

T cells stimulated *in vitro* have a suppressive function but do not contain only regulatory T cells

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Summary

The generation of regulatory T cells (T_{regs}) *in vitro* represents an attractive possibility to set up cellular therapies that could prevent and cure autoimmune disorders. Different methods have been proposed to generate T_{regs} *in vitro* and to evaluate their phenotype and function. Moreover, the overlap between generation of activated and regulatory cells could often be underestimated. We showed that *in vitro* treatment of $CD4^+ CD25^-$ lymphocytes with different stimuli leads to a good expression of CD25 and forkhead box P3 (FoxP3) on most cells, but to a full T_{reg} phenotype (including CD127 negativity) in only a minor percentage of cells, ranging from 17–38% of cells treated with phytohaemagglutinin (PHA) to 50–91% of cells treated with T cell receptor (TCR) stimulation in association with transforming growth factor (TGF)- β . Some suppressive activity was demonstrated for T cells activated with all the different stimuli. However, while suppression mediated by TCR/TGF- β treated T cells was associated with an inhibition of both interleukin (IL)-2 and interferon (IFN)- γ in the co-culture supernatant, the suppression observed for PHA-activated cells occurred in the presence of large amounts of these cytokines. In conclusion, also taking into account other recent publications, caution should be taken in interpretation of data in the field of regulatory T cells.

Keywords: activation, cytokines, proliferation, regulatory T cells

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Introduction

Regulatory T cells (T_{regs}) represent the most important cell lineage responsible for active immune tolerance [1]. In a transgenic system, forced expression of the transcription factor forkhead box P3 (FoxP3) was able to convert murine effector T cells ($CD4^+ CD25^-$) into T_{regs} [2]. FoxP3 is now considered the most specific phenotypic marker of T_{regs} [3], even if the study of additional antigens, including glucocorticoid-induced tumour necrosis factor receptor-related gene (GITR), CD62 ligand (CD62L), CD103, cytotoxic T lymphocyte-associated protein antigen (CTLA)-4, human leucocyte antigen D-related (HLA-DR), chemokine (C-C motif) receptor 7 (CCR7), CD39, CD73 and CD127, may define these cells more clearly [4–7]. Moreover, the key role of FoxP3 in tolerance was confirmed further by the evidence that mutations in this protein are associated with a severe syndrome characterized by multiple autoimmune disorders and allergy both in humans and mice [X-linked autoimmunity–allergic dysregulation syndrome (XLAAD) or immunodysregulation, polyendocrinopathy, enteropathy,

X-linked (IPEX) or scurfy] [8–10]. Since then, a defect in T_{regs} has been hypothesized and/or demonstrated in many autoimmune disorders, including autoimmune polyendocrinopathies, diabetes and multiple sclerosis [11–14]. As a consequence, methods to induce T_{regs} *ex vivo* or *in vivo* represent a very attractive opportunity in preventing or curing severe autoimmune disorders [15]. Cells with some T_{regs} features have been generated *ex vivo* by different authors, but it is still doubtful whether they represent similarly stable-lineage naturally occurring T_{regs} [16–23]. The induction of T_{regs} directly *in vivo* by means of super-agonist anti-CD28 antibodies [24,25] seemed to be an even more promising approach but, unfortunately, a trial in human volunteers resulted in an almost fatal activation of lymphocytes and cytokine storm instead of allowing a tolerance increase [26].

In our study we tried to generate regulatory T cells *in vitro*. By means of different stimuli we obtained a mixture of cells with activated and regulatory phenotypes. These cells were not anergic, but exerted a suppressive function on both naive and memory T cells. These results strengthen the notion that methods for the generation of T_{regs} are still far from being

standardized. As a consequence we suggest that, although some enthusiastic results have been published, great care should be taken when experimental data are brought from bench to bedside.

Materials and methods

Samples

For each experiment, blood samples were obtained from two unrelated donors 5 days apart from each other (first and second donor); informed consent was requested and obtained. For both samples, peripheral blood mononuclear cells were extracted by means of centrifugation on Histo-paque 1077 (Sigma Aldrich, Milan, Italy) at 560 *g* for 30 min at room temperature. CD4⁺ cells were then separated by negative selection using magnetic sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). CD25⁺ cells were depleted from the samples using anti-CD25-coated beads (Miltenyi Biotec) in order to obtain CD25⁻ cells with a purity > 99%. CD25⁻ cells were divided further into RA and R0 subpopulations using the memory CD4⁺ T cell kit (Miltenyi Biotec).

The study was approved by the local independent bioethical committee.

Stimulation of CD4⁺ CD25⁻ cells

CD4⁺ CD25⁻ from the first donor were incubated in X-vivo 15 (Cambrex Bio Science, Milan, Italy). In order to use standardized conditions, for each stimulus 1×10^6 cells were split into 10 wells of a U-bottomed 96-well plate (Corning, NY, USA) with the following stimuli: (i) not stimulated (n.s.); (ii) 1 µg/ml of coated anti-CD3 (cCD3) plus 1 µg/ml of soluble anti-CD28 (sCD28) (Caltag Laboratories, Burlingame, CA, USA); (iii) 1 µg/ml of cCD3 plus 1 µg/ml of sCD28 plus 10 ng/ml of transforming growth factor (TGF)-β (Euroclone, Milan, Italy); (iv) 5 µg/ml phytohaemagglutinin (PHA) (Sigma Aldrich, Milan, Italy). After 5 days, all cells treated using the same set of stimuli were put into a single vial and 1×10^5 cells were analysed by flow cytometry. CD4 TRI-COLOR® (TC) (Caltag Laboratories, Burlingame, CA, USA), CD25 allophycocyanin-cyanin 7 (APC-Cy7) (BioLegend, San Diego, CA, USA), CTLA4 phycoerythrin (PE) (Pharmingen, BD Biosciences, San Jose, CA, USA), CD39 PE (abcam, Cambridge, UK), CD127 Alexa Fluor 647 (BioLegend), CCR7 fluorescein (R&D Systems Inc., Minneapolis, MN, USA) and FoxP3 fluorescein isothiocyanate (FITC) (human regulatory T cell staining kit; eBioscience, San Diego, CA, USA) were used to evaluate the phenotype of these cells.

Evaluation of the proliferation of stimulated T cells upon restimulation

For the proliferation assay, stimulated cells were labelled with carboxy fluorescein diacetate succinimide ester (CFSE,

Molecular Probes, Eugene, OR, USA) according to the Aids Clinical Trials Group (ACTG) protocol (<http://aactg.s-3.com/labmanual.htm>) and their proliferation was evaluated by means of the dye dilution at each cell division. The number of cells for each generation was measured using FlowJo software (Tree Star Inc., Ashland, OR, USA). The percentage of proliferated precursors was calculated using the following formula: $\% = (G1/2 + G2/4 + G3/8) / (G0 + G1/2 + G2/4 + G3/8)$. Results expressed the average of two independent experiments.

Evaluation of the suppression activity of stimulated T cells

The above-mentioned pretreated cells were then incubated with CD4⁺ CD25⁻ target cells from the second donor at different ratios (first/second 1 : 0, 2 : 1, 1 : 1, 1 : 2, 0 : 1) in a 96-well plate coated with 1 µg/ml anti-CD3 (cCD3) plus 1 µg/ml soluble anti-CD28 (sCD28) for 5 days. Labelling of target cells with CFSE and calculation of the percentage of proliferated precursors were performed as described above. Proliferation of target cells in co-culture was expressed as a percentage of the target cells alone. Results expressed the average of two independent experiments.

Cytokine production by stimulated T cells and in co-culture

Cell culture supernatants were collected from each experiment and the production of interleukin (IL)-2 and interferon (IFN)-γ was measured by means of the cytometric bead array (CBA, Beckton Dickinson, San Diego, CA, USA) according to the manufacturer's instructions.

Results

Expression of T_{reg} phenotype after stimulation *in vitro* of CD4⁺ CD25⁻ cells

Stimulated cells showed positive expression of CD25, CTLA4, CD62L, CCR7, CD39 and FoxP3 irrespective of the received stimulus (Fig. 1a). PHA-stimulated cells showed higher mean fluorescence intensity (MFI) for CD25 (MFI = 138·91) and FoxP3 (MFI = 11·61) compared with cCD3/sCD28-stimulated cells (CD25 = 24·42, FoxP3 = 8·6) and with cCD3/sCD28-TGF-β-stimulated cells (CD25 = 14·65, FoxP3 = 9·60). On the other hand, expression of the activation marker CD127 was higher in PHA-stimulated cells (MFI = 58·97) than in the other two groups which showed, respectively, a CD127 MFI of 15·48 and 4·61. Among CD25⁺ cells, a combination of high expression of FoxP3 and low expression of CD127 was more common in cells treated with cCD3/sCD28-TGF-β (50·91%) compared with cells stimulated with cCD3/sCD28 (29·60%) or with PHA (17·38%) (Fig. 1b).

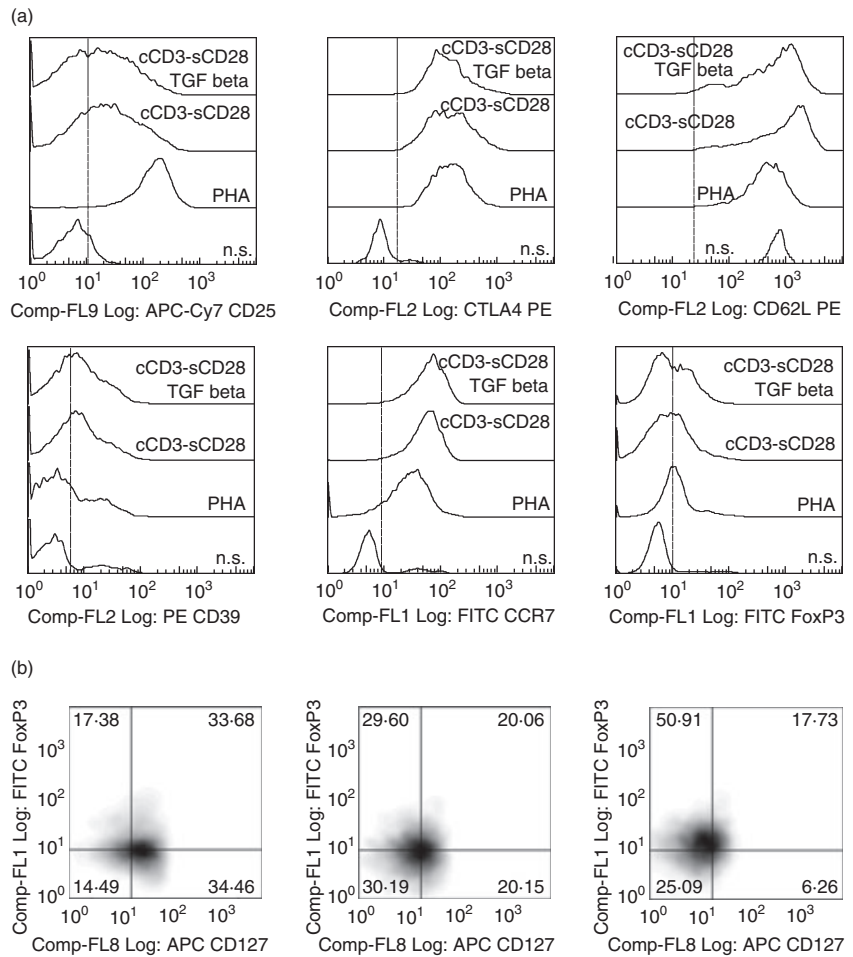


Fig. 1. Evaluation of T regulatory (T_{reg}) immunophenotype on T lymphocytes after 5 days of culture with different stimuli: (a) histograms of expression of CD25, cytotoxic T lymphocyte-associated protein antigen-4 (CTLA4), CD62 ligand (CD62L), CD39, chemokine (C-C motif) receptor 7 (CCR7) and forkhead box P3 (FoxP3); (b) percentage of FoxP3⁺ CD127⁻ cells in a CD25 positive gate in the different groups. All dot plots were obtained after lymphocyte scatter and CD4⁺ gating.

Proliferation and cytokine production after restimulation

Cells prestimulated with PHA or with cCD3/sCD28 showed a good proliferative response when restimulated with cCD3/sCD28 antibodies (41.25% and 38.77%, respectively), while the proliferative response of TGF- β -treated cells was clearly lower (13.95%). Non-prestimulated cells responded very poorly to stimulation (1.45%) (Fig. 2a). The production of IFN- γ upon restimulation was higher with cCD3/sCD28 stimulus (13660 pg/ml) compared with PHA (5109 pg/ml) and cCD3/sCD28-TGF β (925 pg/ml). Larger amounts of IL-2 were produced with PHA (4371 pg/ml) than with cCD3/sCD28 stimulus (105 pg/ml) and cCD3/sCD28-TGF β (80 pg/ml) (Fig. 2b, c).

Suppressive function of stimulated cells on heterologous CD4⁺CD25⁻ cells

The proliferation of CFSE-labelled target cells was suppressed to various extents by the addition of prestimulated T cells in co-culture. At a ratio of 2 : 1 (prestimated to target cells), the proliferation of target cells diminished to 68%,

47%, 21% and 27% in co-culture, respectively, with n.s., PHA-stimulated, cCD3/sCD28-stimulated and cCD3/sCD28-TGF- β -stimulated cells. The effect was similar on naive and memory T cells, apart from the PHA-stimulated cells which appeared to be more effective in suppressing naive T cells. The production of IL-2 was increased strongly in co-culture with PHA-stimulated T cells (Fig. 3).

Discussion

The generation of regulatory T cells *in vitro* is an attractive opportunity in the prevention and cure of autoimmune disorders [15]. Unfortunately, different experimental studies for generation of T_{reg} *in vitro* obtained controversial results, due to slight differences in culture media, stimuli, time of incubation and cytokines used, and there is no agreement on the best method for generating T_{reg} *in vitro*. Only few researchers have pointed out the apparent paradox that the stimuli used to generate regulatory T cells are the same used to activate T cells, and that the expression of CD25 is not a prerogative of only regulatory T cells [27–29]. This apparent paradox continues when the generation of T_{reg} is also attempted *in vivo*, obtaining opposite results with the induction of regulatory T

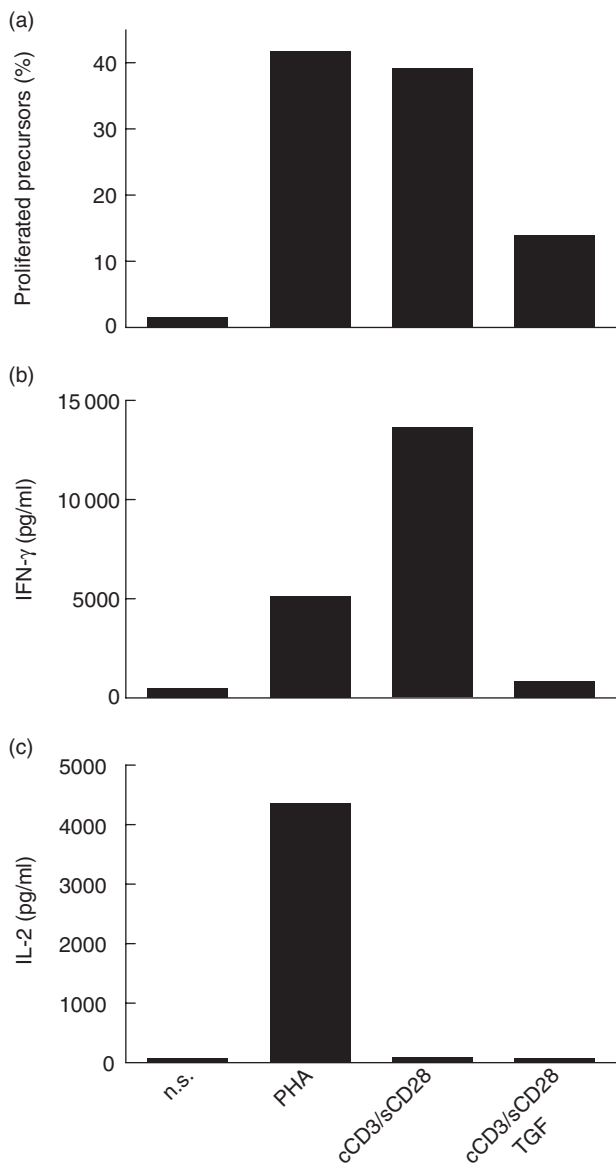


Fig. 2. Behaviour of stimulated cells upon restimulation with cCD3/sCD28: percentage of proliferated precursors (a); production of interferon- γ (IFN- γ) (b); production of interleukin-2 (IL-2) (c).

cells [30] or activated T cells [26]. We still do not know what is responsible for distinguishing the two cases. Moreover, recent data have suggested: (i) that a T_{reg} phenotype is normally acquired transiently by activated T cells [27] and (ii) that *in vitro*-generated T_{regs} require further stimulation to maintain their regulatory function [31]. Thus, *in vitro*-generated T_{regs} appear to be largely different from the naturally occurring T_{regs} , which represent a stable lineage.

We characterized the immune phenotype and the regulatory activity of lymphocytes after 5 days activation. After treatment with different stimuli, most cells expressed typical T_{reg} markers, including CD25 and FoxP3. However, only minor percentages of cells showed a T_{reg} phenotype as defined by the presence of CD25 and FoxP3 and the lack of

CD127. These results are in agreement with the observations of Wan *et al.*, who identified only a minority of FoxP3⁺ cells after TCR stimulation in the presence of TGF- β in a transgenic mouse model [32]. We suggest that, *in vitro*, T cell stimulation always leads to the generation of a mixture of activated and regulatory T cells, with higher percentages of the latter occurring after TCR stimulation in the presence of TGF- β . In fact, activated cells were not anergic after restimulation, even if the proliferative response appeared poorer for TCR/TGF- β -stimulated cells. These results are in partial agreement with recent data from Tran *et al.*, who showed that TCR/TGF- β -stimulated naive T lymphocytes, in spite of the expression of CD25 and FoxP3, still retain the capacity of proliferating and secreting lymphotropic cytokines upon restimulation [33].

The high levels of lymphotropic cytokines produced (in particular after PHA stimulation) demonstrated that the mixture contains true activated T cells. The high production of cytokines in FoxP3⁺ cells is not surprising, as it has been shown recently that FoxP3 is also expressed transiently in activated T cells [27,28]. What is surprising is that some suppressive function could also be demonstrated for PHA-stimulated cells. This effect occurred with the production of increasing levels of cytokines, that tended to be even higher as the percentage of prestimulated cells in the co-culture increased. A possible explanation of this suppression at the presence of high levels of cytokines could reside in competition for nutrients and cytokines by prestimulated T cells. We cannot exclude that among prestimulated cells a small population of true regulatory T cells exist; however, this seems unlikely, as the PHA-stimulated cells did not show very high levels of FoxP3, while it has been argued recently that true regulatory cells display even higher levels of FoxP3 expression [28]. That the greater suppressive effect of PHA-prestimulated cells was on naive T cells has no clear explanation, but it could be consistent with a mechanism of suppression different from that of typical T_{regs} .

It is noteworthy that the suppressive function of preactivated cells could be seen only if we use methods able to measure the proliferation of target cells in the co-culture (e.g. CFSE dilution assay), rather than those based on the measure of the whole co-culture proliferation (e.g. [³H]-thymidine). The fact that other authors failed to demonstrate a suppressive capacity of activated T cells may depend upon small differences in the experimental procedure (e.g. the stimulation of naive T cells in the work by Tran *et al.* [33]) or in the method used to measure the proliferation of target cells ([³H]-thymidine, instead of dye dilution methods).

Our results highlight the possible problems and pitfalls in *in vitro* generations of T_{regs} . Walker proposed that the timing after stimulation could be crucial for the choice between the fate of activated and regulatory T cells, the latter requiring as long as 10 days to be generated [18]; however, other authors argued later that regulatory T cells generated *in vitro* are not

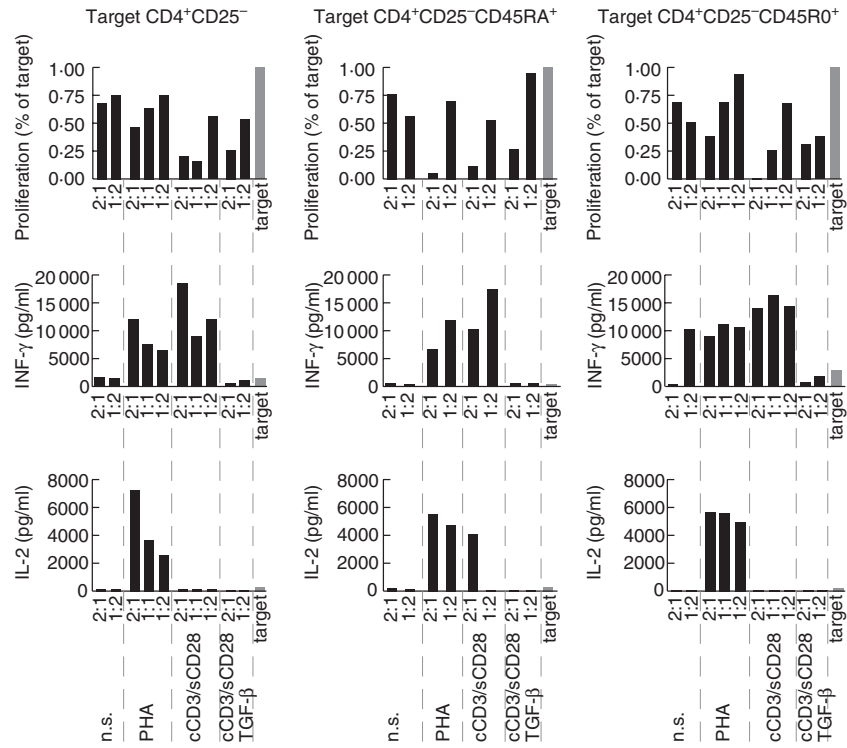


Fig. 3. Proliferation of carboxy fluorescein diacetate succinimide ester-labelled target T cells co-cultured with prestimulated cells at different ratio (prestimated : target) and production of cytokines in the co-culture supernatants. The results are compared with target cells alone (grey bar).

a stable lineage [31]. We still lack evidence on the possibility of generating stable regulatory T cells *in vitro* and it is possible for most attempts to result only in the description of a particular stage of lymphocyte activation and development. As a consequence, we suggest that we still need to be extremely cautious in translating transient results *in vitro* to experimentation *in vivo*.

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