Modulation of the TCR stimulation strength can render human activated CD4⁺ T cells suppressive

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Abstract
In this study, we explored the potential of human naive CD4⁺ T cells to acquire regulatory properties upon stimulation. We demonstrated that, in vitro, pre-activated naive CD4⁺CD25⁻CD45RA⁺ T cells could become anergic and suppressive CD4⁺CD25⁺ T cells upon lower intensity TCR stimulation. These CD4⁺CD25⁺ T cells generated in vitro potently suppress the proliferation of allogenic CD4⁺CD25⁻ T cells independently of cytokines and in a contact-dependent manner. Our data indicate that expression of Foxp3 is not necessary to induce the suppressive T cell activity. We demonstrate that these CD4⁺CD25⁺ T cells are unresponsive upon re-stimulation and that their suppressive activity is transient. However, we showed that the anergy and the suppressive function could be reversed by increasing the stimulus and their level of activation. We concluded from our data that these anergy and suppressive activities are related to a fine tuning of TCR activation threshold.

Introduction
Proper regulation of the immune system is essential to control autoimmunity or excessive inflammatory immune responses. Over time, it became clear that some T-cells, named naturally occurring regulatory T cells (nTreg), were important in dampening the immune response. These cells with a typical phenotype CD4⁺CD25hi (1, 2) play a crucial role in peripheral tolerance and the prevention of autoimmunity (3–6). A member of the forkhead/winged helix tran- 
scription factors, Foxp3, has been shown to be essential for the development, as well as for the suppressive function of nTreg (7–9). These cells inhibit the IL-2 transcription and the proliferation of naive and memory CD4⁺ and CD8⁺ T cells (10–14). Their mechanisms of suppression are not yet well understood but the nTreg have been shown to suppress T cell responses in a contact-dependent and cyto- 
kine-independent manner upon TCR activation (14, 15). Furthermore, this population subset constitutes a minority of T cell population and their numbers constantly range be- 
tween 5 and 10% of the CD4⁺ T cells in human peripheral blood (11–13). However, it appears that Foxp3 expression could also be induced in human peripheral CD4⁺CD25⁻ T cells (R cells) upon activation (16–18), suggesting that Foxp3 is not specific for nTreg. Several studies have also demonstrated that these cells could be converted into sup- pressor cells (19, 20). Interestingly, the increase of human regulatory T cells (Treg) has been reported to be associ- ated with the reduction of R cells in tumors suggesting that new Treg could be generated during an immune response in order to control the spread of the response but the prov- enance and the mechanisms that contribute to increase Treg remain elusive (21). In mice, CD4⁺ T cell conversion could be induced after TCR activation in the presence of transforming growth factor-β (TGF-β) that results in the expression of Foxp3 and induction of suppressive function (15, 22–24). Unlike mice, human T cells require multiple activi- 
vations with TGF-β to become nTreg like (25, 26). However, other groups showed an unexpected expansion of induced regulatory T cells (iTreg) from human naive T cells, which were not dependent of TGF-β neither IL-10 for their differ- 
entiation nor their regulatory function (16, 27, 28). The

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possibility to isolate iTreg from human R cells in vitro, after a short period of expansion, would be useful for cell-based therapy in autoimmune diseases and would also allow to study easily the mechanism of suppression.

Overall, our results demonstrate that naïve R cells can become polyclonal effector CD4*CD25+ T cells acquiring energic and suppressive properties after TCR re-stimulation of lower strength.

Methods

Antibodies and flow cytometry

Anti-human CD4-FITC, CD25-PE, CD25-PECy5, CD25-PECy7, CD127-PE, CD69-PE, CD45RA-PECy5, CD45RO-PE, CTLA-4-PECy5, CD80-PE and CD86-PE were purchased from BD Pharmingen (San Jose, CA, USA). Purified (NA/LE) anti-human CD28, anti-human CTLA-4 antibodies were also purchased from BD Pharmingen. Foxp3-PE, Foxp3-Alexa-Fluor 488, Foxp3-Alexa-Fluor 700 antibodies were purchased from eBiosciences (San Diego, CA, USA). CD69-PECy5 was purchased from Beckman Coulter (Fullerton, CA, USA). Pelicluster CD3 (clone CLB) was purchased from Sanquin (Amsterdam, Netherlands). Purified (NA/LE) anti-human CD3 clone (UCHT1) was purchased from R&D systems (Minneapolis, MI, USA). Purified anti-human IL-12p35 and APC-conjugated anti-mouse IgG antibodies were purchased from R&D systems for the intracellular staining of the IL-35 subunit. PE-coupled anti-human IL-10 was purchased from R&D systems and APC-conjugated anti-mouse IgG antibodies were purchased from R&D systems for the intracellular staining of the IL-35 subunit. PE-coupled anti-human IL-10 was purchased from AbD Serotec (Raleigh, NC, USA). Protein Transport Inhibitor (GolgiPlug) used from the cytokine intracellular staining was purchased from BD Pharmingen. Identification of apoptotic and dead T cells, after 24 or 48 h of activation, were detected using the Apoptosis Detection kit from BD Pharmingen.

Isolation of CD4*CD25hi CD127low/- T cells (nTreg) from peripheral blood (PBMC)

PBMC were obtained from peripheral blood of healthy volunteers by Ficoll–Histopaque density gradient centrifugation (Sigma, Saint Louis, MO, USA). CD4* T cells were purified using the StemSep™ CD4* T cell enrichment kit (StemCell Technologies, Vancouver, Canada). Isolation of CD4*CD25* T cells was obtained by positive selection of CD25* T cells from CD4* T cells using CD25 MicroBeads II kit (Miltenyi Biotec SAS, Paris, France). The CD4*CD25* T cells were stained with FITC-coupled anti-CD4, PE-conjugated anti-CD127 and APC- or PE-Cy7-labeled anti-CD25 antibodies and sorted using a FACSARia™ cell sorter and FACSDiva™ software (BD Biosciences).

Isolation of effector R cells from PBMC

PBMC were obtained from peripheral blood of healthy volunteers by Ficoll–Histopaque density gradient centrifugation (Sigma). T cells were purified from PBMC using the StemSep™ CD4* T cell enrichment kit (StemCell Technologies) and depletion of CD25* T cells were performed using CD25 MicroBeads II kit (Miltenyi Biotec SAS). Purity was determined to be >90–95% CD4*CD25-.

Generation of iTreg

CD4*CD25-CD45RA* T cells were purified from PBMC of healthy volunteers using the StemSep™ CD45RO TAC kit (StemCell Technologies). The CD4*CD45RA* T cells obtained were stained with CD4-FITC, CD25-PECy7, CD45RA-PECy5 and CD45RO-PE before sorting the CD4*CD25-CD45RA* T cells with a purity >97% with a FACSARia™ cell sorter. These cells were activated with 5 µg ml-1 plate-bound anti-CD3 (clone UCHT1) and 1 µg ml-1 soluble anti-CD28 (clone CD28.2, Pharmingen). These cells were removed from the plate after 24 h and cultured in complete RPMI medium (RPMI 1640 supplemented with 10% pooled human AB serum, 2 mM l-glutamine, 1 mM sodium pyruvate and 100 U ml-1 penicillin-streptomycin, all from Invitrogen, Cergy-Pontoise, France). After 6–9 days of culture, T cells were used in different assays and activated with soluble anti-CD3 (clone CLB) and anti-CD28.

Suppression of T cell proliferation

Two types of functional assays were performed: thymidine incorporation and carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution. Effector R cells were seeded in U-bottom well plates at 25,000 cells per well for the thymidine assay and for the CFSE dilution assay. They were activated with soluble anti-CD3 (clone CLB) and anti-CD28 with or without nTreg or iTreg. A 1 µCi per well of [3H]-thymidine (Amersham Pharmacia, Piscataway, NJ, USA) was added to the cultures for the last 16 h. The cultures were harvested at day 5 and counted in a β-scintillation counter (Packard–Perkin Elmer, Courtaboeuf, France). The CFSE dilution was assessed after 3–5 days of culture on a FACScalibur™ (BD Biosciences). The data were analyzed using CellQuest Pro™ software. For transwell experiments, cells were cultured in 96-well plates (flat-bottom) with or without a 0.4 µm transwell insert separating Treg (25,000 cells per well) from R CFSE* cells (25,000 cells per well).

Isolation of CD4*CD25*CTLA-4* cells from peripheral blood

As previously described by Birebent et al. (29), CD4* T cells were prepared from healthy volunteers’ PBMC using the StemSep™ CD4* T cell enrichment kit (StemCell Technologies). CD4*CD25* were obtained by positive selection using the CD25 MicroBeads II kit (Miltenyi Biotec SAS) and stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 antibodies. They were then incubated for 1 h at 37°C in the presence of ionomycin (Sigma; 20 µg ml-1) and stained with PE-Cy5-conjugated anti-CTLA-4 antibody. The stained cells were sorted using a FACSARia™ cell sorter and FACSDiva™ software (BD Biosciences).

CD4*CD25*CTLA-4* suppression assay of T cell proliferation

PBMC (50,000 cells per well) were seeded in U-bottom 96-well plates and stimulated for 7 days with 50,000 allogeneic-irradiated (2500 rad) PBMC in the presence or absence of 25,000 autologous CD4*CD25*CTLA-4* T cells. For the blockade experiment, 10 µg ml-1 of anti-CTLA-4 antibody was added to the co-cultures. To determine the cell proliferation, 1 µCi per well of [3H]-thymidine (Amersham Pharmacia) was added in the last 16 h of the culture. The cells were harvested...
and the thymidine uptake counted on a β-scintillation counter (Packard–Perkin Elmer).

**CFSE labeling**

The cells were re-suspended in PBS at 10^7 cells ml^{-1} and stained with the same volume of 2 μM CFSE (Molecular Probes, Invitrogen) for 10 min at 37°C. Staining was stopped by addition of the same volume of FCS. The cells were then washed twice in complete RPMI medium and re-suspended at the desired concentration.

**IL-2 assays**

iTreg, nTreg and R cells were activated with soluble anti-CD3 (clone CLB) and anti-CD28 for 4 and 12 h. Then the cells were harvested and the total RNA was isolated using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA). Complementary DNA (cDNA) was synthesized using the SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technologies, Rockville, MD, USA). Real-time PCR was conducted using 2 μl of cDNA (corresponding to 1:10 of the total RNA of the cells used). Real-time PCR for IL-2 was performed using Taqman Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) and ribosomal RNA control reagent (Applied Biosystems) was used as the endogenous control. ABI Prism 7700 Sequence BioDetector was used for the real-time PCR for a total of 40 cycles.

**Membrane and intracellular immunofluorescence staining**

The staining of surface antigens was performed by incubating the cells for 30 min in 5% mouse serum at 4°C. The cells were then stained with the appropriate dilution of the antibody (anti-CD80-PE, anti-CD86-PE and anti-CTLA-PECy5) in buffer PBS/0.5% BSA/1 mM EDTA. For the determination of intracellular expression of CTLA-4, IL-10 and IL-12p35, the cells were stained as indicated in the figure legends. In some experiments, GolgiPlug (BD Pharmingen) was added in the last 4 h of stimulation. The cells were then harvested and fixed for 20 min at 4°C using the BD Cytofix/Cytoperm™ buffer. (BD Pharmingen). After two washes in BD Perm/Wash™, they were re-suspended in the same buffer containing the optimal concentration of the antibody and incubated at 4°C for 30 min in the dark. Samples were washed twice in PBS–BSA–EDTA buffer and run on a FACScalibur™ flow cytometer (BD Biosciences). The data were analyzed using the WinMDI software (Scripps, San Diego, USA).

**Generation of monocyte-derived immature and mature human dendritic cells**

Monocytes were prepared from PBMC obtained from peripheral blood of healthy volunteers by Ficoll–Histopaque density gradient centrifugation (Sigma). They were seeded at 2.10^6 cells per well in six-well plates for 5 days in the presence of complete medium supplemented with 800 U ml^{-1} granulocyte macrophage colony-stimulating factor (GM-CSF) (Cellgenix, Clermont l’Hérault, France), 250 U ml^{-1} IL-4 (Cellgenix). Additional supplemented complete medium was added at day 2. To obtain mature dendritic cells, the cells were harvested at day 5 and seeded at 10^6 cells per well in 48-well plates in the presence of complete medium supplemented with 800 U ml^{-1} GM-CSF (Cellgenix), 250 U ml^{-1} IL-4 (Cellgenix), 10 ng ml^{-1} IL-1β (Cellgenix), 10 ng ml^{-1} TNF-α (R&D systems) and 1 μg ml^{-1} prostaglandin E2 (Pfizer, Groton, CT, USA) for 24 h. The cells were then harvested and stained with fluorescent markers in order to determine their maturation state.

**Statistical analysis**

Data were analyzed using an unpaired two-tailed t-test by Prism 5.0 (GraphPad Software, San Diego, CA, USA). All P values <0.05 were considered significant.

**Results**

**Treg can be generated from naive CD4+ lymphocytes**

It has been described that naïve T cells could become Treg following activation (28). Because naïve T cells represent a major circulating population and are easy to isolate, we found interesting to be able to generate Treg in vitro and wanted to better characterize them. Since the literature was not clear on how these so-called ‘iTreg’ can be generated, we started our study by performing stimulation assays based on the conditions used by Walker et al. (16). CD4^+CD25^+CD45RA^- naïve T cells were freshly isolated from PBMC by FACS sorting and stimulated at 5 μg ml^{-1} plate-bound anti-CD3 (clone UCHT1) and 1 μg ml^{-1} soluble anti-CD28 (clone CD28.2) for 24 h. The cells were then harvested and expanded in complete medium for 9 days. On day 10, they were re-activated with 0.1 μg ml^{-1} of soluble anti-CD3 and 0.1 μg ml^{-1} of soluble anti-CD28. We observed, as previously described, that they were unable to proliferate and were able to suppress the proliferation of freshly isolated R cells when added to the cultures at a ratio of 1:1 (Fig. 1A). In order to rule out the possibility that iTreg could die following re-activation, we stained the cells with Annexin V and 7AAD. We tested whether iTreg as well as R cells were alive following 48 or 96 h of activation (data not shown) and whether suppression of the proliferation could be a consequence of cell death. To confirm the suppression, the R cells were CFSE labeled and were activated for 4 days with 0.1 μg ml^{-1} of soluble anti-CD3 and anti-CD28. The R cells proliferated and divided four to six times (Fig. 1B, left panel). However, when iTreg were added to the cultures, at a cell ratio of 1:1, dramatic inhibition of proliferation was observed (Fig. 1B, middle panel). As already indicated in the Fig. 1(A), the CFSE^-labeled iTreg did not proliferate for 4 days upon activation (Fig. 1B, right panel). Surprisingly, iTreg expressed almost no IL-2 messenger RNA (mRNA) after 12 h of activation, whereas R cells showed a significant production of IL-2 mRNA. When both cells were cultured together at a ratio 1:1, the IL-2 mRNA production of R cells was significantly decreased (P < 0.004) (Fig. 1C). These data suggested that we generated cells that were anergic and had the properties of nTreg.

**The suppressive effect of iTreg is independent of Foxp3 expression**

We could not rule out that these iTreg could be derived from an amplification of a small subset of CD25^+Foxp3^+ cells that
became CD25\textsuperscript{+}Foxp3\textsuperscript{+} upon activation. In order to verify this hypothesis, we decided to investigate if Foxp3\textsuperscript{+} cells could be found in our starting population of CD4\textsuperscript{+}CD25\textsuperscript{−}/CD45RA\textsuperscript{+}naive T cells. A <0.5% of the population was Foxp3\textsuperscript{+} when analyzed by flow cytometry (data not shown). We, then, followed the frequency of Foxp3\textsuperscript{+} cells in iTreg, during their \textit{in vitro} generation, and observed a maximum of Foxp3\textsuperscript{+} cells 7 days after activation (\(n=5\)) (Fig. 2A, left panel). At this time point (day 7), 27\% (±3\%) of the iTreg were Foxp3\textsuperscript{+} that was reduced to 10\% after 10 days of activation. The follow-up of the CD25 marker indicated that 85–95\% were CD25\textsuperscript{+} after only 2 days of activation and remained positive during all the period tested (Fig. 2A, right panel). Because the expression of Foxp3\textsuperscript{+} was maximum at day 7, we decided to harvest iTreg at this time point and used them to determine if they were suppressive. As already shown, cell surface expression of CD127 could be used as an alternative to the Foxp3 transcription factor to purify nTreg (1, 2). We noticed that iTreg also inversely expressed Foxp3 and CD127. The cells were sorted based on the CD4, CD25 and CD127 markers (Fig. 2B, upper left panel). Group I (CD4\textsuperscript{+}CD25\textsuperscript{−}CD127\textsuperscript{+}) represented ~50\% of the iTreg with ~20\% of cells expressing Foxp3 with a mean fluorescence intensity (MFI) of 40. The group II (CD4\textsuperscript{+}CD25\textsuperscript{−}CD127\textsuperscript{low/−}) represented 34\% of iTreg with almost 70\% of Foxp3\textsuperscript{+} cells (with a MFI of 30) and the group III (CD4\textsuperscript{+}CD25\textsuperscript{+}CD127\textsuperscript{+}) represented <10\% of iTreg and included only 4\% of Foxp3\textsuperscript{+} cells (with a MFI of 22) (Fig. 2B, lower panels). The CD4\textsuperscript{+}CD25\textsuperscript{−}CD127\textsuperscript{−} subset was negative for the Foxp3 expression (data not shown).

The comparison between the group II and the nTreg showed that these two populations were phenotypically close for the usual markers CD4\textsuperscript{+}CD25\textsuperscript{+}CD127\textsuperscript{low} and Foxp3\textsuperscript{+} (Fig. 2B, upper right panel). However, no cells were CD127\textsuperscript{−} as observed for nTreg. In order to determine whether one group of these cells was responsible for the suppressive effect, we tested each of them in a T cell proliferation-based assay. Freshly isolated R cells, labeled with CFSE, proliferated when stimulated for 4 days with 0.1 \(\mu\)g ml\(^{-1}\) of soluble anti-CD3 and anti-CD28 (Fig. 2C, upper panel). However, when cells of the groups I, II or III were added to the cultures, complete suppression of the CFSE\textsuperscript{+} R cells proliferation was observed (Fig. 2C, middle panels).

These data were confirmed by the quantification of the IL-2 mRNA (Fig. 2C, lower panel). iTreg (Fig. 2C, lower panel) as well as group I, II or III tested separately (data not shown).
Fig. 2. Foxp3 expression is not necessary for the suppressive function of iTreg. (A) Naive CD4+ T cells were stimulated for 24 h with 5 µg ml⁻¹ plate-bound anti-CD3 and 1 µg ml⁻¹ soluble anti-CD28 and then removed and cultured for 1, 3, 6 or 9 days in complete medium (2, 4, 7 or 10 total days) before staining for Foxp3-PE and CD25-PECy5. Each graph represents the frequency of Foxp3⁺ or CD25⁺ cells of five independent experiments. Time point 0 was measured before 24 h stimulation. (B) After 7 days of culture, iTreg were stained with CD4-FITC, CD25-APC and CD127-PE. FACS plots are gated on CD4⁺ T cells. Quadrants were set up according to the isotype controls for CD25⁻ and CD127⁻/low. Three groups were sorted based on CD25 and CD127. CD4⁺CD25⁺CD127⁺ was named group I, CD4⁺CD25⁻CD127⁻/low was named group II and CD4⁺CD25⁻CD127⁻ was named group III by convenience and are shown in the FACS plot. Each group sorted was, then, stained for Foxp3-Alexa 700 and the frequencies of Foxp3⁺ cells (gray peaks) are shown in the lower histograms. nTreg were sorted based on CD4⁺CD25hiCD127⁻/low from PBMC and then stained with Foxp3 and the frequency of positive cells is shown in the upper histogram (gray peak). For each experiment, isotype control is represented with white peak. (C) In a CFSE suppression assay, freshly isolated R cells were stained with CFSE. R cells were activated with soluble 0.1 µg ml⁻¹ anti-CD3 and anti-CD28 for 4 days without (upper panel) or with each sorted group (I, II or III) of iTreg (lower panels) at a ratio 1:1. Quantitative real-time PCR on IL-2 mRNA from iTreg, R cells or R cells mixed with cells from group I, II or III, at a ratio 1:1, after 12 h of activation with soluble 0.1 µg ml⁻¹ anti-CD3 and anti-CD28. Data are representative of three experiments. (D) In the CFSE suppression assay, as shown in Fig. 2(C), the cells were stained with Foxp3-PE after 2 days of stimulation and gated on CFSE⁻ cells. Frequency of Foxp3⁺ (gray peak) is indicated on each histogram as well as the isotype control (white peak).
did not produce any IL-2 mRNA by real-time PCR. R cells expressed IL-2 mRNA after 12 h of activation with 0.1 μg ml⁻¹ of soluble anti-CD3 and anti-CD28 (Fig. 2C, lower panel). However, each group of iTreg suppressed the production of IL-2 mRNA of R cells when mixed together (Fig. 2C, lower panel).

In order to determine if Foxp3 could be expressed upon re-stimulation and responsible for the suppression, we stained for Foxp3 expression in each group after 2 days (Fig. 2D) or 4 days (data not shown) of re-activation. Though the Foxp3 expression remained quite stable in each group (Fig. 2D; MFI of 50 for the group I, 40 for the group II and 16 for the group III), the frequency of Foxp3⁺ cells decreased progressively. It was particularly noticeable for the group II that Foxp3⁺ cells frequency was ~70% before activation and reduced to 53% (Fig. 2D) and 38% (data not shown) after 2 and 4 days, respectively, of activation. In accordance with these results, no Foxp3 expression was observed in the CD4⁺CD25⁺CD127⁻ subset during activation though it suppressed the R cell proliferation (data not shown). These data indicated that iTreg could suppress the IL-2 mRNA production and the proliferation of effector T cells by a mechanism independent of the Foxp3 expression and on the amplification of a Foxp3⁺ cell subset.

iTreg suppress effector R cells proliferation to the same extent than nTreg

We then evaluated the suppressive activity of the different groups of iTreg by performing some titrations (Fig. 3A). The results indicated that the Foxp3⁺ fraction (group II) (Fig. 3A, middle panels) was able to suppress to the same extent than the Foxp3⁻/low fractions (groups I and III; Fig. 3A, left and right panels), whatever the ratio of iTreg to the R cells used. Comparing these titrations with the nTreg dilutions (Fig. 3B), we observed that iTreg were able to suppress R cells proliferation to the same extent than nTreg. Indeed, no suppression of R cells proliferation was observed when iTreg or nTreg were added to R cells at ratios lower than 1:4 (Fig. 3A and B). As a control, R cells were able to proliferate in the presence of an equal number of FACS-sorted-activated T cells after 4 days of co-culture (Fig. 3C). These latter were shown to proliferate in the co-cultures in contrast to iTreg (data not shown).

Effector R cells are transiently but not permanently suppressed by iTreg

One explanation for the suppression we observed could be that both iTreg and R cells compete for activation. We tested...
the expression of CD69 in each population 24 h following activation. We showed that CFSE$^+$ R cells were CD69 negative (Fig. 4A, left upper) before activation and up-regulated CD69 (79%) 24 h after activation with 0.1 μg ml$^{-1}$ of soluble anti-CD3 and anti-CD28 (Fig. 4A, left lower panel). Mixed iTreg and CFSE$^+$ R cells did not express CD69 before activation (Fig. 4A, upper middle panel) but up-regulated it to the same extent after activation (Fig. 4A, lower middle panel). As a control, nTreg co-cultured with CFSE$^+$ R cells did not express CD69 before activation but up-regulated it following activation (Fig. 4A, right upper and right lower panels). These data clearly showed that R cells were activated in the presence of iTreg or nTreg. Furthermore, an increase in the number of R cells in the wells did not alter their proliferation (data not shown), indicating that the suppression we observed was not linked to the competition for activation by the cells.

As already described, nTreg are also able to render R cells anergic after re-stimulation (30). To better understand the mechanism of suppression of iTreg, we tested whether these cells were able to anergize R cells. CFSE$^+$ R cells, cultured alone or mixed with iTreg or nTreg (ratio 1:1), were stimulated with 0.1 μg ml$^{-1}$ of soluble anti-CD3 and anti-CD28 for 24 h. The CFSE$^+$ R cells were then sorted based on the CFSE staining. They were rested for 3 days in complete RPMI medium and re-stimulated for 3 days with 0.1 μg ml$^{-1}$ of soluble anti-CD3 and anti-CD28. Their state of activation was followed according to the CFSE staining and the staining of CD25 (Fig. 4B). As indicated in Fig. 4(B), CFSE$^+$ R cells, which have been previously activated, were 90% CD25$^+$ and most of them proliferated. Similarly, 87% of the CFSE$^+$ R cells, which have been suppressed by iTreg, were CD25$^+$ and proliferated. However, CFSE$^+$ R cells, which have been in contact with nTreg during the first stimulation, were 70% CD25$^+$ and did not proliferate. These data indicated that iTreg, in spite of their ability to suppress CFSE$^+$ R cells, failed to anergize permanently the R cells in contrast to nTreg.

The iTreg suppression is mediated by a contact-dependent mechanism

We next wanted to determine if the suppression by iTreg could be mediated by IL-10 and/or IL-35 cytokines (31). We performed a kinetic of the intracellular production of IL-12p35 (a subunit of the IL-35 cytokine) in iTreg after 0, 6, 24 and 48 h of stimulation in the co-cultures. Our results showed that iTreg did not produce any IL-12p35 molecules at these time points (supplementary Figure 1A is available at International Immunology Online) although the protein could be detected by intracellular staining in mature monocyte-derived dendritic cells (supplementary Figure 1C is available at International Immunology Online). Similarly, no IL-12p35-producing cells were detected in nTreg after their purification or after a 6 h activation in a suppression assay (supplementary Figure 1B is available at International Immunology Online). These findings are in agreement with the recent work of Bardel et al (32) who showed that the EBI3 subunit mRNA and protein were not detected by real-time PCR or western blotting in resting or nTreg activated for 40 h with polyclonal stimulus.

A kinetic was then performed to assess for the intracellular production of IL-10 by iTreg. No IL-10-producing cells were detected in the co-cultures after 0, 6 and 24 h of stimulation (supplementary Figure 1D is available at International Immunology Online). As a positive control, IL-10 could be detected by intracellular staining in immature monocyte-derived dendritic cells (supplementary Figure 1E is available at International Immunology Online).

We then performed a transwell experiment in order to verify whether the iTreg suppression could be mediated by other cytokines or a contact-dependent mechanism. CFSE$^+$ R cells cultured in the lower chamber alone or in presence of iTreg seeded in the upper chamber, proliferated. In contrast, CFSE$^+$ R mixed with iTreg in the lower chamber did not (Fig. 4C, upper panels). We used nTreg as a control (Fig. 4C, lower panels) and showed that both regulatory populations acted very similarly. We concluded from this experiment that iTreg were mediating their suppression by a contact-dependent mechanism.

In order to determine whether CTLA-4 could play a role in the iTreg suppression, an intracellular and membrane staining of this molecule was realized after 0, 6, 24, 48 and 96 h of stimulation in the co-cultures (supplementary Figure 2 is available at International Immunology Online). With no stimulation, 26% of iTreg were shown to express CTLA-4 intracellularly. This intracellular expression increased with time and reached a plateau after 48 h of stimulation (supplementary Figure 2A is available at International Immunology Online). CTLA-4 molecules could also be detected on the surface of 65% of iTreg after 24 h of stimulation in the co-cultures (supplementary Figure 2B is available at International Immunology Online). We then performed a surface staining of the CTLA-4 ligands (CD80 and CD86) on the CD4$^+$CD25$^+$ effector T cells in the co-cultures after 0, 6, 24 and 48 h of stimulation. Results indicated that effector R cells did not express these ligands at these time points (supplementary Figure 2C is available at International Immunology Online) though mature monocyte-derived dendritic cells, used as a positive control, stained positive (supplementary Figure 2D is available at International Immunology Online). Ultimately, a neutralizing anti-CTLA-4 antibody was added to the co-cultures for 4 days. This antibody has been previously shown to partially abrogate the CD4$^+$CD25$^+$CTLA-4$^+$ T cell suppression in mixed lymphocyte reactions (MLR) (29). Although we confirmed the reversion of the CD4$^+$CD25$^+$CTLA-4$^+$ T cell suppression in MLR (supplementary Figure 2E is available at International Immunology Online), no abrogation of the iTreg suppression was observed (supplementary Figure 2F is available at International Immunology Online). We concluded from these experiments that iTreg were able to exert their suppressive activity through a mechanism independent of CTLA-4.

**Effect of CD4$^+$ T lymphocytes acquire their suppressive activity after TCR re-stimulation of lower strength**

Because we found some differences between nTreg and iTreg, particularly in their capacity to suppress effector T cells, we tested whether iTreg were always anergic regardless of the intensity of the activation. As shown in Fig. 5(A) (left upper and lower panels), CFSE$^+$ nTreg stimulated for...
Fig. 4. iTreg suppress effector R cells transiently. (A) Freshly isolated R cells were stained with CFSE and co-cultured without (left panels) or with iTreg (middle panels) or nTreg (right panels) at a ratio 1:1. Cells were stained with anti-CD69-PECy5 upon activation with soluble 0.1 µg ml⁻¹ anti-CD3 and anti-CD28 at the beginning of the culture, before the culture (upper panels) or at 24 h (lower panels). Frequencies of each population were indicated according to the isotype control antibody. (B) Freshly isolated R cells stained with CFSE were co-cultured with or without iTreg or nTreg at a ratio 1:1 for 24 h upon activation with soluble 0.1 µg ml⁻¹ anti-CD3 and anti-CD28. CFSE⁺ R cells were sorted and cultured alone for 3 days in complete medium for resting. Resting CFSE⁺ R cells were activated for 3 days with 0.1 µg ml⁻¹ anti-CD3 and anti-CD28 and then stained with anti-CD25-PECy5. FACS plots of CD25 and CFSE on R cells are shown. R cells that were cultured alone during the first activation are presented in the left panel. The middle panel is showing iTreg co-cultured with CFSE⁺ R cells and CFSE⁺ R cells seeded with nTreg are presented in the right panel. (C) CFSE⁺ R cells were cultured alone in the lower chamber of a transwell, for 4 days, upon activation with soluble 0.1 µg ml⁻¹ anti-CD3 and anti-CD28 (left panels). CFSE⁺ R cells were co-cultured in the lower chamber in presence of iTreg or nTreg at a ratio 1:1 for 5 days of activation (middle panels). CFSE⁺ R cells, placed in the lower chamber, were co-cultured with iTreg or nTreg in the upper chamber, at a ratio 1:1 for 5 days upon activation (right panels). CFSE profile histograms of cells cultured in the lower chamber are shown. These data (A, B and C) are representative of at least three independent experiments.
4 days with 0.1 μg ml⁻¹ of soluble anti-CD3 and anti-CD28 (low TCR activation) or with 1 μg ml⁻¹ of soluble anti-CD3 and anti-CD28 (high TCR stimulation) did not proliferate. Similarly, iTreg showed almost no proliferation (only one cell cycle) upon low activation (Fig. 5A, right upper panel), but surprisingly, these cells proliferated after higher TCR stimulation and completed three to six cycles (Fig. 5A, right lower panel), showing that they behaved differently than nTreg. We concluded from these data that iTreg could be pre-activated T cells that became anergic T cells when they were activated in lower stimulation conditions. In order to verify this hypothesis, we stimulated naive T cells with either 5 μg ml⁻¹ plate-bound anti-CD3 (clone UCHT1) and 1 μg ml⁻¹ soluble anti-CD28 (iTreg⁵) or with 0.5 μg ml⁻¹ plate-bound anti-CD3 and 1 μg ml⁻¹ soluble anti-CD28 (iTreg⁰.⁵) for 24 h and rested for 6 days. Quantitative real-time PCR on IL-2 mRNA from iTreg⁵, iTreg⁰.⁵ or freshly isolated R cells co-cultured with or without iTreg⁵ or iTreg⁰.⁵ at a ratio 1:1 for 12 h upon activation with soluble 0.1 μg ml⁻¹ anti-CD3 and anti-CD28 (left panel). R cells were stained with CFSE and used in a suppressive proliferative test. Histograms were gated on CFSE⁺ R cells co-cultured with iTreg⁵ (middle panel) or iTreg⁰.⁵ (right panel) at a ratio 1:1, stimulated 4 days with soluble 0.1 μg ml⁻¹ anti-CD3 and anti-CD28 (gray peaks). As a control, non-stimulated CFSE⁺ R cells were used (white peaks). Data are representative of three independents experiments. (C) Quantitative real-time PCR on IL-2 mRNA from iTreg⁵, iTreg⁰.⁵ or R cells co-cultured with or without iTreg⁵ or iTreg⁰.⁵ at a ratio 1:1 for 12 h upon activation with soluble 0.02 μg ml⁻¹ anti-CD3 and 0.1 μg ml⁻¹ anti-CD28. (D) Quantitative real-time PCR on IL-2 mRNA from R cells, iTreg⁵, co-culture (R+iTreg), R cells sorted from the co-culture after addition of IL-2 (R⁺) and iTreg⁵ (iTreg⁵) sorted from the co-culture after addition of IL-2. Co-cultures were realized using iTreg⁵ and R cells at a ratio 1:1 and activation with soluble 0.1 μg ml⁻¹ anti-CD3 and anti-CD28.
It was shown that iTreg were also able to inhibit the proliferation of R cells upon activation in a proliferation assay, whereas iTreg did not (data not shown). Because iTreg were not able to suppress R cells, we hypothesized that iTreg were not anergic. In order to test this postulate, iTreg were stained with CFSE and re-stimulated for 3 days with 0.1 μg ml\(^{-1}\) of soluble anti-CD3 and anti-CD28 and compared with CFSE* iTreg. As shown in Fig. 5(B), iTreg proliferated (middle panel); whereas iTreg did not (right panel), showing that iTreg were in a different activation state compared with the same cells generated with a 10-fold higher stimulation (5 μg ml\(^{-1}\)). In order to test if iTreg could become regulatory upon lower re-stimulation, the cells were then stimulated 12 h with 0.02 μg ml\(^{-1}\) of soluble anti-CD3 and 0.1 μg ml\(^{-1}\) anti-CD28. In Fig. 5(C), iTreg and iTreg showed no IL-2 mRNA, whereas R cells expressed the IL-2 cytokine mRNA upon this weaker stimulation. iTreg and iTreg both significantly inhibited the IL-2 mRNA of R cells. At least 73% of the IL-2 mRNA was suppressed when iTreg were used (n = 3, P < 0.008). Unfortunately, proliferation of R cells could not be tested at this lower activation as they did not divide.

In order to determine if the addition of co-stimulation signals could influence the iTreg suppression mechanism, we added exogenous IL-2 (100 U ml\(^{-1}\)) in the co-cultures. Both R cells and iTreg were proliferating in the co-cultures (data not shown). We sorted iTreg (iTreg) and R cells (R) after 12 h of co-cultures and quantified the IL-2 mRNA in both subsets by real-time PCR. We observed that the iTreg suppression was abrogated (Fig. 5, panel D). Thus, we concluded from these data that iTreg are pre-activated T cells, which could, according to the strength of the TCR signal given, become anergic and acquire a suppressive potential upon re-stimulation.

Discussion

It was shown that T cells with a CD25*Foxp3* phenotype and regulatory properties could be generated after stimulation of R cells (16, 22, 28). However, it was widely proposed that these cells could originate from the expansion of a contaminating population of nTreg (33–35). In this study, we have demonstrated that naive CD4 CD25 T cells, cultured in vitro, could generate effector CD4 CD25 T cells with a regulatory function. In our model, <0.5% of the initial population expressed Foxp3 and we observed an increase of Foxp3 cells over the expansion. We also have shown that the suppressive activity was independent of the expression of Foxp3, suggesting that Foxp3 was not necessary for their mechanism of suppression. Our findings are in agreement with Hansen et al. (36) study that showed that an in vivo population of CD25 Foxp3 T cells was able to suppress CD8 T cell cytotoxic activity/IFN-γ production, suggesting that Foxp3 expression was not a prerequisite for the suppressive capacity. Thus, these in vitro and the in vivo production of Foxp3 cells presenting a suppressive activity is arguing against the amplification of the nTreg subset. Recent studies have shown that Foxp3 could be induced in virtually all activated CD4 CD25 Foxp3, that this expression was transient, and that only a subset of this population remained Foxp3* after several days of activation (27, 37). One could then postulate that this transient expression of Foxp3 is necessary to induce a regulatory activity. However, during the generation of these so-called iTreg, we achieved a kinetic of the Foxp3 expression and did not detect Foxp3 (unlike iTreg) after 2, 4, 7 or 10 days of activation (data not shown). This population was however able to suppress freshly isolated R cells as observed in Fig. 4(C), indicating that Foxp3 was not essential for the regulatory function.

The CD4 CD25* T cells we generated in vitro were able to suppress the proliferation, as well as the IL-2 mRNA expression of allogenic effector R cells, supporting the finding that all naive R cells have the capacity to acquire a suppressive function.

We hypothesized that the anergy we observed could be related to a tuning of the TCR activation threshold as it was proposed by Grossman and Paul in their tunable activation threshold (TAT) model (38, 39). In this TAT model, mature T cells could maintain tolerance to an antigen on persistent stimulation and can proliferate if this stimulus exceeds the steady state. However, this threshold could be tuned by the stimulatory experience of the cell (40). Because of the use of a polyclonal population, we cannot exclude the possibility that a selection of tolerant T cells to the stimulus occurred. However, the stimulus was not sustained and all the cells divided during the culture arguing that we were able to generate populations with different thresholds of unresponsiveness to diverse stimuli as proposed in the TAT model. But more interestingly, we observed that this state of anergy was associated with a suppressive function. We showed that these cells behave very differently of nTreg as their suppression was transient, reversible after the addition of exogenous IL-2 and dependent of the TCR signal strength. These data support the ‘Civil Service’ model proposed by Graca et al. whereby T cells upon inefficient antigen presentation and stimulation could acquire regulatory properties (41, 42).

Such observation is significant as Chen et al. (43) demonstrated, in an in vivo murine transplantation model, that transgenic T cells specific for an epitope of a male antigen could behave as Treg after ‘incomplete’ TCR activation by an altered peptide ligand. In accordance with this finding, it would also be interesting to investigate if anergic T cell clones generated after in vitro stimulation with low concentrations of ligands or altered peptides (44) would present a suppressive function.

In conclusion, such a model could explain the discrepancy observed between several studies indicating that Foxp3 expression is required for the regulatory function of CD4 T cells and other data describing a distinct population of CD4* T cells with regulatory capacity in the absence of Foxp3 expression (36, 37, 45). Our model would predict that hypo-responsiveness and regulatory function of T cells could be reversed by increasing the level of TCR activation.

It would be interesting to better understand their mechanism of suppression. Indeed, Knoechel et al. (46) showed that the pattern of gene expression in nTreg correlated with functional responses and was very different in anergic T cells. Gene expression profiling may be an approach for
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Supplementary data
Supplementary Figures 1 and 2 are available at International Immunology Online.

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Abbreviations
CFSE: carboxyfluorescein diacetate succinimidyl ester
cDNA: complementary DNA
GM-CSF: granulocyte macrophage colony-stimulating factor
iTreg: induced regulatory T cells
MFI: mean fluorescence intensity
MLR: mixed lymphocyte reactions
mRNA: messenger RNA
nTreg: naturally occurring regulatory T cells
R cell: CD4\(^+\)CD25\(^-\) T cell
TAT: tunable activation threshold
TGIF-β: transforming growth factor-β
Treg: regulatory T cells

References