Chapter 2

Generation of stable, functional regulatory T lymphocytes *in vitro* within 4 days by a two step activation protocol

Sander van der Marel, Anna Majowicz and Valerie Ferreira

Abstract

The significance of regulatory T cells in the control of tolerance shows promise for new therapeutic applications. Cell therapy with regulatory T cells has the potential to become a new patient tailored approach in the treatment of allergic, autoimmune or inflammatory disorders. Our objective was to define a new, straightforward protocol to generate sufficient numbers of inducible regulatory T cells *in vitro* applicable for future clinical trials.

With the use of a novel, two-step T cell activation protocol we generate *in vitro*, within 4 days regulatory T cells. The phenotype and functionality of those cells assessed by flow cytometry and MLR's were sustainable over a period of at least 30 days in culture. These cells could be applied either alone or together with naturally occurring regulatory T cells in the treatment of allergic, autoimmune or inflammatory disorders.

Introduction

Regulatory T cells (Treg) are key players in maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammation [1]. Therefore, strategies that aim for therapeutic tolerance induction may take advantage of the functions of Treg [2].

The transcription factor FOXP3 is generally considered as the master regulator in the development and function of Treg [3-5]. However FOXP3 expression can also be induced in human T effector cells which do not have suppressive activity [6]. Thus, FOXP3 expression is necessary, but not sufficient to identify functional Treg and other markers have to be taken in consideration as well [6].

Treg are able to suppress an immune response both by cell contact (*e.g.* killing or functional modulation of antigen presenting cells or effector T cells) and soluble factor dependent mechanisms (*e.g.* secretion of immunosuppressive cytokines or deprivation of cytokines necessary for the expansion/survival of responder T cells) [7]. They are considered to be an attractive tool for the treatment of a broad range of diseases [8-12]. Recently, different groups have been able to expand functional, naturally occurring regulatory T cells (nTreg) *ex vivo* [13-15]. In Phase I clinical trials a favorable safety profile was shown for nTreg in the treatment of acute graft versus host disease [16, 17]. However it was reported by the same group [18] that cellular therapy using Treg induced *in vitro* has several potential advantages over nTreg, including ease of isolation, increased number of the starting population, greater proliferation potential and reduced production costs [14]. Additionally multiple defects in nTreg in patients with autoimmune diseases have been described [19] which could prevent the use of these cells for the treatment of autoimmunity. This problem could be overcome by using Treg induced *in vitro*. These induced Treg (iTreg) like nTreg, have been shown to suppress auto-, and alloreactivity *in vivo* [18, 20]. Furthermore, it has been demonstrated that the physiologic generation of iTreg from naive T cells *in vivo*, is essential for establishing oral tolerance [21] and that iTreg generated *in vitro* can act in synergy with nTreg from the same donor to restore and maintain tolerance *in vivo* [22]. It is there-

fore postulated that iTreg could have an important, non-redundant role in establishing tolerance [21-23]. Consequently iTreg generated from $CD4^+CD25^-$ T cells *in vitro* have potential, either alone or together with nTreg, for the treatment of allergy, autoimmune or inflammatory disorders. This approach has been explored before, whereby stimulation with anti-CD3 was an important element [24, 25], as anti-CD3 up-regulates FOXP3 expression *in vitro* [26]. However, in contrast to nTreg FOXP3 expression seems to decline over time in iTreg [26] resulting in a loss of regulatory function. Apart from activation with anti-CD3 [24, 25], several other groups have used different strategies to generate iTreg *in vitro*, including use of TGFβ, all-trans-retinoic acid and the recently discovered regulatory cytokine IL-35 [27, 28]. All these technologies require more or less extensive cell handling and processing and it remains uncertain whether these methods will enable generation of sufficient iTreg under *Good Manufacturing Practices* (GMP).

We have established a new, simple and robust method to generate stable and functional iTreg from human $CD4^+CD25^-$ T cells *in vitro*. This method is composed of two steps of activation with an initial PMA/ionomycin activation step followed by anti-CD3 stimulation in presence of a low dose of Interleukin-2 (IL-2). This approach has the advantage of promoting the conversion of $CD4^+CD25^-$ T cells to a high number of functional iTreg and could be suitable for application under GMP. Furthermore, by making use of substances that are well known, simple to obtain and relatively low priced this protocol could be easy to employ in a routine laboratory setting.

Material and methods

Samples

Buffy Coats from healthy adults were obtained from the blood bank (Sanquin, the Netherlands). The study was reviewed and approved by the ethical board of Sanquin Blood Supply Foundation. Peripheral Blood Mononuclear Cells (PBMC's) were extracted using cell preparation tubes with sodium citrate (BD Vacutainer® CPT™, BD Biosciences).

Isolation and stimulation of $CD4^+CD25^-$ T cells

$CD4^+CD25^-$ T cells were obtained from PBMC's by means of negative selection using the naive $CD4^+$ T cells isolation kit (Miltenyi Biotec®) and CD25 microbeads (Miltenyi Biotec®) for additional CD25 depletion to ensure that no $CD4^+CD25^+$ cells remained, as described before [29]. Both isolation steps were performed according to the instructions of the manufacturer with the use of LD columns (Miltenyi Biotec®). Routine evaluation of the obtained $CD4^+$ $CD25^-$ population analyzed by flow cytometry showed a purity of >95%.

$CD4^+CD25^-$ T cells were re-suspended in X-vivo15 human medium (Lonza) containing 5% human serum (Lonza) at 2.5 x 10^5 cells/ml and subsequently activated with PMA (10 ng/ml, obtained from Sigma-Aldrich) and ionomycin (250 ng/ml, obtained from Sigma-Aldrich). After 48 h cells were washed twice with PBS, re-suspended in medium and activated with 10 μg/ml anti-human CD3 (Clone OKT3, eBioscience) in the presence or absence of IL-2 (50 U/ml, obtained from eBioscience). Cells were cultured in 96-well, flat bottom plates. IL-2 (50 U/ml) was added every 72 h.

Phenotypical characterization

Cells were analyzed using flow cytometry (FACSCalibur, BD Biosciences) and CellQuest Pro software (BD Biosciences). For Treg staining the human regulatory T cell staining kit was used (PE-conjugated FOXP3, clone PCH101, FITC-conjugated CD4, clone RPA-T4 and APC-conjugated CD25, clone BC96) obtained from eBioscience. To assess the specificity of the PCH101 clone we performed FOXP3 staining with clone PCH101 (eBioscience), 259D/C7 (BD biosciences) and 3G3 (Miltenyi Biotec®) in parallel, according to the instructions of the manufacturer (experiments in triplicate, n=2, *data not shown*). Overall little (<5%), non significant differences were observed in our experimental setup and therefore we continued with PCH101 as this clone has been validated for FOXP3 detection [30].

Additional monoclonal anti-human antibodies used in this study were as follows: anti-CD127 (PerCP Cy5.5-conjugated, clone eBioRDR5), anti-CD4 (PE-conjugated, clone RPA-T4), anti-CD152 (CTLA-4, PE-conjugated, clone 14D3), anti-AITR/GITR (PE-conjugated, clone eBioAITR), anti-OX40

(CD134, FITC-conjugated, clone ACT35), anti-ICOS (APC-conjugated, clone ISA-3) and anti-IL-10 (APC-conjugated, clone JES3-9D7) obtained from eBioscience. The viability dye 7-AAD (BD Biosciences) and the scatter dot plot were used to exclude dead cells from analysis. For intracellular staining (FOXP3, CTLA-4 and IL-10), cells were fixed and permeabilized using the FOXP3 staining buffer set from eBioscience. The IL-10 staining was performed intracellularly, as it has been described before [29]. High expression of a marker is defined as fluorescence intensity greater than 100, as it has been described before [31]. Regulatory markers were followed over time up until 30 days in culture, with or without supplementation of a low dose of IL-2 (50 U/ml, every 72 h). As control, $CD4^+CD25^-$ T cells activated with PMA (10 ng/ml)/ionomycin (250 ng/ml) followed by IL-2 (50 U/ml) or anti-CD3 (10 μg/ml) followed by IL-2 (50 U/ml) were analyzed by flow cytometry after 9 days or after 30 days in culture.

Viability and proliferation

Viability of the cells was determined by staining with 7-AAD (BD Biosciences) followed by flow cytometry analysis. Cell number was determined by use of the NucleoCounter to assess proliferation rate in culture (NucleoCounter, Chemometec).

Functional characterization

Regulatory function was analyzed using Mixed Leukocyte Reactions (MLR's) with CFSE stained responder cells [32], described previously as a valuable tool to study suppressor T cells [33]. Shortly CFSE labelling: The CellTrace CFSE cell proliferation Kit (Molecular Probes, Invitrogen) was used to stain PBMC's from the same donor as the experimental cells according to the manufacturer's instructions. These cells are referred to as responder cells. A final working concentration of 5 μM CFSE was used per 5 x 10^6 PBMC's, to obtain a $CFSE^{high}$ stained responder population, as determined by flow cytometry using different titrations of CFSE. CFSE expression was routinely evaluated after staining by flow cytometry.

MLR

CFSE labelled PBMC's (responder cells) were cultured in 96-well round bottom plates at 2.5 × 10^4 cells/well with 5 × 10^4 cells/well irradiated allogenic PBMC's (as feeder cells) in the presence of varying amounts of experimental T cells. Autologous PBMC's as feeder cells for the autologous control, $CD4^+CD25^-$ T cells as negative control and rapamycin (100 ng/ml) as positive control were plated out to assess their effect on proliferation. Cell cultures were stimulated with 2 µg/ml anti-human CD3. All culture settings were performed in triplicate.

Experimental T cells, generated with the two-step activation protocol, were analyzed for their suppressive function in the described MLR's after 9 days in culture or after 30 days in culture. The MLR was set, after 5 days cells were harvested, washed with PBS and the CFSE signal was analyzed by flow cytometry. Routine evaluation during titration experiments showed that proliferating cells loose their $CFSE^{high}$ staining and become $CFSE^{low}$. Therefore suppression of $CFSE^{low}$ represents the suppression of proliferating responder cells compared to control (see: **Figure 4.B**), as described before [33]. Cell cultures were set with or without a 0.4 µm transwell permeable support system (Corning), in order to determine cell contact or cytokine dependence in the Treg functionality assay.

Results

Induction of a regulatory phenotype in $CD4^+CD25^-$ T cells

$CD4^+CD25^-$ T cells were activated with PMA/ionomycin and subsequently with anti-CD3, in the presence of IL-2 (50 U/ml). After 9 days in culture, the T cells from four different donors were analyzed by flow cytometry and 55% to 83% of the cells were shown to express the classical regulatory phenotype ($CD4^+CD25^{high}FOXP3^+$, **Figure 1.A**). Inter-donor variation was observed, but for every analyzed donor more than 50% of the $CD4^+CD25^-$ T cells differentiated into ($CD4^+CD25^{high}FOXP3^+$) Treg. We will refer to this inducible regulatory T cell population as 'TregPMA' cells.

In order to demonstrate the specificity and effectiveness of our two-step activation procedure, $CD4^+CD25^-$ T cells from three of the four donors were

activated with PMA/ionomycin followed by IL-2 or activated with anti-CD3 followed by IL-2, as control groups. The cells were analyzed by flow cytometry after 9 days in culture. Between 0.1% and 2% of the cells in the anti-CD3/IL-2 group and between 13% and 31% in the PMA/ionomycin/IL-2 group were shown to express the classical regulatory phenotype ($CD4^+CD25^{high}FOXP3^+$) after 9 days in culture (**Figure 1.B**).

Expression of regulatory markers by the TregPMA

High CD25 expression [34] and down-regulation of CD127 [35, 36] are established characteristics of the Treg phenotype in humans. Therefore expression of those markers on the surface of the TregPMA was monitored by flow cytometry. Furthermore, we analyzed the expression of CTLA-4, which is linked to Treg function [37, 38], glucocorticoid-induced TNF receptor (GITR), linked to Treg survival [1], ICOS, linked to Treg function [39, 40] and OX40, also known as CD134, which is linked to Treg homing and survival [41, 1]. Additionally, we analyzed the expression of IL-10, a regulatory cytokine that down-regulates the expression of T helper cell cytokines, MHC class II antigens, co-stimulatory molecules on macrophages and has been associated with Treg function [42].

The TregPMA demonstrate down-regulation of CD127 and up-regulation of CD25, FOXP3, IL-10, CTLA-4, GITR, ICOS and OX40 when compared to the PMA/ionomycin/IL-2 control group and to non-activated T cells (**Figure 2**).

To monitor the acquisition of the regulatory phenotype over time by the TregPMA in culture, the profile of expression of the established regulatory markers CD25, CD127, FOXP3 and IL-10 was analyzed by flow cytometry at different time points (**Figure 3**). A clear up-regulation of the Treg lineage transcription factor FOXP3 was observed over time, as well as of the markers CD25 and IL-10. CD127 was down-regulated, further confirming the regulatory phenotype [35, 36]. For these markers the PMA/ionomycin and anti-CD3 steps seemed to act synergistically to achieve the expression of a regulatory profile.

It has to be noted that the expression of CD4 at the cell surface was down-regulated after the PMA/ionomycin activation step, but was gradually restored after anti-CD3 activation, as it was described before [43, 44, 45].

The expression profiles, determined by flow cytometry of $CD4^+$, $CD25^{high}$, $CD127^{-/low}$ and $FOXP3^+$ demonstrate that within 4 days in culture the TregPMA obtain their regulatory phenotype (**Figure 3**).

Using the described TregPMA protocol we are able to generate a mean of 2.2 x 10^6 TregPMA cells/ml (SD 1.0 x 10^6; N=3) after 9 days in culture starting from 2.5 x 10^5 $CD4^+CD25^-$ T cells/ml. The mean cell viability was >95% as confirmed by both NucleoCounter and 7-AAD staining (*data not shown*).

TregPMA suppress the proliferation of responder cells *in vitro*

Since the main functional characteristic of Treg is the capacity to suppress the proliferation of responder cells *in vitro*, the capacity of the TregPMA to suppress the proliferation of responder cells was analyzed by MLR's, after 9 days in culture. A fixed number of CFSE labelled responder cells was co-cultured with a varying amount of TregPMA (responder cells to TregPMA ratio 1:0, 1:0.33, 1:0.67 and 1:1.67). The cells of three different donors were analyzed. Inter-donor variations were observed, but TregPMA always suppressed the proliferation of responder cells in co-culture with an efficacy between 25% and 43% (**Figure 4.A**). The observed effect was proportional to the number of TregPMA added in the assay, demonstrating a dose dependent inhibition. The separation of TregPMA from the responder cells, in a transwell assay, abrogates their suppressive function demonstrating contact-dependent suppression of the proliferation of responder cells (*data not shown*).

It has to be noted that the inhibition of proliferation was not due to depletion of nutrients or accumulation of waste products as in the negative control the addition of an equal amount of $CD4^+CD25^-$ T cells, instead of the TregPMA caused no inhibition of the CFSE division (**Figure 4.A and B**).

All together the obtained data show that the TregPMA suppress the proliferation of responder cells *in vitro* in a dose and contact dependent manner.

Phenotype and function of TregPMA are stable in long term culture

Since the sustainability of TregPMA phenotype and functionality over time is important for possible clinical applications [46], the TregPMA were kept in culture for 30 days and analyzed by flow cytometry and MLR's.

Expression of regulatory markers after long term culture

To monitor the sustainability of the regulatory phenotype over time of the TregPMA, the profile of expression of the regulatory markers, CD127, CD25, FOXP3, IL-10, CTLA-4, GITR, ICOS and OX40 was analyzed by flow cytometry after 30 days in culture. After following our two-step protocol, the TregPMA of two different donors demonstrated a sustainable down-regulation of CD127 as well as up-regulation of CD25, FOXP3, IL-10, CTLA -4, GITR, ICOS and OX40 when compared to the PMA/ionomycin/IL-2 control group and to non-activated T cells (*data not shown*).

Stable regulatory function of the TregPMA after 30 days in culture

The capacity of the TregPMA, after 30 days in culture, to suppress the proliferation of responder cells was analyzed by MLR's. The TregPMA from two different donors cultured for 30 days were shown to inhibit the proliferation of responder cells (mean inhibition up to 40%). Therefore it can be concluded that the regulatory function of the TregPMA is maintained over time in culture.

IL-2 contributes to FOXP3 transcription through activation of Stat5 and is thereby of importance both for Treg homeostasis and for maintaining their suppressive program [47]. Therefore we cultured the generated TregPMA in the presence or absence of IL-2 and examined their regulatory properties. Cells of two different donors were analyzed by flow cytometry and it was determined that 65% and 70% of the TregPMA express FOXP3 when cultured in the presence of a low dose of IL-2, while less than 5% of cells express FOXP3 without IL-2 supplementation. Furthermore, the TregPMA, cultured in the absence of exogenous IL-2, were not able to suppress the proliferation of responder cells in MLR's (*data not shown*).

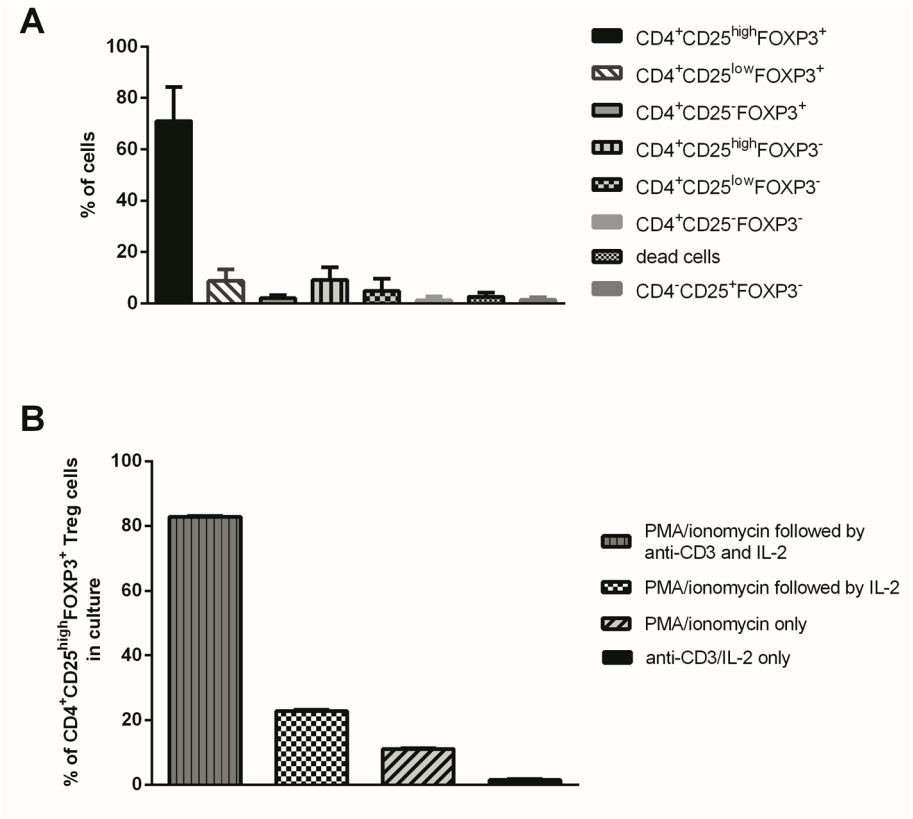

Figure 1. CD4$^+$CD25$^-$ T cells acquire a regulatory phenotype

A. CD4$^+$CD25$^-$ T cells expressing the regulatory phenotype CD4$^+$CD25^{high}Foxp3$^+$, activated with our two-step protocol, after 9 days in culture. Experiments were performed in triplicate with four different donors. Mean of 71.06% (SD 13.27, N=4) of the CD4$^+$CD25$^-$ T cells acquired a regulatory phenotype (CD4$^+$CD25^{high}Foxp3$^+$). Results are presented as means +/- standard deviation (SD) of all four donors.

B. CD4$^+$CD25$^-$ T cells expressing the regulatory phenotype CD4$^+$CD25^{high}Foxp3$^+$ after activation with our two-step protocol as compared to control groups. Experiments were performed in triplicate with three different donors. One representative experiment is shown. Depicted data are means +/- standard deviation (SD).

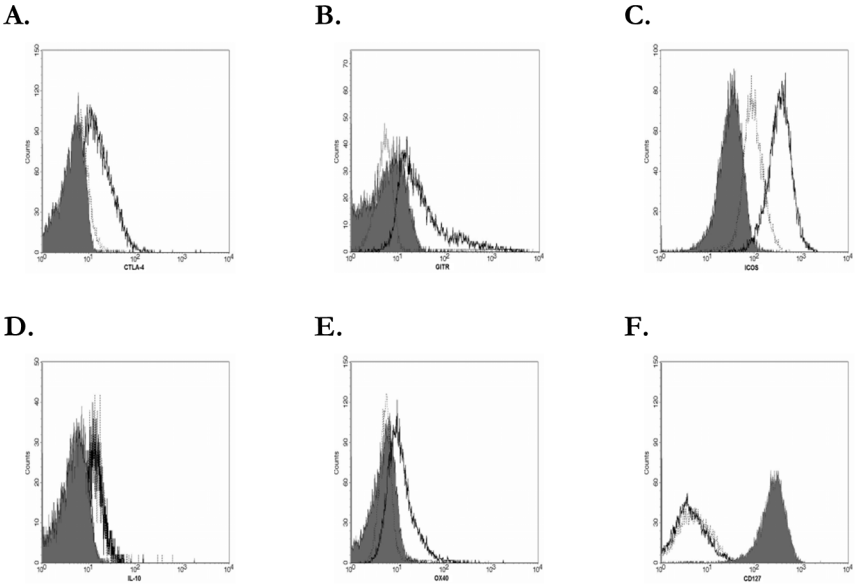

Figure 2. The TregPMA up-regulate CTLA-4, GITR, ICOS, OX40, IL-10, CD25, and FOXP3 and down-regulate CD127 as compared to control groups
TregPMA (black continuous line), show up-regulation of markers CTLA-4 (**A**), GITR (**B**), ICOS (**C**), OX40 (**D**), IL-10 (**E**), CD25 (**F**) and FOXP3 (**G**) and down-regulation of CD127 (**H**) after 9 days in culture as compared to not activated T cells (grey, filled in) and compared to PMA/ionomycin/IL-2 alone (non filled in, dotted line) control groups.
Experiments were performed in triplicate and with three different donors. One representative experiment is shown.

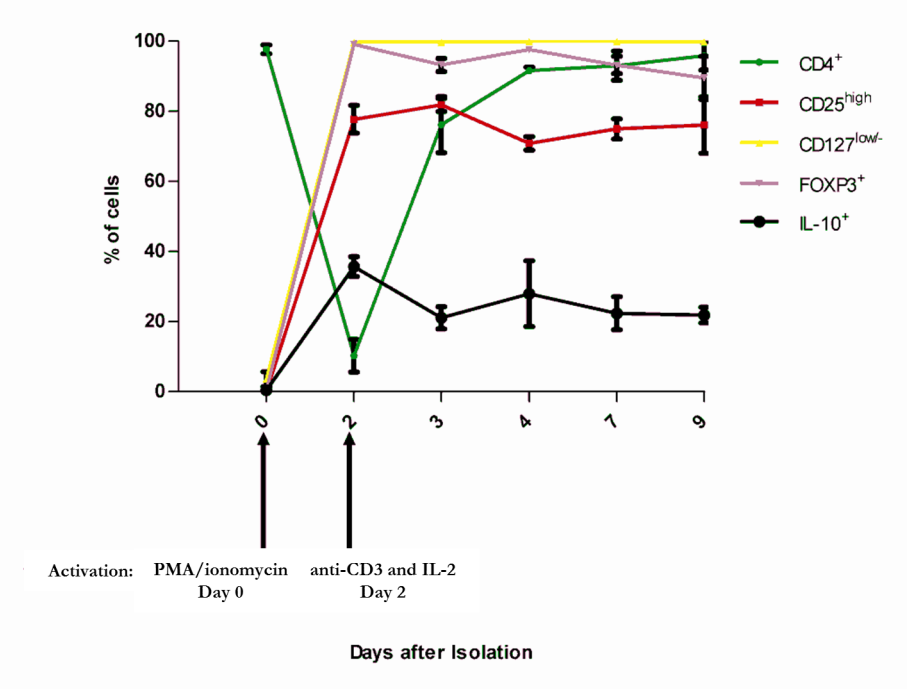

Figure 3. The TregPMA expression profile of $CD4^+$, $CD25^{high}$, $CD127^{low/-}$, $FOXP3^+$ and $IL\text{-}10^+$ the first 9 days in culture

TregPMA were analyzed at day 0, 2, 3, 4, 7 and 9 after activation. The marker CD127 was down-regulated, the markers CD25, FOXP3 and IL-10 were up-regulated and we show a synergy over time with the expression of FOXP3. Experiments were performed in triplicate and with three different donors. Results are presented as means +/- standard deviation (SD) of all three donors.

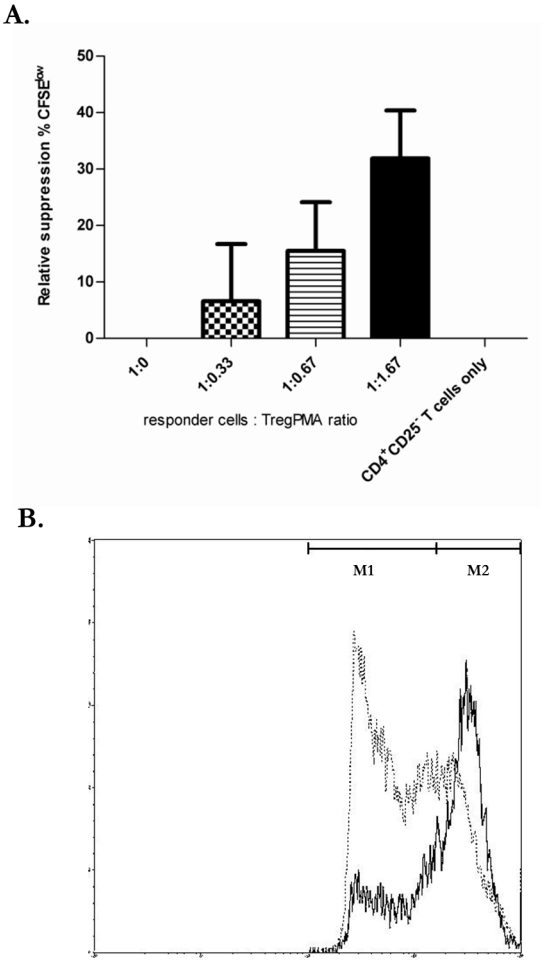

Figure 4. The regulatory phenotype of the TregPMA correlates with suppressive activity *in vitro*

A. TregPMA, after 9 days in culture suppress the proliferation of responder cells in response to irradiated allogenic feeder cells in a CFSE based MLR. Suppression of $CFSE^{low}$ represents the suppression of proliferating responder cells as compared to control groups. Overall we show a mean inhibition up to 31.87% (SD 9.38, N=3). $CD4^{+}CD25^{-}$ T cells caused no inhibition of the CFSE division. Experiments were performed in triplicate. Results are presented as means +/- standard deviation (SD) of all three donors.

B. Histogram of one representative flow cytometry experiment

The dotted line is the control group without Treg, showing the overall proliferation ($CFSE^{low}$, represented in M2). The continuous line represents the experimental group, with 1:1.67 TregPMA added showing less proliferation of responder cells (non-proliferating cells in M1).

Discussion

This paper describes a new and simple method to generate *in vitro*, within 4 days, stable and functional $CD4^+CD25^{high}FOXP3^+CD127^{-/low}$ iTreg. This approach could be easily adapted to clinical application under *Good Manufacturing Practices* (GMP) and thereby allow the preparation of a sufficient quantity of cells to enable use of iTreg for both prevention and subsequent therapy [48]. Other techniques to generate iTreg have been reported and are available [27, 28]. However, the protocol described in this paper presents a straightforward and robust alternative, by making use of substances which are well known, easy to obtain and relatively low priced.

Our protocol consists of two steps. The first step is the activation of $CD4^+CD25^-$ T cells with PMA/ionomycin. This combination provides a potent stimulation allowing us to bypass the T cell receptor activation essential for Treg development and prevent the emergence of $CD4^-CD8^+$ cells in the culture [49]. PMA activates protein kinase C [50] while ionomycin is a Ca^{2+} mobilizing agent [51]. This combination has shown to up-regulate CD25 on T lymphocytes [49] and a high CD25 expression is a marker of the Treg phenotype [34]. Additionally, it was demonstrated that Ca^{2+} signaling is required for the development and function of Treg [52, 53]. Therefore we expected that raising the intracellular levels of Ca^{2+} in $CD4^+CD25^-$ T cells using ionomycin would have a synergistic effect with PMA in starting a regulatory developmental program in $CD4^+CD25^-$ T cells. The expression profiles, analyzed by flow cytometry, after activation with PMA/ionomycin, show a clear up-regulation of the Treg lineage transcription factor FOXP3, as well as of the markers CD25 and IL-10. Conform to the regulatory phenotype [35, 36], CD127 was down-regulated.

The second step in our protocol is a step of activation with anti-CD3 in the presence of a low dose of IL-2. This second stimulation is needed to maintain the regulatory developmental program, since the initial up-regulation of FOXP3 expression, as well as the expression of CD4 on the cell surface declines in time after only PMA/ionomycin stimulation. Anti-CD3 was used for this purpose since it has been shown to maintain and expand Treg [54, 55]

and restore CD4 expression [45]. We show here that, for the established regulatory markers, $CD25^{high}$, $CD127^{-/low}$, and $FOXP3^{+}$ [34-36] the PMA/ionomycin and anti-CD3 steps seem to act synergistically to achieve the expression of a regulatory profile within 4 days in culture. Furthermore, the anti-CD3 activation step in our protocol restores the CD4 expression, which is down-regulated after the PMA/ionomycin activation step.

IL-2 contributes to FOXP3 transcription through activation of Stat5 and is thereby of importance both for Treg homeostasis and for maintaining their suppressive program [47]. Therefore we cultured the generated TregPMA in the presence or absence of IL-2 and examined their regulatory phenotype and function. We demonstrate that the expression of regulatory markers and capability of suppressing proliferation of responder cells *in vitro* are dependent on exogenous IL-2. Furthermore we show that in the presence of IL-2 the phenotype and function are stable over a long period of time.

Conclusions

Generating sufficient numbers of iTreg from human PBMC's and preserving their regulatory function for clinical application still proves to be difficult. In this paper we describe a protocol which permits to induce a stable regulatory phenotype and function in $CD4^{+}CD25^{-}$ T cells *in vitro*, within 4 days. We present a good alternative to previously established methods [27, 28] by making use of substances which are well known, simple to obtain and relatively low priced. Therefore, our two-step activation protocol could be easily adapted to routine laboratory settings, for future clinical applications, using iTreg either alone or together with nTreg in the treatment of allergic, autoimmune or inflammatory disorders.

REFERENCES

1. Sakaguchi, S, Miyara, M, Costantino, CM and Hafler, DA (2010). FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol*; 10: 490-500.

2. St Clair, EW, Turka, LA, Saxon, A, Matthews, JB, Sayegh, MH, Eisenbarth, GS, et al. (2007). New reagents on the horizon for immune tolerance. *Annu Rev Med*; 58: 329-346.

3. Fontenot, JD, Gavin, MA and Rudensky, AY (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*; 4: 330-336.

4. Hori, S, Nomura, T and Sakaguchi, S (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science*; 299: 1057-1061.

5. Khattri, R, Cox, T, Yasayko, SA and Ramsdell, F (2003). An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol*; 4: 337-342.

6. Chen, X and Oppenheim, JJ (2011). Resolving the identity myth: Key markers of functional CD4(+)FoxP3(+) regulatory T cells. *Int Immunopharmacol*;

7. Sakaguchi, S, Yamaguchi, T, Nomura, T and Ono, M (2008). Regulatory T cells and immune tolerance. *Cell*; 133: 775-787.

8. Allan, SE, Broady, R, Gregori, S, Himmel, ME, Locke, N, Roncarolo, MG, et al. (2008). CD4+ T-regulatory cells: toward therapy for human diseases. *Immunol Rev*; 223: 391-421.

9. Allez, M and Mayer, L (2004). Regulatory T cells: peace keepers in the gut. *Inflamm Bowel Dis*; 10: 666-676.

10. Li, Z, Arijs, I, De, HG, Vermeire, S, Noman, M, Bullens, D, et al. (2010). Reciprocal changes of Foxp3 expression in blood and intestinal mucosa in IBD patients responding to infliximab. *Inflamm Bowel Dis*;

11. Gagliani, N, Ferraro, A, Roncarolo, MG and Battaglia, M (2009). Autoimmune diabetic patients undergoing allogeneic islet transplantation: are we ready for a regulatory T-cell therapy? *Immunol Lett*;

12. Leishman, AJ and Bundick, RV (2005). Immunology: turning basic research into a therapy. *Immunol Lett*; 99: 1-7.

13. Feng, G, Nadig, SN, Backdahl, L, Beck, S, Francis, RS, Schiopu, A, et al. (2011). Functional regulatory T cells produced by inhibiting cyclic nucleotide phosphodiesterase type 3 prevent allograft rejection. *Sci Transl Med*; 3: 83ra40.

14. Hippen, KL, Merkel, SC, Schirm, DK, Sieben, CM, Sumstad, D, Kadidlo, DM, et al. (2011). Massive ex Vivo Expansion of Human Natural Regulatory T Cells (Tregs) with Minimal Loss of in Vivo Functional Activity. *Sci Transl Med*; 3: 83ra41.

15. Sagoo, P, Ali, N, Garg, G, Nestle, FO, Lechler, RI and Lombardi, G (2011). Human regulatory T cells with alloantigen specificity are more potent inhibitors of alloimmune skin graft damage than polyclonal regulatory T cells. *Sci Transl Med*; 3: 83ra42.

16. Brunstein, CG, Miller, JS, Cao, Q, McKenna, DH, Hippen, KL, Curtsinger, J, et al. (2010). Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*;

17. Trzonkowski, P, Bieniaszewska, M, Juscinska, J, Dobyszuk, A, Krzystyniak, A, Marek, N, et

al. (2009). First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+C. *Clin Immunol*; 133: 22-26.

18. Hippen, KL, Merkel, SC, Schirm, DK, Nelson, C, Tennis, NC, Riley, JL, et al. (2011). Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. *Am J Transplant*; 11: 1148-1157.

19. Buckner, JH (2010). Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. *Nat Rev Immunol*; 10: 849-859.

20. Akbar, AN, Vukmanovic-Stejic, M, Taams, LS and Macallan, DC (2007). The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. *Nat Rev Immunol*; 7: 231-237.

21. Fukaya, T, Takagi, H, Sato, Y, Sato, K, Eizumi, K, Taya, H, et al. (2010). Crucial roles of B7-H1 and B7-DC expressed on mesenteric lymph node dendritic cells in the generation of antigen-specific CD4+Foxp3+ regulatory T cells in the establishment of oral tolerance. *Blood*; 116: 2266-2276.

22. Haribhai, D, Lin, W, Edwards, B, Ziegelbauer, J, Salzman, NH, Carlson, MR, et al. (2009). A central role for induced regulatory T cells in tolerance induction in experimental colitis. *J Immunol*; 182: 3461-3468.

23. Thompson, LJ, Valladao, AC and Ziegler, SF (2011). Cutting edge: De novo induction of functional Foxp3+ regulatory CD4 T cells in response to tissue-restricted self antigen. *J Immunol*; 186: 4551-4555.

24. Walker, MR, Kasprowicz, DJ, Gersuk, VH, Benard, A, Van, LM, Buckner, JH, et al. (2003). Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+. *J Clin Invest*; 112: 1437-1443.

25. Walker, MR, Carson, BD, Nepom, GT, Ziegler, SF and Buckner, JH (2005). De novo generation of antigen-specific CD4+CD25+ regulatory T cells from human CD4+. *Proc Natl Acad Sci U S A*; 102: 4103-4108.

26. Allan, SE, Crome, SQ, Crellin, NK, Passerini, L, Steiner, TS, Bacchetta, R, et al. (2007). Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol*; 19: 345-354.

27. Lu, L, Zhou, X, Wang, J, Zheng, SG and Horwitz, DA (2010). Characterization of Protective Human CD4CD25 FOXP3 Regulatory T Cells Generated with IL-2, TGF -beta and Retinoic Acid. *PLoS ONE*; 5: e15150.

28. Collison, LW, Chaturvedi, V, Henderson, AL, Giacomin, PR, Guy, C, Bankoti, J, et al. (2010). IL-35-mediated induction of a potent regulatory T cell population. *Nat Immunol*; 11: 1093-1101.

29. Taylor, AL and Llewelyn, MJ (2010). Superantigen-induced proliferation of human CD4+. *J Immunol*; 185: 6591-6598.

30. Law, JP, Hirschkorn, DF, Owen, RE, Biswas, HH, Norris, PJ and Lanteri, MC (2009). The importance of Foxp3 antibody and fixation/ permeabilization buffer combinations in identifying CD4+CD25+Foxp3+ regulatory T cells. *Cytometry A*; 75: 1040-1050.

31. De Groot, AS, Moise, L, McMurry, JA, Wambre, E, Van, OL, Moingeon, P, et al. (2008). Activation of natural regulatory T cells by IgG Fc-derived peptide "Tregitopes". *Blood*; 112: 3303-3311.

32. Nikolaeva, N, Bemelman, FJ, Yong, SL, van Lier, RA and ten Berge, IJ (2006). Rapamycin does not induce anergy but inhibits expansion and differentiation of alloreactive human T cells. *Transplantation*; 81: 445-454.

33. Venken, K, Thewissen, M, Hellings, N, Somers, V, Hensen, K, Rummens, JL, et al. (2007). A CFSE based assay for measuring CD4+CD25+ regulatory T cell mediated suppression of auto-antigen specific and polyclonal T cell responses. *J Immunol Methods*; 322: 1-11.

34. Baecher-Allan, C, Brown, JA, Freeman, GJ and Hafler, DA (2001). CD4+CD25high regulatory cells in human peripheral blood. *J Immunol*; 167: 1245-1253.

35. Liu, W, Putnam, AL, Xu-Yu, Z, Szot, GL, Lee, MR, Zhu, S, et al. (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*; 203: 1701-1711.

36. Seddiki, N, Santner-Nanan, B, Martinson, J, Zaunders, J, Sasson, S, Landay, A, et al. (2006). Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*; 203: 1693-1700.

37. Bour-Jordan, H and Bluestone, JA (2009). Regulating the regulators: costimulatory signals control the homeostasis and function of regulatory T cells. *Immunol Rev*; 229: 41-66.

38. Eagar, TN, Turley, DM, Padilla, J, Karandikar, NJ, Tan, L, Bluestone, JA, et al. (2004). CTLA-4 regulates expansion and differentiation of Th1 cells following induction of peripheral T cell tolerance. *J Immunol*; 172: 7442-7450.

39. Vocanson, M, Rozieres, A, Hennino, A, Poyet, G, Gaillard, V, Renaudineau, S, et al. (2010). Inducible costimulator (ICOS) is a marker for highly suppressive antigen-specific T cells sharing features of T(h)17/T(h)1 and regulatory T cells. *J Allergy Clin Immunol*;

40. Coquerelle, C, Oldenhove, G, Acolty, V, Denoeud, J, Vansanten, G, Verdebout, JM, et al. (2009). Anti-CTLA-4 treatment induces IL-10-producing ICOS+ regulatory T cells displaying IDO-dependent anti-inflammatory properties in a mouse model of colitis. *Gut*; 58: 1363-1373.

41. Griseri, T, Asquith, M, Thompson, C and Powrie, F (2010). OX40 is required for regulatory T cell-mediated control of colitis. *J Exp Med*; 207: 699-709.

42. Moore, KW, de Waal, MR, Coffman, RL and O'Garra, A (2001). Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*; 19: 683-765.

43. Baran, J, Kowalczyk, D, Ozog, M and Zembala, M (2001). Three-color flow cytometry detection of intracellular cytokines in peripheral blood mononuclear cells: comparative analysis of phorbol myristate acetate-ionomycin and phytohemagglutinin stimulation. *Clin Diagn Lab Immunol*; 8: 303-313.

44. Daculsi, R, Vaillier, D, Carron, JC and Gualde, N (1998). Effect of PGE2 on the cell surface molecule expression in PMA treated thymocytes. *Immunol Lett*; 60: 81-88.

45. Wiegers, GJ, Stec, IE, Klinkert, WE and Reul, JM (2000). Glucocorticoids regulate TCR-induced elevation of CD4: functional implications. *J Immunol*; 164: 6213-6220.

46. Roncarolo, MG and Battaglia, M (2007). Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nat Rev Immunol*; 7: 585-598.

47. Malek, TR and Castro, I (2010). Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity*; 33: 153-165.

48. Tolar, J, Hippen, KL and Blazar, BR (2009). Immune regulatory cells in umbilical cord blood: T regulatory cells and mesenchymal stromal cells. *Br J Haematol*; 147: 200-206.

49. Takahama, Y and Nakauchi, H (1996). Phorbol ester and calcium ionophore can replace TCR signals that induce positive selection of CD4 T cells. *J Immunol*; 157: 1508-1513.

50. Frey, MR, Leontieva, O, Watters, DJ and Black, JD (2001). Stimulation of protein kinase C-dependent and -independent signaling pathways by bistratene A in intestinal epithelial cells. *Biochem Pharmacol*; 61: 1093-1100.

51. Dadsetan, S, Shishkin, V and Fomina, AF (2005). Intracellular Ca(2+) release triggers translocation of membrane marker FM1-43 from the extracellular leaflet of plasma membrane into endoplasmic reticulum in T lymphocytes. *J Biol Chem*; 280: 16377-16382.

52. Oh-Hora, M, Yamashita, M, Hogan, PG, Sharma, S, Lamperti, E, Chung, W, et al. (2008). Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. *Nat Immunol*; 9: 432-443.

53. Oh-Hora, M and Rao, A (2009). The calcium/ NFAT pathway: role in development and function of regulatory T cells. *Microbes Infect*; 11: 612-619.

54. Nishio, J, Feuerer, M, Wong, J, Mathis, D and Benoist, C (2010). Anti-CD3 therapy permits regulatory T cells to surmount T cell receptor-specified peripheral niche constraints. *J Exp Med*; 207: 1879-1889.

55. Penaranda, C, Tang, Q and Bluestone, JA (2011). Anti-CD3 Therapy Promotes Tolerance by Selectively Depleting Pathogenic Cells while Preserving Regulatory T Cells. *J Immunol*;