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Characterization and effective expansion of CD4$^-$CD8$^-$ TCRαβ$^+$ T cells from individuals living with type 1 diabetes

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CD4$^-$CD8$^-$ TCRαβ$^+$ (double-negative [DN]) T cells represent a rare T cell population that promotes immunological tolerance through various cytotoxic mechanisms. In mice, autologous transfer of DN T cells has shown protective effects against autoimmune diabetes and graft-versus-host disease. Here, we characterized human DN T cells from people living with type 1 diabetes (PWT1D) and healthy controls. We found that while DN T cells and CD8$^+$ T cells share many similarities, DN T cells are a unique T cell population, both at the transcriptomic and protein levels. We also show that by using various cytokine combinations, human DN T cells can be expanded *in vitro* up to 1,000-fold (mean >250-fold) and remain functional post-expansion. In addition, we report that DN T cells from PWT1D display a phenotype comparable to that of healthy controls, efficiently expand, and are highly functional. As DN T cells are immunoregulatory and can prevent T1D in various mouse models, these observations suggest that autologous DN T cells may be amenable to therapy for the prevention or treatment of T1D.

INTRODUCTION

Conventional T cells, including helper (CD4$^+$) and cytotoxic (CD8$^+$) T lymphocytes, account for the majority of T cells in humans and mice. Still, multiple other T cell subsets, termed non-conventional, have been identified, such as T cell receptor γδ$^+$ (TCRγδ$^+$) T cells, CD1d-restricted natural killer T (NKT) cells, MR-1-restricted mucosal-associated invariant T (MAIT) cells, CD8αα$^+$ intra-epithelial lymphocytes (IELs), and CD4$^-$CD8$^-$TCRαβ$^+$ cells (double-negative [DN] T cells).[1–3] These T cell populations play multi-faceted and specialized functions, ranging from epithelium repair (TCRγδ$^+$ T cells) to initiation of immune responses (NKT cells) and gut homeostasis (CD8αα$^+$ IELs).[4–7] The role of DN T cells, however, remains controversial and unclear.

In many settings such as transplantation and autoimmunity, DN T cells display immunosuppressive properties by eliminating autoreactive immune cells.[8–15] This process is mediated, at least in part, via FAS or perforin/granzyme-dependent direct cytotoxicity.[9,10,13–15] Other surface receptors are also implicated in the cytotoxic activity of DN T cells, such as NKG2D, DNAM-1, Ly-6A, and Lag-3.[10,16–18] We and others have shown that in individuals that have received hematopoietic stem cell grafts, heightened DN T cell numbers and DN T cell activation status inversely correlate with graft-versus-host disease (GVHD) severity.[19,20] In other settings, DN T cells are viewed as pro-inflammatory. In MRL-*lpr* mice and systemic lupus erythematosus (SLE) patients, DN T cell numbers are greatly increased, and these cells produce interleukin-17 (IL-17),[21,22] a pro-inflammatory cytokine that contributes to the pathology of SLE.[23] These conflicting properties likely reflect the heterogeneity of DN T cells. Indeed, DN T cells are isolated based on the absence of CD4 and CD8 expression and often comprise a mix of different T cell subsets. While some groups exclude type I NKT cells using CD1d-tetramers, in most studies DN T cells also include γδ T cells, MAIT cells, and type II NKT cells. As these three cell types can produce pro-inflammatory cytokines, including IL-17,[1,2] this may partially explain the discrepancy in the description of DN T cell function. Still, even after excluding most other unconventional T cell subsets, recent single-cell RNA sequencing (RNA-seq) data revealed heterogeneity in mouse DN T cells, where, even at steady state, some DN T cells display a cytotoxic phenotype, while others have a high potential for cytokine production.[24] This heterogeneity is further reflected in the fact that some DN T cells originate from CD4$^+$ or CD8$^+$ T cells that have downregulated the co-receptor,[25–28] while others originate from thymic precursors.[29] This functional heterogeneity could explain why in different contexts, DN T cells seemingly play opposite roles. Regardless, DN

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T cells convincingly contribute to immune tolerance in multiple settings.[9,10,30–33]

NOD mice are prone to develop autoimmune diseases and spontaneously develop autoimmune diabetes.[34–36] Interestingly, adoptive transfer of DN T cells in NOD mice is sufficient to confer protection from diabetes.[12,28,37–39] These results suggest that transfer of DN T cells in humans may be beneficial to treat or prevent type 1 diabetes (T1D) or other immune pathologies.

The objective of this study was to better define human DN T cells and their propensity to expand in culture. By carefully excluding γδ T cells, MAIT cells, and NKT cells, we characterized DN T cells both at transcriptomic and protein levels. When compared to $CD4^+$, $CD8^+$, and γδ T cells, DN T cells exhibit a unique transcriptomic profile. Moreover, we showed that DN T cells can be expanded *in vitro* over 1,000-fold (mean >250-fold), while retaining their high cytotoxic properties, associated with immunosuppression. Finally, we found that DN T cells from healthy individuals and people living with T1D (PWT1D) are comparable, both at phenotypic and functional levels. Overall, our data suggest that DN T cells could be used in the context of autologous cell therapies.

RESULTS

DN T cells are a unique T cell population sharing similarities with $CD8^+$ T cells

To gain insights into the similarities and differences between DN T cells and other T cell subsets, we performed RNA-seq of *ex vivo* DN T cells, as well as $CD4^+$, $CD8^+$, and $TCR\gamma\delta^+$ T cells, all sorted from the peripheral blood mononuclear cells (PBMCs) of healthy donors. In a principal-component analysis (PCA) of *ex vivo* T cells, DN T cells segregated separately from all other T cell populations (Figure 1A, left panel), suggesting that the transcriptomic signature of DN T cells differs from that of other T cell populations. Indeed, while the transcriptome of DN T cells most closely resembled that of $CD8^+$ T cells based on PC1, 2,262 genes were differentially expressed between the two populations (Figures 1B–1D). This is consistent with previous RNA microarray data showing that DN T cells (without NKT and MAIT cell exclusion) are more similar to $CD8^+$ T cells than to $CD4^+$ T cells in terms of gene expression.[40]

To investigate the biological properties of DN T cells, we performed pathway analysis using gene set enrichment analysis, which identified 4,622 significant gene sets that were different between DNT cells and the other three T cell subsets. We used EnrichmentMap to generate modules based on the similarity of the gene set leading edge genes using a Jaccard similarity index cutoff of 0.25 to reduce functional redundancy. We identified 43 modules that were specifically enriched in DN T cells, relative to the three other T cell subsets (Figure 1E). This analysis revealed a strong enrichment for gene sets related to proliferation (cell cycle, FoxM1 pathway, MYC targets) and activation (antigen response, T cell activation, synaptic signaling, TCR activation) (Figure 1E). DN T cells also displayed an enrichment of signatures associated with cytokine signaling (IL-1, IL-2, IL-4, IL-6, IL-10, and IL-12) (Figure 1E), suggesting that their highly activated profile may also be driven by cytokines *in vivo*. While we observed enrichment for gene sets associated with regulatory T cells (Figure 1E), DN T cells do not express FoxP3 (Figure S1A), similar to mouse DN T cells[12,41] and consistent with previous reports in humans.[42] Interestingly, multiple modules related to anti-microbial responses (type I interferon [IFN] signaling, NK and NKT cell signatures, signal transducer and activator of transcription1 [STAT1] signaling, NOD-2 and Toll-like receptor [TLR] signaling, chronic viral infection) were enriched in DN T cells. This suggests that similar to other non-conventional T cells, DN T cells may possess cytotoxic and innate-like properties. As many non-conventional T cell populations display restricted TCR repertoires (NKT and MAIT cells), we assessed the diversity of the DN T cell TCR repertoire. CDR3 diversity analysis revealed that DN T cells are polyclonal and that their TCR repertoire is highly diverse (Figures S1B and S1C). This is consistent with a previous analysis of the TCRVβ repertoire of human DN T cells, which showed high diversity of TCRVβ gene segment usage.[43] We also compared the similarity between the repertoires of DN, $CD4^+$, and $CD8^+$ T cells. Based on the quantification of unique combinations of VDJ segments, we observed that the repertoire of DN T cells more closely resembled that of $CD8^+$ T cells than $CD4^+$ T cells (Figure S1D). However, subjecting the three cell types to the Bray-Curtis index for beta diversity did not reveal any statistically significant comparison (permutational multivariate analysis of variance; $p > 0.05$; data not shown), likely because of the small sample size.

As the gene signatures separating DN and $CD8^+$ T cells in our PCA analysis (PC2) were related mainly to activation status and proliferation (Figure S2A), we also performed transcriptomic analysis after *in vitro* activation and expansion of the same T cell populations. In this condition, DN T cells closely clustered with $CD8^+$ T cell samples but remained distinct from $CD4^+$ and $\gamma\delta^+$ T cells (Figure 1A, right panel). Still, 163 differentially expressed genes (DEGs) were identified between DN and $CD8^+$ T cells (Figures 1B and 1C), 51 of which were expressed more by DN T cells (Figure S2B). Examples of these genes include *KLRB1* (CD161) and *NCR3* (Nkp30), classical NK cell receptors also expressed by subsets of highly cytotoxic T cells[44,45]; *TNFRSF25*, coding for the co-stimulatory receptor DR3[46]; and *BLK* (B lymphoid kinase), a Src family kinase for which expression had been observed in B cells and a subset of γδT cells, but not yet in αβT cells.[47] Differentially expressed transcription factors include *ZBTB16* (PLZF), classically associated with non-conventional T cells; *GATA3*, *RUNX2*, and *NFATC1*, all of which promote the activation and cytotoxic functions in T cells[48–51]; and *IKZF2* (Helios), which has been implicated in the maintenance of the regulatory function of both $CD4^+$ and $CD8^+$ T cells.[52,53] This is consistent with the expression of Helios in DN T cells at the protein level.[54,55] Interestingly, Helios expression is highest in DN relative to other T cell subsets in mice,[24] and loss of Helios expression in DN T cells has been associated with disease severity in two mouse models of lupus.[56] IL-10R was also highly expressed in DN T cells, consistent with the autocrine impact of IL-10 that was previously described in mouse DN T cells.[41] In addition, relative to other populations, DN T cells

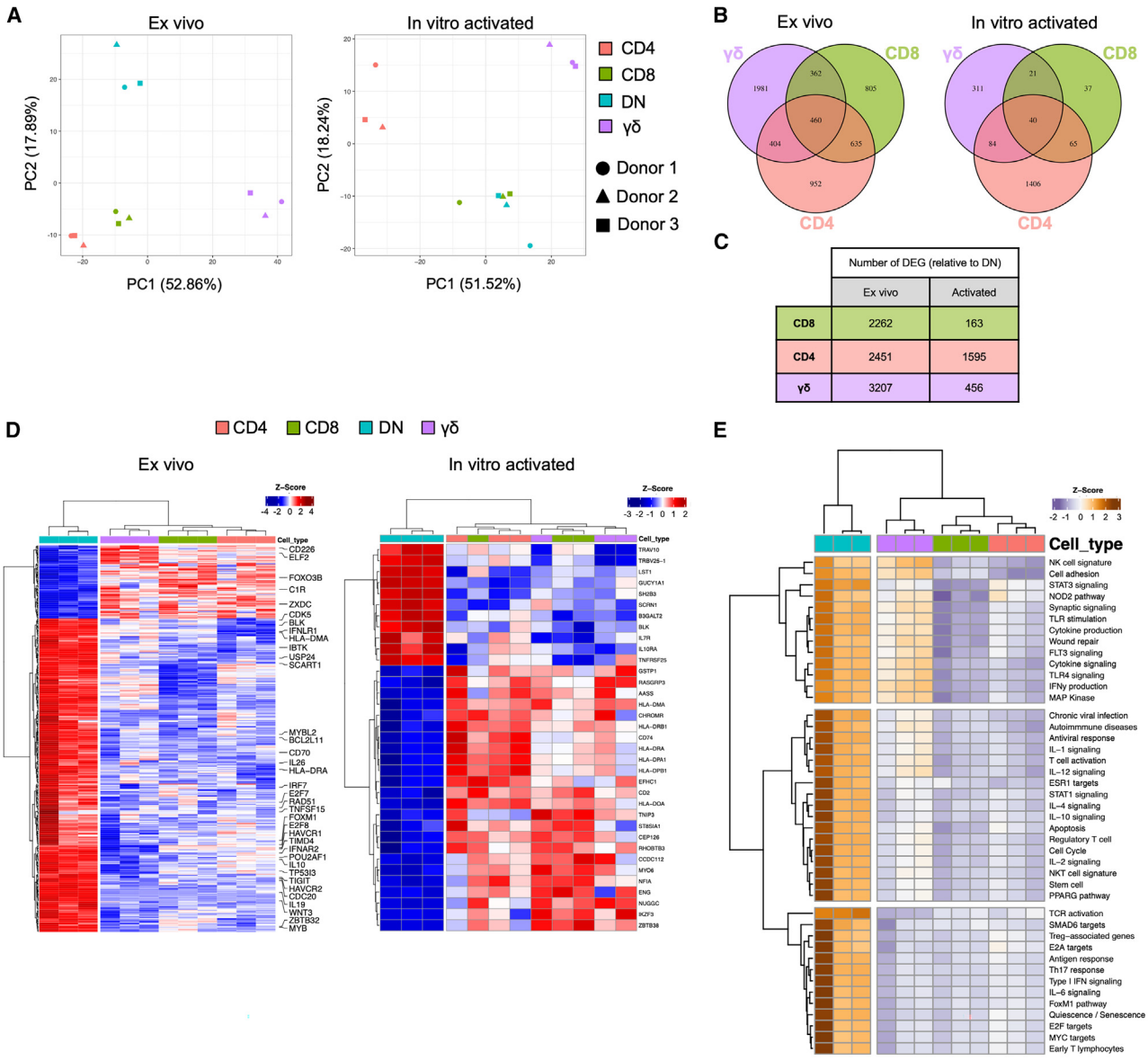

Figure 1. Human DN T cells represent a unique T cell population with an innate T cell signature

(A) PCA plot of $CD4^+$ ($CD56^-TCR\alpha\beta^+TCR\gamma\delta^-CD4^+CD8\alpha^-$), $CD8^+$ ($CD56^-TCR\alpha\beta^+TCR\gamma\delta^-CD4^-CD8\alpha^+$), DN ($CD56^-TCR\alpha\beta^+TCR\gamma\delta^-CD4^-CD8\alpha^-$), and $TCR\gamma\delta^+$ ($CD56^-TCR\alpha\beta^-TCR\gamma\delta^+$) T cells, *ex vivo* (left) and after *in vitro* expansion (right), for the 1,000 top DEGs. (B) Venn diagram of DEGs ($p < 0.05$) from *ex vivo* (left) and expanded (right) DN T cells and other T cell populations. (C) Summary of DEG number, relative to DN T cells. (D) Heatmap presenting DEGs between *ex vivo* (left) and expanded (right) DN T cells and other T cell populations. Columns represent individual samples, whereas rows represent normalized gene expression, from blue to red representing low to high expression, respectively. (E) Heatmap representing pathway EnrichmentMap analysis for immune transcriptomic genes sets for *ex vivo* samples, where each column represents individual samples, whereas rows represent normalized pathway module scores, with a gradient from purple to orange denoting low to high expression.

expressed lower levels of several human leukocyte antigen molecules, including classical (DR and DP) and non-classical (DM and DO) major histocompatibility complex class II (MHC class II) molecules (Figure 1D, right panel). Of note, we did not detect differential expression of IL-17 by *ex vivo* or expanded DN T cells (data not shown). These data suggest that DN T cells represent a unique polyclonal T cell population and that while they share many similarities with conventional $CD8^+$ T cells, DN T cells retain unique features that are characteristics of non-conventional and highly cytotoxic T cells.

Cytokine receptor expression in DN T cells

To characterize DN T cells in healthy individuals and in the context of autoimmunity, we quantified DN T cells derived from the blood of PWT1D and healthy controls. We found no difference in the

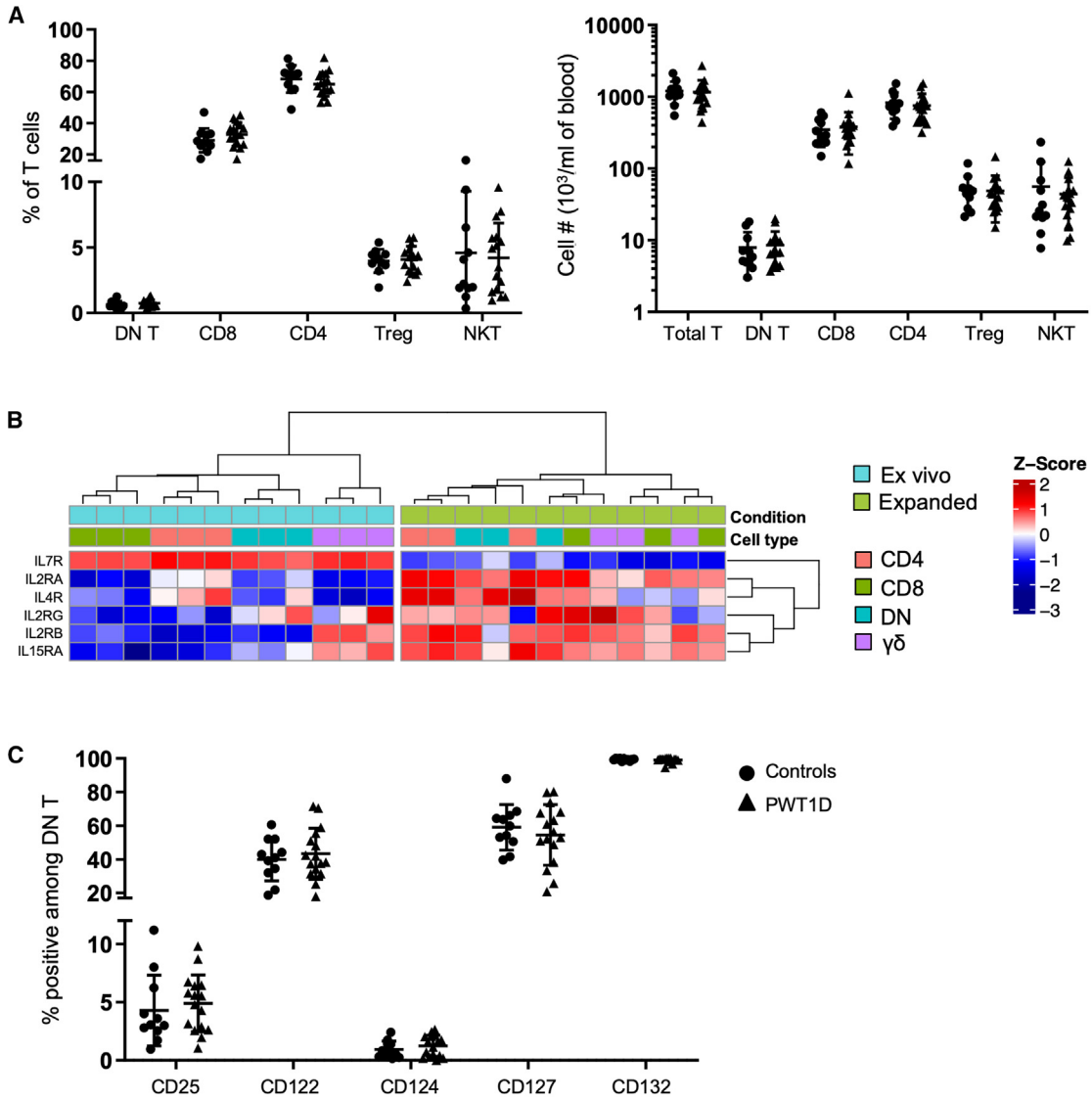

Figure 2. DN T cells from PWT1D and controls do not differ in proportion, number, and expression of cytokine receptor chains

(A) Proportion (left) and absolute number (right) of T cell populations in the blood of control donors (circle, *N* = 11) and PWT1D (triangle, *N* = 16). Mean and SD are shown. DN T cells were gated as MR1-tet$^-$TCR$\alpha\beta^+$CD8α^-CD8β^-CD4$^-$CD56$^-$ (see Figure S3). Statistical significance was tested using Mann-Whitney tests. (B) Gene expression heatmap showing expression of various cytokine receptors from *ex vivo* and expanded T cell populations. Columns represent individual samples, whereas rows represent normalized gene expression, from blue to red representing low to high expression, respectively. (C) Protein expression of selected cytokine receptor chains by DN T cells *ex vivo*, measured by flow cytometry. *N* = 11 (controls) and 16 (PWT1D). Mean and SD are shown.

proportion or numbers of DN T cells, CD8$^+$, CD4$^+$, T regulatory cells, and NKT cells between controls and PWT1D (Figures 2A and S3A), and, as previously described,[43] DN T cells account for less than 2% of total T cells in the blood of healthy donors (Figure 2A). Considering the low number of DN T cells, it is obvious that DN T cell-based therapy will require robust *in vitro* T cell expansion. Current expansion protocols for T cell-based therapies typically use combinations of cytokines such as IL-2, IL-7, and IL-15.[57,58] To gain insight into the cytokines that may promote DN T cell expansion, we took advantage of our RNA-seq data. Relative to conventional CD4$^+$ and CD8$^+$ T cells, *ex vivo* DN T cells expressed higher levels of *IL2RG* (CD132) and *IL15RA* (CD215) and lower levels of *IL2RA* (CD25) and *IL4R* (CD124) than CD4$^+$ T cells (Figure 2B). We confirmed the expression of cytokine receptors on DN T cells at the protein level by flow cytometry and observed a high proportion of *ex vivo* DN T cells expressing CD122, CD127, and CD132, while few DN T cells expressed CD25 and CD124 (Figure 2C). This phenotype is distinct from all T cell subsets tested, including CD8$^+$ T cells (Figure S3B). While the expression of

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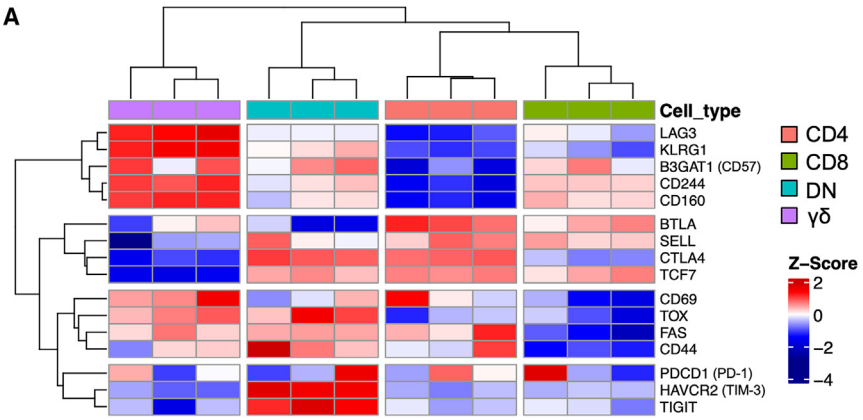

B

	DN		CD8		CD4		*P*-value, DN vs.	
	Control	T1D	Control	T1D	Control	T1D	CD8	CD4
2B4+	27.4	25.7	30.1	31.4	2.1	1.2	NS	***
CD57+	29.6	26.9	17.8	16.8	3.7	2.1	*	***
FAS+	46.5	45.8	21.6	24.2	28.3	29.5	***	***
KLRG1+	46.2	42.3	37.2	35.3	15.4	12.9	NS	***
LAG-3+	0.6	0.9	0.3	0.4	0.3	0.3	NS	NS
PD-1+	24.0	18.7	19.6	17.2	8.4	7.7	*	***
TIM-3+	23.5	24.5	4.7	5.8	1.8	2.3	***	***
CD69+	4.0	4.9	2.2	2.9	0.7	1.0	NS	***
Naïve	46.0	55.5	56.1	63.0	51.7	60.0	*	NS
CM	9.9	6.6	7.2	4.9	35.5	29.0	**	***
EM	27.6	20.7	15.7	13.5	10.7	9.8	***	**
EMRA	15.9	16.9	20.6	18.2	1.7	0.8	NS	***

Figure 3. DN T cells display an activated phenotype

(A) Gene expression heatmap representing the expression of activation and exhaustion markers on *ex vivo* T cell populations. Columns represent individual samples, whereas rows represent normalized gene expression, from blue to red representing low to high expression, respectively. (B) Protein expression heatmap representing the surface protein expression of selected activation and exhaustion markers, measured by flow cytometry. Each box shows the mean frequency of positive cells for a specific marker in controls ($N = 11$) and PWT1D ($N = 16$). Blue and red represent low and high expression, respectively. Only significant differences between DN T cells and other populations are indicated, in the last columns. No significant difference was observed for samples from controls and PWT1D for any marker. Naive cells are gated as $CCR7^+CD45RA^+$, CM, central memory ($CCR7^+CD45RA^-$), EM, effector memory ($CCR7^-CD45RA^-$), and EMRA, effector memory $CD45RA^+$ ($CCR7^-CD45RA^+$). Statistical significance was tested using a one-way ANOVA (Friedman test) followed by Dunn's multiple comparisons test; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

IL4R (CD124) was low in DN T cells *ex vivo*, we observed an induction of *IL4R* transcription during culture (Figure 2B), suggesting that its expression may be induced by *in vitro* activation in DN T cells. Apart from a non-statistically significant reduction of CD25 expression in T cells from PWT1D (Figure S3B), consistent with previous reports,[59,60] no difference was observed between the samples of healthy controls and PWT1D (Figures 2C and S3B). Together, these results suggest that DN T cells express the receptors to respond to various γ chain family cytokines, including IL-2, IL-4, IL-7, and IL-15.

DN T cells express markers associated with activation and exhaustion

HAVCR2, coding for the activation/exhaustion marker TIM-3, is listed among the most DEGs in *ex vivo* isolated DN T cells (Figure 1D, left panel). We therefore assessed the expression of different markers associated with activation/exhaustion on DN T cells and other T cell populations in our RNA-seq dataset. At the transcriptomic level, most selected activation/exhaustion markers were highly expressed in DN T cells (Figure 3A). In contrast, the expression of other markers classically associated with $CD8^+$ T cell exhaustion, such as *BTLA* and *CD160*,[61–64] was lower in DN T cells relative to $CD8^+$ and γδ T cells (Figure 3A). This suggests that the DN T cell signature may reflect an activated phenotype rather than exhausted cells, consistent with the enrichment of modules related to TCR activation and cytokine signaling gene sets (Figure 1E). We confirmed the expression of some of these markers at the protein level and observed the expression of all tested markers in at least a portion of DN T cells, including 2B4, CD57, FAS, KLRG1, LAG-3, PD-1, and TIM-3 (Figures 3B and S4A). While the percentage of DN and $CD8^+$ T cells expressing KLRG1 and 2B4 was similar, a greater proportion of DN T cells expressed CD57, FAS, PD-1, and TIM-3 relative to both $CD4^+$ and $CD8^+$ T cells (Figure 3B). These observations are consistent with a previous report of TIM-3 and PD-1 expression by DN T cells.[65] No phenotypic difference was observed between DN T cells from healthy controls and PWT1D (Figure 3B). While expression of these markers by $CD8^+$ T cells has been well correlated with exhaustion,[66,67] the functional significance of these markers on DN T cells is unknown and do not necessarily indicate an exhausted state, as observed for other T cell populations.[68] In addition, we classified our different T cell populations into naive and memory subsets using the classical markers CCR7 and CD45RA (Figure S4B). Human T cells can be divided into naive ($CCR7^+CD45RA^+$), central memory (CM; $CCR7^+CD45RA^-$), effector memory (EM; $CCR7^-CD45RA^-$), and EM $CD45RA^+$ (EMRA; CCR7-$CD45RA^+$).[69] Consistent with

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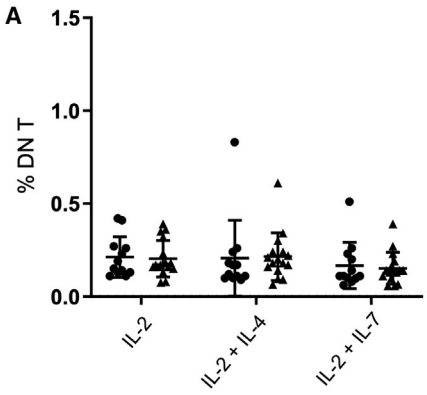

B

	IL-2		IL-2 + IL-4			IL-2 + IL-7		
	Control	T1D	Control	T1D	*P*-value vs. IL-2	Control	T1D	*P*-value vs. IL-2
2B4+	26	30	28	31	NS	28	31	NS
CD57+	25	24	25	23	NS	19	18	*
KLRG1+	14.1	18.0	11.3	10.9	***	9.2	13.9	**
PD-1+	13.6	12.7	13.2	13.5	NS	13.6	13.5	NS
CCR7+	78	76	87	88	***	79	73	NS
CD45RA+	67	66	49	56	***	65	67	NS
CD45RO+	69	62	80	72	***	72	66	NS

	IL-2		IL-2 + IL-4			IL-2 + IL-7		
	Control	T1D	Control	T1D	*P*-value vs. IL-2	Control	T1D	*P*-value vs. IL-2
FAS MFI	1853	1594	1570	1433	**	1954	1716	*
LAG-3 MFI	1770	1810	1567	1423	NS	2159	2372	**
TIM-3 MFI	5021	4550	3636	3301	*	6978	6497	***

Figure 4. Cytokine combinations alter DN T cell phenotype during *in vitro* expansion

PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies and IL-2, IL-2 and IL-4, or IL-2 and IL-7 for 6 days. (A) The frequency of DN T cells from healthy donors (circle, N = 11) and PWT1D (triangle, N = 16) among total T cells is plotted. Mean and SD are shown. (B) Expression of selected markers were analyzed by flow cytometry on activated DN T cells from healthy donors and PWT1D from A. Protein expression heatmaps for selected activation and exhaustion markers. Each box shows the mean frequency of positive cells (top heatmap) or the mean fluorescence intensity (MFI; bottom heatmap) for a specific marker. Markers with bimodal expression are shown in frequency of positive cells and others are shown with MFI. Blue and red represent low and high expression, respectively. Only significant differences between cells expanded with IL-2 alone and cells expanded with IL-2 + IL-4 or IL-2 + IL-7 are indicated. No significant difference was observed for samples from controls and PWT1D for any marker. Statistical significance was tested using a one-way ANOVA (Friedman test) followed by Dunn's multiple comparisons test (paired); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

previous reports,[70,71] we observed a reduction in T cells with a CM phenotype in PWT1D relative to healthy controls (Figure S4C). This observation applied to both conventional ($CD4^+$ and $CD8^+$) T cells as well as DN T cells. While DN T cells displayed a similar proportion of naive cells relative to conventional T cells, we observed more DN T cells with an EM phenotype relative to both $CD4^+$ and $CD8^+$ T cells (Figures 3B and S4B), suggesting a rapid response to stimulation and a potentially different tissue distribution. These observations highlight the distinct surface phenotype of DN T cells, characterized by the expression of a unique combination of markers of activation and an increased proportion of EM cells.

In vitro culture conditions impact DN T cell phenotype

As *ex vivo* DN T cells express CD122, CD127, and CD132 (Figures 2B and 2C) as well as *IL4R* following activation (Figure 2B), we tested whether DN T cell expansion would be enhanced relative to other T cell subsets in the presence of cytokines. After 6 days in culture, the frequency of DN T cells remained low in the presence of IL-2 or combinations of IL-2 with IL-4 or IL-7 (below 0.5% for most samples) (Figure 4A). No cytokine combination was therefore able to preferentially expand DN T cells from total PBMCs. Still, by assessing the expression of the previously described activation/exhaustion markers, we identified cytokine-driven phenotypical differences (Figure 4B). For instance, CD57 was lower in the presence of IL-7, while FAS, LAG-3, and TIM-3 were increased. In addition, the expression of CCR7, CD45RA, and CD45RO, which is associated with subsets of memory T cells, was modulated by the cytokines used during expansion, where the percentage of $CD45RA^+$ cells was reduced and CD45RO was increased in the presence of IL-4 (Figure 4B). Therefore, different cytokine combinations lead to distinct phenotypes on expanded DN T cells and thus possibly to functional differences. For this reason, we performed all the subsequent expansions using the three different cytokine conditions. Of note, the addition of IL-15 was also tested but did not result in increased expansion of DN T cells or changes in phenotype compared to other combinations (data not shown). Therefore, IL-15 was not tested further.

Rapid *in vitro* expansion of DN T cells

DN T cells represent a rare population in human blood. Based on similar cell products,[72,73] robust *in vitro* expansion protocols would be required to obtain a sufficient number of cells for DN T cell-based

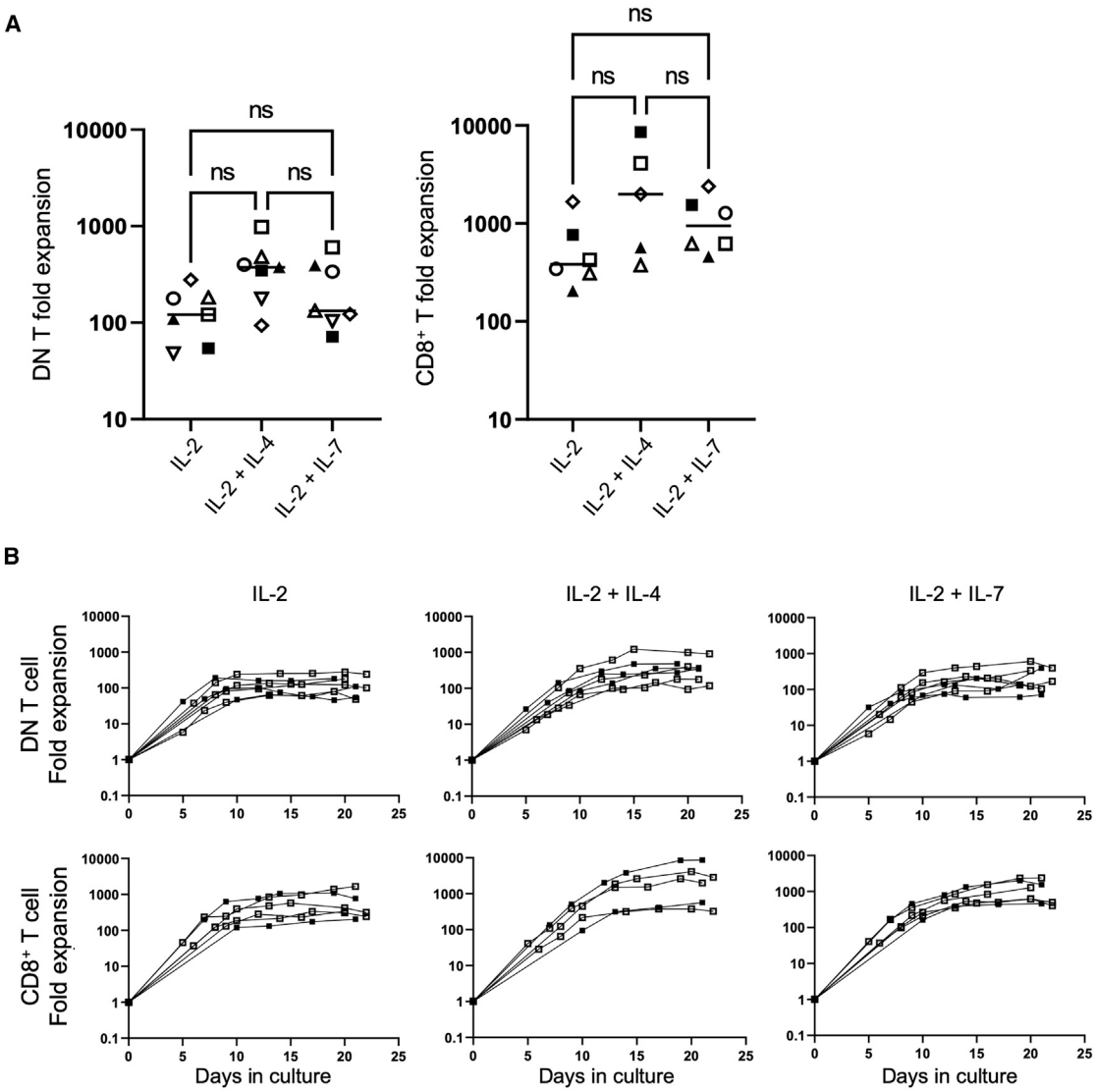

Figure 5. Sorted DN T cells can be expanded in the presence of IL-2, IL-4, and IL-7

DN and CD8$^+$ T cells were sorted from PBMCs, stimulated with anti-CD3 and anti-CD28 antibodies, and cultured with IL-2, IL-2, and IL-4 or IL-2 and IL-7. (A) Graphs show the fold expansion after 21 days in culture for DN (left) and CD8$^+$ T cells (right). Filled symbols are from controls and open symbols are from PWT1D. Mean is shown. Statistical significance was tested using a one-way ANOVA (Friedman test) followed by Dunn's multiple comparisons test. (B) Individual expansion curves of sorted DN and CD8$^+$ T cells. Cell counting and media changes were performed at each point. Each line represents a donor (controls: filled symbols; PWT1D: open symbols).

therapies. We therefore sorted DN T cells (Figure S5A) and subjected them to stimulation with aCD3/aCD28 antibodies with different cytokine combinations to allow for expansion. We compared the expansion of DN T cells to CD8$^+$ T cells, as those two cell types share the most similarities. DN T cells from all donors rapidly expanded *in vitro* and reached an expansion fold between 100 and 1,000 (mean 266) at day 21 of culture (Figures 5 and S5B). While all cytokine combinations allowed strong expansion, we observed a non-statistically significant trend for an increased expansion using a combination of IL-2 and IL-4 for both DN T cells and CD8$^+$ T cells (Figures 5 and S5B). Again, no difference was observed between DN T cells from PWT1D and healthy controls (Figures 5 and S5B). Most DN T cells (>80%) retained a CD4$^-$CD8$^-$TCR$\alpha\beta^+$ phenotype after expansion (Figures S5C and S5D).

Expanded DN T cells are functional and display potent cytotoxic activity

Our transcriptomic analysis revealed a strong cytotoxic signature in DN T cells (Figure 1), and we and others have previously reported cytolytic activity of mouse and human DN T cells.[13,41,74] To test whether our expanded DN T cells remained functional after culture, we performed an *in vitro* cytotoxicity assay as a surrogate marker of

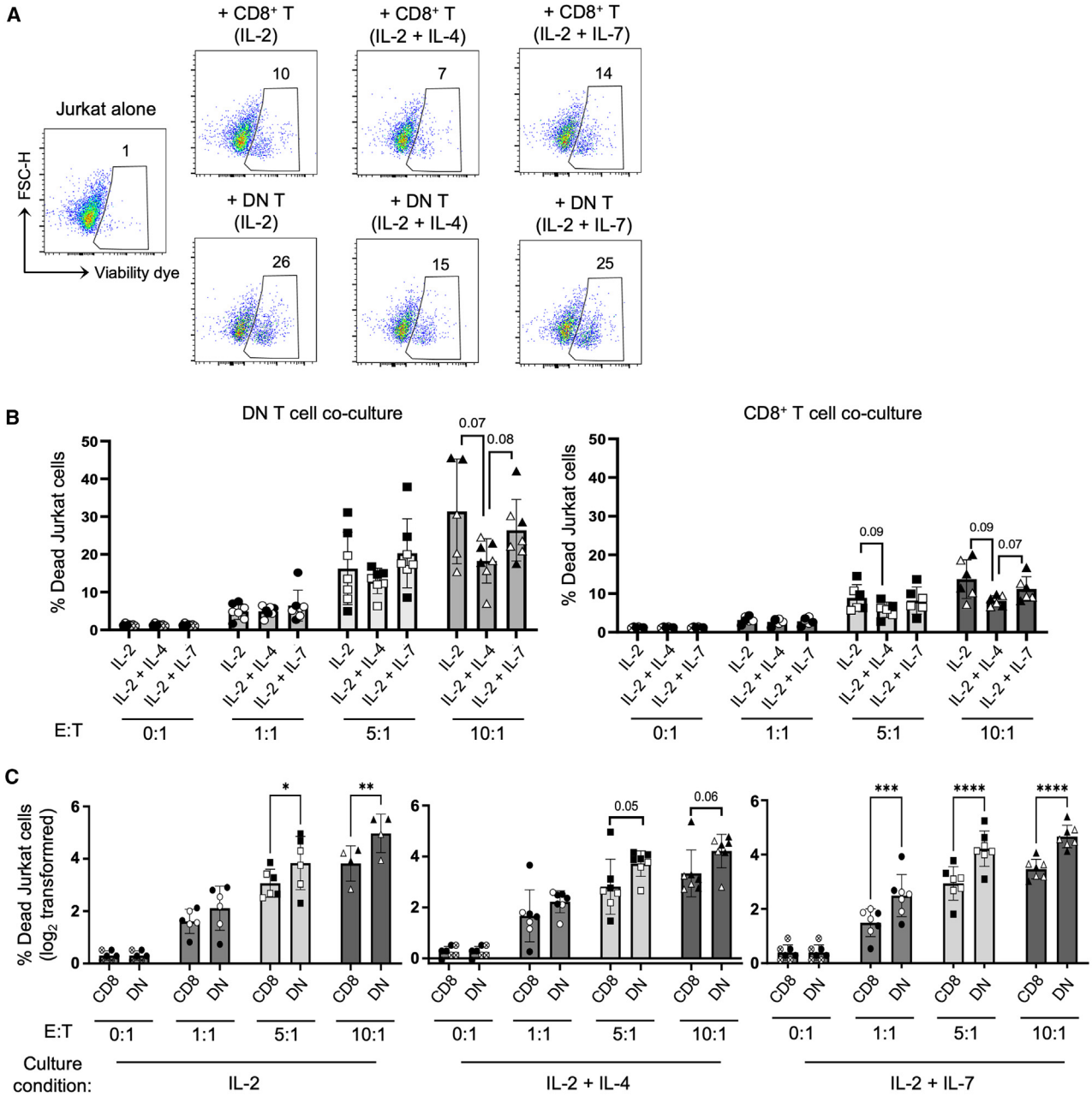

Figure 6. Expanded DN T cells remain functional and display higher cytotoxicity than donor-matched CD8⁺ T cells

DN and CD8⁺ T cells were expanded for 21 days in the presence of IL-2, IL-2, and IL-4 or IL-2 and IL-7 prior to co-culture with Jurkat cells for 4 h. (A) Representative flow profiles showing the frequency of dead Jurkat cells (viability dye⁺), alone (left) or co-cultured with CD8⁺ (top) or DN (bottom) T cells (E:T ratio of 5:1). (B) Compilation of the frequency of dead Jurkat cells after co-culture with DN or CD8⁺ T cells, separated by cytokine combination and E:T ratio. Controls are shown with black symbols and PWT1D with white symbols. Mean and SD are shown. (C) Comparison of cytotoxic potential between DN and CD8⁺ T cells for all culture conditions. Controls are shown with black symbols and PWT1D with white symbols. Data were log2 transformed. Mean and SD are shown. Statistical significance was tested using a two-way ANOVA, followed by Sidak's multiple comparisons test (paired); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

their cytotoxic function. We used Jurkat cells as they are efficiently targeted by DN T cells *in vitro*.[75,76] Using our expanded cells as effectors, we observed significant cytotoxic activity for both CD8⁺ and DN T cells, regardless of the cytokine condition used during expansion (Figures 6A and 6B). No difference was observed in the cytotoxic potential between DN T cells from healthy donors and PWT1D.

Interestingly, when compared to donor-matched $CD8^+$ T cells, DN T cells showed significantly higher cytolytic activity under all cytokine conditions tested (Figure 6C). These data reveal that DN T cells remain functional after robust *in vitro* expansion and that they display superior cytolytic abilities relative to conventional $CD8^+$ T cells.

DISCUSSION

DN T cells represent a rare population of unconventional T cells in both mice and humans. While the development and function of DN T cells has been described in mice,[9,10,29,77] the relationship between DN T cells and other T cell populations in humans remains unclear. In the present study, using transcriptomic data, flow cytometry analysis, and functional assays, we show that DN T cells are a unique polyclonal population that share many characteristics with conventional $CD8^+$ T cells. Still, DN T cells bear a unique surface phenotype typically associated with an activated state, and their transcriptomic signature is associated with important cell cycling, TCR activation, and TLR and cytokine signaling. As DN T cells show preclinical potential for the treatment of a variety of diseases, including T1D, we developed expansion protocols and found that DN T cells can be rapidly expanded up to 1,000-fold (mean >250-fold), while remaining functional. Finally, we demonstrated that PWT1D display a similar number of peripheral DN T cells and that these cells exhibit an unaltered surface phenotype, expansion capacity, and functional potential. These findings suggest that autologous DN T cell transfer should be explored as a therapeutic option in the context of T1D prevention or treatment.

Many unconventional T cell populations have been described in mice and humans, including γδ T cells, IELs, NKT, and MAIT cells. Most of these populations can be identified based on the expression of surface markers (γδTCR or CD8αα) or TCR restriction (CD1d or MR1). The lack of specific markers for DN T cells, combined with a polyclonal repertoire, has complicated the study of these cells, hence, our poor understanding of their origin and properties. Here, we used transcriptomic analysis to uncover the main features of human DN T cells and highlight the characteristics of DN T cells when compared to γδ T cells and conventional $CD4^+$ and $CD8^+$ T cells. We observed that similar to mice, human DN T cells are highly polyclonal. This contrasts with the well-documented invariant NKT and MAIT cell populations, which display oligoclonal TCR repertoires and TCR restriction to a single MHC molecule, CD1d and MR1, respectively.[78] In addition to TCR diversity, our transcriptomic analysis of *ex vivo* samples revealed an enrichment for gene sets related to antiviral responses in DN T cells, including STAT1, TLR, and type 1 IFN pathways, as well as IFN-γ production and some transcriptomic similarities with NK cells. This is consistent with reports that DN T cells can respond to viral infections in mice.[79] Enrichment of gene sets related to pathways of microbe sensing (TLR4, NOD2, IL-1), as well as the expression PLZF, the master regulator of innate T cell development and function,[80,81] also suggest that DN T cells may possess some innate-like properties, which have been described for other unconventional T cells such as γδT, NKT, and MAIT.[82,83] Reminiscent of other unconventional T cells such as NKT and MAIT cells,[84,85] we observed that DN T cells were enriched for cells with an EM phenotype ($CCR7^-CD45RA^-$). This suggests that DN T cells may possess a low threshold for stimulation, either through cytokines, their TCR, or pattern recognition receptors, which would be consistent with their highly activated gene signature *ex vivo*.

We observed that DN T cells share most similarities with conventional $CD8^+$ T cells. Indeed, while over 2,000 genes were differentially expressed between DN and $CD8^+$ T cells *ex vivo*, the transcriptome of DN and $CD8^+$ T cells closely segregated together after *in vitro* expansion. This suggests that most differences between *ex vivo* samples were driven by activation state, which disappeared after *in vitro* activation. This is consistent with a strong enrichment of gene sets related to TCR activation, cytokine signaling, and TLR signaling in *ex vivo* DN T cells. Still, expanded DN T cells retain some unique properties, highlighted by 163 DEGs between expanded DN and $CD8^+$ T cells. Interestingly, many of those were related to cytotoxicity, such as *KLRB1* (CD161), *NCR3* (Nkp30), and *TNFRSF25* (DR3), all of which are typically expressed by NK cells and subsets of T cells and promote cytotoxic functions. This is consistent with previous reports of DN T cells being able to efficiently eliminate cancer cells in mice and humans.[65,74,76,86–88] Note that in these previous studies, DN T cells were defined as $CD3^+CD4^-CD8^-$, such that the cellular preparation of DN T cells included both $\alpha\beta TCR^+$ and $\gamma\delta TCR^+$ DN T cells. Nevertheless, the observations made here suggest that the cytotoxic properties of the mixed $\alpha\beta TCR^+$ and $\gamma\delta TCR^+$ DN T cells are not solely attributed to the γδT cell fraction.

It has been reported that a portion of DN T cells may originate from $CD8^+$ T cells that have downregulated their CD8 co-receptor in response to activation or inflammation.[25,26,54,55] Our transcriptomic analysis supports this possibility. Indeed, after *in vitro* activation, DN and $CD8^+$ T cells cluster together on the PCA plot, with 163 DEGs between the two cell types. Previous evaluation of CDR3 sequences from $CD3^+CD4^-CD8^-$ cells from patients with autoimmune lymphoproliferative syndrome suggested that the DN T cell population is composed of expanded clones sharing sequences with $CD8^+$ T cells.[89] In contrast, our TCR analysis in healthy donors did not reveal reduced TCR diversity in DN T cells relative to $CD4^+$ and $CD8^+$ T cells, which argues against the notion of clonal expansion in the DN T cell population. In addition, we did not observe donor-specific matching between DN and $CD8^+$ T cells in the usage of specific VDJ segments. Together, these data suggest that in healthy individuals (i.e., in the absence of an inflammatory condition), the DN T cell population is clonally diverse and does not primarily originate from $CD8^+$ T cell clones. Our small sample size and the use of bulk RNA-seq limit our interpretation of the TCR repertoire. More in-depth analysis of the TCR repertoire of DN T cells in health and disease is needed to best understand the similarities and differences relative to other T cell populations.

Our characterization of human DN T cells is concordant with many of the previously reported characteristics of mouse DN T cells. Indeed, mouse DN T cells display a polyclonal αβTCR repertoire,

high cell-cycling activity in the absence of immune challenge, and the capacity to respond to diverse TLR stimulation.[29,77] Mouse DN T cells also express the transcription factor Eomes[77] and produce the cytotoxic protein granzyme B and the anti-inflammatory cytokine IL-10,[41] all of which were observed in this study with human DN T cells. At the cell surface, *ex vivo* mouse and human DN T cells also share a similar phenotype, with expression of multiple NK cell receptors and high expression of many activation markers.[77,79] This strong resemblance between DN T cells in mice and humans suggests that many of the potential therapeutic benefits described in mouse models, such as in autoimmune diabetes, may successfully translate to human pathologies.

In contrast to conventional T cells, DN T cells do not appear to induce GVHD or any severe cytokine release syndrome once transferred *in vivo*.[65,90] In addition, transferred DN T cells show significant persistence,[65,90,91] which represents a key limitation of current NK cell-based immunotherapies.[92] Therefore, because of their safety profile and their immunomodulatory properties, DN T cell-based therapies have been suggested in various settings, such as transplantation, autoimmunity, and cancers.[10,93–95] Considering the multiple reported benefits of DN T cells in mouse models of autoimmune diabetes,[12,28,37,38] we are interested in exploring the feasibility of autologous DN T cell transfers in the context of T1D. While DN T cell abundance is not reduced in the blood of PWT1D, their frequency in PBMCs remains low (under 2%). Any cellular therapies using DN T cells would therefore require robust expansion prior to cell injection. As we observed *ex vivo* expression of CD122 (IL-2Rβ), CD127 (IL-7Rα), and CD132 (IL-2Rγ), as well as an upregulation of CD124 (IL-4Rα) upon *in vitro* activation, we tested the expansion of DN T cells in the presence of a combination of IL-2, IL-4, IL-7, and IL-15. No cytokine combination was able to preferentially expand DN T cell in a heterogeneous T cell pool. Cell isolation prior to expansion is therefore essential to obtain a pure population of DN T cells after expansion. All cytokine combinations resulted in robust DN T cell proliferation, with most samples showing expansion levels of 100 to 1,000-fold, over the course of 3 weeks. Based on the typical dose from other regulatory T cell therapies,[72] this protocol would allow us to generate a sufficient number of DN T cells for cell therapy. The bulk of the expansion was achieved in the first 2 weeks, suggesting that shorter expansion protocols may be sufficient. The presence of IL-4 led to a trend for higher DN T cell expansion, although it did not reach statistical significance. The choice of cytokine therefore does not seem to greatly impact the expansion capacity of DN T cells, except for IL-2, which is critical for long-term *in vitro* T cell expansion.[96,97] Still, flow cytometry analysis revealed that different cytokine combinations lead to altered phenotypes post-expansion, such as differential expression of FAS, TIM-3, LAG3, and CD45 isoforms. It is still unclear how those phenotypic differences relate to cellular function. More work is needed to understand the properties of human DN T cells, both *in vivo* and after expansion.

Importantly, even after 3 weeks in culture, DN T cells remained functional, as observed by their ability to lyse Jurkat cells, a known target of human DN T cells.[74,76] This observation contrasts the expression of exhaustion markers observed on *ex vivo* DN T cells by RNA-seq and flow cytometry. Markers classically associated with exhaustion on $CD8^+$ T cells may therefore not indicate a state of exhaustion when expressed on other T cells such as DN T cells. More work is needed to understand the function of such surface receptors on DN T cells. Interestingly, when compared to donor-matched $CD8^+$ T cells, DN T cells displayed higher levels of cytotoxicity. This was true for cells expanded in all cytokine conditions and both for healthy controls and PWT1D, highlighting the unique functional properties of DN T cells.

In conclusion, our data reveal that DN T cells represent a unique polyclonal T cell population in humans, distinct from both conventional and other unconventional T cells. It also suggests that DN T cells from PWT1D are phenotypically and functionally similar to those of healthy controls and that they are present in similar numbers. We finally showed that DN T cells can be isolated from the blood and efficiently expanded *in vitro* using different cytokine combinations, resulting in a large pool of pure DN T cells retaining functional properties. This study strongly suggests that DN T cell-based therapy is a feasible option in the context of T1D and potentially for other autoimmune disorders.

MATERIALS AND METHODS

Donor samples

PBMCs were obtained from healthy donors and PWT1D attending the Maisonneuve-Rosemont Hospital. Patient clinical data including age, sex, age at diabetes onset, glycosylated hemoglobin (HbA1c) levels, and insulin delivery method are indicated in Tables 1 and 2. PBMCs were isolated using Lymphoprep (STEMCELL Technologies) and used for culture and *ex vivo* phenotyping or cryopreserved in 10% DMSO and later thawed for cell sorting. The study was approved by the Research Review Office of the CIUSSS de l'Est-de-l'Île-de-Montréal institutional review board and was conducted according to the Declaration of Helsinki (protocol no. 2011-463). All subjects gave informed written consent before participation.

RNA-seq sample preparation

Isolated PBMCs from three healthy donors were stained with antibodies against the following markers: CD4, CD8α, CD56, TCRαβ, and TCRγδ (all from BioLegend) (see Table S1). Cell populations were sorted using an Aria III cytometer (BD Biosciences) as follows: $CD4^+$ T cells ($CD56^-TCR\alpha\beta^+TCR\gamma\delta^-CD4^+CD8\alpha^-$), $CD8^+$ T cells ($CD56^-TCR\alpha\beta^+TCR\gamma\delta^-CD4^-CD8\alpha^+$), TCRγδ cells ($CD56^-TCR\alpha\beta^-TCR\gamma\delta^+$), and DN T cells ($CD56^-TCR\alpha\beta^+TCR\gamma\delta^-CD4^-CD8\alpha^-$). Cells were stained in PBS containing 2% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1 mM EDTA. A total of 10^5 cells were resuspended immediately in TRIzol (Thermo Fisher Scientific) and are referred to as *ex vivo* samples. The remaining sorted cells were resuspended in ImmunoCult-XF T cell expansion medium (STEMCELL Technologies) (0.5×10^6 cells/mL) with 1% penicillin-streptomycin (50 U/mL–50 μg/mL) (Wisent) and added to plates coated with anti-CD3 (1 μg/mL) and anti-CD28 (5 μg/mL)

Table 1. Clinical data of donors for immunophenotyping and T cell expansion

	Controls	PWT1D
Sample size	11	16
Age, y		
Median	31	22
Mean	31	24
Range	26–36	18–37
p, mean controls vs. PWT1D	0.0012	
Sex		
Female (%)	5 (45)	6 (38)
Male (%)	6 (55)	10 (62)
P, sex distribution	0.6794	
Mean age at diagnosis, y	NA	10
Insulin delivery		
Injected (%)	NA	9 (56)
Pump (%)	NA	7 (44)
HbA1c		
≥8 (%)	NA	6 (38)
<8 (%)	NA	8 (50)
Unknown (%)	NA	2 (12)
Smokers (%)	1 (9)	3 (19)
Ethnicity		
White (%)	10 (91)	12 (75)
Hispanic (%)	1(9)	0
Afro Caribbean (Haitian) (%)	0	1 (6)
Middle Eastern (%)	0	3 (19)

See Figures 2 and 4. NA, not applicable; PWT1D, people living with type 1 diabetes.

Table 2. Clinical data of donors for DN T cell sorting and cytotoxic assay

	Controls	PWT1D
Sample size	5	5
Age, y		
Median	29	27
Mean	30	27
Range	24–42	22–38
P, mean controls vs. PWT1D	0.3968	
Sex		
Female	2	3
Male	3	2
P, sex distribution	0.5271	
Mean age at diagnosis, y	NA	10
Insulin delivery		
Injection	NA	3
Pump	NA	2
HbA1c		
≥8	NA	1
<8	NA	3
Unknown	NA	1
Smokers	0	1
Ethnicity		
White	3	4
Hispanic	1	0
Middle Eastern	1	1

See Figures 5 and 6. NA, not applicable; PWT1D, people living with type 1 diabetes.

antibodies (both BioLegend). After 24 h, the following human cytokines were added: IL-1β (560 U/mL), IL-2 (30 U/mL), IL-7 (200 U/mL), and IL-15 (125 U/mL) (from Miltenyi Biotec). Cells were cultured for 14 days, with fresh medium containing cytokines being added every 2–3 days. At the end of the culture, cells were washed twice with PBS, resuspended at 5×10^5 cells/mL in TRIzol (Thermo Fisher Scientific) and stored at −80°C prior to sequencing. RNA was isolated using TRIzol. Libraries were prepared using the KAPA RNA HyperPrep PolyA Single-End Read Kit. Libraries were sequenced using Illumina NextSeq 500 in two sequencing runs.

RNA-seq preprocessing

FASTQ files were trimmed for sequencing adapters and low-quality 3′ bases using Trimmomatic version 0.38[98] and aligned to the reference human genome version GRCh38 (from Ensembl build 106) using STAR version 2.7.6a.[99] Aligned reads were deduplicated using UMI-Tools version 1.01.[100] Read counts were extracted from BAM files at the gene level using HTSeq version 0.12.3.[101] For immune repertoire quantification, deduplicated BAM files were converted to FASTQ format using the bamtofastq function from bedtools version 2.29.2[102] and then aligned with MiXCR version 3.0.13[103] for the TCR repertoire only. GEO: GSE285617.

Transcriptomic downstream analysis

Raw counts from HTSeq were normalized with the R DESeq2 package.[104] A donor-driven batch effect was detected using principal variation component analysis, and DonorID was added to statistical models as a covariate for a paired design. Differential expression analysis was performed as pairwise comparisons between cell types using the Wald test, with Bayesian log fold change shrinkage. It was also performed across cell culture conditions (*ex vivo* versus expanded) for each cell type in parallel. Donor effects were corrected using ComBat with the sva package[105] for visualization purposes, such as for PCA and heatmaps. Pathway enrichment analysis was performed using the fgsea package,[106] with shrunken fold changes as a ranking metric, for the hallmark, c2, c5, and c7 MSigDB collections.[107,108] An implementation of the EnrichmentMap approach[109] was used to consolidate functionally 4,622 redundant significant gene sets (adjusted $p < 0.05$ and nominal $p < 0.05$) into modules using a Jaccard distance of ≤ 0.25. Module-level p values and normalized enrichment scores (NESs) are reflective of the minimal p-value and maximal NES, respectively, across gene sets in each module. The GSVA package[110]

was then used to generate sample-level pathway scores from the leading-edge genes of each module.

A ClueGO (Cytoscape plugin)[111] network enrichment analysis was performed on the top 155 genes from the PCA loadings associated with PC2. Enrichment was performed for Gene Ontology Terms and Reactome Pathways, with a $p < 0.05$.

Immune repertoire quantification: MiXCR clonotype reports were loaded into R. CDR3 clonotype alpha diversity was quantified with the Shannon index using the vegan R package.[112] Additional details and all original code generated for this analysis is available on Github. The DOI accession number from Zenodo for Github repository is https://doi.org/10.5281/zenodo.14564379.

Flow cytometry

Cells were stained at 4°C for 30 min in staining buffer (PBS containing 2% FBS and 1 mM EDTA) with various antibodies (see Table S1). After washing, stained cells were fixed with intracellular fixation buffer (Thermo Fisher Scientific) for 20 min at 4°C. Cells were analyzed using a Fortessa X-20 5-laser flow cytometer (BD Biosciences) and data with the FlowJo version 10 software (BD Biosciences).

Total T cell expansion

Freshly isolated PBMCs were suspended in ImmunoCult-XF T cell expansion medium (STEMCELL Technologies) with 1% penicillin-streptomycin (Wisent) and added to a flat-bottom 96-well plate (Sarstedt) pre-coated with anti-CD3 (1 μg/mL) and anti-CD28 (5 μg/mL) antibodies (both from BioLegend). A range of 20–50 $\times$ 10^3 cells were initially seeded per well in 200 μL medium (10–25 $\times$ 10^4 cells/mL). The plates were incubated at 37°C, 5% CO_2. After 24 h, cytokines (IL-2: 30 U/mL, IL-4: 30 U/mL, and IL-7: 200 U/mL, all from Miltenyi Biotec) were added. At day 3, cells were transferred in new culture plates without antibodies, with fresh media and cytokines. At day 6, expanded T cells were stained as described above and analyzed by flow cytometry.

DN and CD8$^+$ T cell sorting and expansion

Thawed PBMCs were stained with antibodies as described above. DN T cells were sorted as CD56$^-$CD19$^-$MR1-tet$^-$TCRαβ$^+$CD8α$^-$CD8β$^-$CD4$^-$. CD8$^+$ T cells were sorted as CD56$^-$CD19$^-$MR1-tet$^-$TCRαβ$^+$CD8α$^+$CD8β$^+$CD4$^-$. Cells were sorted on a Sony SH800 (Sony Biotechnology) cell sorter. Sorted cells were expanded, as described above, for 21 days. Cells were resuspended at 1 $\times$ 10^6 cells/mL in fresh medium with cytokines every 2–3 days.

Cytotoxic assay

Jurkat cells were cultured in complete RPMI-1640 medium (10% FBS, 50 μM 2-mercaptoethanol, 10 mM HEPES, and 1% penicillin-streptomycin). Jurkat cells were stained with eFluor 450 cell viability dye (1 μM) (Thermo Fisher Scientific). Stained Jurkat cells and expanded DN or CD8$^+$ T cells were seeded together in a 96-well V-bottom plate at different effector:target (E:T) ratios in complete RPMI-1640 medium. The plates were incubated for 4 h at 37°C, 5% CO_2. Cells were then stained with antibodies, and the frequency of Zombie aqua (viability dye) positive Jurkat cells was quantified using a Fortessa X-20 5-laser flow cytometer (BD Biosciences).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 9 (GraphPad Software). The statistical analyses and post hoc tests are indicated in each figure legend. The differences were considered significant for values of $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

DATA AND CODE AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

J.E.F.-D. contributed to the investigation, formal analysis, and methodology. F.L.-V. contributed to the formal analysis, methodology, and writing of the original draft. A.-N.P. contributed to the formal analysis, methodology, writing, and editing. A.O. contributed to the investigation and formal analysis. L.B. provided resources. S.P. provided supervision and contributed to the writing and editing. V.J. contributed to the methodology and formal analysis. L.L. contributed to the conceptualization and provided resources. J.-S.D. provided supervision and contributed to the methodology, writing, and editing. E.E.H. participated in the conceptualization, writing, and editing. L.C. contributed to the formal analysis, writing, and editing. S.L. contributed to the funding acquisition, project administration, writing, and editing and provided supervision and resources.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101400.

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