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T-REGULATORY CELLS AND FLT3-LIGAND IN THE THERAPY OF COCKROACH ANTIGEN-INDUCED ASTHMA

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A THESIS

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Dedicated to my beloved wife,
Mrs. Geneva Marie McGee

to my loving mother, late father and step-father,
Mrs. Darlene McGee, Mr. Jessie McGee Jr, and Mr. TJ Robinson

to my mother-in-law and late father-in-law,
Mrs. Mary Montgomery-Wright and Robert Wright

to my adoring and tenderhearted late Grandparents,
Mr. Sim Curtis and Mrs. Dora Curtis

to my supportive brothers, sisters, sister-in-law and brother-in-law,
Mr. Jessie McGee, III, Mr. Jerry McGee, and Mr. Avery Robinson and Mrs. Valma Robinson, Mrs. Charmaine Davis-McGee and Mr. Lawrence Davis, and Mrs. Monique McGee

and I thank the Almighty Lord,
for blessing me with perseverance, drive and fortitude in achieving my Doctorate of Philosophy
ABSTRACT

Asthma is a chronic disease of the airways, characterized by airway hyper-responsiveness (AHR) and airway inflammation. Many inflammatory cells, including mast cells, eosinophils, neutrophils, and lymphocytes, are critical in the initiation of the pathophysiological changes in asthma. The mobilization and recruitment of these inflammatory cells are primarily due to the release of pro-inflammatory cytokines (IL-4, IL-5, IL-9 and IL-13) released from TH2 cells. Normally, TH1 responses are presumed to protect from invasive bacterial, protozoa and viral infections, while TH2 responses protect from extracellular, parasites and helminths. One mechanism by which the ratio of TH1 and TH2 cells is maintained is by the immune T cell subset T-regulatory cells (Tregs) (CD4+CD25+). Tregs are divided into two categories: “naturally occurring” CD4+CD25+ T regulatory cells (NTregs), that constitutively express Foxp3 and CD25, and “inducible” CD4+CD25- T regulatory cells (iTregs), which up-regulate CD25 and Foxp3 after exposure to inhaled antigen in the lung. However, how these cells regulate allergic airway inflammation and AHR in asthma remains ill-defined.

In this study, the effect of NTregs and iTregs, isolated from mouse lung and spleen (BALB/c), was examined by adoptive transfer into cockroach (a common inner-city allergen)-sensitized and challenged mice. The adoptive transfer of either of the two subtypes of Tregs reversed AHR and airway inflammation. The cells from the spleen had longer-lasting effect than those of lung cells. Tregs isolated from the lungs of the adoptively-
transferred recipients expressed significantly high levels of IL-10, TGF-β, and Foxp3 transcripts. Bronchoalveolar lavage fluid (BALF) of adoptively-transferred mice had decreased levels of Th2 cytokines, IL-4, IL-5, IL-13, and increased levels of IL-10 compared to cockroach-sensitized and challenged mice without adoptive transfer. The lung T cells isolated from the adoptively-transferred recipients expressed significantly higher levels of programmed death-1 (PD-1), a negative regulator of immune responses. These data suggest a critical role of PD-1 in the reversal of AHR and airway inflammation by Tregs. To confirm the role of PD-1, the effect of anti-PD-1 antagonist was examined. The effects of the adoptive transfer of Tregs were blocked after administration of the anti-PD-1 antagonist. Tregs isolated from the lungs of the mice that received the anti-PD-1 antagonist exhibited a significant decrease of Foxp3 transcripts, an increase in BALF IL-4, IL-5 and IL-13 levels, and a decrease in BALF IL-10 levels. Additionally, CTLA-4, a negative regulatory of T cell activation, was significantly decreased in the lung Tregs of mice that received the anti-PD-1 antagonist.

Flt3-ligand (Flt3-L), a hematopoietic growth factor, dramatically increased the number of dendritic cells in the lungs of Ova-sensitized and challenged mice. Therefore, the effect of Flt3-L on CD4+CD25+ T cells in cockroach-sensitized and challenged mice was examined. These mice received three i.p. injections of anti CD25 antibody (PC61) (250 µg) and Flt3-L (3µg) daily for ten days. Cytokines and immunoglobulins levels in the serum were measured and differential BALF cell counts were examined. Flt3-L reversed AHR to methacholine to the control level. Flt3-L significantly decreased levels of BALF IL-5, IFN-γ, eosinophilia and substantially increased IL-10 and the number of CD4+CD25+Foxp3+IL-10+ T-cells
in the lung. Administration of PC61 antibody blocked the effect of Flt3-L and substantially increased AHR, eosinophilia, BALF IL-5 and IFN-\(\gamma\) levels and decreased BALF IL-10 levels and the number of CD4\(^+\)CD25\(^+\)Foxp3\(^+\)IL-10\(^+\) T-cells. Flt3-L significantly decreased the expression of cell surface receptor CD62L, increased expression of cell surface receptor ICOS and Foxp3 transcripts in CD4\(^+\)CD25\(^+\) T-cells isolated from lungs of CRA-sensitized and challenged mice. Flt3-L significantly inhibited the effect of CRA sensitization to decrease GATA3 expression in lung CD4\(^+\)CD25\(^+\) T-cells.

Finally, the functional effect and migration of Tregs was examined by adoptively transferring naturally occurring CD4\(^+\)CD25\(^+\) T-regulatory cells (NTregs) and CD4\(^+\)CD25\(^-\) inducible T-regulatory cells (iTregs) from lung and spleens of GFP-transgenic BALB/c mice into cockroach-sensitized and challenged mice. Adoptive transfer of either NTregs or iTregs from lung or spleen reversed airway inflammation and AHR to methacholine, and the effect lasted for at least four weeks. GFP-labeled iTregs up-regulated CD25 and Foxp3, and migrated to lymph node and lung. Lung CD4\(^+\)CD25\(^+\) T-cells isolated from each group of recipients mice were ICOS\(^\text{high}\) and PD-1-positive; however, higher expression of PD-1 was found in the spleen iTregs (S25\(^-\)) and lung iTregs (L25\(^-\)) groups. Higher levels of TGF-\(\beta\) and IL-10 transcripts, and BALF IL-10 and INF-\(\gamma\) levels were observed in lung CD4\(^+\)CD25\(^+\) cells from the L25\(^-\) and S25\(^-\) cell-recipient mice than from lung NTregs (L25\(^+\)) and spleen NTregs (S25\(^+\)) cell recipient mice. Adoptive transfer of either cell type significantly reduced BALF IL-4, IL-5 and IL-13 levels.

In conclusion, the findings from this study demonstrated the potential therapeutic properties of T-regulatory cells and the underlying cellular
mechanisms in reversing the deleterious effects of allergen-induced airway inflammation and AHR in a murine model of cockroach antigen-induced asthma. Furthermore, the immunomodulatory effect of Flt3-L in allergic asthma involves an increase in lung CD4^+CD25^+ICOS^+Foxp3^+ T cells with a decrease in CD4^+CD25^+CD62L^- T-cells. The findings from this study may be useful in developing better therapeutic modalities applicable to allergic asthma.
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PRESENTATIONS AT REGIONAL AND NATIONAL MEETINGS


# TABLE OF CONTENTS

Abstract .......................................................................................................................... i
Acknowledgements ......................................................................................................... v
Publications and Abstracts ............................................................................................. vii
Presentations at Regional and National Meetings ....................................................... viii
Table of Contents ......................................................................................................... ix
List of Figures ............................................................................................................... xi
List of Tables ............................................................................................................... xii
Abbreviations .............................................................................................................. xiii

## Chapter 1: Introduction ............................................................................................... 1

### 1.1 Epidemiology of Asthma ..................................................................................... 2
#### 1.1.1 Defining Asthma .......................................................................................... 2
#### 1.1.2 Cockroach Allergen and Asthma ................................................................. 3

### 1.2 Pathophysiology and Development of Asthma .................................................. 4
#### 1.2.1 Sensitization ............................................................................................... 4
#### 1.2.2 Early Phase Response ................................................................................. 4
#### 1.2.3 Late Phase Response .................................................................................. 5

### 1.3 TH2 Cytokines and the Development of Asthma ............................................. 8
#### 1.3.1 Interleukin-4 .............................................................................................. 8
#### 1.3.2 Interleukin-9 .............................................................................................. 9
#### 1.3.3 Interleukin-13 ........................................................................................... 9
#### 1.3.4 GATA-3 and T-bet Transcriptional Factors of TH2 and TH1 cells ............ 10
#### 1.3.4.1 GATA-3 and T-bet ............................................................................... 10

### 1.4 Asthma Development and Control ..................................................................... 10
#### 1.4.1 CD4+CD25+ T-regulatory cells ................................................................... 12
#### 1.4.2 Cell Surface Markers on Tregs ................................................................... 14
##### 1.4.2.1 Programmed Death One (PD-1) ......................................................... 14
##### 1.4.2.2 Cytotoxic T-lymphocyte Antigen ...................................................... 15
##### 1.4.2.3 L-Selectin (CD62-L) .......................................................................... 15
##### 1.4.2.4 Inducible Co-stimulatory Molecule (ICOS) ..................................... 16
##### 1.4.2.5 Neuroplin-1 (Nrp-1) .......................................................................... 17
#### 1.4.3 Cytokines Secreted by Tregs ....................................................................... 18
##### 1.4.3.1 Interleukin-10 .................................................................................. 18
##### 1.4.3.2 Transforming Growth Factor beta ...................................................... 19
##### 1.4.3.3 Interferons ....................................................................................... 19

### 1.5 Foxp3 and the Development of Tregs ................................................................. 21
#### 1.5.1 Foxp3 ........................................................................................................ 21

### 1.6 Role of Fms-like Tyrosine Kinase 3 Ligand in Asthma .................................... 22
#### 1.6.1 Origin of Flt3-L ....................................................................................... 22
3.4 Results of the Initial Adoptive Transfer Study using Donor cells
From Naïve Mice .............................................................................................................. 57

3.4.1 Establishment of AHR in CRA-sensitized and
Challenged mice ............................................................................................................. 57
3.4.2 Purity of Isolated Lung and Spleen CD4+CD25+ T-cells for
Adoptive Transfer ............................................................................................................. 59
3.4.3 The effect of adoptive transfer of NTregs and
CD4+CD25- T-cells .......................................................................................................... 59
3.4.4 Histological changes in the lung tissue post adoptive
Transfer .............................................................................................................................. 60
3.4.5 Cytokines in the BALF ............................................................................................. 65
3.4.6 Inflammatory Cells in the Bronchoalveolar Lavage
Fluid (BALF) ..................................................................................................................... 67
3.4.7 Expression of PD-1 and ICOS in CD4+CD25+ cell
Isolated lungs and spleens recipient mice
Post-adoptive transfer ...................................................................................................... 68
3.4.8 Expression of PD-1, ICOS and Foxp3 in Lung CD4+CD25-
Cells from Recipients of L25- and S25- cells
Post-adoptive Transfer ...................................................................................................... 73
3.4.9 Foxp3 mRNA Transcripts in Donor Cells Prior to
Adoptive Transfer ............................................................................................................. 75
3.4.10 Foxp3 mRNA Transcripts in CD4+CD25+ cells
Isolated from Lungs of Recipient Mice Post-adoptive
Transfer .............................................................................................................................. 75
3.4.11 mRNA Transcripts of Neuropilin-1 (Nrp-1) in
T-regulatory Cells ............................................................................................................. 77
3.4.12 mRNA Transcripts of GATA3 and T-bet Post-adoptive
Transfer .............................................................................................................................. 78
3.4.13 mRNA Expression of IL-10 and TGF-β in Donor cells ........................................... 80
3.4.14 TGF-β and IL-10 mRNA Expression in Lung CD4+CD25+ Cells
Post-adoptive Transfer ...................................................................................................... 80
3.4.15 Flow Cytometric Analysis of Foxp3 Expression in
CD4+CD25+ T-cells Post-adoptive Transfer ..................................................................... 83

3.5 Results of the Subsequent Study Using Donor cells from
GFP-Transgenic for adoptive transfer ................................................................................. 85

3.5.1 Establishment of AHR in CRA-sensitized and
Challenged Mice ................................................................................................................. 85
3.5.2 Number of Adoptively Transferred Tregs Required to
Reverse Existing AHR and Airway Inflammation in
Cockroach-Sensitized and Challenged Mice .................................................................. 86
3.5.3 Effect of Adoptive Transfer of GFP-labeled NTregs
and iTregs cells .................................................................................................................... 95
3.5.4 Migration of GFP-labeled Adoptively Transferred Tregs to Lung, Spleen and Lymph Nodes ........................................... 99
3.5.4.1 GFP-labeled Lung NTregs isolated From Lung and Spleen Tissue ................................................................. 99
3.5.4.2 GFP-labeled Lung iTregs isolated From Lung and Spleen Tissue ................................................................. 102
3.5.4.3 GFP-labeled Spleen NTregs isolated From Lung and Spleen Tissue ................................................................. 105
3.5.4.4 GFP-labeled Spleen iTregs isolated From Lung and Spleen Tissue ................................................................. 108
3.5.4.5 GFP-labeled Tregs Isolated From Lymph Nodes of Adoptively Transferred Recipients ................................. 111
3.5.5 Suppressive Effect of Tregs on the Proliferation of Naïve Splenocytes ................................................................. 113
3.5.6 Number of Lung CD4\(^{+}\)CD25\(^{+}\) Tregs Before and After Adoptive Transfer ........................................ 115

3.6 Discussion .................................................................................. 117

Chapter 4: The Inhibition of PD-1 on Naturally Occurring and Inducible T-Regulatory Cells Exacerbates Airway Hyper-responsiveness and Inflammation in Asthma ........................................... 124

4.1 Abstract .......................................................................................... 125
4.2 Introduction ....................................................................................... 126
4.3 Methods ........................................................................................... 128
4.3.1 Mice ............................................................................................ 128
4.3.2 AHR was Induced by Sensitization and Challenge with Cockroach Antigen (CRA) ................................................ 128
4.3.3 Assessment of Specific Airway Resistance ................................ 128
4.3.4 Isolation of T-regulatory cells for Adoptive Transfer ............... 129
4.3.5 CD4\(^{+}\)CD25\(^{+}\) and CD4\(^{+}\)CD25\(^{-}\) T-cells were Adoptively Transferred with the Administration of the aPD-1\(^{ab}\) into Cockroach-sensitized and Challenged mice .................................................. 129
4.3.6 FACS Analysis ......................................................................... 129

4.4 Results ............................................................................................ 131
4.4.1 Establishment of AHR in CRA-sensitized and Challenged Mice ........................................................................ 131
4.4.2 Effect of aPD-1\(^{ab}\) on AHR Post-Adoptive Transfer ............... 133
4.4.3 Effect of aPD-1\(^{ab}\) on Inflammation in the Airways Post Adoptive ................................................................. 138
4.4.4 Expression of CTLA-4 on CD4\(^{+}\)CD25\(^{+}\) T-cells Isolated
from the Lungs of Recipient Mice Post-adoptive Transfer ..........140

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4.5 The Effect of aPD-1ab Plus Adoptive Transfer on Leukocytes in BALF</td>
<td>142</td>
</tr>
<tr>
<td>4.4.6 Expression of Foxp3 transcripts from CD4+CD25+ T-cells Isolated from Lungs of Mice With and Without aPD-1ab Plus an Adoptive Transfer T-regulatory cells</td>
<td>144</td>
</tr>
<tr>
<td>4.4.7 Measuring Cytokines Levels in the BALF</td>
<td>146</td>
</tr>
</tbody>
</table>

4.5 Discussion .................................................................................................. 148

Chapter 5: Flt3-L Increases CD4+CD25+Foxp3+ICOS+Cells in the Lung of Cockroach-Sensitized and Challenged Mice ........................................................ 152

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1 Abstract</td>
<td>153</td>
</tr>
<tr>
<td>5.2 Introduction</td>
<td>154</td>
</tr>
<tr>
<td>5.3 Materials and Methods</td>
<td>157</td>
</tr>
<tr>
<td>5.3.1 Animals</td>
<td>157</td>
</tr>
<tr>
<td>5.3.2 Sensitization, Treatment and Pulmonary Function</td>
<td>157</td>
</tr>
<tr>
<td>5.3.3 Flow Cytometry and Antibodies</td>
<td>159</td>
</tr>
<tr>
<td>5.3.4 Serum IgE analysis</td>
<td>159</td>
</tr>
<tr>
<td>5.3.5 Serum and Anti-CRA IgE and IgGs analysis</td>
<td>159</td>
</tr>
<tr>
<td>5.3.6 Bronchoalveolar Lavage Fluid (BALF)</td>
<td>160</td>
</tr>
<tr>
<td>5.3.7 Cytokine Assays</td>
<td>160</td>
</tr>
<tr>
<td>5.3.8 Secretion of IL-10 by CD4+CD25+ T-cells from Flt3-L-treated Mice</td>
<td>161</td>
</tr>
<tr>
<td>5.3.9 RT-PCR</td>
<td>161</td>
</tr>
</tbody>
</table>

5.4 Results ........................................................................................................ 162

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4.1 Assessment of AHR to Methacholine in Cockroach-sensitized and Challenged Mice after Flt3-L Treatment</td>
<td>162</td>
</tr>
<tr>
<td>5.4.2 Effect of Flt3-L Treatment on Total and Differential Cells in the BALF</td>
<td>164</td>
</tr>
<tr>
<td>5.4.3 Effect of Flt3-L Treatment on Serum Immunoglobulins in Cockroach Pre-sensitized and Challenged Mice</td>
<td>165</td>
</tr>
<tr>
<td>5.4.4 Effect of Flt3-L Treatment on BALF Cytokines in Cockroach-sensitized and challenged mice</td>
<td>166</td>
</tr>
<tr>
<td>5.4.5 Effect of Flt3-L and PC61 on CD4+CD25+ T-cells Secreting IL-10 in Cockroach-Sensitized and Challenged Mice</td>
<td>167</td>
</tr>
<tr>
<td>5.4.6 Effect of Flt3-L on CD4+CD25+ T-cells Isolated from Lungs of Flt3-L-treated Mice</td>
<td>169</td>
</tr>
<tr>
<td>5.4.7 Effect of Flt3-L on the Expression of CD62L in CD4+CD25+ T-cells Isolated from the Lungs of</td>
<td>169</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

1. Primed Mast Cells cause Smooth Muscle Cell constriction .................................. 5
2. Repeated Antigen Exposure Leads to Late Phase Response ............................... 6
3. TH2 Cytokines Mediate the Progression of Airway Inflammation .................... 8
4. Development and Function of T-cell Subtypes ................................................. 11
5. WBP Diagram .................................................................................................32
6. Penh Algorithm ............................................................................................. 34
7. GFP and Black Light Excitation Spectrum ...................................................... 40
8. Positive Expression of GFP in BALB/c Mice ................................................ 42
9. Cockroach-sensitized and Adoptive Transfer Protocol ..................................... 50
10. Day 33 Non-invasive and Invasive Pulmonary Analysis .................................. 58
11. Purity of Lung and Spleen CD4⁺CD25⁺ T-cells .............................................. 59
12. Pulmonary Evaluation Post-adoptive Transfer ............................................... 60
13. Histological evaluation post adoptive transfer .............................................. 62
14. Cytokines secreted in the BALF Post-adoptive Transfer Day 68 .................. 66
15. The Expression of PD-1 in Lung and Spleen CD4⁺CD25⁺ T-cells Post-adoptive Transfer ................................................................. 70
16. The Expression of ICOS in Lung and Spleen CD4⁺CD25⁺ T-cells Post-adoptive Transfer ................................................................. 72
17. Expression of PD-1, ICOS, and Foxp3 in lung CD4⁺CD25⁻ T-cells post-adoptive transfer ................................................................. 74
18. Expression of Foxp3 transcripts (420 bp) in CD4⁺CD25⁺ T-cells from Donor Mice and Post-adoptive...
Transfer Recipients.................................................................76

19. Expression of Neuropilin-1 transcripts (375 bp) in Lung CD4+CD25+ Cells Post-adoptive Transfer..................................................77

20. Expression of GATA3 (710 bp) and T-bet (425 bp) transcripts in Lung CD4+CD25+ T-cells Post-adoptive Transfer.........................79

21. Expression of TGF-β (400 bp) and IL-10 (610 bp) Transcripts in the Lung CD4+CD25+ donor cells and in lung CD4+CD25+ T-cells post-adoptive transfer .................................................................81

22. Intracellular Protein Expression of Foxp3 in Lung CD4+CD25+ T-cells post-adoptive Transfer.........................................................84

23. Non-invasive and Invasive Pulmonary functions.................................85

24. Dose-Dependent Study to Determine the Optimal Number of CD4+CD25+ and CD4+CD25+ T-cells Needed for Adoptive Transfer to Reverse AHR and Airway Inflammation.................................87

25. Pulmonary evaluation post adoptive transfer of GFP-labeled Tregs........96

26. Flow Cytometric Analysis of Isolated GFP-Positive Lung CD4+CD25+ NTregs from Lung and Spleen Tissue ..............................................100

27. Flow Cytometric Analysis of Isolated GFP-Positive Lung CD4+CD25+ iTregs from Lung and Spleen Tissue.................................................103

28. Flow Cytometric Analysis of Isolated GFP-Positive Spleen CD4+CD25+ NTregs from the lung and spleen tissue...............................106

29. Flow Cytometric Analysis of Isolated GFP-Positive Spleen CD4+CD25+ iTregs from the Lung and Spleen tissue.................................109

30. Flow Cytometric Analysis of Isolated GFP-Positive Lung and Spleen
CD4\(^+\)CD25\(^+\) Tregs from Lymph nodes ................................................................. 112

31. Suppression of Naïve Splenocytes by Lung and Spleen GFP-labeled
and Naïve Tregs .................................................................................................. 114

32. Number of Lung CD4\(^+\)CD25\(^+\) Tregs Before and After Adoptive
Transfer ........................................................................................................ 115

33. Protocol to Sensitize BALB/c Mice with Cockroach
Antigen ........................................................................................................ 131

34. Examine AHR to Methacholine in Sensitized Mice.............................. 132

35. Pulmonary Evaluation Post Adoptive Transfer with aPD-1 therapy ...... 134

36. Lung sections were Evaluated after aPD-1\(^{ab}\) Treatment Day 68 .......... 139

37. Expression of CTLA-4 on Lung CD4\(^+\)CD25\(^+\) T-cells
Post-adoptive Transfer ................................................................................ 141

38. Leukocytes in BALF Post-adoptive Transfer ...................................... 143

39. Expression of Foxp3 in Lung CD4\(^+\)CD25\(^+\) T-cells
Post-adoptive Transfer with and without aPD-1\(^{ab}\) ...................................... 145

40. Protocol for Administration of PC61 and Flt3-L Treatment in Cockroach-
sensitized and Challenged Mice............................................................... 158

41. AHR and Specific Airway Resistance to Methacholine...................... 162

42. Evaluate AHR to Methacholine After Treatment with PC61 and
Flt3-L .............................................................................................................. 163

43. Effect of Flt3-L and PC61 Treatment on BALF and Cellularity in
CRA-sensitized mice .................................................................................. 164
44. BALF Cytokine Levels After Treatment with PC61 and Flt3-L in Cockroach-
sensitized and Challenged Mice ................................................................. 166

45. Effect of Flt3-L and PC61 Treatment on IL-10-Secreting
    CD4^+CD25^+ T-Regulatory Cells ............................................................. 168

46. Expression of CD4^+CD25^+ T-cells ......................................................... 169

47. Expression of CD62L on CD4^+CD25^+ T-cells ........................................... 171

48. Expression of ICOS on CD4^+CD25^+ T-cells ............................................. 173

49. The effect of Flt3-L on Lung CD4^+CD25^+ T-cells expressing
    Foxp3 and GATA3 ..................................................................................... 175

50. Intracellular Protein Expression of Foxp3 and the Number of Lung
    CD4^+CD25^+ After Flt3-L and PC61 Treatment ......................................... 177
**LIST OF TABLES**

1. Materials used in experimental procedures............................................................... 30
2. Primers used in RT-PCR reactions............................................................................. 43
3. Absolute Number of BALF Leukocytes...................................................................... 68
4. Measurement of BALF Cytokines after Treatment with aPD-1\textsuperscript{ab}................ 147
5. Serum immunoglobulin levels.................................................................................... 165
ABBREVIATIONS

AHR (Airway hyper-responsiveness)

aPD-1\textsuperscript{ab} (anti-PD1 antibody plus an adoptive transfer)

BALF (Bronchoalveolar Lavage Fluid)

BrdU (Bromodeoxyuridine)

CRA (Cockroach Antigen)

CRA\textsuperscript{AT+} (Cockroach sensitized and challenged mice with Adoptive transfer)

CRA\textsuperscript{AT-} (Cockroach sensitized and challenged mice without Adoptive transfer)

DNA (Deoxyribonucleic acid)

ELISA (Enzyme-Linked Immuno-Sorbent Assay)

Fc\varepsilon R1 (FC Episilon Receptor One)

FITC (Fluorescein Isothiocyanate)

Flt3-L (Fms-like tyrosine kinase 3 ligand)

Foxp3 (Forkhead winged transcription factor box P3)

GATA-3 (GATA3 Binding Protein 3)

GFP (Green Fluorescent Protein)

GITR (Glucocorticoid induced tumor necrosis factor receptor)

ITAM (Immunoreceptor tyrosine-based activation motif)

ITIM (Immunoreceptor tyrosine-based inhibitor motif)

H1 (Histamine 1 receptor)

H and E (Hematoxylin and Eosin)

HES1 (Hairy and enhancer split homologue-1)

HPRT (Hypoxanthine-guanine phosphoribosyl transferase)
ICOS (Inducible costimulatory molecule)
IgE (Immunoglobulin E)
IL-2 (Interleukin-2)
IL-4 (Interleukin-4)
IL-5 (Interleukin-5)
IL-6 (Interleukin-6)
IL-9 (Interleukin-9)
IL-10 (Interleukin-10)
IL-13 (Interleukin-13)
IPEX (Immune dysregulatory, polyendocrinopathy, enteropathy, X-linked syndrome)
iTregs (Inducible CD4+CD25- T-regulatory cells)
KO (Knock-out)
L25+ (Lung NTregs)
L25- (Lung iTregs)
L-Selectin (CD62L)
mRNA (Messenger RNA)
Mch (Methacholine)
NF-κβ (Nuclear factor kappa beta)
Nrp-1 (Neuropilin-one)
NTregs (Naturally occurring CD4+CD25+ T-regulatory cell)
OVA (Ovalbumin)
PAS (Periodic acid-Schiff Stain)
PBS (Phosphate Buffer Saline)
PBSAT+ (PBS control with adoptive transfer)
PBS + aPDab (PBS control with aPD-1ab)
PBS4 (Phosphate Buffer Saline + 4% fetal calf serum)
PC61 (anti CD25 antibody)
PCR (Polymerase Chain Reaction)
PD-1 (Programmed death one)
PEF (Peak expiratory flow)
PIF (Peak inspiratory flow)
Penh (Enhanced Pause)
RNA (Ribonucleic acid)
RT-PCR (Reverse-Transcriptase Polymerase Chain Reaction)
S25+ (Spleen NTregs)
S25- (Spleen iTregs)
STATs (Signal Transducer and Activator of Transcription 4, 5 and 6)
T-Bet (T-box Transcriptional Factor for T-cells)
Te (Total Expiratory phase)
TGF-β (Transforming Growth Factor Beta-1, 2 and 3)
Th1 (T-helper Type One)
Th2 (T-helper Type Two)
TNF-α (Tumor Necrosis Factor Alpha)
TNF-β (Tumor Necrosis Factor Beta)
Tr (Time Relax)
TR1 (Type One T-regulatory cell)
Tregs (T-regulatory cells)
UV (Ultra violet)
Chapter 1

INTRODUCTION
1.1 Epidemiology of Asthma

Asthma is a chronic inflammatory disease of the airways with cardinal features of airway hyper-responsiveness (AHR), reversible airway obstruction, chronic airway inflammation and airway remodeling (1). Asthma is considered a major public health problem(2) particularly for children under the age of 5 (3). Worldwide, this disease afflicts more than 300 million people of all ages and ethnic backgrounds (4, 5). Asthma episodes cause 500,000 hospitalizations, nearly 2 million emergency room visits and more than 5000 deaths per year(6). Asthma is the third ranking cause of hospitalization for children under 15 years old and it is the fourth most common cause for office visits for health care professionals (7). Nationally, this disease is the leading cause for school absenteeism attributed for chronic illness which accounts for more than 14 million lost school days annually (2). Despite advances in research, the worldwide prevalence, morbidity, and mortality of asthma have dramatically increased over the last two decades (8).

1.1.1 Defining Asthma

Asthma causes recurring episodes of wheezing and airway inflammation(9). Chronically inflamed airways are hyper-responsive; they become obstructed and airflow is blocked when the airways are exposed to provoking environmental factors such as allergens, irritants, cold air, and exercise (10). This causes the difficulty of moving air flow in and out of the bronchiole tree (11). The narrowing or obstruction can cause one or a combination of symptoms to include shortness of breath, wheezing, chest tightness (12) particular at night or in the early morning hours(13). Asthma attacks can vary from person-to-person and the exacerbations are episodic; however, airway inflammation is
chronically present. Many people with asthma require daily medications to control symptoms, improve lung function, and prevent attacks.

### 1.1.2 Cockroach Allergen and Asthma

When most people think of allergy "triggers," they often focus on plant pollens, dust, animals and stinging insects; however, cockroaches have been shown to be potent triggers for bronchial asthma and allergic rhinitis in sensitive patients (14). In the 1970s, studies demonstrated that patients with cockroach allergies develop acute asthma(15). Cockroach allergen is derived from feces, saliva and the bodies of these insects(16). The most common cockroaches encountered in the home are Blatella germanica (German cockroach), Periplaneta americana (American cockroach) and Blattella orientalis (Oriental cockroach). The German cockroach, an indoor insect is the most common and immunogenic (16, 17). Cockroach infested homes increase the rate of hospitalization for asthmatics (18). In (37- to- 85 percent) of urban homes, cockroaches are found and among inner city children morbidity was associated with positive skin test and high levels of cockroach allergen in the bedroom(19). Cockroach allergy is a major health problem among people who live in large-cities, the South and are of low socioeconomic status (20, 21). The National Cooperative Inner City Asthma Study recently demonstrated that cockroach allergic children with asthma from U.S. inner city communities are at risk for greater asthma morbidity if elevated amounts of cockroach allergen are present in their homes (22). Cockroach allergy is more common among poor African Americans; however, experts believe that this is not because of racial differences; rather, it is because of the disproportionate number of African Americans living in the inner cities (23).
1.2 Pathophysiology and Development of Asthma: An allergic reaction is a complex condition that includes: (i) Sensitization to environmental antigen, (ii) Early-phase (acute) response and (iii) Late phase (chronic) response after constant exposure to inhaled antigen.

1.2.1 Sensitization: Inhaled antigen such as pollen, molds, house dust mites and cockroach antigen (common-inner city allergen) is presented to TH2 cells by antigen presenting cells (24). Interleukin-4 secreted by activated TH2 cells causes B-cells to isotype to IgE (25). These activated B-cells differentiate into plasma cells that produce high levels of IgE (26). These immunoglobulins bind to high affinity FceR1 receptors on mast cells (27).

1.2.2 Early-phase response: When the immune system is re-exposed, primed mast cells expressing IgE will bind inhaled allergen molecules by crosslinking of an adjacent Fab components of another IgE molecule (28, 29). Once sufficient activation occurs, the mast cells degranulate and release copious levels of histamine and other chemical mediators (30). The secretion of histamine binds to the histamine receptors (H1) on the airway smooth muscle cells(31) this is often characterized as the initial period of broncho-constriction and maximal airway narrowing can occur within 15–30 min (32). Furthermore, mediators such as leukotrienes help contribute to the symptoms of wheezing, sneezing, coughing, watery eyes, runny nose, and shortness of breath(33) (Figure 1).
Figure 1: Primed Mast Cells cause Smooth Muscle Cell Constriction - Upon antigen inhalation IgE is secreted from plasma cells. IgE binds to mast cells and the antigen crosslinks two IgE immunogloblins on the mast cells which causes the process of degranulation.

1.2.3 Late-phase response: Continuous allergen exposure leads to the increase of IgE-coated mast cells to cross the epithelial layer of the airways (34). These allergens will crosslink the bound IgE molecules on tissue mast cells causing more mast cell degranulation (35). Mast cells also secrete inflammatory mediators such as prostaglandin D2 and sulfidopeptidyl leukotrienes C4, D4 and E4(36). The latter causes blood vessels to become more permeable and thus leads to the clinical diagnosis of mucosal edema and watery rhinorrhea (Figure 2).
**Figure 2: Repeated Antigen Exposure Leads to Late Phase Response.** Upon repeated exposure to inhaled antigen mast cells continue to release inflammatory mediators and transverse through the epithelial cell layer.

The morphological changes seen in asthmatic airways caused by the cytokine milieu produced by TH2 cells promotes the recruitment of eosinophils and neutrophils (37). These cells penetrate and disrupt the basement membrane which is the onset of hypertrophy to the epithelial cell layer (38). The constant trafficking of these inflammatory cells results in the sloughing of the columnar epithelial cells called creola bodies. Within this columnar epithelial layer are goblets cells. When these cells are continuously exposed to cytokines, particularly IL-13, they become hyperplastic (39) and
secrete prodigious amounts of mucus. In addition, TGF-β released from eosinophils promotes the development and proliferation of myofibroblasts to differentiate into fibroblasts \(40\). These mesenchymal-derived cells secrete a deleterious source of collagen. This extra-cellular matrix causes thickening of the airways and is postulated that this change prevents smooth muscle cells from relaxing \(41\). In addition, activated fibroblasts secrete eotaxin, a potent eosinophil chemoattractant, increases pathology in the disease (Figure 3) \(42\). These events are defined as airway remodeling and are seen in the airways of postmortem laboratory animals and human\(43\). Asthma is a complex disease, and the current therapies lack the ability to completely prevent or reverse the remodeling of the airways. Hence, the need for new therapeutic strategies to manage, control and prevent increase incidence of allergic asthma.
Figure 3: TH2 Cytokines Mediate the Progression of Airway Inflammation.
The pro-inflammatory cytokines released by TH2 cells causes IgE production, eosinophil recruitment, mast cells survival, epithelial cell hypertrophy and mucus hyper-secretion from goblet cells.

1.3 TH2 Cytokines, GATA-3, T-bet and the Development of Allergic Asthma

1.3.1 Interleukin-4

Interleukin-4 (IL-4) is a pro-inflammatory cytokine that promotes the development of allergic inflammation. IL-4 causes B-cells to undergo isotype switch and differentiate into plasma cells that secrete IgE (44). IgE-mediated immune responses are further enhanced by IL-4 through its ability to cause the upregulation of IgE receptors on the
cell surface: the low-affinity IgE receptor (FcεRII; CD23) on B-lymphocytes and mononuclear phagocytic cells, and the high-affinity IgE receptor (FcεRI) on mast cells and basophils (45). IgE-dependent mast cell activation induced by IL-4 has a pivotal role in the development of immediate allergic reactions. An additional mechanism by which IL-4 contributes to airway obstruction in asthma is through the induction of mucin gene expression and the hypersecretion of mucus (46). IL-4 increases eotaxin and other inflammatory cytokine secretion from fibroblasts that contribute to inflammation and lung remodeling in chronic asthma (47).

1.3.2 Interleukin -9

Interleukin (IL) -9 has pleiotropic activities and has been implicated in asthma pathogenesis(48). Arras and colleagues demonstrated that IL-9 can modulate the development lung fibrosis(49). IL-9 was discovered independently as a growth factor for activated T-cells (49). Subsequently, IL-9 was also shown to recruit and maintain mast cell function (50) particularly in asthmatic patients (51). Studies on transgenic mice indicated that IL-9 induces the accumulation of mast cells in mucosal tissues (52). IL-9 expression is increased in atopic asthma and induced in the lung on local allergen challenge (48, 53).

1.3.3 Interleukin- 13

Interleukin-13 (IL-13) is a TH2 cell cytokine implicated as a key mediator of inflammation in respiratory diseases including asthma (54). In animal models of pulmonary inflammation, IL-13 has been demonstrated to play a key role in the development of airway inflammation and a goblet cell metaplastic phenotype(55). In
addition, IL-13 has been shown to induce the expression of MUC5AC and the density of goblet cells in the airways (55).

1.3.4  **GATA-3 and T-bet Transcriptional factors of TH2 and TH1 cells**

1.3.4.1  **GATA-3 and T-bet**

GATA3 is the master transcription factor for TH2 cell differentiation. GATA3 is selectively expressed in TH2 cells and its ectopic expression induces TH2 cell differentiation even in the absence of STAT6 (56, 57). T-bet a member of the T-box family of transcription factors whose expression is primarily limited to the immune system, is rapidly induced in early developing TH1 cells and is absent in developing TH2 cells (58).

1.4  **Asthma Development and Control**

Asthma is an inflammatory disease of the lungs that is characterized by airway hyper-responsiveness (AHR) and airway inflammation (59). Eosinophils, mast cells and B-lymphocytes are the inflammatory cells critical in the initiating patho-physiological changes in the airways of atopic subjects with asthma (60). However, the recruitment and control of these inflammatory cells is mediated by TH2 cells that have differentiated from naïve CD4+ T-cells (61). Progressive accumulation of activated CD4+ T cells in the lamina propria of the airways is associated with development of the clinical features of asthma in individuals who previously exhibited asymptomatic AHR (62). Increased numbers of activated CD4+ T-cells are recruited into the airways of asthmatics following allergen challenge (62). In asthma there is an imbalance in the ratio of TH1 to TH2 cells in the lung. In general, TH1 cells secrete IL-2 and IFN-γ thereby, activating cell-
mediated immunity to fight against viruses and bacteria (63). On the other hand, TH2 cells secrete IL-4, IL-5, IL-9, and IL-13, to activate humoral immunity to protect against multi-cellular parasites and helminths (64). To achieve a fine balance between these two drastically different immunological outcomes, CD4+CD25+ T-regulatory cells (Tregs) exists to regulate the effector T-cell response (65) (Figure 4).

**Figure 4: Development and Function of T-cell Subtypes.** Differentiation of naïve CD4+ T-cells is dependent on the cytokine environment as well as genetic disposition. The development of a skewed TH2 cell ratio leads to asthma and the balance of TH1, TH2 and possibly TH17 cells is mediated by Foxp3+ T-regulatory cells.
1.4.1 CD4⁺CD25⁺ T-regulatory cells

Historically, the idea of suppressive T-lymphocytes was first observed in the CD8 T-regulatory cells and originated in the laboratory of Richard Gershon at Yale in the 1970s (66). Together with his student Kondo he established that thymus-derived lymphocytes have the ability of exerting a specific negative regulatory effect in immune responses and called these cells suppressor T-cells (67). However, due to not having the technology that we have today Dr. Gershon was unable to characterize this unique T-cell population. Furthermore, in a remarkable series of experiments by Dr. Shimon Sakaguchi of Tokyo, Japan he discovered if a thymectomy wasn’t done for several days in newborn mice, the animals initially survive quite well because sufficient T-cells developed to protect against infection. However, weeks later they found that these mice develop extensive and severe autoimmune disease (68). By the mid-1980s, virtually all researchers had abandoned the concept of suppressor T-cells except Dr. Sakaguchi, who continued his quest to learn more about this elusive T-cell subset. In 1995, Dr. Sakaguchi and his colleagues dubbed the term T-regulatory cells by the discovery of the novel cell surface protein CD25, as well as the ubiquitously expressed surface protein CD4 (69). To date, CD4⁺CD25⁺ T-regulatory cells (Tregs) that express CD25, the alpha chain of the IL-2 receptor complex are considered the cornerstones for immune homeostasis (70). Over the last five years, two distinct subsets of Tregs have been identified: ‘naturally occurring’ CD4⁺CD25⁺ T-regulatory cells (NTregs) that constitutively express the CD25 and ‘inducible’ CD4⁺CD25⁻ T-cells (iTregs) which are the precursors to T-regulatory type 1 cells (T₉₁) that up-regulate CD25 with repeated exposure to immature dendritic cells (71), or activation in the presence of IL-10 alone, or
in combination with TGF-β (72). NTregs represent approximately 5 to 10% of the peripheral CD4+ T-cells in human subjects and mice (73), and constitutively express the Forkhead-winged transcriptional factor box protein 3 (Foxp3) (74). Compelling evidence demonstrate the necessity of Tregs to regulate immune response (75). Mutations in the X-linked Foxp3 gene in scurvy mice leads to an autoimmune disease characterized by multi-organ lymphocytic infiltration that results in enlarged spleen, lymph nodes, and liver, as well as dermatitis and severe runting (76). In human, mutations in FOXP3 have been linked to autoimmune diseases including X-linked autoimmunity allergic dis regulation syndrome (77), X-linked autoimmunity immune deficiency syndrome (78, 79), and Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX) (74, 80). A striking feature of NTregs is that the majority are spawned from the normal thymus as a functionally unique and mature subpopulation of T-cells that exist in circulation with stable function (81).

Conversely, IL-10 producing CD4+ type 1 (T$_{R1}$) cells were originally derived from patients with severe combined immunodeficiency who had undergone successful HLA-mismatched bone marrow transplantation (82). T$_{R1}$ cell precursors are naive CD4+CD25- T-cells, which on encountering antigens presented by immature dendritic cells are induced to differentiate and expand in the microenvironment enriched in inhibitory cytokines, notably IL-10 (83). Although T$_{R1}$ cells are induced through IL-10-dependent mechanisms, their unique feature is secretion of immuno-inhibitory cytokines IL-10 and TGF-β. First described in allergy (84) and autoimmune disorders, T$_{R1}$ cells are also thought to mediate suppression of antitumor immune responses in human cancer (85).
TR1 cells display a unique profile of cytokine production that is distinct from Th0 cells, which secrete both Th1 and Th2 cytokines albeit at very low levels (86-88). Th1 cells secrete IFN-\(\gamma\), TNF-\(\beta\), and IL-2 (86, 87, 89), and Th2 cells secrete IL-4, IL-5, IL-6, IL-9 and IL-13 (86, 88). TR1 cells are characterized by the secretion of copious amounts of immuno-suppressive cytokines IL-10 and TGF-\(\beta\), mild-to-moderate levels of IFN-\(\gamma\) and IL-5, low-to-absent secretion of IL-2 and IL-4 (83, 90), and expression of CTLA-4 and Programmed death one (PD-1).

1.4.2 Cell Surface Markers on Tregs

1.4.2.1 Programmed Death One (PD-1)

PD-1 (CD279) receptor is 55 kDa type I transmembrane protein of the Ig superfamily, with an extracellular region having one V-like domain. Two ligands for PD-1 (CD279), have been identified: PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) (91). Interaction of PD-1 with PD-L1 or PD-L2 has been characterized as a negative modulator for cytokine production and T-cell proliferation. The intracellular region contains two tyrosine residues: (1) an immuno-receptor tyrosine inhibitor motif (ITIM), and (2) an immuno-receptor tyrosine switch motif (ITSM). PD-1–PD-L1 belongs to the CD28–B7 signaling family, and this interaction mediates the down-regulation of T-cell activity (92). Latchman et al, showed that the interaction of PD-L1 and PD-1 leads to cell cycle arrest in G0/G1 phase, but it does not increase cell death (93). In addition, PD-L2 has been shown to have an affinity for PD-1 that is two to six times higher than that of PD-L1 (94, 95). Independent investigators have reported that tyrosine phosphorylation of PD-1 in the ITSM, rather than in the ITIM, was responsible for
inhibitory signaling via recruitment of Src homology domain 2-containing tyrosine phosphatase-1 (SHP-1) and SHP-2 (93, 96, 97).

1.4.2.2 CTLA-4 (CD152)

CTLA-4 (CD152) is a member of the CD28-family receptors and is expressed on CD4+ T-cells (98). It is a type 1 trans-membrane glycoprotein of the immunogloblin superfamily, 223 amino acids (aa) in length, with a 35 aa signal peptide and exist as a covalent homo-dimer of 41-43 kDa (99). The extracellular architecture of CTLA-4 is characterized by a single IgV-like domain (100). CTLA-4 binds the same ligands as CD28 (CD80 and CD86) on B-cells and dendritic cells but, with higher affinity than CD28. CTLA-4 inhibits T-cell proliferation, unlike CD28 which delivers an activation signal to stimulate cytokine production and cell survival (101). Unlike CD28, CTLA-4 down-regulates IL-2 production and inhibits cell-cycle progression in T-cells, thereby depressing T-cell proliferation (102). CTLA-4 is also found on T-regulatory cells and may be important for their function. NTregs constitutively express CTLA-4, which only happens after activation in other T-cell subsets, and evidence has suggested that Foxp3 controls the expression of CTLA-4 in Tregs (103)

1.4.2.3 L-selectin (CD62L)

CD62L is of the L-selectin family of adhesion molecule and is a single-pass type-1 334 amino acid glycoprotein (104). Extravasation and mobilization of leukocytes to inflammatory sites are driven by differential expression of multiple surface adhesion molecules. These molecules have been classified into several families, of which the selectins are one (105). In general, high expression of CD62L on naïve T-cells allows
these cells to migrate into the lymph nodes by binding chemokines CCR6 and/or CCR7 (106), where they encounter antigen presented by dendritic cells (107). Conversely, down-regulation of CD62-L allows activated T-cells to leave the lymph node and migrate to the site of inflammation (108). Studies have shown that CD62L\textsuperscript{high} and CD62L\textsuperscript{low} Treg subsets have been reported in several studies to harbor a similar in vitro suppressive capacity (109, 110), therefore, the \textit{in vivo} regulatory capacity of the CD62L\textsuperscript{high} subset in these models most probably reflects differences in homing properties, rather than suppressor potential. Cockroach-sensitized and challenged mice treated with Flt3-L caused a significant reduction of CD62L expression on lung CD4\textsuperscript{+}CD25\textsuperscript{+}ICOS\textsuperscript{+}Foxp3\textsuperscript{+} Tregs and these cells reversed established AHR and allergic airway inflammation (111).

\textbf{1.4.2.4 Inducible Co-stimulatory Molecule}

ICOS is a marker that is up-regulated on activated T-cells in the presence of antigen(112). ICOS has been shown to play an important role in the Th2 polarized responses in the airways, as inhibition of ICOS suppresses lung inflammation and Th2 cytokine production (113). Similarly, anti ICOS monoclonal antibody for blocking B7RP-1 the ligand for ICOS, increases allergic airway inflammation (114). These studies parallel with studies examining the role of ICOS in memory or recall responses in the lung, which appear to be ICOS-independent and suggest that other molecules are more important (114). More recently, it has been reported that, similar to CD28, ICOS may regulate the function of TR1 cells secreting IL-10 (115). The mechanism for ICOS and IL-10 has not been clearly defined; however, it has been suggested that B7RP-1 engaging CD4\textsuperscript{+}ICOS\textsuperscript{+} T-cells induces high expression of p50\alpha a regulatory subunit of PI3K,
carries stronger lipid kinase activity than p85α on the synapse after DC/T-cell interaction. CD4+ICOS+ T-cells express significantly high levels of PI3K(116). In addition, CD4+ T-cells that have differentiated into CD4+Foxp3+ Tregs and the expression of AKT has been implicated in the development of Tregs (117). Additionally, PI3K-Akt pathway has been shown to contribute to activation of NF-kB by the phosphorylation of IkBα(118). In addition, it has also been suggested that PI3K-Akt can phosphorylate IKKα and activate IKK complex, leading to NF-kB activation(119). The report that T-regulatory cells require ICOS to be able to suppress immune responses may provide a mechanism for the dramatic augmentation of disease severity observed in EAE when ICOS is inhibited (120). A study in EAE demonstrated the role of ICOS by using blocking antibody for ICOS during immune response (9-20 days after immunization) and this blocked disease development. However, blockade of ICOS during antigen priming (1-10 days after immunization) caused the disease progression. Interestingly, these data suggest that inhibition of a positive costimulatory molecule such as ICOS may augment inflammation by inhibition of regulatory or suppressor subsets of cells (121). These data suggest that the expression of ICOS on Tregs regulates the secretion of IL-10; therefore, blocking ICOS appears to inhibit the suppressive function of Tregs.

1.4.2.5 Neuropilin-1 (Nrp-1)

Nrp-1 was initially described as a cell surface glycoprotein expressed on axons in the developing nervous system and was shown to be a receptor for semaphorin (SEM3D) a family of transmembrane and secreted glycoproteins that act as mediators of neuronal guidance(122). Recently, Neuropilin-1 was found in primary immune response(123). Nrp-1 caused polarization of T cells after the interaction between DCs and resting T cells
in the initiation of the primary immune response (51). Furthermore, Bruder and colleagues demonstrated Nrp-1 expression is significantly down-regulated after T cell activation (52). Moreover, Nrp-1 expression on Tregs correlates with the expression of Foxp3, thus representing a novel cell surface marker to identify Tregs and their separation from recently activated T cells (124).

1.4.3 Cytokines secreted by Tregs

1.4.3.1 Interleukin-10

Interleukin 10 (IL-10) was first identified in 1989, as a cytokine synthesis inhibitor produced by activated TH2 cells (125). It is an 18.5 Kd cytokine with a broad immuno-regulatory activity(126). It is produced by several cell types, including monocytes, macrophages, B-cells, dendritic cells, mast cells and various tumor cell lines(127). Recently over the last five years is been shown to be released from T-regulatory cells (88). It’s main biological functions seem to limit and terminate inflammatory responses, block the pro-inflammatory cytokine secretion and regulate the differentiation and proliferation of several immune cells such as T-cells, B-cells, natural killer cells, antigen-presenting cells, mast cells, and granulocytes (128). IL-10 binds as a 2-fold symmetric homodimer to a functional tetrameric complex of two receptors (129), consisting of two α- or R1 chains which bind to IL-10, and of two CRF2-4 chains (β- or R2) which initiate the IL-10-induced signal transduction events. CRF2-4 is a member of the class II cytokine receptor family (CRF-2), which includes the IFN receptors and is encoded by the CRFB4 gene on chromosome 21 (130). IL-10R signals through the JAK1-STAT3 pathway and activates the SOCS-3 gene (Suppressor of cytokine signalling-3) which results in inhibition of JAK/STAT-dependent
IL-10 inhibits the synthesis of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6), and the Th2 cell-derived cytokines (IL-4 and IL-5) (127).

1.4.3.2 Transforming Growth Factor Beta

Transforming growth factor-β (TGF-β) is a multifunctional cytokine regulating T-cell growth and development (132). TGF-β has three isoforms to include TGF-β1, TGF-β2, and TGF-β3. Evidence has shown that TGF-β has pro-inflammatory and anti-inflammatory effects (133). Marinova-Mutafchieva et al. showed in patients with arthritis TGF-β1 and TGF-β2 were significantly decreased during disease remission (134). However, in pulmonary fibrotic diseases, TGF-β causes the accumulation of extracellular matrix and induces trans-differentiation of fibroblasts to myofibroblasts (133). TGF-β1 has also been shown to inhibit IL-2 production, up-regulate cell-cycle inhibitors, and has potent anti-proliferative effects on CD4+ T-lymphocytes (135). TGF-β induces differentiation of naïve CD4+CD25- T-cells into CD4+CD25+ Foxp3+ T-regulatory cells (TR1) (136). In addition, TR1 cells secrete TGF-β and IL-10 to suppress auto-reactive T-cells to restore immune tolerance (137-140). Luttman et al. showed eosinophils treated with low concentrations of TGF-β1 promotes the chemotaxis of eosinophils; however, high concentrations of TGF-β1 leads to a reduction of eosinophil survival even in the presence of IL-3, IL-5, and GM-SCF (141).

1.4.3.3 Interferons

The interferons are a large family of multifunctional proteins involved in antiviral defenses, regulating cell growth, and activation of the immune system (142, 143). Type I interferons (IFNs) consist of IFN-α and β, which are the first line of defense against
viruses (144). Both IFN-α and β protect against viruses directly by blocking virus replication in the cells and indirectly by activating the innate and adaptive immune responses (144). IFN-α and β, directly inhibit viral activity by a several mechanisms such as, preventing virus from entering the cell, regulating viral transcription, cleavage of RNA, and inhibit translation (145, 146). Indirectly, IFN-α and β, by virtue of their immunoregulatory properties, stimulate the innate and adaptive immune responses. For example, stimulating cytotoxicity levels from natural killer cell and up-regulate MHC class I molecules various cells and costimulatory molecules on APCs (147, 148). In addition, IFN-α and β increase cross-presentation of exogenous antigen in MHC class I molecules and promote the expansion of T-cells (149). Type II IFN is represented by IFN-γ instead of being activated by virus infection, IFN-γ is produced in response to the recognition of infected cells by activated T lymphocytes and natural killer (NK) cells(143). IFN-γ secretion by macrophages and DCs acting locally may be required for self-activation of cells and the stimulation of other cells in close proximity. IFN-γ secretion by NK cells and possibly professional APCs is likely to be important in early host defense against infection, whereas TH1 cells become the dominant source of IFN-γ in the adaptive immune response(143). Type III IFNs were recently discovered and represented by IFN-λ, or (IL-28 and IL-29), and have been classified as cytokines with IFN-like activity. IFN-λ is produced in response to certain virus infections and to mediate antiviral activity in vitro (129, 150). However, activation of IFN-λ in response other viruses and how they respond in vivo is ill-defined (151). EAE was exacerbated in an IFN-γ knock-out murine model, and the number, and function of CD4+CD25+Foxp3+ T-cells were substantially reduced. However, in vitro treatment of IFN-γ has been shown to induce development of CD4+CD25− T-cell into CD4+CD25+ T-regulatory cells
and suppression of EAE with adoptive transfer (152). Tregs have also been shown to secrete moderate amount of IFN-γ in response to effector T-cells (153). In a study, using non-obese diabetic mouse model demonstrated that pathogenic CD4⁺TH1 cells isolated from these mice were converted into T-regulatory cells by lentiviral transduction with Foxp3 under the control of IFN-γ promoter. After transduction, IFN-γ promoter mediated Foxp3 expression in diabetic CD4⁺TH1 cells. These differentiated CD4⁺Foxp3⁺ T-cells were anergic upon in vitro stimulation by antigen. Adoptive transfer of these cells into diabetic recipients substantially suppressed the incidence and progression of diabetes (154). How Tregs utilize IFN-γ for their development and function is unclear and warrants further investigation.

1.5  **Foxp3 and the Development and Function of Tregs**

1.5.1  **Foxp3**

The forkhead (FKH) (155) box protein FOXP3 is a member of the P-subfamily of Fox transcription factors, which as a group are characterized by the presence of a highly conserved winged-helix/FKH DNA binding domain (DBD) (156). The development of T-regulatory cells is critically dependent on Foxp3 and the expression of this protein, prevents self-destructive immune responses (157). Despite the importance of Foxp3 for NTregs development, the molecular and functional features of Foxp3 for Tregs remain unknown. It has been postulated that Foxp3 expression is required for both survival of Tr1 precursors as well as their inability to produce interleukin IL-2 and independently proliferate after T-cell-receptor engagement (158, 159). Although its function is required for Tregs suppressor activity, to a large extent Foxp3 amplifies molecular features of Tregs, including anergy and dependence on paracrine IL-2. Furthermore,
Foxp3 solidifies lineage of Treg stability through modification of cell surface molecules (PD-1, CTLA-4 and ICOS), and signaling molecules (ITIM, ITSM, SHP1 and SHP2), resulting in adaptation to the signals required to induce and maintain Tregs (160). Currently, new research has focused on associating abnormal numbers of Tregs with immune disorders. However, the quality of Tregs is also critical for their function (161). Studies show that intra-islet Tregs had low levels of Foxp3 expression compared with Tregs from other peripheral lymphoid organs in diabetic NOD mice, whereas the frequencies of Foxp3-expressing Tregs among different compartments were comparable (161). However, this drastic decrease of Foxp3 expression was not observed in non-diabetes prone C57BL/6 mice (161). Indicating a correlation that reduced number and function of Tregs leads to the development of autoimmune disease such as diabetes. This deficiency of Tregs is also seen in the airways of asthmatics indicating the urgency for therapeutic strategies for modulating Tregs for the management of asthma and autoimmune diseases.

1.6 Role of Fms-like Tyrosine Kinase 3 Ligand (Flt3-L) in Asthma

1.6.1 Origin of Flt3-L

Fms-like tyrosine kinase 3 (Flt3) or Flk2 is a member of the class III tyrosine kinase receptor family. The murine Flt3 or Flk2, was independently cloned by two groups of investigators. Flt3 was cloned from murine placenta based upon its similar sequence homology to c-fms (162). The c-fms (cellular) oncogene is a homolog of v-fms (viral) oncogene which was originally encoded by the Susan McDonough strain of feline sarcoma virus (163). The human homolog of murine Flt3 gene was also cloned (76, 164, 165) and found to be expressed in CD34+ progenitor cells and in some leukemic cells.
Therefore, administration of Flt3-L could be possible therapy for increasing the numbers of T-cells, B-cells or dendritic cells in individuals that are deficient in these cell types.

1.6.2 Functional Effects of Flt3-L

Fms-like tyrosine kinase 3 ligand (Flt3-L) stimulates the expansion and differentiation of hematopoietic progenitor cells to generate mature immune cells of both myeloid and lymphoid lineages(166). Flt3-L is ubiquitously expressed in human and mouse tissues and Flt3-LR is expressed almost exclusively in progenitor and stem cells in the bone marrow, thymus, and spleen, and it is not present on mature cells, mast cells, or erythroid progenitors (167). Flt3-L has been shown to promote the expansion of dendritic cells (DC), natural killer (NK) cells, and moderate stimulation on B and T lymphocytes (168). Transgenic mice lacking Flt3-L have reduced numbers of bone marrow progenitor cells, NK cells, and myeloid- and lymphoid-related DC (169). In vivo administration of Flt3-L in PBS control mice for 5 days causes a substantial increase in the number of progenitor cells in the bone marrow, spleens, and peripheral blood of the mice(170). When Flt3-L is administered for 10 days, there are significantly greater numbers of progenitors, as well as mature DC, NK cells, NK/T cells, and T cells in the spleens, liver, and peripheral blood. These expanded populations are functional and responsive to appropriate stimuli (170). Flt3-L treatment has been shown to significantly increase the resistance of mice to lethal infections with herpes simplex virus, with decreased amounts of latent virus in neurons and elevated numbers of hepatic and splenic NK lymphocytes and DC (171). The effects of Flt3-L are transient, and hematopoietic parameters begin to return to baseline levels within one week after
withdraw of therapy (172). Administration of Flt3-L to human subjects results in dramatic increases in the number of dendritic cells in peripheral blood, and no toxicity is observed (173). The murine and human ligands (Flt3-L) for the Flt3/Flk2 receptor were cloned, and demonstrated to share structural similarities with c-kit-L and M-CSF-L (174, 175).

1.6.3 Flt3-L as a Potential Therapeutic Remedy

These findings suggest that Flt3-L maybe a potential therapy to enhance immune function in patients with inadequate cell number and function. Clinical trials are underway to examine the potential of Flt3-L as a therapy to manage and control immunologically responsive cancers (176, 177), and other diseases. The development of distinct populations of DCs by Flt3-L suggest there is a regulation of T_{H1}/T_{H2} cell profile in allergic asthma and this action maybe by the induction of CD4^{+} CD25^{+} T-regulatory cells.

1.7 Green Fluorescent Protein (GFP)

1.7.1 GFP as a Protein Tag

Green fluorescent protein (GFP), is a spontaneously fluorescent protein isolated from coelenterates, such as the Pacific jellyfish, *Aequoria Victoria* (178). Its role is to transduce, by energy transfer, the blue chemi-luminescence of another protein, aequorin, into green fluorescent light. GFP has been expressed in bacteria (179), slime mold (180), drosophila (181) and zebrafish (181) and it can function as a protein tag, as it tolerates N- and C-terminal fusion to a broad variety of proteins many of which have
been shown to retain native function (182). When expressed in mammalian cells, fluorescence from wild type GFP is typically distributed throughout the cytoplasm and nucleus, but excluded from the nucleolus and vesicular organelles (183). Recently, a number of investigators have used GFP-labeling for tracking the migration of Tregs and understanding their development and function after adoptive transfer studies (184).
1.8 Hypothesis and Specific Aims

Central Hypothesis

Adoptive transfer of naturally occurring CD4+CD25+ T-regulatory cells and inducible CD4+CD25- T-regulatory cells into cockroach-sensitized and challenged mice reverses airway hyper-responsiveness and airway inflammation.

Sub-Hypothesis

Flt3-L can reverse airway hyper-responsiveness and airway inflammation by increasing the number of naturally occurring CD4+CD25+ T-regulatory cells in the lung of cockroach-induced mouse model allergic asthma.

Specific Aims

1. To examine if an adoptive transfer of naturally occurring CD4+CD25+ T-regulatory cells and inducible CD4+CD25- T-regulatory cells isolated from lung and spleen tissue of healthy BALB/c mice into cockroach-sensitized and challenged mice can reverse airway hyper-responsiveness and airway inflammation.

2. To examine the migration of adoptively-transferred T-regs in the lung and draining lymph nodes of cockroach-sensitized and challenged mice.

3. To evaluate the role of PD-1 expressed on Tregs by adoptively transferring naturally occurring CD4+CD25+ T-regulatory cells and inducible CD4+CD25- T-regulatory cells isolated from lung and spleen tissue of healthy BALB/c mice into cockroach-sensitized and challenged mice.
4. To examine the effect of Flt3-L on airway hyper-responsiveness and airway inflammation in cockroach-sensitized and challenged mice, and the phenotypic expression of CD4+ T-cells the lung.
Chapter 2

GENERAL MATERIALS AND METHODS
The materials and methods used for the experiments described in the following chapters are listed within the appropriate chapters. The present chapter will describe in more details the Buxco Whole Body Plethysmography and several methods used for experimentation.

2.1 Materials: Reagents, solutions, and buffers

A list of reagents, buffers, and other solutions used in the experimental methods is provided in Table 1. All antibodies used are listed with source and the dilution used in flow cytometry within each chapter. Anti PD-1 antibody was provided as a gift from Dr. Hideo Yagita, Department of Immunology Tokyo, Japan. Anti CD25 antibody (PC61) was purchased from BioLegend San Diego, CA.

9 GFP-transgenic BALB/c mice; 6 females and 3 males were purchased from Jackson Laboratories Bar Harbor, Maine and caged as breeding trios. Four-to-eight week old GFP-transgenic female offspring was used as donor mice for adoptive transfer of Tregs. In addition, four-to-five week old female BALB/c mice were purchased from Harlan Laboratories Indianapolis, IN.
Table 1: Materials used in the Experimental Procedures

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<td>Miltenyi Biotec</td>
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</table>
2.2 Methods

2.2.1 Whole Body Plethysmography

Whole body plethysmographys (WBP) purchased from Buxco Electronics Inc, (Troy, NY) were used to measure pulmonary function in conscious, free-moving mice (185). The several advantages of using this system over the invasive method is WBP chambers allow animals to move freely within the chamber while respiratory function is measured, thus eliminating the potential problems of anesthesia(186). More importantly, the mice are not sacrificed in order to conduct the experiments thus permitting repeated testing of animals and up to 10 mice can be tested simultaneously. The WBP system layout is set-up as shown in the diagram (Figure 5). Each WBP chamber is connected to a bias flow regulator to supply a smooth and constant flow of air during testing. A nebulizer delivers aerosol to each chamber via the nebulizer mixing unit. A transducer attached to each chamber detects pressure changes that occur as the animal breaths. Pressure signals are amplified by a MAX II Strain Gauge preamplifier and analyzed by the Biosystem XA software supplied with the Buxco system. Pressure changes within each chamber are calibrated prior to testing as explained in the following section.
**Figure 5: WBP diagram.** This is a schematic of an unrestrained and freely moving mouse that is sending a signal gathered from a whole body plethysmography and relayed via the Pre-amplifier. This data is interpreted by the Buxco Biosystem XA software.
2.2.2 Enhanced Pause (Penh)

Penh is a dimensionless value that characterizes the expiratory shape change (187). The algorithm for Penh compares the average amplitude of the early part of the expiratory phase to the average amplitude of the later part of the expiratory phase; and the peak amplitude of the expiratory phase to the peak amplitude of the inspiratory phase (188). This algorithm has been used successfully to follow the pulmonary function in mice. The methacholine challenge elicits a very characteristic waveform in the WBP, with a large deflection early in expiration, followed by duration of very low amplitude (pause) for the rest of expiration (188). The Pause measures the changes in the box pressure occurring during expiration (189). Expiration during bronchoconstriction occurs against the resistance of constricted airways. Comparing the relaxation time (Tr), the time required for the pressure during expiration to decay to 36% of the total expiratory pressure to the total expiratory phase (Te) calculates the value known as Pause. During bronchoconstriction the peak expiratory flow (PEF) increases to a greater degree than the peak inspiratory flow (PIF) as a result of expiration through increasing constricted airways. When the ratio of PEF/PIF is multiplied by the ‘Pause’, the unit ‘Enhanced Pause’ is found (Figure 6) (189).
Figure 6: Penh Algorithm. Unrestrained Whole Body Plethysmography with representative waveform and parameters used in the calculation of Penh (Buxco Electronics, Inc)

2.2.3 Calibrating the Buxco System

One millimeter of air is pushed into the plethysmograph for calibration before conducting an experiment. To begin go to the top of the menu and click system, then calibrate. In the calibration screen, select the ‘freeze trace’ and scroll down and select the average period to three seconds. The “reading to sample” toggle was set to low with “0” entered in the “low” reading box and the appropriate lead number was highlighted. The AC/DC switch on the amplifier was set to DC, the “balance” knob on the pre-amplifier was adjusted to produce a “0” voltage reading on the computer screen. The “Take sample” button was pressed to calibrate the “low reading”, which is the “F3” key. To take the “high” reading, a value of -10 was entered in the “high” reading box, which is
by pressing the “F4” key. Using a 1 ml syringe connected to the plethymograph, push in the 1 ml of air slowly, but with consistency, then press “F7” and enter. If the “Effective Range” reading was within 17-to-27 volts as recommended by Buxco Electronics, switch the preamplifier switch to AC. However, if the effective range is not optimal, adjust the gain knob accordingly and/or use the fine adjustment button then repeat the steps above. The entire procedure was repeated for all chambers that will be used in the experiment.

2.2.4 Invasive Tracheostomy for Assessment of Established Airway Responsiveness

To confirm AHR to methacholine, several randomly selected sensitized and non-sensitized mice were anesthetized, and cannulated via tracheostomy. Mice were placed in supine position and the cannula was connected to the ventilation port in the single chamber plethysmograph for anesthetized animals PLY3111 (Buxco Electronics, Wilmington, North Carolina). Mice were mechanically ventilated with Harvard Rodent Ventilator model 683 (Harvard Apparatus, Holliston, Massachusetts). After mice were stabilized, they were challenged with aerosolized PBS followed by increasing doses of methacholine (3.1, 6.25, 12.5, 25, 50, and 100 mg/ml). Pressure and flow data was continuously recorded by pulmonary software (BioSystemXA, Buxco Electronics, Inc) to calculate specific airway resistance.

2.2.5 Histology and Staining

2.2.5.1 Processing lung and H and E Staining

After lung tissue has been embedded, blocks are made and sectioned with Microtome
(IMEB, San Marcos, CA) at a size of five microns in thickness. Sections are submerged in a water bath at 39 – 40 °C and placed on a slide. Slides are deparaffinized using Xylene and ethanol. Once the tissues have been deparaffinized they are stained with a Hematoxylin and Eosin staining kit (Newcomer Supply, Middleton, WI) to examine morphology.

2.2.5.2 Lung sections showing Collagen Deposition
Deparaffinized lung sections are incubated in Bouin solution, then rinsed in tapped water. Sections were placed in Biebrich’s Scarlet acid fuchsin solution for 15 minutes and rinsed. These rinsed sections were submerged in Phosphotungstic and Phosphomolybdic acid solution for 30 – 45 minutes and stained with Hematoxylin solution for 5 minutes. Aniline blue was the last stain applied for 12 – 15 minutes (IMEB, St Louis, MO), rinsed and mounted with Permount Glue (Sigma-Aldrich, St Louis, MO). The slide was examined under a microscope.

2.2.5.3 Lung Sections showing Mucus Deposition
Lung sections were submerged in periodic acid for 30 minutes. Schiff's reagent was microwaved to a slight boil to allow the reagent to become a bright purple. Slides were immersed in Schiff’s reagent for 15 minutes at room temperature (18–26°C) and washed in tap water for 5 minutes. Slides were counterstained in Hematoxylin Solution, Gill No. 3, for 90 seconds. Slides were dehydrated, cleared and mounted using xylene and alcohol gradients.
2.2.6 T-cell Proliferation Assay

Splenocytes from naïve mice were seeded (100,000 cells per well) in anti-CD3 antibody pre-coated plates (Ebioscience) and 2.5 µg/ml anti CD28 antibody was added (Ebioscience). CD4⁺CD25⁺ T-cells from lung and spleen of adoptively transferred mice and naïve CD4⁺CD25⁺ T-cells were added at varying ratios (1:1, 1:0.5, 1:0.25 and 1:0.125). Cells were incubated for 48 hours at 37 °C in RMPI 1640 medium. BrdU (20 µl) (1 ml BrdU labeling solution is required if the cells were cultured in 100 µl/well (10 µl/well) and 2 ml BrdU labeling solution is required if the cells were cultured in 200 µl/well (20 µl/well (Roche Applied Science) was added to each well for an additional 12 hours followed by analysis of T-cell proliferation using ELISA plate reader.

2.2.7 Dose-Dependent Assay with Tregs

CD4⁺CD25⁺ and CD4⁺CD25⁻ T-cells were isolated from lung and spleens of naïve BALB/c mice and beginning at 200,000-to-30,000 decreasing doses were adoptively transferred into cockroach-sensitized and challenged mice. AHR to methacholine was examined each week for 4 consecutive weeks. We found that 100,000 Tregs of both subtypes effectively suppressed AHR for four weeks.

2.2.8 RNA Isolation

Isolated and purified CD4⁺CD25⁺ and CD4⁺CD25⁻ T-cells were centrifuged and the supernatant discarded. The pellet was placed in 1 ml of Trizol Reagent and incubated for 15 minutes at room temperature. 100µL 1-Bromo-3-Chloropropane was added to
each eppendorf tube, vigorously shaken for about 5 seconds and incubated for 15 minutes at room temperature. Sample was centrifuged @ 12000g for 15 minutes at 4°C. The aqueous phase (top clear layer) was transferred into a new tube and 500µL isopropanol was added and mixed, and incubated for 5-10 minutes at room temperature, then centrifuged @12000g for 8 minutes at 4°C. The RNA pellet was mixed with 1mL of 70% ethanol and centrifuged @ 7500g for 5 minutes at 4°C (Molecular Research Center, Inc. Cincinnati, OH ). The RNA pellet was air-dried for 2-4 minutes and dissolved in 20 – 50 µl RNase free water. RNA samples (2µl) were diluted in water (98 µl) and the concentration was quantified using the GeneQuant 1300 spectrophotometer (GE Healthcare Bio-sciences Corp., Piscataway, New Jersey).

2.2.9 Semi-quantitative conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The ImProm-11 Reverse Transcription System was purchased from Promega (# A3800) and manufacturer's instructions were as followed. Experimental RNA (up to 1 µg/reaction) (3.0 µL), Oligo (dt) (1 µL) and Nuclease-free water (1.0 µL). Each tube was placed into a preheated 70 °C block for 5 minutes and chilled in ice-water for 5 minutes. Each tube was centrifuged to collect the condensate and maintained in the original volume 5 µL. Then 5X Reaction Buffer (4 µL), MgCl2 2.5 mM (3.2 µL), dNTP mix (0.5 mM), recombinant Rnasin ribonuclease inhibitor (0.5 µL), ImProm-II reverse transcriptase (1.0 µL) and Nuclease-free water (5.3 µL) were used for the reverse-transcription reaction mixture which was placed on the heat at 25 °C for 5 minutes, 42 °C for 60 minutes, 70 °C for 15 minutes, and then 4 °C for 5 minutes.
Each PCR reaction mixture contained PCR Master Mix (25 µL), cDNA (2 µL), forward primer (5 µL; 1 µM), reverse primer (5 µL; 1 µM), and RNase/DNase Free Water (13 µL). The PCR reaction mixtures were placed in the PCR machine heat block and the cDNA was initially denatured at 95 °C for 15 minutes. PCR amplification was performed by denaturation for 60 seconds at 94 °C, annealing for 60 seconds at 55 °C, primer extension for 60 seconds at 72 °C, and a final extension for 10 minutes at 72 °C. See Table 2 for number of cycles and annealing temperatures. For all experiments, HPRT was used as an internal control.

The PCR product was loaded onto a 1.5 % agarose gel. After electrophoresis, the gels were photographed under UV light using the UVP Bioimaging System (Upland, CA). Each experiment was performed three times.

2.2.10 Identifying GFP Expression

To identify mice expressing GFP we purchased a Black light from The Blacklight Shop, Pittsburgh, PA. This black light has a wavelength of 380 – 385 nm. The peak excitation for GFP is 488 nm (Figure 7). Therefore, black light is able to excite the lower end of the GFP spectrum which is enough energy to prompt the visible effects of GFP expression in the eyes, ears, tail, and paws in GFP positive mice compared to no expression in the control mouse (Figure 8 A and B).
**Figure 7: GFP and Black Light Excitation Spectrum.** Excitation of GFP is 488 nm and excitation of black light is 380 – 385 nm. Therefore, black light is able to excite the lower end the GFP spectrum (Invitrogen Corporation, Carlsbad, CA).
Figure 8: Positive Expression of GFP in BALB/c Mice.  A. Black light allows the naked eye to see the visual effects of GFP expression in two positive mice. GFP is highly expressed causing the eyes, ears, nose, tail and paws to glow green.  B. One mouse is positive for GFP expression and the second mouse has no expression of GFP, which serves as control.
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Chapter 3

NATURALLY OCCURRING AND INDUCIBLE T-REGULATORY CELLS MODULATING IMMUNE RESPONSE IN ALLERGIC ASTHMA
3.1 Abstract

Rationale: T-regulatory cells (Tregs) are potent immunomodulators in allergic asthma. Objectives: We evaluated the functional effects of Tregs on AHR and airway inflammation, by adoptively transferring naturally occurring CD4\(^+\)CD25\(^+\) T- regulatory cells (NTregs) and CD4\(^+\)CD25\(^-\) inducible T-regulatory cells (iTregs) from lung and spleens of GFP-transgenic BALB/c mice into cockroach-sensitized and challenged mice. Methods: GFP-labeled NTregs and iTregs were adoptively transferred into cockroach-sensitized and challenged mice. AHR to methacholine was examined using a single-chamber, whole-body plethysmograph and invasive tracheostomy. Measurements and main results: Adoptive transfer of either NTregs or iTregs from lung or spleen reversed airway inflammation and AHR to methacholine, and the effect lasted for at least four weeks. GFP-labeled iTregs up-regulated CD25 and Foxp3, and migrated to lymph node and lung. Lung CD4\(^+\)CD25\(^+\) T-cells isolated from each group of recipients mice were ICOS\(^{\text{high}}\) and PD-1-positive; however, higher expression of PD-1 was found in the spleen iTregs (S25\(^-\)) and lung iTregs (L25\(^-\)) groups. Higher levels of TGF-\(\beta\) and IL-10 mRNA transcripts, and BALF IL-10 and INF-\(\gamma\) levels were observed in lung CD4\(^+\)CD25\(^+\) cells from the L25\(^-\) and S25\(^-\) cell-recipient mice than from lung NTregs (L25\(^+\)) and spleen NTregs (S25\(^+\)) cell recipient mice. Adoptive transfer of either cell type significantly reduced BALF IL-4, IL-5 and IL-13 levels. Conclusion: Tregs reverse AHR and airway inflammation; however iTregs that differentiated into T\(_{\text{REG}}\) cells in the lung exert their suppressive activity likely by higher levels of TGF-\(\beta\), IL-10, IFN-\(\gamma\) and elevated levels of PD-1 compared to NTregs. Hence, PD-1 may be a conduit for reversing AHR by Tregs and a plausible target for treating asthma.
3.2 Introduction

Asthma is a chronic inflammatory lung disease of the airways believed to reflect the cumulative effects of a skewed TH2 cell polarized response to inhaled antigens (190). Airway hyper-responsiveness (AHR) and airway inflammation are believed to be mediated by Th2 cells and the release of their signature cytokines, IL-4, IL-5, IL-9, and IL-13 (191). This cytokine milieu causes pulmonary eosinophilia, chronic inflammation, leading to mucus cell hyperplasia, destructive airway tissue remodeling (192), and contraction of smooth muscle cells (193).

CD4+CD25+ T-regulatory cells (Tregs) have been shown to be a plausible strategy to modulate the effects of dysregulated type 2 immune response. This specialized subset of CD4+ T-cell is characterized into two categories: Naturally occurring CD4+CD25+ Tregs (NTregs) (90, 194) and inducible CD4+CD25− T-regulatory cells (iTregs) that includes T_R1 and T_H3 cells (195). NTregs constitutively express CD25, the α-chain of the IL-2R (159, 196, 197), and are vital in suppressing autoimmune T-cell responses and maintaining peripheral tolerance (198). NTregs represent approximately 5 to 10% of the peripheral CD4+ T-cells in humans and mice (73) and constitutively express the Foxp3 (74). Compelling evidence demonstrates the necessity of Tregs for normal immune response(75). Mutations in the X-linked Foxp3 gene in scurfy mice leads to an autoimmune disease characterized by multi-organ lymphocytic infiltration that results in enlarged spleen, lymph nodes, and liver, as well as dermatitis and severe runting (76). In human, mutations in FOXP3 have been linked to autoimmune diseases including X-linked autoimmunity allergic disregulation syndrome (77), X-linked autoimmunity immune deficiency syndrome (78, 79), and Immune Dsyregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome (IPEX) (74, 80).
IL-10 producing CD4+ type 1 (TR1) cells were originally derived from patients with severe combined immunodeficiency who had undergone successful HLA-mismatched bone marrow transplantation (82). TR1 cells are spawned from naïve CD4+CD25- T-cells activated through T-cell receptor, CD28, and IL-2 receptors and can suppress antigen-driven proliferation of CD4+ T-cells in vivo and in vitro (199). TR1 cells display a unique profile of cytokine production that is distinct from that of naïve T-cells (TH0), which can secrete both TH1 and TH2 cytokines albeit at very low levels (86-88), TH1 cells secrete IFN-γ, TNF-β, and IL-2 (89, 200, 201), and TH2 cells secrete IL-4, IL-5, IL-6, IL-9 and IL-13 (86, 88). TR1 cells are characterized by the secretion of high levels of IL-10, TGF-β, and mild-to-moderate levels of IFN-γ, IL-5, and little-to-no release of IL-2 and IL-4 (90, 200, 201). IL-10- secreting T-regulatory cells are the major T-cell subset that responds to non-pathogenic antigen in healthy individuals. However, in subjects with atopic asthma there is a reduction of TR1 cells and an increase in TH2 cells. Therefore, a skewed TH2 ratio initiates the development of AHR and airway inflammation in allergic asthma(140). However, TR1 cells are characterized by their ability to secrete immunosuppressive cytokines IL-10 and TGF-β that modulate immune response. In addition, it has been suggested that suppression by TR1 cells maybe through the expression of the cell surface marker programmed death-one (PD-1) (140) and possibly other co-stimulatory molecules; however, the mechanism and which other molecules participate is unknown. Never-the-less, a shift in this delicate balance of T-cells subsets may be pivotal in tolerance or immunopathology as seen in allergic asthma. Currently three possible outcomes exist from effector T-cells: (i) The TH1 response, which is the normal outcome after exposure to viruses or intracellular bacteria (such as Salmonella typhimurium and Listeria monocytogenes), yeast, and cancers (ii) The TH2 response,
resulting in the milieu of IL-4, IL-5, IL-9, and IL-13 cytokines in the lung or accumulation of eosinophils and mast cells as a reaction from parasite (Helminths) and extracellular bacteria (i.e. helicobacter pylori) or inappropriate Th2 response to non-pathogenic environmental antigens, and (iii) The most prevalent response is tolerogenic or T-regulatory cells producing immunosuppressive cytokines, IL-10 and TGF-β, in steady state conditions (202).

Therefore, using a GFP-transgenic mouse model we investigated and compared the functional effect of NTregs and iTregs on AHR and allergic airway inflammation, and would there be differences depending on whether these cell types were derived from spleen and lung tissue. We adoptively transferred indigenous lung and spleen tissue NTregs and iTregs from naïve GFP-transgenic BALB/c mice into cockroach (CRA)-sensitized and challenged mice. We found complete reversal of AHR and airway inflammation in all recipients of either Treg type and the effect lasted for at least four weeks. In addition, the adoptively transferred CD4+CD25− T-cells migrated to the lymph node, lung and spleen and differentiated into CD4+CD25+Foxp3+ with significantly higher levels of transcripts of IL-10, TGF-β and PD-1 than in lung CD4+CD25+ cells isolated from adoptively transferred NTregs group.
3.3 Methods

3.3.1 Mice.

GFP-transgenic BALB/c breeder mice were purchased from Jackson laboratories (Bar Harbor, ME) and 4-8 weeks old offsprings were used in this study. The initial studies used four- to five-week old BALB/c female mice purchased from Harlan Laboratories (Indianapolis, IN) and were housed in separate cages according to treatment protocol. Food and water were provided *ad libitum*. According to National Institutes of Health guidelines, the research protocol of this study was approved by the Institutional Animal Care and Use Committee of Creighton University.

3.3.2 Sensitization and challenge with cockroach antigen

Allergic airway inflammation was induced in naïve BALB/c mice in the (initial study) and (subsequent study) by the administration of an i.p. injection of CRA antigen 10 µg a mixture of (American) *Periplaneta Americana* (Linnaeus) and (German) *Blattella germanica* (Linnaeus) (Hollister Stier laboratories LLC, Spokane, WA) emulsified in alum (Pierce, Rockford, IL) in a total volume of 100 µl per mouse on day 0 and 14 (Figure 9). Subsequently, animal received aerosol challenge with CRA antigen (50 mg/ml) on days 28-30 and 32. On day 33, AHR was examined using a single-chamber, whole-body plethysmograph (Buxco Electronics, Troy, NY) and aerosolized acetyl β-methylcholine (Sigma-Aldrich) in a dose-dependent manner (0.031 g, 0.062 g, 0.125 g, 0.25 g, 0.50 g, and 1.0 g in 10ml PBS) (Figure 9).
Figure 9: Cockroach-sensitization and Adoptive Transfer Protocol. Day 0 to 33, mice are sensitized and challenged with cockroach allergen. Day 33, GFP-labeled Tregs were adoptively transferred and AHR to methacholine was evaluated each week for four weeks until day 68.

3.3.3 Tissue preparation and Isolation of T-Regulatory cells for adoptive transfer

To isolate CD4⁺CD25⁺ T-cells and CD4⁺CD25⁻ T-cells from naïve or GFP-transgenic mice, lungs and spleens were harvested from balb/c mice. The tissues were cut into fragments, followed by digestion using Collagenase D (Roche laboratories) (1mg/1ml) and 5 mls of RPMI 1640 (Cambrex). The samples were incubated at 37 degrees in a CO2 incubator for 90 minutes. Tissue was disrupted with a 1 ml syringe. After the tissue was
disrupted the cell suspension was poured over 40 micron filter (BD Biosciences, San Jose, CA) and collected into 15 ml tubes and labeled accordingly. Red blood cells were lysed using Tris Buffered Ammonia Chloride solution and suspension was neutralized with PBS4 solution. The suspension was centrifuged at 350g for 15 minutes. The supernatant was discarded and pellet washed in 10 mls Hanks Balanced Buffered Solution (HBSS) and centrifuged and resuspended in AutoMACS running buffer. CD4+ T-cells were pre-enriched by depleting unwanted cells by using a cocktail of antibodies (CD8a (Ly-2), CD11b (Mac-1), CD45R (B220), CD49b (DX5) and Ter-119). Then the CD25+ cells were positively selected from the enriched CD4+ T-cell fraction (CD4+ CD25+ T-regulatory cell Isolation Kit, Miltenyi Biotec, Auburn, CA). Remaining CD4+ T-cell were designated as CD4+CD25- cells.

In the initial studies the purity of the CD4+CD25+ T cell population of the lung (L25+) was 71 – 77 % with 1% of the cells determined to be CD4-CD25+ T cells and 23% of the cells being CD4+CD25- T cells. The purity of the CD4+CD25+ T cell population of the spleen (S25+) was 94% with 1% of the cells being identified as CD4-CD25+ T cells and 5% of the cells CD4+CD25- T cells. The purity of the CD4+CD25- T cell population of the lung (L25-) was 97% with 3% of the cells being CD4+CD25+ T cells. The purity of CD4+CD25-T cell population isolated from spleen (S25-) was 98% with 2% of the cells being identified as CD4+CD25+. However, in the subsequent study GFP-labeled CD4+CD25+ and CD4+CD25- were used and the same steps were followed for isolating CD4+CD25+ and CD4+CD25- T-cells from the lung and spleen with the exception of GFP-transgenic BALB/c mice and these cells were further purified and sorted by FACS Aria (BD Biosciences, San Jose, CA) cell sorter by expression of CD4 antibody conjugated with PercP and CD25 antibody conjugated with PE on CD4+CD25+ and CD4+CD25- T-
cells. The purity and viability of the CD4+CD25+ and CD4+CD25- T-cell populations from both lung and spleen tissue were > 99.0% and >98%, respectively.

3.3.4 Adoptive transfer therapy

Mice with established AHR to methacholine were subjected to adoptive transfer of the cells in this study (adoptive transfer of GFP-labeled Tregs) and in the initial study (adoptive transfer of Tregs from naïve BALB/c mice). In the initial study, starting day 33, cockroach-sensitized mice were randomized into four groups; (i) L25+ group mice that received lung NTregs; (ii) L25- group mice that received lung iTregs, (iii) S25+ group mice that received spleen NTregs, and (iv) S25- group mice that received spleen iTregs. Cells were injected intravenously into the dorsal tail vein of sensitized recipients at 400,000 cells/50 µl of sterile PBS. Twenty four hours later these animals received an additional i.p. injection of CRA antigen to prime the adoptively transferred cells. Beginning at day 37 animals were weekly challenged for four weeks with aerosolized cockroach antigen (50 mg/ml) and AHR to methacholine was evaluated at the end of each week. Non-sensitized control mice were sham treated with sterile PBS as a vehicle. In this study, the same experimental groups and time points were used; however, the Tregs were GFP-labeled and were injected intravenously into the dorsal tail vein of sensitized recipients at 100,000 cells/50 µl of sterile PBS.

3.3.5 Flow Cytometry and Antibodies

A FACScan (Becton Dickerson) was used for analytical Flow cytometry and data was processed with CellQuest Pro (Becton Dickerson)(28) using protocol for cell preparation. Each antibody was diluted in PBS4 using the established amount for each
antibody. Cells were stained with PercP CD4 (L3T4) (1:400 dilution; 1 µl of antibody and 399 µl of PBS4), Ftc CD69 (H12F3) (1:200 dilution; 1 µl of antibody and 199 µl of PBS4), Ftc CD62L (MEL-14) (1:200 dilution; 1 µl of antibody and 199 µl of PBS4) all purchased from BD bioscience. Ftc GITR (DTA-1) (1:400 dilution; 1 µl of antibody and 399 µl of PBS4), Apc ICOS (7e.17G9) (1:500 dilution; 1 µl of antibody and 499 µl of PBS4), and Ftc PD-1 (J43) (1:400 dilution; 1 µl of antibody and 399 µl of PBS4). 100 µl of cells and equal amount of the each antibody was added to the appropriate labeled tube and incubated for 30 minutes in the dark at 4 °C. Suspension is centrifuged for 2 minutes at 300g at 4°C. Supernatant was aspirated using vacuum and a pasteur pipette. This step was repeated two more times. Cells were resuspended in 500 µl of FACSfix (4% formalin in PBS). Samples were covered and refrigerated prior to analysis. In addition, appropriate controls were made that included cells only (used to determine autofluorescence) and fluorochrome only (This was a control for each fluorochrome and was used as compensation control to determine the extent of fluorochrome spectra overlap. In addition, cells were also stained with FITC anti-mouse/rat Foxp3 using a staining kit(eBioscience, San Diego, CA). 100 µl of prepared cells was added (maximum of 1 million) to each tube. Centrifuged and decanted. After the supernatant was removed the cells were vortexed. Then 1ml of Foxp3 Fixation/Permeabilization Buffer was added to each sample and vortexed again. Samples were incubated for 1 hour in the dark at 4°C. Samples were washed once by adding 2 ml 1X Permeabilization Buffer and were centrifuged and decanted. 100µl of Foxp3 (PCH101) antibody (1:200 dilution) was added to sample and incubated for 30 minutes in the dark at 4 °C. Samples were centrifuged and washed 2 times in 2 ml Permeabilization Buffer 3 times. Samples were resuspended in FACS Fix for analysis the next day.
3.3.6 Mice euthanization and Lung preparation

Mice were euthanized with a lethal injection of 100µl of pentobarbital. Lungs were removed and a section of the left lobe was placed in 4% formalin. The formalin was removed and tissue was placed in 70% ethanol and paraffin embedded by a Sakura Tissue-Tek VIP paraffin processor (IMEB, San Marcos, CA).

3.3.7 Bronchoalveolar lavage Fluid (BALF) and Cytokine Measurements

After euthanization, lungs were gently lavaged with 1 ml of warm saline (37°C) via a tracheal cannula. Total cell counts were performed coulter counter (Beckman and Coulter). All samples were centrifuge at 400 rpm, for 10 minutes, and the supernatant was stored in -80°C freezer until ELISA assays were performed. Mouse IL-4, IL-5, IL-10, and INF-γ were conducted using a TH1/TH2 and IL-5 ELISA Detection, Ready-Set-Go kits (Ebioscience) according to Manufacturer’s protocol. Capture antibody in coating buffer was added (100 µl/well) to NUNC Maxisorp 96 well ELISA plate. The plate was sealed and incubated overnight at 4°C. Wells were aspirated and washed 5 times with PBS and Tween 20 (250 µl per well) and allowed to soak for one minute. Plate was blotted on absorbent paper to remove any residual buffer. Diluent assay was diluted with 1 part 5X concentrated diluent assay and 4 parts DI water. Wells were blocked with 200 µl/well of 1X assay diluent and incubated at room temperature for 1 hour. Wells were aspirated and washed as before. Using 1X assay diluent, standards were diluted according to certificate of analysis. 100 µl/well of standard was added to the appropriate wells. Two-fold serial dilutions were performed to make standards and to
make the standard curve. Sample was added (100 µl/well) to the appropriate wells. The plate was covered and incubated at room temperature for 2 hours. The wells were aspirated and washed for a total of 5 washes. Detection antibody was added (100 µl/well), and plate was sealed, and incubated at room temperature for 1 hour. Wells were aspirated and washed for a total of 5 washes. 100 µl/well of Avidin-HRP* diluted in 1X Assay Diluent was added to each well and plate was sealed and incubated at room temperature for 30 minutes. Wells were aspirated and washed for a total of 7 washes. Substrate solution was added (100 µl/well) and incubated at room temperature for 15 minutes. 50 µl of stop solution was added to each well and the plate was read at 450 nm and analyzed.

### 3.3.8 RT-PCR

To analyze expression of Foxp3, Neuropilin-1, GATA3, IL-10, TGF-β and T-bet messenger RNA was prepared from isolated T-regulatory cells using Trizol (Sigma-Aldrich) reagent protocol. Gene Amp PCR System 2400 (Perkin Elmer) was used 32 cycles for Foxp3, GATA-3, and T-bet, 30 cycles for neuropilin-1, 31 cycles for IL-10 and 33 cycles for TGF-β. *Foxp3:* Forward 5’- TAC ACC CAG GAA AGA CAG CAA CCT – 3’, Reverse 5’ – TCT GAA GTA GGC GAA CAT GCG AGT – 3’ T<sub>m</sub>: 55.0° C. *Neuropilin-1:* Forward 5’ – GAA AGA GGG AAA TAA AGC CA – 3’, Reverse 5’ – TCC CAC CCT GAA TGA TGA CA – 3’, T<sub>m</sub>: 50.0° C  *HPRT:* Forward 5’ – GAT ACA GGC CAG ACT TTG TTG – 3’, Reverse: 5’ – GGT AGG CTG GCC TAT AGG CT – 3’, T<sub>m</sub>: 50.0° C was used at 28 cycles.  *GATA-3:* Forward 5’ – AGG CAA GAT GAG AAA GAG TGC CTC -3’, Reverse 5’ – CTC GAC TTA CAT CCG AAC CCG GTA – 3’, T<sub>m</sub>: 55.0° C  *T-bet:* Forward: 5’ – GAT CGT CCT GCA GTC TCT CC – 3’, Reverse: 5’ - AAC TGT GTT CCC GAG GTG TC – 3’,
Tm: 52.0° C. IL-10: Forward: 5’–ACTGCTATGCTGCTGCTTTACT–3’, Reverse: 5’–TGGCCCTTGTAGACA CCTTGGTCTT–3’, Tm: 60.2° C. TGF-β: Forward: 5’–TAAAGAGGTCAACCCGCGTGCTAAT–3’, Reverse: 5’–TGTAACGTGTGTCAGGCTCCA–3’ Tm: 60.2° C.

3.3.9 Data analysis

Data were analyzed using GraphPad Prism statistical analysis and graphing software. Unpaired Student’s t-test was used to determine differences between two groups by Microsoft Excel. Multiple group comparison was made using ANOVA. A p value of <0.01 was considered significant.
3.4 Results of the Initial Adoptive Transfer Study using Donor cells from Naïve Mice

3.4.1 Establishment of AHR in CRA-sensitized and challenged mice

The AHR to methacholine was examined and established with non-invasive whole body plethysmograph and confirmed with a more rigorous invasive method involving tracheostomy and the measurement of specific airway resistance. Administration of 100 mg/ml methacholine exhibited Penh values of 3.94 ± 0.75 (n = 24 mice) (Figure 10A). Specific airway resistance induced by 100 mg/ml methacholine exhibited mean values 7.85 ± 0.75 cm H₂O.s/ml (Figure 10B). After tracheostomy several representative animals were sacrificed; lungs were harvested and sectioned, then stained with H&E to demonstrate histological hallmarks of asthmatic airways. The PBS control animals displayed normal airway morphology (Figure 10C). In contrast, histology from the CRA-sensitized and challenged mice without adoptive transfer (CRAAT-) exhibited inflammatory cell infiltration, epithelial cell hypertrophy and smooth muscle cell hyperplasia in the airways (Figure 10D).
Figure 10: Day 33 Non-invasive and Invasive Pulmonary Analysis. (A) Pulmonary function was evaluated by non-invasive technique of unrestrained single chamber whole body plethysmograph. (B) Invasive tracheostomy was a measure of specific airway resistance indicated by (Upright triangles) by CRAAT- mice. The results are presented as means ± SE of Penh values of six mice per group. (C) At day 33, several recipient mice were sacrificed, lungs sectioned, then stained with H&E. PBS control animals displayed normal lung parenchyma. (D) At day 33, sensitized animals exhibited the cardinal features of asthma.
3.4.2 The Purity of Isolated Lung and Spleen CD4+CD25+ T-cells for Adoptive Transfer

Mice were euthanized and lung and spleen tissue was processed by AutoMACS to collect lung and spleen CD4+CD25+ T-cells. These cell populations were analyzed by expression of CD4 conjugated to FITC and CD25 conjugated to PE. The purity of the cells isolated from the lung tissue was 74 – 76 percent and the spleen tissue was 90 – 96 percent (Figure 11).

Figure 11: Purity of Lung and Spleen CD4+CD25+ T-cells. A. Flow cytometric analysis of lung CD4+CD25+ T-cells from naïve BALB/c mice. B. Analysis of CD4+CD25+ T-cells isolated from spleen tissue of naïve BALB/c mice that were donors for adoptive transfer into cockroach-sensitized and challenged mice.

3.4.3 The effect of adoptive transfer of NTregs and iTregs

The cockroach-sensitized and challenged mice with established AHR were randomized into their respective groups to receive an adoptive transfer of designated T-regulatory
cells and CD4+CD25− T-cells (400,000 cells/mouse). The recipient mice of L25+ cells showed reversal of AHR for two weeks and the therapeutic effect gradually declined by week three (Figure 12A). The recipients of L25− cells exhibited AHR reversal for at least three weeks (Figure 12B). In contrast, the recipients that received S25+ cells (Figure 12C) and S25− cells (Figure 12D) experienced a remarkable therapeutic effect of attenuating AHR for at least 4 weeks.
Figure 12: **Pulmonary evaluation post adoptive transfer.** A. (L25+) These recipient mice received 400,000 naturally occurring CD4⁺CD25⁺ T-cells (NTregs) isolated from lungs of naïve BALB/c mice. B. (L25⁻) Recipients of inducible CD4⁺CD25⁻ T-cells isolated from the lungs of naïve BALB/c mice. C. (S25⁺) Recipient mice of NTregs isolated from the spleen of naïve BALB/c mice. D. (S25⁻) Recipients of iTregs purified from the spleen of naïve BALB/c mice. Data is shown as mean ± SEM of Penh values of six mice in each group. *p <0.01; **p <0.001.
3.4.4 Histological changes in the lung tissue post adoptive transfer:

The lung of all CRA\textsuperscript{AT}-groups exhibited extensive peri-bronchial and peri-vascular inflammation, epithelial cell hyperplasia (as shown by H&E stain; Figure 13A), collagen deposition (as shown by blue color using trichrome stain; Figure 13B) and mucus hypersecretion (as shown by pink color using PAS stain; Figure 13C). However, the adoptive transfer therapy of Tregs in the CRA\textsuperscript{AT+} mice nearly restored these features to the PBS control level (Figures 13 A, B and C). There was no apparent difference in the lung histology between the PBS control and the CRA\textsuperscript{AT+} groups.

<table>
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B.  

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</table>
Figure 13: Histological Evaluation of Airway Inflammation Post-adoptive transfer. (A) H & E staining was used to examine inflammation in the airways (B) Yellow arrows pointing to collagen deposition indicated in blue by trichrome staining around the airways. (C) Yellow arrows pointing to mucus secretion indicated in pink by PAS staining. These data are for six mice per group and magnification of 40x.
3.4.5 Cytokines in the BALF

In the BALF of all PBS control and CRA\textsuperscript{AT+} groups there were minimal levels of IL-4 compared to significantly elevated levels in the CRA\textsuperscript{AT-} groups (p\textless .001) (Figure 14A). In the BALF of mice in all CRA\textsuperscript{AT-} groups we observed substantially high levels of IL-5 compared to significantly reduced levels in the CRA\textsuperscript{AT+} and the PBS control groups (Figure 14B). These data indicate higher amount of T\textsubscript{H2} cytokines in the lungs of CRA\textsuperscript{AT-} groups than in the PBS control and CRA\textsuperscript{AT+} groups. High levels of IL-10 were observed in the BALF of all CRA\textsuperscript{AT+} groups compared to substantially low levels in both CRA\textsuperscript{AT-} and PBS control groups (Figure 14C). IL-10 is produced by T\textsubscript{H2} cells but, Treg cells have been shown to produce IL-10 at significantly high levels (194). In addition, in the CRA\textsuperscript{AT-} groups there was a significant increase in BALF IL-13 levels compared to significantly reduced levels in the CRA\textsuperscript{AT+} and PBS control groups (Figure 14D). The concentration of IFN-\(\gamma\) in the BALF of the CRA\textsuperscript{AT+} groups was significantly increased compared to the CRA\textsuperscript{AT-} and PBS control groups (Figure 14E).
Figure 14: Cytokines secreted in the BALF Post-adoptive transfer Day 68 Analyzed by ELISA. Animals were aerosol challenged for four consecutive weeks with cockroach antigen and euthanized on day 68. BALF was obtained and analyzed for cytokine levels in each experimental group. (A) IL-4, (B) IL-5, (C) IL-10, (D) IL-13 and (E) IFN-γ. These data are presented as means ± SE values of six mice per group. (ψ p <0.05, * p <0.01, ** p <0.001).

3.4.6 Inflammatory Cells in the Bronchoalveolar Lavage Fluid (BALF)

In the BALF of all (CRAAT+) there was a significant reduction in eosinophils and lymphocytes compared to CRAAT- group. The number of leukocytes in the BALF of the CRAAT+ group was nearly restored to the level observed in PBS control mice (Table 3).
Table 3: Absolute Number of BALF Leukocytes

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<th>Group II</th>
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<td>.71 ± .072</td>
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* p <0.01 and ** p <0.001; The CRA group values are compared with the PBS group; the CRA-Adoptive group values are compared with the CRA group.

3.4.7 Expression of PD-1 and ICOS in CD4+CD25+ cell isolated lungs and spleens recipient of mice post-adoptive transfer

Lung CD4+CD25+ cells were gated for PD-1 expression to compare between PBS control, CRAAT- group, and the CRAAT+ group. The cells from the recipients of L25- and S25- cells demonstrated a significant increase in both PD-1 or ICOS expression in CRAAT+ group compared to significantly low levels in PBS control and CRAAT- groups (Figure 15A
The left panels show PD-1 (Figure 15B) or ICOS (Figure 16B) expression in lung CD4+CD25+ cells post adoptive transfer in each experimental group. The lung CD4+CD25+ cells in the CRAAT+ in the recipients of L25- and S25- cells expressed significantly higher levels of PD-1 (Figure 15B) and ICOS (Figure 16B) compared to recipients of L25+ and S25+ cells. Lung CD4+CD25+ cells from the recipient mice of S25- cells expressed substantially higher levels of PD-1 than the recipients of S25+ cells (Figure 15). However, there was no significant difference in the ICOS levels between the S25- and S25+ groups (Figure 16B). The panels on the right show PD-1 (Figure 15B) or ICOS (Figure 16B) expression in spleen CD4+CD25+ cells post adoptive transfer in each experimental group. The expression of PD-1 was increased in all CRAAT+ groups except in the recipients of L25+ cells, compared to PBS control and CRAAT- (Figure 15B). However, the expression of ICOS was increased in all CRAAT+ groups except in the recipients of S25+ cells, compared to PBS control and CRAAT- (Figure 16B).
A. 

B. 

L25^+ group

L25^- group

S25^+ group

S25^- group

Lung tissue  Spleen tissue
Figure 15: The expression of PD-1 in lung and spleen CD4⁺CD25⁺ T-cells post-adoptive transfer. A. Contour plots that are representative of the cells in the L25⁻ and S25⁻ groups. B. Left panels are lung CD4⁺CD25⁺ cells of all experiment groups. Right panels are spleen tissue of the same. These data are presented as means ± SE values of six mice per group. * p <0.01, ** p <0.001.
A. 

B. 

L25+ group

L25- group

S25+ group

S25- group

% of cells expressing ICOS

Lung tissue

Spleen tissue

** **
Figure 16: The expression of ICOS in lung and spleen CD4+CD25+ T-cells post-adoptive transfer

A. Contour plots that are representative of the cells in the L25- and S25- groups. B. Left panels CD4+CD25+ T-cells from lung tissue of all experiment groups. The right panels are from spleen tissue of the same animals. These data are presented as means ± SE values of six mice per group. * p <0.01, ** p <0.001.

3.4.8 Expression of PD-1, ICOS and Foxp3 in Lung CD4+CD25- cells from Recipients of L25- and S25- cells Post-adoptive Transfer

The expression of PD-1, ICOS, and Foxp3 is shown in a representative contour plot for each set of cells in the L25- and S25- groups (Figure 17 A-C) respectively. CD4+CD25- cells in the PBS control were PD-1low compared to PD-1int cells in the CRAAT- and CRAAT+ groups (Figure 17A). In contrast, ICOS expression of CD4+CD25- in the PBS control and CRAAT+ groups were ICOSlow compared to ICOSint in the CRAAT- group (Figure 17B). In addition, the lung CD4+CD25- T-cells in the PBS control, CRAAT-, and CRAAT+ groups showed minimal or no expression of intracellular Foxp3 protein (Figure 17C). Statistical bar graphs showing the expression of ICOS and PD-1 on CD4+CD25- T-cells isolated from the lungs of adoptively transferred mice (Figure 17D). These data are presented as means ± SE values of six mice per group. Intermediate = cell population has shifted to the second decade but, below the third decade.