Naïve regulatory T cells: a novel subpopulation defined by resistance towards CD95L-mediated cell death

Running Head: Naïve T_{reg} and apoptosis resistance

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Abbreviations: AICD (Activation induced cell death), FACS (Fluorescence activated cell sorting), lo (low), hi (high), intermediate (int), T_{reg} (regulatory T cells), T_{eff} (effector T cells), T_{conv} (conventional T cells), rT_{reg} (CD95L-mediated apoptosis resistant T_{reg})
Abstract

The majority of CD4+CD25hiFOXP3+ regulatory T cells (T_{reg}) from adult peripheral blood expresses high levels of CD45RO and CD95 and is prone to CD95L-mediated apoptosis in contrast to conventional T cells (T_{conv}). However, a T_{reg} subpopulation remained consistently apoptosis-resistant. Gene micro array and 6-color flow cytometry analysis including FOXP3 revealed an increase in naïve T cell markers on the CD95L-resistant T_{reg} compared to the majority of T_{reg}. In contrast to T_{reg} found in adult humans, most CD4+CD25+FOXP3+ T cells found in cord blood are naïve and exhibit low CD95 expression. Furthermore, the great majority of these newborn T_{reg} is not sensitive towards CD95L similar to naïve T_{reg} from adult individuals. After short-time stimulation with anti-CD3/28 mAbs cord blood T_{reg} strongly upregulated CD95 and were sensitized towards CD95L. This functional change was paralleled by a rapid upregulation of memory T cell markers on cord blood T_{reg} that are frequently found on adult memory T_{reg}. In summary, we show a clear functional difference between naïve and memory T_{reg} that could result in different survival rates of those two cell populations in vivo. This new observation could be crucial for the planning of therapeutic application of T_{reg}. 
Introduction

There is now clear evidence that CD4+ T cells contain a population of naturally immunosuppressive T cells characterized by constitutive expression of CD25, CTLA-4 and FOXP31. As not all potentially autoreactive T cells are deleted in the thymus2,3, peripheral control of T cell responses by naturally occurring CD4+CD25+FOXP3+ immunoregulatory T cells (T<sub>reg</sub>) is crucial to prevent autoimmunity1. Depletion of T<sub>reg</sub> contributes to the induction of severe autoimmune diseases in animal models and several studies have reported a defect of T<sub>reg</sub> in various human autoimmune diseases1,4,5.

Despite extensive research to unravel the immunosuppressive function of T<sub>reg</sub>, the exact molecular mechanism of immunosuppression is still elusive. Promising targets for pharmacological mimicking of T<sub>reg</sub> function remain undefined. Consequently, direct use of T<sub>reg</sub> for therapy is currently under examination. Therapeutic expansion or depletion of T<sub>reg</sub> with defined antigen-specificity offers new treatment options for human diseases6. Since accumulation of T<sub>reg</sub> has been shown to be detrimental in cancer7, new insights into mechanisms of T<sub>reg</sub> homeostasis are required.

In order to gain new insight into the modulation of T<sub>reg</sub> numbers as well as their antigen specificity, the development of natural T<sub>reg</sub> during ontogeny and in the adult needs to be explored. The peripheral T<sub>reg</sub> compartment consists mainly of thymus derived T<sub>reg</sub>1. However, under specific circumstances T<sub>reg</sub> can also be generated out of conventional T cells (T<sub>conv</sub>), e.g. if the antigen is targeted to immature DC8-10. It has yet to be established
how much “converted” T_{reg} contribute to the total number of T_{reg} in the periphery of adult mice and men.

At a given time, the overall number of T_{reg} is defined by a balance of generation and demise of T_{reg}. At the end of an immune reaction, T cells are depleted by apoptosis. Activation-induced cell death (AICD) through CD95/CD95L has been described as such an apoptosis-inducing mechanism\(^{11}\). While naïve T cells are CD95 negative and resistant to apoptosis induction, activated T cells (CD45RO\(^{\text{hi}}\)) upregulate CD95 and become sensitive to apoptosis. Upon TCR restimulation, activated effector T cells (T_{eff}) upregulate CD95L and induce AICD through crosslinking of CD95 by CD95L. AICD eliminates T_{eff} after an immune response and contributes to T cell homeostasis. We have previously shown that the majority of T_{reg} constitutively express CD95 and can easily be killed via crosslinking of this death receptor by CD95L\(^{12}\). Together with the fact that most T_{reg} express high levels of CD45RO, we hypothesized that most T_{reg} resemble memory/recently activated T cells rather than naïve T cells. Only very recently has the presence of naïve T_{reg} of CD4^{+}CD25^{+}CD45RA^{hi} phenotype in addition to memory T_{reg} been appreciated in peripheral blood\(^{13-15}\). However, as naïve T_{reg} express less CD25 (CD25\(^{+}\)) than memory T_{reg} (CD25\(^{\text{hi}}\)) and because those studies did not include FACS-analysis of FOXP3 expression, a clear separation between CD25\(^{+}\) T_{reg} and CD25\(^{+}\) T_{eff} was not possible. Therefore, analysis of CD4^{+}CD25^{+}CD45RA^{hi} T cells might have included contaminating T_{eff}. Moreover, no functional differences between naïve and memory T_{reg} were described as both cell types were reported to be immunosuppressive\(^{14,15}\). In contrast, memory T_{conv} cells are highly differentiated cells with distinct biological
properties from those of naïve T_{conv}. As analogies, naïve and antigen-primed/memory T_{reg} should exhibit different functional features.

Here we analyzed CD95 expression levels and apoptosis sensitivity of T_{reg} subpopulations. We showed that naïve T_{reg} are resistant to CD95L-mediated apoptosis in contrast to memory T_{reg}. First, gene microarray analysis of CD95L-resistant T_{reg} (rT_{reg}) revealed a naïve T cell signature of rT_{reg}. A 6-color FACS analysis was performed to detect FOXP3 and typical memory/naïve T cell markers on T_{reg} from adult peripheral blood. Naïve T_{reg} constitute only 0.2-0.8 % of total human CD4 cells, whereas in cord blood most T_{reg} belong to this naïve apoptosis-resistant subpopulation. Short term TCR stimulation \textit{in vitro} converted these naïve T_{reg} into CD95L-sensitive T_{reg} similar to adult memory (CD4^{+}CD25^{hi}) T_{reg}. \textit{In vivo}, recognition of self-antigens by T_{reg} might lead to the conversion of most T_{reg} into memory cells, and previous studies focused on CD25^{hi} memory T_{reg} to avoid T_{eff} cell contamination of the T_{reg} pool. However, under pathogenic conditions like human autoimmunity we expect alterations of adult memory T_{reg} frequencies, e.g. due to enhanced CD95L-mediated apoptosis as reported for (CD4^{+}CD25^{hi}) T_{reg} derived from SLE patients \textsuperscript{16}. In such situations the analysis of the so far neglected naïve T_{reg} pool might be of great importance.
Material and Methods

Human samples

Peripheral blood was obtained from healthy adult donors. Cord blood samples were obtained from the placentas of normal full-term newborns delivered at the Department of Gynaecology and Obstetrics at the University of Heidelberg. All samples were obtained after informed consent and approval by the University of Heidelberg ethics committee according to the Declaration of Helsinki.

Abs and reagents

The monoclonal Abs (mAbs) against human CD4, CD45RO, CD45RA, CD31 were obtained from BD Pharmingen (Heidelberg, Germany), αCD25 Ab from Miltenyi Biotech (Auburn, CA). CD95L was produced as a Leuzin zipper-tagged ligand of CD95, which binds both murine and human CD95 17. The monoclonal αCD3 Ab (OKT3), αCD28 mAb and the agonistic monoclonal αCD95 Ab (anti-Apo-1) were purified from hybridoma supernatants by Protein A affinity purification as described 17. The monoclonal αFoxP3 mAb (clone PHC101) was obtained from eBioscience (San Diego, CA, USA). Annexin V Alexa Fluor 488 from Molecular Probes (Eugene, OR), propidium iodide (PI) and protein A from Sigma-Aldrich (St. Louis, MO).

Lymphocyte separation

Human PBL were purified from peripheral blood by Biocoll™ (Biochrom, Berlin, Germany) gradient centrifugation followed by plastic adherence to deplete monocytes. Adult peripheral CD4⁺CD25⁺ cells were first enriched using MACS beads (Miltenyi
Biotech) and subsequently CD4^{+}CD25^{high} cells were sorted with a FACS-Diva™ cell sorter (BD Biosciences). CD4^{+}CD25^{hi} T_{reg} cells contained only the 1-2% of human CD4 cells with the highest CD25 expression, as previously reported 18. Similarly, CD4^{+}CD25^{+} or CD4^{+}CD25^{+}CD45RO^{lo/hi} T cells were FACS-sorted from cord blood derived lymphocytes. FACS sorted CD4^{+}CD25^{−} cells were used as T_{conv}.

**Dead cell removal**

Dead cells were depleted using the MACS™ Annexin V MicroBead kit (Miltenyi Biotech (Auburn, CA)). Briefly, cells were incubated with Annexin V microbeads and passed over a MS column to magnetically remove Annexin V positive cells. Purity of viable cells was determined by FSC/SSC analysis and Propidium Iodide staining.

**Cell culture and cytotoxicity assays**

Freshly isolated human T cells were cultured in IL-2 (100 IU) containing ex Vivo-15™ medium (Cambrex, Verviers, Belgium) supplemented with 1% glutamine (Invitrogen, Karlsruhe, Germany). For apoptosis induction T cells were stimulated with αCD95 Ab and 10 ng/ml Protein A or different concentrations of CD95L (maximal 1:20 dilution) as previously described 12. Unstimulated cells were incubated with an isotype control Ab or CD95L-free control medium. To expand T_{reg}, cells were incubated with 0.1 μg/ml αCD3 Ab and 1 μg/ml αCD28 Ab in combination with irradiated JY feeder cells (kind gift from C. Falk, Munich) and 300 IU IL-2 12. Cell death was assessed by AnnexinV/Propidium Iodide co-staining and forward-to-sideward-scatter profiles. Specific cell death was calculated as described previously 12.
Cell proliferation assay

When expanded T_{reg} were used, they were washed extensively to remove IL-2, but no further resting of T_{reg} occurred prior to cell proliferation assays. T_{conv} (1 x 10^4) were incubated in 96-well plates (Nune, Wiesbaden, Germany) alone or in co-culture with T_{reg} (1 x 10^4 T_{reg}) together with irradiated T cell depleted PBMC (1x10^5) and were stimulated with soluble αCD3 mAb (0,2 µg/ml) and αCD28 mAb (0,005 µg/ml). APC and T_{conv} were always derived from the same blood sample. After four days at 37°C in 5% CO2, 1 µCi [^3]H thymidine per well was added for additional 16h and proliferation was measured in cpm with a scintillation counter. Inhibitory capacity (%) of T_{reg} in coculture experiments was defined as (1 - [^3]H thymidine uptake [cpm] of coculture of T_{reg} with T_{conv} divided by cpm of T_{conv} alone) x 100%. Triplicate wells were used in all suppression experiments.

FACS staining

FOXP3 staining was performed according to the manufacturer’s protocol. For 6-color FACS, cells were first stained with surface mAb of interest followed by FOXP3 intracellular staining. FACS-acquisition was performed immediately with a FACS Canto™ cytometer and analyzed with FACS-Diva software (BD Biosciences).

RNA preparation and quantitative RT-PCR

Total RNA was isolated using the Absolutely RNA Microprep kit (Stratagene, Heidelberg, Germany) and cDNA was prepared using random oligo(dT) primers (Invitrogen). The FOXP3 message was quantified by detection of incorporated SYBR®
Green using the ABI Prism 5700 sequence detector system (Applied Biosystems, Foster City, CA). The relative expression level was determined by normalization to GAPDH and results are presented as fold expression of T_{conv} mRNA levels. FOXP3 primer sequences were as follows: 5`- AGC TGG AGT TCC GCA AGA AAC (forward) and 5`- TGT TCG TCC ATC CTC CTT TCC (reverse). GAPDH primer sequences were as follows: 5`- GCA AAT TCC ATG GCA CCG T (forward) and 5`- TCG CCC CAC TTG ATT TTG G (reverse).

**DNA microarray hybridization and analysis**

Quality and integrity of 500 ng total RNA were controlled by running all samples on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). For RNA-amplification the first round was done according to Affymetrix without biotinylated nucleotides using the Promega P1300 RiboMax Kit (Promega, Mannheim, Germany) for T7 amplification. For the second round of amplification the precipitated and cleaned aRNA was converted to cDNA using random hexamers (Pharmacia, Freiburg, Germany). Second-strand synthesis and probe amplification was performed as in the first round, with two exceptions: there was an incubation with RNAse H before first strand synthesis to digest the aRNA, and T7T23V oligo was used for initiation of the synthesis of the second strand. The concentration of biotin-labeled cRNA was determined by UV absorbance. In all cases, 12.5 µg of each biotinylated cRNA preparation were fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix HG-U133A 2.0
for 16 hours. After hybridisation the GeneChips were washed, stained with SA-PE and read using an Affymetrix GeneChip fluidic station and scanner.

**Microarray data analysis**

Analysis of microarray data was performed using the Affymetrix GCOS 1.2 software. For global normalization all array experiments were scaled to a target intensity of 150, otherwise using the default values of the GCOS 1.2 software. Filtering of the results was done as follows: genes were considered as regulated when their fold change was greater than or equal to 1.2 or less than or equal to –1.2 and the statistical parameter for a significant change (change call) was not NC (no change). The entire MIAME-compliant data set of the microarray experiments will be posted at the GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/).
Results

**CD4^+CD25^{hi} T_{reg} contain a subpopulation of CD95L-mediated cell death resistant T_{reg}**

We have previously shown that most CD4^+CD25^{hi} regulatory T cells (T_{reg}) are highly susceptible to CD95L-mediated cell death in contrast to conventional CD4^+25^{lo} T cells (T_{conv}) \(^{12}\). Interestingly, a subpopulation of T_{reg} remained consistently apoptosis-resistant to the treatment with CD95L. This apoptosis resistant population (rT_{reg}) represents a minor subpopulation (10%-30%) within the sorted CD4^+CD25^{hi} T_{reg} \(^{12}\). To further analyze this population, FACS-sorted CD4^+CD25^{hi} T_{reg} from multiple donors were pooled and incubated with CD95L. Dead T_{reg} were removed by Annexin V microbeads and the cell death-resistant rT_{reg} were recovered (for experimental setup see Figure 1A). During a second challenge with CD95L (Figure 1B) or αCD95 mAb (data not shown) these rT_{reg} remained apoptosis resistant, confirming that they display a stable phenotype. As a control, CD4^+CD25^{hi} T_{reg} were left without CD95L, incubated with Annexin V microbeads, passed over MS columns, washed and were subsequently incubated with CD95L. As expected, specific cell death for these total CD4^+CD25^{hi} T_{reg} remained high (Figure 1B).

**CD45RO^{lo} T_{reg} are resistant to CD95L-mediated cell death**

To further characterize rT_{reg} we pooled rT_{reg}-cDNA derived from FACS-sorted CD4^+CD25^{hi} T_{reg} of eight healthy blood donors and performed gene chip microarray analysis. In this screen we compared cDNA from rT_{reg} with cDNA derived from all CD4^+CD25^{hi} T_{reg}. Interestingly, rT_{reg} showed a significant decrease of mRNA expression
of genes related to T cell memory like CTLA4, various HLA genes and CD103, whereas mRNA for molecules expressed mainly on naïve cells such as CCR7 or CD127 (IL7R) was more prevalent in rTreg than in total CD4+CD25hi Treg (see supplemental table 1 and 2). This finding suggested that rTreg are more naïve in contrast to the majority of Treg found in the CD4+CD25hi T cell population which are known to exhibit an antigen-primed/memory-like phenotype. To test this hypothesis, we incubated FACS-sorted CD4+CD25hi Treg with increasing concentrations of CD95L. The remaining viable rTreg were then analyzed by 4-color FACS for the expression of the typical memory marker CD45RO19 (Figure 1C-E) and the naïve marker CD3120,21 (data not shown). In contrast to Treg cultured in control medium (Figure 1C), rTreg surviving CD95L-stimulation were mostly CD45ROlo (>85%) (Figure 1D). The number of CD45ROhi cells progressively declined with increasing concentrations of CD95L (Figure 1E). The naïve cell marker CD31 increased correspondingly on rTreg (data not shown). We conclude that CD95L preferentially kills CD45ROhi Treg cells, leaving behind rTreg with a naïve phenotype and slightly lower CD25 expression levels. Furthermore, FACS analysis for intracellular FOXP3 expression demonstrated that all rTreg were FOXP3+ and the anergy and suppressive capacity of in vitro expanded rTreg (data not shown) further supported their classification as Treg.

Human adult CD4+CD25hiFOXP3+ Treg consist of memory and naïve Treg

Consequently human Treg should comprise a heterogeneous population with memory and naïve Treg. To prove that naïve CD4+CD25hi T cells are indeed Treg, we performed FOXP3 FACS-analysis in PBL. In the absence of further highly specific Treg markers,
FOXP3 is considered to be the most reliable marker to identify T\textsubscript{reg} in human peripheral blood to date\textsuperscript{22}. Therefore, we established 6-color FACS analysis including mAbs against CD4, CD25 and FOXP3 for T\textsubscript{reg} detection together with mAbs against CD45RO and CD45RA to distinguish memory and naïve T\textsubscript{reg}. Naïve T\textsubscript{reg} were initially considered as CD4\textsuperscript{+}FOXP3\textsuperscript{+}CD45RO\textsubscript{lo} cells (R2 in Figure 2A, right panel) and memory T\textsubscript{reg} were characterized as CD4\textsuperscript{+}FOXP3\textsuperscript{+}CD45RO\textsuperscript{hi} cells (R3 in Figure 2A, right panel). Using multicolor analysis one can ascertain that memory T\textsubscript{reg} (blue) are mainly CD25\textsuperscript{hi} while naïve T\textsubscript{reg} (red) are mainly CD25\textsuperscript{int} and overlap with CD25\textsuperscript{int} Teff cells. Accordingly, pure FACS-based isolation of T\textsubscript{reg} is limited to the CD25\textsuperscript{hi} memory T\textsubscript{reg}-enriched cell pool, as surface expression of CD25 is quite similar between naïve T\textsubscript{reg} and FOXP3-negative CD4\textsuperscript{+}CD25\textsuperscript{+} Teff (Figure 2B). Naïve T\textsubscript{reg} showed a higher expression of CD45RA than memory T\textsubscript{reg} (Figure 2C). Analysis of the naïve cell marker CD31 also showed increased expression (data not shown). Comparison of CD95L-resistant cells in Figure 1D with the CD45RO\textsubscript{lo} naïve T\textsubscript{reg} (red) from the 6-color staining (Figure 2 B and C), revealed a similar phenotype, supporting that apoptosis-resistant T\textsubscript{reg} represent naïve T\textsubscript{reg} in adult peripheral blood. Since sensitization towards CD95L-mediated cell death includes upregulation of CD95 we compared CD95-expression of naïve and memory T\textsubscript{reg} by 6-color FACS for expression of CD4, CD25, FOXP3, CD45RO, CD45RA and CD95 (Figure 2D). Indeed, naïve CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+}CD45RO\textsubscript{lo} T\textsubscript{reg} (red) expressed very low levels of CD95 in comparison to the highly CD95-positive memory T\textsubscript{reg} (CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+}CD45RO\textsuperscript{hi}, blue, Figure 2D). Collectively, we define naïve T\textsubscript{reg} as CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+}CD45RO\textsubscript{lo}CD95\textsuperscript{lo} T cells and memory T\textsubscript{reg} as CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+}CD45RO\textsuperscript{hi}CD95\textsuperscript{hi} T cells. Furthermore we conclude, that rT\textsubscript{reg}
represent naïve T_{reg} in adult peripheral blood. The frequency of naïve T_{reg} varied between 10-50% of total T_{reg} in adult peripheral blood and clearly declined with increasing age (Figure 2E).

**Cord blood CD4^{+}CD25^{+} T cells are FOXP3^{+} and express low levels of CD95**

Whereas the frequency of CD45RO^{lo} T_{reg} in adult peripheral blood is rather low, newborns should exhibit high numbers of naïve T_{reg}. Therefore, we extended our functional analysis on cord blood derived T_{reg}. Nearly all CD4^{+}CD25^{+} human cord blood T cells expressed FOXP3 as determined by FACS-analysis (Figure 4A) and quantitative RT-PCR (data not shown). The percentage of FOXP3^{+} T cells within the CD4^{+} compartment was only slightly higher in cord blood (5.9\%\pm3.1\%, n=6) than in adult peripheral blood (4.8\%\pm1.7\%, n=6). T_{reg} were sorted from adult and cord blood according to CD25 expression as depicted in Figure 3B, and showed significant suppressive capacity *in vitro* (Figure 3C). Thus, all CD4^{+}CD25^{+} T cells in human cord blood constituted CD4^{+}CD25^{+}FOXP3^{+} T_{reg} similar to murine T_{reg}. In contrast, only a minor fraction of CD4^{+}CD25^{+} T cells in adult peripheral blood contained CD4^{+}CD25^{+}FOXP3^{+} T_{reg} (Figure 3A). Further, FACS-analysis revealed that most cord blood T_{reg} showed low expression of CD45RO (Figure 4B), but high expression of CD45RA and CD31 (data not shown) confirming their naïve status. Interestingly, most cord blood T_{reg} showed a low expression of CD95 (Figure 3D) similar to adult naïve T_{reg}. High expression of CD95 was limited to the minor subpopulation of CD45RO^{hi} T_{reg} in cord blood (Figure 3E). To summarize, both naïve T_{reg} from cord blood as well as naïve T_{reg} from adult peripheral blood show a lower expression of CD95 than memory T_{reg}. In
addition, both exhibit a similar level of CD25 expression (compare Figure 2B and Figure 3B), further indicating that cord blood Treg and adult naïve Treg represent a similar cell population.

**Freshly isolated cord blood Treg are resistant to CD95L-mediated apoptosis**

Next, we tested if cord blood-derived Treg were resistant to CD95L-mediated apoptosis similar to adult naïve Treg. In contrast to freshly isolated adult CD4^+^CD25^hi^ Treg, cord blood Treg were nearly resistant towards CD95L-mediated cell death (Figure 4A). This correlates with the CD95^hi^ phenotype of memory Treg in the adult CD4^+^CD25^hi^ Treg fraction (Figure 2D) and the rather low presence of such memory Treg among freshly isolated cord blood Treg (Figure 4B, arrow). Interestingly, the few CD45RO^hi^ Treg present in cord blood showed not only increased CD95 expression, but also slightly enhanced sensitivity to CD95L-mediated apoptosis (Figure 4A, right side).

**TCR-stimulated cord blood Treg are sensitive to CD95L-mediated apoptosis and acquire a memory phenotype similar to adult Treg**

During an immune response naïve Tconv are stimulated via their TCR. They clonally expand and differentiate into Teff. One hallmark of peripheral T cell depletion is that Teff upregulate CD95 and become sensitive to CD95L-mediated cell death to allow depletion of the expanded T cell pool by CD95L. We have shown that TCR-stimulation of Tconv from adult peripheral blood with anti-CD3 mAb and IL-2 can be used to mimic this critical process *in vitro*. Accordingly, we questioned if naïve Treg could also upregulate CD95 and be sensitized to CD95L-mediated apoptosis in response to TCR-stimulation.
For this purpose, we stimulated freshly isolated cord blood T\textsubscript{reg} with anti-CD3/28 mAbs and IL-2 and analyzed their phenotypes at various time points (Figure 5). Soon after TCR-stimulation CD95 was upregulated on cord blood T\textsubscript{reg} and reached a CD95 expression plateau after 3-4 days (Figure 5A and B). This was notable because adult human T cells take 5-6 days to reach a plateau of CD95 expression (data not shown). However, murine T cells were also sensitized towards CD95L faster than adult T cells after TCR-stimulation \textsuperscript{23}, which suggests that naïve human cord blood T cells share similarities with murine T cells. Simultaneous FACS analysis of memory cell markers during the \textit{in vitro} stimulation of cord blood T\textsubscript{reg} revealed that CD45RO increased more gradually than CD95 (Figure 5 B) and that CD45RA and CD31 were gradually lost until day 6 (data not shown). Thus, this \textit{in vitro} induced phenotype of cord blood T\textsubscript{reg} (CD95\textsuperscript{hi}CD45RO\textsuperscript{hi}CD45RA\textsuperscript{lo}) was identical with the phenotype of most adult CD4\textsuperscript{+}CD25\textsuperscript{hi} T\textsubscript{reg} \textit{in vivo} (Figure 3).

Most importantly, prestimulated cord blood T\textsubscript{reg} were sensitive to CD95L treatment in contrast to freshly isolated cord blood T\textsubscript{reg} (Figure 5C). To rule out outgrowth of a few pre-existing cord blood CD45RO\textsuperscript{hi}CD95\textsuperscript{hi} T\textsubscript{reg}, we aimed to exclude CD45RO\textsuperscript{hi}CD95\textsuperscript{hi} T\textsubscript{reg} by FACS-sorting. However, FACS-sorting with anti-CD95 mAb could induce isolation-based apoptosis. Therefore we defined an expression level of CD45RO which allowed separation of CD95\textsuperscript{hi} from CD95\textsuperscript{lo} cells without costaining for CD95 (dotted line in Figure 4B). This was performed using an aliquot of the FACS-sorting cells stained for CD45RO and CD95. After defining the CD45RO “cut off”, the sorting cells were separated into CD45RO\textsuperscript{hi} (correspondingly CD95\textsuperscript{hi}) cells (Figure 4B, R1) and CD45RO\textsuperscript{lo} (correspondingly CD95\textsuperscript{lo}) cells (Figure 4B, R2). When we stimulated the
CD45RO^{lo}CD95^{lo} cord blood T_{reg} for 6 days, they still upregulated CD95 and became sensitive towards CD95L-mediated apoptosis (Figure 5C). Therefore outgrowth of CD45RO^{hi}CD95^{hi} T_{reg} could be ruled out.

Likewise, isolated rT_{reg} from adult blood upregulated CD95 in response to TCR-stimulation (anti-CD3/28 mAbs plus IL-2) and became sensitive to CD95L-mediated cell death (Figure 5E and F). Finally, freshly isolated adult CD4^{+}CD25^{hi} T_{reg}, naïve cord blood T_{reg} and prestimulated cord blood T_{reg} showed similar immunosuppressive capacity \textit{in vitro} (Figure 5D and Figure 3C). In contrast, upregulation of CD95 and sensitization towards CD95L-mediated cell death clearly distinguished memory T_{reg} from naïve T_{reg} on a functional basis.
Discussion

In our efforts to characterize CD95L-resistant T_{reg} (r T_{reg}) within the population of adult CD4^{+}CD25^{hi} T_{reg}, we found an increased expression of molecules defining naïve T cells and a decreased expression of memory-cell associated molecules by microarray analysis. Further FACS-analysis confirmed this finding and revealed that naïve T_{reg} from adult and cord blood express low levels of CD95 and are resistant to CD95L-mediated cell death. This is in contrast to in vitro prestimulated naïve T_{reg} or in vivo activated CD4^{+}CD25^{hi}CD45RO^{hi} T_{reg} which express high levels of CD95 and are highly sensitive to death receptor ligation. The number of T_{reg} in the organism is tightly regulated and we propose that elimination of T_{reg} after an immune response contributes to the low frequency of T_{reg} in the adult CD4^{+} fraction.

CD95L-mediated apoptosis of T cells has mainly been studied in naïve T_{conv} that were activated in vitro. Analysis of apoptosis sensitivity of in vivo primed/memory T_{eff} has been hampered by their low frequencies in the healthy human individual. By contrast, continuous in vivo stimulation of naïve T_{reg} with self-antigens seems to keep the proportion of CD95^{hi}T_{reg} relatively high. In fact, incubation of naïve T_{reg} with autologous APC and IL-2 has been shown to stimulate naïve T_{reg} in contrast to unreactive naïve T_{conv}^{14}, and Romagnoli and coworkers have shown a high degree of self-reactivity within the T_{reg} population^{25}. Thus, a constant activation of T_{reg} in vivo could conceivably lead to a very high proportion of memory T_{reg} in adults. This high frequency of memory T_{reg} allowed us to demonstrate the high apoptosis sensitivity towards CD95L of these antigen-experienced T_{reg} with a memory-like phenotype directly ex vivo.
Whereas naïve cord blood T\textsubscript{reg} are CD95\textsubscript{lo} similar to naïve T\textsubscript{conv}, TCR-stimulation \textit{in vitro} revealed a rapid upregulation of CD95. Simultaneously, CD45RO was upregulated, whereas expression of the naïve marker molecule CD31 declined in slower kinetics (data not shown). Six days after TCR-stimulation \textit{in vitro} most cord blood-derived naïve T\textsubscript{reg} had acquired the phenotype (CD95\textsuperscript{hi}CD45RO\textsuperscript{hi}CD45RA\textsuperscript{lo}) known from adult CD4\textsuperscript{+}CD25\textsuperscript{hi} T\textsubscript{reg} in peripheral blood and lymphoid tissue \textit{in vivo}\textsuperscript{15}. In summary, naïve T\textsubscript{reg} show the same phenotype in cord blood and adult peripheral blood, but are much more frequent in cord blood. By contrast, memory T\textsubscript{reg} predominate in adult peripheral blood and are rare in cord blood. However, short-time TCR-stimulation \textit{in vitro} transformed cord blood-derived naïve T\textsubscript{reg} into T\textsubscript{reg} of a memory-like phenotype presented by most adult CD4\textsuperscript{+}CD25\textsuperscript{hi} T\textsubscript{reg}.

This \textit{in vitro} induction of memory T\textsubscript{reg} might mimic the interaction of naïve T\textsubscript{reg} with self-antigens \textit{in vivo}, which generates a pool of self-antigen-primed/memory-like T\textsubscript{reg} very early in fetal ontogeny. T\textsubscript{reg} can be detected as early as 14 weeks of human gestation in the periphery\textsuperscript{26} and T\textsubscript{reg} in fetal lymph nodes (<17 weeks old) already show a higher expression of CD95 and CD45RO in comparison to T\textsubscript{conv}\textsuperscript{27}. Since the healthy fetus is usually not challenged with foreign antigens, activation of T\textsubscript{reg} might reflect the early interaction of T\textsubscript{reg} with self-antigens in the pathogen-free organism. Interestingly, T\textsubscript{reg} in the fetal and adult thymus express lower levels of CD25 and are of naïve phenotype, while T\textsubscript{reg} in the fetal/adult lymphoid organs as well as in the adult peripheral blood express higher levels of CD25 (CD25\textsuperscript{hi}), and are of memory phenotype (e.g. CD95\textsuperscript{hi}, CD45RO)\textsuperscript{15,27}. Thus, after their emigration from the thymus, T\textsubscript{reg} recognize self-
antigen in the periphery and convert to memory T<sub>reg</sub>. During adult life ongoing self-antigen specific activation of naïve T<sub>reg</sub> might trigger the immunosuppressive function of T<sub>reg</sub> and thereby continuously supply the pool of memory CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>reg</sub>. Upregulation of CD95 and high sensitivity towards CD95L-mediated cell death would then allow for constant elimination of antigen-experienced T<sub>reg</sub>. Both, the high turnover of mouse self-specific T<sub>reg</sub><sup>28-30</sup> and the larger proportion of activated T<sub>reg</sub> than T<sub>conv</sub> in the human periphery<sup>26</sup> strongly support this model.

Previously, only CD4<sup>+</sup>CD25<sup>hi</sup> T cells have been accepted to represent human T<sub>reg</sub> and many studies have focused on this population since pure isolation of FOXP<sup>+</sup> T<sub>reg</sub> is limited to this population in humans. With the availability of specific FOXP3 mAb for flow cytometry, we now detected naïve T<sub>reg</sub> with lower CD25 expression in adult peripheral blood. This confirmed recent reports that suggested the presence of naïve T<sub>reg</sub> within the CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>hi</sup> T cell population of adult peripheral blood<sup>14,15</sup>. However, our multicolor FOXP3-FACS analysis also revealed that CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>hi</sup> T cells consist of both FOXP3<sup>+</sup> and FOXP3<sup>-</sup> T cells (data not shown). Therefore, FACS-sorting for CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>hi</sup> T cells from adult peripheral blood yields a significant contamination with FOXP3-negative T<sub>conv</sub> cells. In fact, these contaminating T<sub>conv</sub> might contribute to the reported relatively high proliferative potential of adult naïve T<sub>reg</sub> in contrast to the profound anergy of CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>reg</sub><sup>14</sup>. 
It has long been appreciated that cord blood contains a distinct population of CD25+CD45RA^{hi} regulatory T cell with suppressive function. Although PCR analysis of these CD25^+ CD45RA^{hi} cells has revealed FOXP3 expression^{31-33}, it remained unclear if all cord blood CD25^+ cells express FOXP3 and are T_{reg}. Our flow cytometric analysis clearly established that essentially all cord blood CD25^+ cells coexpress FOXP3. Thus, FOXP3^+ cells can easily be isolated from cord blood on the basis of their CD25 expression. Cord blood derived T_{reg} are immunosuppressive and show proliferative anergy like adult CD4^+CD25^{hi} T_{reg}^{15,31,33,34}. Most importantly, expression of FOXP3 seems to be more specific for naïve T_{reg} than for recently activated T_{reg}. Activation-induced upregulation of FOXP3 mRNA has been reported for \textit{in vitro} stimulated T_{eff} and triggered a controversial discussion on FOXP3-specificity for human T_{reg}^{35-38}. Interestingly, we also observe a slightly higher FOXP3 expression in memory T_{reg} than in naïve T_{reg}, which might reflect the recent activation of memory T_{reg} by self-antigen \textit{in vivo}. However, naïve FOXP3^+ CD4^+ T cells should unequivocally represent natural T_{reg} since T_{eff} with activation-induced FOXP3 expression (e.g. peripheral generated T_{reg}) do not exhibit a naïve phenotype. Furthermore, strict gating for CD4^+CD25^{hi} T cells had led to false low T_{reg} numbers (1-2%) in human adult peripheral blood in many previous studies. With the detection of naïve T_{reg}, total T_{reg} frequencies in mice (5-10%) and man (3-7%) are actually comparable.

Modulation of the naïve T_{reg} pool could be an interesting target for therapeutical intervention. Sereti et al. recently showed reconstitution of CD4^+CD25^+CD45RO^- T cells in lymphopenic HIV-patients after retroviral treatment. In response to IL-2 treatment
CD4⁺CD25⁺CD45RO⁻ T cells rapidly expanded 3⁹. Similarly, CD4⁺CD25⁺CD45RO⁻ T cells have been reported to expand during rheumatoid arthritis 4⁰. Although CD4⁺CD25⁺CD45RO⁻ T cells expressed high amounts of FOXP3, Sereti and coworkers considered these cells to be different from T_{reg} as they were CD45RO^{lo}, CD25^{int} and also weak suppressors unlike CD4⁺CD25^{hi} T_{reg}. Moreover, CD4⁺CD25⁺CD45RO⁻ T cells were reported to be CD95^{lo} and resistant to CD95L-mediated apoptosis 3⁹. Since, these properties are all features of naïve T_{reg}, we consider it highly likely that the reported CD4⁺CD25⁺CD45RO⁻ T cells in treated HIV patients predominantly contained naïve T_{reg}. As FOXP3 was not be analyzed at as single cell level by flow cytometry, contamination of naïve T_{reg} cells with T_{conv} within the analyzed CD4⁺CD25⁺CD45RO⁻ T cells could not be detected. Such a contamination with T_{conv} could explain the low IL-2 production and weak suppressive capacity of the CD4⁺CD25⁺CD45RO⁻ T cell population derived from HIV patients that was observed.

Altogether, several lines of evidence support the existence of naïve FOXP3⁺ T_{reg} (CD4⁺CD25⁺FOXP3⁺CD95^{lo}CD45RO^{lo}CD45RA^{hi}) and warrant thorough investigation of these cells in human disease. Naïve and memory T_{reg} frequencies should be considered as a result of different regulating mechanisms including generation, elimination and transformation of naïve T_{reg} into memory T_{reg}. It is important to delineate the predominating processes for T_{reg} homeostasis in detail to allow a rationale translation into human pathogenesis. This report provides a functional assay for the distinction between naïve and antigen-primed T_{reg}, as resistance towards CD95L-mediated apoptosis clearly separates naïve T_{reg} from their in vivo preactivated CD4⁺CD25^{hi} T_{reg} counterparts.
Pathogenetically this difference might be relevant, since Miyara et al. recently showed that reduced T_{reg} frequencies in active SLE resulted from enhanced CD95-mediated cell death of systemic lupus erythematosus (SLE)-derived CD4^{+}CD25^{hi} T_{reg}^{16}. However, only CD25^{hi} T_{reg} were studied, which are mainly comprised of memory T_{reg}. Therefore it remains possible that naïve T_{reg} numbers are increased in SLE to compensate for a relative loss of memory-like CD4^{+}CD25^{hi} T cells in SLE. As for other diseases with altered frequencies of CD4^{+}CD25^{hi} T_{reg}, an analysis of naïve T_{reg} should accompany that of the quantity and function of T_{reg}. Altered distribution of both cell types within the T_{reg} population might not only reflect age dependency but also fluctuating disease activity. Careful reanalysis of the previously neglected naïve T_{reg} pool might help to optimize novel therapies based on the expansion of naïve and apoptosis-resistant T_{reg}.
Figure 1

Resistance to CD95L-mediated apoptosis of a T\textsubscript{reg} subpopulation. (A) Isolation of apoptosis-resistant T\textsubscript{reg} (rT\textsubscript{reg}). FACS-sorted T\textsubscript{reg} were incubated with CD95L (1:20) and rT\textsubscript{reg} were recovered after removal of dead cells using Annexin V microbeads as described in Material and Methods. (B) rT\textsubscript{reg} were incubated with different concentrations of CD95L and specific cell death rates were compared to those of T\textsubscript{reg} cultured in control medium. (C) and (D): FACS-sorted T\textsubscript{reg} (CD4\textsuperscript{+}CD25\textsuperscript{hi}) were incubated alone (C) or with CD95L (1:20) (D) and the remaining cells were analyzed for the expression of CD25 and CD45RO. (E) The percentage of CD45RO\textsuperscript{lo} and CD45RO\textsuperscript{hi} subpopulations among the total surviving cell population was determined for various concentrations of CD95L.

Figure 2

Detection of memory and naïve T\textsubscript{reg} among human adult CD4\textsuperscript{+}CD25\textsuperscript{+}FOXP3\textsuperscript{+} T\textsubscript{reg}. (A) Freshly isolated adult PBL were stained directly with fluorochrome-labeled mAbs against CD4, CD25, CD45RO, CD45RA followed by intracellular staining with anti-FOXP3 mAb (clone PHC101) and were analyzed with a FACS Canto\textsuperscript{TM} cytometer. After gating for CD4\textsuperscript{+} T cells (R1), CD45RO\textsuperscript{hi}FOXP3\textsuperscript{+} T cells (R3, blue) as well as CD45RO\textsuperscript{lo}FOXP3\textsuperscript{+} T cells (R2, red) were defined. Identical populations (blue1\textsuperscript{1}red) are shown in (B) and (C). (B) Multicolor analysis allocates CD45RO\textsuperscript{hi}FOXP3\textsuperscript{+}T cells (blue) as well as CD45RO\textsuperscript{lo}FOXP3\textsuperscript{+} T cells (red) within the CD4\textsuperscript{+}CD25\textsuperscript{+} population. (C) CD45RO\textsuperscript{hi}FOXP3\textsuperscript{+}T cells (blue) as well as CD45RO\textsuperscript{lo}FOXP3\textsuperscript{+} T cells (red) were analyzed for expression of CD45RA and compared to T\textsubscript{conv}. (D) PBL were stained with
directly fluorochrome-labeled mAbs against CD95, CD4, CD25, CD45RO, CD45RA followed by intracellular staining with anti-FOXP3 mAb (clone PHC101) to determine expression of CD95 on CD45RO^{hi}FOXP3^{+} (R3 as in (B), blue) as well as CD45RO^{lo}FOXP3^{+} (R2 as in (B), red) cells. The dashed line represents isotype control. (E) Note the significant inverse correlation between the percentage of naïve T_{reg} (CD4^{+}CD25^{+}FOXP3^{+}CD45RO^{lo}CD95^{lo} T cells) among total T_{reg} and age. Dots correspond to individual samples tested. The solid line shows linear regression.

Figure 3
Expression of FOXP3 and CD95 on T cells derived from cord blood. (A) Freshly isolated adult PBL as well as cord blood lymphocytes were stained with fluorochrome-labeled mAbs against CD4 and CD25 followed by intracellular staining with anti-FOXP3 mAb (clone PHC101) and were analyzed with a FACS Canto™ cytometer. Cells were gated on viable CD4^{+} T cells. (B) Adult CD4^{+}CD25^{hi}, cord blood CD4^{+}CD25^{+} T cells and their respective T_{conv} counterparts were FACS-sorted using the indicated gates and (C) inhibitory capacities of FACS-sorted T_{reg} were analyzed by proliferation assays. For this purpose, T_{conv} were FACS-sorted from a third party adult donor and incubated with cord blood T_{reg} or adult T_{reg} as described in Material and Methods. (D) Freshly isolated adult PBL as well as cord blood lymphocytes were stained with fluorochrome-labeled mAbs against CD4, CD25 and CD95 followed by intracellular staining with anti-FOXP3 mAb (clone PHC101) and were analyzed on a FACS Canto™ cytometer. Histogram overlays indicate expression of CD95 on CD4^{+}CD25^{+}FOXP3^{+} T cells from cord blood versus adult blood. The isotype control for CD95 is shown for cord blood and was similar
to isotype staining of adult PBL. (E) Cord blood lymphocytes were stained as in (D) plus anti-CD45RO mAb. Histogram overlays indicate the expression of CD95 on CD4⁺CD25⁺FOXP3⁺ T cells as well as on CD4⁺CD25⁺FOXP3⁺CD45ROhi T cells. Isotype control for CD95 staining is shown.

Figure 4

Resistance of cord blood Treg towards CD95L-mediated cell death. (A) FACS-sorted adult Treg (CD4⁺CD25hi), cord blood Treg (CD4⁺CD25⁺) and their respective Tconv were incubated with CD95L (1:20) for 10 hours and specific cell death rates were determined by FACS-analysis as described previously.¹² Similarly, CD4⁺CD25⁺CD45ROlo T cells (R2) as well as CD4⁺CD25⁺CD45ROhi T cells (R1) were FACS-sorted as shown in (B) and all cells were incubated with CD95L (1:20) for 10 hours. (B) Enrichment of naïve or memory cord blood Treg by FACS-sorting. Cord blood lymphocytes were stained with fluorochrome-labeled mAbs against CD4, CD25 and CD45RO. Previous to FACS-sorting, one aliquot from the sort sample was additionally stained with anti-CD95 mAb to detect CD4⁺CD25⁺CD95hi T cells. Since CD4⁺CD25⁺CD95hi T cells co-segregate with CD45ROhi T cells (arrow), the sorting gate was set on CD45ROhi cells to separate CD4⁺CD25⁺CD45ROhi CD95hi memory T cells in R1 from the naïve cord blood Treg in R2. See the text for further explanation. One representative experiment of at least three independent experiments is shown.
Figure 5

Sensitization of cord blood T<sub>reg</sub> towards CD95L-mediated cell death in response to TCR-stimulation. (A-E) FACS-sorted cord blood T<sub>reg</sub> (CD4<sup>+</sup>CD25<sup>+</sup>) and adult T<sub>reg</sub> (CD4<sup>+</sup>CD25<sup>hi</sup>) were incubated with anti-CD3/28 mAbs and IL-2 as described in Material and Methods. CD95 expression (A and B) and CD45RO expression (B) were determined at the points in time indicated. (C) Six days after TCR-stimulation, cord blood T<sub>reg</sub> were incubated with CD95L (1:20) for 10 hours and specific cell death rate was determined and compared to the specific cell death rate of freshly isolated cord blood T<sub>reg</sub>. Likewise, freshly isolated FACS-sorted CD45RO<sup>lo</sup> cord blood T<sub>reg</sub> without CD95<sup>hi</sup> memory T<sub>reg</sub> (see Figure 4B) were incubated with CD95L (1:20) for 10 hours. (D) Proliferation assays were used to measure the inhibitory capacities of freshly isolated cord blood T<sub>reg</sub> or 6 days activated T<sub>reg</sub> (anti-CD3/28 mAbs plus IL-2). For this test, T<sub>conv</sub> were FACS-sorted from an adult donor and incubated with cord blood T<sub>reg</sub> as described in Material and Methods. (E-F) Apoptosis-resistant T<sub>reg</sub> were incubated with anti-CD3/28 mAbs plus IL-2 as described in Material and Methods. After six days, CD95 sensitivity towards CD95-mediated apoptosis (E) and expression of CD95 (F) were determined. One representative experiment of at least three independent experiments is shown.
References


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Naive regulatory T cells: a novel subpopulation defined by resistance towards CD95L-mediated cell death

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