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Commentary

$CD3^+CD4^-CD8^-$ (double negative) T cells: Saviours or villains of the immune response?

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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.

1. 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 (αβTCR) 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].

Thus it seems that immune cells, like human beings, like to transgress from their role and change their function or phenotype in specific settings. We have also learned that these T cells differ from their "conventional" siblings not just in their make-up of cell surface markers, but also in their function (Fig. 1) since they appear to behave more like innate rather than adaptive immune cells.

In this commentary we will be focusing our attention on $CD3^+CD4^-CD8^-$ double negative (DN) T cells, and on their pathophysiological functions. In particular, we will provide an

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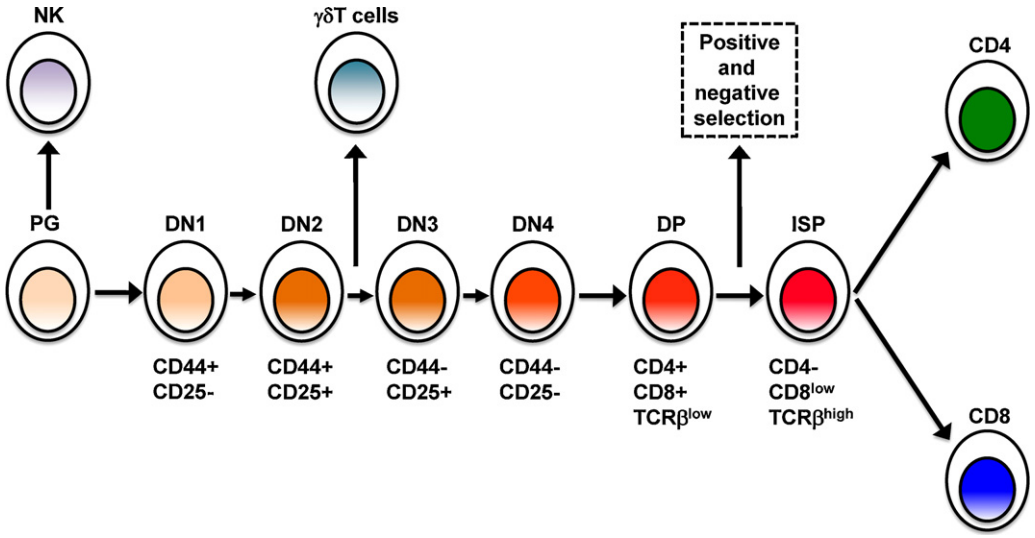

Fig. 1. Schematic representation of T cell development in the thymus. Progenitor (PG) cells from the bone marrow colonize the thymus where they commit to natural killer cells (NK) or T cell lineage. Here they undergo progressive differentiation from double-negative (DN) to double-positive (DP) to single-positive CD4 or CD8 thymocytes. The immature DN thymocytes can be further divided into DN1 to DN4 based on their expression of CD44 and CD25. The $\gamma\delta$ lineage diverge from the $\alpha\beta$ at the DN2/DN3 stage. Progression from DP stage to SP can occur through an immature CD8+ single positive (ISP) cell intermediate.

overview of their origin and development as well as of their role as suppressor (DN regulatory T cells; DN Tregs) or effector (DN T cells) of the immune and inflammatory response.

2. A very brief overview of T cell development

Most T cells develop in the thymus, which provides a specialized environment that supports the maturation of lymphocyte precursors into functional T-cells [7]. The thymus is seeded by blood-borne progenitor cells from the foetal liver or adult bone-marrow which develop along a well-characterized programme, dependent on bidirectional signals between the developing thymocytes and the thymic epithelial cells (TEC) [8]. During αβ T-cell development, thymic $CD4^{-}CD8^{-}$ DN cells give rise to the $CD4^{+}CD8^{+}$ double positive (DP) population, which differentiate to mature $CD8^{+}CD4^{-}$ or $CD8^{-}CD4^{+}$ single positive (SP) cells. The DN population can be subdivided by cell surface expression of CD25 and CD44. $CD44^{+}CD25^{-}$ (DN1) cells differentiate to become $CD44^{+}CD25^{+}$ (DN2) cells, which then differentiate to become $CD44^{-}CD25^{+}$ (DN3). The DN3 population gives rise to the $CD44^{-}CD25^{-}$ (DN4) subset, which undergo a phase of rapid proliferation before differentiation into the DP population, in general *via* a cycling immature $CD8^{+}$ intermediate single positive (ISP) cell (Fig. 1).

Maturation from the DP population to the mature SP T-cell populations involves positive selection of the αβTCR repertoire to ensure appropriate MHC restriction and negative selection of potentially self-reactive clones. Many models have been proposed to describe how DP thymocytes commit to the CD4 and CD8 lineages, and to explain how positive selection ensures that selected CD4 and CD8 SP populations express TCR appropriately restricted by MHC Class II and Class I, respectively. TCR repertoire selection is dependent on interactions between the TCR on the developing thymocyte and its MHC/peptide ligand on TEC [9–11]. Strength and duration of TCR signal are thought to broadly determine the DP cell's fate with the strongest signals leading to negative selection and apoptosis, (i.e. in the case of TCR recognizing self antigens), intermediate signals leading to positive selection, and, weaker signals or lack of TCR signaling leading to DP cell death by neglect [12]. For DP thymocytes that are undergoing positive selection, TCR signal strength and duration have been proposed to influence CD4 and CD8 lineage commitment, with those cells receiving stronger TCR signals tending to be biased towards the CD4 fate, and weaker/more transient signals tending to favour differentiation to CD8 SP T cells [10]. Transcription factors, such as Th-POK, play essential roles in lineage commitment, and the ways in which the transcriptional regulation of lineage commitment relate to TCR signaling require further study [11]. In addition, other secreted factors from TEC, such as the morphogen Sonic hedgehog influence TCR repertoire selection and differentiation from DP to SP cell [13,14].

3. Thymic and extrathymic origin of DN T cells

DN T cells have been proposed to originate in the thymus by escaping negative selection followed by migration in the periphery where they expand upon experiencing antigen [15]. Similar to T helper cell lineage commitment, the strength of TCR signaling seems to be an important factor for the development of DN T cells. According to this hypothesis, lower intensity TCR signals would lead typically to the generation of mature $CD4^{+}$ and $CD8^{+}$ T cells while high TCR strength that borders deletion would promote the survival and escaping of DN T cells from the thymus [16]. Using a model of thymocyte reaggregated culture Wang et al. [17] demonstrated that stimulation of cells with high concentrations of high affinity antigens leads to the conversion of DP thymocytes into DN T cells *via* down-modulation of both CD4 and CD8 coreceptors expression. It has alternatively been proposed that some DN T cells arise from the pre-TCR+ DN population (late DN3 population). Under this model, strong signal transduction through the pre-TCR/CD3 complex of γδTCR complex favours differentiation to DN T cell, whereas "normal/average" pre-TCR/CD3 complex signal transduction promotes differentiation to DP cell and commitment to the αβT cell lineage [18].

Several line of evidences also support the theory that DN T cells are generated in the periphery rather than in the thymus. Using CD8-deficient mice and sublethally irradiated thymectomized mice reconstituted with T cell-depleted bone marrow cells Ford et al. demonstrated that functional regulatory DN T cells exist in CD8-deficient mice, hence they are not derived from antigen-stimulated $CD8^{+}$ T cells. In contrast, a reduction in DN T cell number was observed in reconstituted thymectomized mice

compared with nonthymectomized recipients. Interestingly, DN T cells that develop in thymectomized mice showed an increased suppressing activity compared to those that develop in sham-thymectomized mice [19].

Mice lacking the expression of Fas/APO-1/CD95 (*lpr*) or Fas-ligand (*gld*) have been widely used to investigate the developmental origin of DN T cells. These mice show a peculiar accumulation of DN T cells in the lymph nodes and other organs [20,21] as a result of an impaired induction of apoptosis leading to lack of deletion of autoreactive T cells that is seen in normal mice [22]. *In vivo* antigenic stimulation of TCR transgenic/*lpr* mice resulted in peripheral deletion of T cells and in a nine fold increase in the percentage of DN T cells in the lymph nodes of the CD95-deficient but not the CD95-intact mice [23] suggesting a peripheral origin of these cells during immune stimulation. The clinical relevance of these observations has been confirmed in human. Patients suffering from autoimmune lymphoproliferative syndrome (ALPS) have mutations in the Fas apoptotic pathway that are associated with impaired apoptosis after TCR ligation of single positive (CD4 or CD8) activated mature T cells [24]. Interestingly, these patients share similar clinical features with *lpr* and *gld* mice and a selective accumulation of DN T cells in peripheral blood [25–29].

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

4. DN T cells as the villains of the immune system

Most of what we know about the pathogenic functions of DN T cells derives from the study of mice expressing a transgenic TCR or from autoimmune-prone genetically modified mice. The artificial expression of the transgenic $\alpha\beta$TCR at the double negative stage of T cell development has been proposed to mimic signals normally conveyed by the $\gamma\delta$TCR and thus lead to the generation of an artificial subpopulation of T cells with a unique profile of surface markers and cytokine production. One example of these mice is the HY-TCR transgenic mice. HY mice express a transgenic TCR that recognizes the male HY antigen presented by MHC class I molecule. In female mice the HY transgenic T cells are positively selected to the CD8 lineage. Conversely, male TCR transgenic mice show a strong reduction of the DP thymocytes and $CD8^+$ transgenic T cells [31,32]. Interestingly, in male mice a relatively small number of DN T cells escape thymic selection and can be recovered in the periphery or as intraepithelial gut lymphocytes [31,33].

Analysis of the gene fingerprint of these cells by microarray demonstrated that, similar to γδTCR or NK T cells, HY-TCR+ DN T cells showed a "hair-trigger" reactivity that place them in an intermediate position between innate and adaptive cells [34,35]. In fact these cells showed an unexpected upregulation of genes associated with the innate immune system and an increased production of IL-2 and IFN-γ when compared to female CD8+ T cells. These findings *in vitro* have been recently confirmed *in vivo* by our group using a classical model of delayed type hypersensitivity (DTH): the λ-carrageenan-induced paw oedema. Male HY mice showed an exacerbated inflammatory response accompanied by accumulation of HY-TCR+ cells in the inflamed paw tissues. *Ex vivo* analysis of the cytokine profile expressed by activated T cells present in the draining lymph nodes of male HY mice showed an increased production of classical DTH cytokines such as IL-2, IFN-γ and TNF-α or other "autoimmune" cytokines such as IL-17A and IL-22 compared to female HY-TCR+ cells [36].

The proinflammatory phenotype of mature DN T cells has also been observed during their development in the thymus. Interestingly, $CD44^+$ DN thymocytes have been found to produce high levels of IL-2, TNF-α and IFN-γ upon stimulation with PMA/Ionomycin while $CD44^-$ DN did not produce any of the cytokines mentioned. Similarly, stimulation of $\alpha\beta$TCR expressing DN thymocytes and DN isolated from the spleen with anti-CD3 revealed a similar pattern with IL-4, IFN-γ and TNF-α being the main cytokines released upon activation [37].

A growing number of recent studies suggest that DN T cells are not just an artificial subsets of cells occurring in transgenic or genetically modified mice but key players in autoimmunity and inflammation in humans. Several studies provided a direct link between DN T cells and the development of autoimmune diseases. DN T cells have been found in the blood of myasthenia gravis patients with lymphofollicular hyperplasia and have been shown to participate in the immunoregulation and increased antibody production [38]. Patients suffering from systemic lupus erythematosus (SLE) have a significantly expanded population of DN T cells and these cells are the major producers of IL-17 [39], one of the key inflammatory cytokine involved in SLE [40,41]. These results confirmed previous observations from the same group showing that DN T cells from MRL/lpr mice produce high levels of IL-17 as the disease progresses. In addition, *in vitro* treatment of MRL/lpr lymph node cells with IL-23, a cytokine required for sustained Th17 cell differentiation [42], induces the generation of highly pathogenic DN T cells causing nephritis following adoptive transfer in lymphocyte deficient $Rag1^{-/-}$ mice [43,44]. In a study conducted on patients suffering from ALPS [45] (also known as Canale-Smith syndrome) the authors demonstrated that the percentage of DN T cells increased from 1% (observed in physiological conditions) to 40% [46]. These results together with other evidence [26–29] suggest that the increased number of DN T cells may represent a sensitive test or biomarker to screen patients who should undergo testing for ALPS. In this regard it is interesting to mention that the analysis of DN T cell levels has been used to distinguish ALPS from other autoimmune disease such as Evans syndrome [27] or sarcoidosis [47].

In line with their ability to produce high levels of IL-17, DN T cells have been reported to play a key role in various models of infections. DN T cells are the major responding T cell subset in the lung of mice infected with live vaccine strain (LSV) of the intracellular bacterium *Francisella tularensis*. Temporal analysis of cytokine production in infected lung showed that DN T cells release large amount of IL-17A during the early stages of infection (7 days) while CD4+ T cells start to produce this cytokine later (14 days) [48]. Considering that IL-17-deficient mice die at day 8–10 post LSV infection [49], it is tempting to suggest that DN T cells rather than CD4+ T cells are major contributors to the development of LSV infection. Marodon et al. provided further evidence of the importance of DN T cells in infectious diseases. In this study the authors showed that DN T cells of HIV-infected patients represent up to 20% of the cellular viral load in T cells; moreover, successful antiviral therapy was associated with a significant decrease in viral load in DN T cells suggesting that infected DN T cells, like $CD4^+$ cells, contribute to viral production and are sensitive to highly active antiretroviral therapy [50].

5. DN T cells as the saviours of the immune system

T cells with immunoregulatory activity have always attracted the attention of scientists for their potential clinical application in a variety of pathological conditions ranging from autoimmunity to tumour immunology [51,52]. These cells act as sentinels of the immune system and thus are essential in the maintenance of immune homeostasis and self-tolerance [53].

The subtypes of T cells with immunoregulatory function has significantly increased in the last few years and we now know that

there are Treg other than the originally identified $CD4^+/FoxP3^+/CD25^+$ T cells. These include NO-Tregs that derive from $CD4^+CD25^-$ T cells and are induced by nitric oxide (NO) [54], Tr1 cells that do not express FoxP3 and release a large amount of IL-10 [55,56] and TGF-β-producing $CD4^+$ Th3 cells [57,58]. As if this field of research was not already "crowded", DN T cells have also been added to this list. These cells have been described as the only subtype of immunoregulatory cells able to suppress antigen specific T cell responses [59,60] as well as a number of other features that make them different from the "conventional" regulatory T cells [61–63]. To avoid confusion with the other DN subset, we will refer to these cells as DN Tregs.

DN Tregs have been isolated and identified in mouse, rat and human. One of the first studies conducted in mice described the isolation of natural suppressor DN T cells derived from the spleen of adult mice [64,65]. A subsequent study on rat DN T cell clones demonstrated that these cells did not elicit disease but rather inhibited the development of experimental autoimmune encephalomyeltis (EAE) [66]. Studies in human identified a population of DN T cells in a patient with immunodeficiency, lymphocytosis, lymphadenopathy, and hepatosplenomegaly. These cells showed an impaired proliferative response upon stimulation with mitogens or anti-CD3 plus anti-CD28 but were not tested for their immunosuppressive activity [67].

As in the case of inflammatory DN T cells, TCR transgenic mice contain a significant number of DN Tregs. These cells present a number of features including a similarity with naive $CD8^+$ T cells that have undergone homeostatic proliferation and conversion into memory-like T cells, lower threshold of activation [68] and functional anergy as they hypoproliferate and produce little or no IL-2 in response to antigen stimulation [69]. Controversies exist on the possible mechanisms by which DN Tregs down-regulate immune responses. According to one study, DN Tregs from TCR transgenic mice acquire the antigen from antigen presenting cells *via* trogocytosis and they then re-express these newly acquired molecules on their cell surface to mediate antigen-specific suppression of activated alloreactive $CD8^+$ T cells [62]. Further studies have confirmed these findings and have shown that DN Tregs acquire alloantigen *in vivo via* trogocytosis [70]. Like classical CD4+ CD25+FoxP3 Treg they require direct cell contact for suppression and express a unique combination of cell surface markers that makes them distinguishable from any previously described T-cell subsets [62]. According to another study, the killing of CD8+ T cells by DN Tregs depends on the direct recognition of the antigen on the target cell by the TCR and this enables DN Tregs to respond to a greater variety of self antigens and hence, to regulate the response of a larger repertoire of T cells [15]. Recent studies on human DN Tregs have revealed some discrepancies with their murine counterparts. Human DN Tregs are not believed to eliminate effector T cells by Fas/FasL-mediated apoptosis, modulation of antigen presenting cells or competition for growth factors but to suppress by an active cell contact-dependent mechanism [71].

Several investigations have shown that DN Tregs play an important role in the development of tolerance after transplantation. DN Tregs have been described to prolong both allo- and xenograft survival compared with untreated controls when adoptively transferred into syngeneic mice. Initial studies in transgenic mice suggested that DN Treg cells can be generated *in vitro* and protect cardiac allograft from rejection when infused into recipients prior to transplantation [72]. However, in a subsequent study by Lee et al. [73] the authors showed that although single-dose infusion of DN Treg clones from normal mice induces permanent survival of MHC class I-mismatched cardiac allografts, their suppressing activity was lost after *in vitro* culture and clonal expansion. Similarly in another study using rat to mouse cardiac xenotransplant it was found that DN Tregs harvested from the spleens of the recipient mice dose-dependently inhibit the proliferation of syngeneic antidonor T cells [74].

DN Tregs also play a homeostatic role in autoimmune diseases. Diabetes-prone mice carry fewer DN Tregs and this contributes to the increased susceptibility of these mice to develop the disease. Different mechanisms have been proposed to contribute to this effect. Using a transgenic TCR mouse model Hillhouse and colleagues have shown that *in vivo* antigenic stimulation of transgenic T cells induced a 10 fold increase in the number of DN Tregs in diabetes-prone $NOD.H^{2k}$ but not in B10.Br control mice. They also showed that IL-10 produced by these cells favours their apoptosis and hence helps to regulate their number [75]. In another study by Duncan et al. [76] the authors proposed a correlation between DN Tregs and the late onset of diabetes in NOD mice. Young NOD mice have a high frequency of DN Tregs that declines in adulthood and their adoptive transfer in Nod/Scid mice provided a long-lasting protective effect. Consistent with the previous study, the authors also demonstrated that the anti-diabetogenic effect of DN Tregs relied mainly on the ability to differentiate into IL-10-secreting Tr-1 cells [76].

6. DN T cell "reservoirs" in the body

DN T cells seem to preferentially dwell in specific organs or tissues (summarized in Fig. 2). In MRL-lpr/lpr mice DN T cells were

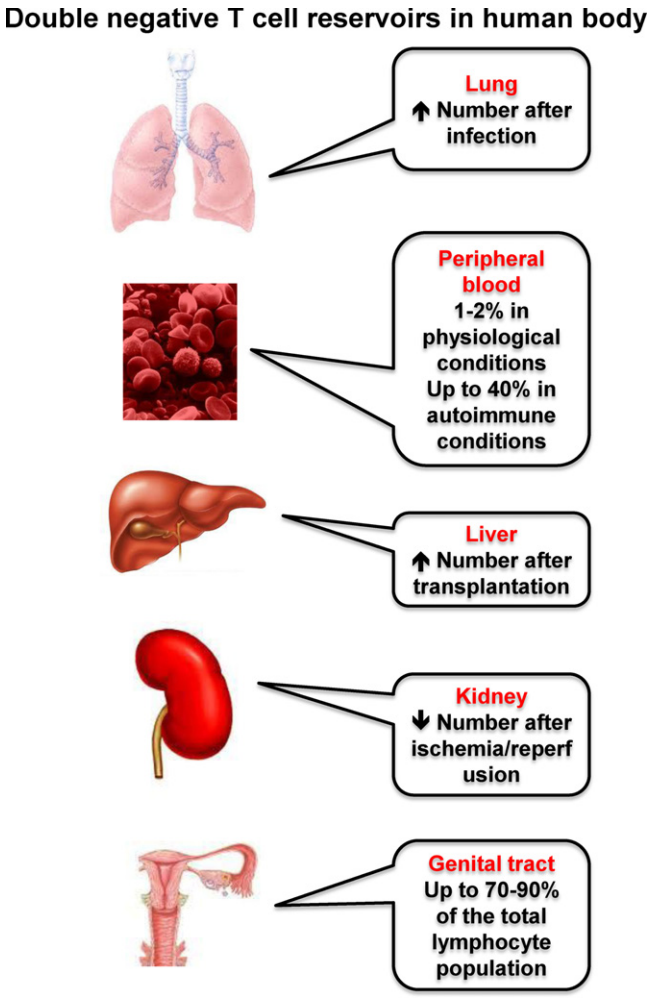

Fig. 2. Schematic representation of favourite tissue localization of DN T cells in murine and human tissues and their relative frequency in health and disease conditions.

found in close vicinity to Kupffer cells or endothelial cells of the hepatic sinusoids. Most interestingly, this liver-specific accumulation of DN T cells is not a unique feature of MRL-lpr/lpr but it occurs in several other autoimmune-prone strains including C3H/HeJ-gld/gld, BXSB, NOD and NZB/W F1 mice with their proportion increasing as mice become old and diseased [77]. The clinical relevance of these findings has been confirmed in a study where an analysis of the number of peripheral blood DN T cells in patients after liver transplantation was conducted. Patients with proven acute cellular rejection showed a significant increase in DN T cells and a significant reduction after pulse corticosteroid therapy [78]. The kidney is another favourite hub for DN T cells. However, unlike the liver, the number of DN T cells in the kidney decreases during inflammatory conditions such as after ischemia and reperfusion [79,80]. These results are in contrast with the aforementioned study in which it was shown that patients with lupus nephritis have a significant number of DN T cells infiltrating the kidney [39].

An interesting study investigated the phenotype, distribution, and function of T lymphocytes in the female genital tract of naive, pregnant, or *Chlamydia trachomatis*-infected C57BL/6 mice. Unexpectedly, the authors observed that DN Tregs were the dominant lymphocyte population (70–90%) in the genital tract [81]. This might be of clinical importance considering that DN T cells have been associated with pathogenesis of Behcet's disease [82], a complex mucosal inflammatory disorder with neural and vascular manifestations that includes oral aphtosis and genital ulcers [83].

A unique distribution of DN T cells has been described in CD18-deficent mice. Comparative analysis of cervical, axillary and inguinal lymph node populations in wild-type and CD18-deficent mice showed an expected six fold decrease in the total lymphocyte number in axillary and inguinal lymph nodes, together with a fourfold increase in the cellularity of cervical lymph nodes, of the latter compared to the former. These cells are massively generated within the lymph nodes upon antigen stimulation and display typical features of antigen-experienced cells including lower threshold of stimulation, heightened proliferation rate and increased re-circulation to non-lymphoid organs [84].

7. Conclusions and perspectives

The increasing number of studies on the role and function of DN T cells over the last decade have significantly expanded our knowledge of this new subset of immune cells in health and disease. Most importantly, we now know that these cells are not just an artefact that occurs in genetically modified or transgenic TCR mouse models but that they are also found in a variety of human conditions spanning from autoimmune disease to inflammation, infection and cancer [26,39,59]. Tables 1 and 2 summarize the main functions of pathological DN T cells and DN Tregs described in this commentary.

One of the most intriguing aspects of the biology of DN T cells is their dual identity i.e. their pathogenic or immunosuppressive phenotype. In this context, a number of questions need to be addressed. For instance, how do DN T cells decide to be villains or saviours of the immune system? Do DN T cells and DN Tregs derive from the same progenitor cell? And most importantly, can DN Tregs convert into inflammatory DN T cells? This very last aspect is of great importance before we even begin considering to use DN Tregs in cell immunotherapies.

A great deal of attention has been recently given to the concept of T cell plasticity. According to this theory, Th effector cells can readily switch from one phenotype to another especially when

Table 1
Summary of the main functions of human and murine pathogenic DN T cells.

Mouse model or human disease	Phenotype	Reference
HY-TCR transgenic mice	"Hair-trigger reactivity Production of high levels of IL-2 and IFN-γ Exacerbated inflammatory response *in vivo*	[34,35]
	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 highlevels 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	[50]
	Successful antiviral therapy was associated with a significant decrease in viral load in DN T cells	[25–29]

Table 2
Summary of the main functions of human and murine DN Tregs cells.

Mouse model 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	[68]
	Hypoproliferation and little or no production of IL-2 in response to antigen stimulation	[69]
$2C_{F1}$(H-$2^{b/d}$, L^{d-}, anti-L^d 1B2-TCR+) transgenic mice	Antigen-specific suppression of activated alloreactiveCD8+ T cells *via* trogocytosis	[62,70]
Anti-L(d) transgenic TCR+ (2C × 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.H^{2k} mice	Increase in number after antigenic stimulation *in vivo*	[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]

adoptively transferred *in vivo*. Thus, Th17 cells can convert in Th1 cells, classical FoxP3$^+$ Treg can become Th17 and Th2 cells can change into an IL-9 producing Th9 cells [85–87]. Whether or not inflammatory DN T cells can acquire an immunosuppressive phenotype and *vice versa* has not been explored yet and it would certainly be very interesting to investigate. Clearly, a number of factors contribute to their phenotype and more investigations are needed to fully appreciate the intrinsic and extrinsic factors influencing their fate. In any case, several observations indicate that, like for Th effector cells, the microenvironment plays a key role in determining DN T cell functions and/or expansion.

Beside these considerations, we think that there are important aspects other than their origin or development that have not been fully explored. If one wants to bring DN T cells under the spotlight of "translational science", there are several gaps that need to be filled in. First of all, the identification of specific markers that allow distinction between inflammatory DN T cells from DN Tregs would be of great clinical and therapeutical importance. A first attempt has been carried out by Lee and colleagues by comparing gene expression differences between functional DN Treg cells and nonfunctional mutants [73] but clearly more extensive proteomic analyses are needed. In other studies it has been found that *lpr* DN lymph node cells are homogenous for expression of high level of CD44, B220 (the isoform of CD45 found on B cells) [88,89], CXCR5 [90] and lack the expression of CD2 [91] and IL-2 receptor [92].

The availability of further specific markers will facilitate the isolation and analysis of these cells and, at the same time, might provide novel diagnostic tools that can be used to predict disease progression and possibly remission. Further insights into the mechanisms of activation of DN T cells could be used to investigate at the biochemical level what makes these cells so hyperreactive and pathogenic. This information could be used for the design of specific drugs able to limit inflammatory DN T activation or, possibly, favour DN Treg development and/or expansion.

We recognize that these are not simple and straightforward processes, especially if one aims at ultimately targeting human DN T cells. However, there are small steps that can (and should) be taken up by pharmacologists interested in immune and inflammatory diseases. In our view, the availability of relatively simple experimental systems to study DN T cells or DN Tregs would provide a significant boost in translational research in this area. For instance, in our recent study we have used the HY-TCR transgenic mice to assess the contribution of DN T cells to the early and late stage of the inflammatory reaction [36]. The high frequency of DN T cells in male mice makes these animals an ideal system to test novel therapeutic strategies targeting these cells. Similarly, given the high number of DN Treg in young NOD mice [76], this system could be an ideal platform for the testing of novel immunosuppressive therapies.

Along these lines, it is somehow surprising that very little attention has been given to the possible effects of commonly used anti-inflammatory and immunosuppressive drugs on DN T cells or DN Tregs. Evidence that popular pharmacological therapies might modulate CD4 and CD8 expression in T cells were reported more than 20 years ago [93–95] and thus the possibility that these drugs might act on DN T cells and DN Treg development and/or activation is not far fetched. In one interesting recent studies by Okazaki et al. [96] the authors showed that FTY720, a novel immunosuppressant that modulates sphingosine 1-phosphate receptor, induced apoptosis in more than 70% of DN T cells from the spleen of MRL/lpr mice *in vitro* and *in vivo*.

To conclude, we hope that this commentary will help the readers of Biochemical Pharmacology to appreciate the importance of DN T cells as therapeutic targets since we think that this is a neglected area of research that needs much attention and which has great potential for pharmacological intervention. We also hope that this overview will invite investigators to look back at their cytofluorimetric or immunohistochemistry data with new eyes that are not obfuscated by the analysis of solely CD4 or CD8 single positive T cells.

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