UNIVERSITY OF CALIFORNIA SAN DIEGO

Human Eosinophils Inhibit Regulatory T Cell Induction From Naïve T Cells

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2018

The Thesis of Neiman Liu is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2018

DEDICATION

This thesis is dedicated to all the millions of cells who sacrificed their lives for the greater good, be it eosinophil, neutrophil, T, red blood, or neural.

I would like to thank my dad for becoming the best VC any investment could ask for. I would like to thank my mom for helping me solve the most challenging puzzles.

I would like to thank Ha Mieu for being the best partner any man could ask for. I would like to thank Srujan for being the best lab partner any man could ask for. Thank you to all the undergraduates in the lab, Alice, Austin, and Faatimah, without you all I would have gotten carpal tunnel a long time ago. Thank you Howard for the political perspective we all are faced with. Thank you Jiayi for saving me in my experiment. Thank you Sam and Mouang for letting me into your hearts and homes.

Thank you Dr. Akuthota for all your lab resources, an amazing scientific journey, and your efforts into raising me into a successful graduate student; still eager to face the future under your guidance. Thank you Dr. Zuniga and Dr. Lu for giving me an A+ in immunology even though I barely passed the undergraduate course.

Thank you to all the friends and family who have been a part of my life.

EPIGRAPH

There is no “I” in team, but there is an “I” in Neiman.

Praveen Akuthota

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LIST OF ABBREVIATIONS

Th2 = T Helper Cell type 2

TGF-b = Transforming Growth Factor Beta-1

PMBC = Peripheral Blood Mononuclear Cell

FOXP3 = Forkhead Box P3

APC = Allophycocyanin

PE = Phycoerythrin

Ag = Antigen

Eos = Eosinophil

iTreg = Induced Regulatory T cell

RPMI/RPMI 1640 = Roswell Park Memorial Institute Medium

HBSS = Hank's Balanced Salt Solution

OVA = Ovalbumin

IL-2 = Interleukin 2

IL-4 = Interleukin 4

MHC = Major Histocompatibility Complex

IgG = Immunoglobulin G Antibody

HLA-DR = Human Leukocyte Antigen – Antigen D Related

GM-CSF = Granulocyte-Macrophage Colony-Stimulating Factor

Asp. f = Aspergillus fumigatus

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ABSTRACT OF THE THESIS

Human Eosinophils Inhibit Regulatory T Cell Induction From Naïve T Cells

by

Neiman Liu

Master of Science in Biology

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Professor Praveen Akuthota, Chair
Professor Elina Zuniga, Co-Chair

This study presents an *in vitro* model of regulatory T cell induction and explores the impact of eosinophils on developing naïve T cell populations. The research is relevant to the type 2 helper T cell mediated allergic response. As regulatory T cells are normally

responsible for the suppression of other helper T cells, including type 2 cells, it serves an essential role in preventing allergic responses. The polarization between helper and regulatory T cell is only one part of allergy, which first needs an antigen presenting cell to process and present allergens to naïve T cells. In either case, eosinophils seem to have a limited scope in allergy compared to T cells. However, eosinophils have a unique feature, in that they can be induced to present antigen to CD4 T cells. Aspergillus fumigatus, a common fungal allergen, is a prime candidate to study the effects of an allergic response. Along with its ubiquitous nature, it has been known to generate varying effects on eosinophils and regulatory T cells. Eosinophils themselves have been established as mere indicators of allergy, but the actual influence that eosinophils have on other T cells in an allergic response is still unclear.

CHAPTER 1 - INTRODUCTION

T-helper type 2 mediated allergy refers to a specific allergic reaction characterized by rampant amounts of Th2 cells, which secrete a large amount of inflammatory cytokines.[1] Th2 cells are a subset of CD4+ T cells, which indirectly fight pathogens by secreting cytokines, recruiting other white blood cells, and developing immunological memory. Regulatory T cells only respond to and target other T cells, critical in preventing autoimmune and allergic disease. In allergic patients, one or more processes are impaired, which could include the ability of regulatory T cells to suppress Th2 cells. By definition, regulatory T cells are CD4+CD25+FOXP3+. CD4 being a specific type of T cell, CD25 an activation marker, and intracellular FOXP3 being the most important in granting suppressor function.[2] Some studies on allergy show defects with Th2 allergic phenotype, or lack of memory in some T cells.[3,4] Regardless, T regulatory cells are present in our bodies to reduce and suppress the effects of unusual T cell behavior.[5]

Induced T regulatory cells refer to an *in vitro* method of inducing CD4+ naïve T cell to express CD25 and FOXP3. The *in vivo* equivalent refers to peripheral T regulatory cells, and would suggest that it is possible to generate regulatory T cells outside the thymus, possibly in response to an antigen.[6] At the very least, harvested naïve T cells need IL-2 and TGF-B to drive iTreg differentiation and to have suppressive function.[7] The addition of anti-CD3 and anti-CD28 helps expand and proliferate iTregs.[8] Once naïve T cells have had appropriate culture conditions and time to develop, they can be

assayed for FOXP3 and CD25 expression, to determine the amount of regulatory T cell induction.

Eosinophils are generally associated with asthma and parasitic infections.[9] There is limited research on eosinophil and T cell interaction, particularly when comparing the specific subsets of CD4+ T cells.[10] Eosinophils can, of course, modulate immune responses by cytokines and granules.[11] In one study, peripheral eosinophils seemed to inhibit regulatory T cells, while the opposite was true in the lamina propia.[12] Another article found eosinophils inhibited FOXP3 expression by IL-4.[13] These suggest eosinophils themselves can modulate regulatory T cells. More interestingly is that Eosinophils can act as antigen presenting cells. GM-CSF induced expression of HLA-DR, a MHC class 2 receptor, paralleling dendritic cell priming.[14] This could prove another mechanism is possible for eosinophils to modulate T cells.

Aspergillus fumigatus is one of the most common fungal allergens, and would likely be able to be recognized by GM-CSF primed eosinophils. Asp. f seems to cause an increase in numbers of eosinophil, even causing cases of eosinophilia in mice.[15, 16] However this may not correspond to the process of antigen presentation. Aspergillus fumigatus can also drastically change regulatory T cell function, as seen in mouse models infected with the conidia.[17] While lysate samples of Aspergillus fumigatus may trigger allergic cellular reactions, it is important to note that the specific parts and proteins of the fungus are not being analyzed. Recent data on this allergen suggests that subtle differences at the protein level may actually dictate levels of antigen-specfic conventional and regulatory T cells[18,19]

CHAPTER 2 - METHODS

Cell Purification of Eosinophils

All cells used for research were purified from blood taken from healthy human donors, under an approved Institutional Review Board protocol. For each experiment, between 80-160 ml of peripheral blood was drawn. 60 ml syringe tubes were prefilled with 10 ml of sodium citrate to prevent coagulation. After 40 ml of blood per tube was taken, 10 ml of STEMCELL HetaSep was added to deplete red blood cells. Tubes were allowed to sit upright at room temperature for approximately 40 minutes, until 30-35ml of red blood layer settled at the bottom. The remaining, straw-colored top layer was collected in 50 ml centrifuge tubes filled with 23 ml of Ficoll-Paque. Straw-colored layer was gently layered on top of the Ficoll-Paque layer, at a 1:1 ratio for each tube. Tubes were spun at 300 x g, at room temperature, for 20 minutes. A granolycte pellet formed at the bottom, below the Ficoll-Paque layer, containing eosinophils. A buffy coat layer, above the Ficoll-Paque layer and below a plasma layer, was used for PBMC collection and isolation of T cells. The buffy coat layer was collected into 50 ml centrifuge tubes and set aside for later T cell isolation. After removal of top layers, the granulocyte pellet was resuspended in 1 ml of 4°C HBSS. 1 ml pellet solutions were combined into a single 50 ml tube filled with 30 ml of 4°C HBSS. Centrifuge tube was topped up to 50 ml of HBSS. Granulocytes were spun at 300 x g, at 4°C for 5 minutes. Supernatant was decanted and resuspended in 50 ml of 4°C HBSS. Granulocytes were diluted in Turk Blood Diluting Fluid and counted using a hemacytometer. Cells were spun at 300 x g, at 4°C, for 5 minutes, decanted, and resuspended in 1 ml of HBSS with 0.5% Grade V

ovalbumin, per 50 million cells. Eosinophils were purified by negative selection using STEMCELL StemSep column based magnetic isolation system. Eosinophils were spun at 300 x g, at 4°C for 5 minutes, decanted, and resuspended in complete medium, consisting of RPMI 1640 with 10% fetal bovine serum and 10% Penicillin Streptomycin. Eosinophils were diluted in Turk Blood Diluting Fluid and counted using a hemacytometer.[20]

Eosinophil purity was measured by microscopy. Eosinophils were immobilized on microscope slides by cytospin. 0.5 ml of eosinophils were spun at 340 rpm, at medium acceleration, for 4 minutes. Once affixed to the slide, eosinophils were stained using Hema 3 kit. Pure, stained eosinophils showed a purple color and bilobed nuclei.

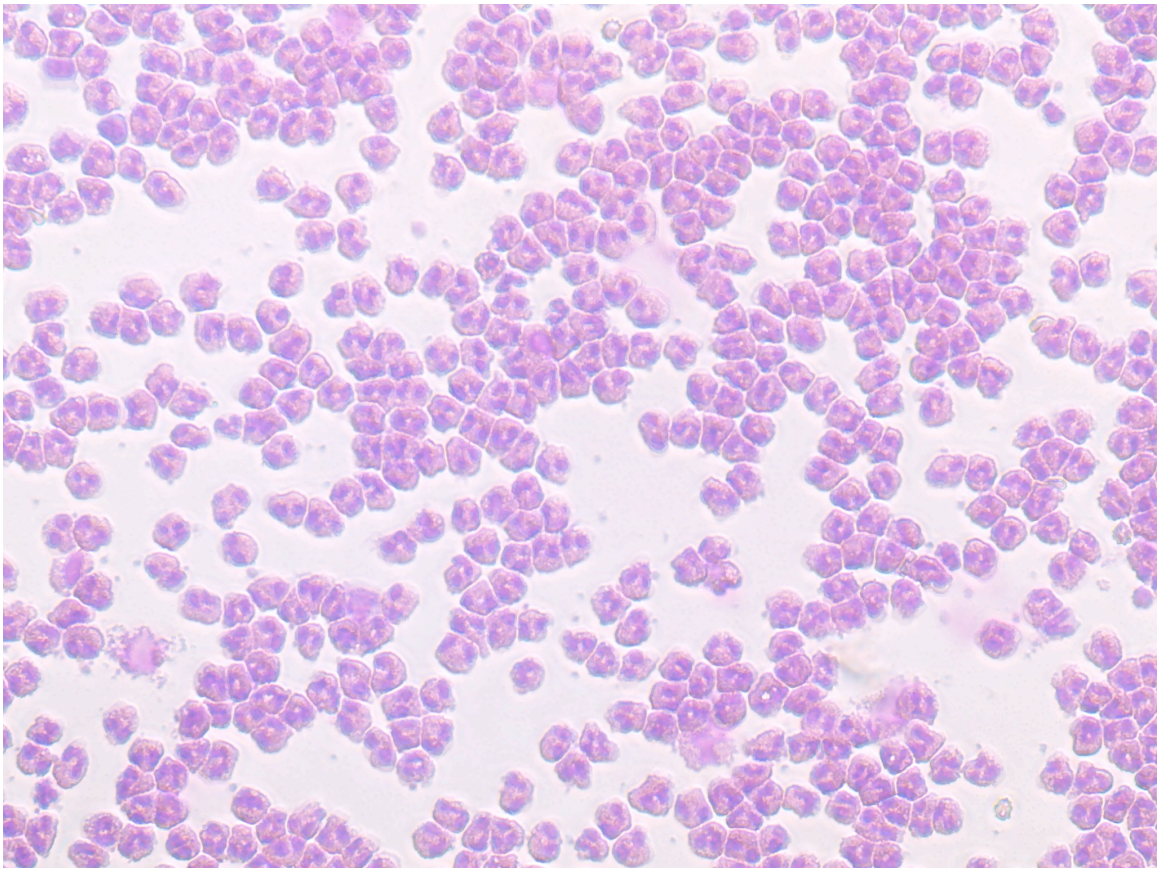

Figure 1. Stained human eosinophils, 20X.

Cell Purification of Naïve T cells

The buffy coat layer from aforementioned eosinophil purification was used to isolate CD4+ naïve T cells. Due to the mixture of Ficoll-Paque contamination, the layer was spun at 1000 x g for 10 minutes. The PBMC supernatant was decanted into a separate 50 ml and spun again as necessary, after cell counting. PBMCs were resuspended in 1-2 ml of RPMI 1640. PBMCs were diluted in trypan blue and counted using a hemocytometer. Cell viability was verified using trypan blue. Approximately 25 PBMCs were needed for every 1 naïve T cell. PBMCs were spun at 300 x g at room temperature for 8 minutes. PMBCs were decanted and resuspended in 50 million cells per ml of RPMI 1640. Naïve T cells were isolated by negative selection using STEMCELL EasySep column-free magnetic isolation system. Naïve CD4+ T cells were spun at 300 x g at room temperature for 8 minutes, decanted, and resuspended in 1 ml of complete medium. T cells were diluted in trypan blue and counted using a hemocytometer.

The purity of T cells was measured by flow cytometry. 5 x 104 - 2 x 105 naïve T cells, split into two samples, were used for FACS. Cells were spun at 400 x g, 4°C for 5 minutes, decancted and resuspended in 200 ul of HBSS with 0.5% OVA. This was repeated once. For surface staining, each sample was resuspended in 100-200 ul of HBSS with 0.5% OVA in a 12 x 75 mm, 5 ml tube polystyrene tube. One sample was used as an isotype control, and 1 ul each of Biolegend antibodies PerCP/Cy5.5 Mouse IgG1 κ, APC/Cy7 Mouse IgG1 κ, FITC Mouse IgG2b κ, and APC Mouse IgG2a κ were added to the tube. The other sample was stained with 1 ul each of Biolegend antibodies PerCP/Cy5.5 conjugated anti-CD4, APC/Cy7 conjugated anti-CD3, FITC conjugated

anti-CD45RA, APC conjugated anti-CD197. Both samples were incubated for 20 minutes on ice. Cells were then spun at 400 x g, 4°C for 5 minutes, decanted and resuspended in 200 ul of HBSS. This was repeated once. OVA was omitted for the last washes to minimize background. Cells were covered from light, placed on ice, and analyzed by flow cytometry (Figure 2).

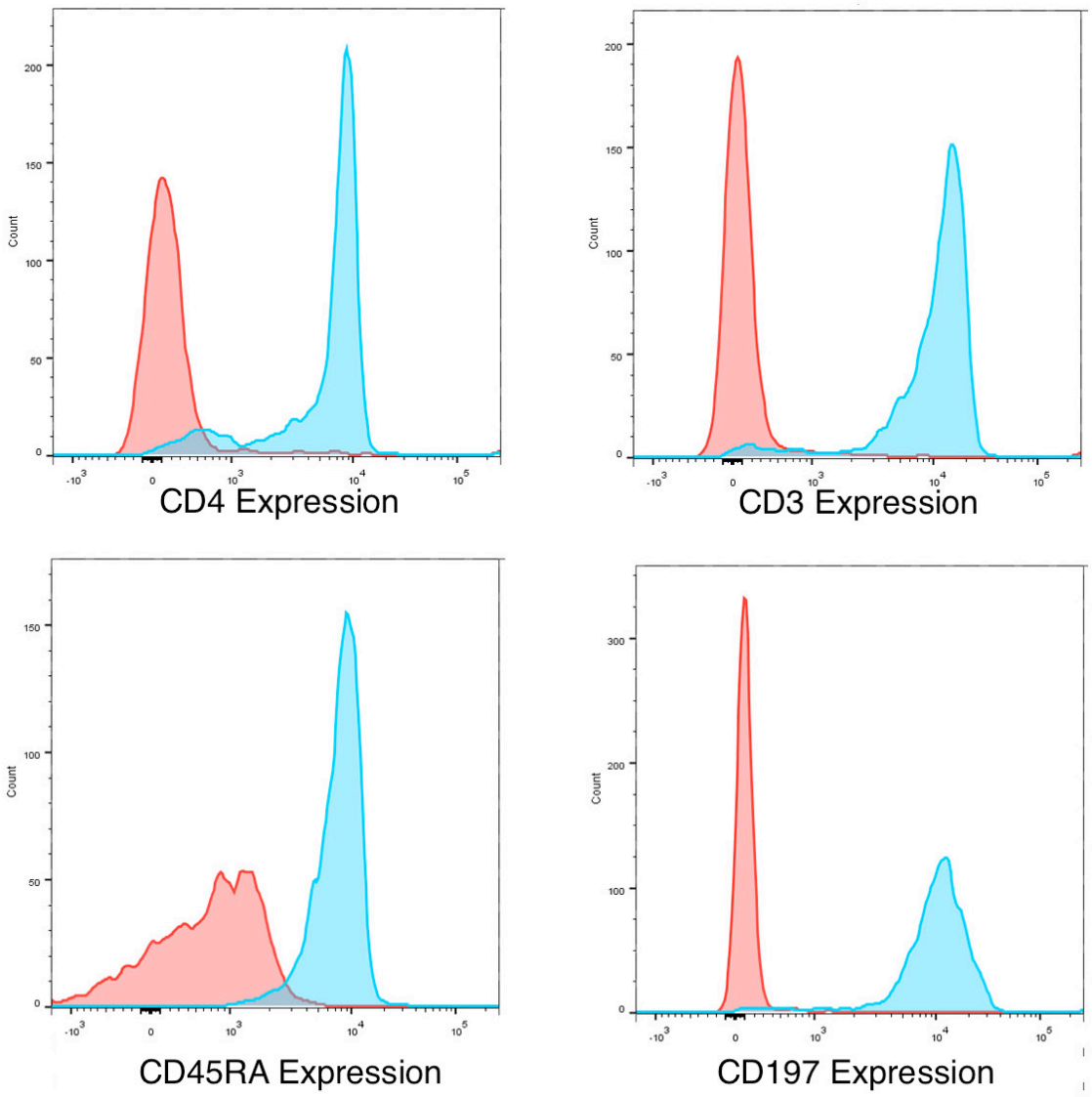

Figure 2. Histogram plots of human naïve T cells with CD4, CD3, CD45RA, and CCR7 surface markers compared to their respective isotype controls. Isotype controls, shown in red, are overlayed with the sample, shown in blue.

HLA-DR Induction in Eosinophils

Eosinophils were cultured overnight in complete medium at 106 cells/ml, either unstimulated without GM-CSF, or with 10 ng/ml of GM-CSF, at 37°C . Cells were transferred into 12 x 75 mm, 5 ml tube polystyrene tubes. Tubes were spun at 400 x g, 4°C for 5 minutes, decanted, and resuspended in 200 ul of PBS. Cells were spun again at 400 x g, 4°C for 5 minutes, decanted, and resuspended in 4% paraformaldehyde in PBS. Cells were fixed for 20 minutes in the dark. If permeabilized, cells were spun at 400 x g, 4°C for 5 minutes, decanted, and resuspended in Polysorbate 20. Cells were permeabilized for 10 minutes. After staining, cells were spun at 400 x g, 4°C for 5 minutes, decanted, and resuspended in 200 ul of PBS. For surface HLA-DR expression, cells were stained after fixation, skipping permeabilization procedure to the last wash. For intracellular HLA-DR expression, cells were stained after permeabilization. The Isotype control was incubated with 2 ul of Alexa Fluor 488 Mouse IgG2b κ, in 200 ul of cell staining buffer. The sample was incubated with 2 ul of Alexa Fluor 488 anti-human HLA-DR in 200 ul of cell staining buffer. Cells were covered from light, placed on ice, and analyzed by flow cytometry (Figure 2).

iTreg Experiment

Induced regulatory T cells were developed from a pure naïve T cell population. After purification, between $5 \times 10^4 - 2 \times 10^5$ naïve T cells were cultured in each well in a 96 well plate for 4-6 days. All wells contained 50 U/ml of IL-2, 25 ul/ml of soluble anti-human CD3, and 25 ul/ml of soluble anti-human CD28, with or without 2ng/ml of TGF-

b. Depending on the experiment, additions to cell culture included, eosinophils at a 1:1 ratio to naïve T cells, eosinophils at a 1:1 ratio to naïve t cells with 20 ug/ml of Aspergillus fumigatus lysate with 10 ng/ml of GM-CSF, 10 ug/ml of Aspergillus fumigatus lysate, 2 ug/ml of anti-human IL-4, 10 ng/ml of recombinant human IL-4. Wells were topped up to 200 ul with complete media and placed in a 37°C incubator. After 4-6 days, cells were fixed and permeabilized, with extracellular and intracellular staining. Plates were spun at 400 x g, 4°C for 5 minutes, decanted, and resuspended in 200 ul of HBSS with 0.5% OVA. This was repeated once. Wells used for isotype control staining were incubated with 1 ul each of Biolegend antibodies APC Mouse IgG2b κ and PE Mouse IgG1 κ, in 100 ul of HBSS with 0.5% OVA. Wells used for sample staining were incubated with 1 ul each of Biolegend antibodies APC anti-human CD4 and PE anti-human CD25 in 100 ul of HBSS with 0.5% OVA. The plate was incubated on ice for 20 minutes, in the dark. Cells were spun at 400 x g, 4°C for 5 minutes, decanted, and resuspended in 200 ul of HBSS. This was repeated once. Fixation and permeabilization was carried out using Biolegend True-Nuclear Transcription Factor Buffer Set. 1 ul of Alexa Fluor 488 Mouse IgG1 κ, in 100 ul of 1X Perm Buffer, was used for each isotype well during permeabilization. 1 ul of Alexa Fluor 488 anti-human FOXP3, in 100 ul of 1X Perm Buffer, was used for each sample well during permeabilization. After fixation and permeabilization, cells were spun at 400 x g, 4°C for 5 minutes, decanted and resuspended in 200 ul of HBSS. Cells were spun again at 400 x g, 4°C for 5 minutes, decanted and resuspended in 100 ul of HBSS. Cells were covered from light, placed on ice, and analyzed by flow cytometry.

iTreg Experiment Gating Strategy

Foxp3 and CD25 expression was measured using the geometric mean of CD4+ T cell populations. First, T cells were gated along forward scatter height and side scatter height, to omit background noise and eosinophil populations, if applicable (Figure 3). Next, the gate would be applied to the corresponding isotype control. The CD4 fluorescence of isotype and stained T cell populations were overlaid on a histogram plot (Figure 4). The stained T cell population was bisected by comparing the negative isotype to the stained cells. The CD4 positive population would be used to measure the mean fluorescence of Foxp3 and CD25. For positive and negative controls of TGF-b, a quadrant gate was also analyzed (Figure 5).

Flow cytometry for all samples was performed using a FACSCanto II, with voltages set at FSC: 250, SSC: 350, Alexa Fluor 488: 700, PE: 480, APC: 600, FITC: 500, PerCP-Cy7: 600, and APC-Cy7: 500.

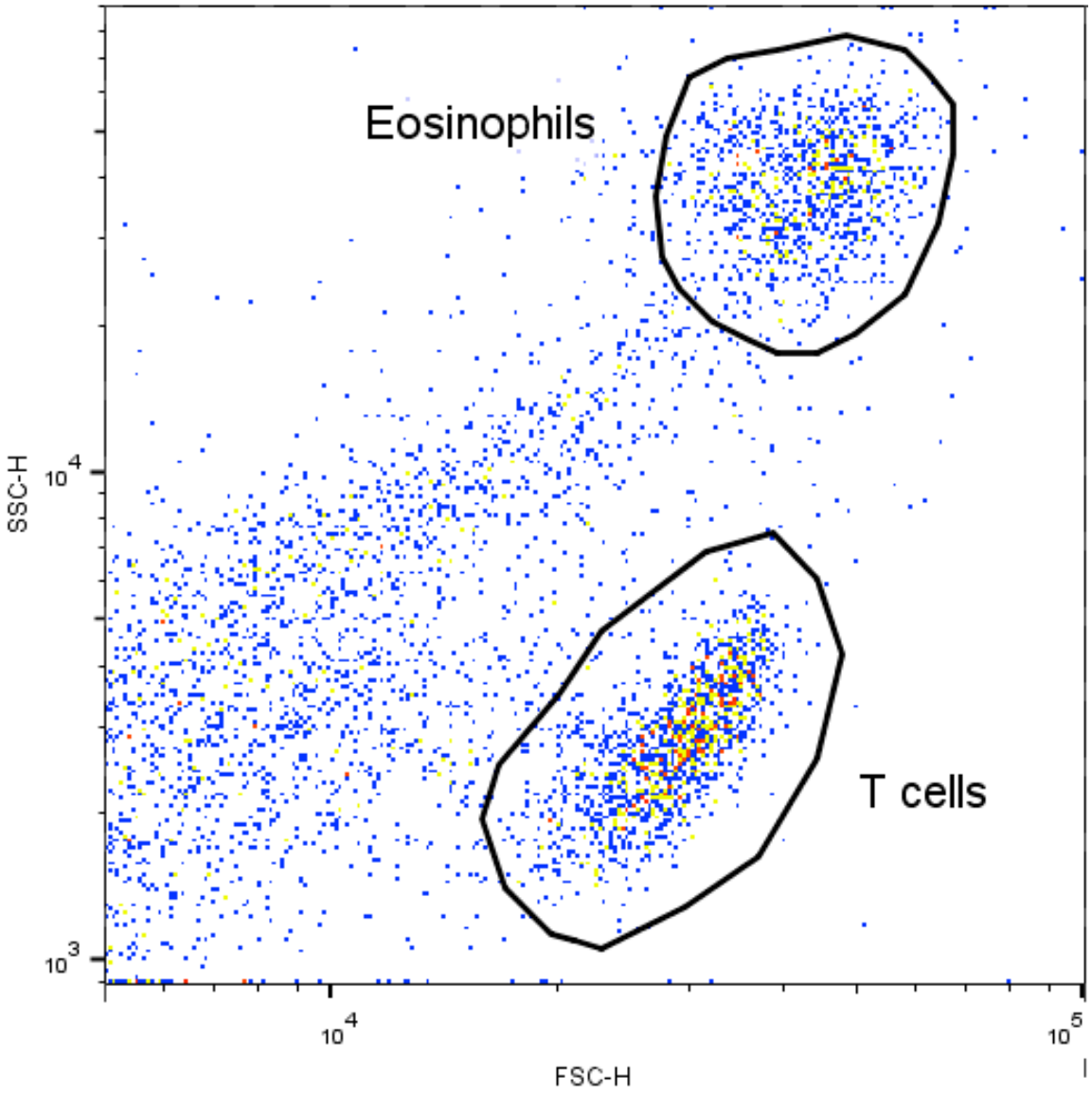

Figure 3. Scatter plot of a mixed cell sample. Forward scatter height and side scatter height are compared to find the population of T cells. Eosinophil population is shown for reference.

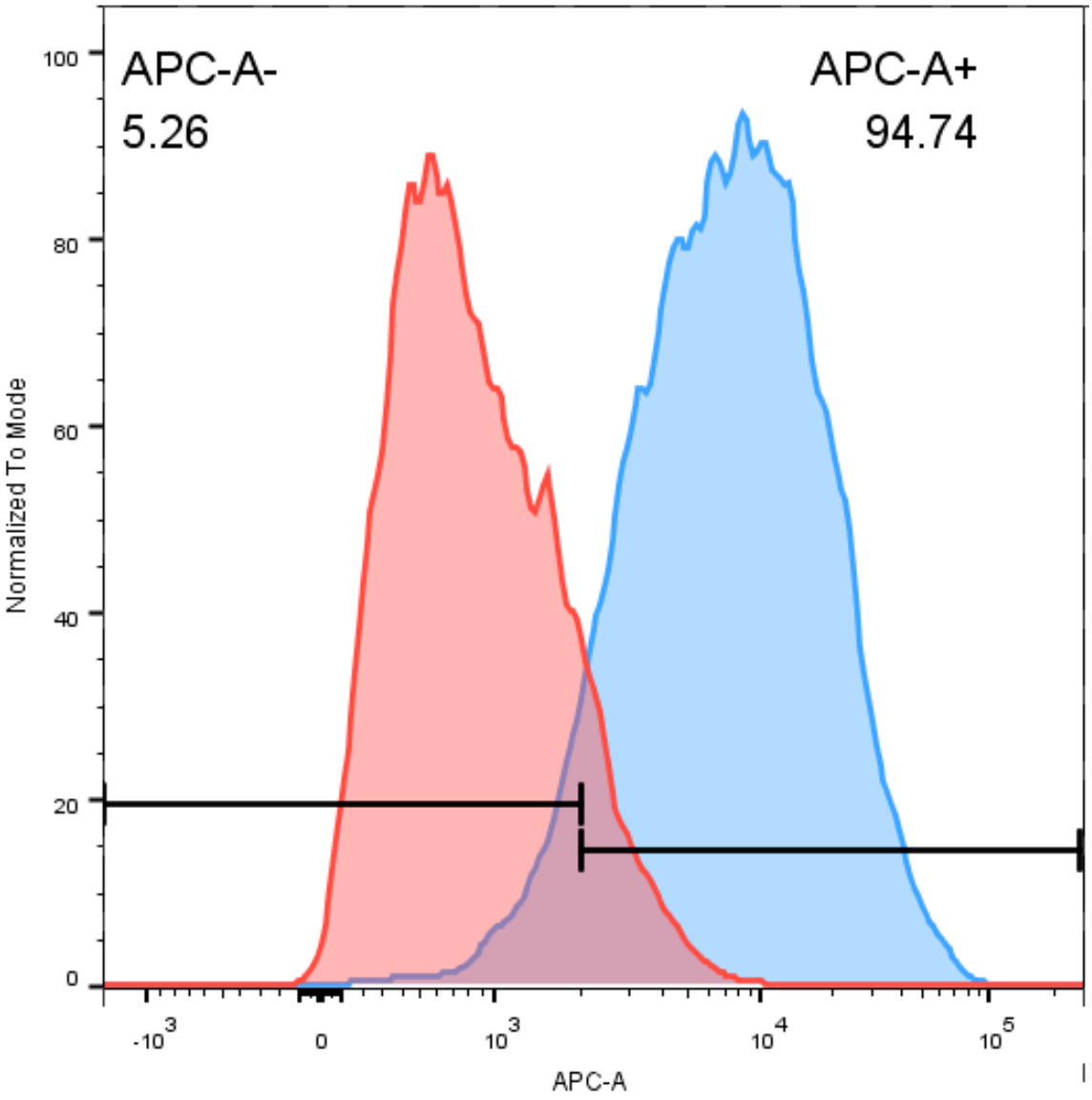

Figure 4. Histogram plot of surface CD4+ expression, as APC fluorescence, in gated T cells. Isotype control, shown in red, is overlaid with sample, shown in blue. Gated T cells are bisected based on overlay to determine CD4+ population.

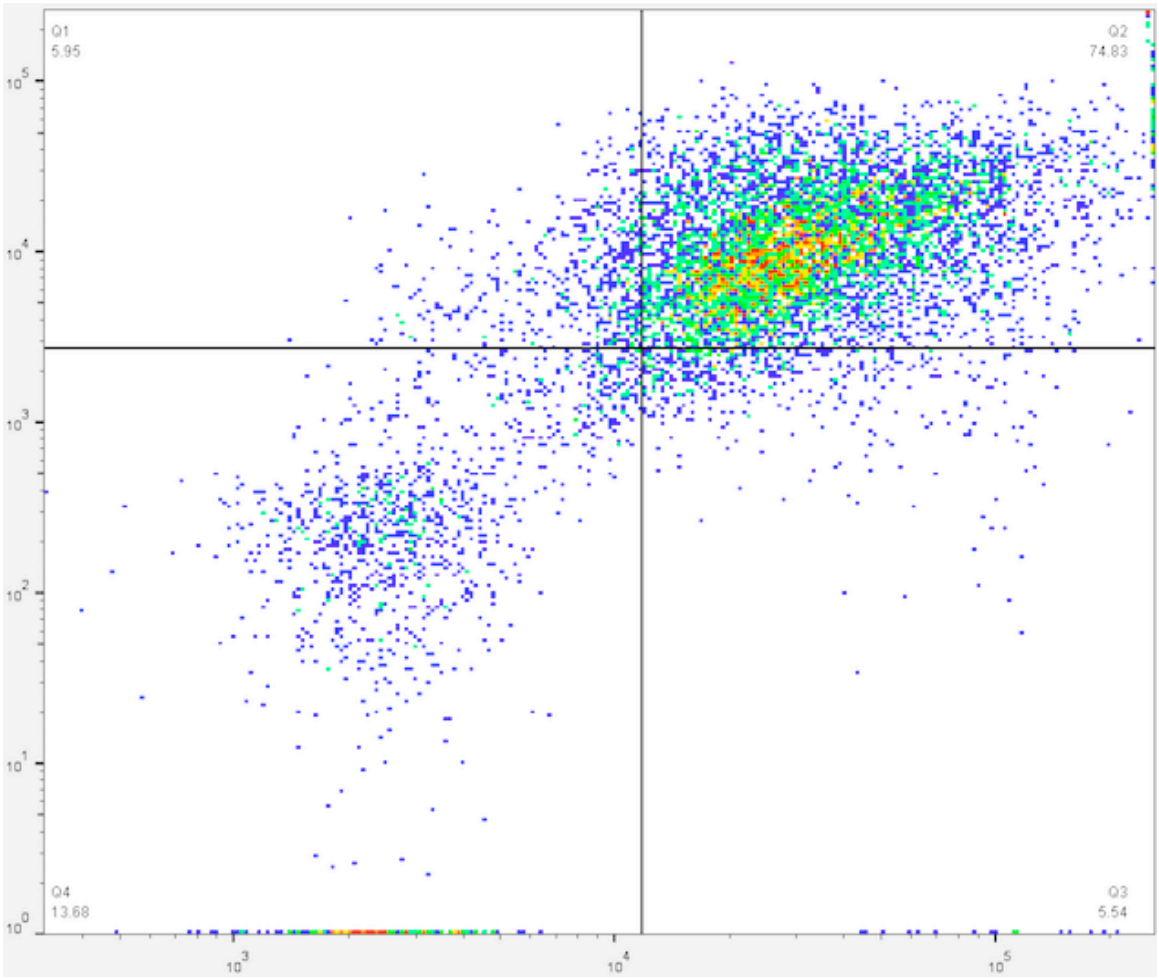

Figure 5. Scatter plot of CD4+ T cells. A quadrant gate was used to analyze CD25+ and FOXP3+ T cells (Q2) for initial control conditions. FOXP3 was plotted along the X-axis and CD25 along the Y-axis. MFI used for quadrant gate parameters were taken from overlays of isotype and sample of CD25 (PE) and FOXP3 (Alexafluor 488), identical to methods described in Figure 4.

CHAPTER 3 - RESULTS

iTreg Experiment

CD4+ naïve T cells were cultured with IL-2, anti-CD3, anti-CD28. The effects of TGF-b on regulatory T cell development were measured by FOXP3 and CD25 expression on CD4+ T cells, after 4-6 days. Naïve T cell purity was over 90%, verified by flow cytometry. In the scatter plot, 'Tn, TGFB+' is the result of naïve T cells cultured with TGF-b. 'Tn, TGFB+' is a duplicate condition, but without any TGF-b added. Comparing FOXP3 expression, the T cells with TGF-b have a much larger average mean fluorescence intensity, and thus more FOXP3 expression (Figure 6a). The same trend also occurs for CD25 expression (Figure 6b). Results were also shown as percentages from a quadrant gate. (Figure 7) After adding TGF-b to CD4+ naïve T cells, the amount of FOXP3 and CD25 increased.

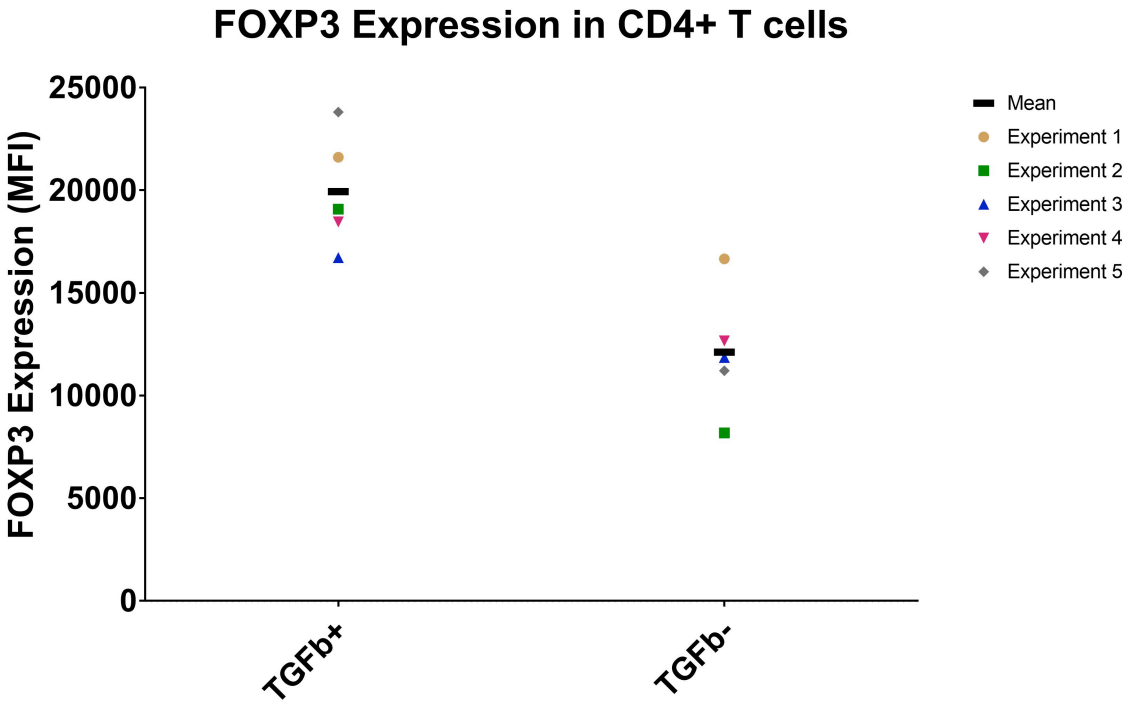

Figure 6a. Scatter plot of intracellular FOXP3 expression in CD4+ T cells. TGFb+ represents T cells assayed after 4-6 days of cell culture with IL2, anti-CD3, anti-CD28, and TGF-b. TGFb- represents duplicate cell culture conditions, omitting the addition of TGF-b. The geometric mean was recorded in each experiment from CD4+ gated T cells.

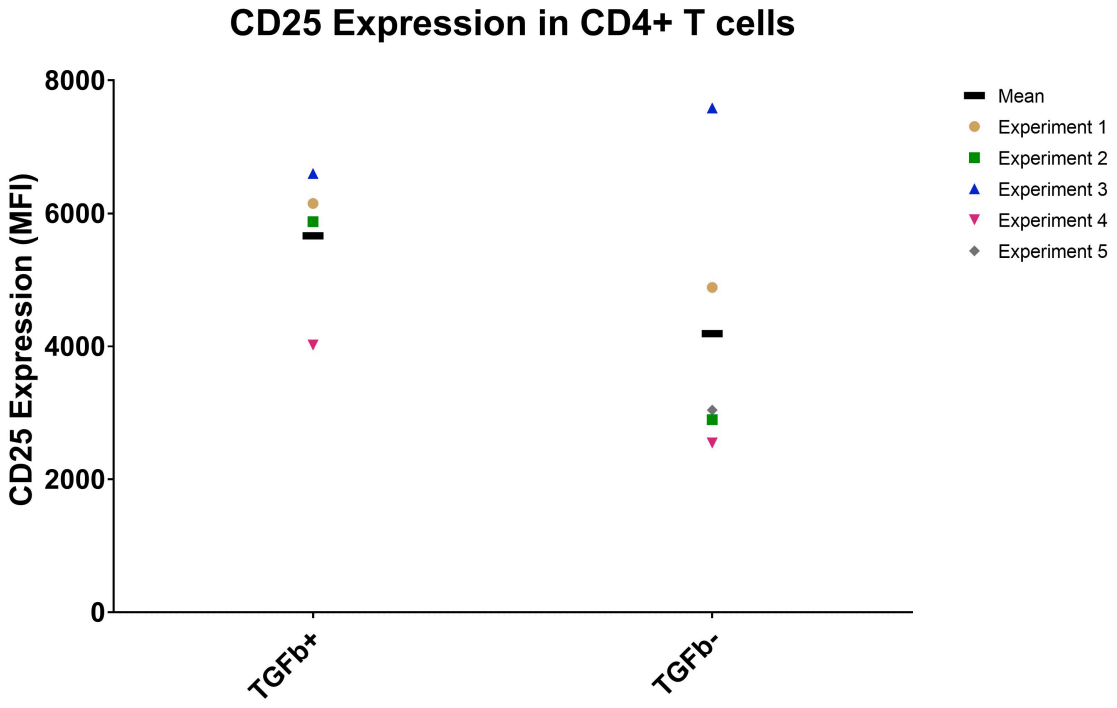

Figure 6b. Scatter plot of surface CD25 expression in CD4+ T cells. Cell populations and experiments are identical to Figure 5a. The geometric mean was recorded in each experiment from CD4+ gated T cells.

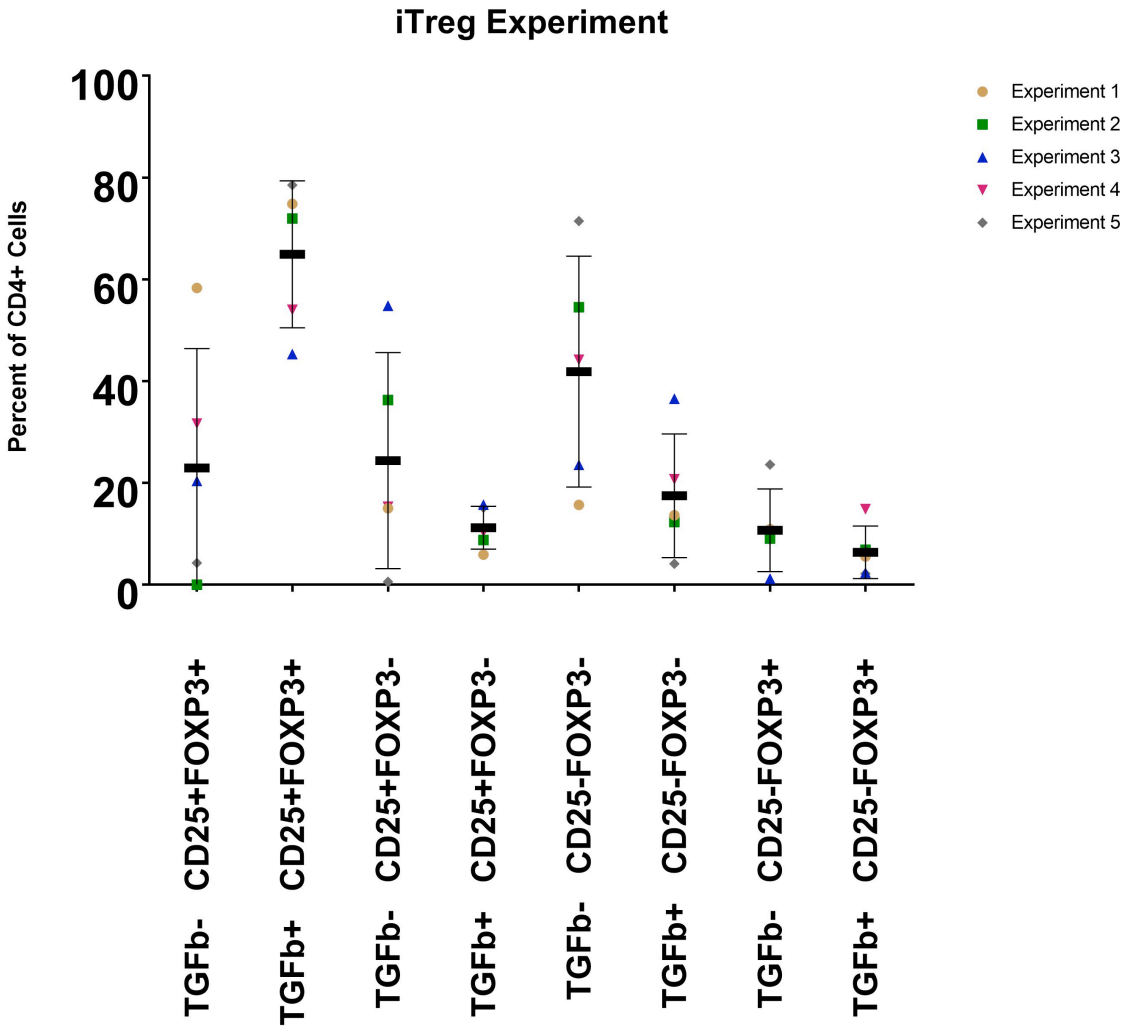

Figure 7. Percentage of CD25+FOXP3+ cells within CD4+ T cells. CD25+FOXP3-, CD25-FOXP3-, CD25-FOXP3+ populations shown for reference.

iTreg Experiment with Eosinophils

The expression of HLA-DR on unstimulated eosinophils was compared to eosinophils cultured with GM-CSF. The level of surface HLA-DR expression in unstimulated eosinophils was almost none, overlapping closely with the isotype control (figure 8). In contrast, the GM-CSF-primed eosinophil population resulted in an increase in HLA-DR expression, although a notable fraction of the cells still overlapped with the isotype control. The increase in HLA-DR expression for eosinophils cultured with GM-CSF was much more prominent after cell permeabilization and comparing intracellular levels (figure 9). After adding GM-CSF, both the amount of intracellular and surface HLA-DR on eosinophils increased.

To test the effects of eosinophils on iTreg development, naïve T cells were cultured in the same conditions as the first iTreg experiment, with or without TGF-b. Eosinophil purity was over 90%, verified by microscopy. Either eosinophils alone or GM-CSF-primed eosinophils with antigen were added to the naïve T cells. In either case, the levels of foxp3 were clearly lower when compared to the naïve T cells with TGF-b (Figure 10a) For experiments 1 and 4, in the 'Eos, TGFb+' and 'Eos+Ag, TGFb+' conditions, the foxp3 expression was almost reaching their respective amounts seen in 'TGFb+'. However, within each experiment, the naïve T cells with TGF-b consistently showed the highest level of foxp3 expression. Overall, the level of foxp3 in conditions with eosinophils, antigen or not, was near or below the level of FOXP3 naïve T cells without TGF-b. For CD25, expression varied widely in the conditions without antigen added, but was mostly decreased. In the conditions with eosinophils, antigen, and GM-

CSF, the spread between CD25 experiments was moderately lower, along with the mean expression.

An additional control condition, with no eosinophils and only antigen added, was tested, but only performed in duplicate (Figure 11a). Compared to each condition's respective experiment, the level of FOXP3 expression was higher. In experiment 4, the FOXP3 expression even exceeded its corresponding TGFB+ control. CD25 expression was also higher than other conditions (Figure 11b).

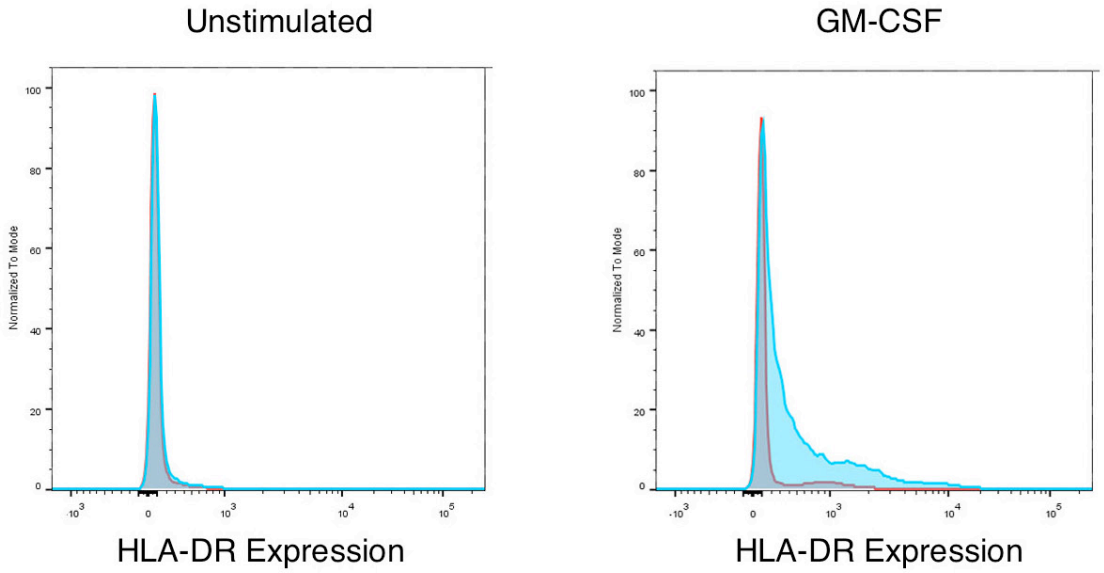

Figure 8. Histogram plots of surface HLA-DR expression in eosinophils after one day of culture, unstimulated or with GM-CSF added. For each condition, the expression of an isotype control, in red, was overlaid over the stained sample, in blue.

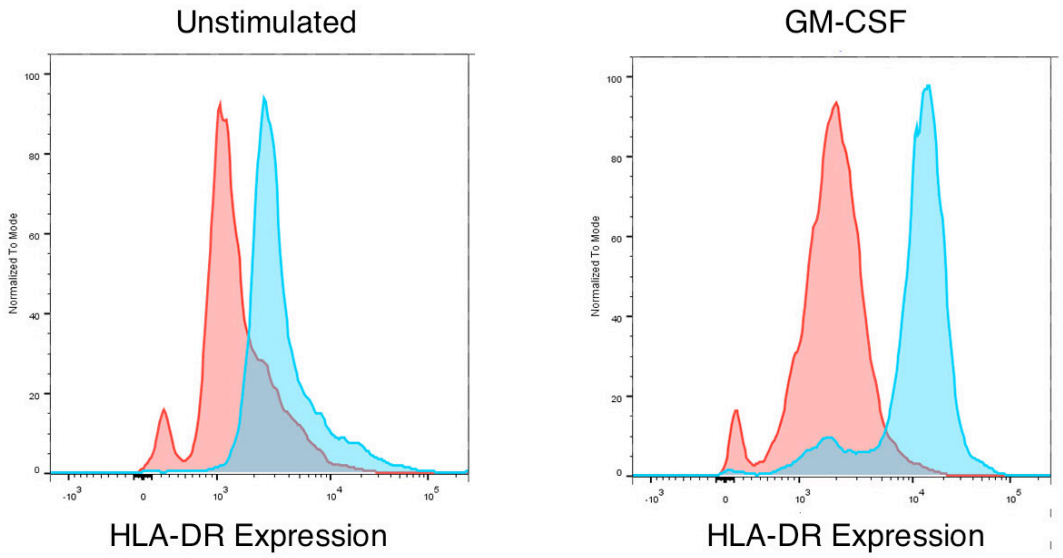

Figure 9. Histogram plots of intracellular HLA-DR expression in eosinophils after one day of culture, unstimulated or with GM-CSF added. For each condition, the expression of an isotype control, in red, was overlaid over the stained sample, in blue.

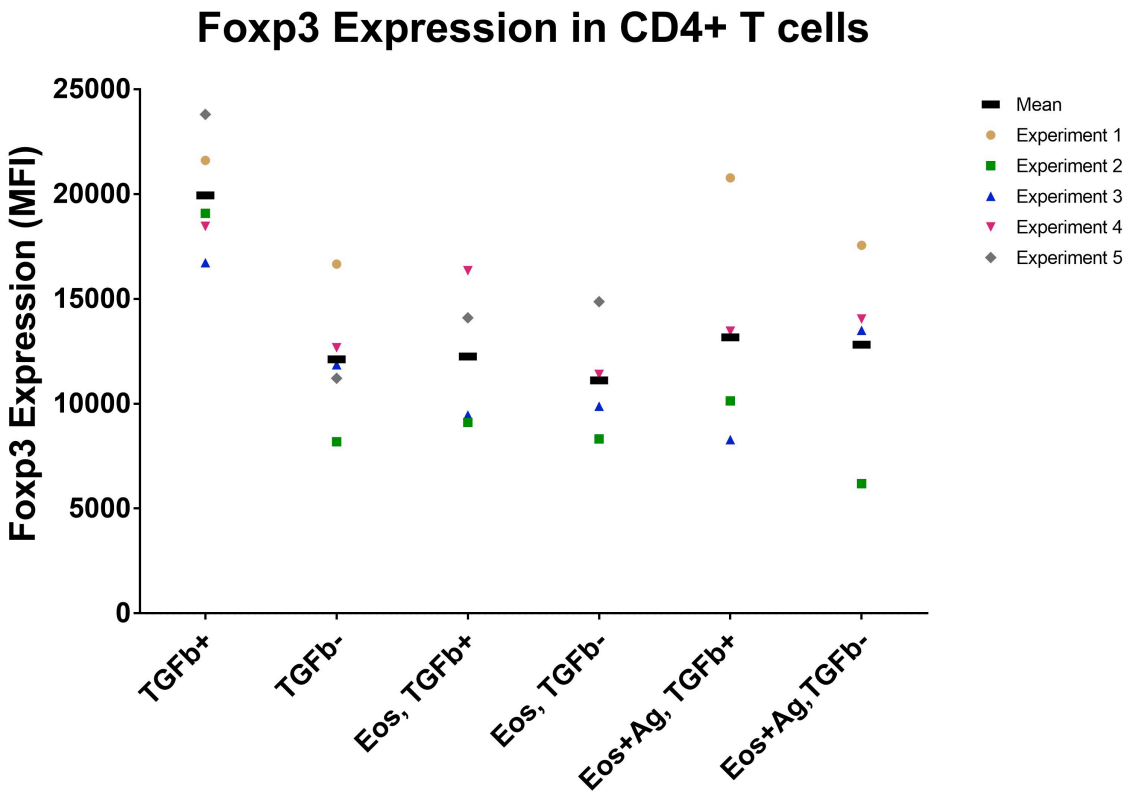

Figure 10a. Scatter plot of intracellular FOXP3 expression in CD4+ T cells with eosinophils and antigen. TGFb+ represents T cells assayed after 4-6 days of cell culture with IL2, anti-CD3, anti-CD28, and the addition of TGF-b. Conditions with TGFb- represent duplicate cell culture conditions, omitting the addition of TGF-b. Eos, TGFb+ and Eos TGFb- represent replicate cell culture conditions, with Eosinophils added at a 1:1 ratio. Eos+Ag, TGFb+ and Eos+Ag TGFb- represent replicate cell culture conditions, with Eosinophils added at a 1:1 ratio, Aspergillus fumigatus lysate, and GM-CSF. The geometric mean was recorded in each experiment from CD4+ gated T cells.

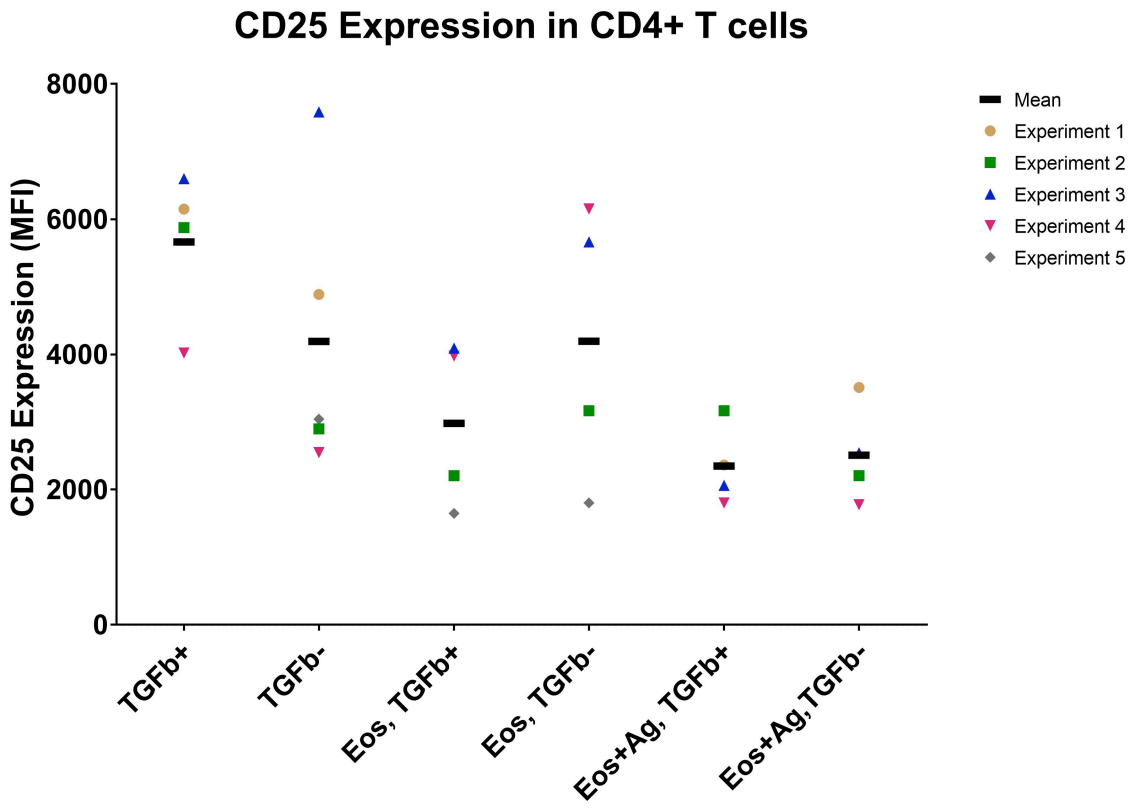

Figure 10b. Scatter plot of surface CD25 expression in CD4+ T cells with eosinophils and antigen. Cell populations and experiments are identical to Figure 8a. The geometric mean was recorded in each experiment from CD4+ gated T cells.

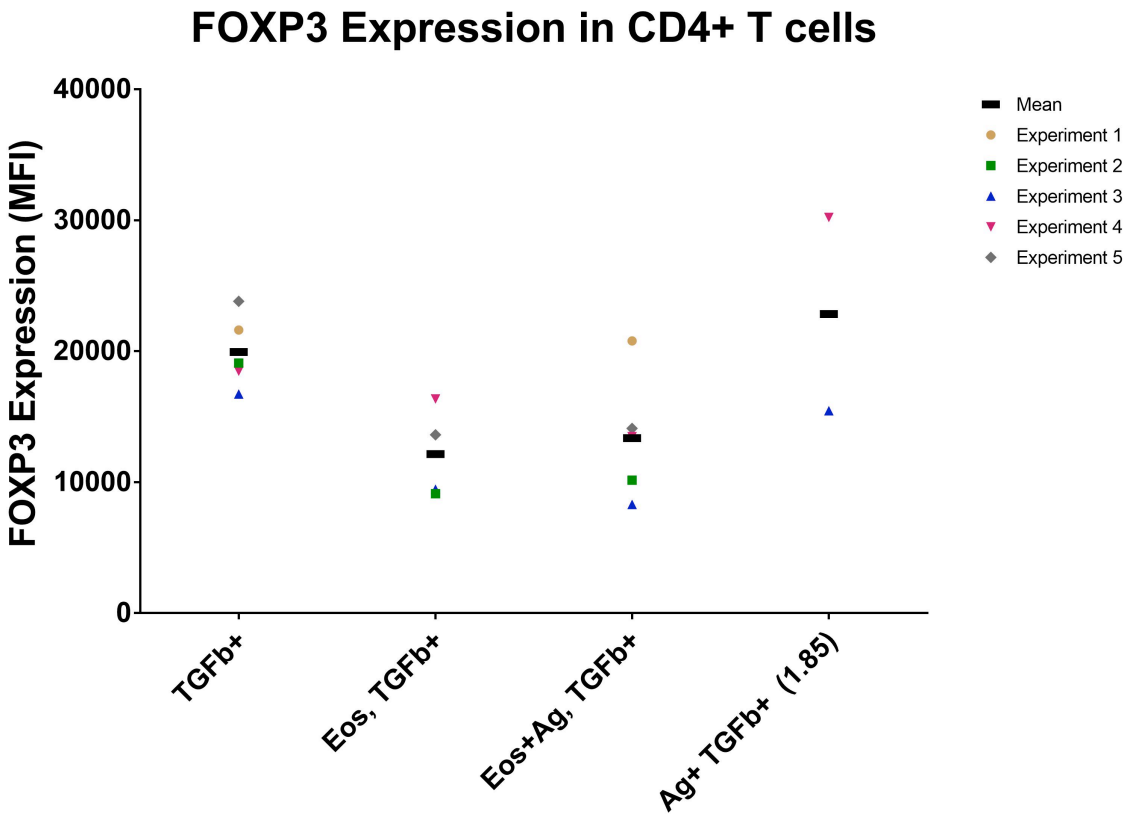

Figure 11a. Scatter plot of intracellular FOXP3 expression in CD4+ T cells with TGF-b, eosinophils, and antigen. TGFb+ represents T cells assayed after 4-6 days of cell culture with IL2, anti-CD3, anti-CD28, and the addition of TGF-b. Eos, TGFb+ represents replicate cell culture conditions, with Eosinophils added at a 1:1 ratio. Eos+Ag, TGFb+ represents replicate cell culture conditions, with Eosinophils added at a 1:1 ratio, Aspergillus fumigatus lysate, and GM-CSF. Ag, TGFb+ represents replicate cell culture conditions with Aspergillus fumigatus lysate added. The geometric mean was recorded in each experiment from CD4+ gated T cells.

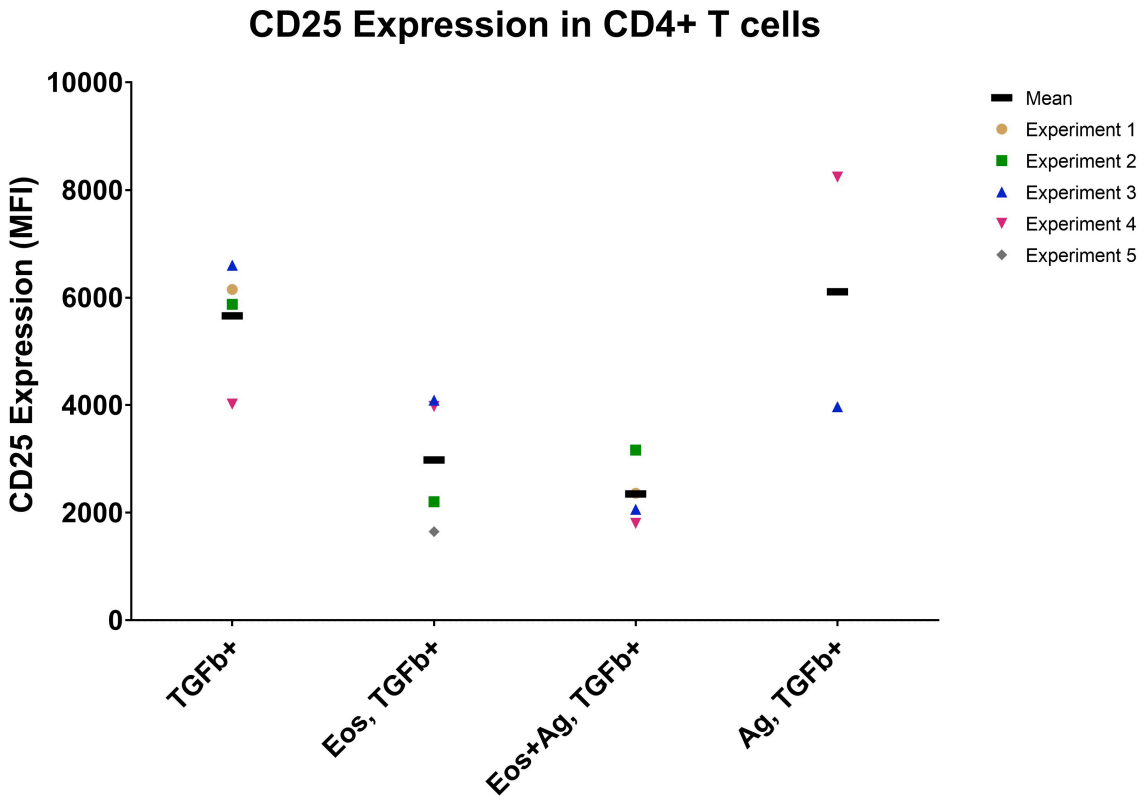

Figure 11b. Scatter plot of intracellular CD25 expression in CD4+ T cells with TGF-b, eosinophils, and antigen. Cell populations and experiments are identical to Figure 9a. The geometric mean was recorded in each experiment from CD4+ gated T cells.

iTreg Experiment with IL-4

The conditions tested so far in producing iTreg cells were performed again with the addition of anti-IL-4 or IL-4. Only two experiments for each new anti-IL-4 or IL-4 condition were tested. Levels of FOXP3 and CD25 expression were still low in conditions with eosinophils added, however experiment 4 actually showed an increase for both, only in the conditions with IL4 added. The same trend was not shown in experiment 5, with low levels of FOXP3 and CD25 expression across all conditions, except for a minor increase in naïve T cells only with anti-IL4.

A table of means, standard deviations, and samples sizes of Foxp3 and CD25 expression across all conditions was created (Table 1).

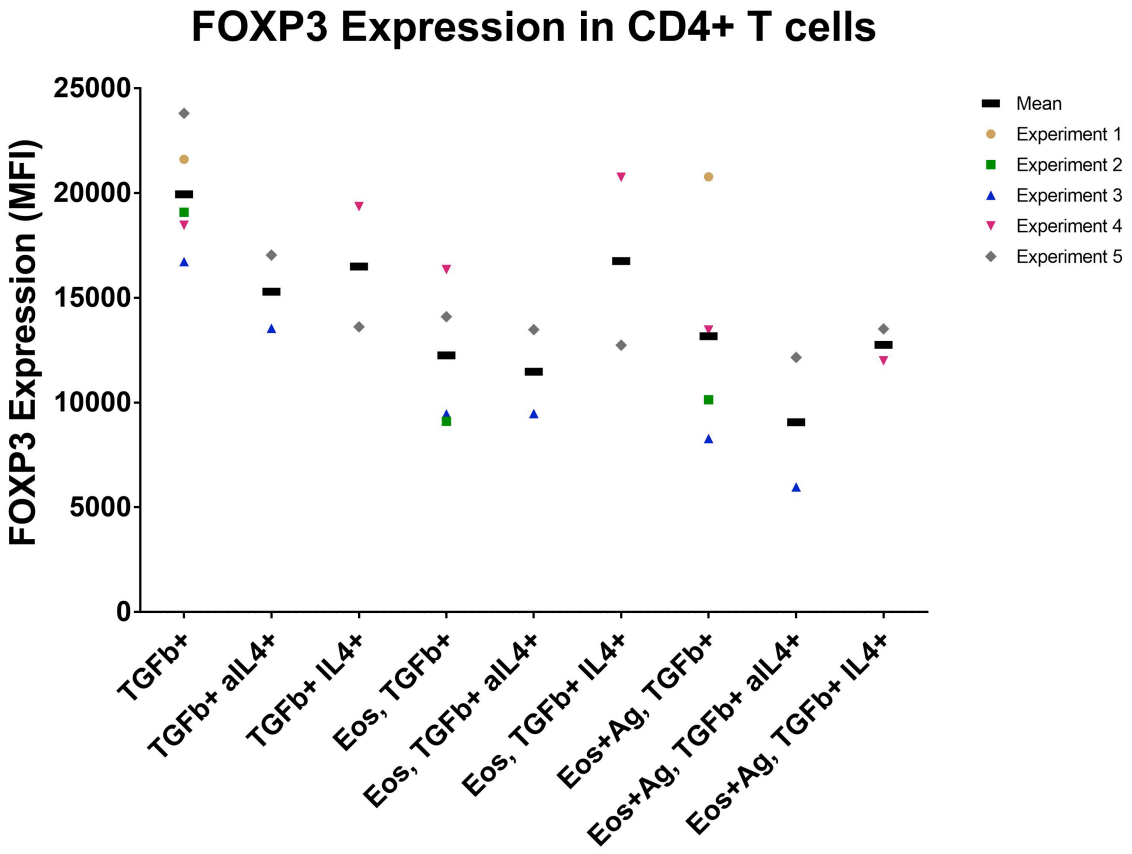

Figure 12a. Scatter plot of intracellular FOXP3 expression in CD4+ T cells with additional IL-4 conditions. TGFb+ represents T cells assayed after 4-6 days of cell culture with IL2, anti-CD3, anti-CD28, and the addition of TGF-b. Conditions with TGFb- represent duplicate cell culture conditions, omitting the addition of TGF-b. Eos, TGFb+ and Eos TGFb- represent replicate cell culture conditions, with Eosinophils added at a 1:1 ratio. Eos+Ag, TGFb+ and Eos+Ag TGFb- represent replicate cell culture conditions, with Eosinophils added at a 1:1 ratio, Aspergillus fumigatus lysate, and GM-CSF. TGFb+ aIL4+ and TGFb+ IL4+ represent replicate cell culture conditions, with the addition of anti-IL-4 or IL-4, respectively. Eos, TGFb+ aIL4+ and Eos, TGFb+ IL4+ represent replicate cell culture conditions, with Eosinophils added at a 1:1 ratio and the addition of anti-IL-4 or IL-4, respectively. Eos+Ag, TGFb+ aIL4+ and Eos+Ag, TGFb+ IL4+ represent replicate cell culture conditions, with Eosinophils added at a 1:1 ratio, Aspergillus fumigatus lysate, GM-CSF, and the addition of anti-IL-4 or IL-4, respectively. The geometric mean was recorded in each experiment from CD4+ gated T cells.

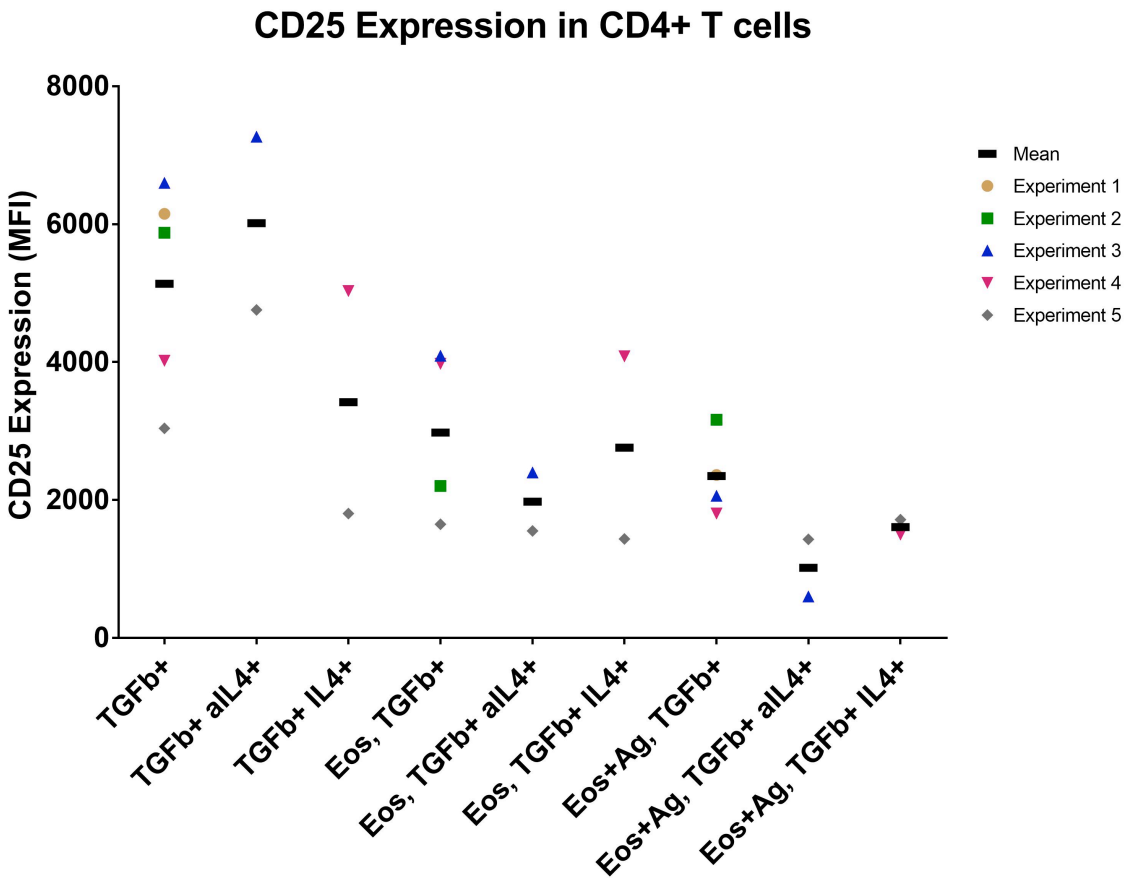

Figure 12b. Scatter plot of surface CD25 expression in CD4+ T cells additional IL-4 conditions. Cell populations and experiments are identical to Figure 13a. The geometric mean was recorded in each experiment from CD4+ gated T cells.

Table 1a. Mean, standard deviation, and sample size of all conditions tested for FOXP3 expression. The mean fluorescence level from each experiment is used to calculate the mean and standard deviation.

	Mean	Standard Deviation	N
TGFb+	19937.42	2784.04	5
TGFb-	12117.41	3056.48	5
TGFb+ aIL4+	15292.61	2468.35	2
TGFb+ IL4+	16489.80	4060.97	2
Eos, TGFb+	12251.92	3554.51	4
Eos, TGFb-	11118.05	2800.09	4
Eos, TGFb+ aIL4+	11476.33	2828.98	2
Eos, TGFb+ IL4+	16747.48	5668.44	2
Ag, TGFb+	22828.42	10447.55	2
Eos+Ag, TGFb+	13167.55	5507.56	4
Eos+Ag, TGFb-	12821.68	4776.35	4
Eos+Ag, TGFb+ aIL4+	9060.87	4373.87	2
Eos+Ag, TGFb+ IL4+	12754.42	1079.42	2

Table 1b. Mean, standard deviation, and sample size of all conditions tested for CD25 expression. The mean fluorescence level from each experiment is used to calculate the mean and standard deviation.

	Mean	Standard Deviation	N
TGFb+	5293.65	1854.51	4
TGFb-	4486.82	1909.89	5
TGFb+ aIL4+	6014.06	1776.35	2
TGFb+ IL4+	3418.56	2281.28	2
Eos, TGFb+	2980.60	1238.53	4
Eos, TGFb-	4992.96	1601.90	3
Eos, TGFb+ aIL4+	1976.02	598.13	2
Eos, TGFb+ IL4+	2760.57	1873.28	2
Ag, TGFb+	6106.63	3021.57	2
Eos+Ag, TGFb+	2508.80	738.22	4
Eos+Ag, TGFb-	2348.64	590.29	4
Eos+Ag, TGFb+ aIL4+	1017.61	585.34	2
Eos+Ag, TGFb+ IL4+	1606.85	152.90	2

CHAPTER 4 – DISCUSSION

iTreg Experiment

iTregs were produced from naïve T cells. The naïve T cells were assayed for CD4, CD3, CD45RA, and CD197 expression. The expression of CD45RA was particularly important in identifying naïve T cells, but all markers showed pure naïve T cells. Depending on CD197, or CCR7, expression, and the absence of CD45RA, Memory or effector T cells could instead be characterized. Regulatory T cells were assayed for CD4, CD25, and FOXP3 expression. The presence of TGF-b was clearly required for regulatory T cell character, as shown by the drop in FOXP3 and CD25 expression without TGF-b. In one case, CD25 expression was drastically increased even without TGF-b in the cell culture. This is still consistent with the chosen experimental conditions, as IL2, anti-CD3, and anti-CD28 can plausibly cause increased T cell activation. Thus, the first experiment set up control levels, for reference in the next following experiments.

A quadrant gate would be best to quantify how many CD4+ T cells were both FOXP3+ and also CD25+, thus officially quantifying how many iTregs are produced. However, there is significant variability among donors, and using the naïve T cells with TGF-b as controls for a quadrant gate, results in large variability between each condition, and unusual cut-off values. This could be alleviated by comparing mature periphery regulatory T cells from the same donor, although that would not take into account in vitro conditions over multiple days, and would require more depletion of precious PBMCs. It seems sufficient to analyze the data based off mean expression of individual FOXP3 and CD25 levels.

iTreg Experiment with Eosinophils

Expression of HLA-DR in eosinophils shows how eosinophils can be induced to present antigen. The overnight stimulation demonstrated this and showed an additional mechanism for eosinophils to impact T cell development. Compared to surface expression, intracellular HLA-DR was significantly larger. More research on MHC Class II presentation is needed to make an accurate assessment on the quantity of surface expression, especially considering eosinophils are not professional antigen presenting cells. Regardless, GM-CSF resulted in a noticeable increase in HLA-DR.

When eosinophils, and eosinophils primed to present antigen, were cultured with naïve T cells, the level of FOXP3 expression decreased dramatically (Figure 8). Since there was little expression regardless of the addition of TGF-b or not, increasing the concentration of TGF-b, or conversely, decreasing the amount of eosinophils, may restore FOXP3 expression to levels seen with only naïve T cells. Eosinophils are regarded as part of the type 2 response, and could inhibit naïve T cells from developing into regulatory T cells. The minute differences in FOXP3 between the addition of either eosinophils or primed eosinophils with antigen was quite small and brings into question how much of an effect antigen presentation had. However, the level of CD25 was somewhat extinguished in the eosinophils primed to present antigen (Figure 9b). If the trend continues in future experiments, this could mean that this specific antigen presentation causes not only regulatory T cell suppression, but overall T cell suppression. Interestingly, after adding only antigen to naïve T cells, there seems to be relatively high levels of FOXP3, showing

a lack of T cell suppressive ability from soluble antigen alone (Figure 9a). This may indicate that eosinophils are most responsible for inhibiting iTregs, while the introduction of GM-CSF and antigen inhibits T cell activation altogether. More replicates are need for the naïve T cells with TGF-b and antigen, to see if Aspergillus fumigatus really increases FOXP3 and CD25 expression, or if the results simply match naïve T cells, with TGF-b.

This experiment needs more controls to tease out the actual effects of eosinophils and antigen presentation on T cell development. More cell markers for type 2 cells, and appropriate cell culture conditions, would uncover whether eosinophils actually favor polarization between CD4+ T cell subsets, or simply inhibit regulatory T cell development. In addition, there should be another set of control experiments comparing macrophage and dendritic cell effects, to see if this would normally be applicable . As this only uses eosinophils and Aspergillus fumigatus, these effects may only be applicable to a narrow range of conditions. Further data is needed to see if these trends apply to other fungal antigens and typical antigen presentation.

iTreg Experiment with IL-4

All conditions with TGF-b added were replicated with the addition of anti-IL-4 and IL-4. These experiments were meant to see if eosinphils were inhibiting T regulator cell development by IL-4 secretion. This would explain the decrease in FOXP3 seen across the board, with all of the conditions with eosinophils added. However, the results from two experiments show wide variance in both cases. IL4 seems to have mixed effects with experiment 3, significantly increasing FOXP3 expression past that of the naïve T

cell with TGF-b. Surprisingly, the addition of anti-IL-4 did not cause an increase in FOXP3 expression. These results seem to suggest that a transient level of IL4 may be required for iTreg Foxp3 expression. More importantly, when analyzing the conditions with eosinophils added, this could mean that eosinophils inhibit iTreg development in a manner unrelated to IL4. No major trend could be seen in CD25 expression besides an increase in naïve T cells with anti-IL-4(Figure 10b). Depending on future experiments, there may be an alternate mechanism of IL4-mediated iTreg FOXP3 expression, however the effects so far are unclear.

More experiments required to analyze an effect of IL-4 on naïve T cell development. As stated before, an additional assay for type 2 helper cells would prove helpful in examining how much of a polarizing effect IL-4 has on T cells. Anti-IL4 might be more effective in returning to control levels of FOXP3 expression, if it were applied multiple times. A more thorough titration of varying anti-IL-4 and IL-4 antibody concentrations might also uncover the effects of this important cytokine. Another experiment should be performed with the same conditions, but measuring levels of IL-4 in addition to CD4, CD25, and FOXP3.

Conclusions

These data showed that eosinophils inhibit FOXP3 expression in CD4+ naïve T cells, suggesting the inhibition of their *in vitro* induction into regulatory T cells. The steps taken to purify and isolate eosinophils and naïve T cells were successful, with populations of over 90% purity, and the priming of eosinophils resulted in HLA-DR+ cells, showing plausible antigen presentation capabilities. The inhibitory effects of antigen presentation and IL-4 are still unclear. The expression of FOXP3 in conditions with antigen presentation were similar to the results with unstimulated eosinophils, This trend suggests that eosinophils may be primarily responsible for inhibiting regulatory T cells, and antigen presentation may have a smaller effect. The effects of not just antigen presentation, but Asp f. or GM-CSF by independent cellular mechanisms, may also explain some minor differences, such as the decrease in CD25, compared to unstimulated eosinophils. The trends seen in FOXP3 expression of T cells incubated with anti-IL-4 were unexpected, as anti-IL-4 was known to increase FOXP3+ in regulatory T cells.[13] In comparison, the effects of IL-4 seemed to suggest anti-IL-4 had more of an inhibitory effect. More replicates are needed to verify this trend and show a significant effect. Overall, eosinophils may have an inhibitory effect on regulatory T cell differentiation.

However, there are many limitations, which arise from the methods used for analysis. As the primary quantitative measure was mean fluorescence intensity among CD4+ T cells, the data did not assert a FOXP3+CD25+ phenotype on the T cells. Although the quadrant gate was established by comparing the isotype controls to samples, a more well-established metric would be to gate along positive and negative

FOXP3 and CD25 populations. The clear, polarizing populations were not seen in the other conditions, and thus the induction of regulatory T cells may be questionable. Using multiple markers to distinguish between subsets of T cells is crucial in establishing whether or not a cell has differentiated into a regulatory T cell. A comparative analysis of natural regulatory T cells or CD4+ T cells from the same donor would be helpful in establishing a CD4+CD25+FOXP3+ defined population. In addition, the role of antigen presentation in eosionophils could be further expanded on, beyond the induction of HLa-DR. There were no controls comparing professional antigen presenting cells, such as dendritic cells, and there was no verification of antigen specificity. As the donors were all healthy , the frequency of antigen-specfic CD4+ T cells, would be low. Without a way to analyze antigen-presentation, there might be alternate mechanisms affecting the naïve T cells, with either interactions from Aspergillus fumigatus lysate or GM-CSF. Therefore, further assays should be performed to support the results seen in this thesis.

In the future, an improved induced regulatory T cell model will have to be implemented. Plate bound or coated bead versions of anti-CD3 and anti-CD28 have been known to be a more effective activator than their soluble counterparts.[21] This may be a likely explanation for the issues with characterizing CD4+FOXP3+CD25+ T cells, and the unexpected result with anti-IL-4 and IL-4. Rapamycin or retinoic acid, may also aid in driving iTregs to mimic natural regulatory T cells and express relevant surface markers.[22] Further titration of cytokines and cell activators will have to be performed to compare the effects and their physiological applications. The role of antigen presentation should be further explored, either by adding a comparison of professional antigen

presenting cells, or creating an expanded cell line of antigen-specific T cells. The use of a general antigen specific marker such as CD154 may be helpful in identifying antigen specific cells after exposure to Aspergillus fumigatus.[18] Otherwise, selecting for patients allergic to Asp f. may also increase the frequency of antigen-specific T cells, and thus those likely to show an significant effects as a result of antigen presentation.

The conclusions in this thesis could prove useful, especially in allergy and infections relating to eosinophils and regulatory T cells. By looking beyond the role of regulatory T cells as immunosuppressive cells, we can see how other cells like eosinophils can act in a similar manner. Granulocytes such as neutrophils can also modulate T cell activity, in addition to professional antigen-presenting cells such as dendritic cells regulating T cells as well.[23,24] Antigen specific regulatory T cells can also be indispensible in certain autoimmune diseases.[25] Eosinophilia, a disease characterized by increased number of eosinophils has also been shown to be closely related to regulatory T cells, while also revealing regulatory functions in eosinophils themselves.[26,27] The regulatory role of eosinophils and T cells still needs to be explored, as their interactions could prove pivotal in helping to uncover allergy and autoimmune diseases.

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