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Impact of culture medium on CD4$^+$ CD25^{high}CD127$^{lo/neg}$ Treg expansion for the purpose of clinical application

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ABSTRACT

A recently discovered population of lymphocytes, called T regulatory cells (Tregs), is characterized by expression of transcription factor Forkhead box P3 (FoxP3). These cells have been successfully used as therapeutic treatments and prophylaxis for graft-*versus*-host disease (GVHD) and diabetes and might become an attractive alternative to traditional immunotherapy. Here we evaluated how the type of culture medium and the type of serum can influence yield and quality of Tregs after *in vitro* expansion. We compared Treg fold of expansion and their phenotypical characteristics including expression of FoxP3, CD25, CD127, CD62L and CD45RA in three commercially available culture media (RPMI 1640 (Cellgro; Manassas VA, USA), SCGM (Cellgenix; Freiburg, Germany), and X-VIVO 20 (Lonza; Walkersville, MD, USA)) with addition of human serum (HS, 10%) or fetal bovine serum (FBS, 10%). Among the tested media, X-VIVO 20 supplemented with HS produced the highest yield after 17 days of *in vitro* expansion (a median of 86-fold expansion, range 30–1365) and highest level of FoxP3 expression (a median of 66.8% of positive cells, range 56–84.8%) in CD4$^+$ CD25^{hi}CD127$^{lo/neg}$ FACS sorted polyclonal Tregs. There was no difference in Tregs yield whether HS or FBS serum was used. In conclusion, the yield of the *ex vivo* expanded Tregs is related to the type of media applied. Supplementation of the culture with FBS or human serum is equally beneficial.

1. Introduction

T regulatory cells (Tregs) [1] have been proved to play pivotal role in immune homeostasis. Numerous studies explored the feasibility of using T regulatory cells as a therapeutic factor in animal models of autoimmune diseases and allogeneic transplantation. Infusion of *in vitro* expanded Tregs reverses autoimmune diabetes in NOD mice [2], produces tolerance to allogeneic transplant [3,4] and alleviates graft-*versus*-host disease (GVHD) [5]. Moreover, recently published reports of using T regulatory cells in clinical trials have been released. Our group achieved alleviation of chronic GVHD symptoms following infusion of *in vitro* expanded Treg, demonstrating the therapeutic potential of Tregs [6]. Later, other groups confirmed the safety and effectiveness of the adoptive transfer of Tregs as prophylaxis against GVHD [7,8]. Only recently, we have reported that the transfer of expanded autologous Tregs is safe and effective in delaying the onset of Type 1 diabetes in children [9]. For the past decades isolation and expansion methods have been developed to overcome scarcity of Tregs available from peripheral blood of a single donor. Despite many advancements such as: new, very specific surface markers for isolation [10–12] and introduction of clinical grade artificial antigen presenting cells for proliferation activation [7], the outcome of Treg expansion and subsequent immunotherapy is hard to predict. There are various conditions in the method of expansion of Tregs that could affect their quality and yield after expansion. Among these factors, the type of culture medium and the type of serum used for medium supplementation are particularly important. In 2000, Carlens et al. reported large differences in cell expansion rate and subsequent quality of *in vitro* expanded CD4$^+$ T cells depending on the type of culture medium used [13]. In this study, we decided to compare the influence of three different culture media (RPMI 1640 (Cellgro; Manassas VA, USA), SCGM (Cellgenix; Freiburg, Germany), and X-VIVO 20 (Lonza; Walkersville, MD, USA)), supplemented with human serum or fetal bovine serum on Treg proliferation rate, expression of specific markers, and function *in vitro*.

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2. Materials and methods

2.1. Materials

Three culture media were used in this study: SCGM (Cellgenix, Germany), X-VIVO 20 (Lonza, USA), and RPMI 1640 (Cellgro, USA) supplemented with 2 mM L-glutamine (PAA; Dartmouth, MA, USA) and 50 U/ml of penicillin/streptomycin (Life Technologies; Grand Island, NY, USA). These media were supplemented with either 10% AB heat inactivated human serum (HS) (LifeSource; Chicago, IL, USA) or 10% fetal bovine serum (FBS) (Life Technologies; Grand Island, NY, USA). Heat inactivated HS was prepared from 3 units of AB human plasma (LifeSource, USA) derived from 3 different donors and stored in 50 ml conical tubes in −80 °C. IL-2 (interleukin 2) or aldesleukin was purchased from Novartis (New York, NY, USA). Stock solution of 1×10^6 IU/ml of IL-2 was prepared in sterile water for injection, and aliquots were stored in −80 °C and de-frozen before use.

2.2. $CD4^+$ cell isolation and overnight culture

Peripheral blood mononuclear cells (PBMCs) were isolated via a Biocoll density gradient (1.077 g/ml; AG Biochrom; Berlin, Germany), from buffy coats obtained from healthy donors (n = 7) (LifeSource, Chicago, IL, USA). Isolated PBMCs were washed twice with phosphate buffered saline (PBS, Life Technologies; Grand Island, NY, USA) and counted on hemocytometer after staining with 0.4% trypan blue (Sigma Aldrich; St. Louis, MO, USA) to exclude non-viable cells. $CD4^+$ cells were then isolated from 600×10^6 of PBMCs using Human $CD4^+$ T Cell Enrichment Kit (Stem Cell Technologies; Vancouver, BC, Canada) according to the manufacturer's directions.

2.3. Treg sorting

$CD4^+$ cells were counted and stained with antibodies for sorting. The fluorochrome-conjugated antibodies used for sorting were as follows: anti-CD4-allophycocyanin (APC) (IgG^1 clone RPA-T4), anti-CD25-fluorescein isothiocyanate (FITC) (IgG^1 clone M-A251), anti-CD127-phycoerythrin (PE) (IgG^1 clone hIL-7R-M21), and anti-CD3-Pacific Blue (IgG^1 clone UCHT1). Additionally, anti-CD8-peridinin-chlorophyll-protein (PerCP) (IgG^1 clone SK1), anti-CD16-PerCP (IgG^1 clone 3G8), and anti-CD19-PerCP (IgG^1 clone 4G7) antibodies were used for staining T, NK, and B cells to exclude them from sorted populations as the "dump channel". BD Bioscience (San Jose, CA, USA) was the supplier of all the antibodies. The gating strategy used was as follows: FSC-A *vs* SSC-A (gate on lymphocytes) → SSC-W *vs* SSC-H (gate excluding doublets) → FSC-W *vs* FSC-H (gate excluding doublets) → CD4 *vs* CD3 (gate on CD4 and CD3 double pos. cells) → CD4 *vs* 'dump channel' (gate on negative cells) → CD127 *vs* CD25 (two gates, first set on 2–5% of $CD127^{low/neg}CD25^{high}$ — Tregs; second set on $CD127^{high}CD25^{low/neg}$). Cells were then sorted using FACS AriaIII (BD Bioscience, USA) into two populations: Tregs: $CD4^+$ $CD3^+$ $CD25^{high}$ $CD127^{lo/-}$ $CD8^-$ $CD16^-$ $CD19^-$ and T effector cells: $CD4^+$ $CD3^+$ $CD25^-$ $CD127^{high}$ $CD8^-$ $CD16^-$ $CD19^-$.

2.4. Culture and expansion of Tregs after sorting

For the preparation of isolated cells for the expansion phase, Treg and T effector cells were divided into equal portions and re-suspended in appropriate media. Six culture media variants were used: X-VIVO 20 HS, X-VIVO 20 FBS, SCGM HS, SCGM FBS, RPMI 1640 HS, and RPMI 1640 FBS. All cultures were maintained in 1000 IU/ml of IL-2. Cells were cultured on 96-well plates (BD Bioscience, USA) at a concentration of 2×10^5 cells/well and stimulated by anti-CD3 and anti-CD28-coated magnetic beads (Life Technologies, USA) at 0.5:1 bead to cell ratio. Cells were cultured for 17 days. On days 5 and 11 cells from each media condition were collected, counted, re-suspended in the same medium as before, re-stimulated with beads at 0.5:1 bead to cell ratio and re-distributed on sterile 96-well plates.

2.5. Phenotypic analysis of the cells during the culture expansion

On days 8, 11, and 17 Tregs and T effector cells were stained for human FOXP3 using Foxp3 staining buffer set (eBioscience, San Diego, CA, USA) and anti-FoxP3-FITC (IgG^2 clone PCH101) antibody (eBioscience, USA) following the manufacturer's instructions. Additionally on days 1, 11, and 17 staining for CD4, CD3, CD25, CD127, CD45RA, and CD62L was performed. The antibodies used were as follows: anti-CD4-PerCP-Cy5 (IgG^1 clone SK3) (BD Bioscience, USA), anti-CD25-APC (IgG^1 clone 2A3) (BD Bioscience, USA), anti-CD127-PE (IgG^1 clone hIL-7R-M21) (BD Bioscience, USA), anti-CD3-Pacific Blue (IgG^1 clone UCHT1) (BD Bioscience, USA), anti-CD45RA-PE-Cy7 (IgG^1 clone L48) (BD Bioscience, USA), and anti-CD62L-APC-Cy7 (IgG^1 clone 3B5) (Life Technologies, USA). The gating strategy used for analysis of FoxP3 expression was as follows: FSC-A *vs* SSC-A (gate on lymphocytes) → CD4 *vs* CD3 (gate on CD4 and CD3 double pos. cells) → FSC-A *vs* FoxP3 (gate set on FoxP3 positive population, T effector cells were considered FoxP3 negative population). The gating strategy used for analysis of percentages of $CD127^{low/neg}CD25^{high}$ was as follows: FSC-A *vs* SSC-A (gate on lymphocytes) → CD4 *vs* CD3 (gate on CD4 and CD3 double pos. cells) → CD127 *vs* CD25 (gate set on $CD127^{low/neg}CD25^{high}$ cells). The gating strategy used for analysis of percentages of different subsets of CD45RA, CD62L was as follows: FSC-A *vs* SSC-A (gate on lymphocytes) → CD4 *vs* CD3 (gate on CD4 and CD3 double pos. cells) → CD62L *vs* CD45RA (quad gate). Cytometric measurements were done on FACS Aria III cell sorter (BD Bioscience, USA).

2.6. Suppression test

The suppressive activity of expanded Tregs was assessed *in vitro* by performing suppression of proliferation assay, as previously described [15]. Briefly, T effector cells were stained with 5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies, USA) and plated at a concentration of 5×10^4 cells per well on 96-well U-bottom plates in co-cultures with expanded Treg cells in different proportions (Treg:T effector — 1:1, 1:2, 1:8). Cells were cultured in the presence of anti-CD3, anti-CD28-coated magnetic beads (Life Technologies, USA) at 1:1 bead to cell ratio and IL-2 at concentration of 100 U/ml. As positive and negative control, T effector cells were used cultured with or without beads, respectively. After 4 days of culture, cells from each well were collected and CFSE dye dilution was measured using FACS Aria III (BD Bioscience, USA). The gating strategy used was as follows: FSC-A *vs* SSC-A (gate on lymphocytes) → CFSE histogram (range gate set on 90% of negative control events and treated as 100% of suppression of proliferation). Suppression of proliferation was calculated with respect to positive and negative controls.

2.7. Data analysis

All cytometric data were analyzed using FlowJo software (version 9.3.3, Tree Star, Ashland, OR, USA). All figures and statistical analysis were done using GraphPad Prism (GraphPad Software Inc.; La Jolla, CA, USA). p values lower than 0.05 based on nonparametric Mann–Whitney or Wilcoxon matched pairs test were considered statistically significant.

3. Results

3.1. Fold of cell expansion

X-VIVO 20 yielded significantly more efficient cell expansion (median fold expansion 86) than SCGM (median fold expansion 49; $p < 0.05$)

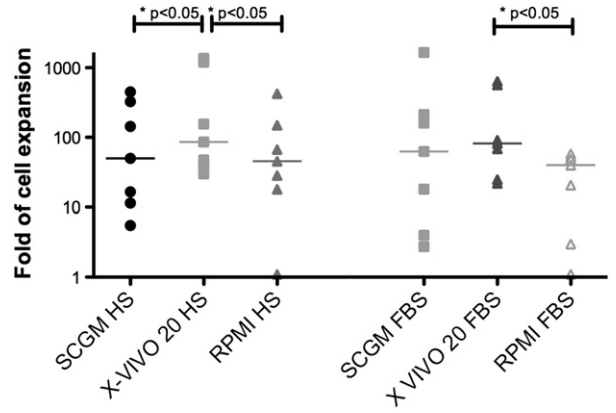

Fig. 1. Fold Treg expansion after 17 days of culture in different medium/serum conditions. Values presented are from 7 independent experiments. Lines represent median fold of cell expansion. p values were calculated based on nonparametric Wilcoxon matched pairs test.

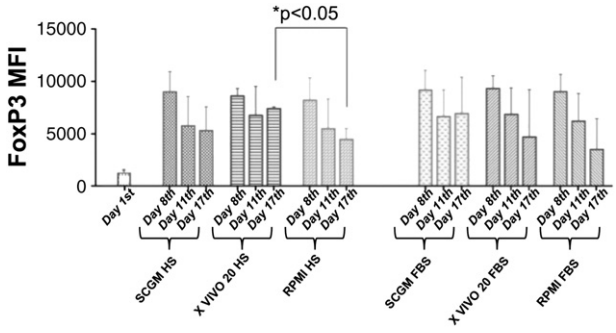

Fig. 2. FoxP3 MFI of Treg cells at days 1, 8, 11, and 17 of expansion. All values are medians with ranges from at least 5 independent experiments. p values were calculated based on a non-parametric Mann–Whitney test.

or RPMI (median fold expansion 45; $p < 0.05$) supplemented with human serum, whereas when cells were cultured in the presence of FBS, X-VIVO 20 (median fold expansion 82) yielded significantly higher when compared to RPMI (median fold expansion 39, $p > 0.05$). There were differences in cell expansion folds at the end of the culture when comparing each medium supplemented with either 10% FBS or HS. However, these differences were not statistically significant (Fig. 1).

3.2. FoxP3 expression

In recent reports on using Tregs in clinical settings, FoxP3 expression was considered to be the most important release criterion [6,7]. We compared FoxP3 expression level in *in vitro* expanded Treg cultures on each medium condition during the 17-day expansions. Mean fluorescence intensity (MFI) of FoxP3 FITC in Tregs expanded in this medium/serum variant was significantly higher at the end of the culture, compared to that in Tregs cultured in RPMI supplemented with HS (Fig. 2). Moreover, X-VIVO 20 supplemented with 10% HS produced the highest mean percentage of FoxP3 positive cells (Fig. 3) at days 11 and 17 among all tested conditions. However, this difference did not reach statistical significance (Table 1). There were no significant differences in the percentage of FoxP3 positive cells or FoxP3 FITC MFI when either serum supplement was used.

3.3. CD25, CD127, CD45RA and CD62L expression

To further compare the quality of expanded cells, we performed immunophenotyping analysis of expanded Tregs. We checked the percentages of cells with high expression of CD25 (alpha chain of IL-2 receptor) and low or lack of expression of CD127 (IL-7 receptor). Following the concept of naïve/effector compartments in the immune system, Tregs were analyzed as proportions of $CD45RA^{+}CD62L^{+}$ naïve, and $CD45RA^{-}CD62L^{+}$ and $CD45RA^{-}CD62L^{-}$ effector subsets [16,17]. Percentages of $CD127^{lo/-}$ $CD25^{hi}$ cells (Fig. 4C,D) together with proportions of different Treg subsets expressing CD62L and CD45RA were similar among all tested conditions indicating that type of serum as well as type of culture medium did not influence the expression of those markers. Drop in percentages of CD45RA positive cells during culture was observed with all tested culture conditions (Fig. 4A,B), which is in agreement with previously published reports [17,18].

3.4. Suppression test

Capacity of expanded Tregs to suppress proliferation of $CD4^{+}CD127^{+}$ $CD25^{-}$ T-cells (T effectors) stimulated with anti-CD3/CD28-coated beads was tested. Tregs expanded on all tested medium conditions were able to suppress proliferation of T effector cells in dose dependent manner. Regulatory cells expanded on X-VIVO 20 medium with 10% HS were characterized by the highest mean percentage suppression of proliferation in CFSE-based proliferation suppression assay (Fig. 5).

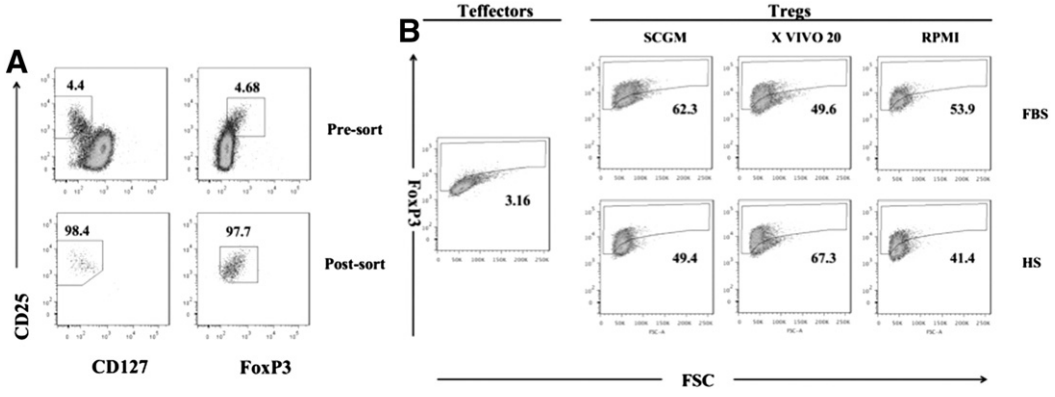

Fig. 3. Sort and post-expansion parameters of FACS-purified $CD127^{low/-}CD25^{high}$ Tregs. (A) Magnetically isolated $CD4^{+}$ cells stained with anti-CD25, anti-CD127, and anti-FoxP3 antibodies and gated like for sorting, are shown in two upper panels. Sorted $CD127^{low/-}CD25^{high}$ Tregs, permeabilized and stained with anti-FoxP3 antibodies are shown in two bottom panels. (B) Sorted Tregs and T effectors were expanded for 17 days using expansion beads, in different medium/serum variants. At the 17th day they were harvested and stained with anti-FoxP3 antibodies after initial permeabilization. The plots show representative example of differences in percentages of FoxP3 positive cells derived from one donor at the end of expansion. T effectors were used to set the cut-off.

Table 1
Percentage of FoxP3 positive cells at 1st, 8th, 11th and 17th days of expansion. All values are medians with range in brackets calculated from at least 5 independent experiments ($p > 0.05$).

	FoxP3$^+$ 1st day	FoxP3$^+$ 8th day	FoxP3$^+$ 11th day	FoxP3$^+$ 17th day
SCGM 10% HS	96 (92–100)	81.9 (72.5–93.3)	66.8 (28.1–90.9)	50.3 (35–85.8)
XVIVO 20 10% HS		78.45 (69.5–96.1)	77.45 (47.9–93.9)	66.9 (56–84.8)
RPMI 1640 10% HS		77.65 (68.7–93.1)	63 (17.2–91.3)	40.4 (30.2–82.5)
SCGM 10% FBS		74.3 (58.2–92.7)	67.85 (59.4–90.8)	62 (46.4–89.2)
XVIVO 20 10% FBS		72.55 (61.7–96)	69.85 (56.1–95.3)	50.8 (13.5–90.7)
RPMI 1640 10% FBS		72.7 (65.7–94.7)	65.3 (53.3–91.8)	53.6 (19–87.5)

4. Discussion

In this study we aimed at improving the method of expansion of T regulatory cells, which has been developing since 2001, when human Tregs were described for the first time [19]. We found that X-VIVO 20 medium supplemented with HS significantly increased the cell expansion rate of CD25^{hi}CD127$^{lo/neg}$ polyclonal Tregs compared to other commercially available culture media that we tested. The cells maintained their regulatory capacities and expressed higher levels of FoxP3 after 17 days of expansion. Recent improvements in Treg expansion, such as *in vitro* use of rapamycin promoting Treg expansion with reduction of conventional T cells in the final product [20], or 50-million-fold expansion of Treg yield achieved after stimulation with cell-based artificial antigen-presenting cells expressing the high-affinity Fc receptor and CD86 [21], could substantially facilitate application of Tregs as an immunotherapy. However, before these methods will be allowed for use in the clinics, currently approved Treg isolation and expansion protocols must be optimized. Expansion protocols used in research as well as in clinical settings differ from each other. Among the variables are types of culture media and types of sera used for cell culture. The most commonly used culture media are RPMI 1640 [6,17], X-VIVO [7,14] and SCGM [8], supplemented with either human serum or fetal bovine serum. Here we show that using different medium/serum configurations may result in significantly different cell expansion rates and final Treg quality, which might be important factor when comparing results obtained by different Treg research groups. Results obtained by us correlate with those published by Carlens et al. in 2000, describing impact of different types of culture media on expansion of CD4$^+$ T cells [13]. In that report X-VIVO medium was proved superior in comparison to RPMI and SCGM. However we did not find significant differences between cell expansion rates of cultures supplemented with HS and FBS. It is worth to mention that we found expansion of Tregs highly inefficient when serum-free culture media were used (results not shown). Obtaining the highest possible yields of fully functional Tregs is also crucial for the clinical application of those cells. Hence, we found that it is necessary to check if and how different types of culture media used for culture could affect Treg expansion.

In our study, from an initial population of more than 90% of FoxP3 positive cells approximately 30% became FoxP3 negative at the end of the expansion (day 17). We speculate that drop in FoxP3 expression is a result of repetitive *in vitro* stimulation, which was already well described by others [17,22]. Currently there is a thrilling discussion regarding Treg plasticity and phenotype stability. Previously it was shown that Foxp3$^+$ Tregs can be converted into Th17-like proinflammatory cells, after *in vitro* exposure to proinflammatory cytokines like IL-1 or IL-6 [23]. On the other hand, recent data of Miyao et al. showed that

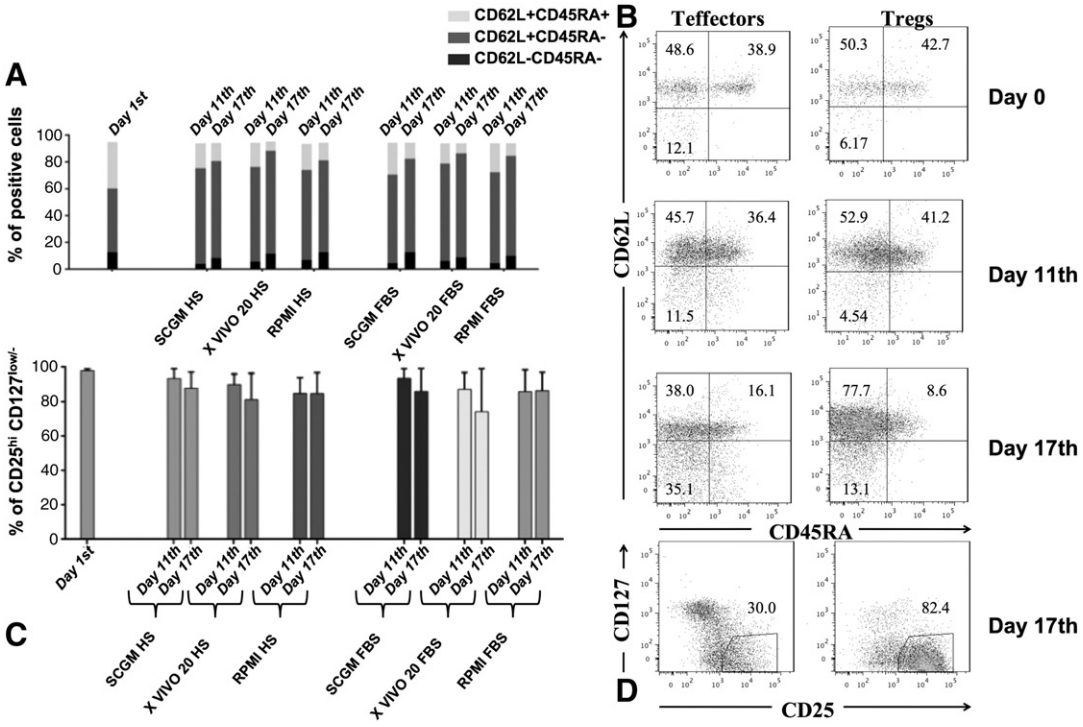

Fig. 4. Expression levels of Treg markers during 17-day *in vitro* expansion. Expanded T effectors and Tregs were stained for CD3, CD4, CD127, CD25, CD45RA and CD62L. Plots represent percentages of expanded Tregs and T effectors from one experiment. In panel A bars represent median values of percentages of CD62L$^+$ CD45RA$^+$, CD62L$^+$ CD45RA$^-$ and CD62L$^-$ CD45RA$^+$ among expanded Tregs at days 1, 11, and 17 of expansion. Median values were normalized to create a total of 100% of all 3 subsets for the purpose of the graph. In panel C bars are median percentages of CD25^{hi} and CD127$^{low/-}$ cells among expanded Tregs. Whiskers represent range. Data presented are from at least 5 independent experiments. Expression level of CD127 and CD25 at the 17th day (D) and changing expression level of CD45RA and CD62L during the 17-day culture (B) were analyzed in T effectors (left column) and Tregs (right column).

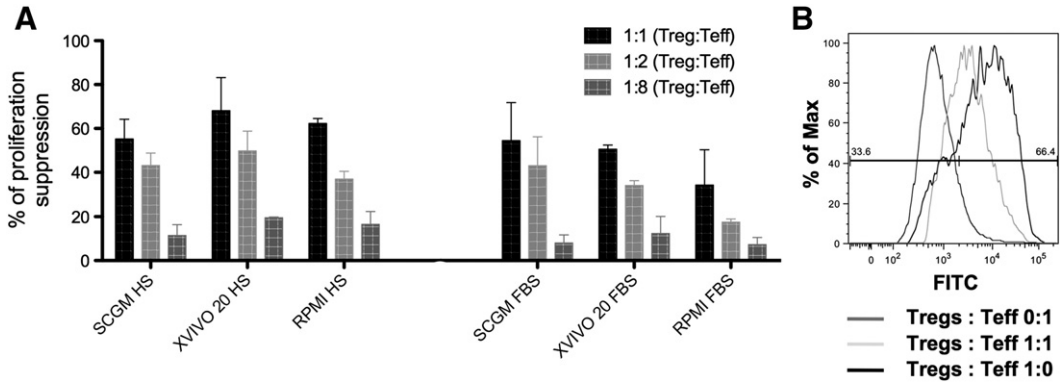

Fig. 5. Suppressive activity of Tregs after 14 days of expansion in different medium/serum conditions. (A) Conventional T cells ($CD4^+CD25^-CD127^+$) were stained with CSFE and co-cultured with Tregs at different ratios in the presence of anti-CD3/CD28-coated expansion beads and IL-2. After four days, CFSE dye dilution was measured by flow cytometry and the percentage proliferation suppression was calculated with respect to positive and negative control. (B) Overlaying histograms depict representative measurement of CFSE dilution in one of the samples together with positive and negative control. All values are medians with range and $p > 0.05$.

"exFoxp3" cells – considered previously as converted Tregs – derive from a minor population of nonregulatory $Foxp3^+$ T cells which up-regulated FoxP3 transiently upon activation. These cells are distinct from committed Tregs which are stable cell lineage regardless of the cytokine environment [24]. In addition, it has been proved that the age of cell donor has a strong impact on the proliferation rate and phenotypic stability of expanded Tregs. The cells from younger donors tend to proliferate more vigorously and express CD45RA at higher level in comparison to Tregs isolated from older donors [15,16]. Nevertheless, Tregs isolated from umbilical cord blood show similarly high differences in cell yields and FoxP3 expression between donors [7], suggesting that there might be other donor specific factors that determine quality and proliferative capacity of expanded Tregs. Strikingly, as reported by Hoffmann et al., different clones of $CD45RA^+$ Tregs from the same donor, produce highly different percentages of FoxP3 positive cells after expansion [22]. Phenotypic instability and differences in proliferation capabilities related to donor age, may not be the only factors affecting Treg expansion. Learning those factors and their influence on Treg expansion would improve safety and effectiveness of the procedure. Controlling the factors required to obtain the high quality Tregs after long *ex vivo* expansion in artificial environment is crucial for the results of clinical trials based on Tregs.

In our study, we demonstrated that proper selection of type of culture media used for *ex-vivo* Treg expansion improves the effectiveness of the procedure, allowing for one to obtain not only higher Treg yields, but also improve the quality of the final Treg product for clinical application. This last feature can be a critical factor in shifting the balance of immune system toward tolerogenic environment after administration of expanded Tregs to the patient. Supplementation of the media with human serum is preferred over equally beneficial FBS, when Tregs are expanded for clinical application. Human serum has lower immunogenicity and carries lower risk for transmission of pathogens to the patient.

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Conflict of interest

All authors have no competing financial interests.

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