In vitro generation of CD4+CD25+ regulatory cells from murine naive T cells

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CD4+CD25+ regulatory T cells (Tregs) are crucial for the maintenance of immunological tolerance. Recent data indicate that Tregs not only develop in the thymus during ontogeny but can also differentiate from naive T cells in the periphery. The following protocol describes a method by which Tregs are generated *in vitro* by stimulation of naive T cells in the presence of transforming growth factor β (Ti-Tregs). *In vitro*-induced regulatory T cells express markers of conventional Treg such as CD25 and the genetic program committing transcription factor FoxP3. Functionally the *in vitro*-generated Ti-Tregs suppress T-cell activation and proliferation while *in vivo* these cells have been proven to control inflammation in different animal models, suggesting a potential use of these cells for immunotherapy. The protocol can be completed within 5 days.

INTRODUCTION

Many lines of evidence sustain that defects of Tregs play a pivotal role in the pathogenesis of many autoimmune diseases. Indeed these cells are thought to prevent the activation and expansion of autoreactive T-cell clones that have escaped the negative selection in the thymus[1]. Tregs are generated in the thymus after the first days of postnatal life and then migrate into peripheral organs where they can prevent the activation of autoreactive T-cell clones. Indeed thymectomy performed in mice at day 3 (3Tx) after birth, when regulatory T cells have not yet been released to the periphery, leads to the development of a multiorgan autoimmune disease that can be prevented by adoptive transfer of syngenic Tregs[2]. Recently, the winged helix transcription factor FoxP3 has been shown to play a key role in determining the regulatory phenotype of Tregs. Indeed, infection of naive T cells with a retroviral vector expressing FoxP3 was sufficient to induce a regulatory phenotype in these cells[3]. Moreover, deletion or loss of function of the FoxP3 encoding gene determines an autoimmune phenotype both in humans (the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome)[4] and mice (Scurfy)[5]. Functionally Tregs have been studied both *in vitro* and *in vivo*. *In vitro*, CD4+CD25+ Tregs have been shown to suppress the activation and expansion of naive cells, and it has been shown that this process does not require soluble factors but a direct contact of regulatory cells with target cells. The regulatory function of these cells has also been tested in different animal models of human disease. In models of colitis, Tregs showed the capacity to prevent and cure gut inflammation[6]. In addition, regulatory cells have been shown to efficiently prevent the development of experimental autoimmune encephalitis in mice, suggesting that these cells might have a potential role in the therapy of human disease[7].

Despite data obtained so far on the function of Tregs, the study and the use of these cells for *in vivo* experiments has been limited by the exiguous number of Tregs normally present in the peripheral blood. Tregs represent only about 5–10% of the circulating CD4+ cells. Moreover, the *in vitro* expansion of these cells is hampered by their anergic phenotype. Indeed, naturally occurring Tregs poorly proliferate in response to TCR stimulation and need exogenous interleukin (IL)-2 to undergo expansion.

We and others have recently shown that cells endowed with regulatory capacity comparable to that of naturally occurring Tregs might be obtained upon activation of naive T cells in the presence of the cytokine TGF-β[8,9]. This protocol describes the methods used in these papers in more detail. In this regard, experiments have been performed to fine-tune and troubleshoot the procedure, thus substantially improving the method initially described.

TGF-β stimulation of CD4+CD25− cells obtained from a double-negative magnetic sorting for CD4 and CD25 has been shown to induce the expression of FoxP3 and many genes normally expressed by naturally occurring Tregs. These cells have been proven to prevent the activation and proliferation of naive cells in coculture experiments *in vitro*, while *in vivo*, Ti-Tregs have been shown to prevent the activation of the immune system in different models of human immune diseases[8,10]. In addition, TGF-β has been implicated, together with IL-6, in the generation of a class of effector cells characterized by the expression of IL-17, called TH17 cells[11,12]. Therefore, TGF-β emerges as a key molecule involved in the polarization of classes of effector cells exerting opposite roles depending on the cytokine milieu. In this paper, we illustrate the methods to generate fully competent Tregs starting from a population of naive T cells (CD4+CD25−). Tregs generated in this way can be used to study the generation and function of this crucial cell population and can further be used to investigate the potential use of Tregs for immunotherapy.

MATERIALS

REAGENTS

- BALB/c mice: 6- to 8-week-old BALB/c mice were obtained from the animal facility of the University of Mainz ❗ **CAUTION** All animal experiments must be performed in accordance with national and institutional regulations.

- Anti-mouse CD3 (145-2C11) and anti-mouse CD28 (37.15) antibody. They were affinity purified from hybridoma cell lines kindly provided by Professor E. Schmitt (Institute for Immunology, Johannes Gutenberg University, Mainz, Germany), using protein G-Sepharose (Pharmacia Biotech)

- Anti-mouse FoxP3-APC, clone FJK-16s (eBioscience)
- FoxP3 intracellular immunofluorescence staining kit (eBioscience)
- ACK cell lysis buffer (Cambrex)
- Recombinant TGF-β1 (R&D Systems)
- Recombinant IL-2 (R&D Systems)
- Serum-free medium X-Vivo15, RPMI supplemented with 10% (v/v) FBS (BioWhittaker)
- CD4 cell isolation kit (Miltenyi Biotech)
- CD25 micro beads kit (Miltenyi Biotech)
- Celltrace CFSE (Invitrogen)
- Mitomycin C (Sigma-Aldrich)
- TRK lysis buffer, for real-time (RT-PCR) only
- Hi-Bind-RNA columns (peqGOLD Total RNA Kit, Peqlab), for RNA isolation for RT-PCR only
- M-MLV reverse-transcriptase (Invitrogen) for RT-PCR only

- iQ sybr green mix (Bio-Rad), for RT-PCR only

EQUIPMENT
- LD columns and LS columns (Miltenyi Biotec)
- MidiMACS Separator (Miltenyi Biotec)
- MACS MultiStand (Miltenyi Biotec)
- FACSCalibur (Becton Dickinson)
- Scissors and forceps
- Tubes 50 ml
- Petri dish
- Microscope glass slides
- Pipettes
- Centrifuge
- Microscope for cell counting
- Burker or Neubauer cell count chambers
- 24- and 96-well plates
- Real-time thermocycler, for RT-PCR only

PROCEDURE
Splenocyte isolation
1| Kill either male or female 6- to 8-week-old mice by cervical dislocation.
▲ **CRITICAL STEP** Perform all steps in the procedure under a sterile laminar flow hood.
▲ **CRITICAL STEP** Clean scissor and forceps properly with water and soap, and keep them in a beaker containing 70% ethanol.

2| Rinse the whole mouse thoroughly with 70% ethanol in order to realize a first line of disinfection.

3| With the mouse in supine position, open the abdomen by a first transversal cut avoiding opening of the peritoneal cavity. Completely expose the peritoneal sac by pulling the skin in opposite directions using your hands or forceps. At this point, the spleen should become visible on the left side of the mouse. With a second cut, open the peritoneal sac and collect the spleen with sterile forceps.
▲ **CRITICAL STEP** Avoid contact between the spleen and the mouse skin to preserve sterility.

4| Clear the spleen from residual debris and fat tissue and place it in a 50 ml tube containing 30 ml of 1× PBS. Wash the spleen twice with fresh PBS and subsequently transfer it into a 10 cm Petri dish containing 20 ml of complete RMPI medium.

5| By using microscope glass slides previously sterilized with 70% ethanol and fire, cut spleens in two halves and squeeze them between the slides to extract the cellular matrix contained within the spleen capsule (**Fig. 1**). Discard the remaining fibrous tissue.

6| Collect the cell aggregates obtained from this step in a 50 ml tube and pipette up and down several times by using a conventional pipettor to obtain a homogeneous cell suspension. Centrifuge the cell suspension at 400g for 6 min at 4 °C in order to pellet the cells. Discard the supernatant.

Lysis of erythrocytes
7| Lyse red cells by resuspending the cell pellet in 5 ml ACK buffer for 90 s.

8| Immediately afterwards, dilute the cell suspension with 45 ml of 1× PBS to neutralize the ACK buffer. Spin cells in a centrifuge at 400g for 10 min at 4 °C in order to pellet the cells and subsequently resuspend the pellet in 10 ml of 1× PBS. The cellular pellet should appear whitish, indicating successful lysis of red cells.

9| Count splenocytes by using a conventional Neubauer chamber to prepare them for the magnetic sorting steps. To do this, pipette 10 μl of cell suspension between the chamber and the cover glass slide. Count the cells contained in the four corner quadrants. The number obtained, divided by 4 gives the number of cells per ml multiplied by 10^4.
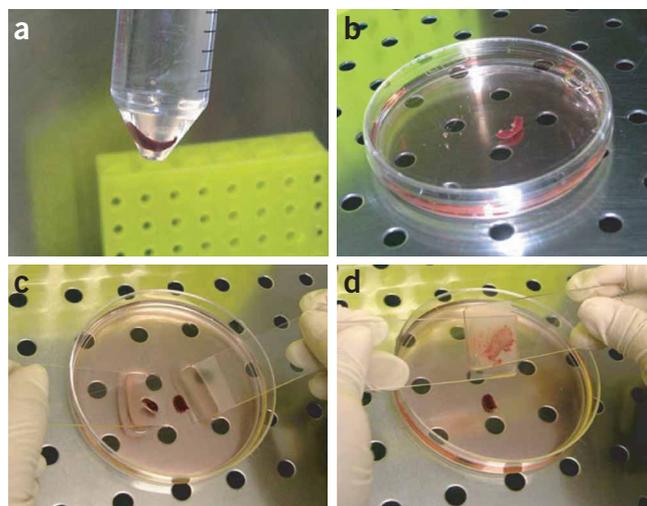
Figure 1 | Splenocytes isolation. Place the spleen in a Petri plate with 20 ml of RPMI 10% FBS, cut in two pieces and squeeze each of them between two sterile glass slides to obtain a single cell suspension. (**a**) The isolated spleen is placed in a 50 ml tube containing PBS. (**b**) The spleen is transferred into a 10 cm Petri dish containing 20 ml of complete RMPI medium. (**c**) By using microscope glass slides previously sterilized with 70% ethanol and fire, the spleens are cut in two halves and (**d**) squeezed between the slides in order to extract the cellular matrix contained within the spleen capsule.

Magnetic sorting of CD4+CD25− cells: part 1

10| If using 10^8 splenocytes as the starting cell number, use the Miltenyi *CD4 cell isolation kit* to label splenocytes from Step 9 with 100 µl of the biotinylated cocktail of antibodies specific for CD4-negative cells diluted in 400 µl of RPMI 10% FBS for 15 min at 4 °C.

11| Add 200 µl of the anti-biotin antibody conjugated with microbeads and 300 µl of RPMI 10% FBS and incubate for 10 min at 4 °C.

12| After labeling, wash cells once with RPMI 10% FBS and resuspend in 6 ml of RPMI containing 10% FBS.

13| Separate untouched CD4+ cells by loading 2 ml of the labeled cells on to an LD separation column (Miltenyi). CD4-negative cells will remain in the column owing to the magnetic beads attached to their surface. CD4-positive cells, in contrast, will flow through the column and can be collected in a tube (**Fig. 2a**). Repeat twice, loading an additional 2 ml of cells each time. Count CD4+ cells as indicated in Step 9.

Magnetic sorting of CD4+CD25− cells: part 2

14| In the second part, label the untouched CD4+ cells (cells that flowed through the column in Step 13) with an anti-CD25PE antibody (Miltenyi; allow 10 µl of antibody for every 10^6 CD4+ cells diluted in 100 µl of RPMI 10% FBS). Incubate the cells for 15 min at 4 °C.

15| Wash cells once with 10 ml of RPMI 10% FBS.

16| Incubate cells with anti-phycoerythrin (PE) antibody conjugated with microbeads (Miltenyi; allow 10 µl of antibody for every 10^6 CD4+ cells diluted in 90 µl of RPMI 10% FBS). Incubate the cells for 15 min at 4 °C.

17| Resuspend cells in 6 ml of complete medium. Separate CD4+CD25− cells from CD4+CD25+ cells by loading an LS positive selection column (Miltenyi) 1 ml at a time. Wait for cells to go through completely before loading the second ml. Collect the flow through containing the untouched CD4+CD25− cells (**Fig. 2a**).
▲ **CRITICAL STEP** Although the use of an LS column in this depletion step is not suggested by the manufacturers, as the number of CD25+ cells to be sorted out does not normally exceed 5–10% of CD4+ population, the use of an LS in place of an LD depletion column still allows an optimal depletion of CD25+ cells while increasing the final yield of CD4+CD25− cells, which is generally lower using an LD depletion column. A typical yield is about 10^6 CD4+CD25− cells per 10^8 splenocytes.

Flow cytometric purity analysis

18| Stain a fraction of cells with anti-CD4 and anti-CD25 antibodies conjugated with fluorescein isothiocyanate and PE. For this we recommend 2.5×10^5 CD4+CD25− cells are sampled, washed once with PBS and resuspended in 100 µl of PBS 0.2% BSA. Add 1 µl of each antibody to the sample (1:100 dilution) and incubate the sample for 10 min at 4 °C.

19| At the end of the incubation, wash the cells and analyze by flow cytometry to quality control your isolation procedure. In general, the purity of CD4+CD25− cells is more than 96% (not shown) according to the manufacturer's data sheet. Note that when sorted cells are analyzed for FoxP3 expression, only 2–3% of the CD4+CD25− cells are FoxP3+ (**Fig. 2b**), as previously described[13]. However, the possibility that this small number of FoxP3-expressing cells could be responsible for the TGF-β-mediated induction of FoxP3+ has been ruled out by experiments performed in TCR transgenic mice on a RAG1 knockout background, which do not have FoxP3-expressing cells, thus demonstrating that TGF-β directly acts on naive cells[14].

Induction of Ti-Tregs

20| Coat a 24-well plate with 10 µg ml^{-1} of anti-CD3 antibody in PBS at 37 °C for 2 h and then wash the plate once with PBS before cell plating.

21| Plate 1 ml of 2×10^6 ml^{-1} CD4+CD25− cells resuspended in X-Vivo15 serum-free medium in the anti-CD3-precoated wells in the absence of antibiotics. Immediately add anti-CD28 antibody (2 µg ml^{-1}) and TGF-β1 (5 ng ml^{-1}) and incubate the cells for 5 days (**Fig. 3**).
? TROUBLESHOOTING
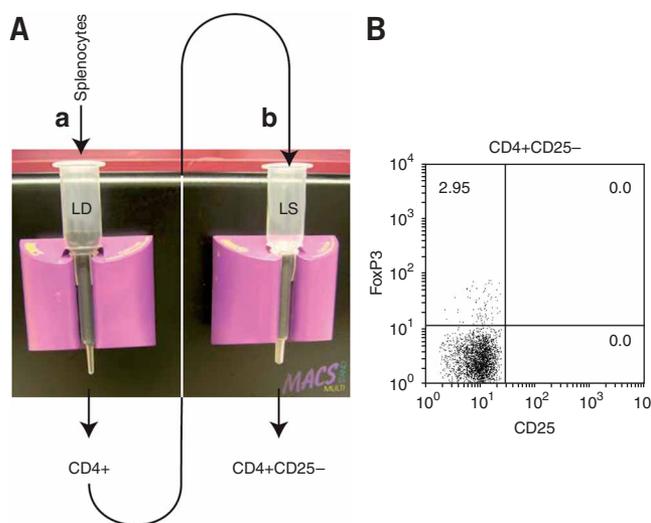
Figure 2 | CD4+CD25− magnetic cell isolation. (**A**) CD4+ cells obtained from freshly isolated splenocytes labeled with the CD4 isolation kit (**a**) are passed through a depletion LD column. The untouched CD4+ cells are further indirectly labeled with an anti-CD25-PE and anti-PE conjugated with microbeads (**b**). Cells are applied on an LS column and CD4+CD25− cells are collected as flow-through. (**B**) Example of FoxP3 staining of freshly isolated CD4+CD25− cells.

Quantitative and qualitative evaluation of Ti-Tregs induction

22| After 5 days of incubation, test an aliquot of Treg culture for Foxp3 induction: we generally evaluate Foxp3 induction quantitatively either at the RNA or protein level by real-time PCR (option A) or flow cytometry (option B), respectively. We also recommend that you perform qualitative evaluation, and the suppressive capacity of Ti-Tregs can be evaluated in a coculture suppression assay (option C).

(A) RT-PCR to detect FoxP3

(i) Lyse 5×10^5 Ti-Tregs in TRK lysis buffer and isolate RNA; we use Hi-Bind-RNA columns according to the manufacturer's protocol.

(ii) Perform reverse transcription; we use M-MLV reverse- transcriptase (Invitrogen).

(iii) Perform real-time PCR. We generally use the following specific primers: mFoxP3 exon 6 sense 5′-GGTACACCCAGGAAAGAC AG-3′ and mFoxP3 exon 9 antisense 5′-ATCCAGGAGATGATCTGCTTG-3′; plus iQ sybr green mix (Bio-Rad). We use an iQ5 real-time thermocycler to perform real-time PCR.

(B) Flow cytometry to detect FoxP3

(i) To determine the amount of FoxP3+ (regulatory) cells in the population, stain 2.5×10^5 cells with a PE-conjugated anti-CD4 as previously described (Steps 18 and 19).

(ii) Fix, permeabilize and stain the cells with a specific APC-conjugated anti-FoxP3 antibody according to the instructions provided in the mouse FoxP3 staining kit (eBioscience).

(iii) Analyze stained cells by flow cytometry (**Fig. 4**).

? TROUBLESHOOTING

(C) Qualitative evaluation of induced Tregs

(i) Use freshly isolated CD4+CD25− cells obtained by magnetic sorting, as described in Steps 10–17.

(ii) Label freshly isolated CD4+CD25− cells with CFSE according to standard protocols: briefly, resuspend cells in PBS at a concentration of 10^6 cells ml^{-1} and stain with 0.2 μM CFSE for 1 h at 37 °C wrapped with an aluminum foil to protect from light.

(iii) Wash cells once in PBS, resuspend at the same concentration in RPMI 10% FBS and incubate for a further hour at 37 °C to remove the unbound CFSE.

(iv) Plate these cells as responder cells at a concentration of 10^5 cells well^{-1} in a 96-well round bottom plate together with MHC-matched APC cells (2×10^4 cells well^{-1}), which have already been either treated with mitomycin C (60 μg ml^{-1}) for 30 min or irradiated (4,000 Rad) (2×10^4 cells well^{-1}).

(v) Add anti-CD3 to each well at the concentration of 1 μg ml^{-1}. Add Ti-Tregs to the wells at different ratios as indicated in **Figure 5a**. Freshly isolated CD4+CD25+ Tregs and CD4+CD25− cells activated in the absence of TGF-β for 5 days can be used as positive and negative controls, respectively.
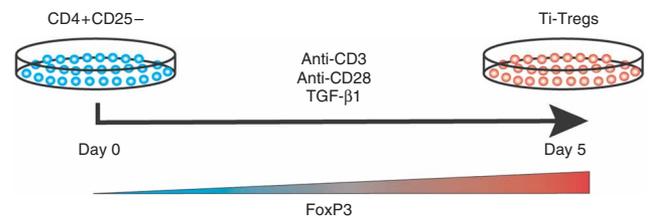
Figure 3 | Ti-Treg induction control. Plate freshly isolated CD4+CD25− cells in serum-free medium (X-Vivo15) and activate with plate-bound anti-CD3 and soluble anti-CD28 (2 μg ml^{-1}) in the presence of TGF-β (5 ng ml^{-1}). Cell cultures should be kept in these conditions for 5 days and then the induced Ti-Tregs can be used for further experiments.

(vi) Analyze cells after 4 days by flow cytometry. As shown in **Figure 5a**, Ti-Treg significantly suppresses the proliferation of CD4+CD25− responder cells at various concentrations indicated by the reduced CFSE dilution in these cultures. Quantification of cells undergoing divisions can be evaluated by determining the proliferation index obtained with the proliferation module of the ModFit LT Macintosh software (**Fig. 5b**).

? TROUBLESHOOTING

● **TIMING**

Steps 1–9, CD4+CD25− cell preparation: 4 h

Steps 10–17, CD4/CD25 staining: 1 h

Figure 4 | Example of results obtained for Ti-Treg induction control. In (**a**), the expression of FoxP3 mRNA was evaluated in CD4+CD25− cell culture activated for 5 days with plate-bound anti-CD3 and soluble anti-CD28 (2 μg ml^{-1}) in the presence or absence of TGF-β. Two representative Ti-Treg inductions are reported, which show an upregulation of FoxP3 in the range of 400- to 700-fold compared with cells activated in the absence of TGF-β as quantified by real-time PCR. The same cells were evaluated by flow cytometry to quantify the number of cells expressing the transcription factor FoxP3. For this, an aliquot of day 5 Ti-Tregs was permeabilized and stained with an anti-mouse FoxP3-specific antibody. In (**b**), a representative flow cytometric analysis of day 5 Ti-Treg culture is shown.

Figure 5 | Example of Ti-Tregs functional quality control. To evaluate the suppressive capacity of Ti-Treg cultures, a proliferation assay was set. Briefly, freshly isolated CD4+CD25− cells were used as responder cells upon CFSE staining. Responder cells were activated in the presence of soluble anti-CD3 (1 μg ml^{-1}) and mytomicin-treated APC cells alone or in the presence of Ti-Tregs (suppressors) at different ratios as indicated. CFSE progressive dilution was used as readout of responder cells proliferation. As shown in (**a**), at all the responder:suppressor ratios, Ti-Tregs were able to suppress proliferation of responder cells. CFSE populations were also analyzed by determining the proliferation index obtained with the proliferation module of ModFit LT Macintosh software (**b**).

Step 21, Ti-Treg culture set up: 1 h
Step 21, Ti-Treg induction: 5 days
Step 22A, RNA isolation: 2 h; reverse transcription: 2 h; real-time PCR: 3 h
Step 22B, CD4/FoxP3 staining: 2 h
Step 22C, CD4+CD25− isolation: 5 h; CFSE staining, 1 h and 30 min; coculture set up: 1 h; proliferation assay: 4 days

? TROUBLESHOOTING
Step 21
Cell death: High cell death in day 5 Ti-Treg culture might be observed upon propidium iodide staining. The use of serum-free medium in the absence of antibiotics increases the general cell viability. Alternatively, dead cells can be eliminated by using the Dead cell removal kit (Miltenyi Biotech), according to the manufacturer's protocol, at the end of the induction phase.

Step 22B
Low FoxP3 induction: Certain variability in the number of FoxP3-expressing cells obtained at the end of the induction phase might be observed in different animal strains (BALB/c versus CB57B/6). This is, in our experience, mainly due to the different capacity of CD4+CD25− cells to propagate *in vitro* for 5 days. To avoid a low yield of Ti-Tregs, we recommend adding 20 ng ml^{-1} of murine IL-2 to the cell cultures. This might improve the yield and viability of Ti-Tregs at the end of the induction phase (refs. 14,15; Fantini *et al.*, unpublished observation).

Step 22C
Contaminating FoxP3-negative cells: To enrich the population of Foxp3-expressing Ti-Tregs, an additional cell sorting for CD25 after resting for 24 h at the end of the induction culture may help eliminate a fraction of non-regulatory cells.

ANTICIPATED RESULTS
This protocol illustrates the procedure to generate CD4+CD25+FoxP3+ regulatory cells from a starting population of naive T cells that are very similar to, if not indistinguishable from, naturally occurring Tregs developing in the thymus. The possibility to have an adequate number of cells available for experiments will help characterize the cellular mechanisms responsible for the suppressive activity of Tregs on target cells.

The peripheral generation of regulatory cells is sustained by several lines of evidence and TGF-β has been implicated in this process. This protocol might represent an *in vitro* model of the process that physiologically leads to the peripheral generation of Tregs *in vivo*. At RNA level, the induction of FoxP3 in Ti-Tregs when compared to CD4+CD25− cells activated in the absence of TGF-β usually varies in a range of 400- to 700-fold while the number of Foxp3+ cells after 5 days of culture usually is around 60% (**Fig. 4**). Overall, the number of FoxP3+ cells recovered after 5 days is one- to twofold greater than the number of CD4+CD25−FoxP3− cells originally plated. Functionally, Ti-Tregs suppress proliferation of freshly isolated CD4+CD25− in a dose-dependent manner, similar to the suppression observed using naturally occurring CD4+CD25+ Tregs. Therefore, the study of the molecular events accompanying the induction of Ti-Tregs *in vitro* might shed light on new intracellular pathways involved in naive T-cell differentiation. The protocol described here can be easily modified to study the induction and function of antigen-specific regulatory T cells both *in vitro* and *in vivo* using T-cell receptor transgenic mice specific for a given antigen. Naturally occurring Tregs physiologically represent 5–10% of circulating CD4+ cells. Owing to the low number of naturally occurring Tregs obtained by sorting peripheral blood cells and because of the scarce expansion obtained *in vitro*, the availability

of an adequate number of Tregs for *in vivo* experiments might often represent a limitation. The *in vitro* generation of Ti-Tregs has already demonstrated that these cells are effective in preventing disease in different animal models. The extension of these experiments to a curative setting will pave the way to a future use of these cells in those human diseases caused by an uncontrolled activation of the immune system. Indeed the capacity to induce regulatory cells in an antigen-specific manner and to transfer them in atopic patients might represent an important advance in the immunotherapy of allergic diseases.

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