TCRαβ+ CD4−/CD8– "double negative" T cells in health and disease—implications for the kidney

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Double negative (DN) T cells, one of the least studied T lymphocyte subgroups, express T cell receptor αβ but lack CD4 and CD8 coreceptors. DN T cells are found in multiple organs including kidney, lung, heart, gastrointestinal tract, liver, genital tract, and central nervous system. DN T cells suppress inflammatory responses in different disease models including experimental acute kidney injury, and significant evidence supports an important role in the pathogenesis of systemic lupus erythematosus. However, little is known about these cells in other kidney diseases. Therefore, it is important to better understand different functions of DN T cells and their signaling pathways as promising therapeutic targets, particularly with the increasing application of T cell–directed therapy in humans. In this review, we aim to summarize studies performed on DN T cells in normal and diseased organs in the setting of different disease models with a focus on kidney.

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Among T lymphocytes, CD4+ and CD8+ T-cell functions are well known. However, there is an unconventional subset of T cells in which both CD4+ and CD8+ receptors are missing. These cells are termed *double-negative (DN) T cells* and are a subject of increasing research interest over the past decade. DN T cells can be found in lymphoid as well as nonlymphoid tissues and comprise 1% to 3% of human T cells in the circulating blood of healthy individuals.[1] The role of DN T cells in systemic lupus erythematosus (SLE) has been well studied, including in lupus nephropathy, and DN T cells have been identified as a major pathogenic player in various autoimmune conditions.[2,3] Protective effects in other diseases, such as acute kidney injury (AKI) and allograft rejection, have been described.[4–6] In addition, innate-like abilities of DN T cells have been demonstrated in the setting of infectious diseases, for example, infection with *Leishmania major*.[7]

These different roles raise questions about the mechanisms by which DN T cells influence health and disease. Apparent differences have been observed between pro-inflammatory cytokines, such as interleukin (IL)-17–producing DN T cells in SLE models, and anti-inflammatory cytokines, for example, IL-10–producing DN T cells with immunosuppressive features.[2] Recently, single-cell RNA sequencing analysis of DN T cells identified specific clusters with 5 DN subsets proposed: resting DN, helper DN, intermediate DN, cytotoxic DN, and innate DN.[8] Plasticity of DN T cells might also explain observed discrepancies. There are different hypotheses for the origin of DN T cells, which we will describe in more detail, and a redifferentiation to the T cell of origin might be possible.[2,9]

An important opportunity is to identify a specific positive marker, given that present investigations of DN T cells are challenged by having to use negative selection techniques. The definition and nomenclature of DN T cells have been inconsistent throughout the literature; thus, different studies could have been investigating divergent populations. Usage of CD3+ antibody labeling instead of T cell receptor (TCR)αβ+ for DN T cells will lead to inclusion of TCRγδ+ in addition to TCRαβ+ cells, confounding interpretation. Furthermore, some publications on DN T cells may have included natural killer (NK) T cells, which have pro-inflammatory characteristics and are therefore extremely important to exclude. A homogeneous DN T-cell population can be best achieved by exclusion of CD1d+-labeled cells targeting NK T cells.[10] Importantly, negative selection of NK1.1+ cells does not result in a pure population, because this marker is less specific and not all NK T cells will be targeted. Recent findings

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Table 1 | Different DN T-cell population definitions found in the literature and included immune cell subsets

Immune-cell subsets	CD3+ CD4− CD8−	TCRαβ+ CD4− CD8−	TCRαβ+ CD4− CD8− CD1d− (*recommended*)	TCRαβ+ CD4− CD8− NK1.1−
TCRαβ+ CD4− CD8−	×	×	×	×
NK T cells	×	×	(CD1d− unrestricted NK T cells)	(CD1d+ NK T cells)
TCRγδ+	×			
CD4− CD8− MAIT cells	(×)	(×)	(×)	(×)

×, not present; (×), potentially present in insignificant numbers; DN, double negative; MAIT, mucosal-associated invariant T; NK, natural killer; TCR, T cell receptor.

described CD1d-unrestricted NK T cells, which might require an additional antibody in the DN T-cell isolation process to achieve pure DN T-cell populations.[11,12] Another type of cell potentially overlapping with DN T-cell gating are DN mucosal-associated invariant T cells (MAIT), but these numbers are considered negligible in kidney and most other organs in mice (Table 1).[13,14]

Differences in technical approaches can have a major impact on DN T-cell population assessment, with differing flow cytometric gating strategies affecting DN T-cell measurements.[15] Thus, we recommend that DN T cells be defined as TCRαβ+CD4−CD8−CD1d− cells in mice and TCRαβ+CD4−CD8−CD56− in humans.[10]

Development of DN T cells

It is important to first review the development of αβ T cells. Progenitor lymphocytes originate from the bone marrow before migrating to the thymus where T-cell differentiation occurs. There, thymocytes lack CD4 and CD8 coreceptors in early stages of maturation, referred to as *DN thymocytes*.[16,17] DN T-cell developmental stages are characterized by expression of cell surface markers such as CD25+ and CD44+. DN thymocyte subsets can be distinguished depending on expression of these receptors. Murine αβ T-cell differentiation includes 4 stages of DN thymocytes, whereas there are 3 stages in humans.[3,18,19] CD44+CD25− (DN stage 1) cells lead to the formation of CD44+CD25+ (DN stage 2) cells. Next, DN2 cells lose CD44 expression and become CD44−CD25+ cells (DN stage 3). Here, murine cells undergo TCRβ selection, which promotes the rearrangement of the TCRα locus to express complete TCRαβ later in development.[17,20] Finally, DN3 T cells lose CD25 and differentiate into CD44−CD25− cells (DN stage 4). DN4 is followed by the CD8+ (CD4+ in humans) immature single-positive stage before DN T cells acquire CD4 and CD8 and become double-positive (DP) T cells.[21] After the DP state, further differentiation into CD4+CD8− and CD4−CD8+ single-positive stages occurs before these cells exit the thymus and into the periphery.[22]

There are 5 main theories regarding the origins of peripheral DN T cells (Figure 1).[4,6,17,20,21,23–25] The first theory claims that DN T cells develop in the periphery. T cells may have committed to either CD4+ or CD8+ T cells in the thymus, but later downregulate their coreceptor. Analysis in lupus-prone MRL/*lpr* mice, a mouse strain with accumulation of DN T cells, suggests that the precursor of DN T cells is CD8+ cells. Cells presenting normal expression of the CD8 coreceptor show demethylation of the *CD8* gene. DN T cells show the same demethylation of this gene; only CD8+ receptors are not present, providing further evidence for the development of DN T cells by downregulation of the CD8 receptor of peripheral CD8+ T cells.[23] Comparison of the adoptive transfer of CD3+TCRαβ+CD4+ and CD3+TCRαβ+CD8+ thymocytes into recombination-activation gene deficient (Rag-$1^{-/-}$) mice revealed that both led to DN T-cell generation. However, CD3+TCRαβ+CD8+ thymocyte injection led to significantly higher DN T-cell numbers than did CD3+TCRαβ+CD4+.[9]

A second theory supports the notion that early DN T cells, after being synthesized in the bone marrow, were unable to differentiate into either CD4+ or CD8+ coreceptor because of cells passing the thymus completely. This theory is supported by the presence of similar numbers of DN T cells in thymectomized B6 wild-type (WT) mice.[6,24] A third theory suggests that the transition from DN to DP was never completed and T cells left the thymus as DN T cells.[2,25] A fourth theory hypothesizes that early T cells made it to the thymus but traversed through an alternate pathway leading to the lack of further development into CD4+ or CD8+ coreceptors.[6,26] Finally, the fifth theory posits that the origin of peripheral DN T cells is variable, and we find a heterogeneous pool of cells.[2] Testing these different theories represents an opportunity to improve our understanding of the origins of DN T cells.

DN T cells in kidney

Kidney DN T cells in steady state. Unlike the DN T-cell population found in lymphoid organs and peripheral blood where they constitute a smaller proportion of T lymphocytes, DN T cells are found in higher quantities in normal kidney tissue (Figure 2).[5,10,27] A total of 20% to 38% of αβ T cells found in normal mouse kidney consists of DN T cells and can vary from 18% to 61% frequency of αβ T cells in human kidney biopsy samples.[4] In steady state, kidney DN T cells tend to express high levels of CD44+ and CD69+ activation subsets and high levels of CD40L+ and CD28+ costimulatory molecules as opposed to CD4+ and CD8+ T cells.[4] Kidney DN T cells have also been found to actively divide during steady state as compared with their CD4+ and CD8+ counterparts. It was found that 36% of mouse kidney DN T cells were proliferating as compared with 1% to 5% of CD4+ and CD8+ cells by measuring expression of the Ki67 nuclear protein.[4] This could be due to the lack of coreceptors on DN T cells because these coreceptors have been found to regulate apoptotic signals via the Fas pathway, thus leading to lack of

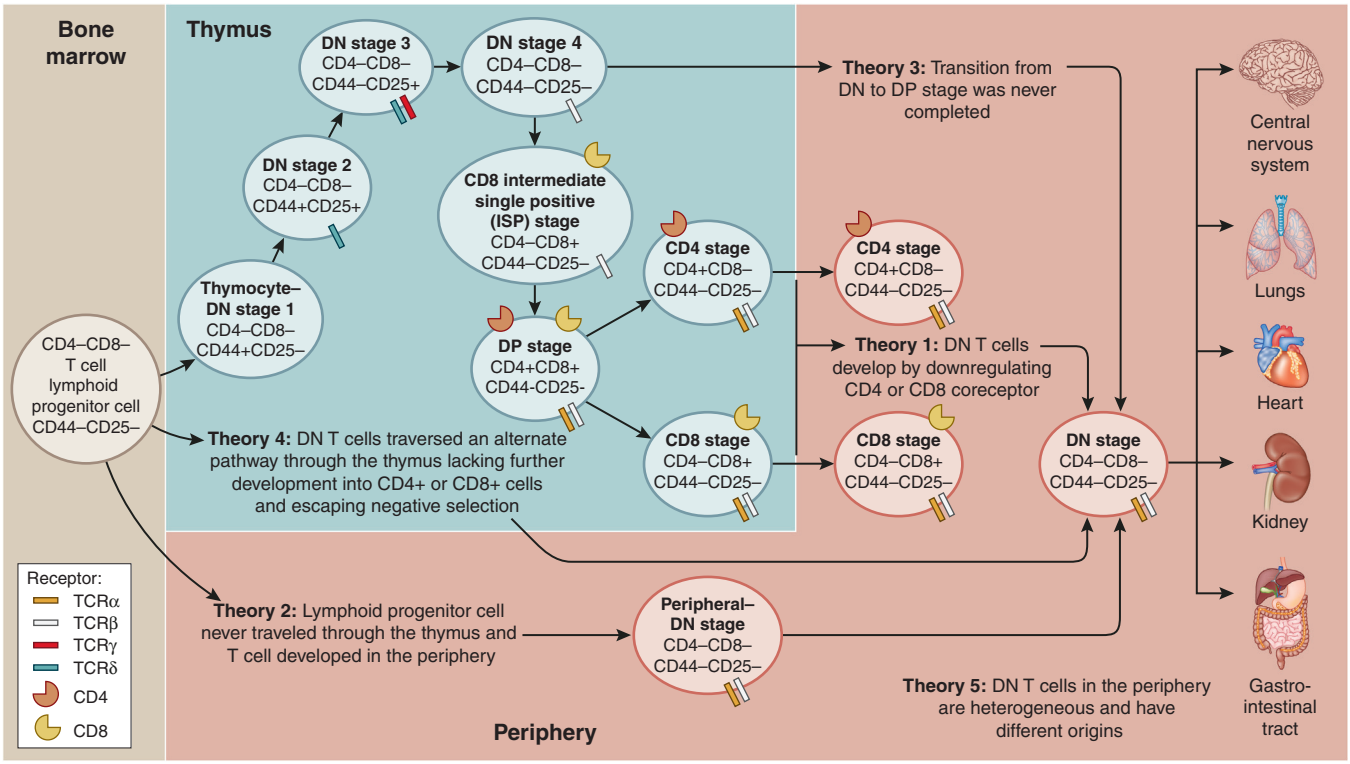

Figure 1 | αβ T lymphocyte maturation and most common theories about the origin of double-negative (DN) T cells in the periphery. The development of murine αβ T cells in the thymus physiologically includes DN stages 1 to 4 of thymocytes that are defined by expression of CD44+ and CD25+. To describe the development of mature T cell receptor (TCR)αβ+ DN T cells in the periphery, there are 5 theories. According to theory 1, CD4+ or CD8+ downregulate their coreceptor in the periphery to become DN T cells.[21,23] Theory 2 hypothesizes that hematopoietic stem cells go directly from the bone marrow and mature into DN T cells in the periphery.[6,24] Theory 3 suggest that DN stage 4 to double-positive (DP) transition in the thymus was never completed and DN T cells traveled to the periphery.[25] Theory 4 suggests an alternate pathway through the thymus.[6] According to theory 5, peripheral DN T cells are a pool of heterogeneous cells with different origins. Peripheral DN T cells have been found in steady state and diseased organ tissue.[4] It is important to note that stages of TCRγ and TCRδ selection are currently unclear; however, it is known that their arrangement occurs before TCRα and TCRβ selection during the DN stage.[17,20]

apoptotic signaling within this population.[19,28,29] Kidney DN T cells depend on nonclassical β2m molecules to maintain their homeostasis in steady state.[10] Moreover, DN T cells in steady state produce high levels of the anti-inflammatory cytokine IL-10, potentially promoted by IL-27, and can suppress CD4+ and CD8+ T cells *in vitro*.[4] In mouse kidneys, 2 key DN T-cell subgroups have been identified. The first subgroup is characterized by a major histocompatibility complex (MHC)–independent programmed cell death protein-1 (PD-1)+ receptor, while the other subgroup is an MHCI-dependent NK1.1+.[10,30]

DN T cells in AKI. DN T-cell subsets in kidney compared with peripheral blood and primary lymphoid organs have been analyzed in AKI mouse models (Figure 3).[4,5,19,31–36] An increase in DN T-cell number and frequency in mouse kidney 24 hours after ischemic-reperfusion injury (IRI) and cisplatin-induced AKI has been described (Table 2).[4,5,10,36,37] Concordant increases in the anti-inflammatory cytokine IL-10 and decreases in pro-inflammatory cytokines interferon-γ (IFN-γ), tumor necrosis factor-α, and IL-17A 24 hours after injury induced by cisplatin supported the hypothesis that the higher DN T-cell population could be important for initial AKI recovery.[5] The DN T-cell population, although increased both in number and in frequency, showed a decrease in the PD-1+ DN T-cell subset in cisplatin-induced AKI.[5] CD69+, an important activation marker, has also shown increase in DN T cells after AKI across studies, also suggesting that these cells may participate in recovery from AKI.[5,36]

Because of the evidence of the correlation of DN T cells with higher anti-inflammatory and lower pro-inflammatory cytokines during AKI recovery, their therapeutic potential was explored in different AKI murine models. DN T-cell transfer from the lymph nodes of generalized lymphoproliferative disease (*gld*) mouse donors, which have high numbers of DN T cells, to B6 WT mice 24 hours before IRI protected against AKI and was IL-10 dependent. The protective effect of adoptively transferred DN T cells was also seen in cisplatin-induced AKI.[4] When composition of different DN T-cell subsets was analyzed in B6 WT mice, an increase in PD-1+ DN T-cell subset at the expense of NK1.1+ DN T-cell subset 24 hours after IRI was found. This might indicate a non–MHC-dependent response to IRI of DN T cells in kidney. DN T cells in kidney are also regulated by IL-2 *in vivo* and *in vitro*.[10] Another study found that pre- and posttreatment with mouse antithymocyte globulin in ischemic murine kidney increased the percentage of DN

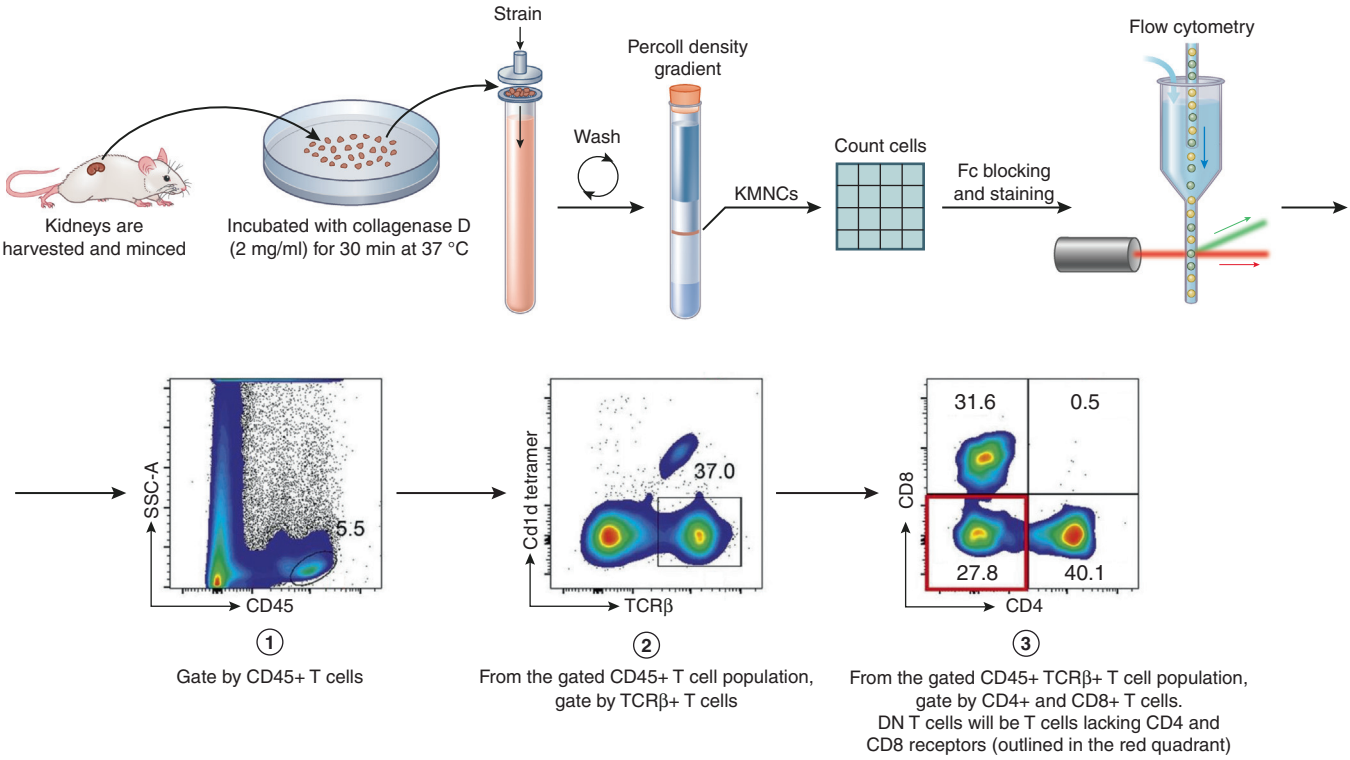

Figure 2 | Isolation and analysis of kidney double-negative (DN) T-cell expansion. To analyze DN T-cell expansion, kidney mononuclear cells (KMNCs) are isolated. After full body exsanguination, kidneys are harvested, minced, and incubated with collagenase D (2 mg/ml) for 30 minutes at 37 °C. Kidney tissue is strained to obtain a single-cell suspension. After rounds of centrifuging and washing, a Percoll density gradient is obtained. KMNCs are then extracted from the gradient and counted using a hemocytometer. After crystallizable fragment (Fc) blocking, fluorochrome-conjugated antimouse monoclonal antibodies are added and followed by flow cytometric analysis. Cells are gated by CD45+, T cell receptor (TCR)β+, Cd1d−, CD4−, and CD8− cells. Finally, DN T-cell expansion is analyzed. SSC-A, side scatter parameter–A. For full gating strategy, see Martina MN, Bandapalle S, Rabb H, Hamad AR. Isolation of double-negative αβ T cells from the kidney. *J Vis Exp*. 2014;(87):51192.[5,27] Gating images (bottom) reprinted with permission from Sadasivam M, Noel S, Lee SA, et al. Activation and proliferation of PD-1^{+} kidney double-negative T cells is dependent on nonclassical MHC proteins and IL-2. *J Am Soc Nephrol*. 2019;30:277–292.[10] Copyright © 2019 by the American Society of Nephrology. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

T cells to become the dominant T-cell population, thus decreasing the CD4+ and CD8+ T-cell percentage within 72 hours of ischemia. However, it is unknown how mouse antithymocyte globulin treatment mechanistically affects DN T-cell expansion after IRI.[37]

Up to 20% of patients diagnosed with AKI can progress to chronic kidney disease within years of hospitalization.[38] Increasing data support the immunomodulatory effect of DN T cells in the recovery phase after AKI.[4,5] Hence, novel therapeutic approaches targeting DN T cells could enhance repair and regeneration, which can prevent AKI to chronic kidney disease transition.

DN T cells in autoimmune diseases. DN T cells have been studied in many different autoimmune diseases, though primarily in SLE (Figure 3). SLE involves various organ systems such as skin, joints, brain, lungs, heart, and kidneys.[39] B cells producing autoantibodies are well-recognized mediators; however, the role of T cells is less well understood. Among T-cell subsets, DN T cells are thought to be important in the pathophysiology of SLE and lupus nephritis and therefore has been investigated as potential treatment targets (Table 3).[2,31,40–52] In patients with SLE, increased numbers of DN T cells with significantly higher expression of activation markers are found in peripheral blood, correlate with disease activity, and therefore might contribute to the pathogenesis of SLE.[42] In lupus nephritis, DN T cells are found to increase in kidney.[43] DN T cells have an impact on antibody production of autologous B cells.[40] There is also evidence for DN T cells inducing an isotype switch in a CD1c-restricted manner.[41] In addition, DN T cells of patients with lupus have been found to release more IL-4 than control cells, correlating with skewing of the B cell compartment and anti-DNA antibody production. This process can be triggered by the mammalian target of rapamycin (mTOR) pathway activation.[46] In addition, it has been shown that DN T cells from patients with SLE constitute a source of pro-inflammatory IFN-γ– and IL-17–producing cells when stimulated *in vitro*.[43] A factor leading to increased CD3+CD8−CD4−CD69+IFN-γ+ in kidney is IFN-α, because IFN-α transgenic mice have been shown to develop SLE manifestations.[51] *In vitro* stimulation of DN T cells from MRL/*lpr* or B6/*lpr* mice by using IL-23 further upregulated IL-17 expression. Adoptive transfer of the stimulated CD3+CD8−CD4− DN T cells caused nephritis in Rag-1$^{-/-}$ mice, which lack T and B cells.[44] Also,

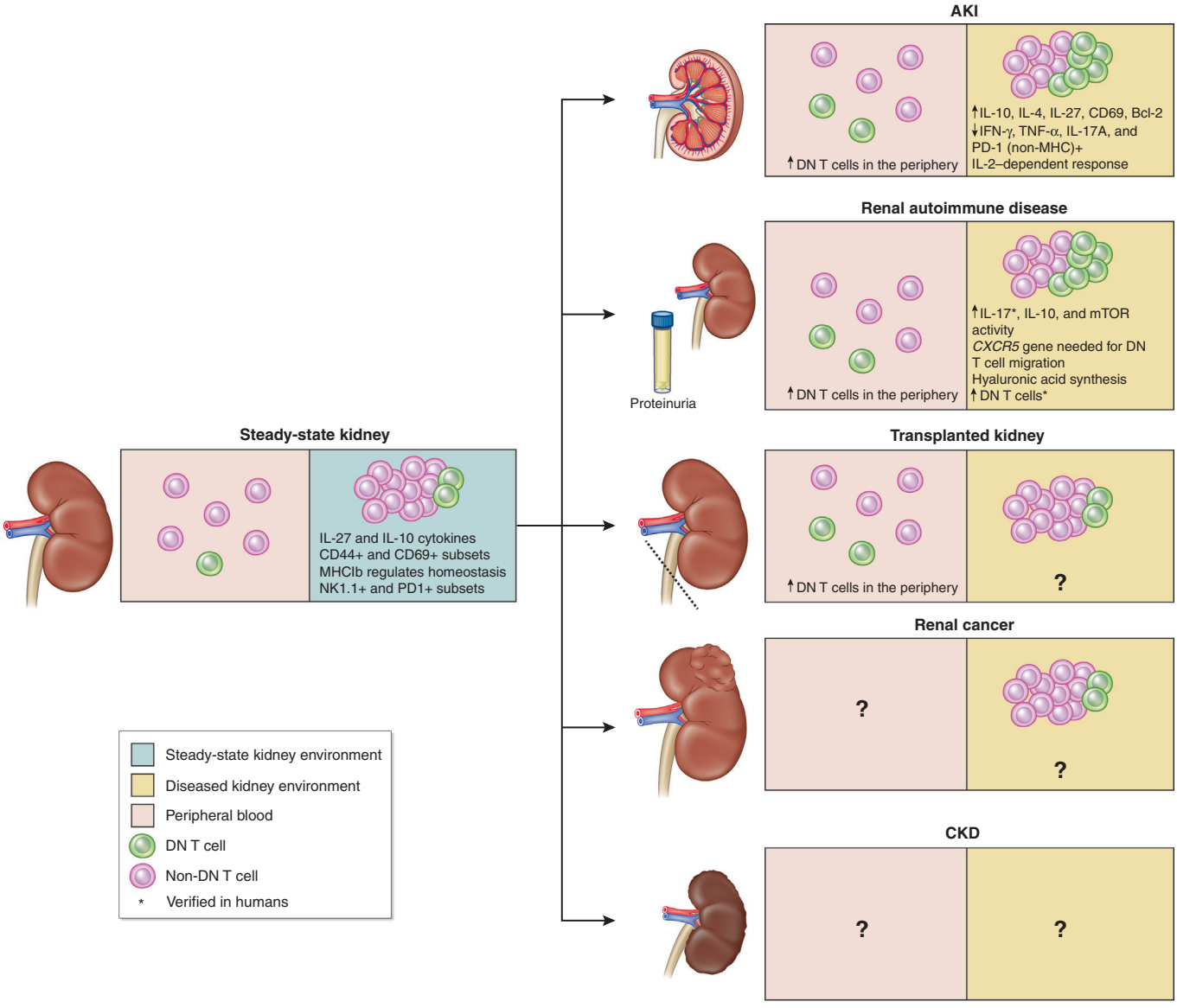

Figure 3 | Role of double-negative (DN) T cells in different kidney disease models. Note: DN T cells are in green, and other αβ T cells are in red. The proportion between DN T and other αβ T cells are not proportional as DN T cells make up a very small percentage of αβ T cells. Therefore, the increase in DN T cells is only for the purposes of showing general increases and decreases in DN T-cell percentage. Steady-state DN T cells in kidney belong to 20% to 38% of αβ T cells in normal murine kidney and show a high frequency of interleukin (IL)-27 and IL-10 cytokines and CD44+ and CD69+ subsets.[4] Major histocompatibility complex (MHC)Ib regulates homeostasis, and 2 DN T-cell subsets have been described: natural killer (NK)1.1+ and programmed cell death protein-1 (PD-1)+ DN T cells in murine and human kidneys. After acute kidney injury (AKI), kidney has shown increased DN T-cell levels 24 hours after injury both in kidney and in the periphery.[4] Autoimmune kidney has shown an increase in DN T-cell population as compared with steady-state kidney; however, DN T cells have been found to further contribute to autoimmune disease progression.[31] After the transplanted kidney, peripheral DN T cells have been found to increase, yet the DN T-cell population is unknown (?).[19] However, DN T-cell adoptive transfer has shown improved survival rates of other cardiac and skin allografts, which leads to the hypothesis that this could improve survival rates of kidney transplants.[32,33] DN T-cell percentage in renal cancer is similar to the adjacent tissue; however, the specific role of DN T cells in renal cancer remains unknown (?).[34] We hypothesize that the treatment of renal cancer with DN T-cell adoptive transfer could mitigate the growth of cancerous cells.[35] The same hypothesis can be made with regard to chronic kidney disease (CKD) because adoptive transfer of DN T cells to ischemic kidney has shown decreased kidney injury in mice and can therefore help mitigate AKI to CKD transition.[5] Bcl-2, B-cell lymphoma 2 protein; CXCR5, CXC chemokine receptor 5; IFN-γ, interferon-γ; mTOR, mammalian target of rapamycin metabolic pathway; TNF-α, tumor necrosis factor-α.

IL-23 receptor knockout (KO) B6/*lpr* mice are protected from SLE development.[45] Another negative effect of IL-23 is limiting the production of IL2, which itself is protective by decreasing CD4−CD8−CD17+ cells in an MRL/*lpr* mouse model.[48,50] IL-17 KO mice not only are protected from pristane-induced SLE but also showed significantly less DN T cells, confirming the pathogenetic role of IL-17–producing DN T cells.[47] CXC chemokine receptor 5 (CXCR5) has been shown to play an important role in DN T cells of lupus-prone mice. $Rag^{-/-}$ recipient mice of adoptively

Table 2 | Mechanistic studies of DN T cells in AKI

Experimental design			
Model	**Species/strain**	**Mechanistic findings**	**Reference**
Murine bilateral renal IRI model	Male C57BL/6J WT mice	- Normal mouse kidney of 8-wk-old mice showed ↑ CD69 on DN T cells as compared with 5-wk-old mice - 3 h after IRI → DN T-cell numbers similar to sham-operated mice - 24 h after IRI → ↓ DN T numbers compared to normal and sham-operated mice → DN T cells had no changes in IFN-γ and TNF-α	Ascon *et al.*[36]
Murine bilateral renal IRI model	Male C57BL/6J WT mice	- Pre- and posttreatment with mouse anti–thymocyte globulin + renal IRI → ↑ DN T-cell percentage and ↓ CD4+ and CD8+ T-cell percentage after 72 h	Jang *et al.*[37]
Murine bilateral renal IRI model	Male C57BL/6J WT mice	- Steady-state kidney → high expression of IL-27 and IL-10 in DN T cells - 3 and 24 h after IRI → ↑ DN T-cell frequency to above baseline levels - 72 h after IRI → ↓ DN T-cell frequency to below baseline levels - 3 h after IRI → ↑ IL-10 gene expression (16-fold) and ↓ IL-27 gene expression (2-fold) in kidney DN T cells - AT of *gld* DN T cells into WT → 24 h after IRI → ↑ protection from AKI compared to PBS-treated controls - AT of *gld* DN T cells into WT + anti–IL-10R neutralizing mAb → 24 h after IRI → ↓ protection from AKI compared to controls - *In vitro* coculture of DN T cells and CD4+ T cells suppressed CD4+ T-cell proliferation	Martina *et al.*[4]
Human "normal" kidney adjacent to RCC		- Presence of DN T cells with a frequency of 18.3%–61%	
Murine bilateral renal IRI model	β2m KO (MHCIa and MHCIb), MHCII KO, and MHCIa KO C57BL/6J WT mice	- Kidney DN T-cell homeostasis depends on nonclassical MHCIb: β2m KO in steady-state → ↓ DN T cells in kidney, potentially owing to ↑ apoptosis and ↓ proliferation (no changes in MHCII KO and MHCIa KO mice) - Lower activation status at steady state in β2m KO mice → ↓ CD69 and ↑ CD62L → maintained high CD44 expression and no change in CD28 expression on DN T cells compared to WT mice - 24 h after IRI in WT → ↑ total DN T-cell frequency in kidney - ↑ PD-1+ DN T-cell subset and ↓ NK1.1+ DN T-cell subset - IRI in β2m KO → ↓ total DN T-cell frequency in kidney - DN T cells can be restored by AT of CD8 T cells or IL-2 injection	Sadasivam *et al.*[10]
Human "normal" kidney adjacent to RCC		- NK1.1+ (MHCI dependent) and PD-1+ (MHC independent) DN T-cell subsets detected - NK1.1+ and PD-1+ DN T-cell subsets detected	
Cisplatin-induced AKI model	Male C57BL/6J WT mice	- 24 h after cisplatin treatment → ↑ DN T-cell frequency and absolute numbers - 72 h after cisplatin treatment → ↓ DN T-cell frequency and absolute numbers - Cisplatin treatment → ↑ apoptotic DN T cells over time with peak at 72 h posttreatment - 24 h after cisplatin treatment → ↑ CD69, ↑ IL-10; ↓ IFN-γ, TNF-α, and IL-17A in kidney DN T cells - 72 h after cisplatin treatment → CD69 in kidney DN T cells returned to baseline - 24 h after cisplatin treatment → ↓ CD62L in kidney DN T cells but no significant difference from controls at 72 h - Cisplatin treatment → ↓ CD44 expression on kidney DN T cells - 24 and 72 h after cisplatin treatment → ↓ NK1.1+ DN T-cell absolute number, but no change in frequency - Adoptive transfer i.v. of *gld* DN T cells into WT mice 24 h before cisplatin treatment → renal functional and structural protection after 72 h compared to controls - 72 h after cisplatin treatment *in vitro* → ↑ PD-L1 in PTECs - Cisplatin treatment of *in vitro* cocultured PTECs + DN T cells → ↓ PD-L1 protein in PTECs	Gong *et al.*[5]

↑, increased; ↓, decreased; AKI, acute kidney injury; AT, adoptive transfer; DN, double negative; IFN-γ, interferon-γ; IL, interleukin; IL-10R, interleukin-10 receptor; IRI, ischemic-reperfusion injury; KO, knockout; mAb, monoclonal antibody; MHC, major histocompatibility complex; NK, natural killer; PBS, phosphate-buffered saline; PD-1, programmed cell death protein-1; PD-L1, programmed death-ligand 1; PTEC, proximal tubular epithelial cell; RCC, renal cell carcinoma; TNF-α, tumor necrosis factor-α; WT, wild-type.

Table 3 | Mechanistic studies of DN T cells in SLE and lupus nephritis

Experimental design			
Model	**Species/strain**	**Mechanistic findings**	**Reference**
SLE	Human blood DN T cells from patients with clinically active SLE	- *In vitro* culture of DN T cells from patients with SLE → ↑ DN T-cell frequency and ↑ IgG class anti-DNA autoantibodies compared to normal and inactive SLE via the IL-2 mechanism - Active SLE → majority (72%) CDw29+ DN T-cell subset	Shivakumar *et al.*[40]
SLE	Human DN T cells from patients with SLE and HC blood samples	- Patients with SLE → ↑ DN T cells compared to HCs - *In vitro* coculture of DN T cells from patients with SLE + CD11+ APCs → IL-4 and IFN-γ - *In vitro* coculture of DN T cells from HCs → IFN-γ, no production of IL-4 - *In vitro* coculture of DN T cells from patients with SLE + CD11+ APCs → IL-4 production and ↑ IgG1 compared to HCs - IgG production from CD1c+ B cells depends on T-cell activation through CD1c → Ab production via the IL-4 and CD40L interaction	Sieling *et al.*[41]
SLE	Human DN T cells from patients with SLE, patients with RA, and HC blood samples	- SLE → ↑ TCRαβ+ DN T-cell percentage of total DN T-cell population compared to RA and HCs - SLE → ↑ HLA-DR+ DN T cells, ↑ CD69+ DN T cells, ↑ CTLA-4+ DN T cells, ↑ CD28+ DN T-cell number compared to RA and HCs	Anand *et al.*[42]
SLE *In vitro* experiments	Human DN T cells from female patients with SLE and HCs. Prednisone was paused at least 24 h before blood draw	- *In vitro* culture of DN T cells from patients with SLE → ↑ IL-17+ DN T cells under basal culture conditions and after 5 d of culture compared with IL-17+ CD4+ T cells - *In vitro* anti-CD3 stimulation of DN T cells from patients with SLE → 5 d → ↑ DN T-cell frequency - *In vitro* cultured DN T cells produce ↓ IL-2, ↑ TNF-α, and ↑ IFN-γ compared with CD4+ T cells - *In vitro* cultured DN T cells from patients with SLE → ↑ IL-17+ DN T cells compared with DN T cells from HCs	Crispín *et al.*[43]
	Kidney biopsies of patients with SLE	- Kidney biopsies of patients with SLE → DN T cells with positive immunofluorescent staining of IL-17 and IL-23	
SLE *In vivo* and *in vitro* experiments	MRL/*lpr* mice	- *In vitro* IL-23 stimulation of DN T cells → ↑ IL-17 expression compared to control cells - AT of stimulated MRL/lpr DN T cells → nephritis in *Rag-1*$^{-/-}$ mice (deposition of Ig and C3d in glomeruli)	Zhang *et al.*[44]
SLE	Mice, IL-23R–deficient C57BL/6	- IL-23R KO B6/*lpr* mice protected from SLE, ↓ DN T-cell number and percentage of IL-17+ DN T cells, ↓ IFN-γ, ↓ IgG, and anti-dsDNA Abs compared with B6/*lpr* mice	Kyttaris *et al.*[45]
SLE	Human DN cells from patients with SLE and HCs	- SLE → ↑ mTOR activity in DN T cells and ↓ CD3+CD4+CD25+FoxP3+ T cells - SLE → ↑ IL-4 production of DN T cells, correlating with the production of anti-DNA Ab - Rapamycin treatment of patients with SLE → ↓ necrosis and ↓ IL-4 production of DN T cells (↓ MFI) - No changes in IL4+ DN T cells or IL17+ DN T-cell percentage	Lai *et al.*[46]
SLE	Mice, C57BL/6 WT and double KO IL-17$^{-/-}$/*Stat1*$^{-/-}$	- IL-17 KO mice protected from pristane-induced SLE, ↓ DN T cells - IL-17 deficiency → protection from SLE independent of Stat1 activity and ↓ DN T cells and ↑ Treg cells	Amarilyo *et al.*[47]
	Pristane lupus induction	- IL-17 → anti-ssDNA, anti-nRNP, and anti-chromatin autoantibody production	
SLE	Mice, MRL/*lpr*−	- IL-2 (usage of IL-2–recombinant adeno-associated virus) → ↓ IL-17+ DN T cells by inducing cell death - IL-2/anti–IL-2 Ab complex and targeting of IL-2 to cytotoxic lymphocytes → ↓ DN T cells and INF-γ, no change in anti-dsDNA antibody production - IL-2 → ↑ DN T-cell death via the CD122 receptor and STAT5 phosphorylation by IL-2 on CD8+ T cells	Mizui *et al.*[48]

(*Continued on following page*)

Table 3 | (Continued) **Mechanistic studies of DN T cells in SLE and lupus nephritis**

Experimental design			
Model	Species/strain	Mechanistic findings	Reference
SLE	Mice, female MRL/*lpr*	- A77 1726 (active metabolite of leflunomide) treatment → ↓ lupus activation (↓ anti-dsDNA, ↓ ANAs, and ↓ inflammation in kidney histological samples) - A77 1726 treatment → Akt-dependent ↑ CD3+CD4+CD25+FoxP3+ T cells → ↓ splenic IL17+ DN T cells	Qiao *et al.*[31]
SLE	Mice, B6/*lpr* CXCR5$^{-/-}$	- AT of DN T cells of B6/*lpr* CXCR5$^{-/-}$ mice → ↓ CD3+ in recipient Rag$^{-/-}$ mice compared with controls receiving B6/*lpr* DN T cells	Wiener *et al.*[49]
	In vitro studies	- *In vitro* transwell studies → ↓ chemotactic response of splenic DN T cells toward CXCL13 compared with control B6/*lpr* DN T cells	
SLE	Human blood samples of patients with SLE	- IL-23 treatment → ↑ SLE DN T-cell numbers independent of Tfh markers and ↑ IL-17 production *in vitro* - IL-23 treatment → ↓ IFN-γ and IL-2 by SLE T cells *in vitro* via the suppression of NFkB p65 (IL-2 enhancer) → ↑ Tfh cells → ↑ anti-dsDNA independent of DN T cells	Dai *et al.*[50]
	Mice, IL-23R$^{-/-}$ MRL/*lpr*	- IL-23R deficiency → ↓ CD45+ DN T-cell infiltration, ↓ DN T cell–dominant T-cell subset, ↓ IL-17 and ↓ IFN-γ in the SLE kidney model - IL-23 treatment → ↑ IL-17+ T cells and ↑ IL-17 production *in vitro* - IL-23$^{+/+}$ MRL/*lpr* → ↑ CD4+ T cells becoming DN T cells compared to IL-23 KO MRL/*lpr* and control MRL/MPJ mice - IL-23$^{+/+}$ MRL/*lpr* → ↑ DN T-cell proliferation compared to IL-23 KO MRL/*lpr* in the spleen and peripheral lymph nodes - IL-23$^{-/-}$ MRL/*lpr* → ↑ IL-2 compared to WT MRL/*lpr in vitro*	
SLE	Mice, IFN-α Tg	- IFN-α Tg mice express ↑ IFN-α and develop SLE manifestations (glomerulonephritis) - ↑ IFN-α leads to ↑ CD69+ IFN-γ+ DN T cells in kidney	Akiyama *et al.*[51]

↑, increased; ↓, decreased; Ab, antibody; ANA, antinuclear antibody; APC, antigen-presenting cell; AT, adoptive transfer; CTLA-4, cytotoxic T-lymphocyte–associated protein 4; CXCL13, CXC chemokine ligand 13; CXCR5, CXC chemokine receptor 5; DN, double negative; dsDNA, double-stranded DNA; HC, normal control; HLA-DR, human leukocyte antigen – DR isotype; IFN-α, interferon-α; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; IL-23R, interleukin-23 receptor; KO, knockout; MFI, mean fluorescence intensity; mTOR, mammalian target of rapamycin metabolic pathway; NFκB, nuclear factor κB; nRNP, nuclear ribonucleoprotein; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; ssDNA, single-stranded DNA; Stat/STAT, signal transducer and activator of transcription; TCR, T cell receptor; Tfh, T follicular helper cell; Tg, transgene; TNF-α, tumor necrosis factor-α; Treg, regulatory T; WT, wild-type.

transferred CD3+CD8−CD4− T cells of B6/*lpr* CXCR5$^{-/-}$ presented significantly lower numbers of renal CD3+ cells than did mice receiving control cells.[49]

Marginal zone macrophages (MZMs) are splenic macrophages at the edge of splenic follicles important for immune tolerance because removing apoptotic cells is important to limit autoimmunity. Both lupus-prone mice and patients with SLE have less MZMs.[53] Recent work demonstrated a mechanistic link between MZM defects and DN T cells in SLE. DN T cells significantly expanded in spleens and infiltrated kidneys after depletion of MZMs in a lupus-prone mouse model. These DN T cells are derived from self-reactive CD8+ T cells, and not from CD4+ T cells. CD8+ OVA TCR (OT)-I (or CD4+ OT-II) TCR transgenic T cells, designed to recognize ovalbumin (OVA) peptide residues, and membrane-bound OVA (m-OVA) transgene-derived apoptotic thymocytes were injected into WT mice with and without depletion of MZM. Autoreactive CD8+ T cells in this model lost CD8+ receptor expression and differentiated into DN T cells after MZM depletion. The authors attributed this mechanism to dead cell debris retention and their associated antigens after MZM depletion. When myeloid cell–specific transforming growth factor β1 (TGF-β1) KO mice were used as recipients of CD8+ OT-I TCR transgenic T cells and m-OVA transgene-derived apoptotic thymocytes, the proliferation of CD8+ OT TCR transgenic T cells was maintained compared to controls. This indicated that tolerogenic cytokine TGF-β1 excreted by MZMs are important for DN T-cell suppression and apoptotic cell clearance, which can reduce autoimmunity.[54]

A77 1726, an active metabolite of leflunomide, led to the reduction in SLE-related antibodies, such as anti–double-stranded DNA (dsDNA), and other antinuclear antibodies (ANAs), and significantly less glomerular and interstitial inflammation in kidney histological samples of MRL/*lpr* mice. As a potential mechanism, the authors invoked a down-regulation of IL17+ DN T cells in the spleen of those mice along with an Akt-dependent upregulation of CD3+CD4+CD25+FoxP3+ T cells.[31]

The role of DN T cells in other rheumatic diseases has also been explored. IL-17–producing DN T cells are expanded in the peripheral blood and salivary glands of patients with Sjöogren syndrome and active disease, further supporting the pathological role of DN T cells in rheumatic diseases.[3,55,56] In autoimmune lymphoproliferative syndrome, high numbers of DN T cells in peripheral blood and secondary lymphoid organs are found. Clinical manifestations are lymphadenopathy with risk of developing lymphoma, splenomegaly, and autoimmunity.[57] The deficiency of Fas or Fas ligand (FasL) in patients with autoimmune lymphoproliferative syndrome is thought to lead to a decrease in apoptosis of DN T cells and therefore their accumulation. Mouse strains that lack Fas (*lpr*) or FasL (*gld*) mimic autoimmune lymphoproliferative syndrome best and are also used as SLE models, as mentioned above.[3] Psoriasis can be associated with systemic inflammation. Psoriatic skin lesions are characterized by keratinocyte proliferation, with DN T cells producing IL-17 and IFN-γ identified as pathophysiological mediators.[58,59]

DN T cells in renal and other cancers. DN T cells have been found in many human tumor types including renal cell carcinoma, melanoma, leukemia, multiple myeloma, and non–small cell lung cancer (Figure 3). Although the role of DN T cells specifically in cancer development is still largely unknown, quantification of these cells in different kinds of cancer tissue and treatment options has been analyzed. In renal cell carcinomas, there are ∼6% of DN T cells. Similar frequencies were found in adjacent normal kidney tissues. However, this was despite an overall increase in T cells in renal cell carcinomas. The presence of DN T cells, which have also been found to express certain immune coinhibitory receptors, such as PD-1, might make them a potential target for immunotherapy.[34]

Much like the population of DN T cells in primary lymphoid organs, those present in lung cancer tissue make up only a small fraction of total T cells (1.4%).[60] In addition, lung adenocarcinoma tissue from patients shows a reduced frequency of the DN T-cell population as compared with normal lung tissue, and DN T cells from patients with lung cancer have a high cytotoxicity against lung cancer cell lines.[61,62] Similarly, DN T-cell populations in human multiple myeloma and monoclonal gammopathy of uncertain significance peripheral blood samples were significantly lower than normal controls, which might contribute to progression.[63] In contrast, the DN T-cell population percentage was doubled in melanoma tumors compared with healthy lymph nodes (7.23% ± 17.18% and 3.57% ± 1.52%, respectively).[64] MHCI-restricted human DN T cells isolated from the peripheral blood of a patient with melanoma secreted IFN-γ and tumor necrosis factor-α cytokines and strong TCR-dependent antigen-specific cytotoxic activity and thus possible antitumor activity.[65]

Other reports have used human DN T cells isolated from peripheral blood and cocultured pancreatic cancer cells (Panc-1). Pancreatic cancer cells cocultured with DN T cells showed reduced cell migration as compared with controls.[66] Similarly in mice, DN T cells had a 35.5% inhibitory rate on pancreatic cancer cells *in vitro*.[67] Moreover, human DN T cells promoted an increase in IFN-γ and FasL levels when cocultured with pancreatic cancer cells.[66] Decreased MHCI chain-related molecule A (MICA) mRNA and protein levels with natural killer group 2 member D (NKG2D) mRNA and protein levels were increased in pancreatic cancer cells with DN T-cell injection i.v. as compared with those without an intervention in a mouse model.[67]

The immunotherapy potential of DN T cells has been explored in different disease models. DN T-cell therapy has shown clinical promise for the treatment of acute myeloid leukemia (AML). Expanded *ex vivo* DN T cells have also been found to eliminate allogeneic and autologous primary CD34+ leukemic blasts *in vitro* after chemotherapy therapy, which can lead to less frequent relapse in patients with AML.[68] Their ability to target AML cells is primarily due to cognate ligands expressed on AML cells binding to the high levels of NKG2D and DNAX accessory molecule-1 (DNAM) receptors on DN T cells.[69] Moreover, IFN-γ and tumor necrosis factor-α were found at high levels in DN T cells expanded during chemotherapy, suggesting an effective ability of DN T cells in tumor immunity. Furthermore, when used as effectors in cytotoxicity assays *in vitro*, DN T cells were able to eliminate leukemic cells and higher doses of DN T cells resulted in higher eradication rates.[68] Healthy donor–derived allogeneic *ex vivo* expanded DN T cells have been found to have anticancer properties when targeting myeloid leukemia *in vitro* and in the immunodeficient mouse AML xenograft model. This was evidenced by cytotoxic activity against leukemic blast markers and decreased AML engraftment levels in the bone marrow by 17.1-fold, respectively.[70] DN T cells can prevent local tumor development in animals when DN T cells were coinfused locally with the A20 lymphoma cell line.[71] The mechanisms of DN T-cell antitumor activity is relatively unknown.

Anti–PD-1 has been shown to increase the ability of DN T cells to attack cancerous lung tissue, therefore leading to higher chances of survival for mice affected.[61] Moreover, IL-15 cytokine has been demonstrated to increase in cytotoxicity against non–small cell lung cancer.[62] It has also been suggested that because of DN T cells promoting increased FasL levels when cocultured with pancreatic cancer cells, the increasing inhibition of cancer cell proliferation occurs via the Fas-FasL pathway, an apoptosis-mediating pathway.[71]

DN T cells in organ transplant

Accumulation of DN T cells has been found in peripheral blood after organ transplantation, suggesting a possible role in alloimmunity.[19] The correlation between high DN T-cell percentage and increased graft survival in humans can be modeled using C57BL/6 *lpr* or C57BL/6 *gld* mice, which accumulate high numbers of DN T cells owing to homozygous lymphoproliferation spontaneous mutation in Fas^{lpr} and Fas^{gld}, respectively. These models have shown high tolerance to skin allografts via antigen-specific syngeneic T-cell

suppression and via the Fas-FasL pathway.[72,73] In patients who received hematopoietic stem cell allografts, higher DN T-cell percentage was correlated with lower graft rejection risk.[74] In *lpr* and *gld* mice, the natural presentation of increased DN T-cell levels, and thus correlated protective behavior against skin allografts, demonstrates the protective role of DN T cells in allograft transplantation. DN T-cell adoptive transfer from normal mice could promote skin allograft, cardiac allograft, and xenograft survival in mice.[75,76] Moreover, DN T-cell adoptive transfer to lethally irradiated mice after bone marrow transplantation can prevent allogeneic T cell–induced graft-versus-host disease.[77] The possible mechanisms by which DN T cells provide graft protection depend on the model. Bone marrow engraftment survival by DN T cells occurs because of perforin (PFN) expression on DN T cells and their subsequent inhibition of NK cells in murine models. The NK cell suppression by DN T cells is via the PFN/FasL-dependent mechanism as evidenced by DN T cells from C57BL/6 and *gld* mice displaying a stronger ability to kill NK cells than do DN T cells from $PFN^{-/-}$ mice *in vitro*.[78] Likewise, the PFN/granzyme-dependent process of NK cell inhibition via DN T cells was found in rat to mouse cardiac xenograft survival. In this study, adoptive transfer of DN T cells was also found to elicit B cell apoptosis via the PFN-dependent pathway while also inhibiting antidonor antibody production.[79] Moreover, DN T-cell suppression of syngeneic CD4+ and CD8+ T cells can occur via various mechanisms in murine models, such as FasL-Fas–dependent and –independent mechanisms, antigen-specific, DN T to effector T-cell contact, and cytotoxicity via TCR-MHC mechanisms.[6] However, these mechanisms of action differ in human DN T cells, where their dependence relies on reversible effector T-cell interactions independent of the FasL-Fas pathway and PFN expression.[80] Data on patients with kidney transplant revealed a correlation between peripheral DN T cells and graft survival. As such, patients with stable transplant function in the first year had increased DN T cells in peripheral blood and late graft dysfunction correlated with a decrease in DN T cells in blood.[81] Thus, the role of DN T cells after kidney transplantation presents an opportunity for further investigation.

DN T cells in nonrenal organs

CNS. The role of DN T cells after ischemic stroke and other pathological conditions of the central nervous system is being increasingly studied. DN T cells have shown to significantly increase to 17% of lymphocytes in post–ischemic cerebral hemisphere in mice.[82] Numbers of DN T cells in the ischemic brain were elevated by 3- to 4-fold after ischemia in the mouse brain using the permanent and transient middle cerebral artery occlusion model.[83] Similar results were found in human postmortem brain sections after stroke. In both ischemic mouse and human samples, DN T cells were found to accumulate near activated microglia over time, suggesting both spatial and temporal cross-talk between these cells.[84] The same study concluded that DN T cells contribute to a pro-inflammatory response by tumor necrosis factor-α secretion via the Fas-FasL pathway, which led to an increase in pro-inflammatory microglia (CD86+) and a decrease in anti-inflammatory microglia (CD206+) after coculture with DN T cells.[84] When analyzing mononuclear cells in the subarachnoid space of rats with experimental autoimmune encephalomyelitis, >50% of TCRαβ cells were DN T cells. This was similar to findings in extrathymic T cells in autoimmune mice.[85,86]

Lung. DN T cells have shown promising outcomes during various types of lung infection models in mice. In a study observing lungs of mice with pulmonary *Francisella tularensis* live vaccine strain intranasal infection, DN T cells were major responders contributing to 11% to 15% of all lung T cells 2 weeks after infection. Pulmonary DN T cells were responsible for high IFN-γ production during the acute phase of infection, and IL-17A production a week after infection.[87] However, DN T cells alone are hypothesized to lack the function required for clearance of live vaccine strain infection *in vivo* because of prolonged infection in mice depleted of all other T cells.[87] DN T cells in murine lungs have also been found to produce IL-5 cytokines after infection with *Toxocara canis*.[88] Similar results were found in mice infected with cilia-associated respiratory bacillus, with a 12% increase in DN T cells of bronchiolar lymph nodes compared to uninfected mice.[89] In addition, the frequency of pulmonary DN T cells showed a significant increase in *Mycobacterium tuberculosis*–infected mice.[90] In the murine *M. tuberculosis* challenge model, vaccination against tuberculosis using the mutant BCG strain (BCG-A4) prepared with DDA/TDB adjuvant (A4/Adj) showed a higher stimulation of DN T cells, a higher frequency of IFN-γ production by DN T cells, and enhanced protection against tuberculosis infection as compared to nonvaccinated controls.[91] However, in another infectious pulmonary model, DN T cells administered to severe combined immunodeficiency disease (SCID)/beige mice infected with *Rhodococcus equi* were unable to clear the infection, unlike CD4+ and CD8+ administration.[92] After influenza A virus infection in mice, NK1.1− DN T cells expand 20-fold. Yet, the capability of these DN T cells to produce IFN-γ cytokines decreased. Moreover, the role of NK1.1− DN T cells in the lung has shown to stimulate the survival of $CD11c^{hi}$ dendritic cells (DCs) in coculture, pointing out their potential role as immunoregulatory cells.[93]

Given the promise of DN T-cell therapy in ischemic AKI, the effects in lung IRI were studied. Using a murine lung ischemia model, DN T cells were found to expand postinjury and increase production of anti-inflammatory cytokines IL-10 and IFN-γ. Moreover, mice treated with DN T-cell adoptive transfer showed significant protection from lung IRI.[94] DN T cells have also shown promise for treating asthma, with adoptive transfer of DN T cells primed with OVA in an OVA-induced allergic airway disease model showed reduced inflammatory cell infiltration of lungs and a reduced proportion of lung DCs.[95]

Heart. Recent studies have examined the efficacy of DN T-cell therapy on cardiac graft-versus-host disease survival

and autoimmune myocarditis. A study in mice found that the adoptive transfer of donor lymphocyte infusion–activated DN regulatory T cells (Treg) pretransplantation prevented CD4+ cells from rejecting cardiac xenografts of Lewis rats. Moreover, DN T cells isolated from donor lymphocyte infusion–treated heart graft recipient mice were able to suppress the proliferation of CD4+ cells *in vitro*.[32] A similar study showed that the infusion of DN T-cell clones before MHCI-mismatched allogenic heart grafts in mice showed a survival of >100 days as compared with the infusion of DN T-cell mutants that had acquired CD8+ expression, which had a survival average of 19.5 days post-transplantation.[96] Rats with experimental autoimmune myocarditis were found to have a higher concentration of DN T cells in mononuclear cells isolated from the pericardial effusion (16.1%) than in the heart (1.7%).[97] It is important to note that work on the heart demonstrates the paradox of the protective role of DN T cells in alloimmunity but a deleterious role in autoimmunity.

Gastrointestinal tract. About 15% to 20% of CD3+ cells are DN in the mucous membranes of the lamina propria of the small and large intestine in euthymic and athymic mice, suggesting that DN T cells may be resident in the gut epithelium and not derived from DP T cells.[98] In addition, the percentages of TCRαβ DN T cells were higher in the cecum and colon than in the duodenum, jejunum, and ileum and were significantly high in the cecum of female mice.[99] In the steady-state intestine, DN and DP T cells were found in similar distributions.[100] In the epithelium of 6-week-old WT mouse colon, duodenum, jejunum, and ileum, TCRαβ DN T cells expressing syndecan-1, a surface marker for intestinal epithelium, have been found and frequency increases with age. By the time WT mice are 24 weeks old, DN T cells form the majority of TCRαβ T cells.[101] The same study found that *gld* DN T-cell proliferation in the gut epithelium is independent of the Fas pathway and occurring rapidly as compared with *gld* DN T-cell proliferation in the periphery, which has allowed DN T cells to be highly resident in the gut epithelium. The authors suggested that the Fas pathway plays a key role in confining *gld* DN T cells to the gut epithelium and preventing lymphoproliferation through Fas-mediated apoptosis. This provides evidence for the importance of the Fas pathway when distributing DN T cells to different organs, including the gut.[101]

When analyzing duodenal biopsies from children with celiac disease, DN T cells were increased 6-fold compared with controls; however, 80% of these DN T cells were TCRγδ and not TCRαβ.[102] In addition, there was an increase in the general DN T-cell population in microscopic colitis and a reduction in DN T cells in Crohn disease while showing no differences in peripheral blood compared to healthy individuals.[100] Although homeostasis of the DN T-cell population in lymphoid tissue has been shown to depend on Fas-mediated apoptosis of DN T cells in the periphery, homeostasis of intraepithelial tissue DN T cells is independent of peripheral Fas-regulated apoptosis.[101]

Conclusion

There are increasing data from experimental studies and limited human samples that DN T cells are important mediators in many diseases. Research in the field has been hampered by challenges in identifying a specific positive surface marker on these cells. In addition, it is important that researchers on DN T cells use uniform methodology to characterize these cells. Understanding how DN T cells engage in nonrenal diseases can aid in understanding how they could be involved in kidney diseases. DN T cells are increasingly felt to be a heterogeneous T-cell subset, which could explain the broad range of activities attributed to them.

Kidney DN T cells are found in relatively high numbers and have unique subsets that are closely regulated. DN T cells play a pathophysiological role in lupus nephritis, protect from ischemic AKI in murine models, correlate with stable kidney transplant function, and can be found in human renal cell carcinomas. With the tremendous recent success and promise for T cell–based human therapeutics such as immune checkpoint inhibitors and cilia-associated respiratory T cells, DN T cells are a promising target in many different kidney diseases. Recent advances in gene editing technology, such as the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9), could allow scientists to engineer pro-inflammatory DN T cells in patients with SLE to slow disease progression. Alternatively, patients at risk for or with AKI could be administered *in vitro* expanded DN T lymphocytes for prevention or improvement of recovery.

DISCLOSURE

The authors declared no competing interests.

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