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T-regulatory cell phenotypes in peripheral blood and bronchoalveolar lavage
from non-asthmatic and asthmatic subjects

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Key Words: asthma, T regulatory cell, Foxp3, peripheral blood, BAL, flow cytometry, suppression assay

Abstract

Background An unresolved issue in T regulatory (Treg) cell biology is the lack of consensus on phenotypic markers that accurately define the natural Treg (nTreg) population.

Objectives To examine nTreg frequency and functional capacity in healthy controls, and their frequency in asthmatic subjects using three different phenotypic strategies. We hypothesized that phenotypically different nTreg are quantitatively and functionally different.

Methods 34 healthy, non-asthmatic and 17 asthmatic subjects were studied. Three nTreg phenotypes were defined: nTreg1 (CD4⁺CD25⁺Foxp3⁺), nTreg2 (CD4⁺CD25⁺CD127^{low}Foxp3⁺), and nTreg3 (CD4⁺CD25^{high}Foxp3⁺). The flow cytometric determination of nTreg frequency in peripheral blood (PB) and bronchoalveolar lavage (BAL) was performed using fluorescently labelled antibodies. PB nTreg functional capacity was assessed using a CFSE-based suppression assay.

Results There was a significantly lower frequency of peripheral blood nTreg3 compared to nTreg2 and nTreg1 ($p < 0.05$). Both nTreg2 and nTreg3 had a significantly greater suppressive capacity than nTreg1 at T responder (Tresp) to nTreg ratios of 16:1 up to 1:1 ($p < 0.01$). Asthmatics exhibited a significantly lower peripheral blood nTreg3 and nTreg1 frequency than healthy controls ($p < 0.05$). There were no differences between healthy controls and asthmatic subjects when comparing BAL nTreg frequency.

Conclusions and Clinical Relevance Phenotypically different nTreg subsets are quantitatively and functionally different and are variably observed in asthma. The CD4⁺CD25^{high}Foxp3⁺ phenotype was the least frequent, but demonstrated the greatest suppression, and was significantly lower in peripheral blood of asthmatic subjects. Consequently, it is imperative that nTreg phenotypes be clearly defined and that the interpretation of their frequency and function be phenotype-specific.

Introduction

T regulatory cells (Treg), originally characterized as CD4⁺CD25⁺, are an independent, specialized subset of T cells that serve to regulate immune responses, thereby maintaining homeostasis and self-tolerance [1,2]. Since their initial identification, Foxp3, a forkhead family nuclear transcription factor, has been identified as a crucial molecule in the development and function of Treg [3,4]. Foxp3⁺ Treg can be divided into two distinct groups, natural Treg (nTreg) derived from the thymus, or induced Treg (iTreg) that develop in the periphery [5-7]. Each are defined phenotypically by the expression of particular cell surface and intracellular markers. Functionally, Treg exhibit potent suppressive capacity that is manifest through several different mechanisms [8,9].

Over the last decade, extensive work in the field of Treg biology has resulted in the description of a number of molecules used to characterize Treg subsets (i.e. Neuropilin-1, CTLA-4, GITR) [10], as well as those used to distinguish nTreg from induced Treg (iTreg)(i.e. Helios)[11]. However, an important unresolved issue in Treg cell biology is the lack of consensus on phenotypic markers that uniquely define the Treg population.

With the invention of new abbreviations and terminology, the Treg nomenclature has become complex and in need of simplification [12]. The Treg population is also becoming increasingly diverse, and is now composed of several phenotypically and functionally distinct subsets [13], resulting in a confusing interpretation of the role of these cells in disease.

Treg have been implicated in immuno-regulatory roles in allergic diseases such as asthma [14,15], where it has been shown that a deficiency in the both the quantity and function of Treg cells play a role in the pathogenesis and propagation of the disease [16]. However, such studies have not adequately addressed Treg heterogeneity, which could impact study conclusions. The aim of the current study was to compare three different nTreg phenotypes with respect to frequency and function. We chose our nTreg phenotypes based on the expression of CD4, CD25, CD127 and Foxp3 as these markers were the most comprehensive and would serve to detect the vast majority of the human nTreg population [17]. We hypothesized that nTreg subsets would not only be expressed at different cellular frequencies, but would also exhibit functional heterogeneity. To examine this, we first used multi-parameter flow cytometry to assess the frequency of the three different nTreg phenotypes in the peripheral blood of healthy, non-asthmatic subjects. Subsequently, we isolated each nTreg phenotype from the peripheral blood of these subjects and measured the functional capacity using an *in vitro* suppression assay. Finally, we measured the nTreg phenotypes in a population of asthmatic subjects. We hypothesized that any difference in nTreg frequency observed between healthy and asthmatic subjects would be a function of nTreg phenotype.

Materials and Methods

Subjects

Thirty four healthy, non-asthmatic and 17 asthmatic subjects between the ages of 18 and 65 years of age were studied (Table 1). Healthy control subjects had no medical history of respiratory symptoms or asthma diagnosis, a negative skin prick test to common aero-allergens, and a $PC_{20} > 32\text{mg/ml}$. All asthmatic subjects had both a history of asthma and a methacholine $PC_{20} \leq 16\text{mg/ml}$. All subjects had no other chronic illness and no lower respiratory tract infection at the time of their study visits. Subjects refrained from using β_2 -agonists or caffeinated beverages for a minimum of eight hours before laboratory visits. Exclusion criteria included those subjects that were pregnant, current smokers, or ex-smokers with a more than 10-pack-year history. This study was approved by the Hamilton Integrated Research Ethics Board and written informed consent was obtained from all study participants.

Study Design

This study was comprised of three phases: Phase 1, we assessed PB nTreg frequency in 14 healthy control subjects; Phase 2, we determined PB nTreg functional capacity using eight healthy control subjects; and Phase 3, we compared PB and BAL nTreg frequency in 12 healthy control subjects versus 17 asthmatic subjects. Healthy control subjects were selected to avoid the confounding issue of chronic disease on the initial quantification of the different phenotypes. For phases 1 and 2, a single laboratory visit was required for a medical history, physical examination, skin prick test, methacholine inhalation challenge, performed as described by Cockcroft *et al.* [18], and blood

collection. For phase 3, subjects were required to attend the laboratory on two non-consecutive days separated by at least one week. On day one, a medical history, physical examination, and methacholine inhalation challenge was performed along with blood collection. On day two, a bronchoscopy was performed with the collection of BAL fluid.

Blood collection and processing

Venous blood was drawn into Vacutainer® blood collection tubes (BD Biosciences, Mississauga, Ontario, CA) containing lithium heparin. For nTreg phenotyping, blood was diluted 1:1 with McCoy's 5A (Gibco, Carlsbad, California, USA) and layered over Lymphoprep density gradient medium (STEMCELL Technologies Inc., Vancouver, British Columbia, CA). Density gradient separation was performed via centrifugation of layered blood at 800g for 20 minutes at room temperature. Peripheral blood mononuclear cells (PBMC) were removed, washed twice with PBS (centrifugation at 250g for 10 minutes at 4°C), re-suspended in FACS buffer (PBS supplemented with 0.1% sodium azide and 0.5% BSA), and subjected to the nTreg phenotyping flow cytometric staining protocol.

For nTreg function, PBMC were separated as described above and used as responder T cell (Tresp) in the suppression assay. In addition, CD4⁺ T cells were indirectly isolated from whole blood by rosetting unwanted cells using a RosetteSep™ CD4⁺ T cell enrichment cocktail (STEMCELL Technologies Inc.) prior to RosetteSep™DM-L (STEMCELL Technologies Inc.) density gradient centrifugation. Isolated CD4⁺ T cells

were then subjected to the nTreg flow cytometric staining protocol for sorting of nTreg used in the suppression assay.

BAL fluid collection and processing

Fibreoptic bronchial bronchoscopies were performed using an Olympus flexible bronchoscope (Tokyo, Japan). The bronchoscope was inserted into the right middle lobe and 3 x 50ml aliquots of room temperature sterile, normal saline were instilled and then aspirated with suction. Pooled BAL fluid was kept on ice and processed within one hour. BAL fluid was then passed through a nylon mesh filter and centrifuged at 250g for 10 minutes at 4°C. The supernatant was removed and the cell pellet washed twice with PBS. BAL cells were re-suspended in FACS buffer and subjected to the nTreg cell phenotyping flow cytometric staining protocol.

Flow cytometric staining

nTreg phenotyping. Freshly processed PBMC or BAL cells in FACS buffer were divided into tubes at $0.5-1 \times 10^6$ cells per tube. Cells were stained first for surface markers using the following fluorescently labelled antibodies: CD3-AmCyan (Clone SK7; BD Biosciences), CD4-eFluor®450 (Clone OKT4; eBioscience, San Diego, California, USA), CD25-PE (Clone M-A251; BD Biosciences), CD127-FITC (Clone eBioRDR5; eBioscience), and relevant isotype controls. Cells were incubated with antibodies for 30 minutes at 4°C in the dark. Cells were washed with FACS buffer (centrifugation at 250g for 10 minutes at 4°C), re-suspended in one mL/tube of 1x fixation/permeabilization solution (eBioscience), and incubated for 30 minutes at 4°C in the dark. Two washes

with Permeabilization buffer (eBioscience) were performed after which cells were re-suspended in 100 μ L of 5% normal rat block and incubated for 15 minutes at 4°C in the dark. This was followed by intracellular staining using Foxp3-APC (Clone 236A/E7; eBioscience), with relevant isotype control, and incubated for 30 minutes at 4°C in the dark. Cells were washed twice with Permeabilization buffer, re-suspended in 400 μ L of Cytotfix (1% paraformaldehyde; BD Biosciences), and stored at 4°C in the dark until flow cytometric acquisition.

nTreg sorting. Enriched CD4⁺ T cells, at 20x10⁶/ml, were stained as per the method outlined in the human regulatory T cell sorting kit (BD Biosciences). Briefly, cells were stained for surface markers using the following fluorescently labelled antibody cocktail: CD4-PerCP-CyTM5.5 (Clone L200; BD Biosciences), CD25-PE (Clone 2A3; BD Biosciences), CD127-Alexa Fluor[®]647 (Clone 40131.111, BD Biosciences), and relevant isotype controls (Note: Foxp3 was omitted as intracellular staining would render the cells non-functional). The antibody cocktail was added at 2 μ L/10⁶cells and incubated for 30 minutes at room temperature in the dark. Cells were washed with PBS containing 1% human AB serum (Life Technologies Inc., Burlington, Ontario, Canada), re-suspended at 10x10⁶/mL in RPMI complete medium, and stored on ice in the dark until flow sorting.

Flow cytometric sorting

Three different PB nTreg phenotypes, not stained for Foxp3 (Treg1 – CD4⁺CD25⁺, Treg2 – CD4⁺CD25⁺CD127^{low}, and Treg3 – CD4⁺CD25^{high}), were sorted from stained CD4⁺ enriched cells using a FACS Vantage SE, three laser, eight colour capable digital

sorter (Becton Dickinson, Mississauga, ON, Canada) in the McMaster University Flow Cytometry Facility. Using a 70 micron nozzle, nTreg were sorted into human AB serum coated tubes containing RPMI complete medium to a purity routinely >95% with a minimum yield of 100,000 cells per phenotype. The sorted nTreg were then washed using complete RPMI medium and re-suspended to 1×10^6 cells/ml for use in the T cell suppression assay.

CFSE labelling

A 5mM stock solution of CellTrace™ CFSE (Life Technologies) was prepared using DMSO. Isolated PBMC were re-suspended to $5-10 \times 10^6$ cells/mL in sterile PBS and 1ml was added to a 14mL conical tube. 110μL of sterile PBS was placed near the top of the tube. To this droplet 1.1μL of 5mM CFSE stock was added, the tube was inverted and immediately vortexed for two seconds. Cells and CFSE were incubated for seven minutes at room temperature in the dark. CFSE labelled PBMC were then washed twice with cold RPMI complete medium and re-suspended to 1×10^6 cells/ml. Cells were kept on ice, protected from light, and used as Tresp in the suppression assay.

Suppression assay

The assay was performed as outlined previously [19,20]. Briefly, CFSE-labelled autologous PBMC were used as Tresp and cultured at 50,000 cells per well in 96 well, round bottom plates (Sigma Aldrich, Oakville, Ontario, Canada). Tresp were then co-cultured with FACS sorted nTreg1, nTreg2, or nTreg3 at Tresp to nTreg ratios of 32:1,

16:1, 8:1, 4:1, 2:1, and 1:1, and relevant controls. Cells were stimulated with plate-bound, purified, functional grade anti-CD3 (eBiosciences) at a concentration of 125ng/ml for five days in a humidified incubator (37°C and 5% CO₂). After five days, cells were harvested, stained for CD4 and then analyzed by flow cytometry. The suppressive capacity of Treg versus Tresp proliferation in co-culture was expressed using the conventional gating method (analyzing only proliferated cells)[21]. The calculation of per cent suppression was as follows: % Suppression = 100 – (Tresp:Treg/Tresp)*100.

Flow cytometry

nTreg phenotyping. Data was acquired using a 15-colour, three laser, LSRII flow cytometer equipped with FACSDiva acquisition software (BD Biosciences). At least 10,000 CD4⁺ events were collected from PBMC whereas as many CD4⁺ events as possible were collected from BAL. Collected data was analyzed using FlowJo analysis software (FlowJo LLC, Ashland, Oregon, USA). Our nTreg gating strategy, as applied to both blood and BAL, is outlined in Fig 1A and Fig 1B. Briefly, a CD3 versus CD4 plot was generated from the lymphocyte population and a gate placed around the CD3⁺/CD4⁺ double-positive population. For the determination of nTreg1 a plot of CD4 vs CD25 was used and a gate placed around cells expressing CD25 above that of the isotype control. These cells were used to plot CD25 vs CD127 with a gate then placed around those cells expressing CD25 and low expression of CD127. These cells defined the nTreg2 phenotype. nTreg3 were identified using CD25⁺ cells on a CD4 versus CD25 plot. A gate was placed on those cells expressing the highest levels of CD25.

Foxp3 expression was determined by plotting cells from each respective nTreg phenotype on a plot of CD4 versus Foxp3. Cells above that of the isotype control were deemed positive for Foxp3 (Fig 1B).

Treg sorting. The three nTreg phenotypes were sorted from enriched CD4⁺ cells as per the above gating strategies with the exception that cells were not stained for CD3.

Suppression assay. As many CD4⁺ events as possible were collected. Briefly, a dot plot of CD4 vs SSC was generated and a gate placed on the CD4⁺ population. These single positive, CD4⁺ cells were used to construct a histogram of CFSE expression.

Proliferated cells were deemed to be those cells to the left of the unstimulated cells and to the right of those cells not stained with CFSE (Fig 3a).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, California, USA). Data normally distributed were expressed as mean $\pm$ SEM. Between phenotype comparisons were performed using unpaired t tests. Repeated measures analysis of variance (ANOVA) was used to compare the between phenotype suppressive capacity.

Results

We assessed the frequency and function of three different nTreg phenotypes using surface markers to CD3, CD4, CD25, CD127 and an intracellular marker to Foxp3. We defined our nTreg as follows: nTreg1 – CD4⁺CD25⁺Foxp3⁺, nTreg2 – CD4⁺CD25⁺CD127^{low}Foxp3⁺, and nTreg3 – CD4⁺CD25^{high}Foxp3⁺ (Fig 1A, 1B).

Healthy control subjects had normal lung function, a normal methacholine PC₂₀, defined as >32 mg/ml (Table I) and were non-atopic. The healthy control and asthmatic subjects were matched for age, but were significantly different with respect to FEV₁ (% predicted) and methacholine PC₂₀ (Table 1).

The frequency of peripheral blood nTreg3 in healthy control subjects, as a percentage of CD4⁺ cells ($1.8 \pm 0.10\%$), was significantly lower when compared to nTreg1 ($3.49 \pm 0.28\%$) or nTreg2 ($3.14 \pm 0.22\%$, $p < 0.05$; Fig 2). This difference was also seen when the frequency of peripheral blood nTreg3, without Foxp3 expression ($2.20 \pm 0.14\%$), was compared to nTreg1 ($18.51 \pm 1.34\%$) or nTreg2 ($4.20 \pm 0.24\%$, $p < 0.05$). When viewed as the percentage of cells expressing Foxp3, nTreg1 ($21.12 \pm 3.24\%$) was significantly lower compared to nTreg2 ($74.62 \pm 2.46\%$) or nTreg3 ($82.40 \pm 1.56\%$) and nTreg2 was significantly lower than nTreg3 ($p < 0.05$). The nTreg populations are phenotypically different but not distinct from one another – nTreg1 subset encompasses both nTreg2 and nTreg3 subsets while nTreg2 also encompasses nTreg3.

All three nTreg phenotypes functionally suppressed Tresp cells in a concentration dependent manner (Fig 3A and Fig 3B). However, for nTreg3 and nTreg2, there were no significant differences in functional capacity, but both demonstrated significantly greater suppression than nTreg1 for all Tresp to nTreg ratios except for 32:1 ($p < 0.05$, Fig 3B).

In the asthmatic subjects, as in healthy control subjects, the frequency of peripheral blood nTreg3 ($0.45 \pm 0.11\%$) was significantly lower when compared to nTreg1 ($2.71 \pm 0.34\%$) or nTreg2 ($2.21 \pm 0.38\%$, $p < 0.05$; Table 2). In addition, asthmatics exhibited a significantly lower peripheral blood nTreg1 and nTreg3 frequency than healthy control subjects ($p < 0.05$, Fig 4A).

Similar to peripheral blood, the frequency of the nTreg phenotypes differed numerically in BAL, but was only significantly different in the asthmatic subjects (nTreg3 was significantly lower when compared to nTreg1 or nTreg2), being $3.98 \pm 1.57\%$ for nTreg1, $2.77 \pm 0.89\%$ for nTreg2, and $1.48 \pm 0.45\%$ for nTreg3 ($p < 0.05$, Table 2). There were no significant differences between asthmatic and healthy control subjects when comparing BAL nTreg frequency (Fig 4B).

Discussion

Our data highlight the heterogeneity that exists in the nTreg population with respect to phenotype and function. We have demonstrated, in a population of healthy, non-asthmatic individuals, that three phenotypically different peripheral blood nTreg subsets are expressed at different frequencies and possess different functional capacities. In addition, we have shown that there are differences in peripheral blood nTreg frequency, when comparing asthmatic to non-asthmatic subjects, is dependent on the phenotype studied.

The characterization of Treg has substantially evolved since Sakaguchi *et al.* proposed the CD4⁺CD25⁺ phenotype two decades ago [1]. As evidence of this, we chose to study the CD4⁺CD25⁺Foxp3⁺ (nTreg1), CD4⁺CD25⁺CD127^{low}Foxp3⁺ (nTreg2), and CD4⁺CD25^{high} Foxp3⁺ (nTreg3) Treg subsets. We have shown that the frequency of peripheral blood nTreg3 is significantly lower than that of nTreg1 and nTreg2. nTreg1 has been shown to be the most inclusive phenotype, essentially encompassing all of the Foxp3⁺ Treg cells [17]. However, this comes with the notable drawback that this phenotype also includes activated T cells that transiently express Foxp3 [22]. The co-expression of CD127, the alpha chain of the IL-7R, and high levels of expression of CD25 has since been shown to discriminate bona fide Treg from activated T cells [23]. The low expression of CD127 on Treg inversely correlated with the expression of Foxp3 [24]. Thus, the inclusion of CD127 in the CD4⁺CD25⁺CD127^{low}Foxp3⁺ (nTreg2) phenotype allows for the study of a more pure nTreg population. The nTreg3 phenotype includes those Treg expressing the highest levels of CD25; on a spectrum of CD25 expression these cells possess the highest suppressive capacity as compared to the CD4⁺CD25^{low} population [25]. By definition, nTreg are inherently suppressive, and nTreg3 therefore allowed us to examine the population of nTreg with the greatest functional capacity. In addition, we have shown that these three phenotypes are functionally different as measured using a CFSE-based *in vitro* suppression assay. Miyara *et al.* [26] elegantly showed that distinct subsets of Treg exist when CD4⁺Foxp3⁺ cells are stratified based on the level of Foxp3 expression. In support of our data, they too concluded that different Treg subsets are functionally heterogeneous. However, our nTreg subsets, while phenotypically different, are not distinct populations.

In addition, our results are in concert with those published by d'Hennezel *et al.* [27] who showed that three different Treg phenotypes (based on CD25^{high}, CD25^{low} or CD25^{neg} expression) expressed different levels of suppression using a tritiated thymidine based suppression assay. Even with the use of different functional assays, it is clear that Treg expressing high levels of CD25 possess the most robust suppressive capacity. Our data also highlight the relationship of Foxp3 and Treg suppressive capacity. We showed that the three different nTreg phenotypes differentially expressed Foxp3 and that the highest Foxp3 expression was associated with the higher suppressive capacity. These data support the essential role of Foxp3 for Treg suppressor function [28,29].

Others have quantified circulating and airway nTreg in an asthmatic population. An increase in circulating CD4⁺CD25⁺CD127^{low} nTreg frequency was observed in asthmatic children compared to healthy controls and was attributable to inhaled corticosteroid (ICS) use and not disease severity [30]. When the asthmatic group was stratified based on inhaled corticosteroid use, comparisons between controls and asthmatics not on ICS yielded no difference in Treg frequency. These data support our finding that there is no difference in nTreg2 frequency between asthmatics and controls. Provoost *et al.* [31] identified no difference in the frequency of peripheral blood CD4⁺CD25^{high} or CD4⁺CD25^{high} Foxp3⁺ Treg between asthmatic and control subjects. This is in contrast to our data which showed a significant decrease in the frequency of nTreg3 (CD4⁺CD25^{high} Foxp3⁺) in asthmatics compared to healthy controls. This discrepancy may be attributable to the method of calculating frequency. We expressed nTreg frequency as a percentage of CD4⁺ cells whereas Provoost *et al.* expressed nTreg as a

percentage of the lymphocyte gate. Smyth et al. [32] evaluated Treg in the airways of healthy, mild asthmatic, and moderate/severe asthmatic subjects and found that differences in BAL Treg frequency between healthy and moderate/severe asthmatic subjects utilizing the CD4⁺CD25⁺CD127⁻ phenotype were not present when using the CD4⁺CD25^{bright} phenotype. Thus, the conclusion that moderate/severe asthmatics have a higher frequency of BAL Treg is only true using the CD4⁺CD25⁺CD127⁻ phenotype. While the phenotypes were not identical, these data support our findings that there may be differences in nTreg frequency depending on the phenotype studied. Of note in the Smyth study was that no differences were observed in Treg frequency, regardless of phenotype, when comparisons were made between healthy and mild asthmatic subjects. Although the numbers of BAL samples from the asthmatic subjects were few, no differences in nTreg frequency were observed comparing asthmatic and healthy control subjects. This may also be because the asthmatic subjects had very mild asthma. Thus, while the conclusions reached about airway nTreg must be very limited, the frequency of nTreg may depend not only on the compartment from which the cells are obtained, but also the phenotype of the subjects studied.

There are several limitations to our study. In the sorting of nTreg for use in the suppression assay, we were unable to use Foxp3 in our phenotypic definition. Foxp3 is an intracellular marker and, as such, staining for it would render nTreg non-functional. Due to insufficient volumes of peripheral blood (limited allowable volume of blood drawn) as well as low cell yields in the BAL, we were unable to assess nTreg function in the comparison of asthmatic and healthy subjects. Also, with no reliable phenotypic or

functional markers that make it possible to distinguish between natural and induced Treg in the periphery [33], we may have enhanced nTreg frequency.

In summary, phenotypically different nTreg cells are quantitatively and functionally different and are variably expressed in states of health and disease. Our results highlight the need for nTreg phenotypes to be clearly defined in studies and for interpretations to be made using consistent and specific phenotypes. Finally, we demonstrate that the $CD4^+CD25^{\text{high}}Foxp3^+$ phenotype exhibited the greatest suppressive capacity and is significantly less frequent in the peripheral blood of mild asthmatic subjects.

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References

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 1995; **155**(3):1151–64.
2. Ng WF. Human CD4+CD25+ cells: a naturally occurring population of regulatory T cells. *Blood* 2001; **98**(9):2736–44.
3. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 2003; **4**(4):330–6.
4. Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, et al. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int. Immunol.* 2004; **16**(11):1643–56.
5. Dhamne C, Chung Y, Alousi AM, Cooper LNJ, Tran DQ. Peripheral and thymic foxp3(+) regulatory T cells in search of origin, distinction, and function. *Front. Immunol.* 2013; **4**:253.
6. Bilate AM, Lafaille JJ. Induced CD4+Foxp3+ regulatory T cells in immune tolerance. *Annu. Rev. Immunol.* 2012; **30**:733–58.
7. Chatenoud L. Natural and induced T CD4+CD25+FOXP3+ regulatory T cells. *Methods Mol. Biol.* 2011; **677**:3–13.
8. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity.* 2009; **30**(5):636–45.
9. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory T cells: how do they suppress immune responses? *Int. Immunol.* 2009; **21**(10):1105–11.
10. Schmetterer KG, Neunkirchner A, Pickl WF. Naturally occurring regulatory T cells: markers, mechanisms, and manipulation. *FASEB J.* 2012; **26**(6):2253–76.
11. Himmel ME, MacDonald KG, Garcia R V, Steiner TS, Levings MK. Helios+ and Helios- cells coexist within the natural FOXP3+ T regulatory cell subset in humans. *J. Immunol.* 2013; **190**(5):2001–8.
12. Abbas AK, Benoist C, Bluestone JA, Campbell DJ, Ghosh S, Hori S, et al. Regulatory T cells: recommendations to simplify the nomenclature. *Nat. Immunol.* 2013; **14**(4):307–8.

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13. Campbell DJ, Koch MA. Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nat. Rev. Immunol.* 2011; **11**(2):119–30.
 14. Larché M. Regulatory T cells in allergy and asthma. *Chest.* 2007; **132**(3):1007–14.
 15. Lloyd CM, Hawrylowicz CM. Regulatory T cells in asthma. *Immunity.* 2009; **31**(3):438–49.
 16. Hartl D, Koller B, Mehlhorn AT, Reinhardt D, Nicolai T, Schendel DJ, et al. Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J. Allergy Clin. Immunol.* 2007; **119**:1258–66.
 17. Fazekas de St Groth B, Zhu E, Asad S, Lee L. Flow cytometric detection of human regulatory T cells. *Methods Mol. Biol.* 2011; **707**:263–79.
 18. Cockcroft DW, Ruffin RE, Dolovich J, Hargreave FE. Allergen-induced increase in non-allergic bronchial reactivity. *Clin. Allergy.* 1977; **7**(6):503–13.
 19. Collison LW, Vignali DAA. In vitro Treg suppression assays. *Methods Mol. Biol.* 2011; **707**:21–37.
 20. Venken K, Thewissen M, Hellings N, Somers V, Hensen K, Rummens J-L, et al. 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 [Internet]* 2007 Apr 30 [cited 2014 Jun 30];**322**(1-2):1–11. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17368474>
 21. McMurchy AN, Levings MK. Suppression assays with human T regulatory cells: a technical guide. *Eur. J. Immunol.* 2012; **42**(1):27–34.
 22. Wang J, Ioan-Facsinay A, van der Voort EIH, Huizinga TWJ, Toes REM. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur. J. Immunol.* 2007; **37**(1):129–38.
 23. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J. Exp. Med.* 2006; **203**(7):1693–700.
 24. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J. Exp. Med.* 2006; **203**(7):1701–11.
 25. Baecher-Allan C, Wolf E, Hafler DA. Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4+ CD25+ T cells. *Clin. Immunol.* 2005; **115**(1):10–8.

- Accepted Article
26. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4⁺ T cells expressing the FoxP3 transcription factor. *Immunity*. 2009; **30**(6):899-911.
 27. d’Hennezel E, Yurchenko E, Sgouroudis E, Hay V, Piccirillo CA. Single-cell analysis of the human T regulatory population uncovers functional heterogeneity and instability within FOXP3⁺ cells. *J. Immunol.* 2011; **186**(12):6788–97.
 28. Wan YY, Flavell RA. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature*. 2007; **445**(7129):766–70.
 29. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat. Immunol.* 2007; **8**(3):277–84.
 30. Singh AM, Dahlberg P, Burmeister K, Evans MD, Gangnon R, Roberg KA, et al. Inhaled corticosteroid use is associated with increased circulating T regulatory cells in children with asthma. *Clin. Mol. Allergy*. 2013; **11**(1):1.
 31. Provoost S, Maes T, van Durme YM, Gevaert P, Bachert C, Schmidt-Weber CB, et al. Decreased FOXP3 protein expression in patients with asthma. *Allergy*. 2009; **64**(10):1539–46.
 32. Smyth LJC, Eustace A, Kolsum U, Blaikely J, Singh D. Increased airway T regulatory cells in asthmatic subjects. *Chest*. 2010; **138**(4):905–12.
 33. Chatenoud L. Natural and induced T CD4⁺CD25⁺FOXP3⁺ regulatory T cells. *Methods Mol. Biol.* 2011 Jan; **677**:3–13.

Table 1. Subject characteristics. Subjects were separated based on those used for assessment of PB nTreg frequency (n=14) or function (n=8) in healthy controls (Phases 1 and 2), and those used for comparison of PB and BAL nTreg frequency between asthmatics and healthy controls (Phase 3). Values expressed as mean $\pm$ SEM.

	Healthy Control Subjects	Asthmatic Subjects
Phases 1 and 2		
Subjects, n	22	NA
Male	10	NA
Female	12	NA
Age, years	34.27 $\pm$ 2.600	NA
FEV ₁ , % predicted	96.12 $\pm$ 2.060	NA
PC ₂₀ , mg/ml	>32	NA
Phase 3		
Subjects, n	12	17
Male	5	7
Female	7	10
Age, years	37.83 $\pm$ 3.988	38.59 $\pm$ 3.657
FEV ₁ , % predicted	100.8 $\pm$ 2.831	82.82 $\pm$ 4.437*
PC ₂₀ , mg/ml	>32	3.808 $\pm$ 1.586*

* - p<0.05 compared to healthy control subjects

NA – not applicable

Table 2. Within group comparisons of peripheral blood or BAL nTreg frequency. Values expressed as mean $\pm$ SEM.

	nTreg1	nTreg2	nTreg3
Healthy Control Subjects			
Peripheral blood frequency (%CD4)	4.43 $\pm$ 0.52	2.45 $\pm$ 0.36 [#]	1.12 $\pm$ 0.14 [*]
BAL frequency (%CD4)	5.12 $\pm$ 0.78 ^{&}	3.29 $\pm$ 0.66	2.63 $\pm$ 0.53
Asthmatic Subjects			
Peripheral blood frequency (%CD4)	2.71 $\pm$ 0.34	2.21 $\pm$ 0.38	0.45 $\pm$ 0.11 [*]
BAL frequency (%CD4)	3.98 $\pm$ 1.57	2.77 $\pm$ 0.89	1.48 $\pm$ 0.45 [*]

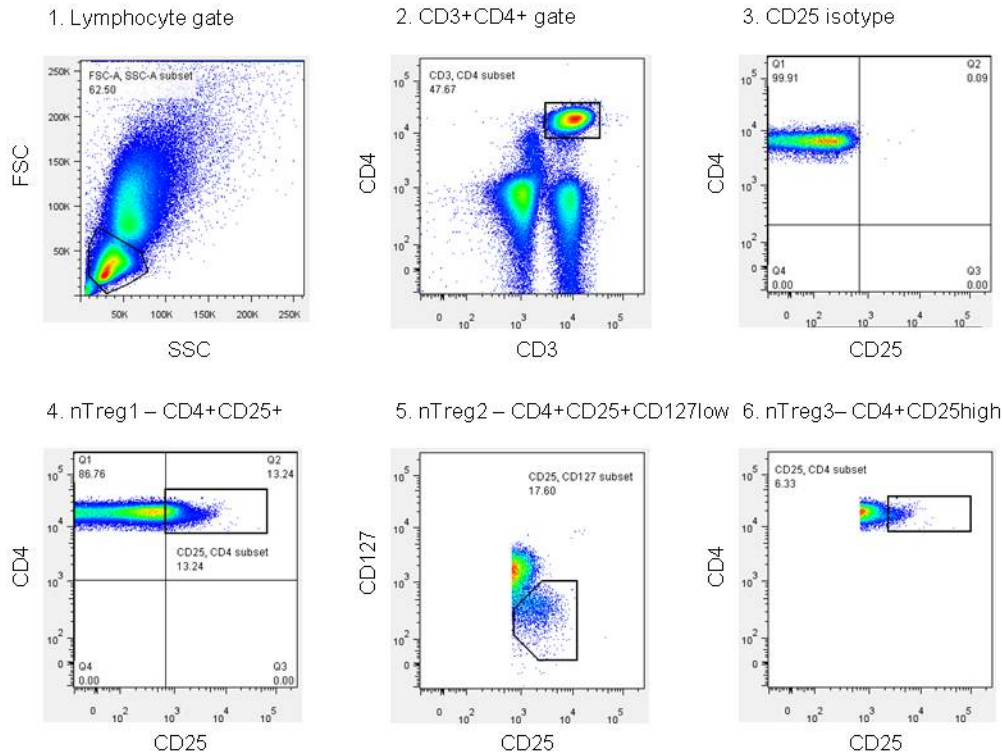
* - p<0.05 versus nTreg1 and nTreg2

- p<0.05 versus nTreg1

& - p<0.05 versus nTreg2 and nTreg3

Fig 1. Flow cytometric dot plots, using a representative peripheral blood sample, outlining the gating strategy for the three nTreg phenotypes. **A.** 1, Lymphocyte gate; 2, CD3⁺CD4⁺ gate; 3, CD4 vs CD25 isotype control; 4, nTreg1 gate; 5, nTreg2 gate; 6, nTreg3 gate. **B.** Determination of Foxp3 expression in each nTreg phenotype – CD4 vs Foxp3 isotype control (left panel) and CD4 vs Foxp3 (right panel).

A.



B.

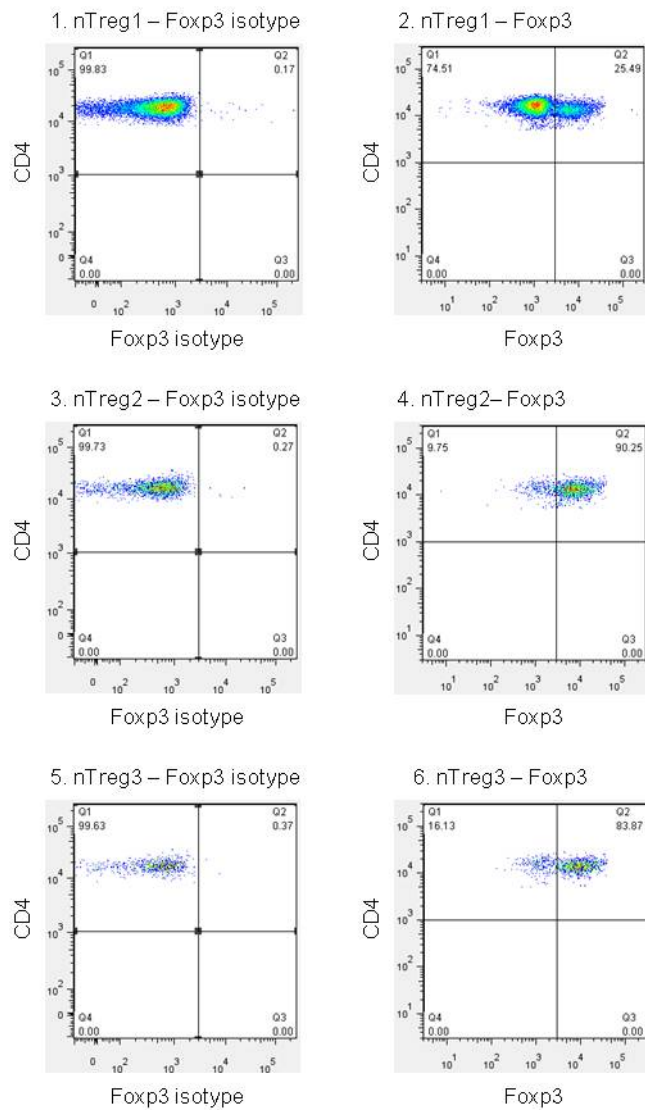


Fig 2. Differences in peripheral blood Treg frequency in healthy control subjects comparing three different nTreg phenotypes (n=14). Values expressed as individual data points with the bar denoting the mean percentage. * denotes $p < 0.05$.

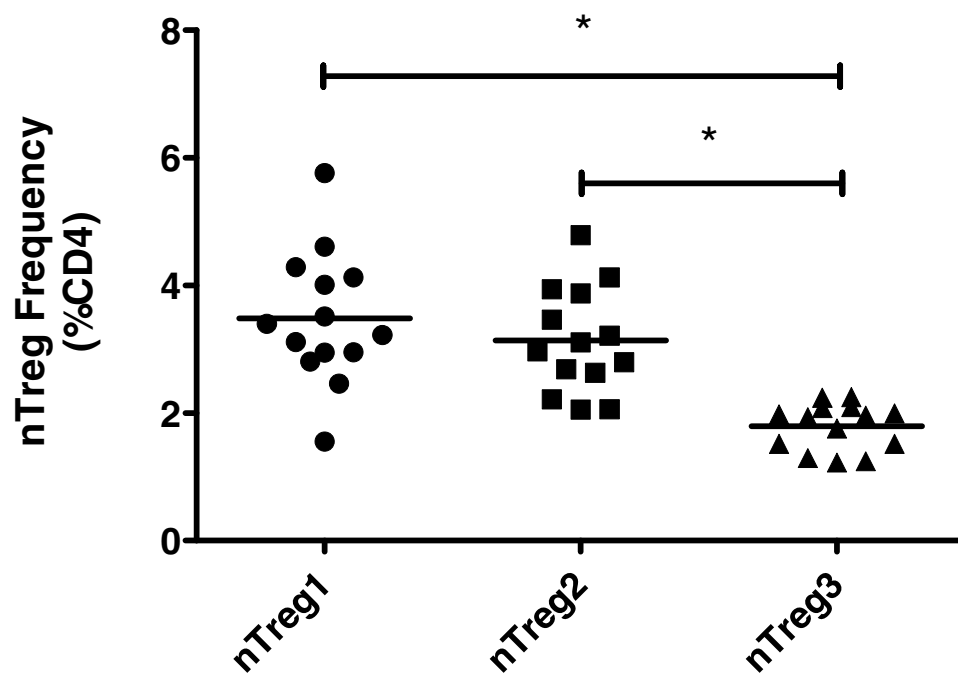


Fig 3. Assessment of peripheral blood Treg function using a CFSE-based suppression assay. A. Representative flow cytometric CFSE histograms – left panel, proliferation profile of Tresp alone; right panel, proliferation profile of Tresp co-cultured with nTreg at a ratio of 1:1. B. Comparison of suppressive capacity of three nTreg phenotypes at six Tresp:nTreg ratios (n=8). Results are expressed as mean + SEM. * denotes $p < 0.05$.

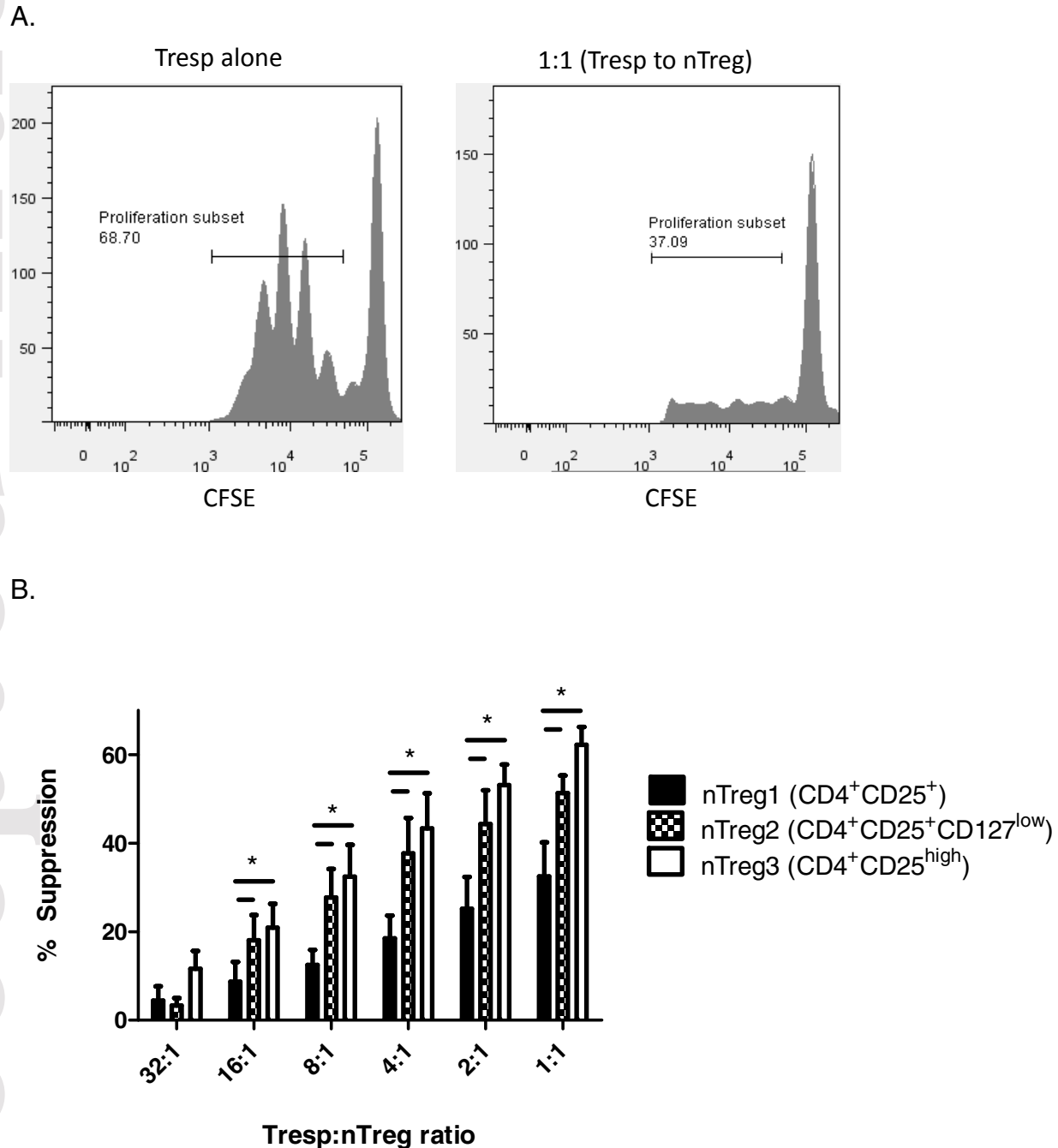
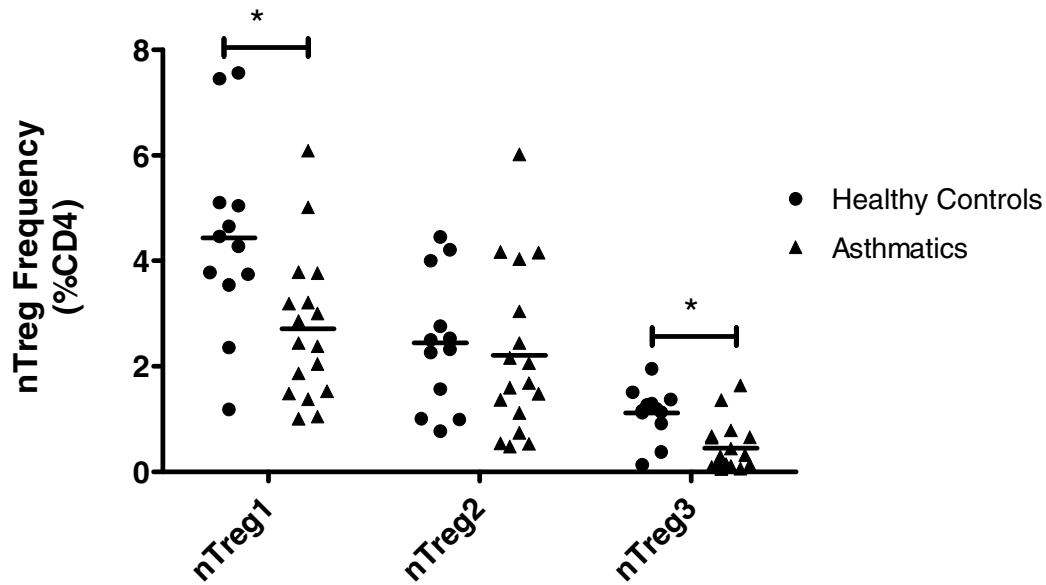


Fig 4. Comparison of nTreg frequency between asthmatic and healthy control subjects. A. Differences in peripheral blood nTreg frequency from asthmatic (n=17) versus healthy control subjects (n=12). B. Differences in BAL nTreg frequency from asthmatic (n=5) versus healthy control subjects (n=11). Values expressed as individual data points with the bar denoting mean percentage. * denotes $p < 0.05$.

A.



B.

