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Isolation and expansion of human natural T regulatory cells for cellular therapy

Rajendra Pahwa^{a,*}, Shashidhar Jaggaiahgari^a, Savita Pahwa^b, Luca Inverardi^a, Andreas Tzakis^c, and Camillo Ricordi^a

^aDiabetes Research Institute, University of Miami, Leonard M. Miller School of Medicine, Miami, FL, USA

^bDevelopmental Center for AIDS Research, Microbiology and Immunology, University of Miami, Leonard M. Miller School of Medicine, Miami, FL, USA

^cDepartment of Surgery, Miami Transplant Institute, University of Miami, Leonard M. Miller School of Medicine, Miami, FL, USA

Abstract

Natural T regulatory cells (nTregs) play a key role in inducing and maintaining immunological tolerance. Cell-based therapy using purified nTregs is under consideration for several conditions, but procedures employed to date have resulted in cell populations that are contaminated with cytokine secreting effector cells. We have established a method for isolation and ex vivo expansion of human nTregs from healthy blood donors for cellular therapy aimed at preventing allograft rejection in organ transplants. The Robosep instrument was used for initial nTreg isolation and rapamycin was included in the expansion phase of cell cultures. The resulting cell population exhibited a stable CD4⁺CD25⁺⁺^{bright}Foxp3⁺ phenotype, had potent functional ability to suppress CD4⁺CD25^{negative} T cells without evidence of conversion to effector T cells including TH17 cells, and manifested little to no production of pro-inflammatory cytokines upon in vitro stimulation. Boolean gating analysis of cytokine-expressing cells by flow cytometry for 32 possible profile end points revealed that 96% of expanded nTregs did not express any cytokine. From a single buffy coat, approximately 80 million pure nTregs were harvested after expansion under cGMP conditions; these cell numbers are adequate for infusion of approximately one million cells kg⁻¹ for cell therapy in clinical trials.

Keywords

Natural T regulatory cells; Rapamycin; IL-2; Expansion; Cytokine; Suppression

*Corresponding author. 1450 NW 10 Avenue, Diabetes Research Institute, University of Miami, Leonard M. Miller School of Medicine Miami, FL 33136, USA. Tel.: +1 305 243 6843; fax: +1 305 243 2813., rpahwa@med.miami.edu (R. Pahwa).

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1. Introduction

Several subsets of T regulatory cells (Tregs) have been described in humans. The CD4 derived major Treg populations include natural T regulatory cells (nTreg) which originate in the thymus (Sakaguchi et al., 2001; Wood and Sakaguchi, 2003; Sakaguchi, 2004; Miyara and Sakaguchi, 2007; Nagahama et al., 2007), induced Tregs (iTreg) derived from naive CD4⁺T cells in the periphery (Dardalhon et al., 2008; King et al., 2008), Tr1 cells which secrete predominantly IL-10 (Roncarolo et al., 2001; Levings et al., 2002) and TH3 cells which secrete predominantly TGF β (Weiner, 2001). Other cell populations such as NKT cells and CD8 T suppressor cells can also mediate immune regulation. Natural T regulatory cells play a key role in inducing and maintaining immunological tolerance and immune homeostasis (Sakaguchi et al., 2001; Wood and Sakaguchi, 2003; Sakaguchi, 2004; Nagahama et al., 2007). This specialized subpopulation of T cells is critical for maintaining unresponsiveness to selfantigens (Wood and Sakaguchi, 2003; Sakaguchi, 2004; Nagahama et al., 2007). These cells are of considerable interest from the viewpoint of cellular therapy in the therapeutic management of autoimmune disorders such as diabetes type 1, for short term immunosuppression in graft versus host disease, and for induction of tolerance in solid organ transplantation to prevent graft rejection. Although clinical trials using Tregs are under way, the procedures for deriving sufficient quantities of cells which have desirable characteristics of nTregs have yet to be optimized. The expanded CD4⁺ T cell population should ideally have the following four characteristic of nTregs: (1) a stable phenotype of CD25^{++bright}Foxp3⁺expression; (2) functional ability to suppress immune reactive T cells by a mechanism that does not involve secretion of IL-10 or TGF- β ; (3) no evidence of conversion to effector T cells or TH17 cells and (4) no secretion of pro-inflammatory cytokines upon in vitro stimulation.

To accomplish the goal of expanding nTregs ex vivo for therapeutic purposes, it is critical to begin with the appropriate starting cell population and to use culture conditions that selectively favor the expansion of Tregs with properties that best characterize nTregs. We have successfully expanded a population of human nTregs ex vivo in cGMP conditions; these expanded CD3⁺CD4⁺T cells fulfill the four required characteristics of nTregs described above. Starting from 0.8 to 1.4 million purified CD4⁺CD25^{++bright} cells from a single buffy coat, we can harvest approximately 50 to 80 million cells at the end of the expansion protocol and this number would yield approximately 1 million cells kg⁻¹ for cellular therapy in an average adult. These expanded cells conform with the FDA requirement which mandates that the identity, purity, potency and sterility of a cell product should be demonstrated before they can be administered. Here we describe the properties of the expanded Treg cells and methods to generate them.

2. Material and methods

2.1. Human buffy coats

Human buffy coats containing approximately 3–4 $\times$ 10¹⁰ cells were obtained from healthy adult donors from Community Blood Center of South Florida, Miami, FL, USA. Informed consent was obtained in accordance with standard policies and procedures. Samples were

processed within 24 h of collection and were required to have a lymphocyte viability of >90% in order to be processed.

2.2. Isolation and culture of natural T regulatory cell (nTregs)

To obtain a purified population of nTregs for subsequent expansion, human CD4⁺CD25^{++bright} cells were isolated from buffy coats in two steps. In the first step CD4⁺T cells were enriched by negative selection using a cocktail of nine monoclonal antibodies. In the second step CD25^{++bright} cells were isolated by positive selection from purified CD4⁺cells using anti CD25 antibody in a Robosep instrument (Stem cell Technologies, Vancouver, BC, Canada).

For the first step, the buffy coat was diluted 1:2 with Ca²⁺Mg²⁺-free phosphate buffered saline (PBS) (Stem Cell Technologies, Vancouver, BC, Canada) containing 2% fetal bovine serum (FBS) (Hyclone, South Logan, Utah, USA) and dispensed in 20 ml volume each in 50 ml conical tubes (Corning Life Sciences, Lowell, MA, USA). RosetteSep human CD4⁺T Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, BC, Canada) which is a mixture of mAbs to CD8, CD16, CD19, CD36, CD56, CD66b, TCR $\gamma\delta$, Glycophorin A and P9 was added to each tube at a concentration of 50 $\mu\text{l ml}^{-1}$ and tubes were incubated at room temperature for 20 min. Thereafter the cell suspension was diluted 1:1 with PBS, and the CD4⁺-enriched cells were harvested by Ficoll Paque Plus (GE Healthcare, Pittsburgh, PA, USA) density gradient centrifugation at 1200 $\times g$ for 20 min at 23 °C. Isolated CD4⁺cells were washed twice with PBS containing 10% FBS and resuspended at a concentration of 5 $\times 10^7$ cells ml⁻¹ and dispersed in 4 ml aliquots in sterile 15 ml round-bottom polyethylene tubes as required for processing on the Robosep instrument. For the second step, the Robosep instrument was primed as per the manufacturer's protocol by loading EasySep human CD25 Positive Selection Cocktail and EasySep Magnetic Nanoparticles (both from Stem Cell Technologies, Vancouver, BC, Canada) using the volume and concentration specified for the selection of CD4⁺CD25^{++bright} cells. The instrument was programmed for automatic separation of CD4⁺CD25^{++bright} cells followed by further separation of the remaining cells into CD4⁺CD25^{dim} and CD4⁺CD25^{negative} cells. To prepare the isolated cells for the expansion phase, they were washed twice and re-suspended in 1 ml of culture medium consisting of X-Vivo 15 (Lonza, Muenchensteinerstrasse, CH, Switzerland), 1% N-Acetylcysteine (American Reagent, Shirley, NY, USA) and 1% Pen-Step (Invitrogen, Carlsbad, CA, USA).

2.3. Ex vivo expansion of Robosep isolated CD4⁺cell populations

The Robosep-isolated population of CD4⁺CD25^{++bright} cells suspended in serum free X-Vivo culture medium was plated in a final volume of 300 μl in flat bottom 48-well microtiter plates (Corning Life Sciences, Lowell, MA, USA) at a concentration of 0.2 $\times 10^6$ cells well⁻¹ with CD3/CD28 T-cell expander Dyna beads at 3:1 ratio (Invitrogen, Carlsbad, CA, USA) and 1000 U ml⁻¹ IL-2 (R&D systems, Minneapolis, MN, USA), in the presence of 100 ng ml⁻¹ of rapamycin (Wyeth, Philadelphia, PA, USA), as has been previously described (Godfrey et al., 2005; Hippen et al., 2008; Putnam et al., 2009). Cells were cultured at 37 °C with 5% CO₂ and 100% humidity. One day after culture initiation, 30 μl human AB serum (Valley Biomedical, Winchester, VA, USA) was added to the wells at a final concentration of

10%. On the second day, 700 μ l X-Vivo culture media with 10% human AB serum with rapamycin 100 ng ml⁻¹ was added to make the final volume to 1 ml in each culture well. On day 5, cells from different wells were pooled, sampled for viability and cultured at a concentration of 0.3×10^6 cells in sterile T-25 tissue culture flasks (Corning Life Sciences, Lowell, MA, USA) in complete X-Vivo culture medium supplemented with 10% human AB serum, 300 U ml⁻¹ of IL-2 and rapamycin 100 ng ml⁻¹. On day 8, of culture, cells from the T-25 tissue culture flasks were pooled, sampled for viable cells and re-distributed into sterile T-75 or T-175 tissue culture flasks at a concentration of 0.3×10^6 cells ml⁻¹ in the culture medium of complete X-Vivo culture medium supplemented with 10% human AB serum, 300 U ml⁻¹ of IL-2 and rapamycin 100 ng ml⁻¹. They were again pooled on day 12 and re-distributed into sterile T-75 or T-175 tissue culture flasks at a concentration of 0.3×10^6 cells ml⁻¹ in the same media as above, and the procedure was repeated on day 15 in a similar manner. For the expansion phase, the cells were cultured for a total of 19 ± 1 days. At the end of the culture period, cells were exposed to the Dyna cell magnetic particle separator (Invitrogen, Carlsbad, CA, USA) for 10 min to remove CD3/CD28 T-Cell expander Dyna beads. Cells were washed twice and were analyzed for cell count, viability, purity, potency of suppression and cryopreserved in 10% DMSO using an automated temperature controlled freezer (T.S. Scientific Kryo 10 Series, Perkasi, PA, USA) in liquid nitrogen. To evaluate properties of all 3 Robosep isolated CD4⁺ cell populations, identical cultures were set up with the other two Robosep isolated cell populations of CD4⁺CD25^{dim} and CD4⁺CD25^{negative} cells. In addition, to evaluate the effect of rapamycin, cultures were also set up without the addition of rapamycin.

2.4. Monoclonal antibody reagents

Flow cytometry panels for 10–12 color polychromatic flow cytometry analyses were utilized to analyze T cell phenotype and functions. Antibodies to IFN- γ -PECy7, IL2-PE, CD3-Amcyan, CD4-PerCPy5–5, TNF- α -Alexafluor700, CD107a-PECy5, CD25-APCCy7, Foxp3-Alexafluor488 or Alexafluor647 (clone 259D/C7), CD127-PE, CD27-APC, CD45RO-FITC, CD14-Alexafluor700 or Pacific blue, CD19-PECy7, and CD8-APCCy7 were obtained from BD Pharmingen, San Jose, CA, USA. IL17-Alexafluor488 and CD56-Pacific blue were obtained from e-Bioscience, San Diego, CA, USA and CD56-Alexafluor488 was obtained from Biolegend, San Diego, CA, USA. CD25-PE was obtained from Stem cell technologies, Vancouver, BC, Canada. CD19-Pacific blue and a violet fluorescent reactive dye used as a viability marker to exclude dead cells from analysis (LIVE/DEAD® Fixable Dead Cell Stain Kit, ViViD) were obtained from Invitrogen, Carlsbad, CA, USA.

2.5. Flow cytometry analysis

Polychromatic flow cytometry for surface and intracellular staining of freshly isolated and expanded nTregs was performed on a BD LSR II Flow Cytometer System (BD Biosciences, San Jose, CA, USA) as described (Perfetto et al., 2004; Lamoreaux et al., 2006; Darrah et al., 2007). The procedure and method for 10–12 color flow cytometry were optimized in key steps which included appropriate concentrations of monoclonal antibodies, use of a dead-cell discriminator and ‘dump’ channel, selection of a cytokine secretion inhibitor, selection of fixation and permeabilization reagents and inclusion of compensation controls as described (Lamoreaux et al., 2006). This assay was used to detect four/five separate

functions (production of three/four cytokines and degranulation) and simultaneous identification of surface markers on the subpopulation of cells. The same method was used for FoxP3 staining and characterization of nTregs. Cells were analyzed by flow cytometry using gated singlets as is usually recommended, as well as open gate to encompass all the cells to ensure that there were no contaminating cells being excluded in the analysis because they would all be included in the expansion phase. It was suggested by the FDA that an open gate analysis should be done on the expanded cell population because all the expanded cells are planned to be infused when used in clinical trials. Open gates encompassing all cells (including doublets, triplets and other cells) were therefore used in addition to cell specific gates to conduct the phenotypic characterization and intracellular cytokine analysis.

2.6. Phenotypic analysis

Phenotypic analysis of freshly isolated nTregs was performed by surface staining of 0.5×10^6 cells with CD3, CD4, CD25, CD8, CD14, CD19, CD56 followed by intracellular staining for Foxp3 according to manufacturing instructions. Surface staining for CD25 was done using CD25-PE from Stem cell Technologies, Vancouver, BC, Canada.

Phenotype analysis of expanded nTregs was performed by surface staining of 1×10^6 cells with CD3, CD4, CD25, CD127, CD27, CD45RO and by using a 'dump' channel for cells stained with ViViD dye, CD14, CD19, CD56, followed by intracellular staining for FoxP3 according to the manufacturer's instructions in a single tube. Cells were also stained in another tube with ViViD dye and for CD4, CD8, CD25, CD14, CD19, CD56 and FoxP3 to trace contamination with monocytes, B cells, NK cells and cytotoxic T cells in the live cells. Flow data were collected on a FACS LSRII cytometer and analyzed with FlowJo software (Mac version 8.6.8, Tree Star).

2.7. Intracellular cytokine analysis

Intracellular cytokine analysis was performed in freshly isolated and expanded nTregs. nTreg populations at a concentration of 1×10^6 cells were cultured with phorbol myristate acetate (PMA) (Sigma-Aldrich, St. Louis, MO) 50 ng ml^{-1} , Ionomycin $1 \text{ } \mu\text{g ml}^{-1}$, Monensin $0.7 \text{ } \mu\text{l ml}^{-1}$ (golgistop, BD Biosciences, San Jose, CA, USA) and Brefeldin-A $10 \text{ } \mu\text{g ml}^{-1}$ (Sigma-Aldrich, St. Louis, MO) in complete media (RPMI 1640 supplemented with 10% heat inactivated FBS, 100 U/ml penicillin G, $100 \text{ } \mu\text{g ml}^{-1}$ streptomycin) for 5 h at 37°C in 5% CO_2 incubator at 100% humidity. Cells were washed twice with RPMI 1640 and intracellular staining for cytokines IL-2, IL-17, IFN- γ , TNF- α and CD107a was performed in a single tube with appropriate controls as described (Lamoreaux et al., 2006). Flow data were collected on a FACS LSRII and analyzed with FlowJo software. Frequency of cytokine expression on per cell basis was analyzed by Boolean gating using FlowJo software.

2.8. Cytokine assay in culture supernatants

Freshly isolated nTregs and expanded nTregs were stimulated with PMA 50 ng ml^{-1} and Ionomycin $1 \text{ } \mu\text{g ml}^{-1}$ for 5 h at 37°C . Cells were centrifuged at $850 \times g$ for 10 min and supernatants were collected and were analyzed in a multiplex format using Quansys multiplex Elisa kit (Quansys Biosciences, West Logan, Utah, USA) for cytokines interferon

(IFN)- γ , IL-2, IL-4, IL5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-1a, IL-16, IL-17, IL-23, TNF- α , LT α , TGF β , TGF β -RII, TGF β -RIII.

2.9. In vitro suppression assay

Frozen autologous CD4⁺CD25^{negative} cells were thawed and washed twice and cell count and viability were assessed by trypan blue dye exclusion. Viable 2×10^6 CD4⁺CD25^{negative} (responder) cells were labeled with Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA, USA) at a concentration of $4 \mu\text{M}/5 \times 10^6$ cells for 10 min at 37 °C. Labeling was terminated by addition of an equal volume of 100% FBS. After 4 washes in 10% FBS in complete media, cells were cultured alone and with unlabeled nTregs at 1:1 and 1:10 (responder: Treg) ratio and stimulated with 50 μl (7.8ul of beads suspended in 1 ml PBS and 4 ml of complete media) of anti CD3/CD28 coated micro beads for 4 days at 37 °C in 5% CO₂ incubator. CD4⁺CD25^{negative} cells in medium alone were also cultured as control. On day 4, cells were washed twice and cell division was analyzed in all culture conditions. Cells undergoing division were identified by the decrease in CFSE, resulting from dilution of dye with each division. The medium-alone culture consisted of non-proliferating cells (CFSE bright) with less than 3.3% CFSE dim (proliferating) cells.

2.10. Sterility of monoclonal antibodies

A custom batch of the RosetteSep human CD4⁺cell enrichment cocktail and EasySep human CD25⁺positive selection cocktail was produced by the manufacturer (Stem Cell Technologies, Vancouver, BC, Canada), in quantities sufficient to cover 60 patients for phase I clinical trials. All the products were tested for sterility and were free of murine retrovirus, adventitious virus (tested with highly sensitive assay, Wuxi Apptec Inc) mycoplasma, bacteria, fungi and endotoxin.

2.11. Statistical analysis

All group results are expressed as mean plus or minus S.D. if not stated otherwise. One way ANOVA-Tukey post test was used for the comparison of group values and discriminatory parameters, where appropriate P values less than 0.05 were considered significant.

3. Results

As the procedure described here is aimed at developing nTreg cell therapies for infusion into patients, the flow cytometry analysis throughout the study utilized an open gate analysis that included the entire cell population without exclusion of doublets, triplets and other potentially contaminating non Treg cells. In addition, conventional analysis of gated singlets was performed.

3.1. Isolation and characterization of freshly isolated n T regulatory cells

Isolation of nTregs was made from buffy coats of healthy donors in two steps. In the first step CD4⁺cells were enriched by negative selection with a cocktail of nine monoclonal antibodies. From eight different buffy coat samples, recovery of CD4⁺cells ranged from $135\text{--}240 \times 10^6$ cells. In the second step, CD4⁺CD25^{++bright} cells were isolated from the CD4⁺cells using positive selection with the Robosep instrument. The yield of

CD4⁺CD25^{++bright} cells ranged from 0.8–1.4×10⁶ cells, which is approximately 0.5–0.71% of the starting population of CD4⁺ cells. We also collected the CD4⁺CD25^{dim} and CD4⁺CD25^{negative} cell fractions. In the flow cytometry open gate data for CD4⁺CD25^{++bright} cell fraction (Fig. 1A), 90% cells were CD3⁺ cells and were composed almost entirely of CD4⁺CD25^{++bright} cells, with 97.4% cells expressing Foxp3 (86.7% bright Foxp3⁺ and 11.3% dim Foxp3⁺) and had insignificant contamination with monocytes (CD14⁺, 0.2%), B cells (CD19⁺, 0.63%), NK cells (CD56⁺, 0.72%) and cytotoxic T cells (CD8⁺, 0.39%). Analysis of the same cell fraction gated on singlets demonstrated similar cell frequencies (Fig. 1B). Among CD4⁺ cells, 99.8% were CD25^{++bright} with 97.4% cells expressing Foxp3 (84.5% bright Foxp3⁺ and 12.9% dim Foxp3⁺) and had minimal contamination with other cellular subsets. The Robosep isolated CD4⁺CD25^{++bright} cell fraction was designated as nTregs.

The Robosep-isolated nTregs were characterized functionally for ability to suppress autologous CD4⁺CD25^{negative} T cells and for their capacity to produce cytokines upon in vitro stimulation. The nTregs mediated modest suppression of proliferation of autologous CD4⁺CD25^{negative} T cells at 1:1 ratio as assessed by CFSE dye dilution with a decrease in CFSE low cells from 91.7% to 70.6% (Data not shown). The cytokine expression profile of the Robosep isolated CD4⁺CD25^{++bright} and CD25^{negative} cell populations was examined following in vitro stimulation with PMA (phorbol myristate acetate) plus Ionomycin for 5 h (Supplementary Fig. 1). To rule out contamination with non-nTreg cells that can be induced to secrete cytokines, we again used an open gate to encompass all cells in the Robosep-isolated CD4⁺CD25^{++bright} cell fraction including doublets, triplets and other possible contaminating cells (Supplementary Fig. 1A). Very low frequencies of cytokine positive cells were identified in the total cell population, consisting of IL-17 (0.27%), IL-2 (3.9%), IFN γ (0.79%) and TNF α (2.9%). A majority of the cytokine expressing cells were CD4⁺T cells, showing the expression of IL-17 (0.16%), IL-2 (2.7%), IFN γ (0.59%), and TNF α (2.63%). Analysis performed on gated singlets (Supplementary Fig. 1B) showed slightly lower cytokine expressing cell frequencies of IL17 (0.12%), IL2 (2.4%), IFN γ (0.63%) and TNF α (2.49%). In contrast, the CD4⁺CD25^{negative} cells expressed very high percentages of all cytokines in both types of analyses (Supplementary Fig. 1C, D). Boolean gating analysis (Supplementary Table 1) of cytokine expressing cells in an open gate for 16 possible profile end points revealed that 94.8% of cells in the CD4⁺CD25^{++bright} cell fraction did not express any cytokine. A single triple positive IFN γ ⁺IL2⁺IL17⁺TNF α ⁻ clone with a frequency of 0.18% was observed consistently in two experiments. Cryopreservation and thawing of CD4⁺CD25^{++bright} and CD4⁺CD25^{negative} cells did not alter the cytokine production (Data not shown). Based on these results we contend that the methodology described here can lead to purification of a CD4⁺CD25^{++bright} cell fraction which is strongly Foxp3⁺ and exhibits preferred attributes of nTregs and constitutes an appropriate source of cells for ex vivo expansion of nTregs.

3.2. Ex vivo expansion of nTregs

Freshly isolated CD4⁺CD25^{++bright} cells were expanded in X-Vivo complete medium containing CD3/CD28 expander beads and rhIL-2, with and without the addition of rapamycin for 19 days and compared with similarly expanded CD4⁺CD25^{dim} and

CD4⁺CD25^{negative} cells (Supplementary Fig. 2). Fold expansion of the cellular fractions in the presence of rapamycin (Supplementary Fig. 2A), shows a mean of 60-fold expansion in CD4⁺CD25^{++bright} cells (10 subjects) whereas the CD4⁺CD25^{dim} (8 subjects) and CD4⁺CD25^{negative} (3 subjects) cells expanded to an average of 216-and 432-fold respectively. Rapamycin is known to selectively block expansion of CD4⁺CD25^{negative} T effector cells, while allowing the growth of CD4⁺CD25^{++bright} Tregs with maintenance of high Foxp3 protein expression and suppressor function (Powell et al., 1999; Kahan and Camardo, 2001; Battaglia et al., 2006; Golovina et al., 2008; Hippen et al., 2008; Zeiser et al., 2008). Cells cultured without rapamycin exhibited tremendous expansion (Supplementary Fig. 2B) and showed marked expansion in all 3 cell populations when rapamycin was omitted from the cultures. Isolated CD4⁺CD25^{++bright} CD4⁺CD25^{dim} and CD4⁺CD25^{negative} cells expanded up to an average of 1200-fold, 64,000-fold and 66,490-fold respectively without the addition of rapamycin.

3.3. Identity, purity and potency of expanded natural T regulatory cells

Phenotypic characterization of expanded nTregs was performed by multicolor flow cytometry analysis on cryopreserved cells (Perfetto et al., 2004; Lamoreaux et al., 2006; Darrah et al., 2007). Because the expansion protocol has been developed for infusion of nTregs into patients, it was suggested by the FDA that open gate analysis be performed on the entire population to include doublets, triplets and other potentially contaminating cells. Using an open gate analysis (Fig. 2A), cell viability was 96%; CD4 purity was 99.2%, with 97.6% CD25^{++bright} cells of which 98.3% cells were Foxp3⁺. The CD3⁺CD4⁺CD25^{++bright} Foxp3⁺ cells were found to be mostly negative for the expression of CD127, and 94.3% of cells were CD45RO⁺CD27⁺ indicating that they were of memory phenotype. These cells were CD57 negative (data not shown) implying that telomeres were preserved in these cells (Darrah et al., 2007; Appay et al., 2008). Similar properties were observed by conventional flow cytometry analysis of singlets (Fig. 2B). The expanded nTregs showed minimal contamination with other cell populations (Supplementary Fig. 3). A representative open gate analysis (Supplementary Fig. 3A), demonstrates extremely low frequencies of contaminating monocytes (CD14, 0.057%), B cells (CD19, 0.16%), NK cells (CD56, 0.09%) and cytotoxic T cells (CD8, 0.099%). Similar results were obtained when cells were gated on singlets (Supplementary Fig. 3B). Means and SD of frequencies of non-Treg contamination observed in expanded CD4⁺CD25^{++bright} cells from four different buffy coats consisted of monocytes (CD14⁺, 0.16±0.09%), B cells, (CD19⁺, 0.19±0.07%), NK cells (CD56⁺, 0.19±0.08%) and CD8 T cells (0.09±0.03%) (Supplementary Fig. 3C).

The expanded nTregs exhibited potent suppressor activity in the classical suppression assay (Brusko et al., 2007). Autologous CD4⁺CD25^{negative} responder cells were labeled with CFSE dye and stimulated with CD3/CD28 dynal beads, and expanded nTregs were mixed with responder cells in 1:1 and 1:10 ratio for 4 days. Proliferation and cell division were analyzed on day 4 (Fig. 3). At a 1:1 ratio of CD4⁺CD25^{++bright} cells with CD4⁺CD25^{negative} responder cells, proliferation (as assessed by CFSE low cells) was reduced from 63.4% to 5.27% (91.6% suppression) whereas addition of CD4⁺CD25^{dim} to CD4⁺CD25^{negative} responder cells at 1:1 ratio only reduced proliferation from 63.4% to 41.4% (34.7% suppression). Suppression was minimal at a 1:10 ratio regardless of the population used.

Summary data from three donors (Fig. 3E) showing significant inhibition of proliferation function at 1:1 ratio of CD4⁺CD25^{++Bright} cells with CD4⁺CD25^{Negative} responder cells which resulted in a decrease of CFSE low cells from 50.9±8.48% to 4.425±1.126% (92.07±0.98% suppression). Suppression/Inhibition of proliferation was statistically insignificant with addition of CD4⁺CD25^{Dim} cells to autologous CD4⁺CD25^{Negative} responder cells.

3.4. Intracellular cytokine expression in stimulated expanded nTreg cells

Assays for intracellular cytokines were performed on frozen and thawed expanded cells as described in the method section. Expanded CD4⁺CD25^{++bright}, CD4⁺CD25^{dim} and CD4⁺CD25^{negative} cells were stimulated with PMA plus Ionomycin for 5 h, stained for surface markers and intracellular cytokines were analyzed by multicolor flow cytometry (Perfetto et al., 2004; Lamoreaux et al., 2006; Darrah et al., 2007). In every experiment we took extreme care to use the ViViD dye in the staining protocol for live/dead cell discrimination. In all the experiments gating was done on live cells where N95% cells were negative for ViViD dye. The analysis on open gate (Fig. 4A) shows that expanded CD4⁺CD25^{++bright} nTregs do not convert to TH17 cells (0.13% IL-17 expression) and have an extremely reduced frequency of cytokine expressing cells for IFN γ (0.51%), TNF α (1.01%), IL-2 (1.53%) and a low frequency of cells with the degranulation marker CD107a (0.06%). In contrast, CD4⁺CD25^{dim} and CD4⁺CD25^{negative} cells expressed high frequencies of cytokine positive cells. The intracellular cytokine analysis using singlet populations in CD4⁺CD25^{bright} cells also revealed minimal cytokine expressing cells (Fig. 4B). Data for the percent cytokine expressing cells in four different experiments are presented (Fig. 4C) and show the minimal cytokine expression in CD4⁺CD25^{++bright} cells in comparison to CD4⁺CD25^{dim} and CD4⁺CD25^{negative} cells. Boolean gating analysis for all the cytokine combinations was determined and 32 possible profile end points were scored and frequencies of more than 0.1% were highlighted (Table 1). Only one triple combination clone of IL-2⁺IFN γ ⁺TNF α ⁺ cells with frequency of 0.16% was observed. The same clone was observed in three additional experiments. Approximately 96% of the cells did not express any cytokines. Interestingly, the cytokine profile of expanded CD4⁺CD25^{++bright} cells cultured without rapamycin (Supplementary Fig. 4) manifests an outgrowth of cytokine expressing cells despite the fact that they were present at very low frequencies in freshly isolated cells. Addition of rapamycin inhibited the growth and expansion of cytokine-secreting cells in this population during the 19 day culture period. Analysis of culture supernatants of three different samples of freshly isolated nTregs and nTregs expanded according to the expansion protocol results in very low or undetectable levels of IL-10, TGF β , or pro-inflammatory cytokines in both fresh and expanded nTreg cell populations (Supplementary Table 2).

4. Discussion

Natural Tregs constitute a minor population in peripheral blood with a frequency of 1–2% of total circulating CD4⁺ cells (Baecher-Allan et al., 2001). Thus expansion of therapeutic quantities of nTregs, particularly under cGMP compliant conditions, is a challenge (Riley et al., 2009). This report describes the methodology for isolation and expansion of a population

of nTregs from peripheral blood in humans. Based on their phenotype and functional characteristics, the expanded Treg population met the criteria that most closely define thymus derived nTregs, thus making them ideally suited for cell therapy in clinical trials. The expanded nTregs had the following properties: (1) stable expression of CD4⁺CD25^{++bright}Foxp3⁺ with N97–98% purity; (2) potent functional ability to suppress CD4⁺CD25^{negative} T cells without secretion of IL-10 or TGF- β ; (3) no conversion into effector T cells or TH17 cells and (4) no production of pro-inflammatory cytokine upon in vitro stimulation with PMA/Ionomycin. Two critical determinants in the quality and quantity of expanded nTregs were first, the successful isolation of purified nTregs from peripheral blood, and second, the use of rapamycin in the expansion protocol.

In humans there is no nTreg-specific cell surface marker for isolation and no clear understanding of how they function to control immune responses in vivo. Majority of CD4⁺CD25^{++bright} nTreg cells are produced by the thymus with a repertoire of antigen specificities that are as broad as that of naïve T cells, and they are capable of recognizing both self and non-self antigens and control various immune responses (Sakaguchi et al., 2001; Wood and Sakaguchi, 2003; Sakaguchi, 2004). A specific role of Foxp3 in the development and function of natural CD4⁺CD25^{++bright} Tregs has been described (Fontenot et al., 2003; Hori et al., 2003; Fontenot and Rudensky, 2004; Miyara et al., 2009). Mutations of the human gene Foxp3, similar to the gene mutated in scurfy mice (Foxp3) were found to be the cause of IPEX syndrome which has X linked inheritance and is characterized by polyendocrinopathy, enteropathy and immune dysregulation (Chatila et al., 2000; Bennett et al., 2001; Khattri et al., 2003). However, expression of Foxp3 is not sufficient to designate cells as nTregs and Foxp3⁺CD4⁺ cells may in fact be composed of a mixture of nTregs derived from the thymus, iTregs generated in the periphery from CD4⁺ naïve cells and activated non-T regulatory CD4⁺ cells which may not have suppressor activity. Functional characteristics that differentiate true nTregs from other subsets are thus critical for defining nTregs that are developed for cell therapy.

For the initial isolation of nTregs we utilized peripheral CD4⁺ cells with bright expression of CD25 as the main marker and used the Robosep magnetic cell isolation method. The choice of markers on which to isolate human nTregs for expansion has been controversial. As Foxp3 is an intracellular protein it cannot be used to isolate viable cells. CD25 represents the α chain of the IL-2 receptor that is essential for the generation and maintenance of nTregs and high expression of CD25 is commonly used in protocols for isolating peripheral Tregs. However CD25 is also upregulated upon cellular activation, thus recently activated effector CD4⁺ T cells may be confused with nTregs and iTregs. Nevertheless there are differences between CD4⁺CD25^{++bright} nTregs and activated T cells with respect to the characteristics of CD25 expression. Human and mouse CD4⁺ cells with potent regulatory properties express high and sustained levels of CD25, whereas recently activated T cells express transient and low levels of CD25 (Kuniyasu et al., 2000; Baecher-Allan et al., 2001). Thus a stable and high expression of CD25 is an essential characteristic of nTregs. In the present report, the expanded nTreg population derived from the CD4⁺CD25^{++bright} cell fraction maintained a stable CD25 bright expression. Other markers, such as latency-associated peptide (LAP) and IL-1 receptor type I & II (CD121a/CD121b) have also been used for Treg characterization. These markers are not expressed on resting Foxp3⁺ Tregs, but are rapidly induced and

expressed on Foxp3⁺Tregs for a short time period after TCR-mediated activation (Tran et al., 2009). Thus these markers can only isolate TCR activated Foxp3⁺Tregs but not resting Foxp3⁺Tregs. Other markers have been ascribed to natural Tregs, e.g. CTLA-4 and GITR, but currently, the most reliable marker for natural Tregs is the transcription factor Foxp3 when applied in conjunction with other properties of nTregs.

A feature that is increasingly used for isolating Tregs from blood is the absence of CD127, the IL-7R α , which is abundantly expressed on naïve cells. We contend that CD127 negativity should not be used to select the initial starting population for nTreg expansion for several reasons. First, by doing so we may also eliminate the thymic derived resting precursors of nTregs which may express CD127. Recent data of Treg expansion using umbilical cord blood which is enriched in naïve cells support this contention. Umbilical cord blood T regulatory cells isolated by positive selection using either AutoMACS or CliniMACS based on CD4⁺CD25⁺ expression, not on the absence of CD127 expression, that were cultured with anti-CD3/CD28 mAb coated Dynabeads with IL2 and rapamycin (Godfrey et al., 2005; Hippen et al., 2008) showed approximately 100-fold (Godfrey et al., 2005) to 199-fold (Hippen et al., 2008) expansion. Foxp3 expression was 72.6% in one report (Hippen et al., 2008) and they exhibited potent suppressor activity of N95% (Tran et al., 2009) and 58 $\pm$ 11% (Hippen et al., 2008) respectively in allogeneic mixed lymphocyte reaction. In the freshly isolated CD4⁺CD25^{++bright} population that we isolated on Robosep, the expression of CD127 was approximately 1%, and the final expanded population was negative for CD127. Thus CD127 negativity may be more useful for characterizing functional expanded nTregs and less so for initial selection of the population to be expanded. Another reason against using the absence of CD127 expression for selecting the initial population is that CD127 negativity as a biomarker cannot discriminate between Tregs and T effector cells (Battaglia et al., 2006). Upon cellular activation CD127 is downregulated in CD4⁺ cells including CD4⁺CD25^{++bright} nTregs. Thus when CD127^{low/-} expression is used in combination with CD25^{++bright} expression for isolating Tregs, it can concentrate a heterogeneous subpopulation of cells consisting of nTregs, iTregs and activated CD4⁺CD25⁺⁺ non Tregs which can be transiently positive for Foxp3. The CD4⁺CD25⁺⁺CD127^{low/-} population may have a greater potential for differentiating into cytokine secreting effector cells. A previous study that has used FACS sorting for isolating CD4⁺Tregs based on CD25 expression and CD127 negativity and subsequent expansion resulted in contamination with effector cells based on their cytokine profile (Putnam et al., 2009). In that report, the expanded cells, despite showing Foxp3 expression of N95%, manifested substantial cytokine producing cells. None of the previous culture systems described in the literature (Hoffmann et al., 2004; Kleinewietfeld et al., 2009; Putnam et al., 2009; Tran et al., 2009) that have had different results of cytokine secreting effector cell-contamination have examined the multiple pro-inflammatory cytokine secreting potential of expanded Tregs on a single cell basis by multi color flow cytometry with proper compensation (Lamoreaux et al., 2006).

The rigorous examination of cytokine expression of the expanded nTreg population described herein has ruled out the presence of contaminating effector cells to a major extent. This is an important criterion because the mechanism of natural Treg function is by cell-to-cell interaction and not via secretion of cytokines IL-10 and TGF β which are rarely found in

the supernatants of in vitro nTreg assays and that the use of anti-IL-10 or anti-TGF- β antibodies fails to abrogate suppression (Sakaguchi, 2004). Another distinct subset of regulatory T cells (Tr-1) suppresses immune responses via cell-to-cell interactions and/or the production of IL-10 and TGF- β (Roncarolo et al., 2001; Levings et al., 2002) for a variety of antigens (Levings et al., 2002). IL-10 is also secreted by other cells like Th-2 cells (Fiorentino et al., 1989; Del Prete et al., 1993), macrophages (Ding et al., 1993), monocytes (Koppelman et al., 1997) and dendritic cells (Banchereau and Steinman, 1998). Thus it is important to demonstrate the suppressor function of nTregs without evidence of cytokine secretion.

The use of rapamycin throughout the culture was critical for ex vivo selective expansion of stable nTregs. CD28 costimulation, IL-2 and rapamycin were required to consistently expand nTregs that had suppressor activity in vitro, in the absence of contaminating cytokine secreting effector cells. Cells expanded in rapamycin have been shown to prevent xenogeneic GVHD (Golovina et al., 2008). Rapamycin selectively blocks the expansion of CD4⁺CD25^{negative} T effector cells, whereas it allows CD4⁺CD25^{++bright} Treg growth (Battaglia et al., 2006). Inhibition of the mTOR pathway in the presence of IL-2 allows Tregs to be constantly activated through the STAT-5 pathways and promotes their preferential expansion and Foxp3 expression (Zeiser et al., 2008). Limited use of rapamycin in the expansion phase is not effective in curtailing the expansion of T effector cells. Thus, in studies where rapamycin was only added early in the culture for 5 days (Tran et al., 2009) or 7 days (Putnam et al., 2009), FACS purified CD4⁺CD127^{low}CD25^{high} cells expanded up to 1500-fold after 14 days in one study (Putnam et al., 2009) and MACS bead sorted CD4⁺CD127^{low}CD25^{high} cells expanded up to 800-fold after 21 days in the other study (Tran et al., 2009). Surprisingly, CD4⁺CD25^{high}CD49⁻CD127⁻ cells used in another study showed very low ex vivo expansion of only 12-fold even in the absence of rapamycin after 33 days of culture (Kleinewietfeld et al., 2009). In all these instances, there was evidence of contamination by cytokine secreting effector cells (Kleinewietfeld et al., 2009; Putnam et al., 2009; Tran et al., 2009). These studies also demonstrate less impressive results of suppression than was observed with cells derived in our expansion protocol. As examples, Foxp3 was 76.4% and exhibited a suppression of 60% of allogeneic CD8⁺ proliferation (Putnam et al., 2009), or Foxp3 was 95.5% and still exhibited only 47% suppression of allogeneic CD4⁺CD25^{negative} cells (Tran et al., 2009), or Foxp3 was 81.4% in bead purified expanded cells (Kleinewietfeld et al., 2009) and showed 70% to 80% suppressive activity on allogeneic CD4⁺effector cells. In our study expanded nTregs were 98.3% Foxp3⁺ and had a suppressor activity of 91.6%. The ability of nTregs to suppress proliferation of responder T cells is mediated through a cell-cell contact-dependent mechanism and they do not secrete either IL-10 or TGF β (Sakaguchi, 2004), whereas stimulation of other regulatory cells results in the secretion of IL10 and TGF β . In our method, analysis of culture supernatant of expanded nTregs did not show detectable levels of TGF β and had extremely low levels of IL10.

In conclusion, our data demonstrate that using an appropriate concentration of nine monoclonal antibodies to isolate CD4⁺cells from buffy coat followed by isolation of CD25^{++bright} cells from CD4⁺cells on automatic Robosep instrument in a custom protocol we can isolate a population of highly purified nTregs from human peripheral blood. These

cells have been successfully expanded ex vivo by 60-fold resulting in an ideal population of human nTregs in sufficient quantities for possible cell therapy. Properties of expanded cells are close to optimal for nTregs, characterized by phenotypically stable expression of CD4⁺CD25^{++bright}Foxp3⁺ population, potent suppression of CD4⁺CD25^{negative} T cells without secretion of IL-10 or TGF- β and no propensity to convert into effector TH17 cells or production of pro-inflammatory cytokine upon in vitro stimulation with PMA/Ionomycin. Based on Boolean gating analysis of all the cytokine combination and frequency analysis, 96% of the cells did not express any cytokine. We propose that these cells are currently the most suitable for evaluating in clinical trials of Tregs. The procedure has the potential for further optimization by incorporating the recently described cell-based artificial antigen presenting cells (aAPCs) preloaded with anti-CD3/CD28 mAbs to achieve a higher level of Treg expansion (Godfrey et al., 2005). Modification of aAPCs to co-express OX40L or 4-1BBL was shown to achieve more than 1250-fold expansion (Godfrey et al., 2005; Hippen et al., 2008) of umbilical cord blood Tregs. These approaches and others can be applied to enhance the basic protocol once the efficacy and safety of the expanded nTregs described herein are demonstrated in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

nTregs	natural T regulatory cells
iTreg	induced Tregs
FBS	fetal bovine serum
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
aAPCs	artificial antigen presenting cells
CFSE	carboxy-fluorescein succinimidyl ester

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Fig. 1.

Purity of freshly isolated CD4⁺CD25⁺⁺bright nTregs showing minimal contamination with other cell populations. CD4⁺CD25⁺⁺bright cells isolated by the two step technique of negative selection of CD4⁺T cells followed by positive selection of CD4⁺CD25⁺⁺bright cells using the Robosep as per protocol were stained and analyzed by flow cytometry. Upper panel shows sequential analysis of frequencies of CD3, CD4, CD25 and Foxp3 expressing cells. Lower panel shows frequencies of individual CD19, CD14, CD56 and CD8 expressing cells on (A) open gate (B) cells gated on singlets (representative of one of the two experiments).

Fig. 2.

Gating strategy and phenotype analysis of ex vivo expanded CD4⁺CD25⁺⁺bright nTregs. CD4⁺CD25⁺⁺bright cells were expanded ex vivo as per protocol and were harvested on day 19. (A) Expanded cells were stained and analyzed on open gate for viability using ViViD dye and for CD3, CD4, CD25 Foxp3, CD27, CD45RO and CD127. The CD3⁺CD4⁺ cells were gated for CD25 and Foxp3. FoxP3⁺ cells were analyzed for expression of CD127 and CD45RO⁺CD27⁺ phenotype demonstrating that they were CD127 negative memory cells.

(B) Cells gated on singlets and analyzed in a similar manner. The figure is representative of 4 experiments.

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figure is representative of 3 experiments showing potent suppression by the CD4⁺CD25^{++bright} cells at 1:1 ratio. (E) Summary data from 3 donors showing the effect of adding CD4⁺CD25^{++bright} and CD4⁺CD25^{dim} cells to autologous CD4⁺CD25^{negative} cells stimulated with anti-CD3/anti-CD28 coated micro beads. Box plots represent mean and 95th percentiles of proliferation response (%CFSE low cells). Asterisks indicate statistical significance (**p<0.01, ***p<0.001).

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Fig. 4.

Ex vivo expanded nTregs exhibit minimal cytokine expression following in vitro stimulation. Robosep isolated CD4⁺CD25⁺⁺bright, CD4⁺CD25^{dim} and CD4⁺CD25^{negative} cells were cultured as per the expansion protocol in X-Vivo complete medium containing CD3/CD28 expander beads and rhIL2 (300 IU ml⁻¹) and rapamycin (100 ng ml⁻¹). Each cell population was harvested on day 19, stimulated with PMA/Ionomycin for 5 h and stained for cytokines IL17, IL2, IFN γ , TNF α and for CD107a. Each cell population was analyzed by FLOWJO using (A) an open gate (B) cells gated on singlets and analyzed in a similar manner. Plots show frequencies of cells positive for each cytokine and for CD107a in CD4⁺CD25⁺⁺bright, CD4⁺CD25^{dim} and CD4⁺CD25^{negative} cells, demonstrating the very low frequencies for all measures in CD4⁺CD25⁺⁺bright cells compared to the other two populations. (C) Summary data from 4 donors showing frequencies of IL17, IFN- γ , IL2, TNF α and CD107a expressing cells in CD4⁺CD25⁺⁺bright cells (blue) in comparison with CD4⁺CD25^{dim} (red) and CD4⁺CD25^{negative} (green) cell populations. Box plots represent mean and 95th percentiles. Asterisks indicate statistical significance p < 0.05 (ANOVA, Tukey post test).

Table 1:

Expanded nTregs: Boolean gating analysis of cytokine expressing cells.

No. of cytokines positive	Cytokine expressing cells	No. of cells	Frequency
5	CD107a + IFN γ + IL2 + IL17+ TNF α +	2	0.00061
4	CD107a-IFN γ + IL2 + IL17+TNF α +	33	0.01
4	CD107a + IFN γ + IL2 + IL17-TNF α +	14	0.0043
4	CD107a + IFN γ -IL2 + IL17+ TNF α +	2	0.0006
4	CD107a + IFN γ + IL2 + IL17+ TNF α -	0	0
4	CD107a + IFN γ + IL2-IL17 + TNF α +	0	0
3	CD107a-IFN γ + IL2 + IL17-TNF α +	516	0.16 [*]
3	CD107a-IFN γ -IL2 + IL17+TNF α +	127	0.039
3	CD107a-IFN γ + IL2 + IL17+TNF α -	45	0.014
3	CD107a + IFN γ -IL2 + IL17-TNF α +	23	0.007
3	CD107a + IFN γ + IL2 + IL17-TNF α -	12	0.0036
3	CD107a-IFN γ + IL2-IL17+TNF α +	10	0.003
3	CD107a + IFN γ + IL2-IL17-TNF α +	8	0.0024
3	CD107a + IFN γ + IL2-IL17 + TNF α -	2	0.0006
3	CD107a + IFN γ -IL2 + IL17+ TNF α -	0	0
3	CD107a + IFN γ -IL2-IL17+TNF α +	0	0
2	CD107a-IFN γ + IL2 + IL17-TNF α -	764	0.24 [*]
2	CD107a-IFN γ -IL2 + IL17-TNF α +	664	0.51 [*]
2	CD107a-IFN γ + IL2-IL17-TNF α +	197	0.061
2	CD107a-IFN γ -IL2 + IL17 + TNF α -	114	0.035
2	CD107a-IFN γ -IL2-IL17 + TNF α +	80	0.025
2	CD107a-IFN γ + IL2-IL17 + TNF α -	55	0.017
2	CD107a + IFN γ + IL2-IL17-TNF α -	50	0.015
2	CD107a + IFN γ -IL2-IL17-TNF α +	39	0.012
2	CD107a + IFN γ -IL2-IL17 + TNF α -	16	0.0049
2	CD107a + IFN γ -IL2 + IL17-TNF α -	8	0.0024
1	CD107a-IFN γ -IL2-IL17-TNF α +	3900	1.2 [*]
1	CD107a-IFN γ -IL2-IL17 + TNF α -	2067	0.64 [*]
1	CD107a-IFN γ -IL2 + IL17-TNF α -	2063	0.63 [*]
1	CD107a + IFN γ -IL2-IL17-TNF α -	1333	0.41 [*]
1	CD107a-IFN γ + IL2-IL17-TNF α -	352	0.11 [*]
0	CD107a-IFN γ -IL2-IL17-TNF α -	311527	95.8

nTregs, expanded as per protocol contain none/minimal single or multiple cytokine or CD107a expressing cells: Boolean gating analysis of a representative experiment of expanded nTregs stimulated with PMA/ Ionomycin for 5 h and stained for intracellular cytokines IL17, IL2, IFN γ , TNF α , and CD107a and analyzed on FLOWJO. Frequencies of cells positive for 1, 2, 3, 4 and 5 measures are depicted.

* Frequencies>0.1%.