Foxp3-Mediated Suppression of CD95L Expression Confers Resistance to Activation-Induced Cell Death in Regulatory T Cells

Eva-Maria Weiss, Angelika Schmidt, Diana Vobis, Natalio Garbi, Katharina Lahl, Christian T. Mayer, Tim Sparwasser, Andreas Ludwig, Elisabeth Suri-Payer, Nina Oberle and Peter H. Krammer

*J Immunol* 2011; 187:1684-1691; Prepublished online 11 July 2011;
doi: 10.4049/jimmunol.1002321
http://www.jimmunol.org/content/187/4/1684
Foxp3-Mediated Suppression of CD95L Expression Confers Resistance to Activation-Induced Cell Death in Regulatory T Cells

Eva-Maria Weiss,*1 Angelika Schmidt,* Diana Vobis,* Natalia Garbi,† Katharina Lahl,‡,2 Christian T. Mayer,‡ Tim Sparwasser,‡ Andreas Ludwig,§,§ Elisabeth Suri-Payer,* Nina Oberle,*,3 and Peter H. Krammer*‡

CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) control self-reactive cells to maintain peripheral tolerance. Treg homeostasis has to be controlled tightly to ensure balanced Treg-mediated suppression. One mechanism that regulates the CD4⁺ T cell pool is activation-induced cell death (AICD). This is mimicked in vitro by TCR restimulation-induced expression of the death ligand CD95L (FasL/APO-1L/CD178) in expanded T cells. These cells express the death receptor CD95 (Fas/APO-1), and binding of CD95L to CD95 results in AICD. In contrast, Tregs do not undergo AICD upon TCR (re)stimulation in vitro despite a functional CD95 cell death pathway. In this study, we show that human and murine Tregs express low levels of CD95L upon stimulation. Knockdown of the transcriptional repressor Foxp3 partially rescues CD95L expression and AICD in human Tregs. Moreover, upon stimulation Foxp3-mutant Tregs from Scurfy mice express CD95L similar to conventional T cells. We further addressed whether exogenous CD95 stimulation provides a mechanism of Treg homeostatic control in vivo and ex vivo. Triggers of CD95 reduced Treg numbers systemically as reflected by in vivo imaging and decreased GFP⁺ Treg numbers ex vivo. Our study reveals that Foxp3 negatively regulates CD95L expression in Tregs and demonstrates that Tregs are susceptible to homeostatic control by CD95 stimulation. The Journal of Immunology, 2011, 187: 1684–1691.

Naturally occurring CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) play an essential role in fine-tuning of immune responses (1). Imbalances in the number or function of Tregs can contribute to various pathologies, including cancer, autoimmune diseases, and transplant rejection (2). A tight control of Treg homeostasis is therefore critical to maintain efficient self-tolerance.

CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) control self-reactive cells to maintain peripheral tolerance. Treg homeostasis has to be controlled tightly to ensure balanced Treg-mediated suppression. One mechanism that regulates the CD4⁺ T cell pool is activation-induced cell death (AICD). This is mimicked in vitro by TCR restimulation-induced expression of the death ligand CD95L (FasL/APO-1L/CD178) in expanded T cells. These cells express the death receptor CD95 (Fas/APO-1), and binding of CD95L to CD95 results in AICD. In contrast, Tregs do not undergo AICD upon TCR (re)stimulation in vitro despite a functional CD95 cell death pathway. In this study, we show that human and murine Tregs express low levels of CD95L upon stimulation. Knockdown of the transcriptional repressor Foxp3 partially rescues CD95L expression and AICD in human Tregs. Moreover, upon stimulation Foxp3-mutant Tregs from Scurfy mice express CD95L similar to conventional T cells. We further addressed whether exogenous CD95 stimulation provides a mechanism of Treg homeostatic control in vivo and ex vivo. Triggers of CD95 reduced Treg numbers systemically as reflected by in vivo imaging and decreased GFP⁺ Treg numbers ex vivo. Our study reveals that Foxp3 negatively regulates CD95L expression in Tregs and demonstrates that Tregs are susceptible to homeostatic control by CD95 stimulation. The Journal of Immunology, 2011, 187: 1684–1691.

Activation-induced cell death (AICD) is involved in the removal of activated T cells in vivo and depends in part on the death receptor CD95 (Fas/APO-1) and its natural ligand CD95L (FasL/APO-1L/CD178) (3, 4). In an in vitro AICD model, resting CD4⁺CD25⁺Foxp3⁺ conventional T cells (Tcons) are expanded by TCR/CD28 stimulation for 6 d. Expanded Tcons express high levels of CD95, whereas resting cells show low expression of CD95. Restimulation of expanded Tcons either through the TCR or by PMA plus ionomycin (P/I) induces CD95L expression (5, 6). Binding of CD95L to CD95 results in AICD of day 6 Tcons, whereas resting (day 0) Tcons are refractory to CD95-mediated apoptosis (5). Tregs differ from Tcons regarding CD95-mediated cell death. A large fraction of Tregs from adult blood shows a CD45R⁺ activated/memory phenotype and displays high expression of CD95 on days 0 and 6 of culture. However, TCR (re)stimulation in vitro does not result in AICD despite a functional CD95 death pathway in Tregs as demonstrated by the induction of apoptosis upon exogenous CD95 stimulation (6). Interestingly, a minority of Tregs from adult blood and most cord blood Tregs express CD45RA, a hallmark of resting/naive T cells (7, 8). The CD45RA⁺ Treg population expresses low surface CD95 and is resistant toward apoptosis induced by exogenous CD95 stimulation (9).

Tregs express the X chromosome-encoded winged/helix transcription factor Foxp3, which together with other transcription factors regulates the Treg-specific gene expression pattern. The activity of Foxp3 represses transcription of cytokines such as IL-2 and IL-4 while simultaneously enhancing expression of the IL-2R α-chain (CD25) (10). Interestingly, binding of Foxp3 in proximity to the CD95L gene was discovered in a genome-wide screen for Foxp3 target genes, but its function remained elusive (11).

The lpr and gld mice harbor mutations resulting in nonfunctional CD95 and CD95L, respectively, which lead to lymphoproliferation and imbalanced lymphocyte homeostasis. Although gld mice
display elevated Treg numbers, the latter was unaltered in lpr mice (12, 13). Moreover, several in vivo studies demonstrated that Treg numbers decline during an immune response as a result of CD95 ligation, whereas others claimed that CD95L expression by Tregs induces responder cell death in vitro (14–17). Therefore, although the CD95/CD95L system seems to play a role in Treg homeostasis, existing discrepancies need to be resolved. Most importantly, the molecular mechanism of CD95L expression by Tregs and the role of CD95 in vivo for Treg homeostasis under steady-state conditions remain enigmatic.

In the present work, we investigated the molecular basis for the resistance of CD4+CD25++Foxp3+ Tregs toward CD95-dependent AICD in vitro. We could demonstrate that Tregs, upon stimulation, displayed low levels of CD95L protein when compared with those of Tcons. Low CD95L levels on Tregs were not caused by increased cleavage of CD95L from the cell surface. However, stimulation-dependent CD95L expression in Tregs was already reduced at the mRNA level. The cellular activation status of Tregs did not play a role in CD95L expression, because neither naive nor memory Tregs could produce high amounts of CD95L. Remarkably, knockdown of Foxp3 in human Tregs could partially rescue CD95L expression and AICD. In addition, murine Foxp3-mutant scurfy (Sf) Tregs produced substantial amounts of CD95L. To address whether exogenous CD95 stimulation controls Treg homeostasis in vivo, we used Foxp3-LuciDTR mice that express Foxp3-promoter-controlled luciferase, diptheria toxin receptor, and GFP. Triggering of CD95 by administration of an agonistic anti-CD95 Ab resulted in a marked systemic decrease of Tregs in vivo and decreased Foxp3+ GFP+ Treg numbers in pooled lymph nodes (LNs) ex vivo. Our data demonstrate that Foxp3 negatively regulates AICD of Tregs by suppressing CD95L expression. However, the sensitivity of Tregs to CD95-mediated apoptosis allows the regulation of their pool size by exogenous CD95 stimulation.

Materials and Methods

**Mice**

C57BL/6, Foxp3-LuciDTR-4, Foxp3-LuciDTR-4 × lpr, and RAG2−/− × lpr mice were housed under specific-pathogen-free conditions at the central animal facility of the German Cancer Research Center. Organs or single-cell suspensions of organs from male DEREG and Sf×DEREG mice were obtained from TWINCORE for the performance of experiments. Human PBLs were obtained from buffy coats or cord blood donations by Ficoll (Biochrom) gradient centrifugation and monocyte depletion. CD4+CD25+ and CD4+CD25− cells were isolated by magnetic cell separation using a limiting amount of CD25 MicroBeads II and the CD4+ T Cell Isolation Kit II (Miltenyi Biotec) (18). Mouse PBLs were obtained from pooled LNs (inguinal, axillary, lateral axillary, mesenteric, and sacral) by single-cell separation. CD4+ cells were negatively isolated by magnetic cell separation (Miltenyi Biotec).

**Cell culture and T cell stimulation**

T cells were used directly after isolation (day 0) or after 1–6 d of culture with soluble anti-CD3 Ab (OKT3, 0.5 μg/ml, purified from hybridoma supernatants (19)) and anti-CD28 Ab (2 μg/ml) and IL-2 (100 U/ml) in X-VIVO 15 medium (Cambrex Corporation) supplemented with 1% GlutaMAX (Invitrogen). For the investigation of CD95L expression or the induction of AICD, T cells were (re)stimulated with 30 ng/ml plate-bound anti-human CD3 Ab (OKT3) or 3 μg/ml plate-bound anti-mouse CD3 Ab (2C11) or respective isotype controls (all from BD Biosciences), with 10 ng/ml PMA and 1 μM monomycin in DM20 or DMSO alone (all from Sigma-Aldrich) for the indicated time. In some experiments, cells were incubated during stimulation with 1 μM of the hydroxamate-based A disintegrin and metalloproteinase-10 (ADAM-10) inhibitor GI254023X (20, 21). Before use for AICD assays, live cells were sorted on the basis of their forward light scatter/side scatter of light profile by flow cytometry to remove dead cells resulting from Amaxa transfection.

**Flow cytometry**

For the investigation of CD95L expression, cells were incubated with biotinylated anti-human (NOK1), anti-mouse (MFL3) CD95L, or isotype control Ab and subsequently stained with allophycocyanin-labeled streptavidin (all from BD Biosciences). After being washed, cells were incubated with the allophycocyanin-specific FASER kit (Miltenyi Biotec) to amplify the signal. Expression of Foxp3 was detected using PE anti-human FOXP3 (259D, Biolegend) or an isotype control Ab (BD Biosciences) with the Foxp3 staining buffer set according to the manufacturer’s instructions (eBioscience). Annexin V (BD Biosciences) and 7-aminominoctinomycin D (Sigma Aldrich) were used according to the manufacturer’s instructions to quantify apoptotic cells. Single-cell suspensions from pooled LNs (or CD4+ cells) for the pooled LNs and spleen of Sf×DEREG and DEREG mice) were investigated with directly labeled Abs against CD3 (eBioscience), CD4, and CD19 (BD Biosciences). Cells were analyzed with a FACScanto II cytometer. The percentage mean fluorescence intensity (MFI) increase was calculated according to the formula: increase in MFI (%) = [(MFIstained − MFIisotype stained)/MFIisotype stained] × 100.

**RNA preparation and quantitative real time PCR**

Total RNA was isolated using the Absolutely RNA MicroPrep Kit (Stratagene), and cDNA was prepared using oligo(dT) primers. mRNA expression was quantified by the detection of incorporated SYBR Green using the 7500 Real-Time PCR System (Applied Biosystems). The relative expression level was determined by normalization to that of GAPDH with results presented as fold expression according to the following formula (where Ct is the threshold cycle value): relative mRNA expression = 2−ΔCt. Primer sequences were as follows: GAPDH 5′-GCA AAT TCC ATG GCA CCG TCC T-3′ (forward) and 5′-TCC CCC CAC TGG ATT TGG G-3′ (reverse); CD95L 5′-TGG AAT TGT CCT GCT TCG TGG-3′ (forward) and 5′-TGG TGC AAC ATT GAC CCC GGC G-3′ (reverse); IL-2 5′-CCA AGT GAC CAT TTA CTG GAT G-3′ (forward) and 5′-TCA GGT GTC TGG CCT TCT TGG G-3′ (reverse); IFN-γ 5′-TTC AGC TCT GCA TGG TTC TGG-3′ (forward) and 5′-TCC CCT GCA TCT GAA TCG GCT T-3′ (reverse).

**RNA interference**

A total of 3.6 × 10^7 T cell-depleted donor bone marrow (BM) cells were injected into sublethally irradiated (4.5 Gray) 5- to 8-wk-old RAG2−/− × lpr mice. Donors were gender-matched Foxp3-LuciDTR-4 or Foxp3, LuciDTR-4 × lpr mice. Experiments were started 6–10 wk after reconstitution. Mice were injected i.p. with 0.25 μg/g body weight anti-CD95 Ab (Jo2) (22) or isotype control Ab (both from BD Biosciences). As a Treg depletion control, 12 ng/g body weight diphtheria toxin (Sigma-Aldrich) was injected i.p.

**Biomimnescence imaging**

Mice were imaged 5 min after i.p. injection of 4.5 mg D-luciferin (Sigma-Aldrich) using the IVIS 100 Imaging System (Xenogen) as described previously (23). Light output was quantified as relative light units (RLU).

**Statistical analysis**

Results represent the mean ± SD. Statistical significance was determined by Student t test.

**Results**

Human Tregs display low CD95L surface protein levels caused by reduced CD95L mRNA levels

Triggering of CD95 induces apoptosis in human Tregs in vitro, but TCR stimulation-induced AICD (characterized by binding of cell-
intrinsically produced CD95L to CD95) is diminished significantly in Tregs (6, 24). Thus, a plausible explanation for the lack of AICD in Tregs is diminished or absent CD95L expression. However, contradictory results regarding CD95L expression by Tregs have been obtained (16, 25–27) that prompted us to investigate CD95L surface protein expression in Tregs compared with that in Tcons by a highly sensitive flow cytometry protocol. Tcons displayed high CD95L surface protein upon stimulation for 20 h with anti-CD3 Ab on day 6 of in vitro culture and upon P/I treatment on both day 0 (freshly isolated) and day 6 of culture (Fig. 1A). In contrast, no substantial stimulation-induced CD95L protein levels could be detected in day 0 or day 6 Tregs after stimulation with anti-CD3 Ab or P/I (Fig. 1A). Thus, using a highly sensitive flow cytometry method, we could see clearly that Tregs show reduced stimulation-induced CD95L protein levels compared with those in Tcons.

One explanation for diminished CD95L cell surface expression in Tregs might be increased cleavage of CD95L from the cell surface by metalloproteinase activity. ADAM-10 can cleave CD95L into a soluble form that has less apoptotic potential (21). To investigate the impact of CD95L cleavage from the cell surface on CD95L expression levels, T cells were incubated with the ADAM-10 inhibitor GI254023X during stimulation. Simultaneous incubation with P/I and GI254023X slightly increased CD95L expression in comparison with that of day 0 Tcons treated with only P/I, whereas no increase in CD95L surface protein was observed for Tcons stimulated with anti-CD3 Ab (Fig. 1B, upper panel). Interestingly, incubation of day 0 Tregs with the ADAM-10 inhibitor during stimulation with anti-CD3 Ab or P/I did not lead to an increase in CD95L expression (Fig. 1B, upper panel). Similarly, on day 6 of culture, Tcons showed an increase of CD95L expression upon cotreatment with P/I and the ADAM-10 inhibitor but not upon simultaneous treatment with anti-CD3 Ab and the ADAM-10 inhibitor (Fig. 1B, lower panel). Remarkably, day 6 Tregs displayed no increase in CD95L expression upon cotreatment with the ADAM-10 inhibitor and anti-CD3 Ab or P/I compared with that of Tregs that were stimulated in the absence of GI254023X (Fig. 1B, lower panel). Taken together, these results exclude CD95L cleavage by ADAM-10 as a reason for the low CD95L surface protein level in Tregs.

Because we could not detect substantial CD95L protein levels in Tregs at different time points of stimulation and after different days of culture (Supplemental Fig. 1A, 1B), we investigated CD95L expression at the mRNA level. In Tcons, CD95L mRNA was

**FIGURE 1.** Human Tregs show diminished CD95L surface protein levels due to low CD95L mRNA expression. A. Freshly isolated (day 0) and in vitro expanded (day 6) Tcons or Tregs were stimulated with plate-bound isotype control Ab (iso), plate-bound anti-CD3 Ab (a-CD3), DMSO (vehicle control), or P/I for 20 h, and CD95L surface protein expression was measured by flow cytometry. Isotype control Tcons (shaded histogram), isotype control Tregs (thin black line); CD95L Tcons (bold black line), CD95L Tregs (bold gray line). One representative of three independent experiments is shown. B. The day 0 and day 6 Tcons or Tregs were stimulated with iso, α-CD3, DMSO, or P/I in the absence or presence of the ADAM-10 inhibitor GI254023X for 20 h. CD95L surface protein expression was measured by flow cytometry. Isotype control staining (shaded histogram), isotype control staining plus inhibitor (thin black line), CD95L staining (bold black line), CD95L staining plus inhibitor (bold gray line). One representative of three independent experiments is shown. C. The day 0 and day 6 Tcons or Tregs were left unstimulated (−) or stimulated with α-CD3, DMSO (D) or P/I for 2 h. RNA was isolated from cell lysates and reverse-transcribed. CD95L mRNA expression was quantified by quantitative PCR. The relative expression level was determined by normalization to that of GAPDH with results presented as fold increase compared with unstimulated Tcons. Tcons (white bars), Tregs (gray bars). One representative of three independent experiments is shown. D. The day 0 and day 6 cord blood Tcons or Tregs were stimulated with iso, α-CD3, DMSO, or P/I for 20 h, and CD95L surface protein expression was measured by flow cytometry. One representative of two independent experiments is shown. Isotype control Tcons (shaded histogram), isotype control Tregs (thin black line); CD95L Tcons (bold black line), CD95L Tregs (bold gray line).
detectable upon P/I stimulation on days 0 and 6 of culture and after stimulation with anti-CD3 Ab on day 6, reflecting the CD95L protein data (Fig. 1C). By contrast, we observed that day 0 as well as day 6 Tregs expressed only minimal levels of CD95L mRNA upon stimulation with anti-CD3 Ab or P/I (Fig. 1C). These data clearly show that human Tregs are impaired in CD95L mRNA expression, which corresponds to results obtained with murine Tregs (28).

In general, most Tregs in adults display an activated/memory phenotype (CD45RO+), whereas human cord blood Tregs, which are not yet sensitive to CD95-mediated cell death, are almost exclusively naive (CD45RA+) (7, 8). To elucidate whether the activation status of Tregs affects CD95L expression, we investigated stimulation-dependent CD95L expression by cord blood Tregs. Similar to their counterparts from adult blood, cord blood Tcons expressed CD95L upon P/I stimulation on day 0 of culture and after treatment with anti-CD3 Ab or P/I on day 6 of culture (Fig. 1D). In contrast, cord blood Tregs, comparable to Tregs from adult blood, produced only marginal amounts of CD95L protein upon stimulation with anti-CD3 Ab or P/I on day 0 as well as on day 6 of culture (Fig. 1D). Thus, the cellular activation status of Tregs does not play a role in CD95L expression.

**Foxp3 knockout in human Tregs increases stimulation-induced CD95L expression**

We wondered which molecular mechanism might be responsible for diminished CD95L expression in Tregs. Because CD95L expression is reduced at the mRNA level in Tregs, the Treg-specific transcription factor Foxp3, which is known to act as a transcriptional repressor, is a potential candidate for CD95L suppression. To investigate the involvement of Foxp3 in the regulation of CD95L expression, we transiently knocked down Foxp3 in human Tregs, which led to diminished Foxp3 expression 72 h after siFoxp3 treatment (Fig. 2A, 2B). Upon stimulation with P/I, CD95L mRNA expression was upregulated 2-fold in siFoxp3-treated Tregs versus that in siCtrl-treated Tregs (Fig. 2C). Accordingly, mRNA expression of IL-2 and IFN-γ, two cytokines that are known to be negatively regulated by Foxp3 (29), was upregulated 2-fold in Tregs transfected with siFoxp3 compared with that of Tregs transfected with siCtrl (Fig. 2C). In Tcons, after treatment with P/I, mRNA levels for all of the genes remained unaffected by Foxp3 knockdown (Fig. 2C). Similar results were obtained by normalization to the housekeeping gene Rps-9 instead of GAPDH (data not shown). CD95L surface protein was upregulated 2-fold in siFoxp3-transfected versus siCtrl-transfected Tregs and not altered in Tcons after siRNA treatment (Fig. 2D, 2E). However, although stimulated siFoxp3-transfected Tregs expressed more CD95L, IL-2, and IFN-γ than stimulated siCtrl-transfected Tregs, they still displayed less compared with stimulated Tcons. This might be due to residual Foxp3 protein bound to regulatory DNA regions of these genes or additional factors involved in their expression control. To investigate if the increase of CD95L expression had a functional effect in Tregs, we stimulated cells by anti-CD3 Ab to trigger AICD and compared the cell death of siFoxp3-transfected versus siCtrl-transfected Tregs. Indeed, after siFoxp3 transfection, Tregs underwent cell death similar to that of Tcons (Fig. 2F). These results show that the negative regulation of CD95L induction by Foxp3 has functional effects on the AICD of Tregs. Because human Tcons were described to transiently upregulate Foxp3 expression upon TCR stimulation, we questioned if this results in transient CD95L downregulation. Therefore, we monitored Foxp3 and CD95L expression in Tcons from days 0 to 6 of stimulation. Foxp3 expression was induced in 75% of cells in day 5 Tcons but to much lower MFI values compared with those of Tregs. On day 5 of culture, when the number of Foxp3-expressing Tcons was maximal, CD95L expression also reached a maximum (Supplemental Fig. 1C, 1D). We conclude that transient Foxp3 expression in Tcons does not interfere with CD95L expression.

**Fboxp3-mutant murine Tregs show CD95L levels comparable to those of Tcons**

To validate the involvement of Foxp3 in CD95L suppression in a different system, we used murine Sf Tregs that have a mutated, unstable (and therefore nonfunctional) Foxp3. Tregs from male Sf × DEREG mice (so-called “would-be” Tregs) express GFP under control of the Foxp3 promoter and can be traced by GFP

**FIGURE 2.** Foxp3 knockout increases CD95L expression and AICD in human Tregs. A, Freshly isolated human Tcons or Tregs were transfected with siCtrl or siFoxp3. Foxp3 protein expression was investigated in Tcons or Tregs 72 h after knockdown by flow cytometry. The percentage of Foxp3+ cells is indicated above the gate. B, Quantification of Foxp3 protein expression by Tcons or Tregs shown in A. Mean ± SD of three experiments is shown. C, Seventy-two hours after siRNA transfection, Tcons or Tregs were left unstimulated or stimulated with P/I for 2 h. IL-2, IFN-γ, and CD95L mRNA expression was investigated by quantitative PCR and normalized to that of GAPDH. Unstimulated Tcons were set to 1. D, Seventy-two hours after siRNA transfection, Tcons or Tregs were treated with DMSO or P/I for 20 h, and CD95L expression was investigated by flow cytometry. Numbers indicate the percentage of CD95L-positive cells. E, Mean of the MFI increase of CD95L expression of three independent experiments is shown. F, Seventy-two hours after siRNA transfection, living cells were sorted on the basis of forward light scatter/side scatter of light profile and stimulated for 20 h with plate-bound anti-CD3 Ab. Annexin V7-aminoaductomycin D double-positive apoptotic cells were quantified by flow cytometry, and specific cell death was calculated. One representative of three independent experiments is shown.
expression (30). Similar to the human system, freshly isolated murine Tcons from wild-type mice express CD95L after stimulation with anti-CD3 Ab or PI, whereas Tregs do not (Supplemental Fig. 2). CD4+ T cells isolated from pooled LNs and spleens of control DEREG and Si × DEREG mice were stimulated with anti-CD3 Ab for 20 h. After stimulation, GFP+ (Tcon) and GFP* (Treg) cells were investigated for CD95L protein expression. As expected, Tregs from control DEREG mice produced only minimal levels of CD95L upon stimulation with anti-CD3 Ab (Fig. 3). Interestingly, “would-be” Tregs from Si × DEREG mice expressed CD95L in amounts comparable to those of DEREG and Si × DEREG Tcons (Fig. 3). Together with the data obtained from the above Foxp3 knockdown studies in human cells, this finding confirms that Foxp3 is involved in the suppression of CD95L induction by Tregs.

Murine Tregs are sensitive to CD95-mediated cell death in vivo

Tregs express the death receptor CD95 on the cell surface and are highly sensitive to apoptosis induced by exogenous CD95 stimulation in vitro (6). In vivo, Tregs can be depleted by CD95 engagement in tumors and in the intestine during colitis (14, 15, 17). However, whether the overall Treg population is sensitive to CD95 engagement in tumors and in the intestine during colitis (14, 15, 17). However, whether the overall Treg population is sensitive to CD95-induced apoptosis (32). Therefore, to investigate the effect of CD95 stimulation on Treg numbers in vivo, we established Treg numbers in vivo upon the injection of anti-CD95 Abs can induce death of CD95-sensitive cells (22). To investigate Treg numbers in vivo upon the injection of anti-CD95 Abs, we made use of the Foxp3LuciDTR mouse strain (hereafter abbreviated as Luci), which expresses GFP, diphtheria toxin receptor, and luciferase under the control of the Foxp3 promoter (23). Almost all of the Foxp3+ cells in these mice express the three gene products, and this allows for the investigation (by bioluminescence and GFP expression) and depletion (by diphtheria toxin [DT]) of Tregs. We could not directly use Luci mice to investigate depletion of Tregs by CD95 stimulation because, like wild-type mice, these mice die by liver failure (due to CD95-induced cell death of hepatocytes) upon anti-CD95 Ab injection (31, 32). The lpr mice survive anti-CD95 Ab treatment due to a mutation in CD95 that renders these mice insensitive to CD95-induced apoptosis (32). Therefore, to investigate the effect of CD95 stimulation on Treg numbers in vivo, we established BM chimeric mice with Rag2−/− × lpr mice as recipients of Luci BM (Luci → Rag2−/− × lpr). As a control, Rag2−/− × lpr mice were reconstituted with BM isolated from Luci × lpr mice (Luci × lpr → Rag2−/− × lpr). The resulting BM chimeras displayed variable degrees of luciferase activity by Tregs in vivo (Fig. 4A). Twenty-four hours after the injection of anti-CD95 Ab, we observed strong depletion of Tregs (reflected by lower luciferase activity) in Luci → Rag2−/− × lpr mice that was partially reversed after 48 h (Fig. 4A, 4B). As expected, the level of luciferase activity only changed minimally in Luci × lpr → Rag2−/− × lpr mice after anti-CD95 Ab injection, proving that a functional CD95 death receptor is needed for depletion (Fig. 4A, 4B). Treatment with an isotype control Ab did not alter luciferase activity significantly (Fig. 4A). Treg depletion by DT served as positive control and decreased luciferase activity in both types of chimeric mice (Fig. 4A, 4B and data not shown). In addition, mice were sacrificed 48 h after treatment, and the amount of GFP+ Tregs in pooled LNs was investigated. We could detect substantially fewer GFP+ Tregs in Luci → Rag2−/− × lpr mice treated with anti-CD95 Ab, whereas the frequency of GFP+ cells remained almost constant in Luci × lpr → Rag2−/− × lpr mice (Fig. 4C). DT injection as a positive control for the depletion of Tregs diminished the frequency of GFP+ cells in Luci → Rag2−/− × lpr and Luci × lpr → Rag2−/− × lpr mice (Fig. 4C and data not shown). Tcon numbers were not altered significantly in mice treated with anti-CD95 Ab or DT (Fig. 4C). In summary, these results demonstrate that Tregs are sensitive to CD95-induced cell death in vivo and can be depleted systemically by anti-CD95 Ab treatment.

Discussion

A balance between Treg and effector cell subsets is important for the initiation, course, and outcome of immune responses. The largest fraction of Tregs in adults, effector/memory Tregs, have elevated expression of CD95 and are highly sensitive to apoptosis triggered by exogenous CD95 stimulation in vitro. However, despite a functional CD95 death pathway, Tregs do not undergo AICD triggered by TCR (re)stimulation-induced CD95L (6). We report two important findings regarding apoptosis of Tregs in the current study. First, we demonstrate that human as well as murine Tregs display reduced stimulation-induced CD95L levels compared with those of Tcons, which can be rescued partially by Foxp3 knockdown or mutation. On a functional level, Foxp3 knockdown increases AICD in Tregs. Second, consistent with in vitro data (6, 33), we demonstrate that Tregs are sensitive to apoptosis induced by CD95 stimulation in vivo in healthy mice.

Our results demonstrate that human as well as murine Tregs are unable to express high surface levels of CD95L upon stimulation. In the case of human Tregs, investigation of freshly isolated (day 0) Tregs as well as in vitro expanded (day 6) cells showed that stimulation-induced CD95L surface expression was diminished compared with that of Tcons. On day 6 of culture, some of the cells in the Treg preparation expressed CD95L; however, these are most likely contaminating Tcons that accumulate from days 0 to 6 of culture due to vigorous proliferation. FACs-based cell sorting after MACS isolation to obtain even purer Treg populations reduced the number of CD95L+ cells in the Treg population on day 6, affirming that these were contaminating Tcons (data not shown).

Cleavage of CD95L into a less active soluble form might contribute to the relative resistance of Tregs to AICD. ADAM-10 plays a crucial role in the cleavage of CD95L protein from the cell surface of T cells (21). However, we could not detect differences in CD95L expression in Tregs incubated with an ADAM-10–specific inhibitor during stimulation, which excludes CD95L cleavage as a reason for low CD95L expression by Tregs. Another factor that could play a role in sensitivity to CD95-induced apoptosis of Tregs is the cellular activation status. Although effector/memory Tregs, the largest Treg fraction in adult blood, are highly sensitive to apoptosis induced by exogenous CD95 stimulation, naive/resting Tregs abundantly found in cord blood are resistant (6, 9). Nevertheless, naive/resting Tregs did not express substantial stimulation-induced CD95L. This establishes low CD95L expression as a characteristic of all Tregs independent of their activation status and apoptosis sensitivity. In addition, our kinetics...
data demonstrate that CD95L expression in Tregs is low irrespective of the time point (days 0 to 6) or duration (0.5 to 40 h) of stimulation, which leads to the conclusion that CD95L expression by Tregs is truly impaired.

In contrast to our results, Baatar et al. (16) demonstrated that human Tregs can express CD95L. However, it is difficult to compare the level of CD95L expression to that of Baatar et al. (16) because Tregs were not TCR stimulated and Tcons were not used as a reference for expression. Nevertheless, Baatar et al. (16) showed that CD95L-expressing Tregs can kill Jurkat T cells in a CD95-dependent manner. In contrast, stimulated Tregs could not induce cell death in CD95-sensitive SKW6.4 B cells in our hands, whereas stimulated Tcons induced cell death (data not shown).

Reduced CD95L expression in human Tregs was not confined to protein levels but also detected at the mRNA level, which prompted us to investigate the involvement of a transcriptional regulator in CD95L expression. The molecular mechanism of CD95L expression by Tregs has not been investigated in detail and might involve the transcription factor Foxp3. Foxp3 acts as a transcriptional activator on some promoters (e.g., IL-2 and IFN-γ), whereas it represses the activation of others (e.g., CD25), whereas it represses the activation of others (e.g., IL-2 and IFN-γ) (10). The presence of Foxp3 in proximity to the CD95L gene has been shown in three independent genome-wide Foxp3 target gene analyses but was not investigated further (11, 34, 35). We employed two experimental systems to elucidate the involvement of Foxp3 in CD95L repression. First, Foxp3 was knocked down transiently in human Tregs, whereas in a second approach, GFP-labeled Foxp3-deficient Tregs isolated from Sf × DEREGR mice were investigated. Stimulation of human Tregs treated with siFoxp3 demonstrated approximately doubled CD95L mRNA and protein expression compared with those of siCtrl-treated Tregs. Nevertheless, Baatar et al. (16) showed that CD95L-expressing Tregs can kill Jurkat T cells in a CD95-dependent manner. In contrast, stimulated Tregs could not induce cell death in CD95-sensitive SKW6.4 B cells in our hands, whereas stimulated Tcons induced cell death (data not shown).

Reduced CD95L expression in human Tregs was not confined to protein levels but also detected at the mRNA level, which prompted us to investigate the involvement of a transcriptional regulator in CD95L expression. The molecular mechanism of CD95L expression by Tregs has not been investigated in detail and might involve the transcription factor Foxp3. Foxp3 acts as a transcriptional activator on some promoters (e.g., IL-2 and IFN-γ), whereas it represses the activation of others (e.g., CD25), whereas it represses the activation of others (e.g., IL-2 and IFN-γ) (10). The presence of Foxp3 in proximity to the CD95L gene has been shown in three independent genome-wide Foxp3 target gene analyses but was not investigated further (11, 34, 35). We employed two experimental systems to elucidate the involvement of Foxp3 in CD95L repression. First, Foxp3 was knocked down transiently in human Tregs, whereas in a second approach, GFP-labeled Foxp3-deficient Tregs isolated from Sf × DEREGR mice were investigated. Stimulation of human Tregs treated with siFoxp3 demonstrated approximately doubled CD95L mRNA and protein expression compared with those of siCtrl-treated Tregs. Despite the enhancement of CD95L expression, CD95L levels comparable to those of stimulated Tcons were not reached. This might be due to 1) low levels of residual Foxp3 after knockdown that could suppress CD95L expression to a certain extent, 2), the high stability/half-life of Foxp3-containing transcription factor complexes bound to DNA, or 3) other factors involved in CD95L gene regulation. Strikingly, expression of the known Foxp3 target genes IL-2 and IFN-γ was affected similarly by Foxp3 knockdown as CD95L expression, strengthening the assumption that Foxp3 controls CD95L expression. In line with these results, Foxp3-deficient Sf Tregs, which completely lack functional Foxp3, displayed elevated CD95L expression that was comparable to that of stimulated Tcons. Although CD95L was induced only 2-fold in siFoxp3-treated Tregs, we observed an effect on AICD that is mediated in part by CD95L. Together, the data obtained from the experiments with human and murine Tregs demonstrate that CD95L expression and AICD are affected by Foxp3 in Tregs. However, whether this effect mainly relies on direct interaction of Foxp3 with the CD95L gene or is achieved by or together with other factors remains to be investigated in detail. Our results are supported by the data of three independent laboratories that show that Foxp3 directly binds in proximity of the CD95L gene (11, 34, 35), although its role as a direct regulator of CD95L expression remains to be investigated in further experiments.

Several groups have reported transient upregulation of Foxp3 in stimulated human Tcons; however, whether this transiently expressed Foxp3 is functional and sufficient to confer Treg properties remains controversial (36, 37). We show that during in vitro culture from days 0 to 6 Tcons simultaneously upregulate Foxp3 and CD95L expression, which implies that transiently expressed Foxp3 is not sufficient to block CD95L expression in Tcons. In vitro, Tregs are sensitive to CD95-induced apoptosis. In the current study, we found that Tregs can be depleted systemically in vivo by CD95 stimulation via a single injection of an agonistic...
anti-CD95 Ab. We observed incomplete depletion of Tregs by CD95 treatment, yet the Treg population in mice is heterogeneous, containing effector/memory as well as resting/naive cells. In humans, only the effector/memory Treg population is sensitive to CD95-induced cell death, whereas resting/naive Tregs are resistant (9). It also is conceivable that in vivo only the effector/memory fraction of Tregs is sensitive to CD95-induced apoptosis, whereas the resting/naive Treg population is resistant. Resting/naive Tregs therefore would be spared from depletion by CD95 engagement and explain the residual Treg population.

Depletion by CD95 was comparable to depletion by DT. Surprisingly, depletion was only transient, because 48 h after injection Treg numbers had increased compared with those at the 24-h time point. This might be due to homeostatic proliferation of surviving Tregs as has been published for the depletion of Tregs by DT (38). However, data describing the depletion of Tregs by DT, albeit in different mouse strains, show that Treg numbers remain low even after 48 h (39, 40). In addition, the recovery of Treg numbers after anti-CD95 Ab (Jo2) injection is unexpectedly fast. Unfortunately, detailed studies on pharmacokinetics for this specific Ab are not available. Because Ab pharmacokinetics depend on the isotype as well as on the Ag due to either internalization after target binding or sequestration to specific organs (e.g., the spleen) (41), it is difficult to estimate the t_{1/2} of the specific Ab used in our study. In addition, different mouse strains might show disparities in Ab half-life. Of note, Treg numbers recover similarly fast in DT- and Ab-treated mice. It is therefore conceivable that the mouse model that we used for the depletion of Tregs (sublethally irradiated RAG2−/− × lpr mice reconstituted with Luci BM) shows different kinetics compared with those of non-reconstituted mice. In addition, the dose of DT and the number of injections, which are different compared with those of previous publications, might alter kinetics. Furthermore, other mechanisms such as conversion of Tcons could lead to the reappearance of Foxp3+ cells, either together with homeostatic proliferation or alone. Further experiments are needed to elucidate the exact mechanism.

Our results demonstrate that Tregs are sensitive to CD95-induced apoptosis in healthy animals. This sensitivity of Tregs to CD95-induced apoptosis is consistent with those in different publications where CD95-mediated apoptosis of Tregs was investigated in different disease settings (14, 15, 17). Chen et al. (15) were interested in the effect of CD95-mediated depletion of Tregs in the tumor environment. They engineered cancer cells to display CD95L on their cell surfaces that could kill Tregs at the tumor site whereas control cells could not. Depletion of Tregs in this tumor model led to a decrease in tumor mass. In addition, a recent report by Gritzapis et al. (17) showed that CD95L-expressing CD4+ Tcons in the tumor can reduce the number of Tregs. In line with these results, it was shown that CD4+ Tcons could kill Tregs in a CD95-dependent fashion in vitro (27). In another study, Reardon et al. (14) investigated Treg numbers during drug-induced colitis. They found that Tregs were depleted from the inflamed tissue site by a yet unidentified cell type expressing CD95L. The mechanism of Treg depletion was only transient but might be a short-term event that allows robust pathogen-clearing immune responses after local injury. The decline of CD95L+ cells after the clearance of the pathogen will allow the repopulation of Tregs and probably help to end the immune response. In the context of autoimmune disease, however, constant elimination of Tregs by hyperactivated self-reactive effector T cells via CD95-mediated apoptosis could result in a hyperactive immune system.

In addition to the above-mentioned studies, our data provide evidence that Tregs are not only sensitive to CD95-induced apoptosis during disease but also in disease-free animals. This implies that Tregs are susceptible to homeostatic control by exogenous CD95 stimulation, although they cannot exert homeostatic self-regulation by CD95-mediated AICD, thus confirming in vitro data (6, 33). Within the CD4+ T cell compartment, this is a unique feature characteristic of Tregs. Therefore, it is conceivable that CD95L-expressing cell subsets are involved in the regulation of Treg numbers. Which CD95L-expressing cell types control the systemic Treg pool in vivo under physiological conditions remains to be clarified.

Our data are in contrast to that of Banz et al. (42) who observed resistance of prestimulated murine CD4+CD25+ T cells toward apoptosis triggered by CD95 stimulation in vitro. However, because a high percentage of Tregs dies spontaneously during in vitro culture, prestimulation might lead to an underestimation of CD95-induced apoptosis. Moreover, compared with our in vivo finding that Tregs die upon CD95 engagement, low CD95L expression by Tregs themselves after stimulation in vitro should be considered as a prerequisite to ensure their survival. CD95L-expressing Tregs would constantly commit suicide or fratricide. Interestingly, there might be a switch from sensitivity to resistance to CD95-induced apoptosis in Tregs. Tregs in head and neck squamous cell carcinoma express CD95L, a finding that remains to be analyzed in other diseases, and are presumably resistant to CD95-induced apoptosis (27). Furthermore, former Foxp3+ T cells (Tregs that lost Foxp3 expression) that have been shown previously to express an altered cytokine pattern should be tested for their ability to express CD95L (43).

For therapy, the sensitivity of Tregs to CD95-mediated apoptosis might be exploited to manipulate these cells in disease. In particular, Treg death might be decreased by CD95/CD95L blocking reagents or by Treg-specific Abs coupled to siRNA against CD95.

Acknowledgments
We thank Rüdiger Arnold, Markus Brechmann, Karsten Gülöw, Daniel Röth, Sabine Sass, and Heiko Weyd for critically reading the manuscript, Günter J. Hämerling, Marie-Christine Kühnle, Tewfik Miloud, and Janine Suffer for providing the Foxp3+LuciDTR mouse strain and for helpful discussions, Benedikt Fritzsching for helpful discussions, Birgitta Maurer for cord blood cells, and Uschi Matiba for excellent technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References

Foxp3 NEGATIVELY REGULATES CD95L EXPRESSION IN Tregs


Foxp3 NEGATIVELY REGULATES CD95L EXPRESSION IN Tregs


Foxp3 NEGATIVELY REGULATES CD95L EXPRESSION IN Tregs


Foxp3 NEGATIVELY REGULATES CD95L EXPRESSION IN Tregs


Supplemental Figure 1: CD95L surface expression by human Treg is low at all investigated time points, and transient Foxp3 expression in Tcon does not correlate with CD95L repression.

Figure S1. **CD95L surface expression by human Treg is low at all investigated time points, and transient Foxp3 expression in Tcon does not correlate with CD95L repression.** (A) Day 0 and day 6 Tcon or Treg were stimulated with plate-bound isotype control (iso), plate-bound anti-CD3 antibody (α-CD3), DMSO or P/I for 0.5, 10, 20 and 40 hours and CD95L surface protein expression was measured by flow cytometry. One of two independent experiments is shown. (B) Day 0-6 Tcon or Treg were stimulated with the reagents described in (A) for 20 hours and CD95L surface protein expression was measured by flow cytometry. One representative of two independent experiments is shown. Tcon (open symbols), Treg (gray filled symbols); isotype control (square), α-CD3 (triangle), DMSO (circle), P/I (diamond). MFI increase is calculated over unstained cells. (C) Foxp3 and (D) CD95L expression by day 0 to day 6 in vitro cultured Tcon was investigated by flow cytometry. Numbers indicate MFI and % Foxp3-positive cells in (C).
Fig. S2. Murine Treg show diminished CD95L surface protein compared to Tcon. Day 0 Tcon or Treg were stimulated with isotype control antibody (iso), anti-CD3 antibody (α-CD3), DMSO or P/I for 20 hours and CD95L surface protein expression was measured by flow cytometry. Isotype control Tcon (shaded histogram), isotype control Treg (thin black line); CD95L Tcon (bold black line); CD95L Treg (bold gray line), One representative of three independent experiments is shown.