2 and IFN- γ when compared to female CD8⁺ T cells. These findings *in vitro*
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20 have been recently confirmed *in vivo* by our group using a classical model of delayed type
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22 hypersensitivity (DTH): the λ -carrageenan-induced paw oedema. Male HY mice showed an
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24 exacerbated inflammatory response accompanied by accumulation of HY-TCR⁺ cells in the
25
26 inflamed paw tissues. *Ex vivo* analysis of the cytokine profile expressed by activated T cells
27
28 present in the draining lymph nodes of male HY mice showed an increased production of
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30 classical DTH cytokines such as IL-2, IFN- γ and TNF- α or other “autoimmune” cytokines such
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32 as IL-17A and IL-22 compared to female HY-TCR⁺ cells [36].
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44 The proinflammatory phenotype of mature DN T cells has also been observed during their
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46 development in the thymus. Interestingly, CD44⁺ DN thymocytes have been found to produce
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48 high levels of IL-2, TNF- α and IFN- γ upon stimulation with PMA/Ionomycin while CD44⁻ DN
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50 did not produce any of the cytokines mentioned. Similarly, stimulation of $\alpha\beta$ TCR expressing DN
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52 thymocytes and DN isolated from the spleen with anti-CD3 revealed a similar pattern with IL-4,
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54 IFN- γ and TNF- α being the main cytokines released upon activation [37].
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1 A growing number of recent studies suggest that DN T cells are not just an artificial subsets of
2 cells occurring in transgenic or genetically modified mice but key players in autoimmunity and
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4 inflammation in humans. Several studies provided a direct link between DN T cells and the
5
6 development of autoimmune diseases. DN T cells have been found in the blood of myasthenia
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8 gravis patients with lymphofollicular hyperplasia and have been shown to participate in the
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10 immunoregulation and increased antibody production [38]. Patients suffering from systemic
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12 lupus erythematosus (SLE) have a significantly expanded population of DN T cells and these
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14 cells are the major producers of IL-17 [39], one of the key inflammatory cytokine involved in
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16 SLE [40, 41]. These results confirmed previous observations from the same group showing that
17
18 DN T cells from MRL/lpr mice produce high levels of IL-17 as the disease progresses. In
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20 addition, *in vitro* treatment of MRL/lpr lymph node cells with IL-23, a cytokine required for
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22 sustained Th17 cell differentiation [42], induces the generation of highly pathogenic DN T cells
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24 causing nephritis following adoptive transfer in lymphocyte deficient Rag1^{-/-} mice [43, 44]. In a
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26 study conducted on patients suffering from ALPS [45] (also known as Canale-Smith syndrome)
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28 the authors demonstrated that the percentage of DN T cells increased from 1% (observed in
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30 physiological conditions) to 40% [46]. These results together with other evidence [26-29] suggest
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32 that the increased number of DN T cells may represent a sensitive test or biomarker to screen
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34 patients who should undergo testing for ALPS. In this regard it is interesting to mention that the
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36 analysis of DN T cell levels has been used to distinguish ALPS from other autoimmune disease
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38 such as Evans syndrome [27] or sarcoidosis [47].
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53 In line with their ability to produce high levels of IL-17, DN T cells have been reported to play a
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55 key role in various models of infections. DN T cells are the major responding T cell subset in the
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57 lung of mice infected with live vaccine strain (LSV) of the intracellular bacterium *F. Tularensis*.
58
59 Temporal analysis of cytokine production in infected lung showed that DN T cells release large
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1 amount of IL-17A during the early stages of infection (7 days) while CD4⁺ T cells start to
2 produce this cytokine later (14 days) [48]. Considering that IL-17-deficient mice die at day 8-10
3 post LSV infection [49], it is tempting to suggest that DN T cells rather than CD4⁺ T cells are
4 major contributors to the development of LSV infection. Marodon *et al.* provided further
5 evidence of the importance of DN T cells in infectious diseases. In this study the authors showed
6 that DN T cells of HIV-infected patients represent up to 20% of the cellular viral load in T cells;
7 moreover, successful antiviral therapy was associated with a significant decrease in viral load in
8 DN T cells suggesting that infected DN T cells, like CD4⁺ cells, contribute to viral production
9 and are sensitive to highly active antiretroviral therapy [50].
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26 **DN T cells as the saviours of the immune system**

27 T cells with immunoregulatory activity have always attracted the attention of scientists for their
28 potential clinical application in a variety of pathological conditions ranging from autoimmunity
29 to tumour immunology [51, 52]. These cells act as sentinels of the immune system and thus are
30 essential in the maintenance of immune homeostasis and self-tolerance [53].
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41 The subtypes of T cells with immunoregulatory function has significantly increased in the last
42 few years and we now know that there are Treg other than the originally identified
43 CD4⁺/FoxP3⁺/CD25⁺ T cells. These include NO-Tregs that derive from CD4⁺CD25⁻ T cells and
44 are induced by nitric oxide (NO) [54], Tr1 cells that do not express FoxP3 and release a large
45 amount of IL-10 [55] [56] and TGF- β -producing CD4⁺ Th3 cells [57, 58]. As if this field of
46 research was not already “crowded”, DN T cells have also been added to this list. These cells
47 have been described as the only subtype of immunoregulatory cells able to suppress antigen
48 specific T cell responses [59, 60] as well as a number of other features that make them different
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1 from the “conventional” regulatory T cells [61-63]. To avoid confusion with the other DN subset,
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4 we will refer to these cells as DN Tregs.
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9 DN Tregs have been isolated and identified in mouse, rat and human. One of the first studies
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11 conducted in mice described the isolation of natural suppressor DN T cells derived from the
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13 spleen of adult mice [64, 65]. A subsequent study on rat DN T cell clones demonstrated that these
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15 cells did not elicit disease but rather inhibited the development of experimental autoimmune
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17 encephalomyelitis (EAE) [66]. Studies in human identified a population of DN T cells in a patient
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19 with immunodeficiency, lymphocytosis, lymphadenopathy, and hepatosplenomegaly. These cells
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21 showed an impaired proliferative response upon stimulation with mitogens or anti-CD3 plus anti-
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23 CD28 but were not tested for their immunosuppressive activity [67].
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31 As in the case of inflammatory DN T cells, TCR transgenic mice contain a significant number of
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33 DN Tregs. These cells present a number of features including a similarity with naive CD8⁺ T
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35 cells that have undergone homeostatic proliferation and conversion into memory-like T cells,
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37 lower threshold of activation [68] and functional anergy as they hypoproliferate and produce little
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39 or no IL-2 in response to antigen stimulation [69]. Controversies exist on the possible
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41 mechanisms by which DN Tregs down-regulate immune responses. According to one study, DN
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43 Tregs from TCR transgenic mice acquire the antigen from antigen presenting cells *via*
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45 trogocytosis and they then re-express these newly acquired molecules on their cell surface to
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47 mediate antigen-specific suppression of activated alloreactive CD8⁺ T cells [62]. Further studies
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49 have confirmed these findings and have shown that DN Tregs acquire alloantigen *in vivo* *via*
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51 trogocytosis [70]. Like classical CD4⁺ CD25⁺FoxP3 Treg they require direct cell contact for
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53 suppression and express a unique combination of cell surface markers that makes them
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55 distinguishable from any previously described T-cell subsets [62]. According to another study,
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1 the killing of CD8⁺ T cells by DN Tregs depends on the direct recognition of the antigen on the
2 target cell by the TCR and this enables DN Tregs to respond to a greater variety of self antigens
3 and hence, to regulate the response of a larger repertoire of T cells [15]. Recent studies on human
4 DN Tregs have revealed some discrepancies with their murine counterparts. Human DN Tregs
5 are not believed to eliminate effector T cells by Fas/FasL-mediated apoptosis, modulation of
6 antigen presenting cells or competition for growth factors but to suppress by an active cell
7 contact-dependent mechanism [71].
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21 Several investigations have shown that DN Tregs play an important role in the development of
22 tolerance after transplantation. DN Tregs have been described to prolong both allo- and xenograft
23 survival compared with untreated controls when adoptively transferred into syngeneic mice.
24 Initial studies in transgenic mice suggested that DN Treg cells can be generated *in vitro* and
25 protect cardiac allograft from rejection when infused into recipients prior to transplantation [72].
26 However, in a subsequent study by Lee *et al.* the authors showed that although single-dose
27 infusion of DN Treg clones from normal mice induces permanent survival of MHC class I-
28 mismatched cardiac allografts, their suppressing activity was lost after *in vitro* culture and clonal
29 expansion [73]. Similarly in another study using rat to mouse cardiac xenotransplant it was found
30 that DN Tregs harvested from the spleens of the recipient mice dose-dependently inhibit the
31 proliferation of syngeneic antidonor T cells [74].
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50 DN Tregs also play a homeostatic role in autoimmune diseases. Diabetes-prone mice carry fewer
51 DN Tregs and this contributes to the increased susceptibility of these mice to develop the disease.
52 Different mechanisms have been proposed to contribute to this effect. Using a transgenic TCR
53 mouse model Hillhouse and colleagues have shown that *in vivo* antigenic stimulation of
54 transgenic T cells induced a 10 fold increase in the number of DN Tregs in diabetes-prone
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1 NOD.H^{2k} but not in B10.Br control mice. They also showed that IL-10 produced by these cells
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3 favours their apoptosis and hence helps to regulate their number [75]. In another study by Duncan
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5 *et al.* the authors proposed a correlation between DN Tregs and the late onset of diabetes in NOD
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7 mice. Young NOD mice have a high frequency of DN Tregs that declines in adulthood and their
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9 adoptive transfer in Nod/Scid mice provided a long-lasting protective effect. Consistent with the
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11 previous study, the authors also demonstrated that the anti-diabetogenic effect of DN Tregs relied
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13 mainly on the ability to differentiate into IL-10-secreting Tr-1 cells [76].
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21 **DN T cell “reservoirs” in the body**

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23 DN T cells seem to preferentially dwell in specific organs or tissues (summarized in Figure 2). In
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25 MRL-lpr/lpr mice DN T cells were found in close vicinity to Kupffer cells or endothelial cells of
26
27 the hepatic sinusoids. Most interestingly, this liver-specific accumulation of DN T cells is not a
28
29 unique feature of MRL-lpr/lpr but it occurs in several other autoimmune-prone strains including
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31 C3H/HeJ-gld/gld, BXSB, NOD and NZB/W F1 mice with their proportion increasing as mice
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33 become old and diseased [77]. The clinical relevance of these findings has been confirmed in a
34
35 study where an analysis of the number of peripheral blood DN T cells in patients after liver
36
37 transplantation was conducted. Patients with proven acute cellular rejection showed a significant
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39 increase in DN T cells and a significant reduction after pulse corticosteroid therapy [78]. The
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41 kidney is another favourite hub for DN T cells. However, unlike the liver, the number of DN T
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43 cells in the kidney decreases during inflammatory conditions such as after ischemia and
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45 reperfusion [79, 80]. These results are in contrast with the afore mentioned study in which it was
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47 shown that patients with lupus nephritis have a significant number of DN T cells infiltrating the
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49 kidney [39].
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1 An interesting study investigated the phenotype, distribution, and function of T lymphocytes in
2 the female genital tract of naive, pregnant, or Chlamydia trachomatis-infected C57BL/6 mice.
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4 Unexpectedly, the authors observed that DN Tregs were the dominant lymphocyte population
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6 (70-90%) in the genital tract [81]. This might be of clinical importance considering that DN T
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8 cells have been associated with pathogenesis of Behcet's disease [82], a complex mucosal
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10 inflammatory disorder with neural and vascular manifestations that includes oral aphtosis and
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12 genital ulcers [83].
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21 A unique distribution of DN T cells has been described in CD18-deficient mice. Comparative
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23 analysis of cervical, axillary and inguinal lymph node populations in wild-type and CD18-
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25 deficient mice showed an expected six fold decrease in the total lymphocyte number in axillary
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27 and inguinal lymph nodes, together with a fourfold increase in the cellularity of cervical lymph
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29 nodes, of the latter compared to the former. These cells are massively generated within the lymph
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31 nodes upon antigen stimulation and display typical features of antigen-experienced cells
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33 including lower threshold of stimulation, heightened proliferation rate and increased re-
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35 circulation to non-lymphoid organs [84].
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43 **Conclusions and perspectives**

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45 The increasing number of studies on the role and function of DN T cells over the last decade have
46
47 significantly expanded our knowledge of this new subset of immune cells in health and disease.
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49 Most importantly, we now know that these cells are not just an artefact that occurs in genetically
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51 modified or transgenic TCR mouse models but that they are also found in a variety of human
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53 conditions spanning from autoimmune disease to inflammation, infection and cancer [26, 39, 59].
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55 Table 1 and 2 summarize the main functions of pathological DN T cells and DN Tregs described
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57 in this commentary.
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1 One of the most intriguing aspects of the biology of DN T cells is their dual identity i.e. their
2 pathogenic or immunosuppressive phenotype. In this context, a number of questions need to be
3
4 addressed. For instance, how do DN T cells decide to be villains or saviours of the immune
5
6 system? Do DN T cells and DN Tregs derive from the same progenitor cell? And most
7
8 importantly, can DN Tregs convert into inflammatory DN T cells? This very last aspect is of
9
10 great importance before we even begin considering to use DN Tregs in cell immunotherapies.
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19 A great deal of attention has been recently given to the concept of T cell plasticity. According to
20
21 this theory, Th effector cells can readily switch from one phenotype to another especially when
22
23 adoptively transferred *in vivo*. Thus, Th17 cells can convert in Th1 cells, classical FoxP3⁺ Treg
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25 can become Th17 and Th2 cells can change into an IL-9 producing Th9 cells [85-87]. Whether or
26
27 not inflammatory DN T cells can acquire an immunosuppressive phenotype and *vice versa* has
28
29 not been explored yet and it would certainly be very interesting to investigate. Clearly, a number
30
31 of factors contribute to their phenotype and more investigations are needed to fully appreciate the
32
33 intrinsic and extrinsic factors influencing their fate. In any case, several observations indicate
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35 that, like for Th effector cells, the microenvironment plays a key role in determining DN T cell
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37 functions and/or expansion.
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45 Beside these considerations, we think that there are important aspects other than their origin or
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47 development that have not been fully explored. If one wants to bring DN T cells under the
48
49 spotlight of “translational science”, there are several gaps that need to be filled in. First of all, the
50
51 identification of specific markers that allow distinction between inflammatory DN T cells from
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53 DN Tregs would be of great clinical and therapeutical importance. A first attempt has been
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55 carried out by Lee and colleagues by comparing gene expression differences between functional
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57 DN Treg cells and nonfunctional mutants [73] but clearly more extensive proteomic analyses are
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1 needed. In other studies it has been found that *lpr* DN lymph node cells are homogenous for
2 expression of high level of CD44, B220 (the isoform of CD45 found on B cells) [88, 89], CXCR5
3
4 [90] and lack the expression of CD2 [91] and IL-2 receptor[92].
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10 The availability of further specific markers will facilitate the isolation and analysis of these cells
11 and, at the same time, might provide novel diagnostic tools that can be used to predict disease
12 progression and possibly remission. Further insights into the mechanisms of activation of DN T
13 cells could be used to investigate at the biochemical level what makes these cells so hyperreactive
14 and pathogenic. This information could be used for the design of specific drugs able to limit
15 inflammatory DN T activation or, possibly, favour DN Treg development and/or expansion.
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26 We recognize that these are not simple and straightforward processes, especially if one aims at
27 ultimately targeting human DN T cells. However, there are small steps that can (and should) be
28 taken up by pharmacologists interested in immune and inflammatory diseases. In our view, the
29 availability of relatively simple experimental systems to study DN T cells or DN Tregs would
30 provide a significant boost in translational research in this area. For instance, in our recent study
31 we have used the HY-TCR transgenic mice to assess the contribution of DN T cells to the early
32 and late stage of the inflammatory reaction [36]. The high frequency of DN T cells in male mice
33 makes these animals an ideal system to test novel therapeutic strategies targeting these cells.
34 Similarly, given the high number of DN Treg in young NOD mice [76], this system could be an
35 ideal platform for the testing of novel immunosuppressive therapies.
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53 Along these lines, it is somehow surprising that very little attention has been given to the possible
54 effects of commonly used anti-inflammatory and immunosuppressive drugs on DN T cells or DN
55 Tregs. Evidence that popular pharmacological therapies might modulate CD4 and CD8
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1 expression in T cells were reported more than 20 years ago [93-95] and thus the possibility that
2 these drugs might act on DN T cells and DN Treg development and/or activation is not far
3 fetched. In one interesting recent studies by Okazaki et al. the authors showed that FTY720, a
4 novel immunosuppressant that modulates sphingosine 1-phosphate receptor, induced apoptosis in
5 more than 70% of DN T cells from the spleen of MRL/lpr mice *in vitro* and *in vivo* [96].
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16 To conclude, we hope that this commentary will help the readers of Biochemical Pharmacology
17 to appreciate the importance of DN T cells as therapeutic targets since we think that this is a
18 neglected area of research that needs much attention and which has great potential for
19 pharmacological intervention. We also hope that this overview will invite investigators to look
20 back at their cytofluorimetric or immunohistochemistry data with new eyes that are not
21 obfuscated by the analysis of solely CD4 or CD8 single positive T cells.
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4 Figure 1. Schematic representation of T cell development in the thymus. Progenitor (PG) cells
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6 from the bone marrow colonize the thymus where they commit to natural killer cells (NK) or T
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8 cell lineage. Here they undergo progressive differentiation from double-negative (DN) to double-
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10 positive (DP) to single-positive CD4 or CD8 thymocytes. The immature DN thymocytes can be
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12 further divided into DN1 to DN4 based on their expression of CD44 and CD25. The $\gamma\delta$ lineage
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14 diverge from the $\alpha\beta$ at the DN2/DN3 stage. Progression from DP stage to SP can occurs through
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16 an immature CD8+ single positive (ISP) cell intermediate
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24 Figure 2. Schematic representation of favourite tissue localization of DN T cells in murine and
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26 human tissues and their relative frequency in health and disease conditions.
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31 Table 1. Summary of the main functions of human and murine pathogenic DN T cells.
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36 Table 2. Summary of the main functions of human and murine DN Tregs cells.
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Table 1

Mouse models or human disease	Phenotype	Reference
HY-TCR transgenic mice	“Hair-trigger” reactivity Production of high levels of IL-2 and IFN- γ	34,35
	Exacerbated inflammatory response <i>in vivo</i> Production of high levels of IL-2, IFN- γ , TNF- α , IL-17A and IL-22	36
MRL/lpr mice	Expansion with disease progression Production of high levels of IL-17	39
	Highly pathogenic following adoptive transfer Expansion following treatment with IL-23	43, 44
Mice infected with F. Tularensis	Release large amount of IL-17A during the early stage of infection	48
Myasthenia gravis patients	Immunoregulatory activity Number correlates with disease activity	38
HIV-infected patients	Represent up to 20% of the cellular viral load in T cells Successful antiviral therapy was associated with a significant decrease in viral load in DN T cells	50
Autoimmune lymphoproliferative syndrome	Increased number/selective expansion	25, 26, 27, 28, 29

Table 2

Mouse models or human disease	Phenotype	Reference
Lewis rats immunized with guinea-pig myelin basic protein	Inhibit the development of experimental autoimmune encephalomyelitis	66
H-2 ^b 2C TCR-transgenic mice	Lower threshold of activation Functional anergy Hypoproliferation and little or no production of IL-2 in response to antigen stimulation	68 69
2C _{F1} (H-2 ^{b/d} , L ^d , anti-L ^d 1B2-TCR+) transgenic mice	Antigen-specific suppression of activated alloreactive CD8+ T cells <i>via</i> trogocytosis	62, 70
Anti-L(d) transgenic TCR+ (2C x dm2)F1 mice	Protect cardiac allograft from rejection	72
Heart grafts from Lewis rats heterotopically transplanted into B6 recipients mice	Dose-dependently inhibit the proliferation of syngeneic antidonor T cells	74
Diabetes-prone NOD.H2k mice	Increase in number after antigenic stimulation <i>in vivo</i>	75
Diabetes-prone NOD mice	Adoptive transfer in Nod/Scid mice provided a long-lasting protective effect	76
Human peripheral blood T cells	Suppress by an active cell contact-dependent mechanism	71

Figure 1

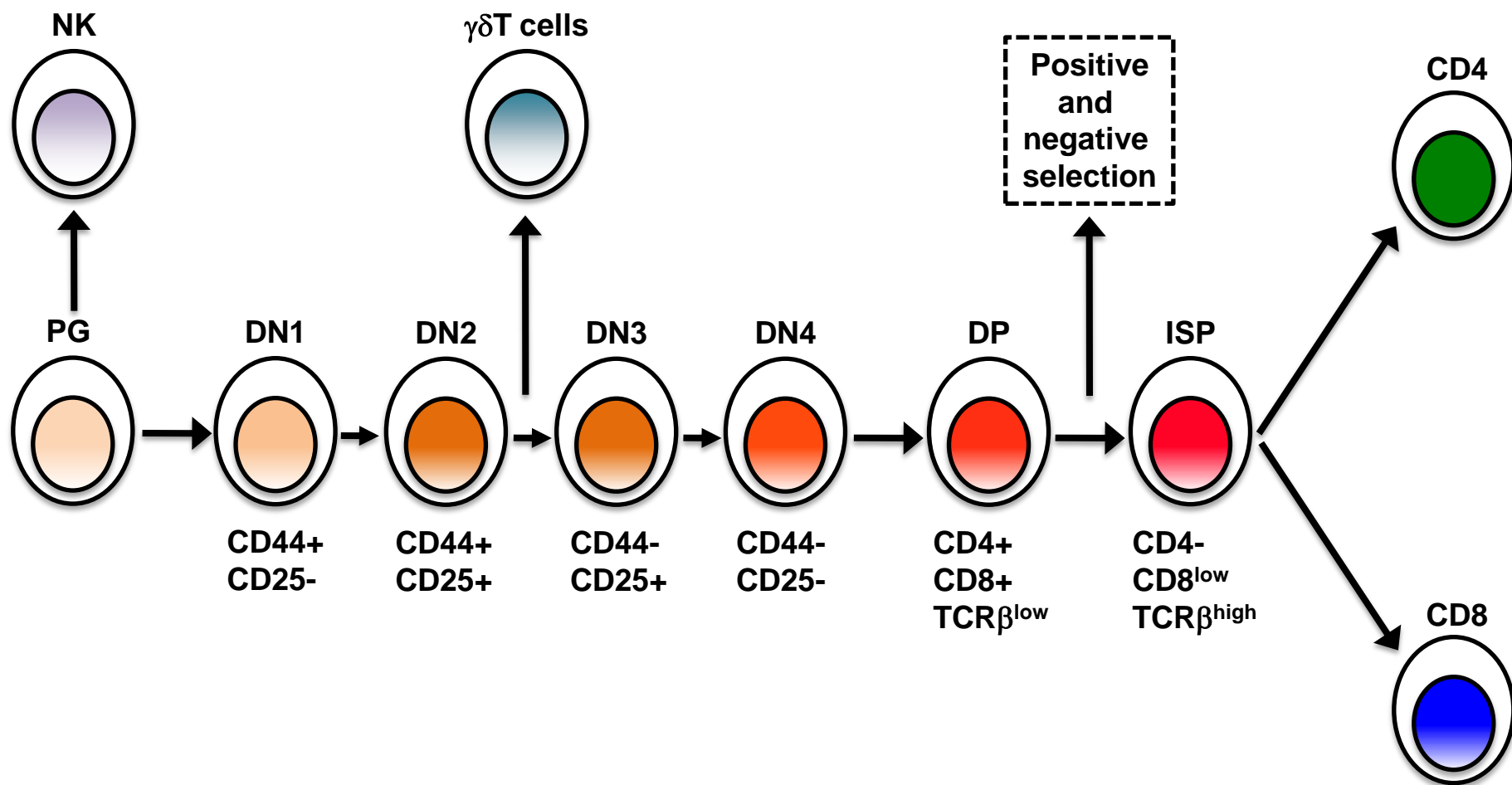
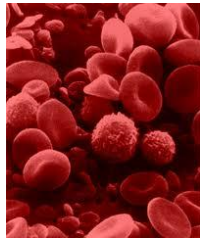


Figure 2

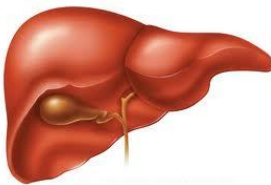
Double negative T cell reservoirs in human body



Lung
↑ Number after infection



Peripheral blood
1-2% in physiological conditions
Up to 40% in autoimmune conditions



Liver
↑ Number after transplantation



Kidney
↓ Number after ischemia/reperfusion



Genital tract
Up to 70-90% of the total lymphocyte population

The double nature of CD3⁺ CD4⁻CD8⁻ T cells

PATHOGENIC

“Hair trigger” reactivity

Release large amount
of IL-17, IL-2 and IFN- γ

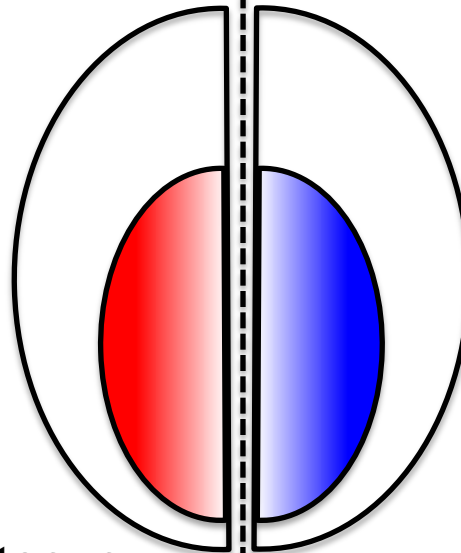
Cellular HIV reservoirs

Increased number
during:

myasthenia gravis

systemic lupus erythematosus

autoimmune lymphoproliferative
syndrome



REGULATORY

Antigen-specific suppression of
alloreactive CD8⁺ T cells

Suppress by an active
cell contact-dependent
mechanism

Protect cardiac
allograft from rejection

Provide a long-lasting
protective effect
in diabetes-prone
NOD mice

Inhibit the development of
experimental
autoimmune encephalomyelitis