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IL-7 Abrogates the Immunosuppressive Function of Human Double-Negative T Cells by Activating Akt/mTOR Signaling

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Recently, a novel subset of TCR αβ⁺ CD4⁻ CD8⁻ double-negative (DN) T cells was described to suppress immune responses in both mice and humans. Moreover, in murine models, infusion and/or activation of DN T cells specifically suppressed alloreactive T cells and prevented the development of graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. We demonstrated that human DN T cells, like their murine counterparts, are highly potent suppressor cells of both CD4⁺ and CD8⁺ T cell responses. After hematopoietic stem cell transplantation and other lymphopenic conditions, IL-7 plays an important role in the reconstitution, survival, and homeostasis of the T cell compartment. Because IL-7 was shown to interfere with T cell functionality, we asked whether IL-7 affects the functionality of human DN T cells. Intriguingly, IL-7 diminished the suppressive activity of DN T cells toward allopositive CD4⁺ effector T cells. Of interest, our studies revealed that IL-7 activates the Akt/mechanistic target of rapamycin (mTOR) pathway in human DN T cells. Importantly, selective inhibition of the protein kinases Akt or mTOR reversed the IL-7 effect, thereby restoring the functionality of DN T cells, whereas inhibition of other central T cell signaling pathways did not. Further analyses suggest that the IL-7/Akt/mTOR signaling cascade downregulates anergy-associated genes and upregulates activation- and proliferation-associated factors that may be crucial for DN T cell functionality. These findings indicate that IL-7 and Akt/mTOR signaling are critical factors for the suppressive capacity of DN T cells. Targeting of these pathways by pharmacological agents may restore and/or enhance DN T cell functionality in graft-versus-host disease. The Journal of Immunology, 2015, 195: 000–000.

Double-negative (DN) T cells are defined by the expression of TCR αβ-chains and the lack of both coreceptors CD4 or CD8. In both mice and humans, these cells make up ~1–5% of all PBLs (1, 2). DN T cells have attracted growing attention as a result of the recent discovery of their potent immunoregulatory function. In murine models, DN T cells were shown to prevent rejection of allogeneic and xenogeneic organ grafts by effectively suppressing activated CD4⁺ and CD8⁺ T cells (1, 3). Furthermore, DN T cells possess the capacity to resolve various inflammatory conditions, including graft-versus-host disease (GvHD) after allogeneic hematopoietic stem cell transplantation (HSCT). Strikingly, murine DN T cells also were able to inhibit onset of GvHD while mediating beneficial antileukemia effects (4, 5). In murine autoimmune diabetes models, adoptive transfer of DN T cells prevented the onset of insulinitis and diabetes mellitus (6, 7). The mechanism by which murine DN T cells mediate suppression is dependent on Fas–FasL interactions or the secretion of perforin/granzyme (1, 8).

Of interest, the first clinical studies on recipients of allogeneic stem cell grafts demonstrated an inverse correlation between the frequency of circulating DN T cells and the severity of acute GvHD (9, 10). The observation that patients with circulating DN T cell frequencies > 1% did not develop severe acute GvHD suggests the therapeutic potential of human DN T cells in HSCT.

We demonstrated that human DN T cells, like their murine counterparts, are highly potent suppressor cells of both CD4⁺ and CD8⁺ T cell responses (11). The suppressive activity of DN T cells is not mediated indirectly by modulation of APCs or by competition for T cell growth factors. Furthermore, DN T cell–mediated suppression toward responder T cells is TCR dependent and requires de novo protein synthesis. However, despite numerous studies on the mechanisms of human DN T cell–mediated suppression, no study considered potential modulators of the immunoregulatory function.

A well-known modulator of T cell responses is the homeostatic cytokine IL-7. IL-7 is essential for thymic T cell development, as well as for proliferation and survival of peripheral lymphocytes. As a growth factor, IL-7 is involved in the maintenance of naive and memory CD4⁺ and CD8⁺ T cells (12–14). Of note, the level of circulating IL-7 is enhanced in response to lymphopenia in a variety of autoimmune disorders and as a result of chemotherapy and irradiation-based conditioning regimens prior to HSCT (15–18). In the context of HSCT, the elevated IL-7 serum concentrations were associated with the occurrence and grade of acute GvHD (19–21). Given the importance of IL-7 for T cell reconstitution,
there has been growing interest in the use of IL-7 to modify T cell homeostasis (22, 23). Although immunoregulatory T cells play a pivotal role in T cell–deficient diseases, little is known about the potential interaction of IL-7 with immunoregulatory DN T cells.

In this study, we investigated the influence of IL-7 on the functionality of human DN T cells. We demonstrate that IL-7 significantly affects suppressive properties of DN T cells. We further show that the Akt/mechanistic target of rapamycin (mTOR) pathway mediates IL-7 effects on the suppressive activity of DN T cells.

Materials and Methods

Medium and reagents

T cells were cultured in RPMI 1640 medium supplemented with 10% human AB-serum (PAN Biotech). The following recombinant human cytokines were used: 100 U/ml IL-2 (Novartis), 20 ng/ml IL-7 (CellGenix), 500 U/ml GM-CSF (Genzyme), 5 ng/ml IL-4 and TGF-β (PeproTech), 10 ng/ml IL-1β and TNF (PromoKine), 1000 U/ml IL-6 (CellGenix), and 1 μg/ml PGE2 (Enzo Life Science).

The following inhibitors were used: Akt inhibitor IV (2 μM; Merck), Erk activation inhibitor peptide I (10 μM; Santa Cruz Biotechnology), p38 inhibitor SB202190 (2 μM; BIOMOL), and JNK inhibitor SP600125 (10 μM; Tocris).

Isolation and culture of T cells

PBMCs were enriched by density gradient centrifugation using Pancoll (PAN Biotech) from leukapheresis products of healthy volunteers. Informed consent was provided according to the Declaration of Helsinki. CD4+ and DN T cells were isolated from PBMCs via magnetic separation, according to the manufacturer’s instructions (Miltenyi Biotec).

Dendritic cells (DCs) were generated from leukapheresis products, as described previously (11). DN T cells (1 × 10^6/well) were cocultured with allogeneic mature DCs (2.5 × 10^5/well) in 96-well plates in complete medium plus IL-2. DN T cells were restimulated weekly with allogeneic DCs for 2–5 wk. Viability and purity of the T cells were monitored by flow cytometry. Further purification via magnetic bead separation was performed if purity was <95%. DN T cells were used for functional assays not earlier than 5 d after the last stimulation.

Flow cytometry

Cells were stained with anti-human anti-Ki67 (B56), anti–annexin V, anti-CD127 (M21), anti-TCRαβ (BW242/412), anti-STAT5(pY694) (47), anti-S6p(S240) (N4–41), anti- glycogen synthase kinase 3β (GSK-3β) (pS9) (D85E12), anti-CD4 (13B8.2), anti-CD132 (TUGh4), anti-CD154 (24-31), anti-CD25 (2A3), anti-CD4 (RPA-T4), and anti-CD3 (UCHT1) mAbs. Intracellular Ki67 was stained with the Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer’s protocol. Lymphocytes were determined by forward scatter (FSC)-A and single scatter–A, doublets were excluded by FSC-W, and cells were gated by the indicated mAbs.

For detection of phosphorylated proteins, DN T cells were stained with violet proliferation dye (VPD450; BD Biosciences) and preincubated in fresh medium in the absence of IL-2. Cells were harvested, washed, and stimulated with DCs in the presence or absence of IL-7. Cells were gently centrifuged to the bottom and incubated for 45 min. Subsequently, cells were fixed with BD Cytofix/Cytoperm, washed, and permeabilized with Perm Buffer III (BD Biosciences) for 30 min. After washing with PBS (2% FCS), cells were incubated with the indicated Abs for 40 min, washed, and fixed with CellFIX (BD Biosciences). Flow cytometry data were acquired on a FACSCanto II (BD), and VPD450* DN T cells were analyzed with FlowJo software (TreeStar).

FIGURE 1. Human DN T cells express a functional IL-7R. (A) Expression of IL-7R on CD4+ T cells. Freshly isolated CD4+ T cells were stained with anti-CD127 and anti-CD132 Abs. A representative line graph with isotype control (shaded) is shown. (B) Phosphorylation of STAT5 in CD4+ T cells after addition of IL-7. Freshly isolated CD4+ T cells were stimulated with 20 ng/ml IL-7 for 20 min. Phosphorylated STAT5 was quantified by flow cytometry. Data show mean fluorescence intensity (MFI) ± SEM of three experiments. (C) Expression of CD127 on CD4+ T cells in the MLR. CD4+ T cells were stimulated with allogeneic DCs in the presence or absence of DN T cells and IL-7. After 24 h, the expression of CD127 on CD4+ T cells was quantified and compared with the expression of CD127 on CD4+ T cells cultured alone. (D) Expression of CD127 and CD132 on DN T cells. Precultured DN T cells were analyzed prior to the MLR setup. One representative line graph with isotype control (shaded) is shown. (E) Phosphorylation of STAT5 in DN T cells after addition of IL-7. DN T cells were stimulated with 20 ng/ml IL-7 for 20 min. Phosphorylated STAT5 was quantified by flow cytometry. Data show MFI ± SEM of five experiments. (F) CD127 expression on DN T cells in the presence or absence of IL-7. The MLR was performed as in (C), and the expression of CD127 was measured by flow cytometry after 24 h. Data are mean relative expression of CD127 (±SEM) of five independent experiments.
T cell–suppression assays

Freshly isolated CD4+ T cells were labeled with CFSE (Sigma) and stimulated with allogeneic DCs at a 2:1 ratio or with human anti-CD3/CD28 Dynabeads (Life Technologies) at a 50:1 ratio, in the presence or absence of IL-7. Precultured DN T cells were used as suppressor cells in a responder/suppressor ratio of 1:1. After 5 d of coculture, cells were harvested, stained with anti-CD4, anti-TCRab, and anti-CD25 mAbs, and measured by flow cytometry. CD4+ T cells were analyzed by first gating on lymphocytes with FSC-A and side scatter–A and on singlets with FSC-W, followed by gating on TCRab+ CD4+ cells. The activation of CD4+ T cells was analyzed with CD25, and proliferation was studied by the decrease in CFSE fluorescence. Unstimulated CD4+ T cells were used as control.

For blocking experiments, DN T cells were labeled with VPD450 and incubated with signaling inhibitors or medium only as control. After 2 h, cells were washed twice and used as suppressor cells in an MLR described above. At day 2 of coculture, cells were harvested and stained with anti-Ki67, anti-CD4, anti-CD25, and anti-CD154 mAbs. Proliferation and activation of the T cells was determined by flow cytometry. DN T cells were analyzed by gating on singlet lymphocytes and on TCRab+ CD4+ VPD450+ cells.

Viability assays

The Cell Meter generic fluorometric caspase activity assay kit (AAT Bioquest) was used to determine T cell cytotoxicity, according to the manufacturer’s instructions. TF2-VAD-FMK was added to the suppression assays after 48 h. After 1 h of incubation, cells were harvested and stained with anti-CD4 and anti-CD3 mAbs. 7-Aminoactinomycin D (7-AAD) was added shortly before the flow cytometric measurement.

Transcriptome analysis

DN T cells were sorted 12 h after adding them to the suppression assay with a MoFlo cell sorter. DN T cells were lysed and RNA extraction was performed using the RNeasy Micro Kit (QIAGEN, Redwood City, CA). Quality of RNA was analyzed using a 2100 Bioanalyzer system (Agilent Technologies). Barcoded RNA-sequencing libraries were prepared from 100 ng total RNA using a Nugen Ovation Human FFPE RNA-Seq system for small amounts of RNA, according to the manufacturer’s instructions. For selective depletion of RNAs, the insert-dependent adapter cleavage method from Nugen was used prior to cDNA synthesis. To control sources of variability during sample and data processing, a common set of external spike-in RNA controls, developed by the external RNA controls consortium, was used (Ambion).

Libraries were subjected to paired-end sequencing (2 × 101 bp) on a HighSeq 2500 platform (Illumina, San Diego, CA). Reads were mapped against the hg19 reference genome, using STAR aligner v.2.3.0 (24), with a unique mapping rate of 85%. Absolute read counts/gene were produced using Subread’s featureCounts program (25) and the gtf annotation file for hg19 by Ensembl. Subsequent analyses were performed using R v3.1.2 (26). In particular, differential expression analysis was performed with the DESeq2 package (27). Functional annotation analysis for the differentially expressed genes was performed using Ingenuity Pathway Analysis (IPA; QIAGEN).

**FIGURE 2.** IL-7 impairs the functionality of human DN T cells. (A) Freshly isolated CD4+ T cells were labeled with CFSE and cultured with allogeneic DCs and DN T cells at an effector/suppressor ratio of 1:1 in the presence or absence of 20 ng/ml IL-7. The proliferative response of CFSE-labeled CD4+ T cells was measured after 5 d by flow cytometry. Representative line graphs of one donor are shown. (B) CFSE-labeled CD4+ T cells were cultured with DN T cells in the presence or absence of IL-7. Either allogeneic DCs or anti-CD3/CD28 beads were used for stimulation. The proliferative response of the CD4+ T cells was determined after 5 d by flow cytometry. Data from ≥10 donors are presented as percentage suppression compared with the controls (+ IL-7) to which no DN T cells were added. (C) CD4+ T cells were cultured with anti-CD3/CD28 beads and DN T cells. CD154 and CD25 expression was quantified on CD4+ T cells at day 2 in the MLRs. Data from seven independent donors are presented. *p < 0.05, **p < 0.01, ***p < 0.005. ns, not significant.
Quantitative PCR analysis

DN T cells were stimulated with anti-CD3/CD28 Dynabeads in the presence or absence of IL-7. After 16 h, cells were lysed and RNA extraction was performed using the RNeasy Micro Kit. A total of 100 ng RNA was transcribed to cDNA with Superscript II reverse transcriptase, according to the manufacturers’ protocol (Invitrogen). Quantitative PCR was performed using SYBR Green PCR Master Mix (QIAGEN) and the StepOnePlus cycler (Applied Biosystems). QuantiTect Primer Assays were purchased from QIAGEN and applied according to the manufacturer’s instructions: Hs_B2M_1_SG (QT00088935), Hs_EGR2_1_SG (QT00000924), Hs_EGR3_1_SG (QT00246498), Hs_CBL_1_SG (QT00070301), Hs_NFATC3_1_SG (QT00052185), and Hs_FOXO4_1_SG (QT00029141). Changes in cycle thresholds were calculated using B2M as housekeeping gene.

Statistical analysis

Data were analyzed with Prism software (GraphPad). Results were compared using the Mann–Whitney U test, unless stated otherwise. The Spearman test was used for correlation analyses. A p value <0.05 was considered significant.

Results

Human DN T cells express a functional IL-7R

Given the pivotal role of IL-7 in T cell reconstitution, we asked whether this cytokine would influence human DN T cell–mediated suppression. Therefore, we first confirmed the expression of IL-7R components on human CD4+ and DN T cells. As expected, CD4+ T cells expressed the IL-7R α-chain (CD127) and the common γ-chain (CD132) (Fig. 1A). Functionality of IL-7R was confirmed by staining for phosphorylated STAT5 protein in CD4+ T cells after cultivation for 45 min in the presence of IL-7 (Fig. 1B). IL-7 is known to be downregulated in response to IL-7 signaling, we analyzed CD127 expression after 2 d of the MLR. CD127 is downregulated on CD4+ T cells by IL-7 independently of DN T cells (Fig. 1C), indicating that functional IL-7 signaling acts on CD4+ T cells during coculture with DN T cells.

We next focused on DN T cells, which have not yet been examined for IL-7R expression. Notably, both resting and cultivated DN T cells express both IL-7R chains (Fig. 1D, data not shown). DN T cells displayed signaling of IL-7R, as shown by STAT5 phosphorylation and downregulation of CD127 (Fig. 1E, 1F). Sensitivity of DN T cells to IL-7 signaling was comparable to conventional CD4+ T cells and reached maximal levels of STAT5 phosphorylation at 20 ng/ml of IL-7 (Supplemental Fig. 1A).

IL-7 impairs functionality of human DN T cells

Because both CD4+ and DN T cells were shown to be susceptible to IL-7, we assessed whether the IL-7 signal modulates the immunoregulatory function of DN T cells. Fig. 2A illustrates that CD4+ T cells exhibit a strong proliferative response upon stimulation with allogeneic DCs that is effectively inhibited by the addition of DN T cells. To study the impact of IL-7, we performed the MLR in the presence of IL-7 and assessed proliferation of CD4+ T cells by CFSE dilution. After 5 d, CD4+ T cells showed a strong proliferation that was not affected by addition of IL-7. However, IL-7 diminished DN T cell–mediated suppression of IL-7.

**FIGURE 3.** IL-7 does not influence viability of CD4+ or DN T cells. (A and B) Freshly isolated CD4+ T cells were cultured in the presence or absence of DC, DN T cells, and IL-7. Annexin V and 7-AAD were measured on CD4+ T cells after 20 h in the MLR. (A) Representative FACS blots are shown. (B) The mean percentage (±SEM) of 7-AAD+ CD4+ T cells is shown for four independent experiments. (C) Freshly isolated CD4+ T cells were cultured in the presence or absence of anti-CD3/CD28 beads, DN T cells, and IL-7. Caspase activity in CD4+ T cells was quantified by TF2-VAD-FMK within the MLR after 2 d. Data are the mean percentage (±SEM) of CD4+ T cells with caspase activity from four independent donors. (D) DN T cells positive for 7-AAD and caspase activity were quantified in the MLR. *p < 0.05, ns, not significant.
CD4+ T cells. Of interest, the DN T cell–mediated suppression proved to be impaired, regardless of stimulation with allogeneic DCs or with anti-CD3/CD28–coated artificial APCs (Fig. 2B). The impairment of the suppressive capacity of DN T cells was observed at IL-7 concentrations as low as 2 ng/ml and reached a maximum at 20 ng/ml (Supplemental Fig. 1B).

We demonstrated recently that human DN T cells restrain proliferation and activation of responder T cells (11). To confirm the impact of IL-7 on DN T cell–mediated suppression, we analyzed the expression of the activation markers CD154 and CD25 on CD4+ T cells (Fig. 2C). Consistent with previous findings, DN T cells were able to suppress activation of CD4+ T cells. However, this suppressive potential was significantly reduced in the presence of IL-7. Taken together, these findings demonstrate that IL-7 represses the DN T cell–mediated suppression toward CD4+ T cell responses.

**IL-7 does not influence viability of CD4+ or DN T cells**

A hallmark of IL-7 is the promotion of cell survival by inducing antiapoptotic molecules. Hence, we asked whether the reduction in DN T cell–mediated suppression by IL-7 is a result of amplified CD4+ T cell viability. We determined the number of annexin-V+ and 7-AAD+ cells within the MLR. As shown in Fig. 3A, CD4+ T cells demonstrated viability >90%, even without stimulation by allogeneic DCs. The addition of DN T cells to the MLR did not result in an increased percentage of annexin-V+ or 7-AAD+ cells, consistent with previous data stating that human DN T cells mediate their suppression not by killing of the responder T cells (11). Of interest, addition of IL-7 did not elicit enhanced CD4+ T cell viability in these settings (Fig. 3A, 3B). To confirm this observation, we analyzed caspase activity in CD4+ T cells after co-culture. Stimulation of CD4+ T cells with IL-7 resulted in a moderate increase in caspase+ cells compared with their unstimulated controls (Fig. 3C). As expected, DN T cells did not augment caspase activity in responder T cells. Moreover, addition of IL-7 to the MLR revealed no impact on caspase activity in CD4+ T cells. We next asked whether IL-7 influences the viability of DN T cells in the MLR. As shown in Fig. 3D, addition of IL-7 did not negatively influence 7-AAD expression or caspase activity in DN T cells. Collectively, these data demonstrate that IL-7 does not influence CD4+ or DN T cell viability.

**IL-7 impairs the suppressive function of DN T cells by selectively activating the Akt/mTOR pathway**

To further explore the mechanism by which IL-7 impairs the suppressive function of DN T cells, we analyzed the IL-7R signaling networks in DN T cells. Key signal transducers downstream of the IL-7R and its associated Janus kinases are the serine/threonine-specific protein kinases Akt and mTOR, which play an important role in regulating cell proliferation, differentiation, and metabolism (28, 29). We analyzed Akt/mTOR signaling in DN T cells and showed that phosphorylation of the Akt downstream kinase GSK-3β, as well as the mTOR-targeted ribosomal protein S6, was induced in DN T cells in response to stimulation. Notably, phosphorylation of GSK-3β and S6 was strongly enhanced by the

**FIGURE 4.** IL-7 impairs the suppressive function of DN T cells by selectively activating the Akt/mTOR pathway. DN T cells were preincubated with Akt and mTOR inhibitors (A), the Akt activator SC79 (B), p38, Erk, or Ink inhibitors (C), or NF-κB inhibitor (D) for 2 h. DN T cells were washed twice and used as suppressor cells in MLRs as described in Materials and Methods. Anti-CD3/CD28 beads were used for stimulation. The proliferation of CD4+ T cells was quantified by Ki-67 staining on day 2. Data for independent donors (n > 6) are presented as percentage suppression compared with controls to which no DN T cells were added. *p < 0.05, **p < 0.01, ***p < 0.005. ns, not significant.
FIGURE 5. Transcriptome analysis identifies key downstream networks in the IL-7–Akt axis in DN T cells. (A) Differentially expressed genes in DN T cells treated with IL-7 compared with control were analyzed with the “Pathway Analysis” tool in IPA software. Pathway enrichment is calculated using the Fisher exact test, and the significance threshold is set at \( p < 0.05 \). The ratio is the number of genes from the dataset that map to a given pathway divided by the number of all known genes ascribed to this pathway. The different shades of gray represent the prediction of an overall decrease or increase in pathway activity calculated with the IPA’s “Pathway Activity Analysis” tool. Arrows indicate signaling networks associated (Figure legend continues).
Addition of IL-7 (Supplemental Fig. 2A). In the absence of TCR stimulation, IL-7 alone failed to induce phosphorylation of Akt and its target proteins (data not shown). Thus, IL-7 markedly increased the TCR-induced activation of the Akt/mTOR signaling cascade in DN T cells.

To investigate the role of Akt/mTOR signaling in the functionality of human DN T cells, we blocked key signaling kinases in DN T cells. As described in Materials and Methods, DN T cells were preincubated with specific signaling inhibitors, washed extensively, and used as suppressor cells in the MLR. Prior to usage, each signaling inhibitor was validated by Phosflow staining (data not shown). The inhibitors revealed no toxic effects on the T cells at the concentrations used (data not shown). Intriguingly, the addition of an Akt inhibitor rendered DN T cells unsusceptible to IL-7 treatment, thereby maintaining their suppressive activity (Fig. 4A). Similarly, blockade of protein kinase mTOR in DN T cells also completely abrogated the IL-7-induced repression of DN T cell–mediated suppression (Fig. 4A). The crucial function of the Akt/mTOR pathway was confirmed by treating DN T cells with a chemical activator of Akt (SC79). Hyperphosphorylation of Akt with SC79 resulted in a significant reduction in DN T cell–mediated suppression (Fig. 4B).

Given that IL-7–induced signaling might also activate MAPKs, such as p38, ERK, and JNK, we examined their potential role in controlling DN T cell function. Importantly, in contrast to blocking the Akt/mTOR pathway, inhibition of p38, ERK, or JNK signaling pathways failed to impede IL-7’s influence on DN T cell–mediated suppression (Fig. 4C). Also, signaling via NF-κB (Fig. 4D) or STAT5 (Supplemental Fig. 2B) revealed to be negligible for IL-7–mediated effects on DN T cell suppressive activity. To summarize, specific activation of the Akt/mTOR pathway by IL-7 impairs the suppressive functionality of human DN T cells.

Transcriptome analysis identifies key downstream networks of the IL-7–Akt axis in DN T cells

Akt/mTOR is known to influence a large variety of cellular functions by regulating numerous downstream targets. In an effort to further unveil the mechanism by which IL-7 impedes the DN T cell–suppressive function, we performed a whole-transcriptome analysis of DN T cells treated with IL-7. DN T cells were cultivated with anti-CD3/CD28–coated artificial APCs and CD4+ T cells in the presence or absence of IL-7 and separated 12 h later by cell sorting. The isolated RNA of the DN T cells was sequenced and analyzed as described in Materials and Methods. The comparison of IL-7–treated and nontreated DN T cell samples showed that 1301 genes were differentially expressed (p value < 0.05; data not shown). Functional IL-7 signaling could be verified by the highly downregulated IL-7R and the several significantly over-represented molecules of the JAK-STAT pathway. Pathway-based functional enrichment analysis with IPA confirmed our previous results showing significant IL-7–mediated PI3K/Akt and mTOR activation in DN T cells, as well as inhibition of PTEN, a negative regulator of the Akt/mTOR signaling pathway (Fig. 5A).

Furthermore, IPA showed that the top enriched molecular and cellular functions are cellular growth, proliferation, survival, and maintenance (Fig. 5B). We further filtered for functions that are annotated specifically for T cells, the most significant of which are presented in Fig. 5B (p ≤ 7.92E-13). Fig. 5C highlights several genes relevant for activation, proliferation, and homeostasis of T cells that were significantly up- or downregulated through IL-7 treatment in DN T cells. Of interest, we found that predominantly genes that are necessary for maintaining an anergic state (30–33) are downregulated in IL-7–treated DN T cells. The abrogated expression of anergy-controlling factors, such as FOXO, NFAT, EGR2, EGR3, and CBL, was verified by quantitative PCR (Fig. 5D). These results raise the possibility that DN T cells lose their suppressive potential as a result of IL-7–mediated reversal of their pivotal anergic state.

Increased activation and proliferation of DN T cells by IL-7 are associated with diminished suppressive capacity

We next hypothesized that the downregulation of anergy-associated genes in DN T cells due to IL-7 may be coincident with an increase in their cellular activity and cell growth. Our analysis of the activation markers CD25 and CD154 revealed that IL-7 significantly amplifies the activation status of stimulated DN T cells (Fig. 6A). Because the transcriptome data additionally indicated that multiple genes associated with proliferation are upregulated in DN T cells in the presence of IL-7, we further examined their mitotic activity on the protein level. As shown in Fig. 6B, measurement of Ki-67 and Cyclin A verified that IL-7 enhances the number of proliferating DN T cells. Finally, we asked whether the increased activation and proliferation of the DN T cells had an effect on their suppressive function. Of interest, we observed that highly activated DN T cells suppressed effector T cells to a lesser extent. Fig. 6C shows this significant negative correlation of the DN T cell activation level with the DN T cell–mediated suppression in the presence or absence of IL-7. Similarly, the increased proliferation of the DN T cells was inversely correlated with their suppressive capacity (Fig. 6D). Overall, these data indicate that IL-7 impedes the functionality of DN T cells by reversing their anergic phenotype while promoting activation and proliferation (Fig. 6E).

Discussion

In this study, we demonstrate that IL-7 is able to alter the immunoregulatory function of human DN T cells. Because DN T cells can regulate T cell responses very effectively, our findings reveal new points of vantage to interfere in peripheral tolerance. In particular, after allogeneic HSCT, the suppressive properties of DN T cells have emerged as attractive modulators of immune reactions. Murine DN T cells were shown to prevent the onset of GvHD while mediating advantageous anti-leukemia effects. In addition, clinical studies pointed to a therapeutic benefit of human DN T cells, because the development and grade of GvHD were negatively correlated with the frequency of DN T cells after HSCT (4, 5, 9, 10). However, our data revealed that IL-7 compromises the suppressive function of DN T cells. This finding is of particular interest because elevated IL-7 levels were associated with the incidence and severity of GvHD (19, 21). Furthermore, plasma IL-7 levels measured early after HSCT were predictive biomarkers of acute GvHD (20). Administration of recombinant human IL-7 (CYT107) was reported to increase circulating CD4+ and CD8+ T cells.
T cell numbers (23, 34); however, several studies recommended careful consideration because of the potential promotion of GvHD with the use of IL-7 after HSCT (19, 20, 35). Our findings support these concerns, because IL-7 impairs DN T cell–mediated suppression of alloreactive T cell responses.

Increased serum IL-7 levels also were described in patients with different autoimmune diseases (15, 16, 36). Of interest, studies on rheumatoid arthritis and juvenile idiopathic arthritis indicated that augmented IL-7 counteracts the suppressive properties of CD4+ CD25+CD127lowFOXP3+ regulatory T cells (Tregs) (37–39). Corresponding to our data revealing a direct impact of IL-7 on the suppressive capacity of DN T cells, IL-7 was recently shown to directly impede the inhibitory function of Tregs (40). In contrast to Tregs that are characterized by their marginal expression of CD127, we demonstrated that both resting and stimulated DN cells express a functional IL-7R, suggesting that DN T cells could

![Image](http://www.jimmunol.org/)

**FIGURE 6.** Increased activation and proliferation of DN T cells by IL-7 are associated with diminished suppressive capacity. (A and B) The activation and proliferation level of DN T cells were analyzed by flow cytometry on day 2 of independent MLRs (n = 12). The relative expression of CD25, CD154, Ki-67, and Cyclin A on DN T cells cultivated with IL-7 is shown in comparison with DN T cells of MLRs without IL-7. (C and D) Activation and proliferation data are plotted against the percentage of DN T cell–mediated suppression and correlation calculated using the Spearman correlation. (E) Proposed model for the impact of IL-7 on DN T cell functionality. Activation of the Akt/mTOR pathway is induced by engagement of the TCR, and it is amplified by IL-7 signals. The simulation of Akt/mTOR signaling above a certain threshold further drives the activation of DN T cells. As a consequence, anergy-associated genes are downregulated so that DN T cells can enter the cell cycle and proliferate; however, this elevated cellular activation and growth result in diminished immunoregulatory function. **p < 0.01, ***p < 0.005.
be sensitive to even low levels of IL-7. Thus, DN T cell–mediated suppression might be modulated already on low IL-7 levels that fail to signal in CD127low Tregs.

Our data on the molecular mechanism revealed that suppression of suppressor cells can be attributed to a single specific signaling pathway, the Akt/mTOR signaling cascade. The impairment of suppression by IL-7 was completely reversed by blocking the Akt or mTOR kinase in DN T cells. In addition, other signaling pathways or transcription factors influenced by IL-7 appeared to be negligible for DN T cell functionality. Notably, we have previously demonstrated that IL-7 was not able to modulate suppressive activity of DN T cells, suggesting that IL-7 mediated Akt/mTOR signaling is highly significant in these cells (11). Several studies investigated the role of Akt/mTOR signaling in Treg cells. Thereby, enforced activation of Akt or loss of PTEN, an upstream inhibitor of Akt-signaling, resulted in reversal of the suppressive function of Treg cells (41, 42). Our results showed that hyper-activation of Akt/mTOR by IL-7 leads to repression of the DN T cell mediated suppression. These findings suggest that the activation level of the Akt/mTOR pathway is not only determining for Treg but also for DN T cell functionality and might conceivably be involved in general establishment and maintenance of regulatory capacity of T cells. The activity of the Akt/mTOR signaling network was recently reported to regulate the expression of FOXP3 in Tregs (43). Of importance, DN T cells lack the expression of this master transcription factor; thus, other downstream factors of Akt/mTOR signaling have to account for the repression of DN T cell–mediated suppression.

The Akt/mTOR pathway was shown to play a central role in regulating T cell proliferation, survival, differentiation, and metabolism (28, 29, 44). Our results revealed that IL-7 crucially amplifies the TCR-induced Akt/mTOR pathway in DN T cells. Consistently, transcriptome analyses showed that cell growth, proliferation, and homeostasis of DN T cells are the most affected molecular functions induced by IL-7. Indeed, our further experiments proved that DN T cells exhibit a more highly activated and proliferating phenotype as a result of IL-7 treatment. This increased activation and proliferation of DN T cells correlated with loss of their suppressive capacity. Previous studies linked Akt/ mTOR signaling to an anergic state of T cells by demonstrating that mTOR inhibition promotes T cell anergy (45–47). Of interest, our findings demonstrated that IL-7 downregulates expression of anergy-controlling factors FOXO, NFAT, p27kip1, EGR, and CBL in DN T cells, suggesting that IL-7/Akt/mTOR signaling abolishes an anergic-like state of the DN T cells.

It is not known whether DN T cells require an anergic phenotype for their suppressive capacity. However, Tregs were demonstrated to show diminished Akt pathway activation and an anergic cell fate (41, 48). In addition, Tregs and type 1 Tregs initially were characterized as T cells with a special anergic phenotype that is necessary for their suppressive activity (49, 50). An IL-7/Akt/mTOR-mediated interruption of the anergic-like state might be critical linked to the loss of suppressive capacity of human DN T cells. Our observations that IL-7 amplifies activation and cell cycle entry of DN T cells would go in line with the reduction of DN T cell anergy. Because the state of anergy depends on mTOR-regulated metabolic activity, future work will need to address the role of IL-7 in the metabolism of DN T cells.

In conclusion, this study identified that IL-7, as well as the Akt/ mTOR signaling pathway, effectively modulates human DN T cell–mediated suppression of alloimmune T cell responses. Pharmacological targeting of the IL-7/Akt/mTOR axis may re-establish the regulatory function of DN T cells in the context of lymphopenic conditions or GVHD.

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Disclosures

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