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Review

TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$ (double negative) T cells in autoimmunity

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ABSTRACT

TCRαβ$^+$CD3$^+$CD4$^-$CD8$^-$ "double negative" (DN) T cells comprise a small subset of mature peripheral T cells. The origin and function of DN T cells are somewhat unclear and discussed controversially. While DN T cells resemble a rare and heterogeneous T cell subpopulation in healthy individuals, numbers of TCRαβ$^+$ DN T cells are expanded in several inflammatory conditions, where they also exhibit distinct effector phenotypes and infiltrate inflamed tissues. Thus, DN T cells may be involved in systemic inflammation and tissue damage in autoimmune/inflammatory conditions, including SLE, Sjögren's syndrome, and psoriasis. Here, the current understanding of the origin and phenotype of DN T cells, and their role in the instruction of immune responses, autoimmunity and inflammation will be discussed in health and disease.

Contents

1. Background

The presence of activated self-reactive T cells is a hallmark of various autoimmune/inflammatory disorders [1]. Through the instruction of inflammatory responses and tissue damage, T cells centrally contribute to the pathophysiology of autoimmune disease through the generation of a sometimes (relatively) disease-specific cytokine milieu, chemo-

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attraction of additional inflammatory cells, and/or the promotion of autoantibody production by B cells [1].

The majority of human and murine T cells express and rearrange the α and β chains of the T cell receptor (TCR) and are therefore referred to as TCRαβ T cells [2]. A rare subset of T cells expresses the γ and δ chains of the TCR, and is mostly $CD4^-$ and $CD8^-$ (DN). $TCR\gamma\delta^+$ T cells represent a separate lineage with distinct phenotype and unique properties [3]. In both humans and mice, approximately 95% of T cells express TCRαβ, whereas 5% of T cells express TCRγδ [4]. Though very complex and beyond the scope of this review, generally, $TCR\alpha\beta^+$ T cells are considered prototypical members of the adaptive immune system, while $TCR\gamma\delta^+$ T cells may be activated by classical triggers of the innate immune system, including heat shock protein derived peptides [5]. Among $TCR\alpha\beta^+$ T cells, $CD8^+$ and $CD4^+$ T cells are the most common subsets. However, $CD4^-CD8^-$, so-called 'double negative' (DN) T cells comprise an additional, usually very small subset that has been suggested to contribute to the pathophysiology of several autoimmune/inflammatory conditions [6,7].

Historically, $TCR\alpha\beta^+$ DN T cells have been considered "abnormal" and/or disease-causing. This assumption was based on massively increased numbers of DN T cells in the peripheral blood and their accumulation in secondary lymphoid organs in Fas-deficient *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferation) mice, resulting in lymphadenopathy and splenomegaly [8–10]. Later on, a phenotypically very similar disease was described in humans several years and termed autoimmune lymphoproliferative syndrome (ALPS) [11,12]. Indeed, in affected humans, as well as aforementioned animals, deficiency of Fas (*lpr* mice; Fas^{lpr}) or Fas ligand (*gld* mice; $FasL^{gld}$) (FasL) was linked with disease expression [13–15]. Our current understanding of pathological DN T cell number expansion includes the hypothesis that usually Fas-mediated apoptosis removes DN T cells from the peripheral blood, which is impaired in ALPS patients and Fas- or FasL-deficient animals [16]. Pathogenic DN T cells exhibit defects in peripheral tolerance and contribute to lymphoproliferation and systemic inflammation. Thus, Fas- and FasL-deficient animals serve not only as model systems for ALPS, but also other autoimmune/inflammatory conditions that are characterized by increased numbers of DN T cells, including Sjögren's syndrome [17] and Systemic Lupus Erythematosus (SLE) [1,7].

Though associated with several inflammatory conditions, DN T cells can also be found in the peripheral blood and tissues of healthy individuals. In humans and rodents, DN T cells represent a relatively small subpopulation of approximately 1–3% of all $CD3^+$ T lymphocytes in the peripheral blood [18,19]. Double negative T cells are also present in secondary lymphoid organs of healthy individuals, but more common in certain non-lymphoid tissues, such as murine and human kidneys [20,21], intestinal epithelium [22], and the murine female genital tract [23]. Thus, more recent studies focusing on the presence and function of DN T cells in humans and rodents suggested DN T cells as components of the "healthy" immune system. Indeed, DN T cells contribute to physiological immunological responses against intracellular bacteria [24–26] or the Influenza A virus [27] in mice, implicating distinct functions of DN T cells in host defense mechanisms.

Despite recent advances, our understanding of DN T cells in health and disease remains incomplete. This is partially based on their low frequencies in (particularly healthy) humans and mice. As a result, origin, differentiation mechanisms, and function of DN T cells remain unclear and are discussed somewhat controversially [6,28]. In the following, recent developments in understanding the origin, phenotype and (patho-)physiological role of DN T cells will be discussed in health and disease.

2. Materials and methods

A PubMed-based literature search was performed using the search stings "Double negative T cells", "$CD4^-CD8^-$ T cells", "T cell differentiation", "T cell generation" and their combination with "gene expression", "surface expression", "pathophysiology", "cytokine", "epigenetic regulation", "inflammation", "therapy", "treatment", "disease", "Sjögren's syndrome", "Systemic lupus erythematosus", and "psoriasis". Furthermore, the authors used their personal collection of PubMed-listed published manuscripts on the topic (that included manuscripts that were to be found under the aforementioned search strings).

3. The origin of DN T cells

Regardless of recent scientific efforts to describe the source and function of DN T cells, their origin and role in immune mechanisms are only incompletely understood. Heterogeneous surface marker expression, as well as cytokine and chemokine expression profiles, suggest that variable sources and/or differentiation pathways may exist [6]. Indeed, even the precise lineage affiliation of DN T cells remains somewhat unclear and may be different between species (humans *vs.* mice). Still, most available data derive from murine systems [10].

3.1. Thymus-derived DN T cells

During "normal" T cell development, thymocytes undergo several differentiation steps, which include four "double negative" stages (DN thymocytes) in mice and three in humans [29]. After passing these DN stages, murine thymocytes subsequently differentiate *via* $CD8^+$ immature single positive stage (ISP) into $CD4^+CD8^+$ "double positive" (DP) cells, before they finally commit to exclusive expression of either CD4 or CD8 [30] (Fig. 1). Human DN thymocytes mature *via* the $CD4^+$ ISP stage into DP cells [31].

As mentioned before, 1–3% of peripheral T cells in healthy individuals are $TCR\alpha\beta^+$ DN T cells, and their number can be increased in autoimmune/inflammatory conditions [6,32,33]. One hypothesis potentially explaining the presence of DN T cells in the peripheral blood includes DN T cells escaping negative selection in the thymus, and their subsequent activation and expansion in the periphery [34–36].

As mentioned above, TCRαβ or TCRγδ lineage commitment in mice and humans is a result of complex TCR gene rearrangement [37]. In mice, later DN thymocyte stages (DN3) during T cell differentiation were suggested to give rise to either $TCR\alpha\beta^+$ DN T cells or $TCR\gamma\delta^+$ DN T cells, depending on the strength of TCR signal [38] or sex steroid-induced release [39] (Fig. 1A). Alternatively, murine DP thymocytes can give rise to DN T cells that then migrate to the intestinal epithelium to become intraepithelial resident $TCR\alpha\beta^+$ DN T lymphocytes (IEL) [36,40] (Fig. 1B). Furthermore, presentation of high-affinity ligands to DP thymocytes by cortical thymic epithelial cells *in vitro* resulted in immunoregulatory $TCR\alpha\beta^+$ DN T cell differentiation [41].

Of note, most studies implicating the thymus as the source of peripheral DNT cells relied on TCR transgenic mice. However, interpreting murine models with transgenic TCR expression can be challenging and should take into account that TCR expression in these models is already present in "early" DN thymocytes. This is in contrast to endogenous TCR expression that is not present before late DN thymocyte stages (DN3/4) or the DP stage [42] (Fig. 1). Indeed, engaging the transgenic TCR with high-affinity ligands may result in abnormal thymic selection processes [43–45].

3.2. Molecular mechanisms of DN thymocyte generation

The engagement or repression of genomic regulatory elements, including promoters and enhancer regions plays a central role during cell fate decisions of T cells while undergoing thymic maturation and also later differentiations steps [46,47]. During T cell differentiation, transcription factor recruitment to regulatory elements regulates stage- and/or lineage-specific gene expression. In addition to the absence or presence of transcription factors, their recruitment to regulatory elements can be regulated through chromatin accessibility. So-called epigenetic events regulate gene expression through chromatin re-arrangement regulating its accessibility to transcription factors and

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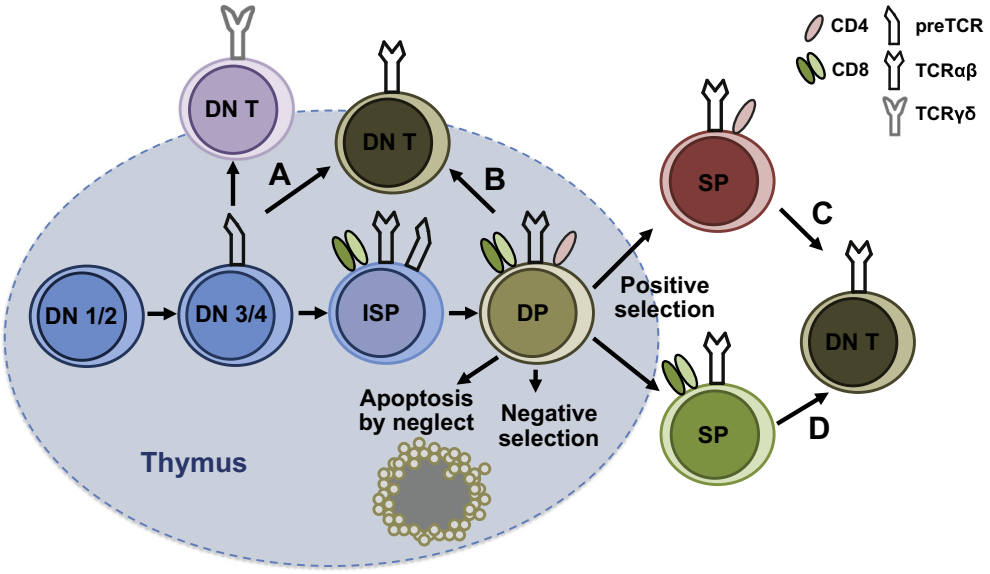

Fig. 1. Origin of DN T cells. Mature "single positive cells" (SP) in the periphery ($CD4^+$ or $CD8^+$ T cells) derive from $CD4^+CD8^+$ "double positive" (DP) cells that derive from immature single positive stages (ISP). (A) Late stage double negative (DN3) thymocytes can give rise to either $TCR\alpha\beta^+$ DN T cells or $TCR\gamma\delta^+$ DN T cells [38,39]. (B) Alternatively, DN T cells may derive from DP thymocytes that then migrate to the intestinal epithelium [36,40]. DN T cells can derive from activated peripheral (C) $CD4^+$ [67,68] and/or (D) $CD8^+$ T cells [69–74].

other elements of the transcriptional complex without altering the underlying DNA sequence. Epigenetic mechanisms comprise several events, namely DNA methylation and post-translational histone modifications [48–50].

Epigenetic mechanisms are involved in the regulation of the *CD8* gene cluster during T cell development in the thymus. Four genomic elements in the murine and six elements within the human *CD8* gene cluster with lineage-specific DNase sensitivity have been identified [51,52] (Fig. 2A). Transgenic murine reporter systems identified four pivotal enhancer elements within the *Cd8* cluster ($E8_I$–$E8_{IV}$) [51–62]. Tight regulation of the resulting enhancer network orchestrates lineage-specific CD8α (encoded by *Cd8a*) and CD8β (encoded by *Cd8b*) expression during T cell development [47]. The heterodimer CD8αβ plays a crucial role during thymic selection, and its expression marks thymus-derived $CD8^+$ T cells [63]. Surface expression of CD8α homodimers is characteristic for peripherally derived $CD8^+$ T cells (*e.g.* gut derived) [64].

To allow stage- and lineage-specific CD8 expression, enhancer elements $E8_I$–$E8_{IV}$ undergo epigenetic remodeling during T cell development either permitting or terminating the expression of CD8A and/or CD8B [52]. Low degrees of DNA methylation along the *Cd8* gene cluster in $CD4^+CD8^+$ and $CD8^+$ T cells allow the expression of murine *Cd8a* and *Cd8b*, whereas increased levels of DNA methylation at the *Cd8a* and *Cd8b* genes in $CD4^+$ and DN T cells prohibit gene expression [65].

3.3. Thymus-independent generation of DN T cells

Studies in humans and mice suggested that at least a subset of DN T cells may be the end product of thymus-independent processes [23,66]. Several lines of evidence support the hypothesis that DN T cells can derive from activated peripheral $CD4^+$ and/or $CD8^+$ T cells. Zhang et al. showed that mature murine peripheral $CD4^+$ ($CD25^+$/$Foxp3^+$ and $CD25^-$/$Foxp3^-$) T cells can convert to MHC II-restricted DN T cells *in vitro* and *in vivo* and exert regulatory function [67]. More recently, Grishkan et al. demonstrated that "chronic" (by the authors defined as more than three weeks) *in vitro* stimulation of murine spleen-derived $CD4^+$ T helper cells results in the generation of DN T cells that show effector phenotypes [68] (Fig. 1C). Conversely, (short term; 5 days) *in vitro* stimulation of human $CD4^+$ T cells did not result in DN T cell generation [69].

Accumulating evidence supports the hypothesis that DN T cells can derive from activated peripheral $CD8^+$ cells through the down-regulation of CD8 surface co-receptor expression (Fig. 1D). In both Fas-deficient MRL.*lpr* mice and human autoimmune lymphoproliferative syndrome patients, the majority of DN T cells have been demonstrated to derive from $CD8^+$ T cells [70–72]. Moreover, in response to *in vitro* TCR complex stimulation, a subset of $CD8^+$ T cells derived from healthy human subjects transformed into DN T cells through the down-regulation of CD8 co-receptor expression [69,72]. These findings are in agreement with studies in mice displaying accumulation of DN T cells in response to antigen-specific *in vivo* stimulation of TCR transgenic $CD8^+$ T cells *in vivo* [73,74].

3.4. Molecular mechanisms of peripheral DN T cell generation

Several molecular mechanisms are involved in activation-induced transformation of peripheral $CD8^+$ T cells into DN T cells. The transcription factor cAMP responsive element modulator (CREM)α recruits to several elements within the human and the murine *CD8* cluster. *Trans*-repression of the *CD8B* promoter and an additional enhancer element (CNS2) through CREMα results in transcriptional silencing and down-regulation of CD8 surface expression [72] (Fig. 2B). Furthermore, CREMα orchestrates recruitment of DNA methyltransferase (DNMT)3a and histone methyltransferase G9a, mediating stable epigenetic silencing through DNA and histone methylation of the *CD8* gene cluster [75] (Fig. 2B). To date, however, it is not clear whether CREMα exclusively regulates the CD8 cluster in peripheral $CD8^+$ T cells in response to TCR activation or whether it also participates in the regulation of chromatin remodeling during the priming and differentiation of T cells in the thymus [75].

Furthermore, the transcription factor Runt-related transcription factor (RUNX)3 may also influence the epigenetic landscape of $CD8^+$ T cells in mice. In activated murine $CD8^+$ T cells, maintenance of CD8 co-receptor expression is dependent on RUNX3 and the Runx/core binding factor-β though their recruitment to the aforementioned enhancer element $E8_I$ [76]. Absence of $E8_I$ results in chromatin remodeling and epigenetic silencing of the entire *Cd8* cluster. Interestingly, $E8_I$ lies within conserved non-coding regions in the human and murine *CD8* cluster that undergo CREMα-instructed epigenetic remodeling in response to TCR activation. Thus, it appears tempting to speculate that

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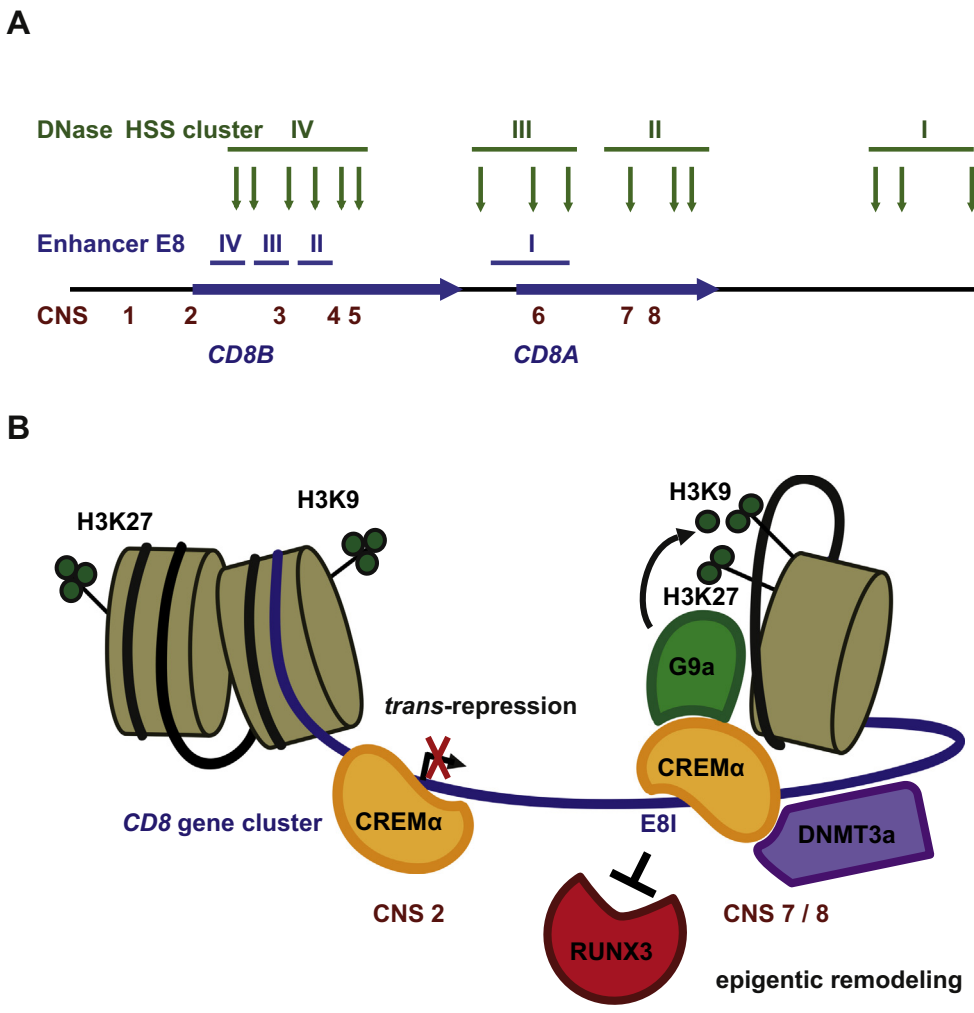

Fig. 2. Transcriptional regulation and epigenetic plasticity of the CD8 gene cluster. (A) Schematic map of the murine *CD8* gene cluster comprising the *Cd8A* and *Cd8B* genes. Previously reported DNAse I hypersensitive sites (HSS) I-IV [51] and CD8 expression regulating enhancer elements $E8_I$–$E8_{IV}$ are displayed [53]. Eight recently reported conserved non-coding sequences (CNS1–CNS8), defined as regions of 200 bp with 70% homology between human and mouse genome [72], are depicted below and (at least partially) map to DNAse hypersensitivity sites and enhancer elements. (B) Model of CREMα-mediated regulation of CD8 expression. CREMα controls transcriptional activity through *trans*-repression of the *CD8B* promoter (CNS2) [72]. Furthermore, CREMα recruits to regulatory elements and co-recruits DNMT3a and histone methyltransferase G9a to the *CD8* cluster (CNS 2,7,8). G9a mediates methylation of histone H3 at H3K9 and H3K27. Resulting DNA and histone methylation results in stable epigenetic silencing of *CD8A* and *CD8B* [48]. Indeed, CREMα-instructed epigenetic remodeling may reduce recruitment of additional transcriptional regulators, such as RUNX3 (required for CD8 co-receptor expression) to enhancer elements (*e.g.* $E8_I$).

CREMα-mediated reduced recruitment of RUNX3 to this region may play a central role in the transformation of $CD8^+$ T cells into DN T cells [75].

Another recent study linked the loss of CD8 expression in $CD8^+$ T cells from OT-I mice to IL-4-induced STAT6 activation *in vitro* and *in vivo* [77]. In the same study, down-regulation of CD8 was linked to increased DNA methylation of the *Cd8a* gene. During the generation of DN T cells, STAT6 may orchestrate transcriptional repression of *Cd8a* and DNA methylation either directly or through the induction of GATA3 expression [78]. Interestingly, the presence of IFN-γ recovered CD8 expression in a subset of DN T cells [77], suggesting that epigenetic silencing of *Cd8* in DN T cells may be reversible. Furthermore, epigenetic plasticity of the *Cd8* gene locus in mature $CD8^+$ T cells strongly supports the hypothesis that DN T cells can derive from $CD8^+$ T cells [75].

Interestingly, studying the methylome of peripheral $TCR\alpha\beta^+$ DN T cells revealed subset-specific DNA methylation patterns when compared to both $CD4^+$ and $CD8^+$ T cells. While regulatory elements of the human CD8 cluster were hypermethylated as previously reported, global DNA methylation was reduced in peripheral $TCR\alpha\beta^+$ DN T cells when compared to $CD4^+$ and $CD8^+$ T cells. In line with these observations, significantly reduced expression of all three DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) were seen in DN T cells. Distinct and highly consistent DNA methylation patterns in genes responsible for cell interaction and adhesion, as well as (usually) Th1 (*IFNG*), Th2 (*IL5*), and Th17 (*IL17F*)-specific genes implicate a unique epigenetic architecture in DN T cells that allows for a broad immune response [79].

4. Phenotypes and functional properties of DN T cells

Variable phenotypes of DN T cells have been described, indicating that DN T cells, just as other T cell lineages, may be divided into subsets [80]. Indeed, depending on activating stimuli or variable developmental histories, DN T cells may exhibit distinct expression patterns of surface co-receptors [80]. Nevertheless, DN T cells also exhibit consistent characteristics observed in several studies. Human peripherally generated DN T cells, for instance, express polyclonal αβ T cell receptor repertoires [19]. Furthermore, DN T cells neither express natural killer (NK) cell markers (CD56, CD16) nor classical markers of regulatory T cells, such as FOXP3, CD25, or CTLA-4 [19,81]. Of note, reduced or even absent expression of the surface co-stimulatory molecule CD28 appears to be representative for the majority of peripheral blood DN T cells [82]. Of note, CD28-deficient T cells are generally considered antigen-experienced and differentiated [83]. Indeed, DN T cells display characteristics of terminally differentiated and "exhausted" T cells that underwent extensive

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proliferation, such as a low content of T-cell receptor excision circles (TRECs) [19] and the expression of the inhibitory molecule Programmed Cell Death Protein 1 (PD-1) [84,85]. In agreement with these observations, DN T cells fail to proliferate in response to TCR complex activation and display increased apoptosis as compared to $CD4^+$ or $CD8^+$ T cells [32,69,85].

Taken together, aforementioned observations suggest effector phenotypes and a terminal differentiation status of DN T cells. Indeed, a significant proportion of freshly isolated or *in vitro* stimulated DN T cells, display surface marker patterns (*e.g.* $CCR7^+$ and $CD45RA^-$) that are characteristic for effector T cells [19,82,85]. Interestingly, in response to TCR complex stimulation, the fraction of $CD45RA^-$ effector DN T cells considerably increased in recent studies, implicating that activated DN T cells favor differentiation into effector memory T cell phenotypes [85,86]. The hypothesis that DN T cells may be terminally differentiated is further supported by the observation that they fail to proliferate in response to TCR complex stimulation and that the pool of DN T cells is maintained or extended by *de novo* generation of DN T cells from $CD8^+$ T lymphocytes [69].

Several studies reported DN T cells to exhibit broad cytokine expression patterns. For instance, DN T cells spontaneously secrete the immune regulatory cytokine IL-10 [19,69]. This observation is of particular interest, since IL-10 is known to on the one hand exert anti-inflammatory functions, *e.g.* through the suppression of T cell responses [87], and on the other hand contributes to B cell activation, differentiation, and antibody production [88,89]. Thus, IL-10 expression may be a key mechanisms connecting effector with regulatory functions (see below) of DN T cells. Effector functions of DN T cells are further suggested by the expression of signature cytokines of effector T cell subsets. We and others observed interferon (IFN-)γ expression in DN T cells in response to TCR complex activation [19,85,69]. As IL-10, IFN-γ may have dual function. While it contributes to tolerance-promoting effects in transplantation, it is generally considered a Th1 specific pro-inflammatory cytokine that centrally contributes to systemic autoimmunity in humans [90]. Both *ex vivo* isolated and *in vitro* generated DN T express an array of pro-inflammatory effector cytokines and chemokines, including IL-1, IL-8, ICXCL3, and CXCL2 [69]. Also, DN T cells express the pro-inflammatory effector cytokine IL-17 (IL-17A) that plays a central role during physiological responses to bacteria and fungi, but also in tissue and organ damage in autoimmune/inflammatory conditions [91]. However, IL-17 is not exclusively expressed by DN T cells in response to infections and autoimmune diseases, but also in DN T cells derived from healthy subjects [24,32,69,92]. Furthermore, activated DN T cells produce the cytolytic protein perforin, but fail to express granzyme B, another protein typical expressed in cytotoxic lymphocytes [19].

Effector T cells are not only characterized by increased expression of lineage-defining cytokines and chemokines (*e.g.* Th1 or Th17 cytokines), but also by reduced expression of others. Double negative T cells fail to express IL-2 when compared to $CD4^+$ or $CD8^+$ T-cells [19, 32, unpublished data from our group]. Indeed, reduced expression of IL-2 is a key feature of effector T cells in SLE [93].

Rodriguez et al. recently identifying a self-reactive subset of DN T in mice, characterized by the expression of PD-1 and the transcription factor Helios that had lost CD8 surface co-receptor expression in response to activation in the periphery [94]. Of note, DN T cells were even found during steady state in healthy animals. However, the absolute number and percentage of $PD\text{-}1^+$ DN T cells was increased in mice that accumulate self-reactive T cells as a consequence of defects in central ($Aire^{-/-}$ mice) and peripheral tolerance (Fas^{lpr} mice) [7]. The cell surface receptor PD-1 is involved in the maintenance of peripheral tolerance by the suppression of activation induced T cell receptor signaling, thereby limiting inflammatory responses [95]. More recently, PD-1 moved into the focus of studies targeting peripheral tolerance and its disruption in autoimmune/inflammatory disease [96]. Indeed, PD-1 expressing DN T cells represent the main source of pro-inflammatory cytokines within the DN T cell compartment [7].

In addition to aforementioned effector functions, (at least) subsets of DN T cells exert regulatory activity. Several murine systems and *ex vivo* isolated human cells were applied to study regulatory functions of DN T cells, reviewed in [80,87]. In response to peptide–HLA complex presentation by antigen-presenting cells, human DN T cells inhibit antigen-specific T cells [19]. In line with data from studies in the murine system, human DN T cells exhibit cell-cell contact dependent suppressive effects on $CD4^+$ and $CD8^+$ T cells *in vitro* [86]. Though the exact molecular mechanisms remain elusive, this was independent of the Fas/FasL pathway or perforin.

Taken together, gene expression patterns and functional properties of DN T cells suggest the presence of various regulatory and effector subpopulations within the DN T cell compartment. However, a majority of cells within the DN T cell compartment displays the phenotype of terminally differentiated effector cells that are capable of pro-inflammatory cytokine production.

5. Autoimmune disorders associated with DN T cell expansion

5.1. Autoimmune lymphoproliferative syndrome

Autoimmune lymphoproliferative syndrome (ALPS) is a rare disease that is characterized by chronic nonmalignant lymphoproliferation, hepatosplenomegaly, and autoimmune symptoms [97]. Chronic or recurrent cytopenia affecting multiple blood cell lines is one of the most common findings, and due to autoimmune reactions (hemolytic anemia, immune-mediated thrombocytopenia, and autoimmune neutropenia) or splenic sequestration [97]. Defective lymphocyte apoptosis is caused by somatic or germline mutations in the *FASL*, or caspase (*CASP*)*10* gene, and results in the accumulation of lymphocytes [15]. Accumulation of DN T cells in the peripheral blood (>2.5% of $CD3^+$ T cells) and lymphoid tissues is a characteristic feature in most of ALPS patients, and therefore part of established diagnostic criteria [15]. Double negative T cells from ALPS patients share a unique CDR3 sequence with $CD8^+$ T cells across several TCRVβ families, suggesting that DN T cells derive from $CD8^+$ T cells [70]. Furthermore, data from studies in SLE support this hypothesis. As cells from patients with SLE, also $CD8^+$ T cells from MRL.*lpr* animals exhibit increased expression of the transcription factor CREMα that contributes to the generation of effector DN T cells from $CD8^+$ T cells (see below under Section 4.2). This may indicate a central contribution of CREMα to DN T cell generation in Fas-deficient animals (and potentially also ALPS patients). However, results require to be interpreted with caution, since in contrast to MRL.*lpr* mice, Fas and FasL expression and function are preserved in SLE patients [98], and additional mechanisms may come into play. However, MRL.*lpr* animals much rather resemble human ALPS (see above) than SLE.

The hypothesis that DN T cells may be involved in the onset of autoimmunological symptoms in ALPS patients is supported by the phenotype of DN T cells that were isolated from patients with ALPS that distinctly differs from the phenotype of DN T cells from healthy individuals. As DN T cells from Fas-deficient MRL.*lpr* mice, cells from ALPS patients abnormally express the B cell antigen B220, an isoform of the CD45 antigen (that used to be referred to as leukocyte common antigen; LCA) [99]. Of note, the B cell marker B220 is alternatively expressed on activated T cells that undergo apoptosis [100].

Furthermore, DN T cells from ALPS patients co-express CD27 and CD28 [101], a characteristic of naïve and central memory T cell subsets that is usually absent in effector T cells [102]. However, also in ALPS patients, DN T cells are mature antigen experienced effector T lymphocytes and certainly not "naïve". Of note, in contrast to DN T cells from healthy individuals [69] or patients with other autoimmune/inflammatory conditions [32,85], DN T cells from ALPS patients can and do proliferate [70].

Another interesting observation that may be (at least partially) caused or promoter by DN T cells is the presence of autoantibodies in most ALPS patients that correlates with the number of DN T cells in

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the peripheral blood. This correlation may be caused by the aforementioned B cell promoting effects of the immune regulatory cytokine IL-10, which is expressed at high levels by DN T cells from ALPS patients [15,70].

5.2. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a prototypical systemic autoimmune disease and facilitated by inappropriate immune responses to self-antigens, resulting in systemic inflammation and organ damage [1]. Various T cell subsets, including effector $CD4^+$ T helper cells, contribute to the pathophysiology, tissue damage and disease expression [1]. Over the past years, DN T cells moved into the focus of scientific studies. Interest in DN T cells in SLE was first generated through the observation that SLE patients exhibit increased numbers of DN T cells in the peripheral blood [32,103] and that the number of DN T cells correlates with disease activity [104]. Currently, we are still at the beginning of understanding the exact contribution of DN T cells to the pathophysiology of SLE. However, first evidence indicates a contribution of effector DN T cells to tissue inflammation and organ damage [105]. Double negative T cells invade the kidneys of patients with SLE-associated nephritis [32], where they produce pro-inflammatory effector cytokines, activate other T cells and induce immunoglobulin production [32,106]. Crispin et al. demonstrated that DN T cells can derive from $CD8^+$ T cells as a result of TCR complex stimulation *in vitro* [32]. Based on these observations others and we concluded that at least a majority of DN T cells derives from activated (self-reactive) $CD8^+$ T cells in SLE.

The pro-inflammatory effector cytokine IL-17A is an important contributor to systemic inflammation and tissue damage in SLE [107]. Besides $CD4^+$ T helper cells, DN T cells represent a major source of IL-17A in SLE patients [32]. The transcription factor cAMP responsive element modulator (CREM)α is expressed at increased levels in T cells from SLE patients (and MRL.*lpr* mice) [108]. We recently demonstrated that CREMα contributes to the down-regulation of CD8 expression and the subsequent generation of DN T cells in cells from healthy controls and (to a larger extent) SLE patients [72,75]. Because CREMα also mediates increased IL-17A and reduced IL-2 expression in $CD4^+$ effector T cells [93], we hypothesized that CREMα may be a central player during the generation of effector DN T cells in SLE.

Several lines of evidence suggest that DN T furthermore contribute to tissue damage through the promotion of anti-dsDNA antibody production by B cells [103,104,109]. This may be caused by increased expression of activated mechanistic target of rapamycin complex 1 (mTORC1) in DN T cells [110]. Activation of mTOR triggers the production of IL-4 by DN T cells, which correlates with B cell activation and the production of dsDNA antibodies [104]. Indeed, treatment with the mTOR inhibitor rapamycin reduces IL-4 production by DN T cells in patients with SLE [104]. Of note, mTOR pathway activation has been linked with the transcription factor CREMα through the CREMα-dependent regulation of the S6 kinase, a substrate of mTORC1 [111,112]. Thus, the transcription factor CREMα more and more appears to emerge as a key element in the pathophysiology of SLE and potentially other autoimmune/inflammatory conditions by its involvement in effector T cell generation and resulting tissue damage [49].

5.3. Sjögren's syndrome

Sjögren's syndrome (SS) is a systemic autoimmune disease characterized by lymphocytic infiltration to exocrine glands (salivary) and lacrimal glands that leads to chronic inflammation, tissue damage, and subsequently impaired secretory function [113]. Sjögren's syndrome may occur either as primary disease, or as a secondary feature in other autoimmune diseases, such as SLE [114], rheumatoid arthritis, or systemic sclerosis [113]. Allunno et al. found DN T cells expanded in numbers in the peripheral blood of SS patients. Furthermore, DN T cells infiltrate salivary glands where they produce IL17A [92]. Indeed, several lines of evidence strongly suggest that secretion of IL-17 is involved in glandular damage in SS [115,116]. Surprisingly, DN T cells from patients with primary SS displayed no response to corticosteroids *in vitro*, which is in line with insufficient therapeutic efficacy of corticosteroids in SS patients [92].

In addition to T cell infiltration to exocrine glands, B cell hyperactivity and autoantibody production are typical features of SS. Aberrant B cell maturation and activation is at least partially mediated by increased expression of the tumor necrosis factor (TNF) family member B cell activating factor (BAFF). The cytokine BAFF is produced by various immune cells, including activated lymphocytes and elevated in several autoimmune conditions (including pSS and SLE) [117,118]. Interestingly, in DN T cells increased BAFF expression and hypomethylation of the BAFF encoding TNFSF13B gene, indicating transcriptional permissiveness, has been reported [79]. Furthermore, BAFF expression is increased in the presence of IFN-γ [117], a cytokine which is also expressed in DN T cells [69].

In summary, DN T cells may contribute to the pathogenesis of SS due through IL-17 production and/or pathological B cell stimulation.

5.4. Psoriasis

Psoriasis is a systemic inflammatory condition that is characterized by immune cell infiltration to the skin and excessive keratinocyte proliferation [119]. IL-17-producing DN T cells have been linked with skin inflammation in a mouse model of psoriasis [120]. Recently, we showed that DN T cells infiltrate the epidermis in patients with plaque-type psoriasis, suggesting a pathophysiological role of DN T cells in tissue inflammation and damage [85]. Furthermore, DN T cells from psoriasis patients exhibited reduced DNA methylation of an enhancer element of the *IFNG* gene, suggesting increased accessibility to transcription factors [85]. Taken results from these studies together, DN T cells may contribute to skin inflammation through the expression of the inflammatory effector cytokines IFN-γ and IL-17.

Following the hypotheses that PD-1 expressing T cells may be crucial players in autoimmune disease [96] and that $PD\text{-}1^+$ DN T cells represent a self-reactive T cell subset [7], it is of special interest that surface PD-1 expression on DN T cells was generally increased in DN T cells from psoriasis patients, however, predominantly enriched in the epidermal layers of psoriatic skin [85]. This highlights the assumptions that plaque-type psoriasis may, in fact be a systemic autoimmune disease [121,122] and that DN T cells may be centrally involvement in its pathophysiology [85].

6. Conclusion and perspective

Double negative T cells comprise a rare and heterogeneous T lymphocyte subset. While some evidence indicates regulatory functions of DN T cells, recent studies report the involvement of self-reactive, pro-inflammatory effector DN T cells in systemic inflammation and tissue damage. Indeed, though rare in the peripheral blood of patients, DN T cells infiltrate inflamed tissues and may contribute to organ damage. Regardless of recent advances in understanding key molecular elements contributing to effector DN T cell generation, we are only beginning to understand the complex mechanisms defining individual phenotypes. Thus, additional studies are warranted targeting the role of DN T cells in health and autoimmune/inflammatory disease, and may bring us closer to an application of DN T cells as disease biomarkers and/or therapeutic targets.

Conflict of interest

The authors declare no conflict of interest in relation to this work.

Author contributions

Both authors contributed equally.

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