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CUTTING EDGE

Cutting Edge: In Contrast to Effector T Cells, $CD4^+CD25^+FoxP3^+$ Regulatory T Cells Are Highly Susceptible to CD95 Ligand- but Not to TCR-Mediated Cell Death[1]

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$CD4^+$ $CD25^+$ FoxP3$^+$ regulatory T cells (T_{reg}) suppress T cell function and protect rodents from autoimmune disease. Regulation of T_{reg} during an immune response is of major importance. Enhanced survival of T_{reg} is beneficial in autoimmune disease, whereas increased depletion by apoptosis is advantageous in cancer. We show here that freshly isolated FACS-sorted T_{reg} are highly sensitive toward CD95-mediated apoptosis, whereas other T cell populations are resistant to CD95-induced apoptosis shortly after isolation. In contrast, TCR restimulation of T_{reg} in vitro revealed a reduced sensitivity toward activation-induced cell death compared with $CD4^+$ $CD25^-$ T cells. Thus, the apoptosis phenotype of T_{reg} is unique in comparison to other T cells, and this might be further explored for novel therapeutic modulations of T_{reg}. The Journal of Immunology,* 2005, 175: 32–36.

The function and manipulation of $CD4^+CD25^+$ FoxP3$^+$ regulatory T cells (T_{reg})[3] during an immune response have gained a lot of attention (1, 2). Depletion of T_{reg} leads to autoimmunity in mice (1), and dysfunction of T_{reg} has been linked to human autoimmune diseases (3–5). Whereas therapeutical expansion of T_{reg} may be advantageous in autoimmunity, accumulation of immunosuppressive T_{reg} in tumors could be detrimental (6). Depletion of T_{reg} should be beneficial in cancer, but specific therapeutic tools such as T_{reg}-depleting Abs are limited. Thus, new strategies to modulate survival or apoptosis of T_{reg} are warranted.

The investigation of apoptotic properties of T_{reg} is important, because they may be used to modulate the ratio of T_{reg} to effector T cells (T_{eff}). Taams et al. (7) reported a high susceptibility of human T_{reg} to spontaneous cell death or cytokine-deprivation death, and murine T_{reg} can be depleted due to their susceptibility to cyclophosphamide toxicity or gamma irradiation (8, 9). Conversely, other groups reported apoptosis resistance of murine T_{reg} when cells were treated with dexamethasone or anti-CD95 Ab (10, 11). While information on the apoptosis sensitivity of murine T_{reg} is inconsistent, CD95-mediated apoptosis of T_{reg} has not been studied in humans.

Apoptosis mediated by the interaction of CD95 (Apo-1/Fas) with CD95 ligand (CD95L) is well characterized in T cells (12). CD95 is widely expressed (13), whereas expression of CD95L is tightly regulated (14). Although 20–60% of naive $CD4^+$ T cells express CD95, >90% of them are resistant to CD95-mediated apoptosis (15). However, several days after activation, they become sensitive toward CD95-mediated apoptosis and up-regulate CD95L after TCR restimulation (12). Subsequently, CD95L triggers apoptosis of CD95$^+$ activated T cells, a phenomenon called activation-induced cell death (AICD). AICD is a major mechanism to eliminate the expanded pool of effector lymphocytes during the contraction phase of the immune response and to maintain lymphocyte homeostasis (12).

To further clarify the physiology of T_{reg}, we studied their apoptosis phenotype ex vivo. We show for the first time that freshly isolated T_{reg} are highly sensitive toward CD95L-mediated apoptosis unlike their resistant T_{eff} counterparts. In contrast, we find that T_{reg} are substantially less sensitive to AICD than T_{eff}.

Materials and Methods

Abs and reagents

The mAbs against CD4, CD62L, and CD95L (Nok-1) were obtained from BD Pharmingen, and anti-CD25 Ab from Miltenyi Biotec. CD95L was produced as a leucine zipper-tagged ligand of CD95 (15, 16). The anti-CD3 Ab OKT3 and the agonistic monoclonal anti-CD95 Ab (anti-Apo-1) were purified from hybdridoma supernatants by protein A affinity purification (15). The monoclonal anti-FoxP3 Ab was a kind gift from A. Banham (Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Oxford, U.K.). The pan-caspase inhibitor zVAD-fmk was obtained from Bachem, annexin V Alexa

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[3] Abbreviations used in this paper: T_{reg}, regulatory T cell; T_{eff}, effector T cell; CD95L, CD95 ligand; AICD, activation-induced cell death; PI, propidium iodide; pb-anti-CD3, plate-bound anti-CD3 Ab.

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Fluor 488 from Molecular Probes, and propidium iodide (PI) and protein A were obtained from Sigma-Aldrich.

Lymphocyte separation

PBL were obtained from healthy individuals. $CD4^+CD25^+$ cells were first enriched using MACS beads (Miltenyi Biotec), and subsequently $CD4^+CD25^{high}$ cells were sorted with a FACS-Diva cell sorter (BD Biosciences).

Cell culture and cytotoxicity assays

Freshly isolated T cells were cultured in IL-2 (100 IU) containing ex Vivo-15 medium (Cambrex) supplemented with 1% Glutamax (Invitrogen Life Technologies). For apoptosis induction, T cells were stimulated with 1 μg/ml anti-CD95 Ab and 10 ng/ml protein A or 1/10 dilution of CD95L (15). Unstimulated cells were incubated with an isotype control Ab or CD95L-free control medium yielding spontaneous apoptosis of 15–30% in 20 h. To enhance viability of T_{reg} and T_{eff}, some experiments were performed in wells coated with anti-CD3 Ab (30 μg/ml). To measure AICD, T cells were expanded for 6 days (day 6 T cells) with 0.1 μg/ml anti-CD3 Ab and 1 μg/ml anti-CD28 Ab in combination with irradiated JY feeder cells (kind gift from C. Falk, Institute for Molecular Immunology, GSF National Research Center for Environment and Health, Munich, Germany) and 300 IU of IL-2 (3, 17). For induction of AICD, day 6 T cells were transferred into wells containing 30 μg/ml plate-bound anti-CD3 Ab (pb-anti-CD3) and were cultured for 24 h. Cell death was assessed by annexin V/PI costaining and forward- to side-scatter profile. Specific cell death was calculated as described previously (15): (% experimental cell death − % spontaneous cell death)/(100% − % spontaneous cell death) × 100.

Cell surface staining

Cells were stained with PE-labeled anti-CD25 Ab or anti-CD62L Ab, PE-Cy5-labeled anti-CD4 Ab, and FITC-labeled anti-CD95 Ab, or their respective isotype control Abs, and analyzed with a FACScan cytometer with at least 10,000 T_{reg} or T_{eff} counted.

RNA preparation and quantitative RT-PCR

Total RNA was isolated using the Absolutely RNA Microprep kit (Stratagene) and cDNA was prepared using random oligo(dT) primers (Invitrogen Life Technologies). FoxP3 message expression was quantified by detection of incorporated SYBR Green using the ABI Prism 5700 sequence detector system (Applied Biosystems). The relative expression level was determined by normalization to GAPDH with results presented as fold expresssion of T_{eff} mRNA levels. FoxP3 primer sequences were as follows: 5′-AGC TGG AGT TCC GCA AGA AAC (forward) and 5′-TGT TCG TCC ATC CTC CTT TCC (reverse).

Results

$CD4^+CD25^+FoxP3^+$ T_{reg} are CD95 positive

Previous reports using magnetic bead-isolated $CD4^+CD25^+$ T cells showed CD95 expression on human and murine $CD25^+$ cells (7, 16). We confirmed these data on $CD4^+$ T_{reg} FACS-sorted for very high CD25 expression. These cells induced strong suppression of both T cell proliferation and cytokine production (data not shown) and expressed 100-fold higher FoxP3 levels than T_{eff} cells (see Fig. 2*B*). Almost all T_{reg} expressed scurfin protein in the nucleus as determined by immunocytochemistry (data not shown). Naive T_{eff} were sorted by gating on $CD4^+CD25^-$ T cells, which were negative for scurfin expression and showed extremely low FoxP3 levels (see Fig. 2*B*). T_{reg} expressed higher levels of CD95 molecules on the cell surface as compared with naive T_{eff} (Fig. 1*A*). T_{reg} remained CD95 positive during short-term (day 6) and long-term (day 20) in vitro expansion (Fig. 1*A*).

$CD4^+CD25^+FoxP3^+$ T_{reg} are sensitive to CD95L-induced apoptosis without TCR prestimulation

To test whether freshly isolated T_{reg} are sensitive to CD95-mediated apoptosis without previous TCR stimulation, we incubated FACS-sorted T_{reg} and T_{eff} with cross-linked anti-CD95 Ab (anti-Apo-1) for 20 h. As reported previously (12), freshly isolated T_{eff} did not die upon addition of anti-CD95 Ab or soluble CD95L. However, T_{reg} treated under the same conditions showed a very high induction of apoptosis to both stimuli (Fig. 1*B*). We have carefully titrated both CD95L (Fig. 1*C*) and anti-CD95 Ab and performed experiments side by side with both reagents. Although both reagents yielded similar results, soluble CD95L might be the more physiological mimic to trigger CD95-mediated apoptosis than anti-CD95 Ab. After 4–6 days of TCR stimulation in vitro $CD4^+$ T cells are usually sensitive toward the extrinsic pathway of apoptosis, which is initiated by the binding of CD95L to CD95 and is then executed by a cascade of caspases (12). To test whether CD95L-induced apoptosis of freshly isolated T_{reg} involves these events, we used a neutralizing Ab against CD95L as well as zVAD-fmk as an

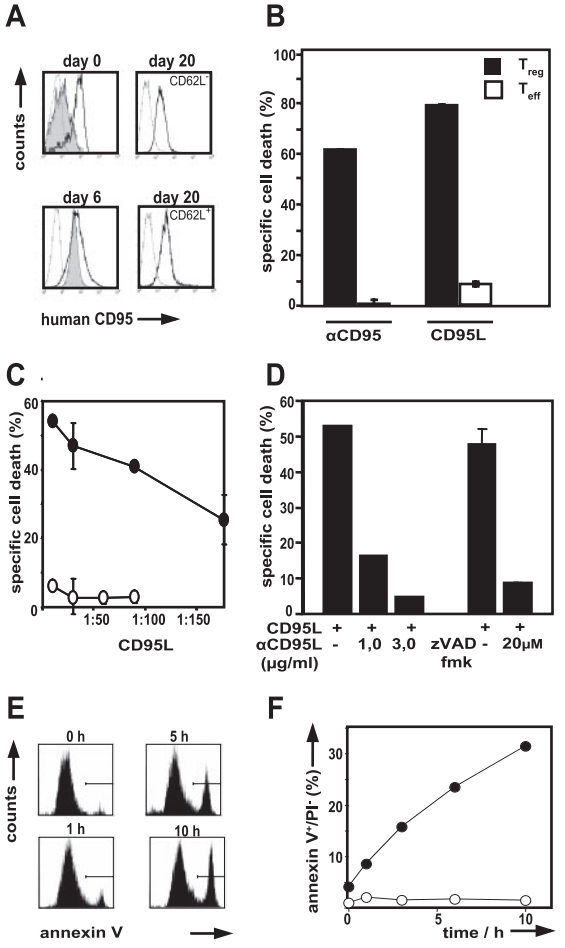

FIGURE 1. $CD4^+CD25^+FoxP3^+$ T_{reg} are sensitive to CD95L-induced apoptosis without prestimulation in vitro. *A*, Freshly isolated (day 0) T_{reg} and in vitro-expanded T_{reg} (day 6 and 20) were stained with anti-CD95-FITC Ab and analyzed by FACS. Dashed line, Isotype control; filled profile, T_{eff} ($CD4^+CD25^-$); open profile, T_{reg} ($CD4^+CD25^{high}$). *B*, T_{reg} (■) and T_{eff} (□) were stimulated with soluble CD95L or anti-CD95 Ab cross-linked with protein A for 20 h, and specific cell death was determined as described in *Materials and Methods*. *C*, Dose-response curve of CD95L-induced cell death of T_{reg} (●) and T_{eff} (○). *D*, For inhibition of CD95L-mediated apoptosis, T_{reg} were incubated with indicated amounts of anti-CD95L Ab or caspase inhibitor zVAD-fmk before CD95L stimulation. *E* and *F*, T_{reg} and T_{eff} were stimulated for the indicated time points with anti-CD95 Ab (1 μg/ml) cross-linked with protein A. *E*, T cells were stained with annexin V/PI, analyzed by FACS, and gated for viable and early apoptotic cells (PI^-), whereas PI^+ necrotic or late apoptotic cells were excluded. Apoptosis was measured at different time points on gated PI^- cells. *F*, Annexin V^+PI^- T_{reg} (●) and annexin V^+PI^- T_{eff} (○) were plotted against time. Error bars are SD of triplicate or duplicate samples.

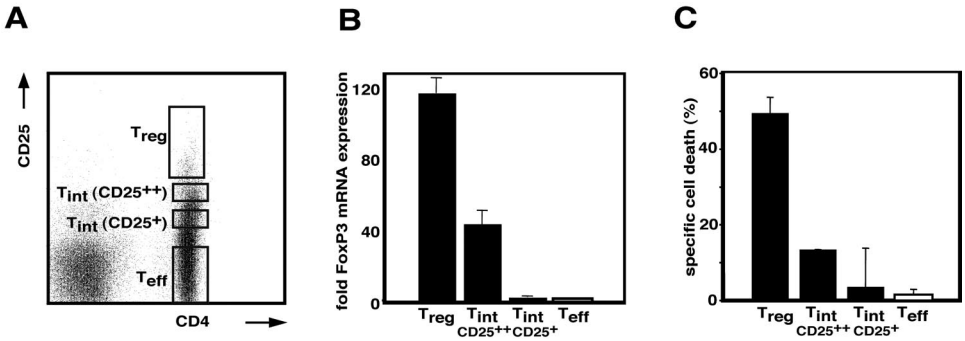

FIGURE 2. Sensitivity to CD95L-induced apoptosis without TCR prestimulation is a unique feature of $CD4^+CD25^+FoxP3^+T_{reg}$. *A*, Using four-way sorting, four different subpopulations with regard to their CD25 expression were simultaneously obtained from human PBL: $CD25^-$ T_{eff}, intermediate T cells with low $CD25^+$ expression (T_{int} $CD25^+$), intermediate T cells with high $CD25^+$ expression (T_{int} $CD25^{++}$), and T_{reg} with the highest CD25 expression. *B*, Sorted subpopulations were analyzed by quantitative PCR for their FoxP3 mRNA content. *C*, Specific cell death was measured after CD95 cross-linking (1 μg/ml) for 20 h. One representative result of three is shown. Error bars are SD of triplicate or duplicate samples.

inhibitor of caspase activity. Both treatments blocked CD95-mediated apoptosis in a concentration-dependent manner (Fig. 1*D*). Once apoptosis has been triggered, annexin V staining and uptake of PI allows distinguishing between early apoptotic and late apoptotic or necrotic cells. Apoptotic cell death started within the first hour after CD95 triggering and led to 30% early apoptotic cells ($annexin^+PI^-$) by 10 h (Fig. 1, *E* and *F*). After 20 h, most of the apoptotic cells had lost membrane integrity ($annexin\ V^+PI^+$), resulting in a total of 40–60% specific cell death, which did not increase further until 48 h (data not shown). Similar observations were also made with freshly isolated $CD4^+CD25^+$ T cells from murine spleen and lymph nodes demonstrating a consistent phenotype between murine and human T_{reg} (data not shown).

Sensitivity to CD95L-induced apoptosis without TCR prestimulation is a unique feature of $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg}

Using high-speed four-way FACS, we simultaneously sorted four different subpopulations from peripheral blood: $CD25^-$ T_{eff}, intermediate T cells with low $CD25^+$ expression (T_{int} $CD25^+$), intermediate T cells with high $CD25^+$ expression (T_{int} $CD25^{++}$), and T_{reg} with the highest CD25 expression (Fig. 2*A*). Quantitative PCR analysis revealed very high FoxP3 mRNA expression in T_{reg}. Low amounts of FoxP3 mRNA were also detectable in the adjacent T_{int} $CD25^{++}$ subpopulation, whereas T_{int} $CD25^+$ and $CD25^-$ T_{eff} cells were essentially $FoxP3^-$ (Fig. 2*B*). Similarly, only the T_{reg} subpopulation showed significant suppressive capacity and anergy as determined by proliferation assays (data not shown). Among the four freshly isolated T cell subpopulations, CD95-induced apoptosis was limited to cells with the highest expression of T_{reg} markers (CD25, FoxP3) (Fig. 2*C*). We suggest that only the $CD25^{++}$ cells are T_{reg} and that cells within the T_{int} $CD25^{++}$ sort gate comprise a mixture of T_{reg} and activated T_{eff}. This "contamination" of T_{reg} in the T_{int} $CD25^{++}$ subpopulation could explain the small increase in apoptosis upon CD95 cross-linking.

AICD is diminished in $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg}

When previously activated lymphocytes encounter a second TCR signal, they express CD95L and kill each other by AICD. To test whether T_{reg} undergo AICD, we prestimulated $CD25^{++}$ cells with anti-CD3/anti-CD28 Abs in combination with FcR-bearing, cross-linking feeder cells and IL-2 (3, 17). This activation protocol allowed proliferation of T_{reg} and resulted in expansion of T_{reg} within 6 days of stimulation. At day 6, T_{reg} were restimulated by pb-anti-CD3 Ab for 24 h. Surprisingly, T_{reg} were significantly less sensitive to AICD than T_{eff}, although both cell types showed similar apoptosis sensitivity toward CD95L treatment (Fig. 3*B*). Because freshly isolated T_{reg} died in response to CD95 stimulation, we tested whether they would also be killed by pb-anti-CD3 Ab. However, stimulation of day 0 T_{reg} with pb-anti-CD3 Ab for 24 h yielded even less cell death compared with T_{reg} cultured in IL-2 medium alone (Fig.

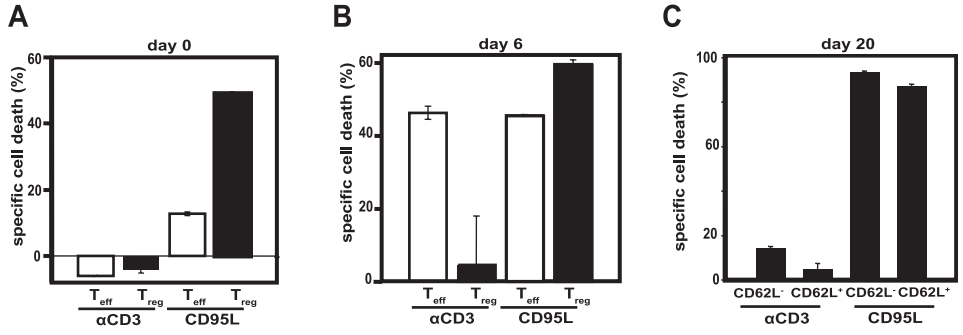

FIGURE 3. AICD is low in $CD4^+CD25^+FoxP3^+$ T_{reg}. *A*, Freshly isolated T_{reg} and T_{eff} were stimulated with pb-anti-CD3 Ab (30 μg/ml) for 24 h or with CD95L for 20 h. *B*, In an in vitro model of AICD, human T_{reg} and T_{eff} were incubated with 0.1 μg/ml anti-CD3 Ab and 1 μg/ml anti-CD28 Ab together with irradiated JY feeder cells and IL-2 for 6 days and then restimulated as described in *A*. *C*, Human T_{reg} were expanded for 20 days similar to *B*, and $CD62L^+$ cells as well as $CD62L^-$ cells were FACS-sorted from expanded T_{reg}. Next, cells were restimulated as described in *A*. Error bars are SD of triplicate or duplicate samples.

3*A*). Next, we tested T_{reg} expanded for 20 days (100- to 1000-fold expansion) before restimulation with pb-anti-CD3 Ab. We repeatedly observed $CD62L^+$ and $CD62L^-$ cells in anti-CD3/anti-CD28-expanded T_{reg} cultures, which were both suppressive. Restimulation of both subpopulations with pb-anti-CD3 Ab did not induce AICD, whereas CD95 killing was preserved (Fig. 3*C*). In summary, we found that freshly isolated T_{reg} are highly sensitive to CD95 cross-linking in contrast to their resistant $CD4^+CD25^-$ T_{eff} counterparts. However T_{reg} are less sensitive to AICD compared with T_{eff}.

Discussion

Upon Ag encounter, T cells are activated, proliferate, and exert their effector functions as T_{eff} (12). Once the Ag is cleared, most of the T_{eff} die and only a few T cells are thought to remain as memory cells. AICD is one of the main mechanisms for T cell contraction in vivo (12, 18). Freshly isolated naive T_{eff} are resistant to CD95-mediated apoptosis in vitro. Four to 6 days after TCR stimulation in vitro, T_{eff} become sensitive toward CD95-mediated apoptosis induced by CD95L or TCR restimulation (12). In contrast, we show for the first time that freshly isolated human T_{reg} are highly sensitive to CD95-mediated apoptosis. We demonstrate that sensitivity of such T cells is a unique feature of $CD25^{++}$ T_{reg} within the $CD4^+$ T cell compartment.

Papiernik and colleagues (11) observed a resistance of prestimulated $CD4^+CD25^+$ T cells derived from C57BL/6 mice toward apoptosis triggered by anti-CD95 Ab. However, this study included neither freshly isolated FACS-sorted T cells nor human T cells. Given the relatively high percentage of T_{reg} dying spontaneously even under conditions of T_{reg} proliferation and expansion (data not shown), some of the CD95-sensitive $CD4^+CD25^+$ cells might have died during their prestimulation phase. This might lead to an underestimation of CD95-triggered cell death. In addition, we used CD95L, which might bind and multimerize CD95 more efficiently than the anti-murine CD95 Ab used by Papiernik and colleagues (11). We consistently observed sensitivity of T_{reg} toward CD95-triggered apoptosis in both freshly isolated human T_{reg} (day 0) and short-term-stimulated T_{reg} (days 2–6) as well as in long-term-expanded T_{reg} (day 20).

In vivo, the apparent selective sensitivity of T_{reg} to CD95L might be an important mechanism to eliminate T_{reg} during the acute effector phase of an immune response at a time when T_{eff} are resistant to CD95-mediated apoptosis. In particular, in the presence of a fulminant acute infection, T_{reg} could be detrimental to the organism and a rapid elimination of T_{reg} in danger situations seems appropriate. In this case, T_{reg} might be killed either by soluble CD95L or adjacent membrane-bound CD95L expressed, e.g., on dying infected cells. Reduced elimination of T_{reg} during acute infection might hamper T_{eff} cells to clear the infection as recently shown in a model of leishmaniasis (19, 20).

Our second finding demonstrates a relative resistance of human T_{reg} to AICD. Neither freshly isolated T_{reg} nor T_{reg} activated for 6 days or expanded for 3 wk showed significant AICD upon TCR stimulation or restimulation. In contrast, activated T_{eff} are AICD sensitive (12) and thus die due to the weekly TCR restimulation (3, 21), whereas T_{reg} expand (data not shown). This observation further supports a reduced AICD sensitivity of T_{reg}. Interestingly, T_{reg} also express CD95L mRNA upon TCR restimulation (data not shown). Further studies including quantitative analysis of CD95L mRNA expression and CD95L protein expression might clarify the reason for reduced AICD sensitivity of T_{reg}. Other mechanisms including proteolytical cleavage of CD95L and CD95 into antagonistic products could further explain the relative AICD resistance of T_{reg} (22, 23).

Because T cell responses to pathogens could not only prime T cells for pathogen elimination, but also induce autoaggressive T_{eff}, prolonged survival of AICD-resistant T_{reg} in the critical down-phase of an immune response is conceivable. In support of this idea, T_{reg} have been described to escape from clonal deletion induced by Ag-specific (re)stimulation in vivo (24–26).

Even in the absence of AICD, T_{reg} numbers might be contracted during the down-phase of the immune response. First, the rapid decline of T_{eff} cells leads to much lower IL-2 levels, which may become limiting, and thus eliminates surplus T_{reg} via death by cytokine deprivation (27). Second, our data offer the possibility that CD95L-expressing T_{eff} might kill neighboring T_{reg}. Although still hypothetical, T_{eff} might keep T_{reg} in check by modulating their survival, whereas T_{reg} might mainly suppress effector function of T_{eff}, resulting in a tightly balanced ratio of both T cell populations. Obviously, further work is warranted to explore the use of the CD95/CD95L system for specifically modulating T_{reg} cell number and function in vivo.

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Disclosures

The authors have no financial conflict of interest.

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