Isolation and characterization of human antigen-specific TCRαβ⁺ CD4⁻CD8⁻ double negative regulatory T cells

Karin Fischer,¹ Simon Voelkl,¹ Jana Heymann,¹ Grzegorz K. Przybylski,²,³ Krishna Mondal,¹ Monika Laumer,¹ Leoni Kunz-Schughart,⁴ Christian A. Schmidt,² Reinhard Andreesen,¹ and Andreas Mackensen¹

¹Department of Hematology and Oncology, University of Regensburg, D-93042 Regensburg, Germany
²Department of Hematology and Oncology, University Medical Center, Ernst-Moritz-Arndt-University, D-17487 Greifswald, Germany
³Institute of Human Genetics, Polish Academy of Sciences, 60-479 Poznan, Poland
⁴Department of Pathology, University of Regensburg, D-93042 Regensburg, Germany

This work was supported by the DFG (MA 1351/5-1), Alfried Krupp von Bohlen und Halbach Stiftung (G.K.P and C.A.S.) and the Committee for Scientific Research, Poland (KBN 2P05A 05726; G.K.P.).

Corresponding author: Andreas Mackensen, M.D., Department of Hematology/Oncology, University of Regensburg, Franz-Josef-Strauss-Allee 11, D-93042 Regensburg, Germany
Tel. +49-941-9445580, Fax. +49-941-9445581,
E-mail: andreas.mackensen@klinik.uni-regensburg.de

Running title: Characterization of human DN regulatory T cells

Word counts: abstract (199), text (4997)

Scientific heading: Immunobiology
Abstract

Down-regulation of immune responses by regulatory T (Treg) cells is an important mechanism involved in the induction of tolerance to allo-antigens (Ag). Recently, a novel subset of Ag-specific T-cell receptor (TCR)αβ⁺ CD4⁺CD8⁻ (double negative, DN) Treg cells has been found to be able to prevent the rejection of skin and heart allografts by specifically inhibiting the function of anti-graft-specific CD8⁺ T cells. Here we demonstrate that peripheral DN Treg cells are present in humans, where they constitute about 1% of total CD3⁺ T cells, and consist of both naïve and Ag-experienced cells. Similar to murine DN Treg cells, human DN Treg cells are able to acquire peptide-HLA-A2 complexes from antigen presenting cells by cell contact-dependent mechanisms. Furthermore, such acquired peptide-HLA complexes appear to be functionally active, in that CD8⁺ T cells specific for the HLA-A2-restricted self peptide, Melan-A, became sensitive to apoptosis by neighboring DN T cells after acquisition of Melan-A-HLA-A2 complexes and revealed a reduced proliferative response. These results demonstrate for the first time that a sizeable population of peripheral DN Treg cells exists in humans that are able to suppress Ag-specific T cells. DN Treg cells may serve to limit clonal expansion of allo-Ag-specific T cells after transplantation.
Introduction

Compelling evidence indicate that regulatory T (Treg) cells play an important role in the maintenance of immune tolerance to self and foreign antigens (Ag). Various subsets of T lymphocytes have been isolated in mice and humans that have the ability to down-regulate the proliferation of autoimmune effector cells. CD4⁻CD25⁺ T cells are the most extensively studied Treg cells. Eliminating CD4⁺CD25⁺ T cells from the periphery of mice leads to the development of systemic autoimmune diseases, and adding them back can ameliorate experimentally induced autoimmune diseases and graft-versus-host disease after allogeneic bone marrow transplantation. Other Treg cells, including CD4⁺CD45Rblow, CD4⁺DX5⁺ T cells, CD8⁺ T cells, T-cell receptor (TCR)γδ⁺ cells, and TCRαβ⁺ CD3⁺CD4⁻CD8⁻ double negative (DN) T cells have also been demonstrated to have a potent role in down-regulating immune responses.

The majority of peripheral TCRαβ⁺ CD3⁺ T cells in normal mice express either CD4 or CD8 molecules. However, approximately 1-3% of peripheral CD3⁺ T cells express TCRαβ but neither CD4 nor CD8, and are thus DN T cells. Strober et al. were the first to describe a natural suppressor activity of DN T cells, that was found to be non-MHC restricted. In man and mouse DN T cells are detected in lymphoid and non-lymphoid tissues (reviewed in ). Clonal or oligoclonal expansion of DN T cells in humans have been reported in healthy individuals, and patients with either autoimmune diseases or combined immunodeficiency with features of autologous graft-versus-host disease.

Zhang and colleagues were the first to identify and characterize Ag-specific DN Treg cells. They initially demonstrated, in mice, that DN Treg cells have a unique phenotype which make the DN Treg cells different from any previously described T-cell. They further demonstrated that: (a) DN Treg cells, as a novel subset of Treg cells, can specifically down-regulate immune responses toward allo-Ag both in vitro and in vivo; (b) both primary
activated and cloned DN Treg cells can specifically kill activated CD4\(^+\) and CD8\(^+\) T cells with the same TCR specificity;\(^{12,19,20}\) infusion of \textit{in vitro} activated DN Treg cells leads to significant prolongation of donor-specific skin\(^{12}\) and heart graft survival.\(^{21}\) Others have shown that DN Treg cells also play an immune regulatory role in autoimmune and infectious diseases.\(^{13}\)

\textit{In vitro} studies have identified a unique mechanism by which DN Treg cells mediate an Ag-specific suppression of syngeneic responder cells. Studies showed that DN Treg cells can use their TCR to acquire allo-MHC-peptides from antigen presenting cells (APC), and utilize them to specifically trap and kill CD4\(^+\) or CD8\(^+\) T cells that recognize the same allo-MHC-peptides through a process that requires cell-cell contact, and Fas/FasL interaction.\(^{12}\)

Although it has been evident that the DN Treg cell population constitutes a unique lineage of professional Treg cells crucial in preventing graft rejection and graft versus host disease in mice,\(^{22}\) it was unclear whether DN T cells with similar functional properties are naturally present in humans. In the present study we show that this indeed is the case, and that the DN T cells in the peripheral blood of healthy adult volunteers are not extrathymically differentiated T cells but rather intrathymically matured Treg cells that exhibit functional properties similar to those discovered in mice.
Materials and methods

Culture medium, cytokines, and peptides

Cells were cultured in supplemented RPMI1640 medium (M' medium). The following recombinant human cytokines were used: 100 U/ml interleukin (IL)-2 (Proleukin™, Chiron, Muenchen, Germany), 800 U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (Leucomax™, Schering-Plough, Brussels, Belgium), 500 U/ml IL-4, 10 ng/ml IL-1β, 1,000 U/ml IL-6, 10 ng/ml tumor necrosis factor α (TNF-α) (all from PromoCell, Heidelberg, Germany), and 1 µg/ml prostaglandin E2 (PGE2) (Minprostin™; Pharmacia, Erlangen, Germany). Preparation of T-cell growth factor (TCGF) was described previously.23 The following HLA-A2-binding peptides were prepared by Clinalfa (Laufenlingen, Switzerland): modified Melan-A26-35 (ELAGIGILTV) and gp100280-288 (YLEPGPVTA). The identity of each peptide was confirmed by mass spectral analysis.

MHC-peptide multimers and mAbs

Phycoerythrin-conjugated (PE)-labeled HLA-A*0201 multimer that had been folded around Melan-A26-35 was synthesized by Beckman Coulter (Fullerton, CA). Monoclonal antibodies (mAbs) were from Becton Dickinson (BD) (San Jose, CA), except anti-CD95-fluorescein isothiocyanate (FITC), anti-CTLA-4-PE (PharMingen, San Diego, CA), anti-CD27-FITC, anti-CD28-FITC, anti-CD45RA-allophycocyanin (Caltag, Burlingame, CA), anti-CD57-PE, anti-TCRαβ-PC5 (Immunotech, Marseille, France), anti-perforin-PE, anti-granzyme B-FITC (Hoelzel Diagnostika, Cologne, Germany), anti-CCR7-FITC (R&D Systems, Abingdon, United Kingdom), anti-HLA class I (W6/32), and anti-HLA-A2 (clone BB7-2, American Type Culture Collection). FITC-labeled annexin-V was purchased from PharMingen. For TCR Vβ-repertoire analysis the IOTest® Beta Mark kit (Beckman Coulter) was used. Flow
cytometry was performed on a FACSCalibur® and data were analyzed with CellQuest® (BD).

**Complementarity-determining region (CDR)3 size analysis of TCR Vβ transcripts**

The CDR3 of the PCR-amplified TCR Vβ1-24 transcripts was analyzed using a run-off procedure.²⁴ The run-off products were run on an automated sequencer in the presence of fluorescent size markers. The length of DNA fragments and the fluorescence intensity of the bands were analyzed by GENESCAN672 software (Applied Biosystems, Foster City, CA).

**Blood samples and preparation of T cells from lymph node (LN) biopsies**

Heparinized blood samples were obtained from 20 healthy donors. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. Fresh LN from patients with non-malignant lymphadenopathy were obtained from the surgery department. The LN were mechanically dispersed into single-cell suspensions. PBMC and cells isolated from the LN were stained directly with anti-CD3, CD4, CD8, TCRαβ, and TCRγδ mAbs and analyzed by flow cytometry. The study was approved by the University of Regensburg Institutional Review Board. Informed consent was provided according to the Declaration of Helsinki.

**Isolation of human T-cell subpopulations, generation of DN T-cell clones and differentiation of DC**

T cells were isolated from leukapheresis products (obtained from healthy donors after informed consent was given) by Ficoll density gradient centrifugation. DN T cells were pre-enriched (up to 70%) from PBMC by depletion of CD4⁺, CD8⁺, CD14⁺, CD19⁺, and CD56⁺ cells with magnetic cell sorting (Dynabeads®, Dynal Biotech, Hamburg, Germany). Cells
were then stained with FITC-conjugated anti-CD4, anti-CD8, and Cy-Chrome™-conjugated anti-TCRαβ mAbs. The TCRαβ⁺ DN T cells were sorted by using a cell sorter (FACStar plus, BD). Isolation of CD4⁺ and CD8⁺ T cells was performed by flow cytometric sorting. T-cell populations were reanalyzed after cell sorting and showed >95% purity. DN T-cell clones were generated by limiting dilution (0.3 and 1 cell/well) in 96-well V-shaped plates in the presence of irradiated allogeneic EBV-transformed B cells (60 Gy, 1.5x10⁴ cells/well), PBMC (35 Gy, 7x10⁴ cells/well) and TCGF (2%).

DCs were generated from leukapheresis products as described previously.²⁵ Briefly, monocytes were enriched by counter-current elutriation and then cultured with M' medium plus 2% autologous serum, supplemented with GM-CSF and IL-4. On day 6, fresh medium containing GM-CSF, IL-4, TNF-α, IL-6, IL-1β and PGE₂ was added to the culture. The culture was continued for an additional 48 h, non-adherent cells were harvested and used for the different experiments.

**Proliferation assays**

To assess proliferation of different T-cell subpopulations, 10⁵ freshly purified DN, CD4⁺, and CD8⁺ T cells were co-cultured in 96-well U-bottom plates either with 10⁵ irradiated (30 Gy) allogeneic PBMC in the presence or absence of IL-2 (100 U/ml) or IL-2 alone. Cultures were incubated for 4 days, labeled with 1.0 µCi/well [³H]thymidine for the last 18 h, and harvested on filter plates (Perkin Elmer, Rodgau-Juegesheim, Germany) and [³H]thymidine incorporation was measured by liquid scintillation counting (Top Count®, Perkin Elmer).

**Acquisition of MHC molecules from APC by DN T cells**

Sorted DN and CD8⁺ T cells from HLA-A2⁺ donors were co-cultured with mature HLA-A2⁺ DC at a ratio of 1:1. At various time points cells were stained with an anti-HLA-A2 mAb
The expression of HLA-A2 on gated DN or CD8+ T cells was analyzed using a flow cytometer. Transwell experiments were performed in transwell chambers (Transwell®, 0.4 µm pore size, Costar-Corning, Acton, MA) on 24-well plates.

**Generation of Melan-A-specific cytotoxic T lymphocyte (CTL) lines from PBMC**

Melan-A-specific CTL lines were generated as described previously.²⁵ Briefly, CD8+ T lymphocytes were enriched from PBMC by MACS® sorting (Miltenyi, Bergisch Gladbach, Germany). DC were pulsed with the Melan-A peptide (30 µg/ml) and human β2-microglobulin (10 µg/ml). 2-5x10⁴ effector cells/well and 5x10³ peptide-pulsed autologous DC/well were co-cultured in 96-well U-bottom plates in M’ medium plus 10% human AB serum and 1-2% TCGF. After 4 cycles of stimulation phenotypic and functional analysis of T cells was performed.

**Assay for recognition of acquired peptide-MHC class I complexes by Ag-specific CTL**

Freshly purified DN T cells or DN T-cell clones from an HLA-A2- healthy donor (donor A) were co-cultured with HLA-A2+ mature DC (donor B) pulsed with Melan-A or gp100 (control) peptides overnight (DN:DC ratio 5:1). DN T cells were then separated from DC by cell sorting and used as effector cells against Melan-A-specific CTL generated from donor B either for induction of apoptosis or suppression of proliferation/cytotoxicity of Melan-A-specific CTL.

Recognition of acquired peptide-MHC complexes on DN T cells by Ag-specific CTL was analyzed either by the onset of apoptosis of the Melan-A-specific CTL, measured by combined Melan-A-multimer/annexin-V or annexin-V/propidium iodide (PI) (Calbiochem, Bad Soden, Germany) staining on CD8+ T cells or by detection of interferon-γ (IFN-γ)-secreting Melan-A-specific CTL (IFN-γ secretion assay, Miltenyi Biotec).²⁵
For proliferation experiments, 5x10^4 Melan-A-specific CTL were co-cultured in 96-well U-bottom plates either with 5x10^4 irradiated (30 Gy) Melan-A-primed DN T cells or Melan-A-pulsed DC. Cultures were incubated for 24 h and labeled with 1.0 µCi/well [3H]thymidine for the last 18 h. The cytotoxic activity of Melan-A-specific CTLs was measured by a conventional 4 h ^51Cr release assay as described.25

**Detection of secreted cytokines by DN T cells**

Supernatants of DN T cells stimulated for 12, 24, and 48 h either polyclonally with plate-bound anti-CD3 (100 ng/ml, OKT3, Orthoclone®, Ortho Biotech, Neuss, Germany) and anti-CD28 (100 ng/ml, CD28.2, BD) mAbs or with allogeneic mature DC were analyzed simultaneously for IL-2, IL-4, IL-5, IL-10, and IFN-γ secretion using the human Th1/Th2 cytokine cytometric bead array kit (BD Pharmingen).

**Analysis of T-cell receptor excision circles (TRECs) by quantitative real-time polymerase chain reaction (QRT-PCR)**

High molecular weight DNA was extracted from the encoded samples of sorted T cells using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The number of TRECs was determined by QRT-PCR using the ABI PRISM 7700 Sequence Detector TaqMan (PE Biosystems, Foster City, CA, USA) as described previously.26
Results

Frequencies of TCRαβ⁺ CD3⁺ CD4⁻/CD8⁻ DN T cells in the peripheral blood and LN of healthy adults

In order to validate if DN Treg cells that exhibit similar functional properties to those found in mice are naturally present in humans, we first examined whether mature DN T cells exist in humans. PBMC were collected from 20 healthy donors and stained with anti-CD3, CD4, CD8, TCRαβ, and TCRγδ mAbs. We observed a resident population of DN T cells that represented 4.1% (range: 1.9-7.7%) of CD3⁺ T cells. Further classification of the DN T-cell pool with regards to the TCRαβ or TCRγδ chain expression revealed a predominant population of TCRγδ⁺ T cells representing on average 3.2% (range: 2.1-3.6 %) as compared to 0.9% (range: 0.4-1.9 %) TCRαβ⁺ T cells within CD3⁺ T cells (see Figure 1A). In contrast, analysis of the DN T-cell pool of non-malignant LN (n = 5) demonstrated a predominance of TCRαβ⁺ T cells representing on average 2.2% as compared to 0.3% TCRγδ⁺ T cells within CD3⁺ T cells (Figure 1A).

As shown in Figure 1B/C, TCRαβ⁺ DN T cells showed a slightly lower CD3 expression level than TCRγδ⁺ DN T cells, which allowed the separation of CD3⁹⁹ TCRγδ⁺ and CD3intermediate TCRαβ⁺ DN T cells (see Figure 1B/C). Human γδ T cells have been shown to express a higher TCR/CD3 complex density than αβ T cells. Comparative analysis of the expression of the TCR/CD3 complex on TCRαβ⁺ DN T cells with TCRαβ⁺ CD4⁺/CD8⁺ T cells revealed a similar density (data not shown) suggesting that DN T cells are not composed of low TCR affinity T cells.

Together, TCRαβ⁺ DN T cells comprised 1-2% of total CD3⁺ T cells in the blood and LN of healthy donors, which is comparable to that seen in normal mice.
Phenotypic analysis of TCRαβ+ DN T cells

To further characterize the phenotype of the TCRαβ+ DN T-cell population we obtained PBMC from 10 healthy donors and analyzed the expression of CD16, CD25, CD27, CD28, CD45RA, CD56, CD57, CD69, CD95, CCR7, and CTLA-4 on the DN-cell population. As shown in Figure 2B TCRαβ+ DN T cells consisted of both CD45RA^bright/CCR7^ naïve cells and Ag-experienced cells with a CD45RA^low/CCR7^- phenotype. The majority of TCRαβ+ DN T cells were in the intermediate (CD27+/CD28-) maturation stage, highly expressed CD95, but were negative for activation markers such as CD25 and CD69 (Figure 2A and data not shown). We also analyzed the intracellular expression of perforin and granzyme-B, two effector molecules in cytolytic activity. The majority of the DN T cells expressed perforin but not granzyme-B (see Figure 2A). Furthermore, DN T cells were negative for NK-cell markers such as CD16 and CD56 (Figure 2A), and lacked CTLA-4 expression (data not shown).

Purification of DN T cells via magnetic beads and flow cytometric sorting and TCR Vβ repertoire of purified DN T cells

In order to purify TCRαβ+ DN T cells, we used a magnetic depletion system to eliminate CD4+, CD8+, CD14+, CD19+, and CD56+ cells, as well as flow cytometric sorting with anti-TCRαβ, anti-CD4, and anti-CD8 mAbs (Figure 3A) and were able to obtain >95% pure populations of DN T cells (see Figure 3A). To assess whether or not the DN T cells consist of polyclonal or clonally amplified T cells, analysis of TCR Vβ gene segment usage was performed on sorted DN and CD4+ T cells from 5 healthy donors. As shown in Figure 3B for 1 representative donor TCR Vβ usage in DN and CD4+ T cells was similar. Molecular analysis of CDR3 distribution of the TCRVβ families in sorted DN cells revealed a polyclonal pattern for most Vβ subfamilies (Figure 3C). However, the molecular pattern was
not Gaussian as was expected for CD4+ T cells, which might be relevant in the context of Ag-specificity of the DN T-cell subset.

**Cytokine profile and proliferation of DN T cells**

Next we analyzed the cytokine profiles of sorted DN and CD4+ T cells after stimulation with either allogeneic mature DC or with plate-bound anti-CD3/anti-CD28 mAbs. CD4+ T cells stimulated with allogeneic DC revealed the classical Th1 cytokine profile with high production of IFN-γ and IL-2 but no production of IL-4 or IL-10 (Figure 4A). In contrast, DN T cells secreted 3-4 times more IFN-γ but no IL-2, with some IL-5, and marginal levels of IL-4 and IL-10 (see Figure 4A). No IL-2 production in DN T cells could be detected at earlier time points (12 and 24 h) after allogeneic stimulation (data not shown). Similar results were obtained after stimulation with anti-CD3/CD28 mAbs (data not shown). This cytokine profile is similar to that seen in mouse DN Treg cells.

A low proliferative potential has been shown to be characteristic of Treg cells, such as CD4+CD25+ Treg cells.32-34 To analyze the proliferative capacity of human DN, CD4+ and CD8+ T cells, cells were sorted and stimulated in vitro. As shown in Figure 4B DN T cells exhibited a strong proliferative response upon stimulation with allogeneic PBMC, similar to CD4+ and CD8+ T cells. Proliferation of the T-cell subpopulations could be further enhanced by the addition of IL-2, while stimulation with IL-2 alone only had a marginal effect.

**Peripheral DN T cells have a long proliferative history**

In order to study whether DN T cells are derived from the same intrathymic maturation pathway as CD4+ and CD8+ T cells we performed QRT-PCR analysis of TREC numbers, as a marker for recent thymic emigrants, in purified DN T cells from 7 healthy adult donors. As controls, autologous purified CD4+ and CD8+ T cells were also analyzed for TREC numbers.
In 4 out of 7 donors the TREC numbers in DN T cells were 2-6 times lower than that in CD8+ and CD4+ T cells, while in the other 3 individuals the TREC numbers were comparable in all T-cell subpopulations (Figure 5). No differences in TREC numbers were observed between the CD4+ and CD8+ subsets (see Figure 5). As found in studies done by us and others,35,36 marked inter-individual variability indicates that the analysis of differences in TREC numbers among cell subsets is more informative when compared within the same individual as opposed to the analysis of the absolute TREC counts. The TREC counts decrease logarithmically with each cell division, therefore the difference in the number of cell divisions between two T-cell subsets can be calculated using the formula: \( N_1 - N_2 = \log_2(T_1 - T_2) \), where N stands for number of cell divisions and T for the TREC content. Comparison of proliferation rates of analyzed T-cell subsets revealed a 1-4 times higher number of cell divisions for DN T cells than for CD4+ and CD8+ cells in 4 out of 7 donors (data not shown). However, in 3 donors no significant difference (<1 division) was observed. These results indicate that DN T cells are not recent thymic emigrants, but rather an expanded T-cell subset.

**Transfer of peptide-MHC molecules from APC to DN T cells**

Since one of the important characteristics of DN Treg cells in mice is their ability to acquire allogeneic MHC molecules from APCs,12 we examined whether the same was true for human DN T cells. Sorted DN and CD8+ T cells from an HLA-A2- individual were incubated with HLA-A2+ DC. Acquisition and expression of HLA-A2 molecules on gated DN and CD8+ T cells was monitored by flow cytometry. A proportion of DN T cells started to express surface HLA-A2 as early as 2 h following incubation with HLA-A2+ DC (data not shown). The mean fluorescence intensity of HLA-A2 on DN T cells increased steadily between 24 and 48 h (Figure 6A/B). Contrary to DN T cells, only a small proportion of CD8+ T cells expressed
HLA-A2 after incubation with DC (Figure 6B). The acquisition of these molecules seems to be an accumulative process as it correlates with the duration of co-incubation with APC. Comparative analysis of different APC-subsets demonstrated that mature DC are much more potent peptide-MHC donors than immature DC and monocytes (data not shown).

In order to define the transfer of peptide-MHC molecules in more detail, we next asked if the transfer requires cell-cell contact. When HLA-A2− DN T cells were co-cultured with HLA-A2+ DC in a transwell system to prevent direct cell-cell contact but maintain diffusion of secreted soluble factors, no MHC transfer was observed (Figure 6A). These results demonstrate that the acquisition of MHC molecules by DN T cells requires cell contact.

The ability of DN T cells to present the acquired HLA-A2-Melan-A complexes to Melan-A-specific CTL could be demonstrated by the specific IFN-γ secretion of Melan-A-specific CTL upon co-culture of both T-cell subsets (data not shown).

**Peptide-MHC complexes that are acquired by DN T cells induce apoptosis and suppress proliferation of Ag-specific CTL**

Murine data have demonstrated that DN Treg cells can present acquired allo-Ag to activated CD8+ T cells and subsequently send death signals to CD8+ T cells. To determine whether the presentation of acquired peptide-MHC complexes by human DN T cells would have a similar effect, we tested the activity of DN Treg cells through their ability to induce apoptosis of Ag-specific CTL. Sorted DN Treg cells or DN T-cell clones from an HLA-A2− donor were co-cultured overnight with allogeneic mature HLA-A2+ DC that had been pulsed with the HLA-A2-binding peptides Melan-A or gp100 as a control, respectively. DN T cells were then separated from the co-cultures by cell sorting and used as effector cells against Melan-A-specific CTL. As shown in Figure 7A annexin-V staining revealed a marked increase in the fraction of Melan-A-specific CTL undergoing apoptosis when in the presence of Melan-A-
primed DN T cells. At a suppressor:target cell ratio of 1:1, 68% of total CD8\(^+\) T cells became annexin-V\(^+\) when co-cultured with Melan-A-primed DN T cells as compared to 29% annexin-V\(^+\) T cells co-cultured with gp100-primed DN T cells. Interestingly, we found a down-regulation of Melan-A-multimer staining upon specific interaction between DN T cells and Melan-A-specific CTL (see Figure 7A, left panels), which has been shown to provide an indirect evidence for the specificity of the peptide-MHC/TCR interaction.\(^{25,37}\)

Calculation of the percentage of apoptotic cells (annexin-V/PI staining) demonstrated that 63% of total CD8\(^+\) T cells underwent apoptosis at a suppressor:target ratio of 5:1 as compared to 12% of CD8\(^+\) T cells in the presence of gp100-specific DN T cells (see Figure 7B).

In a next step we asked whether DN T cells, that had acquired the Melan-A/HLA-A2 complexes from DC, were also able to inhibit the proliferation of Melan-A-specific CTL. Activated Melan-A-specific responder CTL, in contrast to irradiated (30Gy) DN T cells, showed a vigorous proliferative response, that could be further enhanced by restimulation with Melan-A-pulsed APCs (Figure 7C). Proliferation of Melan-A-specific CTL was inhibited up to 50% at a 1:1 ratio upon co-culture with Melan-A-primed DN T cells.

We next asked whether DN T cells can inhibit the cytotoxic activity of Melan-A-specific CTL against HLA-A2\(^+\) Melan-A-expressing target cells. Cytotoxicity of Melan-A-specific CTL against Melan-A-expressing target cells (E:T ratio 1:1) was not significantly inhibited upon co-culture with Melan-A-primed DN T cells (data not shown). However, quantification of the suppressive activity of Treg cells with the \(^{51}\)Cr release assay is critical because of it’s low sensitivity.

Taken together, our findings indicate that similar to DN Treg cells identified in mice, TCR\(\alpha\beta\)\(^+\) DN T cells also exist in humans, are able to acquire allo-Ag from APC, induce apoptosis and suppress proliferation of Ag-specific CTL.
Discussion

Regulatory T (Treg) cells, also referred to as suppressor T cells, have been implicated as important mediators of immune tolerance. It is now clear that Treg cells consist of many distinct T-cell subsets, including CD4+CD25+, Tr1, Th3, CD8+, TCRγδ+, and TCRαβ+ DN cells. Based on *in vitro* studies, several mechanisms have been implicated in Treg cell-mediated suppression. For instance, CD4+CD25+ Treg cell-mediated suppression appears to act by inhibiting IL-2 gene transcription and IL-2 production in the responder cells, which can be reversed by the addition of exogenous IL-2. Some Treg cells, including, Tr1, Th3, and γδ-TCR+ cells can suppress immune responses through the secretion of cytokines, such as TGF-β, IL-10, and IL-4, which may modify costimulatory molecule expression on T cells or APCs. Other Treg cells, such as NKT cells, and possibly subtypes of CD4+ Treg cells, have been shown to suppress immune responses through a non-specific cytotoxic mechanism. Competition with Ag-specific T cells for local IL-2 or surface area on APCs is yet another mechanism that may be involved in Treg cell-mediated suppression.

Treg cells have been shown to down-regulate immune responses in various disease models. DN Treg cells have been found to be able to prevent the rejection of skin and heart allografts by specifically inhibiting CD8+ T cells that carry anti-graft TCR specificity. Here we demonstrate that human DN T cells that previously had been considered to represent extrathymically differentiated T cells in fact appear to be the human counterpart of the DN Treg cells that have been studied in mice. We were able to isolate the DN Treg cells from PBMC of adult healthy volunteers in sizeable quantities (about 1% of total CD3+ T cells). Furthermore we show that TCRαβ+ DN T cells consist of both, naïve and Ag-experienced T cells. This is confirmed by quantitative TREC analysis, revealing lower (more cell divisions) or similar TREC counts in the DN Treg subset as compared to CD4+ and CD8+ T cells. Together, our data indicate that the DN Treg cells are not recent thymic emigrants and in 3
out of 7 healthy donors even have a longer proliferative history than either CD4$^+$ or CD8$^+$ T cells.

The cytokine profile of human DN Treg cells appears to be identical to the murine counterpart possessing a unique array that differ from Th1, Th2 or Th3/Tr1 cells. Others have described either a similar or a Th1 and/or Th2-like phenotype of DN T cells. In contrast to other Treg cells, DN Treg cells showed a marked proliferation in response to allogeneic MNC. Unlike other Treg cells, DN Treg cell-mediated suppression is Ag-specific.

Similar to murine DN T cells human DN Treg cells lack CTLA-4 expression, suggesting that they do not perform their suppressive function through this pathway but through another mechanisms that involve cell-cell contact. Zhang et al. have identified a unique mechanism by which DN Treg cells mediate Ag-specific suppression of syngeneic responder cells. They have shown that murine DN Treg cells can use their TCR to acquire allo-MHC-peptides from APCs, and then use them to specifically trap and kill CD4$^+$ or CD8$^+$ T cells that recognize the same allo-MHC-peptides through a process that requires cell contact, and Fas/FasL interaction. There have been several descriptions of the transfer of molecules, including MHC molecules, from APC to T cells. It has been demonstrated that CD4$^+$ T cells can directly acquire peptide-MHC class II complexes from APC. Furthermore, such acquired peptide-MHC complexes appeared to be functional, as T cells became hyporesponsive and apoptotic after interaction with neighboring T cells following the acquisition of peptide-MHC class II complexes. In addition, Huang et al. reported that CD8$^+$ T cells can also acquire peptide-MHC class I complexes through TCR-mediated endocytosis. During this process those CD8$^+$ T cells were sensitized to peptide-specific lysis by neighboring CD8$^+$ T cells, which then killed each other.

Like their murine counterparts human DN Treg cells are also able to acquire peptide-MHC class I complexes from APCs. The mechanism is unclear, however, our data clearly indicate
that it requires cell contact. Of interest, the acquisition of peptide-MHC molecules on DN Treg cells seems to be an accumulative process: CD8+ T cells maintained the expression of acquired MHC for only 2h,49 in contrast to DN Treg cells on which acquired allo-MHC could be detected for longer than 48h. The fact that human DN T cells use the acquired peptide-MHC complexes to induce apoptosis and suppress the proliferative activity of Ag-specific CTL recognizing the cognate peptide, but not controls, suggests the involvement of specific Ag recognition during suppression. This is in contrast to other Treg cells: although CD4+/CD25+ Treg cells require activation via the TCR to exert their regulatory function, once activated their suppressive activity is Ag non-specific.50 Moreover, it indicates that DN T-cell-mediated suppression is neither due to competition with CD8+ T cells for the surface area on APC nor for growth factors. If human DN Treg cells use a similar molecular pathway to control immune responses as their murine counterpart, has to be defined in further studies.

Studies on the in vivo role of human DN T cells in regulating immune responses are limited. It is well known that Treg cells also play an important role in preventing autoimmune diseases.4-6 Various studies have shown that autoimmune diseases develop because of a lack of, or a malfunction of Treg cells.12,7,9 Interestingly, several murine and human autoimmune disease phenotypes show an increase in the percentage of DN T cells within the T-cell population (summarized in15). For example, DN T cells are present at unusually high frequency and produce IL-4 and IFN-γ in patients with systemic lupus erythematosus.51 In autoimmune lymphoproliferative syndrome (ALPS) type Ia patients have an accumulation of TCRαβ+ DN T cells in the periphery as a result of inherited defects in apoptosis.52,53 Recently, somatic Fas mutations were detected in DN T cells from ALPS patients.54 The function and ontogeny of DN T cells in autoimmune diseases have remained elusive. However, our data indicate that DN T cells may be increased in some autoimmune diseases in an attempt to control autoreactive effector cells.
In summary, a new subset of human Treg cells that down-regulate Ag-specific immune responses in vitro was identified. The identification and characterization of human DN Treg cells will allow for their monitoring in various disease states and may have important implications for understanding and treating autoimmunity and graft rejection.
Acknowledgments

We thank Ms. Vogl, Rehm, and Bahr for excellent technical assistance, and Petra Hoffmann for critical reading the manuscript.
References


Legends to Figures

Figure 1. TCRαβ⁺ DN T cells in healthy individuals. Lymphocytes isolated either from PBMC or LN of healthy donors were stained with anti-CD3-APC, anti-CD4-PerCP, anti-CD8-PerCP, and anti-TCRαβ-PE or anti-TCRγδ-FITC mAbs and analyzed by flow cytometry. The cells were gated on DN T lymphocytes via their forward and side scatter properties and their CD3⁺, CD4⁻, and CD8⁻ expression profile. (A) Results represent mean percentages (± SD) of TCRαβ⁺ and TCRγδ⁺ DN T cells within total CD3⁺ T cells isolated either from peripheral blood or LN. (B,C) CD3⁺ CD4⁻/CD8⁻ DN T cells from peripheral blood lymphocytes can be separated in CD3high TCRγδ and CD3intermediate TCRαβ T cells. (B) The dot plots show staining with APC-conjugated anti-CD3 mAb and PerCP-conjugated anti-CD4/CD8 mAb. The region R1 indicates the population of CD3high DN T cells, the region R2 shows CD3intermediate T cells. (C) Dot plots show staining for anti-TCRαβ or anti-TCRγδ mAbs for cells of R1 (left plot) and R2 (right plot). Numbers in the upper left and lower right quadrants represent percentages of TCRαβ⁺ and TCRγδ⁺ cells within CD3⁺ DN T cells, respectively. One set of representative results of 20 healthy donors is shown.

Figure 2. Phenotypic analyses of freshly isolated peripheral TCRαβ⁺ DN T cells. PBMC from healthy donors were stained with anti-CD3-APC, anti-CD4-PerCP, anti-CD8-PerCP, and anti-TCRαβ mAbs and combinations of mAbs against surface Ag and intracellular proteins. (A) Cells were gated for CD3⁺ CD4⁻/CD8⁻ DN T cells. The right eight dot plots show staining with FITC/PE-conjugated anti-TCRαβ⁺ mAbs and FITC/PE-conjugated mAbs against CD27, CD28, CD25, CD95, CD16, CD56, and intracellular proteins (granzyme-B, perforin). (B) CCR7/CD45RA phenotype of TCRαβ⁺ DN T cells. Numbers in the quadrants
are the percentages of TCRαβ⁺ DN T cells with the corresponding phenotype. Representative data from 1 of 10 healthy donors are shown.

**Figure 3.** Purification of TCRαβ⁺ DN T cells via magnetic beads and flow cytometric sorting and TCR Vβ repertoire of purified DN T cells. (A) Purification of TCRαβ⁺ DN T cells using a Dynabeads®-based depletion system to eliminate CD4⁺, CD8⁺, CD14⁺, CD19⁺, and CD56⁺ cells, as well as flow cytometric sorting with anti-TCRαβ, anti-CD4, and anti-CD8 mAbs. (B) Comparative analysis of the TCR Vβ repertoire of purified DN and CD4⁺ T cells. (C) Total RNA from sorted DN T cells was extracted, reverse transcribed, and amplified by RT-PCR using Vβ primers. Amplified cDNA was copied with a fluorescent Cβ primer in a run-off reaction and subjected to electrophoresis on an automated sequencer. The patterns obtained show the size in amino acids of the CDR3 region (x-axis) and the relative fluorescence intensity (y-axis) of in-frame Vβ-Cβ amplification products.

**Figure 4.** Cytokine secretion and proliferation after allogeneic stimulation of highly purified peripheral T-cell subsets from healthy adults. (A) Cytokine profile of purified DN (black bars) and CD4⁺ (white bars) T cells stimulated with allogeneic mature DC for 48 h. A representative result (± SD) of three independent experiments is shown. (B) Sorted DN, CD4⁺, and CD8⁺ T cells were stimulated with irradiated allogeneic PBMCs in the presence or absence of IL-2 (100 U/ml) or IL-2 alone. [3H]TdT incorporation was measured after 4 d of culture. Bars represent the means (± SD) of triplicate cultures. Data represents one of 3 experiments with similar results using cells from 3 different donors.

**Figure 5.** Quantification of TREC content in highly purified peripheral T-cell subsets from healthy adults. Genomic DNA of sorted subpopulations from seven healthy adults was
isolated from freshly isolated DN (black bars), CD4+ (white bars), and CD8+ (grey bars) T cells and the number of TRECs was determined by quantitative real-time PCR. TREC counts per 1000 cells in individual samples. 1-7, no. of healthy donors.

**Figure 6.** Transfer of HLA-A2 molecules from HLA-A2+ DC to HLA-A2- DN and CD8+ T-cells. FACS-sorted DN and CD8+ T cells from an HLA-A2- donor were co-cultured with mature HLA-A2+ DC at a ratio of 1:1. At indicated time points cells were harvested and analyzed for the expression of HLA-A2 on FSC/SSC-gated lymphocytes. (A) Histograms show the kinetics (before, 24 h, and 48 h after co-culture) of HLA-A2 expression on DN T cells from 2 healthy donors (left panel, donor #1, right panel, donor #2) after co-culture or in a transwell system. (B) Percentage of HLA-A2+ DN and CD8+ T cells from HLA-A2- donors at different time points of coinubation with HLA-A2+ DC are shown.

**Figure 7.** Peptide-MHC complexes acquired by DN Treg cells induce apoptosis and suppress proliferation of Ag-specific CTL. DN T cells from an HLA-A2- donor (donor A) were co-cultured with HLA-A2+ mature DC (donor B) pulsed with either Melan-A or gp100 (control) peptides overnight. DN T cells were then separated from DC by cell sorting and used as effector cells against Melan-A-specific CTL generated from donor B. Induction of apoptosis of Melan-A-specific T cells was measured by combined Melan-A-multimer, annexin-V, and PI staining on CD8-gated T cells. (A) Dot plot of annexin V-FITC/Melan-A-multimer staining of CD3+/CD8+ gated Melan-A-specific CTL after 4 h coincubation with DN T cells stimulated either with Melan-A (left panel) or gp100 (right panel)-pulsed DC at different E/T ratios from 0-5:1. Numbers in the cross represent percentages of cells in the different quadrants within CD8+ T cells. One of three independent experiments is shown. (B) Percentage of different apoptotic target cell subpopulations calculated from annexin-V/PI
staining. (C) Melan-A-specific CTL (CTL<sub>resp</sub>) were cultured either with peptide-pulsed DC or Melan-A-primed or unprimed DN T cells at ratio of 1:1. [3H]TdR incorporation was measured after 24 h of culture. Panels show 1 of at least 3 different experiments, bars represent the means (± SD) of triplicate wells.
Figure 1

Fischer et al.
Figure 2

Fischer et al.
Figure 3

Fischer et al.
Figure 4

Fischer et al.
Figure 5

Fischer et al.
Figure 6

Fischer et al.
Figure 7

Fischer et al.
Isolation and characterization of human antigen-specific TCRαβ+ CD4−CD8− double negative regulatory T cells

Karin Fischer, Simon Voelkl, Jana Heymann, Grzegorz K Przybylski, Krishna Mondal, Monika Laumer, Leoni Kunz-Schughart, Christian A Schmidt, Reinhard Andreesen and Andreas Mackensen