

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

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**Most CD4<sup>+</sup>CD25<sup>hi</sup>FOXP3<sup>+</sup> regulatory T cells (T<sub>regs</sub>) from adult peripheral blood express high levels of CD45RO and CD95 and are prone to CD95L-mediated apoptosis in contrast to conventional T cells (T<sub>convs</sub>). However, a T<sub>reg</sub> subpopulation remained consistently apoptosis resistant. Gene microarray and 6-color flow cytometry analysis including FOXP3 revealed an increase in naive T-cell markers on the CD95L-resistant T<sub>regs</sub> compared with**

**most T<sub>regs</sub>. In contrast to T<sub>regs</sub> found in adult humans, most CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T cells found in cord blood are naive and exhibit low CD95 expression. Furthermore, most of these newborn T<sub>regs</sub> are not sensitive toward CD95L similar to naive T<sub>regs</sub> from adult individuals. After short stimulation with anti-CD3/CD28 monoclonal antibodies (mAbs), cord blood T<sub>regs</sub> strongly up-regulated CD95 and were sensitized toward CD95L. This functional change was paralleled by a rapid**

**up-regulation of memory T-cell markers on cord blood T<sub>regs</sub> that are frequently found on adult memory T<sub>regs</sub>. In summary, we show a clear functional difference between naive and memory T<sub>regs</sub> that could result in different survival rates of those 2 cell populations in vivo. This new observation could be crucial for the planning of therapeutic application of T<sub>regs</sub>. (Blood. 2006;108:3371-3378)**

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## Introduction

There is now clear evidence that CD4<sup>+</sup> T cells contain a population of naturally immunosuppressive T cells characterized by constitutive expression of CD25, CTLA-4, and FOXP3.<sup>1</sup> Because not all potentially autoreactive T cells are deleted in the thymus,<sup>2,3</sup> peripheral control of T-cell responses by naturally occurring CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> immunoregulatory T cells (T<sub>regs</sub>) is crucial to prevent autoimmunity.<sup>1</sup> Depletion of T<sub>regs</sub> contributes to the induction of severe autoimmune diseases in animal models, and several studies have reported a defect of T<sub>regs</sub> in various human autoimmune diseases.<sup>1,4,5</sup>

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 pharmacologic mimicking of T<sub>reg</sub> function remain undefined. Consequently, direct use of T<sub>regs</sub> for therapy is currently under examination. Therapeutic expansion or depletion of T<sub>regs</sub> with defined antigen specificity offers new treatment options for human diseases.<sup>6</sup> Because accumulation of T<sub>regs</sub> has been shown to be detrimental in cancer,<sup>7</sup> new insights into mechanisms of T<sub>reg</sub> homeostasis are required.

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>regs</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>regs</sub>.<sup>1</sup> However, under specific circumstances T<sub>regs</sub> can also be generated out of conventional T cells (T<sub>convs</sub>) (eg, if the antigen is targeted to immature dendritic cells [DCs]).<sup>8-10</sup> It has yet to be established how much “converted” T<sub>regs</sub> contribute to the total number of T<sub>regs</sub> in the periphery of adult mice and humans.

At a given time, the overall number of T<sub>regs</sub> is defined by a balance of generation and demise of T<sub>regs</sub>. 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.<sup>11</sup> While naive T cells are CD95<sup>-</sup> and resistant to apoptosis induction, activated T cells (CD45RO<sup>hi</sup>) up-regulate CD95 and become sensitive to apoptosis. Upon T-cell receptor (TCR) restimulation, activated effector T cells (T<sub>effs</sub>) up-regulate CD95L and induce AICD through crosslinking of CD95 by CD95L. AICD eliminates T<sub>effs</sub> after an immune response and contributes to T-cell homeostasis. We have previously shown that most T<sub>regs</sub> constitutively express CD95 and can easily be killed via crosslinking of this death receptor by CD95L.<sup>12</sup> Together with the fact that most T<sub>regs</sub> express high levels of CD45RO, we

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hypothesized that most  $T_{\text{regs}}$  resemble memory/recently activated T cells rather than naive T cells. Only very recently has the presence of naive  $T_{\text{regs}}$  of  $CD4^+CD25^+CD45RA^{\text{hi}}$  phenotype in addition to memory  $T_{\text{regs}}$  been appreciated in peripheral blood.<sup>13-15</sup> However, because naive  $T_{\text{regs}}$  express less CD25 ( $CD25^+$ ) than memory  $T_{\text{regs}}$  ( $CD25^{\text{hi}}$ ) and because those studies did not include fluorescence-activated cell sorting (FACS) analysis of FOXP3 expression, a clear separation between  $CD25^+$   $T_{\text{regs}}$  and  $CD25^+$   $T_{\text{effs}}$  was not possible. Therefore, analysis of  $CD4^+CD25^+CD45RA^{\text{hi}}$  T cells might have included contaminating  $T_{\text{effs}}$ . Moreover, no functional differences between naive and memory  $T_{\text{regs}}$  were described; both cell types were reported to be immunosuppressive.<sup>14,15</sup> In contrast, memory  $T_{\text{convS}}$  are highly differentiated cells with distinct biologic properties from those of naive  $T_{\text{convS}}$ . Analogously, naive and antigen-primed/memory  $T_{\text{regs}}$  should exhibit different functional features.

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

## Materials and methods

### Human samples

Peripheral blood was obtained from healthy adult donors. Cord blood samples were obtained from the placentas of healthy 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 antibodies (mAbs) against human CD4, CD45RO, CD45RA, and CD31 were obtained from BD Pharmingen (Heidelberg, Germany) and  $\alpha$ CD25 Ab from Miltenyi Biotec (Auburn, CA). CD95L was produced as a Leucin zipper-tagged ligand of CD95, which binds both murine and human CD95.<sup>17</sup> The monoclonal  $\alpha$ CD3 Ab (OKT3),  $\alpha$ CD28 mAb, and the agonistic monoclonal  $\alpha$ CD95 Ab (anti-Apo-1) were purified from hybridoma supernatants by protein A affinity purification as described.<sup>17</sup> The monoclonal  $\alpha$ Foxp3 mAb (clone PCH101) was obtained from eBioscience (San Diego, CA), annexin V Alexa Fluor 488 from Molecular Probes (Eugene, OR), and propidium iodide (PI) and protein A from Sigma-Aldrich (St Louis, MO).

### Lymphocyte separation

Human peripheral blood lymphocytes (PBLs) 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 magnetic-activated cell separation (MACS) beads (Miltenyi Biotec), and subsequently  $CD4^+CD25^{\text{high}}$  cells were sorted with a FACS-Diva cell sorter (BD Biosciences, San Jose, CA).  $CD4^+CD25^{\text{hi}}$   $T_{\text{regs}}$  cells contained only the 1% to 2% of human CD4 cells with the highest CD25 expression, as previously reported.<sup>18</sup> Similarly,  $CD4^+CD25^+$  or  $CD4^+CD25^+CD45RO^{\text{lo/hi}}$  T cells were FACS sorted from cord blood-derived lymphocytes. FACS-sorted  $CD4^+CD25^-$  cells were used as  $T_{\text{convS}}$ .

### Dead cell removal

Dead cells were depleted using the MACS annexin V MicroBead kit (Miltenyi Biotec). Briefly, cells were incubated with annexin V microbeads and passed over a medium-size (MS) column to magnetically remove annexin V-positive cells. Purity of viable cells was determined by forward scatter/sideward scatter (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  $\alpha$ CD95 Ab and 10 ng/mL protein A or different concentrations of CD95L (maximal 1:20 dilution) as previously described.<sup>12</sup> Unstimulated cells were incubated with an isotype control Ab or CD95L-free control medium. To expand  $T_{\text{regs}}$ , cells were incubated with 0.1  $\mu$ g/mL  $\alpha$ CD3 Ab and 1  $\mu$ g/mL  $\alpha$ CD28 Ab in combination with irradiated JY feeder cells (kind gift from C. Falk, GSF-Institute for Molecular Biology, Munich, Germany) and 300 IU IL-2.<sup>12</sup> Cell death was assessed by annexin V/propidium iodide costaining and forward to sideward scatter profiles. Specific cell death was calculated as described previously.<sup>12</sup>

### Cell proliferation assay

When expanded  $T_{\text{regs}}$  were used, they were washed extensively to remove IL-2, but no further resting of  $T_{\text{regs}}$  occurred prior to cell proliferation assays.  $T_{\text{convS}}$  ( $1 \times 10^4$ ) were incubated in 96-well plates (Nunc, Wiesbaden, Germany) alone or in coculture with  $T_{\text{regs}}$  ( $1 \times 10^4$   $T_{\text{regs}}$ ) together with irradiated T-cell-depleted PBMCs ( $1 \times 10^5$ ) and were stimulated with soluble  $\alpha$ CD3 mAb (0.2  $\mu$ g/mL) and  $\alpha$ CD28 mAb (0.005  $\mu$ g/mL). Antigen-presenting cells (APCs) and  $T_{\text{convS}}$  were always derived from the same blood sample. After 4 days at 37°C in 5%  $CO_2$ , 1  $\mu$ Ci (0.037 MBq) [ $^3$ H]-thymidine per well was added for additional 16 hours, and proliferation was measured in counts per minute (cpm) with a scintillation counter. Inhibitory capacity (percentage) of  $T_{\text{regs}}$  in coculture experiments was defined as  $(1 - \frac{[^3\text{H}]\text{-thymidine uptake [cpm] of coculture of } T_{\text{regs}} \text{ with } T_{\text{convS}}}{\text{cpm of } T_{\text{convS}} \text{ alone}}) \times 100\%$ . 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 the 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 reverse transcriptase-polymerase chain reaction (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<sub>conv</sub> mRNA levels. FOXP3 primer sequences were 5'-AGCTGGAGTTCCGCAAGAAAC (forward) and 5'-TGTTTCGTCATCCTCCTTTCC (reverse). GAPDH primer sequences were 5'-GCAAATTCATGGCACCGT (forward) and 5'-TCGCCCACT-TGATTTTGG (reverse).

**DNA microarray hybridization and analysis**

Quality and integrity of 500 ng total RNA were controlled by running all samples on a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). For RNA amplification, the first round was done according to Affymetrix without biotinylated nucleotides using the 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 2 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 was fragmented and placed in a hybridization cocktail containing 4 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 hybridization the GeneChips were washed, stained with streptavidin-phycoerythrin (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 Minimum Information About a Microarray Experiment (MIAME)-compliant data set of the microarray experiments will be posted at the Gene Expression Omnibus (GEO) database.<sup>19</sup>

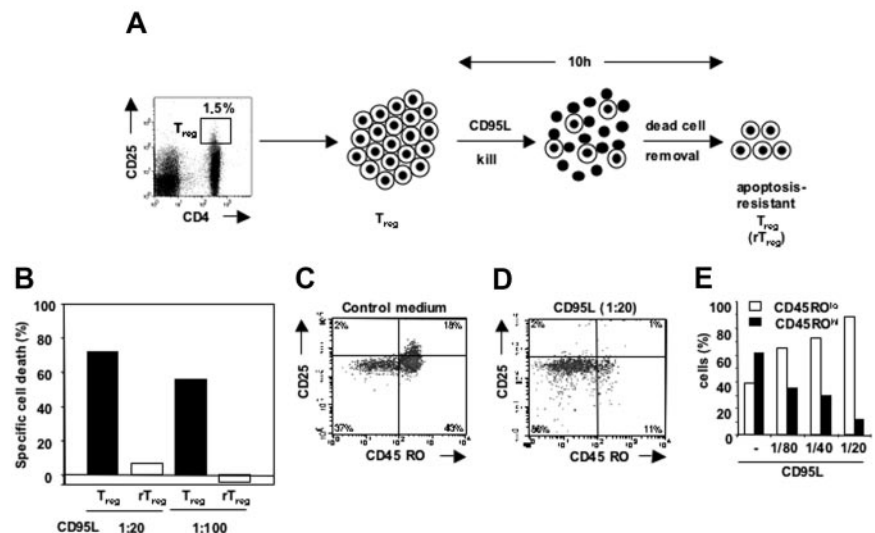
**Results**

**CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub> contain a subpopulation of CD95L-mediated cell death-resistant T<sub>regs</sub>**

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

**CD45RO<sup>lo</sup> T<sub>regs</sub> are resistant to CD95L-mediated cell death**

To further characterize rT<sub>regs</sub>, we pooled rT<sub>reg</sub> cDNA derived from FACS-sorted CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub> of 8 healthy blood donors and performed gene chip microarray analysis. In this screen, we compared cDNA from rT<sub>regs</sub> with cDNA derived from all CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub>. Interestingly, rT<sub>regs</sub> showed a significant decrease of mRNA expression of genes related to T-cell memory like CTLA-4, various HLA genes, and CD103, whereas mRNA for molecules expressed mainly on naive cells such as CCR7 or CD127 (IL7R) was more prevalent in rT<sub>regs</sub> than in total CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub> (Tables S1-S2, available on the *Blood* website; see the Supplemental Tables link at the top of the online article). This finding suggested that rT<sub>regs</sub> are more naive in contrast to most T<sub>regs</sub> found in the CD4<sup>+</sup>CD25<sup>hi</sup> T-cell population, which are known to exhibit an antigen-primed/memory-like phenotype. To test this hypothesis, we incubated FACS-sorted CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub> with increasing concentrations of CD95L. The remaining viable rT<sub>regs</sub> were then



**Figure 1. Resistance to CD95L-mediated apoptosis of a T<sub>reg</sub> subpopulation.** (A) Isolation of apoptosis-resistant T<sub>regs</sub> (rT<sub>regs</sub>). FACS-sorted T<sub>regs</sub> were incubated with CD95L (1:20), and rT<sub>regs</sub> were recovered after removal of dead cells using annexin V microbeads as described in "Materials and methods." (B) rT<sub>regs</sub> were incubated with different concentrations of CD95L, and specific cell death rates were compared with those of T<sub>regs</sub> cultured in control medium. (C-D) FACS-sorted T<sub>regs</sub> (CD4<sup>+</sup>CD25<sup>hi</sup>) 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<sup>lo</sup> and CD45RO<sup>hi</sup> subpopulations among the total surviving cell population was determined for various concentrations of CD95L.

analyzed by 4-color FACS for the expression of the typical memory marker CD45RO<sup>20</sup> (Figure 1C-E) and the naive marker CD31<sup>21,22</sup> (data not shown). In contrast to T<sub>regs</sub> cultured in control medium (Figure 1C), rT<sub>regs</sub> surviving CD95L stimulation were mostly CD45RO<sup>lo</sup> (more than 85%) (Figure 1D). The number of CD45RO<sup>hi</sup> cells progressively declined with increasing concentrations of CD95L (Figure 1E). The naive cell marker CD31 increased correspondingly on rT<sub>regs</sub> (data not shown). We conclude that CD95L preferentially kills CD45RO<sup>hi</sup> T<sub>regs</sub> cells, leaving behind rT<sub>regs</sub> with a naive phenotype and slightly lower CD25 expression levels. Furthermore, FACS analysis for intracellular FOXP3 expression demonstrated that all rT<sub>regs</sub> were FOXP3<sup>+</sup>, and the anergy and suppressive capacity of in vitro-expanded rT<sub>regs</sub> (data not shown) further supported their classification as T<sub>regs</sub>.

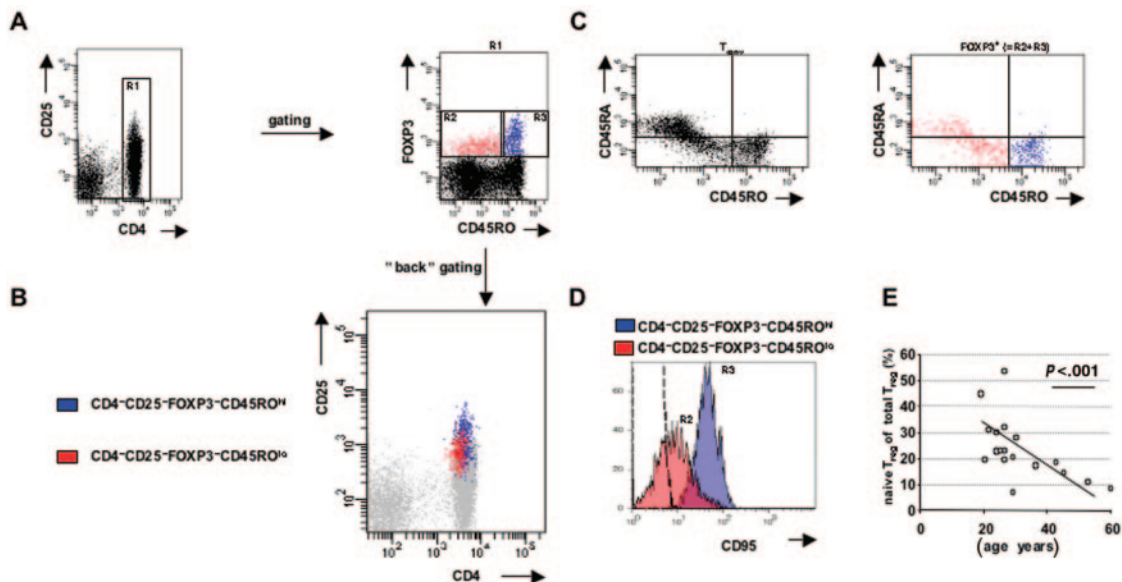
#### Human adult CD4<sup>+</sup>CD25<sup>hi</sup>FOXP3<sup>+</sup> T<sub>regs</sub> consist of memory and naive T<sub>regs</sub>

Consequently, human T<sub>regs</sub> should comprise a heterogeneous population with memory and naive T<sub>regs</sub>. To prove that naive CD4<sup>+</sup>CD25<sup>hi</sup> T cells are indeed T<sub>regs</sub>, we performed FOXP3 FACS analysis in PBLs. In the absence of further highly specific T<sub>reg</sub> markers, FOXP3 is considered to be the most reliable marker to identify T<sub>regs</sub> in human peripheral blood to date.<sup>23</sup> Therefore, we established 6-color FACS analysis including mAbs against CD4, CD25, and FOXP3 for T<sub>reg</sub> detection together with mAbs against CD45RO and CD45RA to distinguish memory and naive T<sub>regs</sub>. Naive T<sub>regs</sub> were initially considered as CD4<sup>+</sup>FOXP3<sup>+</sup>CD45RO<sup>lo</sup> cells (R2 in Figure 2A, right panel), and memory T<sub>regs</sub> were characterized as CD4<sup>+</sup>FOXP3<sup>+</sup>CD45RO<sup>hi</sup> cells (R3 in Figure 2A, right panel). Using multicolor analysis one can ascertain that memory T<sub>regs</sub> (blue) are mainly CD25<sup>hi</sup> while naive T<sub>regs</sub> (red) are mainly CD25<sup>int</sup> and overlap with CD25<sup>int</sup> T<sub>effs</sub>. Accordingly, pure FACS-based isolation of T<sub>regs</sub> is limited to the CD25<sup>hi</sup> memory T<sub>reg</sub>-enriched cell

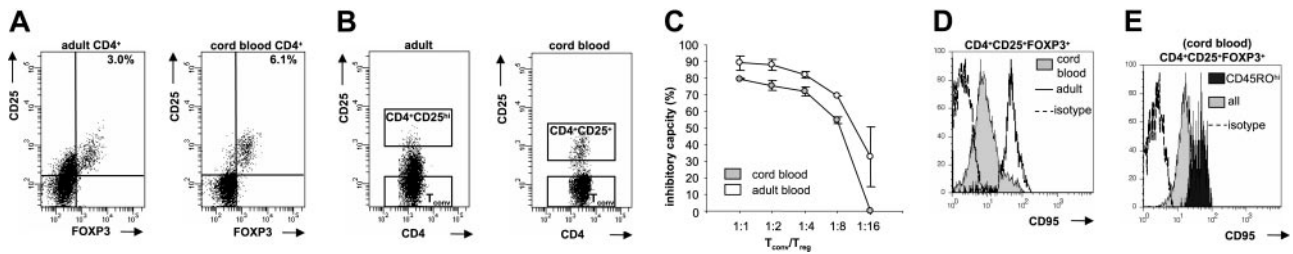
pool, because surface expression of CD25 is quite similar between naive T<sub>regs</sub> and FOXP3<sup>-</sup>CD4<sup>+</sup>CD25<sup>+</sup> T<sub>effs</sub> (Figure 2B). Naive T<sub>regs</sub> showed a higher expression of CD45RA than memory T<sub>regs</sub> (Figure 2C). Analysis of the naive cell marker CD31 also showed increased expression (data not shown). Comparison of CD95L-resistant cells in Figure 1D with the CD45RO<sup>lo</sup> naive T<sub>regs</sub> (red) from the 6-color staining (Figure 2B-C) revealed a similar phenotype, supporting that apoptosis-resistant T<sub>regs</sub> represent naive T<sub>regs</sub> in adult peripheral blood. Because sensitization toward CD95L-mediated cell death includes up-regulation of CD95, we compared CD95 expression of naive and memory T<sub>regs</sub> by 6-color FACS for expression of CD4, CD25, FOXP3, CD45RO, CD45RA, and CD95 (Figure 2D). Indeed, naive CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>CD45RO<sup>lo</sup> T<sub>regs</sub> (red) expressed very low levels of CD95 in comparison with the highly CD95<sup>+</sup> memory T<sub>regs</sub> (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>CD45RO<sup>hi</sup>, blue, Figure 2D). Collectively, we define naive T<sub>regs</sub> as CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>CD45RO<sup>lo</sup>CD95<sup>lo</sup> T cells and memory T<sub>regs</sub> as CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>CD45RO<sup>hi</sup>CD95<sup>hi</sup> T cells. Furthermore, we conclude that rT<sub>regs</sub> represent naive T<sub>regs</sub> in adult peripheral blood. The frequency of naive T<sub>regs</sub> varied between 10% to 50% of total T<sub>regs</sub> in adult peripheral blood and clearly declined with increasing age (Figure 2E).

#### Cord blood CD4<sup>+</sup>CD25<sup>+</sup> T cells are FOXP3<sup>+</sup> and express low levels of CD95

Whereas the frequency of CD45RO<sup>lo</sup> T<sub>regs</sub> in adult peripheral blood is rather low, newborns should exhibit high numbers of naive T<sub>regs</sub>. Therefore, we extended our functional analysis on cord-blood-derived T<sub>regs</sub>. Nearly all CD4<sup>+</sup>CD25<sup>+</sup> human cord blood T cells expressed FOXP3 as determined by FACS analysis (Figure 3A) and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (data not shown). The percentage of FOXP3<sup>+</sup> T cells within the CD4<sup>+</sup> compartment was only slightly higher in cord



**Figure 2. Detection of memory and naive T<sub>regs</sub> among human adult CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T<sub>regs</sub>.** (A) Freshly isolated adult PBLs were stained directly with fluorochrome-labeled mAbs against CD4, CD25, CD45RO, and CD45RA followed by intracellular staining with anti-FOXP3 mAb (clone PCH101) and were analyzed with a FACS Canto cytometer. After gating for CD4<sup>+</sup> T cells (R1), CD45RO<sup>hi</sup>FOXP3<sup>+</sup> T cells (R3, blue) as well as CD45RO<sup>lo</sup>FOXP3<sup>+</sup> T cells (R2, red) were defined. Identical populations (blue, red) are shown in panels B and C. (B) Multicolor analysis allocates CD45RO<sup>hi</sup>FOXP3<sup>+</sup> T cells (blue) as well as CD45RO<sup>lo</sup>FOXP3<sup>+</sup> T cells (red) within the CD4<sup>+</sup>CD25<sup>+</sup> population. (C) CD45RO<sup>hi</sup>FOXP3<sup>+</sup> T cells (blue) as well as CD45RO<sup>lo</sup>FOXP3<sup>+</sup> T cells (red) were analyzed for expression of CD45RA and compared with T<sub>conv</sub>s. (D) PBLs were stained with directly fluorochrome-labeled mAbs against CD95, CD4, CD25, CD45RO, and CD45RA followed by intracellular staining with anti-FOXP3 mAb (clone PCH101) to determine expression of CD95 on CD45RO<sup>hi</sup>FOXP3<sup>+</sup> (R3 as in panel B, blue) as well as CD45RO<sup>lo</sup>FOXP3<sup>+</sup> (R2 as in panel B, red) cells. The dashed line represents isotype control. (E) Note the significant inverse correlation between the percentage of naive T<sub>regs</sub> (CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>CD45RO<sup>lo</sup>CD95<sup>lo</sup> T cells) among total T<sub>regs</sub> 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 PBLs 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 PCH101) and were analyzed with a FACS Canto cytometer. Cells were gated on viable CD4<sup>+</sup> T cells. (B) Adult CD4<sup>+</sup>CD25<sup>hi</sup>, cord blood CD4<sup>+</sup>CD25<sup>+</sup> T cells, and their respective T<sub>conv</sub> counterparts were FACS sorted using the indicated gates, and (C) inhibitory capacities of FACS-sorted T<sub>regs</sub> were analyzed by proliferation assays. For this purpose, T<sub>convs</sub> were FACS sorted from a third-party adult donor and incubated with cord blood T<sub>regs</sub> or adult T<sub>regs</sub> as described in “Materials and methods.” (D) Freshly isolated adult PBLs 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 PCH101) and were analyzed on a FACS Canto cytometer. Histogram overlays indicate expression of CD95 on CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> 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 PBLs. (E) Cord blood lymphocytes were stained as in panel D plus anti-CD45RO mAb. Histogram overlays indicate the expression of CD95 on CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T cells as well as on CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>CD45RO<sup>hi</sup> T cells. Isotype control for CD95 staining is shown.

blood (5.9% ± 3.1%, n = 6) than in adult peripheral blood (4.8% ± 1.7%, n = 6). T<sub>regs</sub> 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<sup>+</sup>CD25<sup>+</sup> T cells in human cord blood constituted CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T<sub>regs</sub> similar to murine T<sub>regs</sub>. In contrast, few CD4<sup>+</sup>CD25<sup>+</sup> T cells in adult peripheral blood contained CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T<sub>regs</sub> (Figure 3A). Further, FACS analysis revealed that most cord blood T<sub>regs</sub> showed low expression of CD45RO (Figure 4B) but high expression of CD45RA and CD31 (data not shown), confirming their naive status. Interestingly, most cord blood T<sub>regs</sub> showed a low expression of CD95 (Figure 3D) similar to adult naive T<sub>regs</sub>. High expression of CD95 was limited to the minor subpopulation of CD45RO<sup>hi</sup> T<sub>regs</sub> in cord blood (Figure 4B, arrow). To summarize, both naive T<sub>regs</sub> from cord blood as well as naive T<sub>regs</sub> from adult peripheral blood show a lower expression of CD95 than memory T<sub>regs</sub>. In addition, both exhibit a similar level of CD25 expression (compare Figures 2B and 3A), further indicating that cord blood T<sub>regs</sub> and adult naive T<sub>regs</sub> represent a similar cell population.

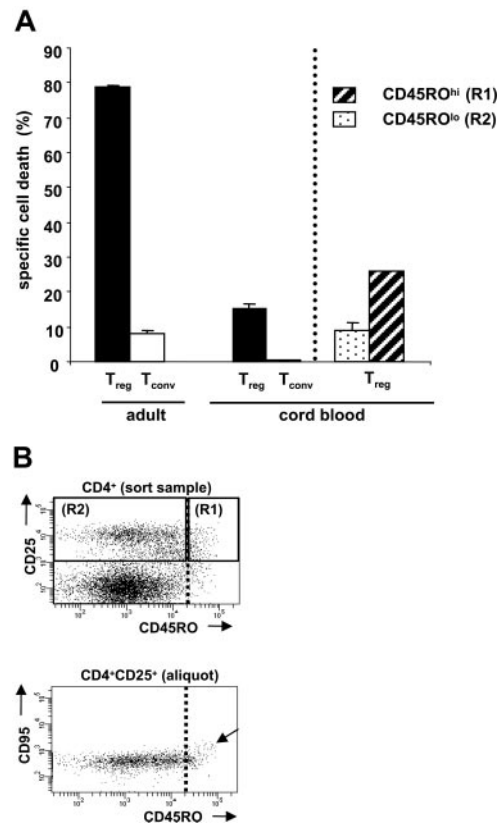
**Freshly isolated cord blood T<sub>regs</sub> are resistant to CD95L-mediated apoptosis**

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

**TCR-stimulated cord blood T<sub>regs</sub> are sensitive to CD95L-mediated apoptosis and acquire a memory phenotype similar to adult T<sub>regs</sub>**

During an immune response, naive T<sub>convs</sub> are stimulated via their TCR. They clonally expand and differentiate into T<sub>effs</sub>. One hallmark of peripheral T-cell depletion is that T<sub>effs</sub> up-regulate CD95 and become sensitive to CD95L-mediated cell death to allow depletion of the expanded T-cell pool by CD95L.<sup>11</sup> We have shown that TCR stimulation of T<sub>convs</sub> from adult peripheral blood with

anti-CD3 mAb and IL-2 can be used to mimic this critical process in vitro.<sup>11</sup> Accordingly, we questioned whether naive T<sub>regs</sub> could also up-regulate CD95 and be sensitized to CD95L-mediated apoptosis in response to TCR stimulation.



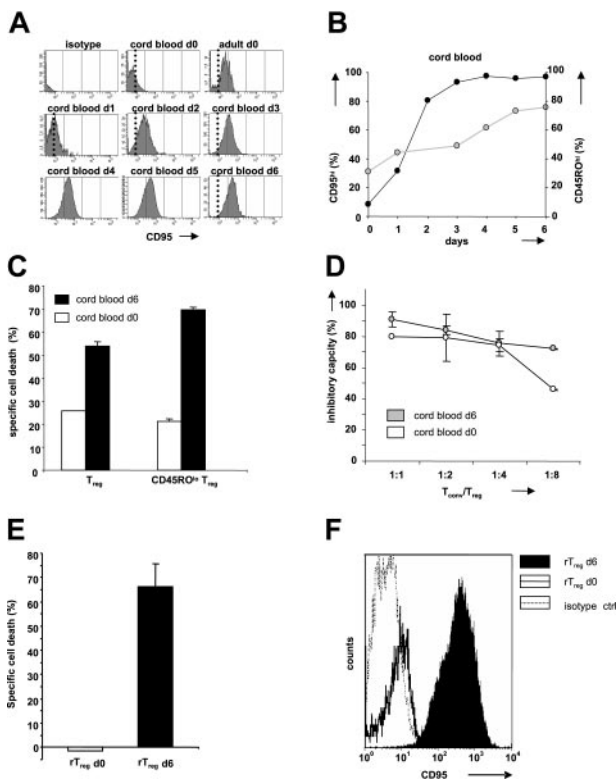
**Figure 4. Resistance of cord blood T<sub>regs</sub> toward CD95L-mediated cell death.** (A) FACS-sorted adult T<sub>regs</sub> (CD4<sup>+</sup>CD25<sup>hi</sup>), cord blood T<sub>regs</sub> (CD4<sup>+</sup>CD25<sup>+</sup>), and their respective T<sub>convs</sub> were incubated with CD95L (1:20) for 10 hours, and specific cell death rates were determined by FACS analysis as described previously.<sup>12</sup> Similarly, CD4<sup>+</sup>CD25<sup>+</sup>CD45RO<sup>lo</sup> T cells (R2) as well as CD4<sup>+</sup>CD25<sup>+</sup>CD45RO<sup>hi</sup> T cells (R1) were FACS sorted as shown in panel B, and all cells were incubated with CD95L (1:20) for 10 hours. Error bars represent SEM. (B) Enrichment of naive or memory cord blood T<sub>regs</sub> 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<sup>+</sup>CD25<sup>+</sup>CD95<sup>hi</sup> T cells. Because CD4<sup>+</sup>CD25<sup>+</sup>CD95<sup>hi</sup> T cells cosegregate with CD45RO<sup>hi</sup> T cells (arrow), the sorting gate was set on CD45RO<sup>hi</sup> cells to separate CD4<sup>+</sup>CD25<sup>+</sup>CD45RO<sup>hi</sup> CD95<sup>hi</sup> memory T cells in R1 from the naive cord blood T<sub>regs</sub> in R2. See the penultimate paragraph of “Results” for further explanation. One representative experiment of at least 3 independent experiments is shown.

For this purpose, we stimulated freshly isolated cord blood  $T_{\text{regs}}$  with anti-CD3/CD28 mAbs and IL-2 and analyzed their phenotypes at various time points (Figure 5). Soon after TCR stimulation, CD95 was up-regulated on cord blood  $T_{\text{regs}}$  and reached a CD95 expression plateau after 3 to 4 days (Figure 5A-B). This was notable because adult human T cells take 5 to 6 days to reach a plateau of CD95 expression (data not shown). However, murine T cells were also sensitized toward CD95L faster than adult T cells after TCR stimulation,<sup>24</sup> which suggests that naive human cord blood T cells share similarities with murine T cells. Simultaneous FACS analysis of memory cell markers during the *in vitro* stimulation of cord blood  $T_{\text{regs}}$  revealed that CD45RO increased more gradually than CD95 (Figure 5B) and that CD45RA and CD31 were gradually lost until day 6 (data not shown). Thus, this *in vitro*-induced phenotype of cord blood  $T_{\text{regs}}$  ( $CD95^{\text{hi}}CD45RO^{\text{hi}}CD45RA^{\text{lo}}$ ) was identical with the phenotype of most adult  $CD4^+CD25^{\text{hi}}$   $T_{\text{regs}}$  *in vivo* (Figure 2).

Most importantly, prestimulated cord blood  $T_{\text{regs}}$  were sensitive to CD95L treatment in contrast to freshly isolated cord blood  $T_{\text{regs}}$

(Figure 5C). To rule out outgrowth of a few preexisting cord blood  $CD45RO^{\text{hi}}CD95^{\text{hi}}$   $T_{\text{regs}}$ , we aimed to exclude  $CD45RO^{\text{hi}}CD95^{\text{hi}}$   $T_{\text{regs}}$  by FACS sorting. However, FACS sorting with anti-CD95 mAb could induce isolation-based apoptosis. Therefore, we defined an expression level of CD45RO that allowed separation of  $CD95^{\text{hi}}$  from  $CD95^{\text{lo}}$  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 “cutoff,” the sorting cells were separated into  $CD45RO^{\text{hi}}$  (correspondingly  $CD95^{\text{hi}}$ ) cells (Figure 4B, R1) and  $CD45RO^{\text{lo}}$  (correspondingly  $CD95^{\text{lo}}$ ) cells (Figure 4B, R2). When we stimulated the  $CD45RO^{\text{lo}}CD95^{\text{lo}}$  cord blood  $T_{\text{regs}}$  for 6 days, they still up-regulated CD95 and became sensitive toward CD95L-mediated apoptosis (Figure 5C). Therefore, outgrowth of  $CD45RO^{\text{hi}}CD95^{\text{hi}}$   $T_{\text{regs}}$  could be ruled out.

Likewise, isolated  $rT_{\text{regs}}$  from adult blood up-regulated CD95 in response to TCR stimulation (anti-CD3/CD28 mAbs plus IL-2) and became sensitive to CD95L-mediated cell death (Figure 5E-F). Finally, freshly isolated adult  $CD4^+CD25^{\text{hi}}$   $T_{\text{regs}}$ , naive cord blood  $T_{\text{regs}}$ , and prestimulated cord blood  $T_{\text{regs}}$  showed similar immunosuppressive capacity *in vitro* (Figures 3C and 5D). In contrast, up-regulation of CD95 and sensitization toward CD95L-mediated cell death clearly distinguished memory  $T_{\text{regs}}$  from naive  $T_{\text{regs}}$  on a functional basis.



**Figure 5. Sensitization of cord blood  $T_{\text{regs}}$  toward CD95L-mediated cell death in response to TCR stimulation.** FACS-sorted cord blood  $T_{\text{regs}}$  ( $CD4^+CD25^+$ ) and adult  $T_{\text{regs}}$  ( $CD4^+CD25^{\text{hi}}$ ) were incubated with anti-CD3/CD28 mAbs and IL-2 as described in “Materials and methods.” CD95 expression (A-B, ●) and CD45RO expression (B, ○) were determined at the time points indicated. (C) Six days after TCR stimulation, cord blood  $T_{\text{regs}}$  were incubated with CD95L (1:20) for 10 hours, and specific cell death rate was determined and compared with the specific cell death rate of freshly isolated cord blood  $T_{\text{regs}}$ . Likewise, freshly isolated FACS-sorted  $CD45RO^{\text{lo}}$  cord blood  $T_{\text{regs}}$  without  $CD95^{\text{hi}}$  memory  $T_{\text{regs}}$  (Figure 3B) were incubated with CD95L (1:20) for 10 hours. (D) Proliferation assays were used to measure the inhibitory capacities of freshly isolated cord blood  $T_{\text{regs}}$  or 6-day activated  $T_{\text{regs}}$  (anti-CD3/CD28 mAbs plus IL-2). For this test,  $T_{\text{convs}}$  were FACS sorted from an adult donor and incubated with cord blood  $T_{\text{regs}}$  as described in “Materials and methods.” (E-F) Apoptosis-resistant  $T_{\text{regs}}$  were incubated with anti-CD3/CD28 mAbs plus IL-2 as described in “Materials and methods.” After 6 days, CD95 sensitivity toward CD95-mediated apoptosis (E) and expression of CD95 (F) were determined. One representative experiment of at least 3 independent experiments is shown. Error bars represent SEM.

## Discussion

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

CD95L-mediated apoptosis of T cells has mainly been studied in naive  $T_{\text{convs}}$  that were activated *in vitro*. Analysis of apoptosis sensitivity of *in vivo*-primed/memory  $T_{\text{effs}}$  has been hampered by their low frequencies in the healthy human individual. By contrast, continuous *in vivo* stimulation of naive  $T_{\text{regs}}$  with self antigens seems to keep the proportion of  $CD95^{\text{hi}}$   $T_{\text{regs}}$  relatively high. In fact, incubation of naive  $T_{\text{regs}}$  with autologous APCs and IL-2 has been shown to stimulate naive  $T_{\text{regs}}$  in contrast to unreactive naive  $T_{\text{convs}}$ ,<sup>14</sup> and Romagnoli and coworkers have shown a high degree of self-reactivity within the  $T_{\text{reg}}$  population.<sup>25</sup> Thus, a constant activation of  $T_{\text{regs}}$  *in vivo* could conceivably lead to a very high proportion of memory  $T_{\text{regs}}$  in adults. This high frequency of memory  $T_{\text{regs}}$  allowed us to demonstrate the high apoptosis sensitivity toward CD95L of these antigen-experienced  $T_{\text{regs}}$  with a memory-like phenotype directly *ex vivo*.

Whereas naive cord blood  $T_{\text{regs}}$  are  $CD95^{\text{lo}}$  similar to naive  $T_{\text{convs}}$ , TCR stimulation *in vitro* revealed a rapid up-regulation of CD95. Simultaneously, CD45RO was up-regulated, whereas expression of the naive marker molecule CD31 declined in slower kinetics (data not shown). Six days after TCR stimulation *in vitro*,

most cord-blood-derived naive T<sub>regs</sub> had acquired the phenotype (CD95<sup>hi</sup>CD45RO<sup>hi</sup>CD45RA<sup>lo</sup>) known from adult CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub> in peripheral blood and lymphoid tissue *in vivo*.<sup>15</sup> In summary, naive T<sub>regs</sub> show the same phenotype in cord blood and adult peripheral blood but are much more frequent in cord blood. By contrast, memory T<sub>regs</sub> predominate in adult peripheral blood and are rare in cord blood. However, short-time TCR stimulation *in vitro* transformed cord-blood-derived naive T<sub>regs</sub> into T<sub>regs</sub> of a memory-like phenotype presented by most adult CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub>.

This *in vitro* induction of memory T<sub>regs</sub> might mimic the interaction of naive T<sub>regs</sub> with self antigens *in vivo*, which generates a pool of self-antigen-primed/memory-like T<sub>regs</sub> very early in fetal ontogeny. T<sub>regs</sub> can be detected as early as 14 weeks of human gestation in the periphery,<sup>26</sup> and T<sub>regs</sub> in fetal lymph nodes (less than 17 weeks old) already show a higher expression of CD95 and CD45RO in comparison with T<sub>conv</sub>s.<sup>27</sup> Because the healthy fetus is usually not challenged with foreign antigens, activation of T<sub>regs</sub> might reflect the early interaction of T<sub>regs</sub> with self antigens in the pathogen-free organism. Interestingly, T<sub>regs</sub> in the fetal and adult thymus express lower levels of CD25 and are of naive phenotype, while T<sub>regs</sub> in the fetal/adult lymphoid organs as well as in the adult peripheral blood express higher levels of CD25 (CD25<sup>hi</sup>) and are of memory phenotype (eg, CD95<sup>hi</sup>, CD45RO).<sup>15,27</sup> Thus, after their emigration from the thymus, T<sub>regs</sub> recognize self antigen in the periphery and convert to memory T<sub>regs</sub>. During adult life ongoing self-antigen-specific activation of naive T<sub>regs</sub> might trigger the immunosuppressive function of T<sub>regs</sub> and thereby continuously supply the pool of memory CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub>. Up-regulation of CD95 and high sensitivity toward CD95L-mediated cell death would then allow for constant elimination of antigen-experienced T<sub>regs</sub>. Both the high turnover of mouse self-specific T<sub>regs</sub><sup>28-30</sup> and the larger proportion of activated T<sub>regs</sub> than T<sub>conv</sub>s 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>regs</sub>, and many studies have focused on this population because pure isolation of FOXP<sup>+</sup> T<sub>regs</sub> is limited to this population in humans. With the availability of specific FOXP3 mAbs for flow cytometry, we now detected naive T<sub>regs</sub> with lower CD25 expression in adult peripheral blood. This confirmed recent reports that suggested the presence of naive T<sub>regs</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<sup>-</sup> T<sub>conv</sub>s. In fact, these contaminating T<sub>conv</sub>s might contribute to the reported relatively high proliferative potential of adult naive T<sub>regs</sub> in contrast to the profound anergy of CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub>.<sup>14</sup>

It has long been appreciated that cord blood contains a distinct population of CD25<sup>+</sup>CD45RA<sup>hi</sup> T<sub>regs</sub> with suppressive function. Although PCR analysis of these CD25<sup>+</sup>CD45RA<sup>hi</sup> cells has revealed FOXP3 expression,<sup>31-33</sup> it remained unclear whether all cord blood CD25<sup>+</sup> cells express FOXP3 and are T<sub>regs</sub>. Our flow cytometric analysis clearly established that essentially all cord blood CD25<sup>+</sup> cells coexpress FOXP3. Thus, FOXP3<sup>+</sup> cells can easily be isolated from cord blood on the basis of their CD25 expression. Cord-blood-derived T<sub>regs</sub> are immunosuppressive and show proliferative anergy like adult CD4<sup>+</sup>CD25<sup>hi</sup> T<sub>regs</sub>.<sup>15,31,33,34</sup> Most importantly, expression of FOXP3 seems to be more specific for naive T<sub>regs</sub> than for recently activated T<sub>regs</sub>. Activation-induced

up-regulation of FOXP3 mRNA has been reported for *in vitro*-stimulated T<sub>eff</sub>s, and triggered a controversial discussion on FOXP3 specificity for human T<sub>regs</sub>.<sup>35-38</sup> Interestingly, we also observe a slightly higher FOXP3 expression in memory T<sub>regs</sub> than in naive T<sub>regs</sub>, which might reflect the recent activation of memory T<sub>regs</sub> by self antigen *in vivo*. However, naive FOXP3<sup>+</sup> CD4<sup>+</sup> T cells should unequivocally represent natural T<sub>regs</sub> because T<sub>eff</sub>s with activation-induced FOXP3 expression (eg, peripherally generated T<sub>regs</sub>) do not exhibit a naive phenotype. Furthermore, strict gating for CD4<sup>+</sup>CD25<sup>hi</sup> T cells had led to false low T<sub>reg</sub> numbers (1% to 2%) in human adult peripheral blood in many previous studies. With the detection of naive T<sub>regs</sub>, total T<sub>reg</sub> frequencies in mice (5% to 10%) and humans (3% to 7%) are actually comparable.

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

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

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