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Treatment with anti-OX40L or anti-TSLP does not alter the frequency of T regulatory cells in allergic asthmatics

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Capsule Summary: *In vivo* treatment of allergic asthmatic subjects with monoclonal antibodies to OX40L or TSLP does not alter the frequency of circulating Foxp3⁺ regulatory T cells.

Key Words: allergic asthma, flow cytometry, OX40L, T regulatory cell, TSLP

Short Title: T regulatory cells in asthma

Abstract

OX40-OX40L interactions and TSLP are important in the induction and maintenance of Th2 responses in allergic disease whereas T regulatory cells (Treg) have been shown to suppress pro-inflammatory Th2 responses. Both OX40L and TSLP have been implicated in the negative regulation of Treg. The effect of anti-asthma therapies on Treg is not well known. Our aim ~~then~~ was to assess the effects of two monoclonal antibody therapies (anti-OX40L and anti-TSLP) on Treg frequency using a human model of allergic asthma. We hypothesized that the anti-inflammatory effects of these therapies would result in an increase in circulating Treg (CD4⁺CD25⁺CD127^{low}Foxp3⁺ cells) frequency. We measured Treg using flow cytometry and our results showed that neither allergen challenge nor monoclonal antibody therapy altered circulating Treg frequency. These data highlight the need for assessment of

airway Treg and for a more complete understanding of Treg biology so as to develop pharmacologics/biologics that modulate Treg for asthma therapy.

The majority of naturally occurring T regulatory cells (Treg) develop in the thymus and characteristically express the forkhead box 3 (Foxp3) transcription factor along with CD4, CD25 and CD127 cell surface molecules (1). Treg possess potent immuno-suppressive properties, making them potentially key regulators of inflammatory responses in many diseases, including asthma (2). Evidence suggests that the balance between effector T cells and Treg is important in the initiation, propagation and resolution of T- helper (Th) 2 inflammatory responses in asthma (3).

Existing asthma therapies focus predominantly on the inhibition of effector cells. Limited research exists examining the effects of these therapies on Treg and results indicate variable effects on effector T cells versus Treg depending on the therapy administered (4,5). Research into novel asthma therapies has recently focused on the development of biologics targeted at specific inflammatory molecules and cytokines (6). Two such targets, OX40 ligand (OX40L) and thymic stromal lymphopoietin (TSLP) have been shown to play a role in allergic responses. OX40/OX40L interactions lead to the induction and maintenance of Th2 inflammation by means of TSLP-activated dendritic cells (7-9). ~~Also,~~ The *in vivo* blockade of OX40L has been shown to inhibit atopic inflammation mediated by TSLP (10).

While the role of OX40L and TSLP in the allergic response has been well characterized, little information exists regarding their effects on Treg. It has been shown that the OX40/OX40L interaction leads to the expansion of effector T cells while inhibiting Treg function (11). Studies examining the effects of TSLP on Treg provided evidence that TSLP inhibited the generation of Treg and also directly impaired Treg function (12,13). This suggests that OX40L and TSLP are not only potent contributors to the allergic inflammatory response, but they may also exert significant negative effects on Treg number and function.

We have previously reported on the effects of anti-OX40L and anti-TSLP monoclonal antibody therapy on allergen-induced airway responses using an inhaled allergen challenge model of human allergic asthma (14,15). In these same studies, we evaluated the effect of anti-OX40L and anti-TSLP monoclonal antibody therapy on the frequency of circulating Treg. We hypothesized that blockade of OX40L or TSLP would result in an increase in PB Treg frequency.

The studies were carried out as two separate randomized, double-blind, placebo-controlled, parallel group clinical trials (ClinicalTrials.gov numbers NCT00983658 and NCT01405963). Studies were approved by the Hamilton Integrated Research Ethics Board and written informed consent was obtained from all study participants. The study design for the anti-OX40L clinical trial has been described by Gauvreau *et al* (14). Briefly, 28 subjects with mild, allergic asthma were recruited and received four doses of treatment (drug or placebo) over three months. Allergen challenges were performed at

baseline and 56 and 113 days after the first treatment dose. Peripheral blood (PB) was drawn at baseline and on days 55, 57, 112 and 114 for assessment of Treg. The study design for the anti-TSLP clinical trial has been described by Gauvreau *et al* (15). Briefly, 31 subjects were recruited and received three doses of treatment (drug or placebo) over two months. Allergen challenges were performed at baseline and 42 and 84 days after the first treatment dose. PB was drawn at baseline and on days 41, 43, 83, and 85 for assessment of Treg.

Venous blood was collected, diluted and layered over Lymphoprep density gradient medium (STEMCELL Technologies Inc., Vancouver, British Columbia, Canada). Peripheral blood mononuclear cells (PBMC) were isolated via density gradient centrifugation. Freshly processed PBMC underwent primary staining 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. Secondary staining for intracellular markers was done using Foxp3-APC (Clone 236A/E7; eBioscience) and its relevant isotype control. Data were acquired on a 15 colour LSRII flow cytometer (BD Biosciences, Mississauga, Ontario, Canada). Treg were identified as CD4⁺CD25⁺CD127^{low}Foxp3⁺ cells (gating strategy outlined in Fig. 1A). These surface markers were used as they are the most comprehensive and would detect the majority of human Treg. The addition of CD127^{low} to the standard CD4⁺CD25⁺ markers helps to discriminate Treg from activated T cells that can transiently express Foxp3. The

frequency of Treg was expressed as a percentage of the CD4⁺ population.

Comparisons were made between pre-treatment and post-treatment values. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, California, USA). Data were analyzed using a two-way repeated measures ANOVA. Data were expressed as mean + SEM.

The clinical outcomes of the two trials were different. Treatment with anti-OX40L MAb did not attenuate the early- or late-phase asthmatic responses at days 56 or 113 compared with placebo (14). By contrast, treatment with anti-TSLP MAb reduced the maximum percent fall in FEV₁ during the late response by 34% compared to the placebo group on day 42 (p=0.09) and by 45.9% on day 84 (p=0.02)(15). Treg frequency ranged between 5%-11% of PB CD4⁺ cells in these allergic asthmatic subjects. PB CD4⁺CD25⁺CD127^{low}Foxp3⁺ Treg frequency did not change after allergen challenge in either study and there was no effect of treatment with either anti-OX40L or anti-TSLP on Treg frequency (Fig. 1B, 1C).

The results of these two studies differed in that treatment with anti-OX40L had no effect on the late asthmatic response or on PB Treg frequency, whereas treatment with anti-TSLP AMG 157 attenuated allergen-induced early and late asthmatic responses, but also showed no effect on PB Treg frequency. Taken together, these studies show that neither an inflammatory stimulus, such as inhaled allergen, nor a pharmacological intervention, such as anti-TSLP, which demonstrated efficacy on allergen-induced responses, induces changes in circulating Treg frequency. This suggests that PB is not

an appropriate compartment to study changes in Treg frequency in asthma. As reported by Kinoshita *et al.* (16), measuring Treg in a more relevant compartment (the airway) may have yielded different results. It is likely that in airways disease, Treg in the relevant compartment (ie. the lung) may be more sensitive to change.

The control of the development, survival, and function of Treg is crucial for the effective regulation of immune responses. Treg have the ability to suppress the activation, proliferation and effector function of other lymphocytes in physiological and pathological immune responses. However, it remains uncertain what ultimately determines the efficacy of Treg, the absolute number of each cell subtype, their frequency compared with other cell subtypes, the location of the cells, their functional capacity or some combination thereof. Thus, a complete understanding of the biological functions of Treg *in vivo* should facilitate the development of pharmacologic and biologic agents that can be used to modulate Treg number/function in a therapeutic setting (17). Additionally, Treg as stand-alone or adjunct cell-based therapy may provide an alternative avenue in the treatment of airway diseases such as asthma (18).

Author contributions: The studies were designed by GM Gauvreau and PM O'Byrne. The experimental work was performed by AJ Baatjes, SG Smith, and B Dua. Analyses were performed by AJ Baatjes. The paper was written by AJ Baatjes and PM O'Byrne. All authors reviewed and approved the manuscript before submission.

Conflicts of interest: The authors declare they have no conflicts of interest.

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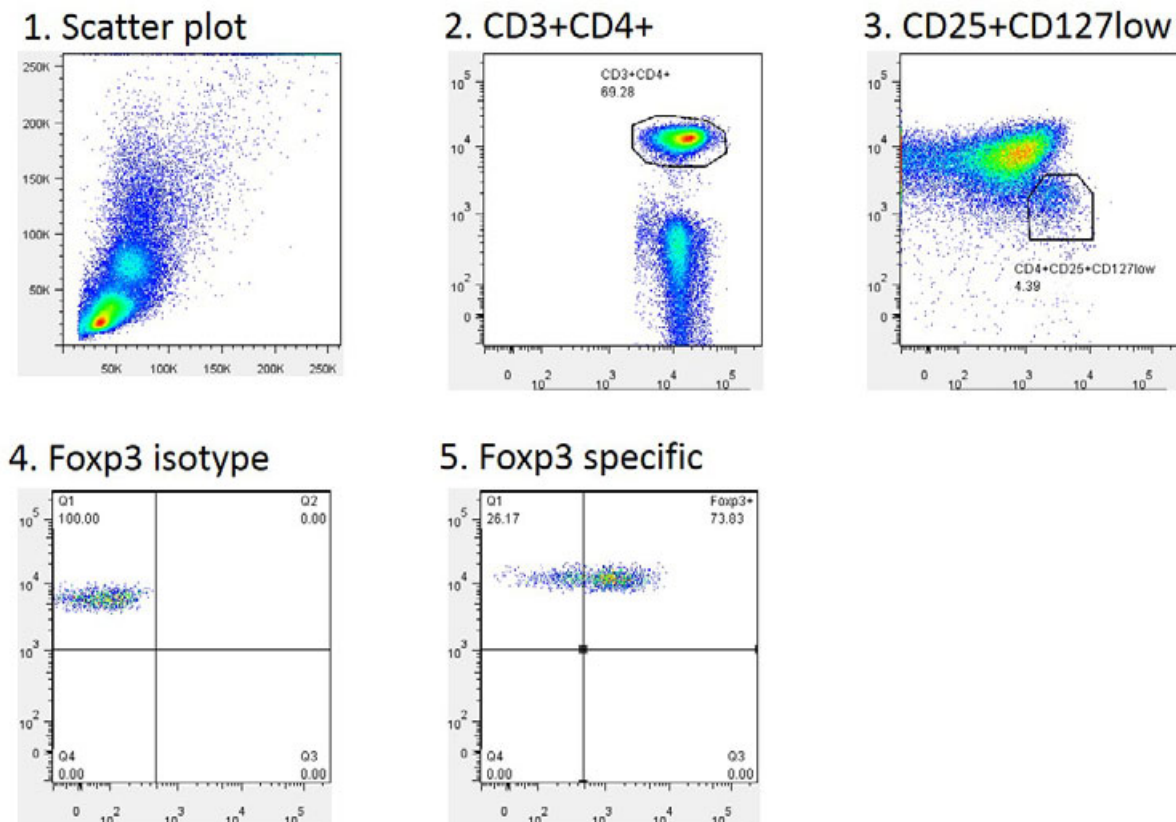
Table I – Subject characteristics summarized from two separate clinical trials (14,15).

Data expressed as mean (SD) or mean $\pm$ SEM. Methacholine PC₂₀ (the provocative concentration causing a 20% fall in FEV₁) expressed as the geometric mean with 95% CI.

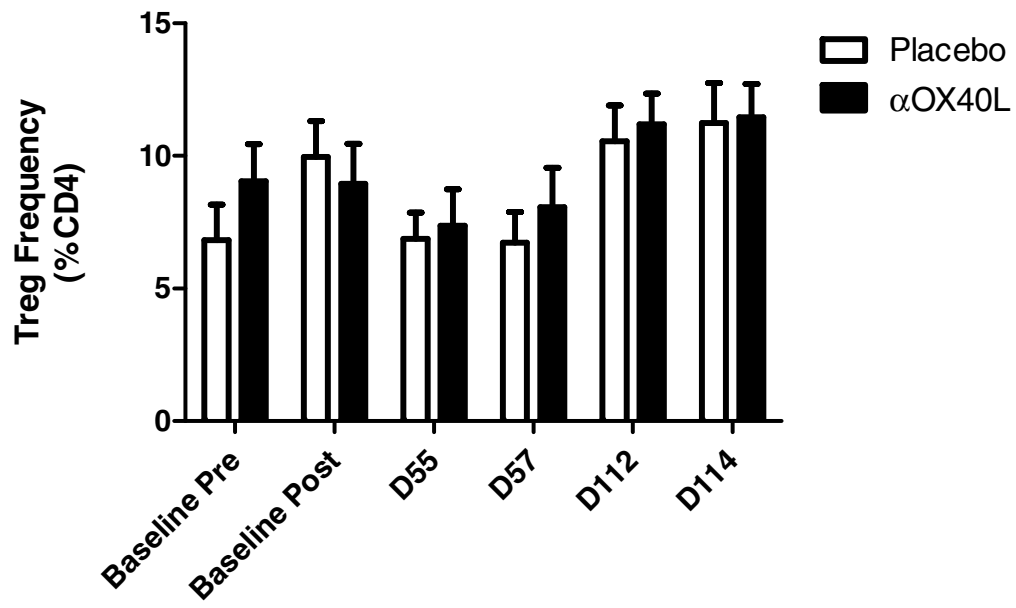
	Anti-OX40L MAb Clinical Trial		Anti-TSLP MAb Clinical Trial	
	Placebo (n=14)	Drug (n=14)	Placebo (n=15)	Drug (n=16)
Age, years	33.9 (12.0)	33.4 (13.3)	31.5 $\pm$ 2.9	30.8 $\pm$ 2.7
Sex (M/F)	7/7	8/6	4/11	6/10
FEV ₁ , % predicted	84.9 (14.7)	91.7 (11.4)	97.6 $\pm$ 3.9	95.4 $\pm$ 3.3
Methacholine PC ₂₀ , mg/ml	0.79 (0.05-13.5)	1.62 (0.3-11.6)	1.87 (0.97-3.61)	1.31 (0.48-3.64)

Figure 1 A. Flow cytometric Treg gating strategy using a representative peripheral blood sample. 1. PBMC forward scatter (FSC) vs side scatter (SSC); 2. T cell CD3⁺CD4⁺ gate; 3. CD25 vs CD127; 4. CD4 vs isotype control; 5. CD4 vs Foxp3. **B.** No changes in PB Treg frequency after anti-OX40L MAb treatment. Data expressed as mean $\pm$ SEM. **C.** No changes in PB Treg frequency after anti-TSLP MAb treatment. Data expressed as mean $\pm$ SEM.

A



B



C

