Large-scale expansion of rat CD4$^+$ CD25$^+$ T_{reg} cells in the absence of T-cell receptor stimulation

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Summary

T-cell receptor (TCR) stimulation is both central to homeostatic maintenance of CD4$^+$ CD25$^+$ regulatory T cells (T_{reg} cells) *in vivo* and a prerequisite for the initiation of suppression by T_{reg} cells, both *in vivo* and *in vitro*. However, TCR-independent stimulation of T_{reg} cells, e.g. with superagonistic CD28-specific monoclonal antibodies (CD28-SA), not only expands these cells *in vivo* but, as we show here, also mediates large-scale expansion of rat T_{reg} cells *in vitro*. Interestingly, CD28-SA stimulation plus interleukin (IL)-2 was even superior to conventional costimulation plus IL-2 in promoting T_{reg} cell growth *in vitro*. Despite their highly activated phenotype suppression by T_{reg} cells expanded in the absence of TCR stimulation remained fully dependent on TCR-triggering for initiation and cell contact was required to exert suppression. With regard to the regulation of suppression by CD28 stimulation we observed that neither the presence of a conventional anti-CD28 monoclonal antibody nor a CD28-SA generally rendered conventional T cells resistant to suppression by preactivated T_{reg} cells. Taken together, we provide a novel protocol for long-term propagation of T_{reg} cells *in vitro* and our data are the first to reveal a difference in the signals required for activation and expansion of T_{reg} cells and those, involving the TCR, necessary for the initiation of suppression.

Keywords: regulatory T cells (T_{reg}); co-stimulation/costimulatory molecules; anergy/suppression/tolerance; proliferation

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Introduction

CD4$^+$ CD25$^+$ regulatory T cells (T_{reg} cells) are key players in the protection against autoimmunity and maintenance of immunologic self-tolerance.[1] These 'naturally occurring', self-reactive[2] T_{reg} cells arise in the thymus under the control of the forkhead-winged transcription factor FoxP3.[3–6] Peripheral T_{reg} cells continue to express FoxP3 independent of CD25 expression or other 'activation markers', like CD152 or CD69, which are also induced on conventional T cells (T_{conv} cells) after stimulation *in vivo*. FoxP3, thus, is the most reliable marker for regulatory T cells *in vivo*. Reduced T_{reg} cell numbers or functional impairment of T_{reg} cells were found to cause autoimmunity in several animal models,[7] indicating that CD4$^+$ CD25$^+$ T_{reg} cells control other autoreactive T cells. Importantly, correlative deficiencies in either T_{reg} cell quality or quantity also characterize many human autoimmune diseases, like multiple sclerosis,[8] rheumatoid arthritis[9] or type 1 diabetes.[10]

Factors promoting proliferation, survival and activation of T_{reg} cells *in vivo* are autoantigen recognition,[11,12] triggering of CD28 on T_{reg} cells[13,14] and CD28-induced interleukin-2 (IL-2) production by conventional autoreactive CD4$^+$ CD25^{low} T cells.[15–17]

To study T_{reg} cells, a number of protocols have been established for *in vitro* culture of these cells using either antigen-pulsed dendritic cells,[18] allogeneic

Abbreviations: APC, antigen-presenting cell; BSA, bovine serum albumin; CD28-SA, superagonistic anti-CD28 mAb; CFSE, carboxyfluorescein succinimidyl ester diacetate; Con A, concanavalin A; c.p.m., counts per minute; IL, interleukin; mAb, monoclonal antibody; NWNA, nylon wool non-adherent; PBS, phosphate-buffered saline; rh, recombinant human; T_{conv} cell, conventional T cell; TCR, T-cell receptor; T_{ind} cells, indicator T cells; T_{reg} cell, regulatory T cell.

antigen-presenting cells (APC)[19] or anti-CD3/anti-CD28 monoclonal antibody (mAb)-coated beads and IL-2.[20,21] Moreover, consecutive expansion of T_{reg} cells *in vitro* and adoptive transfer of expanded T_{reg} cells into, for example, non-obese diabetic mice or into recipients of allogeneic T cells *in vivo* mediated protection from diabetes[18,20] or graft-versus-host disease,[19] respectively.

We have recently shown that superagonistic anti-CD28 antibodies (CD28-SA) are capable of activating rat regulatory T cells both *in vitro*[22] and *in vivo*,[22,23] and of strongly expanding T_{reg} cells *in vivo*.[22,23] Of clinical significance, *in vivo* activation of T_{reg} cells by CD28-SA directly translated into protection from experimental autoimmune encephalomyelitis in two independent models.[23]

In this study we followed up on our previous *in vitro* data by establishing long-term cultures of rat T_{reg} cells using a CD28 superagonist (CD28-SA) and IL-2. Further, we analysed CD28-SA/IL-2-expanded rat T_{reg} cells both phenotypically based on marker protein expression and functionally in surrogate *in vitro* suppression assays.

Materials and methods

Animals

Normal Lewis rats and C57Bl/6 mice were bred at the animal facility of the Institute for Virology and Immunobiology, University of Würzburg, and used for experiments between 6 and 12 weeks of age. All experiments were performed according to the Bavarian state regulations for animal experimentation and approved by the responsible authorities.

Purification of $CD4^+$ $CD25^+$ (T_{reg} cells) and $CD4^+$ $CD25^-$ T cells (T_{conv} cells)

Routinely, single-cell suspensions were prepared form inguinal, axillary, cervical, mesenteric and paraortic lymph nodes of normal Lewis rats and T-cell subsets were purified essentially as described.[22] In brief, lymph node cells were first depleted of B cells and $CD8^+$ cells prior to separation of $CD4^+$ cells into $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ cells using magnetic-activated cell sorting (MACS) beads (MACS®, Miltenyi Biotec, Bergisch Gladbach, Germany) and MACS® separation columns. Cell purities of regulatory $CD25^+$ T cells and conventional $CD25^-$ T cells were on average 85% and 95%, respectively.

In vitro *expansion of T_{reg} and T_{conv} cells*

Purified T_{reg} and T_{conv} cells were resuspended to a density of 5×10^4–5×10^5 cells/ml in *x-vivo* 15 medium™ (Bio Whittaker, Verviers, Belgium) supplemented with 15% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, non-essential amino acids, 100 U/ml penicillin and streptomycin, 30 μM mercaptoethanol and 2 mM L-glutamine (all from Gibco, Gaithersburg, MD) and cultured in flat-bottomed plates coated with sheep anti-mouse-immunoglobulin (0.5 mg/ml in 15 mM Na_2CO_3/35 mM $NaHCO_3$, pH 9.6). Five μg/ml mAb JJ316 and 300 U/ml recombinant human (rh) IL-2 (Chiron, Amsterdam, The Netherlands) were added in solution to stimulate the T cells. For costimulation, anti-TCR mAb R73 (5 μg/ml) was immobilized on sheep anti-mouse immunoglobulin-coated plates and conventional anti-CD28 mAb JJ319 (0.2 μg/ml) was added in solution. Proliferation was determined by [^{3}H]thymidine incorporation (Amersham Biosciences Europe, Freiburg, Germany) for the last 16 hr of culture. The DNA of [^{3}H]thymidine pulsed cells was harvested onto fibreglass filters and radioactive content quantitated using a β-scintillation counter.

For long-term culture, cells were propagated at densities between 5×10^4 and 2×10^6 cells/ml and restimulated on a weekly basis. Long-term costimulation was performed with soluble anti-TCR and anti-CD28 mAbs in the presence of coated sheep anti-mouse immunoglobulin.

In vitro *suppression assays*

To test for suppressor function, fresh indicator T cells were cocultured with different numbers of T_{reg} cells. In case of stimulation with concanavalin A (Con A, 2 μg/ml, Sigma-Aldrich, Taufkirchen, Germany), irradiated (20 Gy) lymph node or spleen cells were added as APC. Proliferation was either measured by determining carboxyfluorescein succinimidyl ester diacetate (CFSE) dye dilution (5 μM; MoBiTec GmbH, Göttingen, Germany) among conventional T cells or by measuring [^{3}H]thymidine incorporation during the final 16 hr of a 3-day culturing period. Counts per minute (c.p.m.) are given as means ± SD.

Transwell cultures

Five $\times 10^5$ CD28-SA-expanded T_{reg} cells were cocultured with 5×10^5 nylon wool non-adherent (NWNA) cells together in the upper well of a transwell chamber (24-well plate with millicell® culture plate insert; Millipore, Bedford, MA) and a further 5×10^5 NWNA cells were cultured in the lower well and stimulated with Con A. After 2 days, cells in both chambers were resuspended and aliquots transferred as triplicates into 96-well round bottom plates before [^{3}H]thymidine was added.[24]

Co-cultures of mouse lymph node cells and rat T_{reg} cells

CFSE-labelled mouse lymph node cells were stimulated by adding αCD3 mAb alone (clone 145-2C11; Pharmingen)

or αCD3 mAb together with αCD28 mAb (clone 37.51; Pharmingen). Co-cultured rat T_{reg} cells were stimulated either with anti-rat TCR mAb alone (R73) or with anti-rat TCR plus anti-rat CD28 (JJ319).

Fluorescence-activated cell sorting (FACS) analysis

The following mAbs were used: anti-rat CD4–CyChrome™ (clone OX35, BD Pharmingen); anti-rat CD25–fluoroscein isothiocyanate (FITC) or –biotin (clone Ox39; Serotec); anti-rat CD152 (cytotoxic T lymphocyte-associated antigen 4, CTLA-4)-biotin (clone WKH203) and anti-mouse FoxP3 (clone FJK-16s, both eBioscience, San Diego, CA).

Staining was performed with up to 1×10^6 cells in 50 µl of phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA)/0.02% NaN_3. Fc-receptors were blocked by incubation with 20 µg/ml of normal mouse immunoglobulin (Sigma-Aldrich). After the blocking step FITC-, phycoerythrin- and CyChrome™-conjugated or biotinylated mAbs were added (15 min, 4°). Bound biotinylated antibodies were detected by incubation with either CyChrome™ or allophycocyanin-conjugated streptavidin (Pharmingen). The cells were analysed on either a FACScan™ or FACSCalibur™ flow cytometer using Cell Quest™ software (all Becton Dickinson, San Jose, CA). Dot plots and histograms are shown as $\log_{10}$ fluorescence intensities on a four-decade scale.

For intracellular staining of FoxP3 and CD152 cells were fixed for 30 min at room temperature with fixation buffer (eBioscience) prior to permeabilization (permeabilization buffer, eBioscience). The cells were blocked with rat serum before staining with anti-CD152 mAb and anti-FoxP3 mAb for 30 min at room temperature. Specificity of anti-CD152 staining was verified by blockade with 100 µg/ml unconjugated anti-CD152 mAb (WKH203).

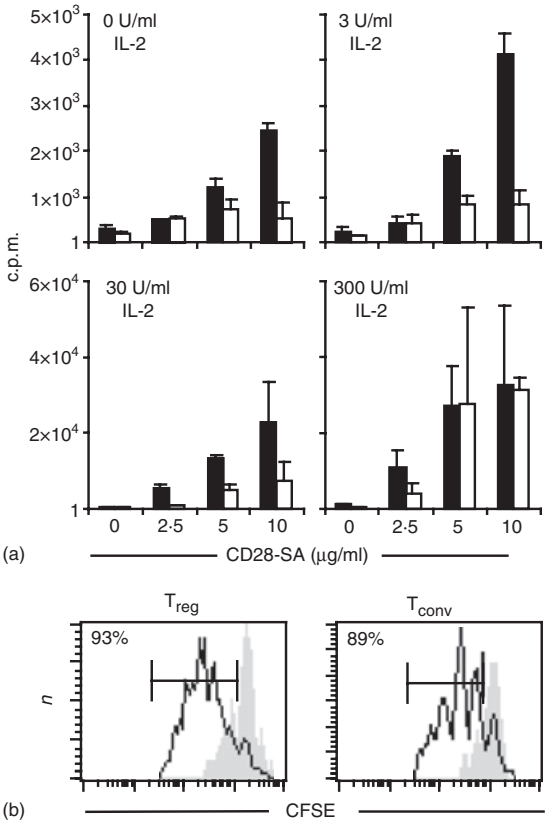

Figure 1. *In vitro* proliferation of T_{reg} and T_{conv} cells after stimulation with the CD28-SA mAb JJ316 in the presence of different amounts of rhIL-2. (a) [^{3}H]thymidine incorporation by 10^4 freshly purified T_{reg} cells (black bars) or T_{conv} cells (white bars) stimulated for 3 days with the indicated amounts of CD28-SA JJ316 and rhIL-2. The bars indicate means of triplicate cultures ± SD. (b) CFSE-labelled T_{reg} cells (left) and CFSE-labelled T_{conv} cells (right) were cultured for 3 days together with irradiated splenic APCs in the presence (black line) or absence (grey shadow) of the CD28-SA added in solution.

Results

Short-term proliferative response of CD4$^+$ CD25$^+$ regulatory T cells after superagonistic anti-CD28 stimulation *in vitro*.

For immunotherapy with *in vitro* expanded T_{reg} cells large-scale expansion is necessary. Therefore, we tried to optimize culture conditions for the expansion of T_{reg} cells with the CD28-SA JJ316 and IL-2. First, we isolated T_{reg} and T_{conv} cells form normal Lewis rats and cultured them for 3 days with different dosages of JJ316 in the absence or presence of exogenous IL-2. Under all these conditions the CD28-SA induced significant proliferation of T_{reg} and also T_{conv} cells as measured by [^{3}H]thymidine incorporation (Fig. 1a). However, T_{reg} cell proliferation clearly was superior to the proliferation of conventional T cells when the cells were stimulated with 10 µg/ml of JJ316 with little or no IL-2-supplementation. Analysis of CFSE dye dilution among CD28-SA-stimulated T_{reg} and T_{conv} cells revealed that the vast majority, if not all, regulatory and conventional T cells could be induced to proliferate upon superagonistic anti-CD28 stimulation (Fig. 1b). Therefore, superagonistic anti-CD28 stimulation *in vitro* induces a strong proliferative response in CD4$^+$ CD25$^+$ T_{reg} cells that is clearly superior to that induced in conventional T cells.

Long-term *in vitro* expansion of T_{reg} cells

To explore whether superagonistic CD28 stimulation is suitable for long-term and large-scale expansion of T_{reg} cells, we compared cell growth obtained with CD28-SA and IL-2 to that obtained with anti-TCR/anti-CD28 stimulation plus IL-2. Within 23 days, CD28-SA/IL-2 stimu-

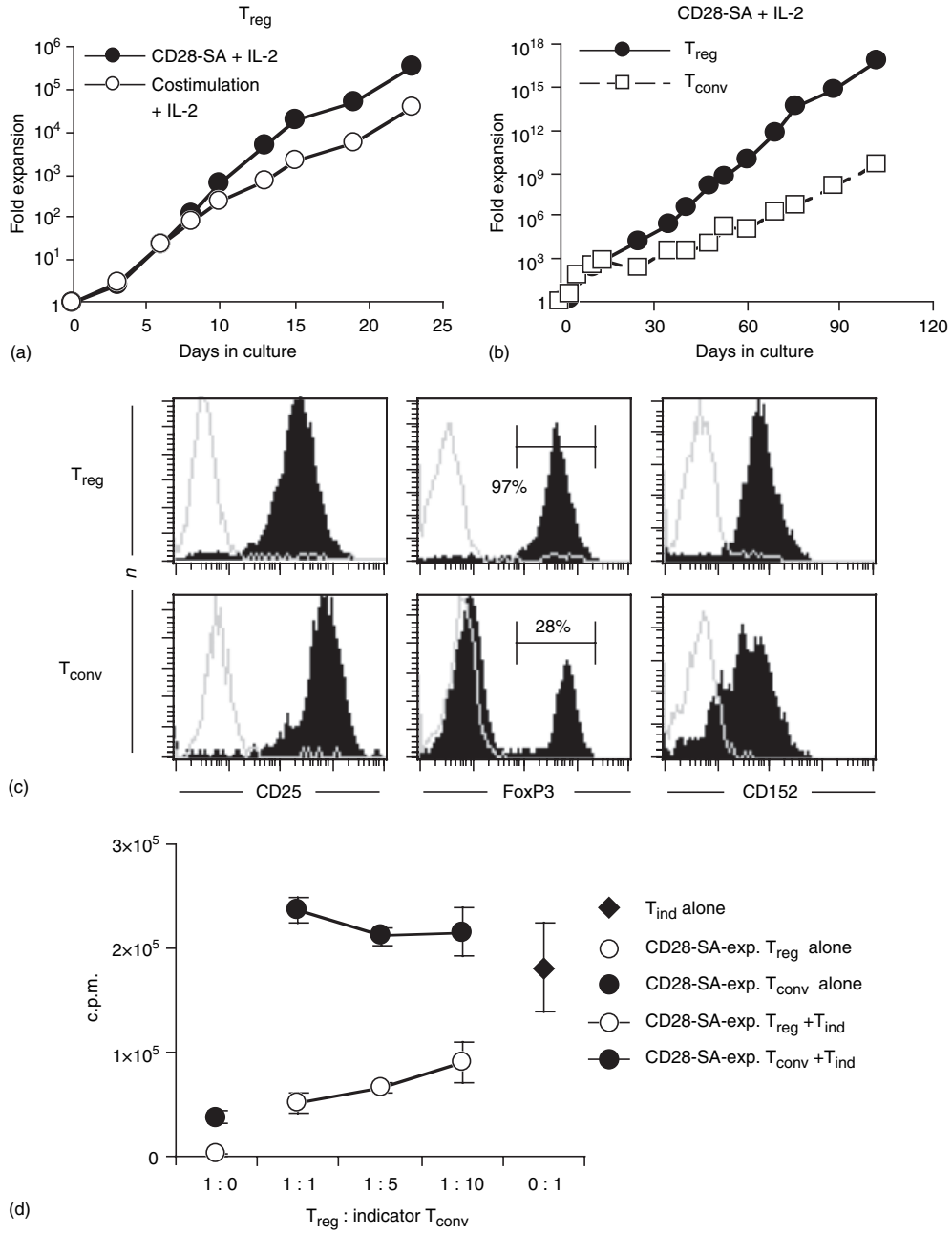

Figure 2. Long-term expansion of bona fide T_{reg} cells. (a) The potency of CD28-SA (5 μg/ml) and rhIL-2 (300 U/ml) to expand freshly isolated T_{reg} cells *in vitro* was compared to that of anti-TCR mAb (R73; 2.5 μg/ml) plus conventional anti-CD28 mAb (JJ319; 2.5 μg/ml) and rhIL-2 (300 U/ml). (b) Freshly isolated T_{reg} and T_{conv} cells were stimulated with CD28-SA and rhIL-2 for up to 102 days. The cultures were re-stimulated on a weekly basis. The fold cell expansion is given on a logarithmic scale. (c) Expression of CD25, CD152 and FoxP3 by CD28-SA-expanded T_{reg} and T_{conv} cells after more than 95 days in culture was determined by triple staining. Specific stainings are depicted in black. Grey shadows show, in the case of CD25 and FoxP3, staining with an isotype-matched control antibody and, in the case of CD152, staining after preincubation with unconjugated anti-CD152 mAb. (d) After 78 days in culture CD28-SA-expanded T_{reg} cells (open circles) or T_{conv} cells (filled circles) were cultured in the absence or presence of 5×10^4 freshly isolated $CD4^+$ indicator T cells (T_{ind} cells) and stimulated with Con A plus APC. Filled diamond: Proliferation of T_{ind} cells in the absence of CD28-SA-expanded cells. Detached circles: Proliferation of CD28-SA-expanded T_{reg} cells (open) or T_{conv} (filled) without T_{ind} cells.

lation led to a more than 10^5-fold expansion while co-stimulation/IL-2 expanded T_{reg} cells 10-fold less (Fig. 2a). To further assess the potential of CD28-SA for long-term T_{reg} cell culture freshly isolated T_{reg} and T_{conv} cells were cultured for up to 110 days in the presence of the CD28-SA and IL-2 (Fig. 2b). T_{reg} cells were preferentially expan-

ded over conventional T cells leading to expansion rates of up to 6×10^{16}-fold for T_{reg} cells and 5×10^{9}-fold for T_{conv} cells within 102 days in culture (Fig. 2b). To phenotypically characterize CD28-SA/IL-2-expanded T_{reg} and T_{conv} cells (see Fig. 2b), we analysed the expression of CD25, CD152 (CTLA-4) and FoxP3 marker proteins by flow cytometry. CD28-SA-expanded T_{reg} and T_{conv} cells cultured for more than 95 days displayed high expression levels of CD25 and CD152 (Fig. 2c). Importantly, all CD28-SA-expanded T_{reg} cells expressed FoxP3 protein (Fig. 2c), identifying them as bona fide T_{reg} cells. FoxP3 expression was, however, not confined to the progeny of T_{reg} cells as also 28% of CD28-SA/IL-2-expanded T_{conv} cells expressed FoxP3 (Fig. 2c). Western blot analysis confirmed FoxP3 expression by CD28-SA-expanded T_{reg} and T_{conv} cells (data not shown). To test whether long-term cultured T_{reg} cells had also functionally retained their suppressor phenotype we performed a standard *in vitro* suppression assay using CD28-SA-expanded T_{reg} cells after 78 days in culture and freshly isolated $CD4^+$ $CD25^-$ T cells as indicator cells. Indeed, these long-term cultured T_{reg} cells significantly inhibited the proliferation of indicator cells upon coculture (Fig. 2d). T_{conv} cells, however, expanded in parallel cultures displayed no suppressive activity (Fig. 2d), despite expression of FoxP3 by 70% of these cells (data not shown). In line with the missing regulatory T cell activity of CD28-SA/IL-2-expanded T_{conv} cells expressing FoxP3, CFSE-tracking experiments using freshly isolated $CD4^+$ $CD25^-$ cells, indeed, indicated induction of 'aberrant' FoxP3 expression in $FoxP3^-$ T_{conv} cells, rather than outgrowth of pre-existing $FoxP3^+$ T_{reg} cells (data not shown). Therefore, FoxP3 expression by the progeny of rat T_{conv} cells does not indicate conversion to a T_{reg} cell phenotype, thus resembling *in vitro*-cultured human $CD4^+$ $CD25^-$ T cells which also express FoxP3 upon activation, but without becoming suppressive.[25] Taken together, CD28-SA-stimulation in conjunction with IL-2 is superior to costimulation plus IL-2 in propagating T_{reg} cell growth *in vitro* and is also suitable for large-scale and long-term expansion of T_{reg} cells.

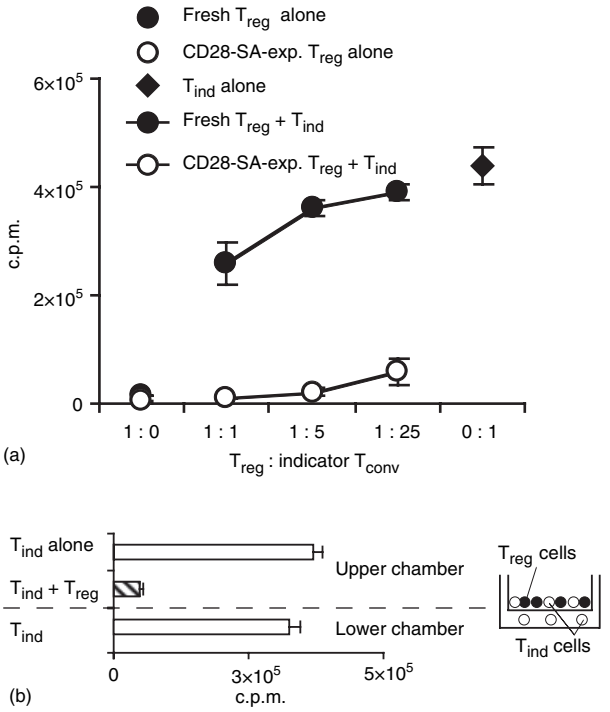

Figure 3. Characterization of suppression by T_{reg} cells after CD28-SA-mediated expansion. (a) After a 12 day culturing period using CD28-SA plus IL-2 T_{reg} cells (open circles; 800-fold expansion) and freshly isolated T_{reg} cells (filled circles) were cocultured with freshly isolated $CD4^+$ $CD25^-$ T_{ind} cells at the given T_{reg} to T_{ind} cell ratios. Filled diamond: Proliferation of T_{ind} cells in the absence of T_{reg} cells. Detached circles: Proliferation of T_{reg} cells without T_{ind} cells. Cultures were stimulated for 3 days with Con A and proliferation was measured by [^{3}H]thymidine incorporation. (b) CD28-SA-expanded T_{reg} cells (53 days in culture; 5.3×10^8-fold expansion) were cultured with nylon wool non-adherent indicator T cells (T_{ind}) of which half had direct cell-contact and the other half was separated from the T_{reg} cells by a millicell® cell-culture insert.

Functional characterization of CD28-SA-expanded T_{reg} cells

To carefully assess the suppressor qualities of CD28-SA/IL-2-expanded T_{reg} cells, we made a side-by-side comparison of their effector function and that of freshly isolated regulatory T cells. CD28-SA-expanded T_{reg} cells displayed greatly enhanced suppressive activity as compared to freshly isolated T_{reg} cells (Fig. 3a), almost completely inhibiting indicator T-cell proliferation at a T_{reg} to indicator T-cell ratio of as little as 1 : 25 (Fig. 3a).

In vitro suppression by 'naturally occurring' regulatory T cells, unlike that of inducible IL-10-producing Tr1 cells[26] or transforming growth factor-β-producing TH3 cells,[27] is limited to settings where T_{reg} cells are in direct contact with the cells they suppress.[28] In coculture experiments using transwell-chambers only the proliferation of indicator cells with direct cell contact to CD28-SA-expanded T_{reg} cells was suppressed, whereas indicator cells separated from the T_{reg} cells by a membrane were not inhibited in their proliferation (Fig. 3b).

Thus, the functional characterization of CD28 superagonist/IL-2-expanded T_{reg} cells revealed a much stronger, but also cell contact-dependent, suppressive activity of these cells as compared to freshly isolated T_{reg} cells.

Suppression by CD28-SA-expanded T_{reg} cells is TCR-dependant, compatible with CD28-SA stimulation and high concentrations of IL-2

Suppression by T_{reg} cells is only initiated after stimulation of their TCR, but not restricted to T_{conv} cells sharing the

same TCR specificity with the T_{reg} cells.[29] Furthermore, it was postulated that 'over-stimulation' of T_{conv} cells by IL-2 or via CD28 constitutes a general mechanism for T_{conv} cells to escape suppression by T_{reg} cells.[30–32] However, both CD28[13,14] and IL-2 are also pivotal for T_{reg} cell homeostasis *in vivo*[33] and IL-2 is known to be a strong activator of T_{reg} cell effector functions both *in vivo* and *in vitro*.[31–34]

Co-cultures of mouse lymph node cells and CD28-SA/IL-2-expanded rat regulatory T cells allowed us to stimulate the TCR complexes and CD28 molecules of indicator and regulatory T cells independently of each other. Neither addition of rat CD28-SA-expanded T_{reg} cells (Fig. 4f) nor anti-rat TCR mAb (Fig. 4b) inhibited mouse $CD4^+$ cell proliferation induced by anti-mouse CD3 mAb (Fig. 4a). Suppression was only achieved when rat regulatory T cells were added together with anti-rat TCR mAb (Fig. 4g). Addition of IL-2 or an anti-mouse CD28 mAb did not greatly enhance proliferation of indicator $CD4^+$ T cells in the absence of CD28-SA-expanded T_{reg} cells (Fig. 4c, e). However, suppression by rat CD28-SA-expanded T_{reg} cells was completely abrogated in the presence of anti-mouse CD28 mAb (Fig. 4h), but not after addition of IL-2 (Fig. 4j). Addition of conventional anti-rat CD28 mAb in the absence of CD28-SA-expanded T_{reg} cells did not hamper mouse indicator T-cell proliferation (Fig. 4d) and did not interfere with suppression (Fig. 4i). These data map the inhibition of suppression elicited by strong CD28 stimulation to the side of the indicator T cells and suggest that, in contrast to T_{conv} cells, T_{reg} cells cannot be 'over-stimulated'.

The TCR-dependency of suppression by CD28-SA-expanded T_{reg} cells was confirmed in syngeneic suppression assays, where no suppression was detectable upon superagonistic anti-CD28 stimulation (compare Fig. 5a and d). Importantly, addition of by itself weakly mitogenic anti-TCR mAb (data not shown) to the CD28-SA, both in solution, allowed very profound suppression by CD28-SA-expanded T_{reg} cells (compare Fig. 5b and e). The slight reduction in the proliferation of indicator $CD4^+$ cells (compare Fig. 5a and b) upon addition of anti-TCR mAb to the CD28-SA can be attributed to the

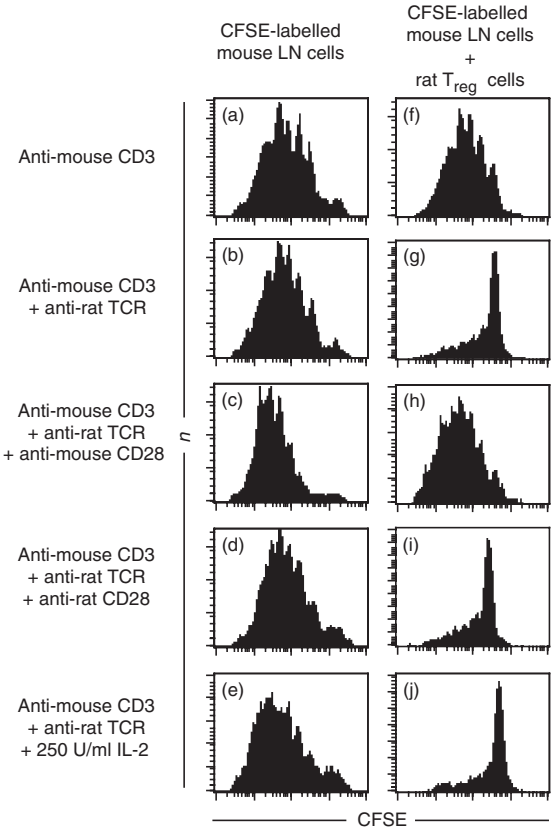

Figure 4. Impact of the TCR, CD28 and IL-2 on suppression by CD28-SA-expanded T_{reg} cells. CFSE-labelled lymph node cells (1×10^5) of C57Bl/6 mice were stimulated with anti-CD3 mAb (0.5 μg/ml) added in solution and cultured either alone (a–e) or in the presence of 5×10^4 CD28-SA-expanded rat T_{reg} cells (f–j). Anti-rat TCR mAb (1 μg/ml), conventional anti-rat CD28 mAb (0.5 μg/ml), anti-mouse CD28 mAb (0.5 μg/ml) or rhIL-2 (250 U/ml) were added where indicated. CFSE-dye dilution was assessed after 4 days in culture.

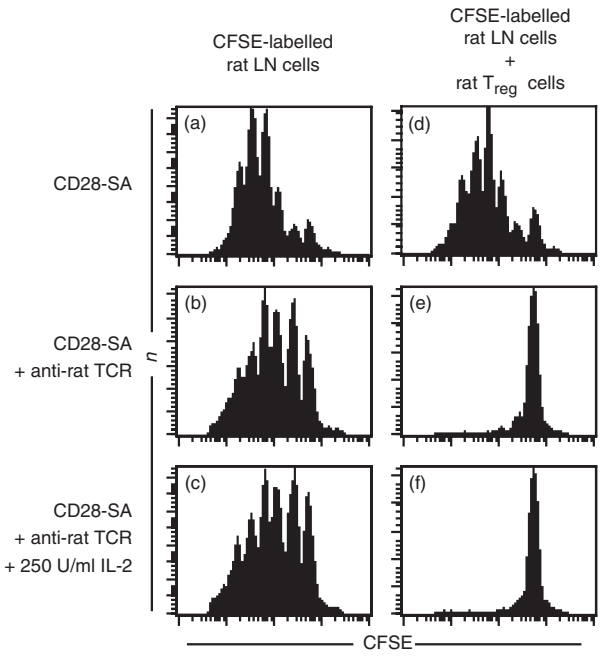

Figure 5. CD28-SA and anti-TCR mAb in solution allow strong suppression by CD28-SA-expanded T_{reg} cells. (a) CFSE-labelled rat lymph node cells (1×10^5) were either stimulated with CD28-SA alone (10 μg/ml) (b) a combination of CD28-SA (10 μg/ml) and anti-TCR mAb (1 μg/ml) or (c) with 250 U/ml of rhIL-2 in addition the stimuli also contained in (b). (d–f) CD28-SA-expanded T_{reg} cells (5×10^4) were added to cultures otherwise set up as in (a–c), respectively. CFSE-dye dilution among $CD4^+$ lymph node cells was determined after 3 days.

10% T_{reg} cells usually found within $CD4^+$ cells. Addition of IL-2 to these cocultures of syngeneic indicator and T_{reg} cells also did not abrogate suppression, confirming the data we had obtained in the xenogenic system (Fig. 4j). Thus, we conclude that CD28-SA/IL-2-expanded and -activated T_{reg} cells strictly depended on triggering of their TCR to exert suppression, which is abrogated by strong costimulation through CD28 but not by addition of IL-2. This, further, implies that strong anti-CD28 co-stimulation does not abrogate suppression by inducing IL-2 production in T_{conv} cells.[31]

CD28-SA/IL-2-expanded T_{reg} cells do not confer infectious tolerance to conventional T cells

In a number of experimental settings *in vivo*[35] and, also, *in vitro*[24,36] induction of T-regulatory activity in conventional T cells by 'naturally occurring' T_{reg} cells has been described. Co-culture of mouse $CD4^+$ $CD25^+$ T_{reg} cells and T helper 2 (Th2)-primed T_{conv} cells, however, induced only anergy in the Th2 cells but no suppressor phenotype.[37] To test whether CD28-SA/IL-2-expanded regulatory T cells render conventional T cells anergic and/or suppressive, we first cocultured CD28-SA-expanded T_{reg} cells with CFSE-labelled conventional T cells in the presence of APC and Con A. After 3 days $CFSE^+$ suppressed T_{conv} cells were separated from $CFSE^-$ T_{reg} cells by FACS. Responder (Fig. 6b, left), suppressed (Fig. 6b, middle) and rested (Fig. 6b, right) T_{conv} cells, were re-stimulated with Con A in the presence or absence of fresh $CD4^+$ $CD25^-$ T_{ind} cells. As depicted in Fig. 6(c), all re-stimulated T_{conv} cells (columns 1–3) responded equally to Con A stimulation, but altogether less than freshly isolated $CD4^+$ $CD25^-$ cells (column 7). Importantly, also the cocultures of each population of preactivated T_{conv} cells, including suppressed T_{conv} cells, with freshly isolated T_{ind} cells produced similar levels of overall proliferation (Fig. 6c, columns 4–6).

These data indicate that the anergy induced in T_{conv} cells by CD28-SA/IL-2-expanded T_{reg} cells is limited to the actual coculturing period and that there is no conversion of conventional T cells into regulatory T cells.

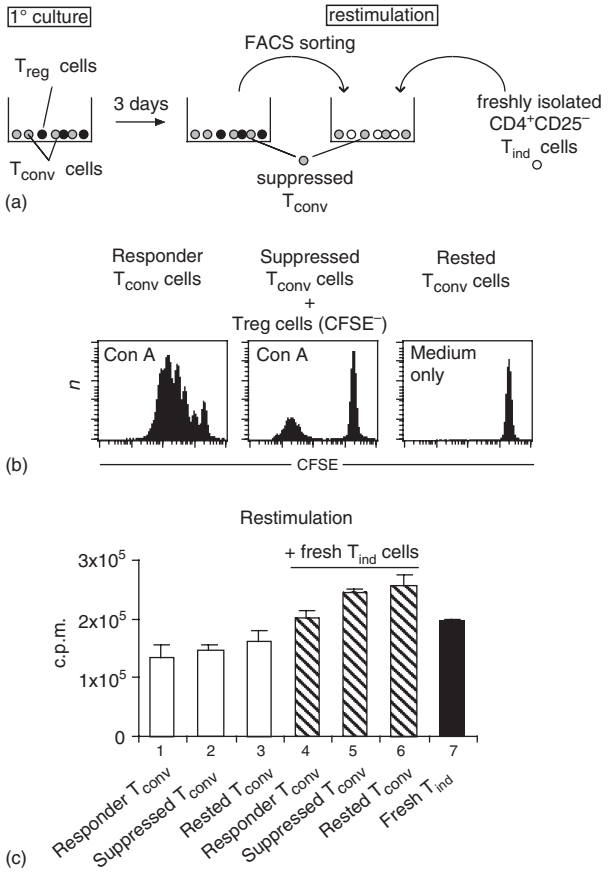

Figure 6. CD28-SA-expanded T_{reg} cells do not induce 'infectious tolerance' in conventional T cells. (a) Schematic diagram of the experiment. (b) CFSE-labelled $CD4^+CD25^-$ T_{conv} cells were stimulated with Con A in the presence ('suppressed T_{conv}') or absence ('responder T_{conv}') of an equal number of CD28-SA-expanded T_{reg} cells or were left in medium only ('rested T_{conv}'). (c) After 3 days, $CFSE^+$ cells were sorted by flow cytometry and fresh $CD4^+$ $CD25^-$ T_{conv} cells (T_{ind}) were purified in parallel. Suppressed T_{conv}, responder T_{conv} and rested T_{conv} cells were re-stimulated with Con A either in the absence (lanes 1–3) or presence (lanes 4–6) of an equal number of T_{ind} cells. Lane 7: Stimulation of T_{ind} cells alone. Proliferation was assayed by [^{3}H]thymidine incorporation.

Discussion

In this study we assessed the potential of CD28-SA together with IL-2 to induce large-scale and long-term expansion of rat regulatory T cells *in vitro*. By carefully titrating the amount of the CD28-SA and IL-2 in a short-term proliferation assay (Fig. 1), CD28 could be identified to substitute for the TCR in providing the 'first signal' and IL-2 the 'second', costimulatory, signal. This suggests that CD28-SA are a super-mimic of physiological CD80/CD86–CD28 interactions, thus inducing T_{reg} cell proliferation by strongly activating a signalling cascade which, *in vivo*, is critically involved in T_{reg} cell homeostasis.[13,14]

On a long-term basis, T_{reg} cells could be expanded dramatically with CD28-SA and IL-2 and kept in culture for more than 3 months (Fig. 2). The long-term expansion of T_{reg} cells was clearly superior to that of freshly purified T_{conv} cells (Fig. 2b), thereby minimizing the risk of outgrowing contaminating T_{conv} cells in T_{reg} cell cultures, as has been observed with other protocols.[20] However, both T_{reg} cells and T_{conv} cells showed better expansion upon CD28-SA/IL-2 stimulation than after costimulation plus IL-2 (Fig. 2a and data not shown). We assume that this difference is, on the one hand, the result of the absence of a strong, pro-apoptotic, TCR signal and, on the other hand, mediated by the induction of anti-apoptotic molecules, like BCL_{XL}, upon CD28-SA stimulation.[38] Import-

antly, long-term culture of freshly isolated T_{reg} cells using CD28-SA and IL-2 gave rise to populations of pure FoxP3$^+$ cells with high CD25 and CD152 expression. Similar to other protocols used for mouse T_{reg} cell expansion *in vitro*,[20,29] this activated phenotype was indicative of the very strong suppressive activity exerted by rat T_{reg} cells after CD28-SA/IL-2 stimulation as compared to freshly isolated T_{reg} cells (Fig. 3).[22] As far as the mode of suppression was concerned, suppression by CD28-SA/IL-2-activated T_{reg} cells, unlike mouse T_{reg} cells expanded with anti-CD3 mAb, APCs and IL-2[29] remained dependent on 'cell-contact' as determined in transwell cell culture systems (Fig. 3). However, the distances between T_{reg} cells and T_{conv} cells were presumably too big in these transwell cell cultures to rule out that the scavenging of IL-2 by T_{reg} cells[32,34] substantially contributes to suppression by CD28-expanded T_{reg} cells.[32] However, even when there were no restraints on the proximity of CD28-SA-expanded T_{reg} cells and indicator T cells[39] the T_{reg} cells had to be activated through their TCR in order to become suppressive (Fig. 4), while IL-2 scavenging also occurs in the absence of TCR stimulation.[34] Therefore, our data generated with CD28-SA-activated rat T_{reg} cells add to the work of others,[31,34] suggesting that suppression by T_{reg} cells *in vitro* is not mediated by mere competition for IL-2. Moreover, the strict dependence of CD28-SA-expanded T_{reg} cells on TCR stimulation for suppression reveals that the molecular programme inducing proliferation and preactivation of T_{reg} cells and that licensing T_{reg} cells for suppression by TCR/CD3 stimulation can be separated.

In contrast to TCR-derived signals, strong CD28 stimulation has been described as a mechanism for T_{conv} cells to escape suppression by T_{reg} cells.[30,31] However, by providing a, by itself, weakly mitogenic TCR stimulus we could show that suppression occurs even in the presence of strong CD28 stimulation, mediated either by a CD28-SA (Fig. 5) or by a conventional anti-CD28 mAb (data not shown). In accordance with published data,[30,31] suppression in the context of strongly mitogenic anti-CD3 stimulation (Fig. 4) was not compatible with additional triggering of CD28 on T_{conv} cells with mAbs. Taken together, these findings suggest that the nature and/or the strength of the TCR signal in T_{conv} cells determines whether additional costimulatory signals transduced by CD28 (Fig. 4),[30,31] and probably also the glucocorticoid-induced tumour necrosis factor receptor[40] or OX40[41] render T_{conv} cells refractory to suppression by T_{reg} cells. Physiological stimulation of T cells by peptide/major histocompatibility complexes also generates a comparatively weak signal, which relies on costimulation via CD28 for full T-cell activation.[42] Therefore, suppression assays using weak TCR stimulation probably resemble the physiological setting more closely than assays employing strongly mitogenic concentrations of anti-CD3 mAb.

Apart from CD28-mediated signals IL-2 has also been described to partially counter anergy of CD4$^+$ CD25$^-$ indicator T cells during suppression assays[31] or to completely abrogate suppression by freshly isolated mouse T_{reg} cells *in vitro*.[32] In contrast, functional analysis of CD28-SA/IL-2-activated T_{reg} cells using either syngeneic (Fig. 5) or xenogeneic indicator cells (Fig. 4) revealed no abrogation of suppression in the presence of high concentrations of IL-2. This, in turn, indicates that it is not induction of IL-2 synthesis by strong costimulation via CD28,[31] which allows conventional T cells to escape suppression by CD28-SA expanded T_{reg} cells (Fig. 4). *In vivo* the cellular source of IL-2 are putatively pathogenic T_{conv} cells that supply the IL-2 to keep T_{reg} cells metabolically fit.[15] Despite continuous IL-2 production by freshly isolated T_{conv} cells cocultured with T_{reg} cells[34], freshly isolated T_{reg} cells are very capable of suppressing these T_{conv} cells. Therefore, our data strengthen the notion that the primary role of IL-2 *in vivo* is to place T_{reg} cells 'ahead' of putatively pathogenic T_{conv} cells.

As a result of suppression by mouse or human T_{reg} cells, T_{conv} cells can be converted into IL-10 producing regulatory Tr1 cells.[24,26,36] Such a form of 'infectious tolerance'[35] unleashes a regulatory cascade originating from CD4$^+$ CD25$^+$ T_{reg} cells. However, even complete suppression of rat indicator T-cell proliferation by CD28-SA/IL-2-stimulated T_{reg} cells did not induce stable anergy or even T_{reg}-cell activity in conventional T cells (Fig. 6). This, together with the licensing of CD28-SA-expanded T_{reg} cells through antigen recognition, could be important to achieve specific suppression of autoimmunity *in vivo* while preserving a pool of conventional effector T cells capable of responding to foreign antigen.

In humans, direct activation of T_{reg} cells *in vivo* or adoptive immunotherapy with cultured T_{reg} cells will certainly turn out to be both feasible and beneficial in, at least, some clinical settings. Therefore, protocols for the expansion of human T_{reg} cells *in vitro* have started to emerge,[21] including superagonistic anti-human CD28 mAb, which are also capable of strongly expanding human T_{reg} cells *in vitro* without loss of function (unpublished data).

Finally, we believe that the availability of CD28-SA-expanded rat T_{reg} cells and the thorough characterization of their suppressive properties *in vitro* will facilitate future research into the underlying mechanisms of CD28-SA therapy *in vivo*, as well as the nature and physiology of T_{reg} cells in general.

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