# Efficient expansion of regulatory T cells *in vitro* and *in vivo* with a CD28 superagonist

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$CD4^+CD25^+$ T cells play a central role in the suppression of autoimmunity and inflammation, making their *in vivo* expansion a highly attractive therapeutic target. By phenotyping with a novel rat CTL antigen-4 (CTLA-4)-specific monoclonal antibody (mAb) and functional *in vitro* assays, we here first establish that rat $CD4^+CD25^+$ T cells correspond to the regulatory T cells (T*reg* cells) described in mice and humans: they constitutively express CTLA-4, produce IL-10 but not IL-2, and are able to suppress the proliferation of costimulated CD25-negative indicator cells. Furthermore, we show that rat T*reg* cells respond less well than $CD25^-$ T cells to conventional costimulation, but are readily expanded *in vitro* with "superagonistic" CD28-specific mAb which are potent mitogens for all T cells without the need for TCR engagement. *In vivo*, functional T*reg* cells are preferentially expanded by CD28 stimulation over other T cell subsets, leading to a 20-fold increase within 3 days in response to a single antibody dose. These data suggest that CD28-driven activation of T*reg* cells may be highly effective in the treatment of inflammatory and autoimmune diseases.

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## 1 Introduction

In mice and humans, a subset of CD4 T cells expressing CD25 and CTLA-4 has been recognized as a potent suppressor cell population counteracting autoimmunity and inflammation (reviewed in refs. [1–3]). The current interest in these "regulatory" T cells (T*reg*) arose from initial observations in post-natally thymectomized mice, which develop multi-organ autoimmunity due to a lack of this T cell subset [4], and from studies first carried out in rats which demonstrated that $CD45R^{low}$ CD4 T cells suppress wasting disease induced by $CD45R^{high}$ CD4 cells [5], as well as experimentally induced autoimmune diabetes [6] and thyroiditis [7]. In the meantime, it is recognized that also in rats, a CD25-expressing subpopulation contained within this $CD45R^{low}$ subset of CD4 cells mediates protective regulatory activity [8]. However, regulatory T cell function has also been observed in the CD25-negative subset of CD4 T cells in some mouse (reviewed in [1]) and rat models of autoimmunity [8]. At the cellular level, two types of $CD25^-$ regulatory cells, termed Tr1 and Th3, have been described based on their cytokine profiles and functional properties. The precise relationship between these, and their relationship to $CD25^+$ T*reg* cells is incompletely defined (reviewed in [1, 3]), although recent evidence in rats [8] and mice [9] suggests that CD25 expression primarily marks T*reg* cells of thymic origin.

There is growing evidence that the functional significance of CD25 expression on T*reg* cells is a requirement for IL-2 in their generation or survival. Thus, the abnormal lymphoproliferation observed in IL-2-deficient mice [10] correlates with their failure to generate this subset [11] and can be suppressed by T*reg* cells from wild-type mice [12]. Furthermore, *in vitro* studies have shown that T*reg* cells rapidly lose viability in the absence of IL-2 [11, 13]. Since mouse [14] or human [15] T*reg* cells do not themselves produce IL-2, this points to their dependence on other activated T cells which thus may support this down-regulating arm of the immune system in a paracrine fashion. Indeed, T*reg* cells do not grow or survive with TCR stimulation alone *in vitro*, but exogenous IL-2 or IL-2 derived from efficiently costimulated $CD25^-$ indicator cells in cocultures partially rescues this defect (reviewed in [1]).

There appear to be several mechanisms by which T*reg* cells suppress the activation of other T cell subsets [1, 3]. Depending on the model studied, IL-10 [16] and TGF-$\beta$ [17] seem to play important roles *in vivo*, most likely via de-activating antigen-presenting cells [18, 19] as well as

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**Abbreviations: CTLA-4:** CTL antigen-4 **CFSE:** Carboxyfluorescein diacetate succininimidyl ester **7AAD:** 7-aminoactinomycin D ***Treg* cells:** Regulatory T cells

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by direct inhibition of pathological T cell responses [20–22]. *In vitro,* the production of IL-10 and TGF-β by T*reg* cells depends on the mode of stimulation employed, and appears most pronounced under conditions of costimulation [23, 24]. Moreover, experiments in cell culture have demonstrated a cell-contact-dependent mechanism independent of known "suppressor" cytokines [14, 25, 26] which also functions in APC-free systems of T cell activation [27].

In T cell activation and clonal expansion, the costimulatory receptor CD28 and its closest structural relative, the de-activating receptor CTLA-4 (CD152) are of central importance [28, 29]. Both bind to CD80 and CD86, members of the B-7 family expressed by APC. While CD28 is constitutively expressed, CTLA-4 is induced as an intracellular protein in response to T cell activation and transported to the immunological synapse where it is thought to dominantly inhibit signaling [29]. Interestingly, $CD4^+CD25^+$ T*reg* cells in mice and humans are the only cells that constitutively express serologically detectable levels of intracellular CTLA-4 (reviewed in [1, 30]). While it is unresolved whether this only reflects the activated state of the T*reg* cells due to continuous confrontation with self-antigens, or whether CTLA-4 functions as a costimulatory receptor or cell interaction molecule important for their function, CTLA-4 expression provides an additional useful marker for this subset in the context of an unstimulated immune system.

In vitro, the physiologic ligands of the TCR and of CD28 can be replaced by mAb, allowing efficient induction of T cell proliferation. This also holds true for the rat [31], where in addition, we have previously described a class of "superagonistic" CD28-specific mAb which are able to induce proliferation of all T cell subsets without the need for TCR engagement [32]. Importantly, CD28 superagonists are also active *in vivo*, where in response to a single mAb injection, CD4 T cell numbers in peripheral lymphoid organs rise fivefold within 3 days before returning to base line levels [32]. Surprisingly, this dramatic polyclonal T cell response occurs without apparent inflammation or discomfort, a finding we have previously attributed to the strong induction of IL-10 but not of pro-inflammatory mediators during *in vivo* activation [33].

Based on these results, we have now investigated whether rat T*reg* cells can be activated and expanded by CD28 superagonists. Using a newly developed mAb to rat CTLA-4 as well as cytokine measurements, we first establish phenotypic identity of rat T*reg* cells with those described in mice and humans, and then demonstrate the suitability of CD28 superagonists for T*reg* stimulation *in vitro* and *in vivo*.

## 2 Results

### 2.1 Identification of $CD4^+CD25^+CTLA\text{-}4^+$ thymocytes and peripheral T cells

As described in Sect. 4, we generated a novel mAb, WKH203, which detects rat CTLA-4-in flow cytometry. Initially, we compared $CD4^+CD25^-$ and $CD4^+CD25^+$ thymocytes and lymph node T cells from adult LEW rats for CTLA-4 expression using intracellular staining with PE-conjugated mAb WKH203 (Fig. 1). In both cases, the $CD25^+$ subset showed a distinct shift in fluorescence intensity as compared to the control blocked with an excess of unlabeled mAb. In contrast, the $CD25^-$ subset of CD4 SP thymocytes and of CD4 T cells did not express detectable amounts of CTLA-4.

Additional phenotyping of $CD4^+$ lymph node T cells showed that the $CD4^+CD25^+$ subset, making up about 5% of CD4 T cells, is $CD45RC^{low}$ and L-selectin (CD62L) high, and that a subset of these cells also expresses the CD8α chain (data not shown).

### 2.2 *In vitro* responses of $CD4^+CD25^+CTLA\text{-}4^+$ T cells

Lymph node CD4 T cells were separated into their CD25-positive and -negative subsets, and compared with respect to survival, proliferative responses and cytokine production. In unstimulated cultures, two thirds of $CD25^+$ cells had lost viability after 2 days as indicated by their sub-diploid DNA content (Fig. 2A), in contrast to only 30% in the $CD25^-$ group. When IL-2 was included, the $CD25^+$ cells were rescued from death (88% viability) but only few of them were in cycle (7% in S and G2/M); as expected, IL-2 had no effect on the $CD25^-$ subset. Thus at least *in vitro*, IL-2 acts as a survival factor for rat $CD4^+CD25^+$ T cells.

Next, proliferative responses of the two CD4 T cell subsets to TCR and CD28 stimulation were compared. In addition to "conventional" TCR- and CD28- specific mAb which effectively costimulate rat T cell responses [31], we also tested whether the putative T*reg* cells defined by their $CD4^+CD25^+CD45RC^{low}CTLA\text{-}4^+$ phenotype can be activated by CD28 superagonists which induce T cell proliferation without TCR engagement [32].

As shown in Fig. 2B, stimulation with immobilized anti-TCR mAb plus conventional anti-CD28 (costimulation), or with the CD28 superagonist, induced equally strong proliferation in both CD25-positive and negative subsets on day 2 of culture. On day 3, however, costimulation-induced proliferation of the $CD25^+$ cells was only one

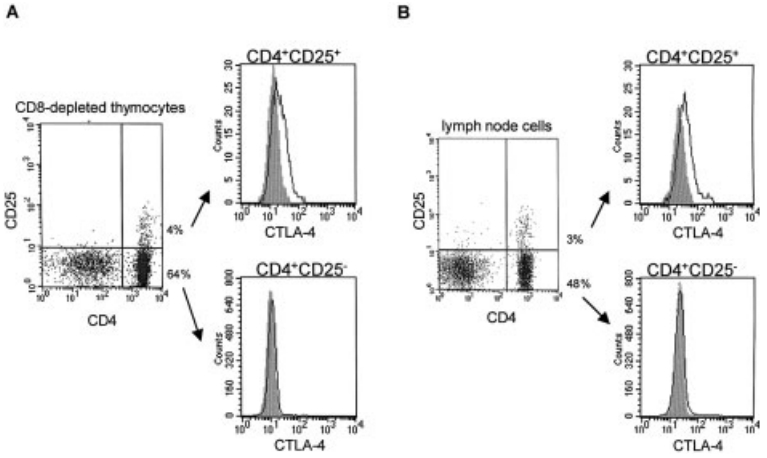


*Fig. 1.* Constitutive expression of CTLA-4 in rat $CD4^+CD25^+$ thymocytes (A) and LN T cells (B). CD8 depleted thymocytes or whole LN T cells were surface stained with anti-CD4 and anti-CD25 mAb, permeabilized, and stained with anti-CTLA-4 mAb (WKH203-PE, solid line). Filled diagrams show staining after blocking with uncoupled WKH203.

third of that observed in the $CD25^-$ subset while both populations continued to proliferate at the same high level when stimulated by the CD28 superagonist. Inclusion of IL-2 restored the response of costimulated $CD25^+$ cells to the level of the CD28 superagonist-activated group. This suggested that rat $CD4^+CD25^+$ T cells are fully reactive to superagonistic CD28 signals but partially defective in their proliferative response to antibody-induced costimulation, and that differences in the production of or dependence on IL-2 in the two modes of T cell activation may explain the superior response to superagonistic CD28 stimulation.

To further investigate this, the IL-2 content in the culture supernatants was determined (Fig. 2C). Costimulation of $CD25^-$ cells resulted in high, and superagonistic CD28 stimulation in even higher levels of IL-2 in the supernatant throughout the 3-day observation period. In contrast, cultures of costimulated $CD25^+$ cells contained no detectable IL-2, in agreement with data obtained with mouse and human *Treg* cells. In cultures of superagonist-activated $CD25^+$ cells, a small amount of IL-2 was detected on day 1 which had disappeared by day 3 (Fig. 2C).

The cytokine IL-10 was found at high concentrations in supernatants from $CD25^+$ cells stimulated by either conventional costimulation or by the CD28 superagonist, while IL-10 production by the $CD25^-$ subset was marginal up to day 2 of culture (Fig. 2C). Interestingly, unseparated CD4 T cells produced much higher levels of IL-10 than purified $CD25^-$ cells, but proliferation was identical in both groups. This is in agreement with findings in mice where APC, which play no role in the present system, were identified as the main target for IL-10 [18, 34].

### 2.3 Suppressive function of *Treg* cells

As an *in vitro* surrogate assay for *Treg* activity, the suppressive effect of *Treg* cells on the polyclonal proliferative response of $CD4^+CD25^-$ T cells ("indicator cells") is well established in the mouse [14]. Since purified rat T cells are unresponsive to TCR stimulation alone [31], we used costimulation of indicator cells as a read-out. When $CD4^+CD25^+$ cells isolated from naive rats were tested in the system at a 1:1 ratio, no suppressive activity was apparent as measured by $[^3H]$thymidine incorporation. However, when the number of cell divisions of the indicator cells was monitored by the CFSE dye dilution method (see below), a small but reproducible suppressive effect was observed (data not shown). This suggested that the assay employed is, in principle, suitable to detect rat *Treg* function, and that, as known from experiments performed in mice, pre-activation of *Treg* cells may be required to obtain more clear-cut effects.

To test this, purified $CD4^+CD25^+$ lymph node cells were cultured for 3 days either in IL-2 alone (to ensure their survival), or additionally in the presence of either a costimulating set of TCR- plus conventional CD28-specific mAb, or of CD28 superagonist. The recovered cells were either cultured alone (Fig. 3A) to test their responsiveness to restimulation, or together with $CD25^-$ indicator CD4 T cells at a 1:1 ratio (Fig. 3B). As shown in Fig. 3A, all three populations of pre-cultured *Treg* cells prolifer-

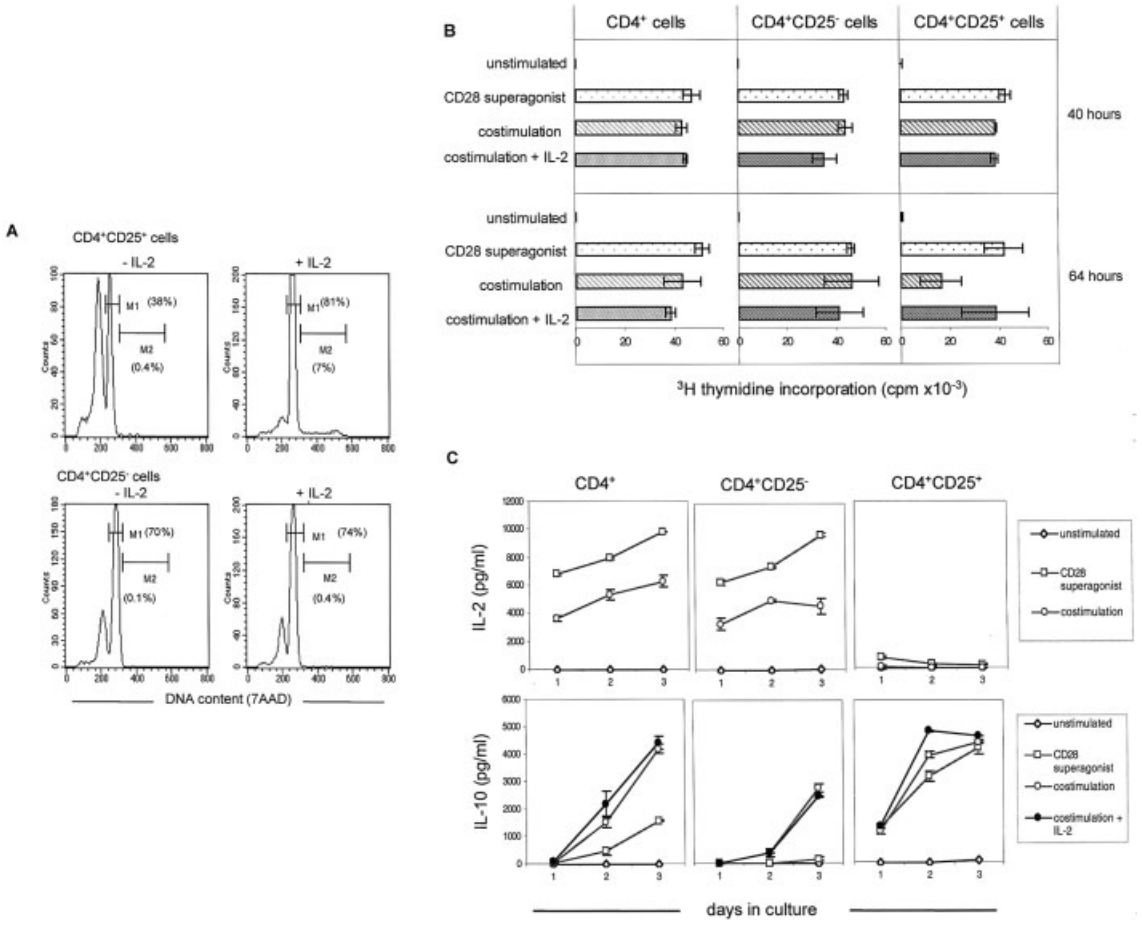


*Fig. 2. In vitro* survival, proliferation and cytokine production of $CD25^-$ *vs*. $CD25^+$ peripheral CD4 T cells. (A) Requirement of IL-2 for $CD4^+CD25^+$ cell survival *in vitro*. Cells were cultured with or without IL-2 for 48 h, permeabilized and stained with 7AAD. G0/G1 (diploid) is indicated by M1, S and G2/M by M2. (B) Proliferative responses. Cells were stimulated with plate-bound anti-TCR plus soluble anti-CD28 (costimulation) in the presence or absence of exogenous IL-2 or with superagonistic anti-CD28. (C) IL-2 and IL-10 secretion. Cells were stimulated as in (B). At the times indicated, supernatants were assayed for IL-2 and IL-10 by ELISA. Results are presented as triplicate means ± SD.

ated poorly or not at all in response to costimulation, but proliferated vigorously when stimulated with the CD28 superagonist. When costimulated together with indicator cells, neither T*reg* cells pre-cultured in IL-2 alone nor those costimulated during pre-culture significantly suppressed [$^3$H]thymidine incorporation below the values expected for the mixture of naive indicator and activated T*reg* cells (Fig. 3B). In contrast, T*reg* cells pre-activated with CD28 superagonist plus IL-2 were highly effective in suppressing T cell proliferation (Fig. 3B). Since activation of the T*reg* cells by CD28 superagonist plus IL-2 during the 3 days of preculture had led to a more than tenfold increase in cell number, this protocol thus seems suitable for the preparation of expanded T*reg* effector populations.

None of the three populations of *in vitro* pre-activated T*reg* cells was able to interfere with the proliferative response of mixed cultures of T*reg* and indicator cells stimulated by CD28 superagonist (Fig. 3B). This was not simply due to the vigorous response of the T*reg* cells themselves that could have obscured suppression of indicator cell proliferation, as no inhibitory effect on the indicator cells was detectable by CFSE dilution analysis (data not shown).

### 2.4 *In vitro* activated $CD4^+CD25^-$ cells exhibit little T*reg* activity

To test whether the pronounced inhibitory effect of T*reg* cells pre-activated *in vitro* with CD28 superagonist plus

IL-2 is a common property of all CD4 cells activated in this manner, $CD4^+CD25^-$ cells were subjected to the same activation protocol. In contrast to anti-CD28 plus IL-2 expanded *Treg* cells which are refractory to costimulation in the secondary cultures (Fig. 3A), these originally $CD25^-$ (but now $CD25^+$) cells were as responsive to costimulation as freshly isolated $CD25^-$ cells (Fig. 4A). Moreover, while the activated *Treg* cells prepared in parallel were highly effective in suppressing the proliferation of naive indicator cells, activated T cells derived by CD28 superagonist stimulation from $CD25^-$ precursors were without effect as assessed by [$^3$H]thymidine incorporation (Fig. 4B). By the CFSE dye dilution method, however, a small reduction in the proliferation of indicator cells was detected. On a per cell basis, this effect was more than tenfold lower than that obtained with *in vitro* activated $CD4^+CD25^+$ cells (Fig. 4C).

### 2.5 Preferential expansion of CD4 T cells with a *Treg* phenotype by *in vivo* stimulation with CD28 superagonist

As shown in Fig. 5A, a single injection of the CD28 superagonist led to a transient increase in the frequency of CD25 expressing cells among CD4 lymph node T cells from an initial 5% to about 18% on day 3 after stimulation. In parallel, a 5-fold rise in total lymph node CD4 T cell number was observed (data not shown, see ref. [32]), resulting in an approximately 20-fold amplification of $CD4^+CD25^+$ cells present in lymph nodes. The expression of CD25 on only a minority of CD4 cells suggested that *in vivo*, not all proliferating CD4 T cells express CD25 but that CD25 expression remains restricted to *Treg* cells during CD28-driven CD4 T cell proliferation. To test whether CD4 T cell proliferation can indeed proceed without CD25 expression, cell cycle analysis of $CD25^+$ and $CD25^-$ $CD4^+$ T cells recovered at different time points after superagonist injection was performed. As

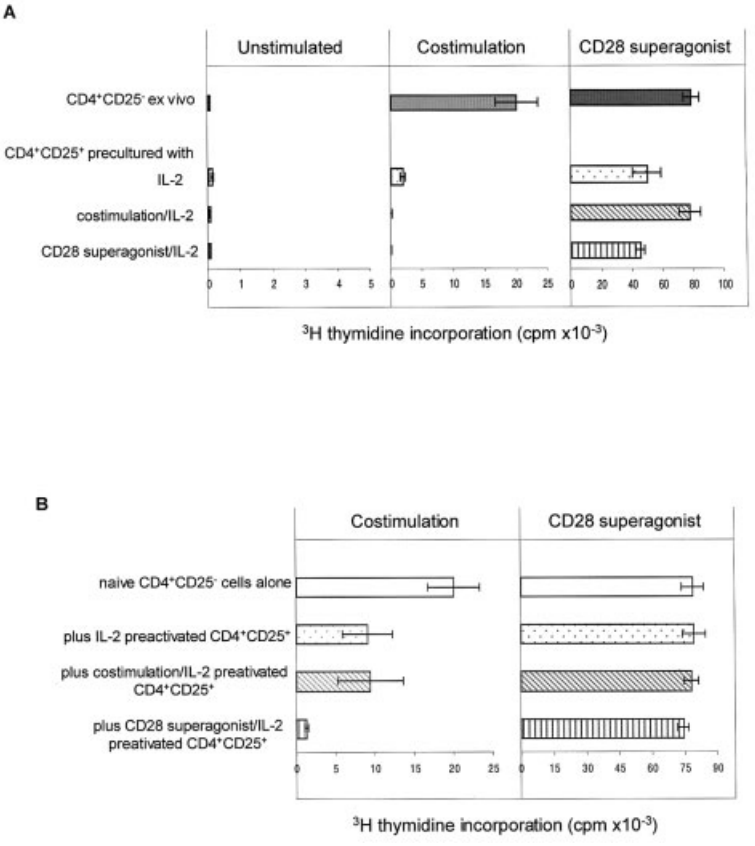


*Fig. 3.* Proliferative responses and suppressive effect of *in vitro* activated $CD4^+CD25^+$ cells. (A) Purified $CD4^+CD25^+$ T cells were pre-cultured for 3 days as indicated, washed and restimulated by TCR/CD28 costimulation or with CD28 superagonist for another 3 days. (B). Coculture of preactivated $CD4^+CD25^+$ T cells with $CD4^+CD25^-$ indicator cells. Conditions as in (A). All cultures contained the same total number of T cells ($10^5$). In coculture, the ratio of $CD25^-$ to $CD25^+$ cells was 1:1.

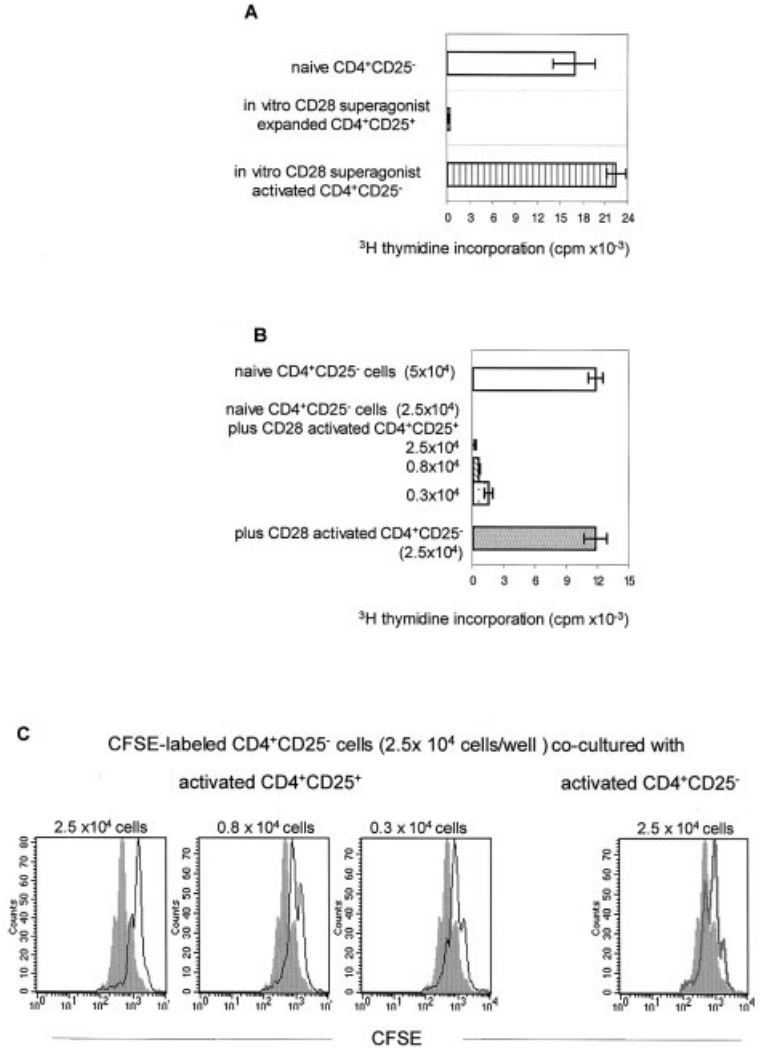


*Fig. 4.* Proliferative capacity and suppressive effect of *in vitro* CD28 superagonist expanded $CD25^+$ and $CD25^-$ cells. Purified $CD25^-$ and $CD25^+$ CD4 T cells were expanded with superagonistic anti-CD28 plus IL-2 for 3 days. (A) Cells were restimulated by plate-bound anti-TCR plus soluble anti-CD28 for 3 days at a total number of $5\times10^4$ cells/well. Freshly isolated $CD25^-$ cells are shown for comparison. (B) Suppressor assay. Freshly isolated $CD25^-$ indicator cells were either costimulated with anti-TCR plus anti-CD28 coated beads alone, or together with pre-activated CD4 cells derived from $CD25^+$ or $CD25^-$ input cells at the cell numbers given. (C) As in (B), but with CFSE-labeled naive $CD25^-$ indicator cells. CFSE fluorescence of naive $CD25^-$ indicator cells cultured alone (filled) or in coculture (solid line) was determined after 48 h.

shown in Fig. 5B, both subsets were cycling 1 day after CD28 stimulation. From day 2 on, however, cycling was restricted to $CD4^+CD25^+$ cells. This could either mean that CD28 superagonist treatment induces more long lasting proliferation in the *Treg* subset, or alternatively that cycling $CD4^+CD25^-$ cells up-regulate CD25, resembling *Treg* cells in phenotype. To test this directly, purified $CD4^+CD25^-$ cells were labeled with CFSE and injected i.v. into normal rats which were then stimulated by CD28 superagonist *in vivo* (Fig. 5C). When analyzed 2 or 4 days later, all of the CFSE-labeled cells had undergone one or several cell divisions, but virtually none of them expressed CD25. Accordingly, $CD4^+CD25^-$ cells which had responded to CD28 stimulation by proliferation did not contribute to the elevated frequency of $CD25^+$ CD4 cells observed at these time points (10 and14% of $CFSE^-$ CD4 cells, respectively), which hence were derived from pre-existing endogenous $CD25^+$ cells.

Additional experiments were performed to investigate whether the expanded population of $CD25^+$ CD4 T cells recovered from CD28-stimulated animals on day 3 of

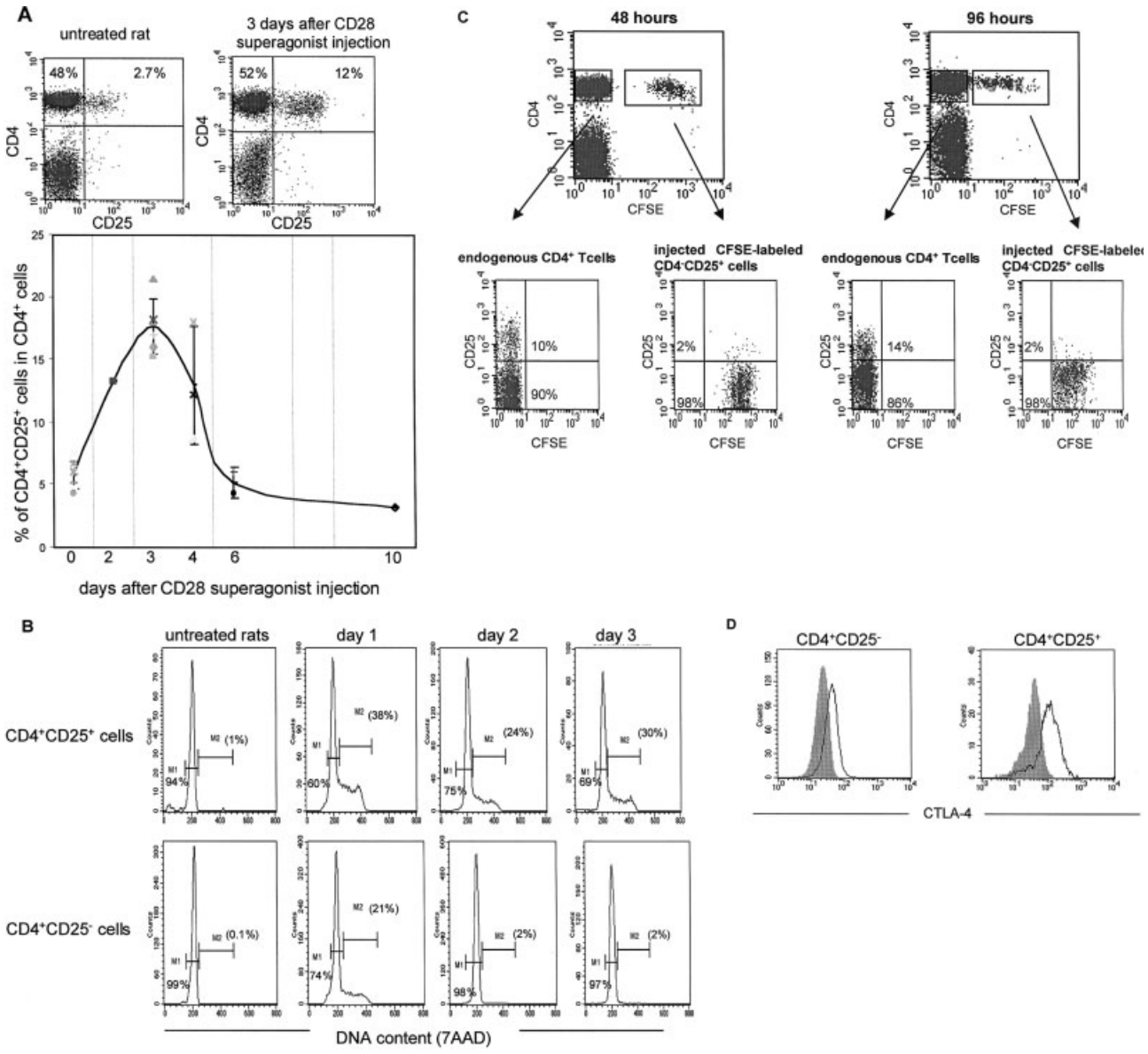


*Fig. 5. In vivo* expansion of CD4$^+$CD25$^+$ cells by superagonistic anti-CD28 mAb. (A) Transient increase of CD4$^+$CD25$^+$ cells in LN from rats injected with 1 mg superagonistic anti-CD28 (B). Cell cycle analysis of CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ cells. (C) CD4$^+$CD25$^-$ cells do not acquire CD25 upon *in vivo* CD28 superagonist stimulation. CFSE-labeled naive CD4$^+$CD25$^-$ T cells were i.v. injected ($5\times10^7$ cells/rat) followed by 1 mg of CD28 superagonist 12 h later. After 2 or 4 days, cells from lymph nodes were analyzed as shown. (D) Expression of CTLA-4 in CD4$^+$CD25$^+$ *vs*. CD4$^+$CD25$^-$ cells from CD28 superagonist stimulated rats. LN cells were collected 3 days after injection of superagonistic anti-CD28, surface-stained with anti-CD4 and anti-CD25, and then stained intracellularly with WKH203-PE without (solid line) or with (filled) pre-incubation with unconjugated WKH203.

*in vivo* activation have the phenotypic and functional properties of T*reg* cells. Initially, their CTLA-4 expression was compared to that of CD25$^-$ cells isolated from the same animals. As expected, CTLA-4 was now detectable in both subsets, confirming that all CD4 cells had been recently activated (Fig. 5D). However, CD4$^+$CD25$^+$ T cells expressed fourfold higher levels of CTLA-4 than the CD25$^-$ CD4 cells. This finding additionally supports the notion that CD4$^+$CD25$^+$ cells recovered from CD28 superagonist-treated rats are activated T*reg* cells, as further up-regulation of CTLA-4 in response to activation is also a characteristic of T*reg* cells from humans and mice.

### 2.6 *In vitro* responses and suppressive function of *in vivo* expanded T*reg* cells

The CD25-positive and -negative subsets of CD4 T cells isolated from rats treated with a single injection of CD28 superagonist 3 days earlier were stimulated *in vitro* by conventional costimulation or with CD28 superagonist and tested for their proliferative response (Fig. 6A) and cytokine production (Fig. 6B). While $CD25^-$ CD4 T cells from CD28 superagonist-treated rats proliferated vigorously to both stimuli, *in vivo* pre-activated $CD4^+CD25^+$ cells failed to proliferate in response to costimulation unless exogenous IL-2 was added which partially restored proliferation. They did, however, proliferate to CD28 superagonist stimulation alone, and this response was further boosted by exogenous IL-2 to the level observed with $CD25^-$ cells.

As expected from their strong proliferative response, $CD25^-$ CD4 T cells isolated from CD28-stimulated rats made copious amounts of IL-2, which decreased in the culture supernatant with time in costimulated but not in CD28-superagonist activated cultures, presumably due to consumption in the former (Fig. 6B). In contrast, IL-2 was not detectable in cultures of T*reg* cells stimulated by either protocol and was also absent in 1:1 mixtures of both subsets, indicating suppression of IL-2 production. Rather, the *in vivo* pre-stimulated $CD25^+$ cells produced high levels of IL-10 when activated by either conventional costimulation or by the CD28 superagonist. $CD25^-$ CD4 T cells from *in vivo* stimulated animals also produced IL-10, although in much lower amounts and with delayed kinetics.

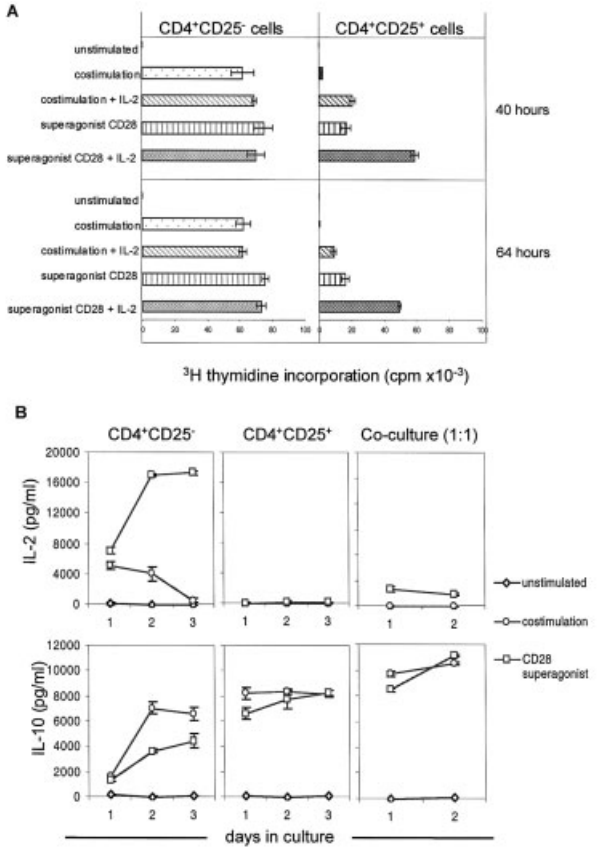


*Fig. 6.* Proliferation (A) and cytokine production (B) of CD4 T cells from CD28 superagonist injected rats. $CD25^+$ and $CD25^-$ $CD4^+$ LN T cells were purified 3 days after injection of 1 mg mAb. For details see legend to Fig. 2.

Importantly, *in vivo* activated T*reg* cells were also effective in suppressing the costimulated response of naive T cells or of $CD25^-$ CD4 T cells isolated from the same CD28-stimulated animals, but had no effect on CD28 superagonist-induced proliferation read out by [$^3$H]thymidine incorporation (Fig. 7A). Furthermore, coculture of *in vivo* pre-activated T*reg* cells with CFSE-labeled indicator cells revealed a strong inhibition of cell division under conditions of costimulation. At the same time, the presence of naive indicator cells rescued the survival of the of *in vivo* pre-activated T*reg* cells which down-regulated CD25 and declined in number when costimulated alone (Fig. 7B). In contrast, when CD28 superagonist restimulation was applied, both the CFSE-labeled naive indicator cells and the *in vivo* pre-activated T*reg* cells proliferated well when cultured either in isolation or together.

## 3 Discussion

The identification of naturally occurring regulatory T cells with the capacity to suppress autoimmunity and inflammation suggests that therapies which activate and expand this subset could be extremely efficient in treating these immunopathological disorders. Both *in vitro* [35], and *in vivo* [36], combinations of immunosuppressive drugs were recently shown to promote T*reg* differentiation during antigen-driven immune responses, suggesting their use as T*reg*-promotors in certain therapeutic settings. However, a polyclonal expansion of the naturally occurring T*reg* population, which would provide the most general approach to treatment of autoimmune disease, has not succeeded so far. We here demonstrate that by stimulating the immune system with superagonistic mAb to CD28, T*reg* cells are disproportionately expanded over other subsets, suggesting that CD28 superagonist therapy will be useful for the treatment of a variety of immunopathological conditions.

First, we report that constitutive expression of CTLA-4 at the level detectable by flow cytometry is restricted to the

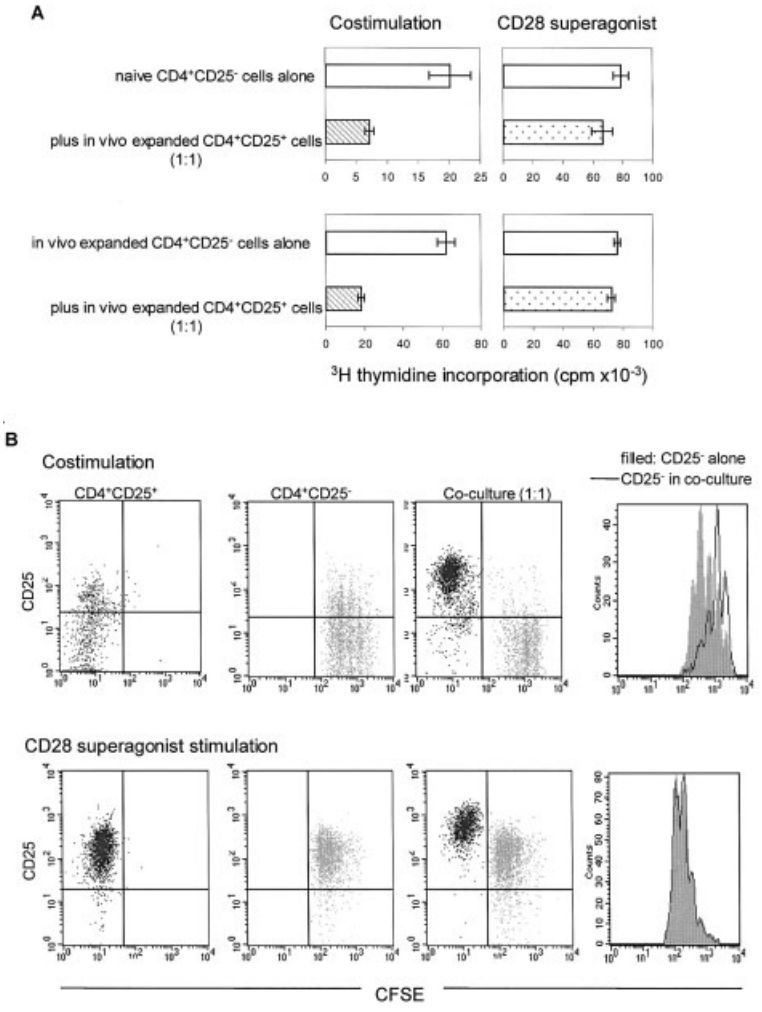


*Fig. 7.* Suppressive effect of CD4⁺CD25⁺ cells from CD28 superagonist treated rats. Indicator CD4⁺CD25⁻ cells from untreated normal rats (A, B) or from superagonistic anti-CD28 injected rats (A) were cultured either alone or with CD4⁺CD25⁺ cells from rats treated with CD28 superagonist 3 days earlier. (A) Suppression of costimulated but not superagonist-stimulated indicator cells. Proliferation was determined at 72 h by thymidine incorporation. (B) Suppression of cell division and CD25 expression of costimulated CFSE-labeled indicator CD4⁺CD25⁻ cells. Cells were analyzed after 48 h of culture.

CD25⁺ subset of CD4 T cells and thymocytes in the rat (Fig. 1). Thus, the peripheral CD25⁻ T*reg* cells which coexist in rats with those expressing CD25 [8] can additionally be distinguished from CD4⁺CD25⁺ cells by their lack of CTLA-4 expression. Furthermore, since rat CD4 SP thymocytes capable of suppressing autoimmunity were previously shown to have differentiated *in situ* rather than having homed back to the thymus from the periphery [37], our present findings demonstrate that CTLA-4 expression is already acquired during thymic differentiation and not a secondary peripheral event resulting from continuous stimulation with autoantigens.

Secondly, we have demonstrated that for *in vitro* survival, rat CD4⁺CD25⁺ cells depend on IL-2 which they cannot make themselves, and that they produce high amounts of IL-10 when activated by conventional costimulation or by a CD28 superagonist (Fig. 2). It is a matter of debate (and of the *in vitro* stimulus employed) whether CD4⁺CD25⁺ T*reg* cells of mice produce high levels of IL-10 (reviewed in [38]), whereas this is well recognized for the CD25⁻ Tr1 population. However, since the latter subset is CTLA-4 negative, our data fit best with the phenotype of CD25⁺ T*reg* cells described in this species.

Finally, we established an *in vitro* assay in which the suppressive effect of CD4⁺CD25⁺ cells on costimulation-induced proliferation of purified CD25⁻ CD4 indicator cells was demonstrated. In this system, pre-activation of

the $CD4^+CD25^+$ cells was required to obtain clear-cut suppression. The requirement for T*reg* pre-activation is most likely due to the known capacity of CD28 costimulation to antagonize T*reg*-mediated suppression [1]. Since purified rat T cells do not respond to TCR stimulation alone, the difficulty of suppressing a costimulated population of indicator cells had to be accepted and was met by increasing the potency of T*reg* cells by pre-activation.

The present experiments were initiated because of our earlier observations that *in vivo*, the CD28 superagonist response is accompanied by high levels of IL-10 mRNA [33], and CD28-specific superagonistic mAb are highly effective in prevention and treatment of autoimmune-inflammatory rat models (unpublished results). As T*reg* cells are hyporesponsive to TCR stimulation with regard to proliferation [14], we therefore hypothesized that circumvention of TCR signaling with CD28 superagonists may provide a protocol for the expansion and activation of this subset.

We found that as compared to conventional costimulation, *in vitro* proliferation of rat T*reg* cells in response to CD28 superagonist is more sustained and less IL-2 dependent (Fig. 2, 3, 6). Moreover, pre-activated T*reg* cells cannot be restimulated by costimulation but still respond to the direct CD28 signal (Fig. 3, 6). Even in the presence of exogenous IL-2, pre-activated Treg cells die during a second cycle of costimulation but are readily expanded by the CD28 superagonist for at least 3 weeks (data not shown).

The most important finding of the present study is that CD28 superagonist potently stimulates the expansion of $CD4^+CD25^+$ cells *in vivo*, and that the recovered $CD4^+CD25^+$ (but not the $CD4^+CD25^-$) cells display all features of T*reg* cells: strong up-regulation of CTLA-4, impaired proliferation, and IL-2 production but very high levels of IL-10 secretion in response to costimulation, and suppression of the proliferative response of costimulated indicator cells (Fig. 5–7). In agreement with our earlier findings that all CD4 T cells undergo DNA synthesis in response to CD28 stimulation [32], we observed that also in the $CD25^-$ subset, a high degree of cycling is observed on day 1 after stimulation (Fig. 5). Since $CD4^+CD25^+$ cells continue to be in cycle for at least another 2 days, whereas $CD4^+CD25^-$ cells stop cycling between day 1 and 2, we hypothesize that the activated T*reg* cells interfere with the proliferative response of the $CD25^-$ subset once they are sufficiently activated. Such an inhibitory effect of T*reg* cells on $CD4^+CD25^-$ cells during CD28 superagonist-driven activation of both subsets is seemingly at odds with our *in vitro* results, where this type of stimulation during coculture did not result in suppression (Fig. 7). It is unclear at present whether this difference lies in the presentation of superagonistic mAb by FcR-positive APC *in vivo* (but not in the *in vitro* system employed), in the differential availability of paracrine cytokines, including IL-2, in the two situations, or in the availability of other, undefined components contributing to the restriction of clonal expansion *in vivo*. Future experiments will address this issue.

The following factors may contribute to the high efficacy of CD28-driven T*reg* expansion: First, direct CD28 stimulation is able to trigger downstream pathways described for costimulation, most notably the NFκB pathway, without proximal TCR signaling [39]. Therefore, superagonist mAb triggering may by-pass the anergic TCR-signaling machinery of T*reg* cells [1]. Secondly, "direct" CD28-stimulation is very efficient in inducing IL-2 production by $CD25^-$ CD4 cells (Fig. 2, 6), leading to high levels of paracrine IL-2 which may support the survival and expansion of T*reg* cells. At the same time, CD28-induced proliferation of both CD25-negative and -positive CD4 subsets is relatively IL-2 independent, as illustrated by CD28-driven cycling of $CD25^-$ cells *in vivo* without conversion to a $CD25^+$ phenotype (Fig. 5), and by CD28 superagonist-induced proliferation of resting and pre-activated T*reg* cells *in vitro* in the absence of detectable IL-2 secretion (Fig. 2, 6). Given the dependence of TCR- or costimulation-induced T*reg* proliferation on exogenous IL-2 *in vitro*, CD28 superagonists may therefore by-pass this requirement when T*reg* cells are activated in isolation. Thirdly, T*reg* cells are highly susceptible to apoptosis (Fig. 2). Since CD28 superagonist stimulation is even more effective than conventional CD28-mediated costimulation in promoting T cell survival (A. Kerstan and T. H., unpublished), CD28 superagonist may render T*reg* cells less dependent on exogenous or paracrine cytokines for survival.

We have recently discovered that targeting the identical structural element of human CD28 by mAb as the one recognized by the superagonist presently employed also results in polyclonal stimulation of human T cells [40]. Hopefully, this will allow to develop a therapeutic reagent suitable for the treatment of human autoimmune and inflammatory diseases.

## 4 Materials and methods

### 4.1 Animals

BALB/c mice and LEW rats were used at 6–12 weeks of age. All animals were maintained in the institute's facility and cared for in accordance with the institutional guidelines for animal welfare.

### 4.2 Generation of anti-rat CTLA-4 mAb

The sequence coding for the extracellular part of rat CTLA-4 cDNA was amplified from a cDNA cloned from ConA-stimulated rat splenocytes using the primers 5'-GGG GGA GCT CAC TAT GGC TTG TCT TGG ACT-3' and 5'-GGC AAG CTT ACT TAC CTG AAT CTG GGC ATG GTT CT-3', and subcloned into the SstI-HindIII sites of the human IgG1 expression vector pHβAPr-1-gpt-Hr1 (kind gift from Dr. P. Lane). Expression of native fusion protein by transfected J558L was confirmed by staining rat B7–1 expressing L929 cells with fusion proteins (rat B7–1 expression vector was kindly provided by K. Okumura, [41]). BALB/c mice received 6-weekly i.p. immunizations of 10 μg alum-absorbed purified rCTLA-4hIg fusion protein, and spleen cells were fused with X63-Ag8.653 myeloma cells 3 days after an i.v. boost in PBS. mAb WKH203 (mouse IgG1) was identified by screening supernatants for reactivity with rCTLA-4hIg fusion protein but not with human Fc (Dianova, Hamburg, Germany) by ELISA and Western blot, and reactivity with native CTLA-4 was confirmed by i.c. staining of CTLA-4-transduced BW thymoma cells. PE conjugation of purified WKH203 was carried out by Serotec Ltd., Oxford, GB.

### 4.3 Purification of T cells

Single-cell suspensions were prepared from all superficial and mesenteric LN from either untreated rats or from rats injected with a single dose (1 mg i.v.) of superagonistic anti-CD28. T cell subsets were purified by magnetic separation with MACS (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. $CD4^+$ T cells were purified by removing B and $CD8^+$ cells using biotinylated anti-CD8 (BD PharMingen, Heidelberg, Germany) and mouse anti-rat Ig (Dianova). To separate $CD25^+$ cells from $CD25^-$ cells, lymphocytes were first incubated with FITC-coupled anti-CD25 mAb (Ox39-FITC, Serotec) and anti-FITC microbeads. Cells coated with microbeads were selected and eluted from the column after removing the magnetic field ($CD25^+$ cells). Unlabeled $CD25^-$ cells were then depleted of $CD8^+$ and B cells. $CD25^+CD4^-$ cells were isolated with an average purity of 85%, $CD4^+CD25^+$ cells of around 95%.

### 4.4 Proliferation assays

T cells were either stimulated with immobilized anti-TCR (clone R73) plus soluble anti-CD28 (clone JJ319) [31] or with superagonistic anti-CD28 (clone JJ316) as described [32]. Cells were plated at an initial density of $5\times10^4$ cells/well in a final volume of 200 μl of medium with or without 300 U/ml recombinant human IL-2 (Strathmann Biotec, Hamburg, Germany). All cultures were performed in X-VIVO-15 medium (Bio Whittaker, Verviers, Belgium) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine.

For assays of T*reg* activity, $2.5\times10^4$/well $CD4^+CD25^-$ indicator cells were incubated together with $CD4^+CD25^+$ cells as indicated in a final volume of 200 μl medium in 96-well F-plates. Costimulation was either performed as given above, or with anti-TCR plus anti-CD28 immobilized at a 2:1 ratio on Dynabeads (Dynal, Hamburg, Germany) employed at a bead to cell ratio of 5:1. Wells were pulsed with [$^3$H]thymidine (0.25 μCi/well; Amersham Pharmacia Biotech) for the last 16 h of incubation. Cells were harvested at the indicated times and [$^3$H]thymidine incorporation into DNA was determined using a scintillation counter.

### 4.5 *In vivo* expansion of T cells by superagonistic anti-CD28

For polyclonal *in vivo* activation of T cells, 6–8-week-old healthy LEW rats received a single i.v. injection of superagonistic anti-CD28 mAb JJ316 in PBS.

### 4.6 ELISA

For detection of IL-2 and IL-10, $5\times10^4$ cells were stimulated with either plate-bound anti-TCR plus conventional anti-CD28 or mitogenic anti-CD28 with or without IL-2 (300 U/ml) for 24, 48 or 72 h and capture ELISA were performed on collected supernatants. Rat IL-2 and IL-10 OptEIA™ ELISA sets (BD PharMingen) were used according to the manufacturer's instructions.

### 4.7 Flow cytometry

Anti-rat TCR (R73), -CD4, -CD8 (Ox8 or G28) and -CD45RC mAb were purchased from BD PharMingen. Expression of IL-2Rα (CD25) was determined by staining with FITC- or biotin-coupled mAb OX39 (Serotec). For staining, cells were incubated first with NMIg (10 μg/ml; Sigma-Aldrich, Taufkirchen, Germany) to reduce unspecific binding (4°C, 10 min), stained for 20 min at 4°C in PBS (0.1% BSA, 0.02% $NaN_3$), washed twice, and analyzed with a FACSCalibur™ flow cytometer using CellQuest™ software (Becton Dickinson, Heidelberg, Germany). Expression of rat CTLA-4 was determined by intracytoplasmic staining with PE-conjugated anti-rat CTLA-4 (WKH203) after staining of cell-surface markers, fixation in Cytofix/Cytoperm™ (BD PharMingen), and permeabilization with saponin buffer (PBS, 0.1% BSA, and 0.5% saponin; Sigma Aldrich) at room temperature for 10 min. CFSE staining was performed in RPMI 1640 without FCS ($1\times10^7$ cells/ml) for 5 min at RT with 2.5 μM CFSE (MoBiTec GmbH, Göttingen, Germany) followed by three washes in RPMI with 5% FCS. DNA content was measured by staining with 3.3 μg/ml 7AAD (Sigma Aldrich) in 0.1% saponin for 30 min at 4°C.

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