The role of IL-15 in response to rhinovirus infections

A thesis submitted for the degree of
Doctor of Philosophy

Annabelle Tan Jayaraman

Department of Respiratory Medicine
National Heart and Lung Institute
Faculty of Medicine
Imperial College London
Norfolk Place
London W2 1PG

2012
Abstract
Rhinoviruses (RV) cause the common cold and are major precipitants of asthma exacerbations. The underlying mechanisms of RV-induced airways disease are unclear. IL-15 is a proinflammatory cytokine produced during viral infections and plays a key role in the regulation of NK cells. Using mouse models of RV infection and RV-induced asthma exacerbation we examined the role of IL-15 and its importance for NK cell responses during RV infections in allergic and non-allergic airways. We demonstrate RV-induced IL-15 upregulation in the airway and lungs of BALB/c mice at day 1 after infection and accumulation of NK cells in the airway and lungs at days 1-2 and 2-4 respectively. The NK cells exhibited an activated phenotype characterised by upregulated CD69, IFN-γ and GranzymeB expression. Blocking IL-15 upon intranasal administration of an IL-15 neutralising antibody inhibited the NK cell response to RV infection, which was associated with deficient IFN-γ production and increased expression of Th2 mediators. IL-15Rα knockout mice lack NK cells and also demonstrated deficient IFN-γ and increased Th2 responses to RV infection; these mice also exhibited deficient CD8+ T cell responses and an increased viral load. Similar results were observed in RV infected IFNAR1 ko mice, which was associated with deficient IL-15 upregulation. We suggest that RV-induced IL-15 is mediated by type I interferon signalling, and is necessary for NK cell responses and early IFN-γ production during RV-1B infection, which drives development of appropriate Th1 antiviral responses. In the absence of this pathway, Th2 responses result and are associated with impaired antiviral immunity. To examine the interaction between allergen driven Th2 immunity and RV infection, we employed a RV-induced asthma exacerbation model. Unexpectedly, RV infected allergen challenged mice, despite having increased viral load, demonstrated increased IL-15 expression and NK cell responses, revealing a novel interaction between allergic responses and antiviral immunity.
# Table of Contents

Abstract ................................................................................................................................. 2

List of Figures .......................................................................................................................... 8

List of Tables .......................................................................................................................... 11

Abbreviations .......................................................................................................................... 12

Statement of Work .................................................................................................................. 15

Acknowledgements ................................................................................................................. 16

1. Chapter 1: Introduction ........................................................................................................ 17
  1.1. Human Rhinoviruses ........................................................................................................ 17
      1.1.1. Epidemiology ........................................................................................................... 17
      1.1.2. Classification ........................................................................................................... 17
      1.1.3. Structure & Life Cycle ............................................................................................... 18
      1.1.4. Disease Pathogenesis & Clinical Features ................................................................. 18
      1.1.5. Therapies ................................................................................................................ 20

1.2. Asthma ............................................................................................................................. 21
      1.2.1. Epidemiology ........................................................................................................... 21
      1.2.2. Definition & Diagnosis ............................................................................................ 21
      1.2.3. Classification ........................................................................................................... 22
      1.2.4. Immunology ............................................................................................................ 22
      1.2.5. Therapy .................................................................................................................. 25

1.3. Asthma Exacerbations ..................................................................................................... 26
      1.3.1. Introduction .............................................................................................................. 26
      1.3.2. Interaction Between Infection & Allergic Asthma ....................................................... 27
      1.3.3. Mechanisms of RV-Induced Asthma Exacerbations ................................................ 27
      1.3.3.1. RV-Modulated Epithelial ICAM-1 Expression ....................................................... 28
      1.3.3.2. RV-Induced Epithelial Expression of Proinflammatory Mediators ....................... 28
      1.3.3.3. RV Infection of the Lower Airways in Asthmatics .................................................. 29
      1.3.3.4. Deficient type I and III Interferon Responses in Asthmatic Airway Epithelial Cells 29
      1.3.3.5. Imbalanced Th1 and Th2 Responses to RV Infection in Asthmatics ..................... 30

1.4. Interleukin-15 ................................................................................................................. 30
      1.4.1. Structure, Expression & Regulation ........................................................................... 30
      1.4.2. Signalling .................................................................................................................. 32
      1.4.3. Role of IL-15 in the Immune System ....................................................................... 35

1.5. Interferons ....................................................................................................................... 37
      1.5.1. Type I Interferons ...................................................................................................... 37
      1.5.2. Expression of Type I Interferons .............................................................................. 37
      1.5.3. Type I Interferon Signalling & Expression of Interferon Stimulated Genes .......... 38
      1.5.4. Type II Interferon ..................................................................................................... 39
      1.5.5. Type III Interferons ................................................................................................. 40

1.6. Natural Killer Cells .......................................................................................................... 41
      1.6.1. Introduction .............................................................................................................. 41
      1.6.2. NK Cell Activating & Inhibitory Surface Receptors .................................................. 41
1.6.3. NK Cell Cytotoxicity & Expression of Soluble Mediators ........................................... 42
1.6.4. Importance of NK Cell Mediated Antiviral Immunity ........................................... 44
1.6.5. IL-15 Mediated Regulation of NK Cells ..................................................................... 45

1.7. Mouse Models of RV Infection and RV-Induced Asthma Exacerbation .................. 46

1.8. Study Rationale, Hypotheses, Aims & Thesis Outline ............................................. 46
1.8.1. Rationale ..................................................................................................................... 46
1.8.2. Hypotheses ............................................................................................................... 47
1.8.3. Aims .......................................................................................................................... 47
1.8.4. Thesis Outline ........................................................................................................... 48

2. Chapter 2: Materials and Methods ................................................................................. 49
2.1. Virological Techniques ................................................................................................. 49
2.1.1. Virus Propagation ...................................................................................................... 49
2.1.2. Virus Purification and Concentration ....................................................................... 49
2.1.3. Virus Titration .......................................................................................................... 50
2.1.4. Virus Inactivation ...................................................................................................... 50

2.2. Mice ............................................................................................................................. 50

2.3. Mouse Models ............................................................................................................ 50
2.3.1. RV Infection Model ................................................................................................. 50
2.3.2. RV Infection Model with Neutralisation of Airway/Lung IL-15 ............................ 51
2.3.3. RV-Induced Asthma Exacerbation Model .............................................................. 52
2.3.3.1. Lung Function: Assessment of Airway Hyperreactivity (AHR) ....................... 53

2.4. Sample Harvesting & Processing ............................................................................... 53
2.4.1. Bronchoalveolar Lavage (BAL) ............................................................................. 53
2.4.2. Cytospins ............................................................................................................... 53
2.4.3. Lung Tissue Processing for Protein Analysis ......................................................... 54
2.4.4. BAL Cell and Lung Tissue Processing for RNA Extraction ................................. 54
2.4.5. Isolating Lung Cells for Flow Cytometry ............................................................... 54

2.5. Flow Cytometry .......................................................................................................... 54
2.5.1. Surface Staining of BAL and Lung Leukocytes ....................................................... 54
2.5.2. Intracellular Cytokine Staining (ICS) of BAL and Lung Leukocytes .................... 55
2.5.3. Flow Cytometry Data Acquisition and Analysis ...................................................... 55

2.6. Taqman Quantitative PCR ......................................................................................... 56
2.6.1. RNA Extraction and Reverse Transcription ......................................................... 56
2.6.2. qRT-PCR ............................................................................................................... 56
2.6.3. qPCR Standards ..................................................................................................... 58

2.7. ELISA .......................................................................................................................... 60

2.8. Immunohistochemistry ............................................................................................... 61

2.9. Statistical Analysis ...................................................................................................... 61

3. Chapter 3: Results - IL-15 expression and the NK cell response during RV-1B infection .................................................................................................................. 68
3.1. Introduction .................................................................................................................. 68
3.2. Hypotheses & Aims .................................................................................................... 69
3.2.1. Hypotheses ............................................................................................................. 69
3.2.2. Aims ......................................................................................................................... 69
3.3. Results ........................................................................................................................ 70
3.3.1. Virus Load ............................................................................................................. 70
3.3.2. Differential BAL Cell Counts .............................................................................. 70
3.3.3. BAL Proinflammatory Mediators ...................................................................... 72
3.3.4. Expression of IL-15 and IL-15Rα ................................................................. 73
3.3.4.1. BAL and Lung IL-15 and IL-15Rα Gene Expression .................................. 73
3.3.4.2. Lung IL-15 and IL-15Rα Protein Expression .............................................. 73
3.3.5. Expression of Type I and III Interferons .......................................................... 76
3.3.6. NK Cell Response to RV-1B Infection .............................................................. 78
3.3.6.1. BAL NK Cells .................................................................................................. 78
3.3.6.2. Lung NK Cells ................................................................................................ 80
3.3.7. T Cell Response to RV-1B Infection ................................................................. 82
3.3.7.1. BAL T Cells .................................................................................................... 82
3.3.7.2. Lung T Cells .................................................................................................. 85
3.4. Chapter Summary ................................................................................................. 88

4. Chapter 4: Results - Immune responses to RV infection in the absence of IL-15
signalling in vivo. ........................................................................................................ 90
4.1. Introduction ............................................................................................................. 90
4.2. Hypotheses & Aims .............................................................................................. 91
4.2.1. Hypotheses ....................................................................................................... 91
4.2.2. Aims ................................................................................................................... 91
4.3. Results – Part 1: Transient and Local Neutralisation of IL-15 During RV Infection 92
4.3.1. Virus Load ......................................................................................................... 92
4.3.2. Differential BAL Cell Counts ......................................................................... 93
4.3.3. Expression of Lung IL-15 and IL-15Rα ......................................................... 95
4.3.4. BAL Proinflammatory Mediators ..................................................................... 95
4.3.5. Expression of Type I IFNs, Type III IFNs and Interferon Stimulated Genes .. 95
4.3.6. Expression of IFN-γ .......................................................................................... 98
4.3.7. Expression of Th2 Mediators .......................................................................... 98
4.3.8. Effect of M96 Antibody Treatment on Splenic and Lung Lymphocytes..... 100
4.3.9. NK Cell Response ............................................................................................ 102
4.3.9.1. BAL NK Cells ............................................................................................... 102
4.3.9.2. Lung NK Cells ............................................................................................. 103
4.3.10. T Cell Response .............................................................................................. 106
4.3.10.1. BAL T Cells ................................................................................................ 106
4.3.10.2. Lung T Cells ............................................................................................... 106
4.4. Chapter Summary – Part 1.................................................................................. 110
4.5. Results – Part 2: RV-1B Infection in IL-15Rα ko Mice........................................ 112
4.5.1. Virus Load ....................................................................................................... 112
4.5.2. Differential BAL Cell Counts ......................................................................... 112
4.5.3. BAL Proinflammatory Mediators ..................................................................... 114
4.5.4. Expression of Type I IFNs, Type III IFNs and Interferon Stimulated Genes . 114
4.5.5. Expression of IFN-γ .......................................................................................... 114
4.5.6. Expression of Th2 Mediators .......................................................................... 117
4.5.7. NK Cell Response ............................................................................................ 119
5. Chapter 5: Results - IL-15 expression and the NK cell responses in the absence of type I IFN signalling ................................................................. 129
  5.1. Introduction .................................................................................. 129
  5.2. Hypotheses & Aims ..................................................................... 130
    5.2.1. Hypotheses ........................................................................... 130
    5.2.2. Aims ...................................................................................... 130
  5.3. Results .......................................................................................... 131
    5.3.1. Virus Load ............................................................................. 131
    5.3.2. Differential BAL Cell Counts .................................................. 131
    5.3.3. BAL Proinflammatory Mediators .......................................... 133
    5.3.4. Expression of Type I and III Interferons ............................... 133
    5.3.5. Expression of Interferon Stimulated Genes ......................... 136
    5.3.6. Expression of IL-15 and IL-15Rα ........................................... 137
    5.3.7. Expression of IFN-γ ............................................................... 137
    5.3.8. Expression of Th2 Mediators .................................................. 138
    5.3.9. NK Cell Response ................................................................. 140
      5.3.9.1. BAL NK Cells ................................................................. 140
      5.3.9.2. Lung NK Cells ............................................................... 140
    5.3.10. T Cell Response ................................................................... 145
      5.3.10.1. BAL T Cells ................................................................. 145
      5.3.10.2. Lung T Cells ............................................................... 145
  5.4. Chapter Summary ........................................................................ 148
6. Chapter 6: Results – IL-15 expression and the NK cell response during RV-induced asthma exacerbation ................................................................ 149
  6.1. Introduction .................................................................................. 149
  6.2. Hypotheses & Aims ..................................................................... 151
    6.2.1. Hypotheses ........................................................................... 151
    6.2.2. Aims ...................................................................................... 151
  6.3. Results .......................................................................................... 152
    6.3.1. Virus Load ............................................................................. 152
    6.3.2. Differential BAL Cell Counts .................................................. 153
    6.3.3. Expression of Th2 Mediators .................................................. 154
    6.3.4. Airway Hyperreactivity ........................................................... 155
    6.3.5. Expression of IL-15 and IL-15Rα ........................................... 156
    6.3.6. Expression of IFN-γ ............................................................... 157
    6.3.7. NK Cell Response ................................................................. 159
      6.3.7.1. BAL NK Cells ................................................................. 159
      6.3.7.2. Lung NK Cells ............................................................... 162
      6.3.7.3. IL-4 Expression in NK Cells ........................................... 165
6.3.8. T Cell Response

6.3.8.1. BAL T Cells

6.3.8.2. Lung T Cells

6.4. Chapter Summary

7.

Chapter 7: Discussion & Future Work

7.1. RV-Induced IL-15 Expression

7.2. NK Cell Responses During RV Infection

7.3. Regulation of NK Cell Responses by IL-15

7.4. Type I IFN Signalling is Necessary for RV-induced IL-15 and the NK Cell Response

7.5. IL-15 is Required for CD8+ T Cell Responses During RV Infection

7.6. Role of Type I IFNs on T cells During RV Infection

7.7. Deficient IFN-γ Responses & Associated Expression of Th2 Mediators

7.8. RV-Induced IL-15 and the NK Cell Response in Allergic Airways

7.9. Summary of Conclusions

7.10. Future Work

7.10.1. The Role of IL-15 and NK cells in RV Replication In Vivo

7.10.2. Cellular Sources of IL-15 Protein in the Lungs

7.10.3. Importance of IL-12 Expression for RV-induced NK Cell Responses

7.10.4. Role of IL-25 in Mediating Th2 Responses to RV infection

7.10.5. Intranasal Administration of rmIL-15 in IFNAR1 ko Mice During RV Infection

7.10.6. Importance of NK Cell Cytotoxic Activity During RV Infection

7.10.7. Role of IL-15 in the Mouse Model of RV-Induced Asthma Exacerbation

7.10.8. IL-15 Expression and NK Cell Responses to RV Infection in Asthmatics and Non-Asthmatics

8. Chapter 8: References
**List of Figures**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Functions of inflammatory mediators and immune cells in asthma</td>
<td>25</td>
</tr>
<tr>
<td>1.2</td>
<td>IL-15 trans-presentation</td>
<td>34</td>
</tr>
<tr>
<td>1.3</td>
<td>NK cell mediated immune functions</td>
<td>44</td>
</tr>
<tr>
<td>2.1</td>
<td>RV infection model with antibody-mediated neutralisation of airway/lung IL-15</td>
<td>51</td>
</tr>
<tr>
<td>2.2</td>
<td>RV-induced asthma exacerbation model</td>
<td>52</td>
</tr>
<tr>
<td>2.3</td>
<td>Representative flow cytometry gating for live, single lymphocytes</td>
<td>63</td>
</tr>
<tr>
<td>2.4</td>
<td>Representative flow cytometry gating for BAL lymphocytes in BALB/c mice</td>
<td>64</td>
</tr>
<tr>
<td>2.5</td>
<td>Representative flow cytometry gating for lung lymphocytes</td>
<td>65</td>
</tr>
<tr>
<td>2.6</td>
<td>Representative flow cytometry gating for intracellular cytokine staining in lung NK cells</td>
<td>66</td>
</tr>
<tr>
<td>2.7</td>
<td>Representative flow cytometry gating for intracellular cytokine staining in lung T cells</td>
<td>67</td>
</tr>
<tr>
<td>3.1</td>
<td>Virus load in the lungs during RV-1B infection</td>
<td>70</td>
</tr>
<tr>
<td>3.2</td>
<td>Differential BAL cell counts during RV-1B infection</td>
<td>71</td>
</tr>
<tr>
<td>3.3</td>
<td>BAL cytokines and chemokines produced in response to RV-1B infection</td>
<td>72</td>
</tr>
<tr>
<td>3.4</td>
<td>RV-1B infection induces IL-15 and IL-15Rα expression in BAL cells and lung tissue</td>
<td>74</td>
</tr>
<tr>
<td>3.5</td>
<td>Immunohistochemistry staining for IL-15 expression in the lungs of BALB/c mice in response to RV-1B infection</td>
<td>75</td>
</tr>
<tr>
<td>3.6</td>
<td>RV-1B infection induces expression of type I and III IFNs in BAL cells and lung tissue</td>
<td>77</td>
</tr>
<tr>
<td>3.7</td>
<td>BAL NK cell response during RV-1B infection</td>
<td>79</td>
</tr>
<tr>
<td>3.8</td>
<td>Total lung leukocyte count during RV-1B infection</td>
<td>80</td>
</tr>
<tr>
<td>3.9</td>
<td>Lung NK cell response during RV-1B infection</td>
<td>81</td>
</tr>
<tr>
<td>3.10</td>
<td>BAL CD4+ T cell response during RV-1B infection</td>
<td>83</td>
</tr>
<tr>
<td>3.11</td>
<td>BAL CD8+ T cell response during RV-1B infection</td>
<td>84</td>
</tr>
<tr>
<td>3.12</td>
<td>Lung CD4+ T cell response during RV-1B infection</td>
<td>86</td>
</tr>
<tr>
<td>3.13</td>
<td>Lung CD8+ T cell response during RV-1B infection</td>
<td>87</td>
</tr>
<tr>
<td>4.1</td>
<td>Effect of IL-15 neutralisation in the airway/lungs on lung virus load during RV-1B infection</td>
<td>93</td>
</tr>
<tr>
<td>4.2</td>
<td>Effect of IL-15 neutralisation in the airway/lungs on total and differential BAL cell counts during RV-1B infection</td>
<td>94</td>
</tr>
<tr>
<td>4.3</td>
<td>IL-15 neutralisation in the airway/lungs does not affect RV-induced IL-15 and IL-15Rα</td>
<td>95</td>
</tr>
<tr>
<td>4.4</td>
<td>Effect of IL-15 neutralisation in the airway/lungs on expression of BAL proinflammatory cytokines and chemokines during RV-1B infection</td>
<td>96</td>
</tr>
<tr>
<td>4.5</td>
<td>Effect of IL-15 neutralisation in the airway/lungs on expression of BAL and lung type I IFNs, type III IFNs and ISGs during RV-1B infection</td>
<td>97</td>
</tr>
<tr>
<td>4.6</td>
<td>IL-15 neutralisation in the airway/lungs results in deficient IFN-γ expression in the lungs during RV-1B infection</td>
<td>98</td>
</tr>
<tr>
<td>4.7</td>
<td>IL-15 neutralisation in the airway/lungs results in increased expression of Th2 mediators in the BAL and lungs during RV-1B infection</td>
<td>99</td>
</tr>
</tbody>
</table>
Figure 4.8 Intranasal administration of M96 IL-15 neutralising antibody does not affect T cell populations but reduces the frequency of lung and splenic NK cells by 48h after administration................................. 101
Figure 4.9 IL-15 neutralisation in the airway/lungs results in deficient BAL NK cell responses during RV-1B infection................................................................. 103
Figure 4.10 Effect of IL-15 neutralisation in the airway/lungs on the total lung leukocyte count during RV-1B infection................................................................. 104
Figure 4.11 IL-15 neutralisation in the airway/lungs results in deficient lung NK cell responses during RV-1B infection................................................................. 105
Figure 4.12 Effect of IL-15 neutralisation in the airway/lungs on the BAL T cell response during RV-1B infection................................................................. 107
Figure 4.13 Effect of IL-15 neutralisation in the airway/lungs on the lung CD4+ T cell response during RV-1B infection................................................................. 108
Figure 4.14 Effect of IL-15 neutralisation in the airway/lungs on the lung CD8+ T cell response during RV-1B infection................................................................. 109
Figure 4.15 IL-15Rα ko mice have an increased lung viral load after RV-1B................. 112
Figure 4.16 Total and differential BAL cell counts during RV-1B infection in IL-15Rα ko mice infection................................................................. 113
Figure 4.17 Expression of BAL proinflammatory cytokines and chemokines in response to RV-1B infection in IL-15Rα ko mice................................................................. 115
Figure 4.18 Expression of BAL and lung type I and III IFNs and interferon stimulated genes in response to RV-1B infection in IL-15Rα ko mice................................................................. 116
Figure 4.19 IL-15Rα ko mice have a deficient IFN-γ response during RV-1B infection....... 117
Figure 4.20 IL-15Rα ko mice demonstrate increased expression of Th2 cytokines and chemokines in BAL fluid and lung tissue during RV-1B infection........................ 118
Figure 4.21 Representative flow plots of BAL and lungs NK cells in B6.129 and IL-15Rα ko mice.................................................................................................................. 119
Figure 4.22 The BAL NK cell response to RV-1B infection in IL-15Rα ko mice is absent.... 120
Figure 4.23 Total lung leukocyte count during RV-1B infection in IL-15Rα ko mice........ 121
Figure 4.24 The lung NK cell response to RV-1B infection in IL-15Rα ko mice is absent.... 122
Figure 4.25 The BAL CD8+ T cell but not CD4+ T cell response during RV-1B infection in IL-15Rα ko mice is deficient................................................................. 124
Figure 4.26 Lung CD4+ T cell response to RV-1B infection in IL-15Rα ko mice............... 126
Figure 4.27 The lung CD8+ T cell response to RV-1B infection in IL-15Rα ko mice is deficient.................................................................................................................. 127
Figure 5.1 IFNAR1 ko mice demonstrate an increased virus load after RV-1B infection..... 131
Figure 5.2 Differential BAL cell counts during RV-1B infection of IFNAR1 ko mice........ 132
Figure 5.3 BAL cytokines and chemokines produced in response to RV-1B infection in IFNAR1 ko mice................................................................................................................. 134
Figure 5.4 Expression of type I and III IFNs in response to RV-1B infection in IFNAR1 ko mice is deficient.................................................................................................................. 135
Figure 5.5 Expression of interferon stimulated genes in response to RV-1B infection in IFNAR1 ko mice is deficient................................................................................................................. 136
Figure 5.6 Expression of IL-15 and IL-15Rα in response to RV-1B infection in IFNAR1 ko mice is deficient................................................................................................................. 137
Figure 5.1 Expression of IFN-γ in response to RV-1B infection in IFNAR1 ko mice is deficient.......................................................................................... 138
Figure 5.2 IFNAR1 ko mice demonstrate increased expression of Th2-associated mediators in response to RV-1B infection...................................................................................................................... 139
Figure 5.3 The BAL NK cell response to RV-1B infection in IFNAR1 ko mice is deficient........................................................................................................ 141
Figure 5.4 Representative flow plots demonstrating deficient BAL NK cell responses at 48h after RV-1B infection in IFNAR1 ko mice.............................................................................................................. 142
Figure 5.5 The lung NK cell response to RV-1B infection in IFNAR1 ko mice is deficient........................................................................................................ 143
Figure 5.6 Representative flow plots demonstrating deficient lung NK cell resposnes at 48h after RV-1B infection in IFNAR1 ko mice.............................................................................................................. 144
Figure 5.7 BAL CD4 and CD8 T cell response during RV-1B infection of IFNAR1 ko mice... 146
Figure 5.8 Lung CD4 and CD8 T cell response during RV-1B infection of IFNAR1 ko mice........................................................................................................ 147
Figure 5.9 RV-1B infected mice with allergic airways inflammation demonstrated an increased virus load compared to non-allergic mice......................................................................................... 153
Figure 5.10 RV-1B infected mice with allergic airways inflammation demonstrated enhanced airway neutrophilia compared to non-allergic mice......................................................................................... 154
Figure 5.11 Expression of lung Th2 cytokines during an RV-induced allergic exacerbation model....................................................................................................................... 155
Figure 5.12 RV-1B infection in allergic mice enhanced airway hyperreactivity............... 156
Figure 5.13 Expression of RV-induced IL-15 in BAL cells and lung tissue was enhanced in mice with allergic airways inflammation compared to non-allergic mice................................................................. 158
Figure 5.14 Expression of lung IFN-γ and BAL IP-10 during an RV-induced allergic exacerbation model....................................................................................................................... 159
Figure 5.15 The BAL NK cell response to RV-1B infection was enhanced in mice with allergic airways inflammation compared to non-allergic mice................................................................. 161
Figure 5.16 Total lung leukocyte counts during an RV-induced allergic exacerbation model....................................................................................................................... 163
Figure 5.17 The Lung NK cell response to RV-1B infection was enhanced in mice with allergic airways inflammation compared to non-allergic mice......................................................................................... 164
Figure 5.18 BAL and lung NK cells expressed IL-4 in resposne to RV-1B infection in mice with allergic airways inflammation....................................................................................................................... 165
Figure 5.19 BAL CD4+ T cell response during an RV-induced allergic exacerbation model....................................................................................................................... 167
Figure 5.20 BAL CD8+ T cell response during an RV-induced allergic exacerbation model....................................................................................................................... 168
Figure 5.21 Lung CD4+ T cell response during an RV-induced allergic exacerbation model....................................................................................................................... 171
Figure 5.22 Lung CD8+ T cell response during an RV-induced allergic exacerbation model....................................................................................................................... 172
Figure 6.1 Role of IL-15 during rhinovirus infections................................................................. 192
List of Tables
Table 1.1 A selection of rhinovirus antiviral therapies............................................................... 20
Table 1.2 Functional effects of IL-15 signalling on immune cells............................................. 36
Table 2.1 RV infection model with neutralisation of airway/lung IL-15, treatment group nomenclature.................................................................................................................. 51
Table 2.2 RV-induced asthma exacerbation model, treatment group nomenclature.............. 52
Table 2.3 Monoclonal antibodies used for flow cytometry (BD Biosciences)......................... 56
Table 2.4 Example of Taqman qPCR Analysis........................................................................ 57
Table 2.5 Taqman quantitative PCR primers and probes......................................................... 59
Table 2.6 Media and Buffers..................................................................................................... 62
Table 4.1 Experimental groups of the RV infection model with neutralisation of airway/lung IL-15................................................................................................................................. 92
Table 6.1 Experimental groups of the RV-induced asthma exacerbation model, and the breakdown of treatments received by mice in each group......................................................... 152
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR</td>
<td>Airway hyperreactivity</td>
</tr>
<tr>
<td>Alum</td>
<td>Aluminium hydroxide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BEC</td>
<td>Bronchial epithelial cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>Cy</td>
<td>Cyanine</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetate acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FEV</td>
<td>Forced expiratory volume</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex virus</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule 1</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroid</td>
</tr>
<tr>
<td>ICS</td>
<td>Intracellular cytokine staining</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFNAR</td>
<td>Interferon-alpha receptor</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon-gamma inducible protein 10kDa/CXCL10</td>
</tr>
<tr>
<td>IRE</td>
<td>Interferon regulatory element</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>ISO</td>
<td>Isotype</td>
</tr>
<tr>
<td>I-TAC</td>
<td>Interferon-inducible T cell alpha chemoattractant</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>kB</td>
<td>Kilobase (pairs)</td>
</tr>
<tr>
<td>ko</td>
<td>knockout</td>
</tr>
</tbody>
</table>
I  litre
LDL  Low density lipoprotein
M  molar
MDA-5  Melanoma differentiation-associated protein-5
MDC  Monocyte-derived chemokines/CCL22
MHC  Major histocompatibility complex
min  Minutes
MIP-2  Macrophage inflammatory protein/CXCL2
mRNA  messenger ribonucleic acid
NaCl  Sodium chloride
NF-κB  Nuclear factor-κB
NK  Natural killer
OAS  Oligoadenylate synthetase
OVA  Ovalbumin
PAMP  Pathogen associated molecular pattern
PBMC  Peripheral blood mononuclear cells
PBS  Phosphate buffered saline
PC_{10/20}  Provocative concentration
PCR  Polymerase chain reaction
PE  Phycoerythrin
PEF  Peak expiratory flow
PEG  Poly ethylene glycol
PenH  Enhanced pause
PerCP  Peridinin-chlorophyll proteins
PKR  Protein kinase R
PMA  Phorbol myristate acetate
PolyIC  Polynosinic-polycytidylic acid
PRR  Pattern recognition receptor
qPCR  Quantitative (reverse transcriptase) polymerase chain reaction
RANTES  Regulated upon activation, normal T cell expressed, and secreted/CCL5
RIG-1  Retinoic acid inducible gene-1
RNA  Ribonucleic acid
RNaseL  Endoribonuclease L
RPMI  Roswell Park Memorial Institute
RSV  Respiratory syncytial virus
RT-PCR  Reverse transcription polymerase chain reaction
RV  Rhinovirus
SEM  Standard error of the mean
Ss  Single stranded
TARC  Thymus and activated-regulated chemokines/CCL17
Tc1/2  Cytotoxic T lymphocyte type 1/2
T cell  T lymphocyte
TCID_{50}  Tissue culture infective dose
Th1/2  T helper lymphocyte type 1/2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl benzidine chromogen</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
**Statement of Work**

I declare that the work presented in this thesis was carried out by myself unless stated otherwise. Specifically, the immunohistochemistry staining of BALB/c mouse lung sections was carried out by Dr. Gaetano Caramori, and measurement of mouse airway hyperreactivity was carried by Peter Sowinski.
Acknowledgements

I must express my gratitude first and foremost to my supervisors Dr. Nathan Bartlett and Professor Sebastian Johnston for the opportunity to undertake this project. In particular, thanks to Nathan for his teaching, guidance and assistance in the laboratory, guidance in writing this thesis and his tough but effective approach in supporting my development into a confident and independent researcher. I am also very thankful for the support received by all members of the Johnston group, the department of respiratory medicine at St. Mary’s campus and the CBS facility. Specifically, thank you to Dr. Corinna Schnöller for her kind support during the difficult times and for all the flow cytometry advice, which was invaluable to my work, and Rebecca Pearson for the memorable banging/ELISA assistance.

Thank you to Imperial College London for the Overseas Research Scholarship and Student Opportunities Fund awards. In addition, I would like to thank all current and previous members of the Beit Hall wardening team whom I have worked with for all the memorable times that made my PhD years very enjoyable.

Finally, I would like to thank my friends and family for their support. My father in particular has been the biggest rock in my life. I would like to thank him for all the work, stress and difficulties he had to overcome to provide me with all the opportunities that have allowed me to be where I am today. His difficult circumstances has taught me to be the confident and strong willed person I am, which has allowed me to endure this difficult process. Last but not least, words cannot express how grateful I am to my boyfriend Eduardo, without whom I would have struggled to keep my sanity during this process. Thank you for all the meals prepared and all the laundry and washing up that you did. Thank you for your generous support that enabled me to get up and continue every single day during my PhD.
1. **Chapter 1: Introduction**

1.1. **Human Rhinoviruses**

1.1.1. **Epidemiology**

Human rhinoviruses (RVs) are the most frequent cause of the common cold and were first isolated and cultured in 1953 from nasal secretions [1]. Viral respiratory tract infections are amongst the most frequent illnesses in humans, and epidemiological studies conducted in the 1960s found RVs to be the causative agent of >60% of all upper respiratory tract infections with cold symptoms [1, 2]. Other aetiological agents of the common cold include respiratory syncytial virus (RSV), influenza viruses A and B, parainfluenza viruses, coronavirus, adenovirus, *Chlamydia pneumoniae* and *Streptococcus pneumoniae* [3].

In the 1990s, reverse transcriptase PCR (PCR) greatly improved diagnosis and detection of RVs, due to increased sensitivity and specificity in comparison to cell culture techniques previously used, demonstrating higher detection rates than earlier epidemiological studies. Transmission of RVs occurs primarily via contaminated surfaces and inhalation of aerosolized particles [4, 5]. On average, it has been observed that adults are infected with RVs 2-3 times per year, and 6-12 times per year in pre-school aged children [6, 7]. Additionally, the rate of symptomatic infection is seen to decrease with age, possibly attributed to development of immune memory and production of protective serum neutralising antibodies [8], though this may be an artefact of observing higher transmission rates in children [9]. RV infections occur all year round; in temperate climates the frequency peaks during early autumn and spring, during which RV infections are the dominant respiratory infection [8]. The peak in tropical areas coincides with the start of the rainy season [10].

More significant to the medical and economic burden caused by RV infections is the discovery of its contribution to development of asthma exacerbations in the late 1990s. RV infections in infants have been linked with development of an asthmatic phenotype. These inflections are estimated to result in ~30 billion US dollars per year in health care costs and lost productivity [11].

1.1.2. **Classification**

RVs belong to the *Picornaviridae* family, literally translated as ‘small RNA viruses’, and are classified within the genus of enteroviruses. Initially, species definition was based on partial genome sequence analysis (of genes VP2 and VP4) that identified two species – A and B, and
sequence analysis of recent RV isolates have detected a third species – C. In 2009, Palmenberg et al. [12] completed sequencing of all classical RV genomes, greatly improving characterisation of HRV-A, HRV-B and the recently identified HRV-C species. Within species A and B, ~100 serotypes have been identified, which fall into 2 categories based on the receptor used for host cell entry: ‘minor group’ RVs (~10%) utilise the low-density lipoprotein receptor (LDL-R) and ‘major group’ RVs (~90%) bind the intracellular adhesion molecule 1 (ICAM-1) [13-15].

1.1.3. Structure & Life Cycle
RVs are small, ~20-30nm in size, non-enveloped viruses. The capsid entails an icosahedral shell of 60 protomers, consisting of 4 capsid proteins VP1-VP4 that are organised such that a deep canyon exists within the protomer [16]. X-ray crystallographic analyses have identified these canyons as ICAM-1 binding sites for virus attachment and entry of host cells [17, 18]. The shell encapsidates an ~7kb single stranded RNA genome containing a single open reading frame, which yields 4 structural capsid proteins and 7 non-structural proteins [19]. Functions of some non-structural proteins remain to be fully elucidated, but are suggested to interfere with host cellular functions including transcription and translation [20].

Upon receptor binding, RV is internalised via receptor-mediated endocytosis and uncoating occurs in the endosome allowing transfer of viral RNA into the cytoplasm. The positive-sense genomic RNA is immediately translated to produce a polypeptide that is cleaved by viral proteases 2A and 2C (included in the polypeptide), generating VP1-4, an RNA dependant RNA polymerase and a small viral protein (Vpg) – the later 2 proteins act to replicate the RNA genome [2]. Progeny genomes are encapsulated by the structural proteins, and the mature virions are released by cell lysis. The growth cycle of RVs is 5-7 hours, and released progeny virions are detectable by 12h post-infection (p.i.) after experimental inoculation in humans [21]; virus titres peak at days 2-3 p.i. and virus shedding continues until total elimination, which can take up to 3 weeks p.i. in otherwise healthy individuals [22-25].

1.1.4. Disease Pathogenesis & Clinical Features
RVs primarily infect epithelial cells of the upper respiratory tract, specifically the nasal mucosa and nasopharynx. Unlike other respiratory viruses, clinical symptoms are primarily caused by the hosts’ inflammatory immune response to RV infection rather than viral cytopathicity, and thus minimal damage to the mucosal epithelial lining occurs. Symptoms
include cough, sore throat, sinusitis, rhinorrhea and headaches in otherwise healthy individuals, and some of these traits are attributed to nasal secretion of bradykinins [26]. Symptom scores tend to reflect virus titres and can arise 10-12h after inoculation in experimental infections, though usually develop 1-2 days p.i., peak between days 2-3 and resolve in days 7-10 [21, 27, 28].

Peak symptom scores are associated with peak nasal virus shedding in addition to peak nasal neutrophilia, of which the later is a hallmark cellular feature of RV infections. Nasal epithelial oedema and mucus hypersecretion also result from viral replication in epithelial cells [29]; alongside production of interferons and proinflammatory cytokines and chemokines, including IL-1, IL-6, IL-8/CXCL8, IL-10, MIP-1α/CCL3, RANTES/CCL5 and TNF-α, favouring a Th1 cytokine profile in otherwise healthy individuals, which limits virus shedding and symptoms [30]. Additionally, early studies demonstrated a 4-fold rise in neutralising antibody levels after RV infection [31-33].

RV mediated common colds are generally acute infections of the upper respiratory tract however, children, the elderly and immunocompromised patients can exhibit severe lower respiratory tract infections [34-36]. In asthmatics, RVs have been detected in the lower airways, which was associated with exacerbations and more severe and persistent infections [37]. RVs are also associated with exacerbations of other chronic airways diseases including COPD and cystic fibrosis, accounting for nearly 50% of COPD exacerbations [38, 39]. Other more serious diseases mediated by RVs include bronchiolitis and pneumonia. The effect of RV infections on the aforementioned diseases highlights the importance of research in the immune responses to RV infections and in models of asthma and COPD in particular.
1.1.5. Therapies

Inactivated RVs were used for vaccine development in the 1990s, which conferred some immunity to re-infection with the same serotype [40, 41]. However, neutralising antibodies produced were found to have limited cross-reactivity, and so vaccine development against the 100+ RV serotypes became less hopeful [41]. Efforts shifted towards antiviral compounds targeting specific virus functions such as capsid binding agents (pleconaril and pirodavir), receptor blockade (soluble ICAM-1), 3C protease inhibitors (Rupintrivir) and interferon-α (summarised in Table 1.1) [42]. However, RV-specific antiviral treatment or prophylactic drugs are not yet available therefore, treatment of symptoms is the most common route, such as use of topical anaesthetics, nasal decongestants, analgesics and antitussives.

<table>
<thead>
<tr>
<th>Antiviral Agent</th>
<th>Mechanism of Action</th>
<th>Route of Delivery</th>
<th>Effect on Virus</th>
<th>Effect on Symptoms</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon-α 2b</td>
<td>Cell susceptibility</td>
<td>i.n. (P)</td>
<td>Reduced infection rates</td>
<td>-</td>
<td>[43]</td>
</tr>
<tr>
<td>Pleconaril</td>
<td>Capsid binding (Prevents viral attachment/ uncoating)</td>
<td>Oral (T)</td>
<td>Reduced virus shedding</td>
<td>Reduced nasal mucus production &amp; total symptom time</td>
<td>[44]</td>
</tr>
<tr>
<td>Pirodavir</td>
<td>Capsid binding (Prevents viral attachment/ uncoating)</td>
<td>i.n. (P)</td>
<td>Reduced virus shedding</td>
<td>No effect on resolution of symptoms or duration of illness</td>
<td>[45]</td>
</tr>
<tr>
<td>Tremacamra (soluble ICAM-1)</td>
<td>Receptor blocking</td>
<td>i.n. (P &amp; T)</td>
<td>-</td>
<td>Reduced illness severity</td>
<td>[40]</td>
</tr>
<tr>
<td>Rupintrivir</td>
<td>Viral protease inhibitor</td>
<td>i.n. (P &amp; T)</td>
<td>Reduced virus load</td>
<td>Reduced illness severity</td>
<td>[44, 46]</td>
</tr>
</tbody>
</table>

Table 1.1 A selection of rhinovirus antiviral therapies.

(P – prophylactic, T – treatment, i.n. – intranasal).
1.2. **Asthma**

1.2.1. **Epidemiology**

It is estimated that approximately 300 million people of all ages worldwide suffer from asthma, making it one of the most prevalent chronic diseases in the world that significantly contributes to morbidity, mortality and health care costs [47]. In the past few decades, the prevalence of asthma has dramatically increased in Westernised countries. Western Europe, North America and Australasia have the highest rates of asthma [47, 48], with approximately 10-33% of children and 5-10% of adults afflicted [49]. In less developed countries, asthma prevalence is rising and is associated with urbanisation and adoption of Western lifestyles [47]. With the projected increase in percentage of the world's urbanised population, the number of asthmatics is expected to rise accordingly, with an estimated additional 100 million persons afflicted with asthma by 2025. In the UK alone, approximately 5.4 million people are currently being treated for asthma (Asthma, UK), one of the highest prevalence rates reported worldwide [47]. Of this figure, 1.1 million are children, also depicted as 1 in 11 children in the UK, thus marking it as the most prevalent chronic disease of children in the UK [50].

The economic burden of asthma is staggering, comprised of hospital admission, pharmaceutical and indirect medical costs. In the UK, treatment of asthma costs the NHS around £1 billion per year, and in 2008-2009 there were over 79,794 emergency hospital admissions, of which 30,740 were attributed to children under age 14 years [50]. Further indirect costs result from missed work days, which were estimated at 1.1 million working days in 2008-2009 [50].

1.2.2. **Definition & Diagnosis**

The word ‘asthma’ itself originally arose from the Greek meaning ‘short of breath’. Asthma is a complex and heterogeneous disease that is characterised by chronic inflammation of the respiratory system, during which airway obstruction is variable and reversible and occurs spontaneously or in response to environmental stimuli [51]. The pathophysiology of asthma results from airway oedema, mucus hypersecretion, cellular infiltration, airway hyperreactivity (AHR) and airway remodelling [48].

Airway obstruction is caused by constriction of airway smooth-muscle and inflammation in the bronchi, resulting in recurrent wheezing, shortness of breath (dyspnea) and chest
tightness, all of which are diagnostic features of asthma [48]. Diagnosis is further defined by lung function tests to assess airflow obstruction by measuring parameters such as forced expiratory volume in one second (FEV$_1$), peak expiratory flow (PEF) and forced vital capacity (FVC) [52]. Additionally, reversibility of airways obstruction following use of an inhaled short acting bronchodilators or oral corticosteroids also supports diagnosis [52]. Furthermore, AHR is a hallmark feature of asthma and is measured based on reduction of the concentration of bronchoconstrictors methacholine or histamine required to reduce airflow by 20% (PC$_{20}$) [52]. Asthma is often associated with atopy – individuals genetically predisposed, thereby contributing to disease expression and severity – thus, a familial history of atopy can be an additional diagnostic factor.

1.2.3. Classification
The Global Initiative for Asthma (GINA) classifies asthmatic patients into four groups – intermittent, mild persistent, moderate persistent and severe persistent – depending on the frequency and severity of symptoms in addition to respiratory function parameters discussed above [53]. Asthma can be further classified by many different phenotypes such as childhood or adult onset asthma, allergic asthma, atopic (extrinsic asthma – associated with more than 100 susceptibility genes) or non-atopic (intrinsic asthma – an uncommon form that is often more severe), steroid resistant asthma and asthma induced by exposure to aspirin, exercise, air pollution or cigarette smoke to name a few [54, 55]. Different phenotypes can coexist and also act in synergy in patients, affecting responses to treatment and prognoses.

1.2.4. Immunology
The chronic airway inflammation resulting in airway obstruction that defines asthma is mediated by numerous immunological pathways involving different inflammatory cells and mediators. The majority of research into the mechanisms of asthma has focused on allergic asthma, which represents the most common form of asthma. Many observations have been acquired from studies assessing the early and late responses to allergen challenge in atopic asthmatics, in addition to mouse studies of allergic airways inflammation.

Histological analysis of bronchial biopsies from asthmatic subjects demonstrates infiltration of eosinophils, activated mast cells on the mucosal surface of the airway and activated T cells. Structural changes that represent airway remodelling include collagen deposition and
thus thickening of the basement membrane under the epithelium, hyperplasia and hypertrophy of the smooth muscle layer and hyperplasia of mucus-secreting goblet cells [56]. Furthermore, epithelial cells in the airway of asthmatics are more easily shed from the basement membrane compared to non-asthmatics, and an increased number of blood vessels is observed in asthmatic bronchial biopsies [57].

Activated mast cells play a key role in bronchoconstriction through the release of inflammatory mediators including histamine, the lipid leukotrienes C₄, D₄, E₄ and prostaglandin D₂. Environmental triggers such as allergens can activate mast cells, via crosslinking of FcεR1 on their surface after binding to IgE-allergen complexes, resulting in synthesis and release of mediators such as the above, that account for the variable bronchoconstriction observed in asthmatics [51]. Mast cells also secrete cytokines IL-4, IL-5 and IL-13 which further potentiates allergic airways inflammation [58]. AHR has been associated with the presence of mast cells in the airway smooth muscle of asthmatics [59].

Eosinophil infiltration of asthmatic airways is promoted by secretion of chemokines such as Eotaxin-1 by airway epithelial cells. In addition to airway infiltration, they contribute to inflammation by release of granule proteins including eosinophil cationic protein, eosinophil peroxidase and major basic protein, which are toxic to a number of cells in the airway [60]. However, their role in AHR is unclear as administration of IL-5 blocking antibody significantly reduced blood and sputum eosinophils, but no effect on AHR or asthma symptoms were detected [61]. Airway neutrophilia is not normally seen in patients with mild asthma however, neutrophils can be detected in individuals with severe asthma, which may be mediated by IL-17 secretion [62].

T cells play a central role in allergic airways inflammation in asthma. Increased numbers of CD4+ T cells, specifically T helper 2 (Th2) cells, are found in the airways of asthmatics [63]. DCs are key regulators of Th2 cells, particularly in allergic asthma, as they act not just at the sensitization phase, but also drive inflammatory responses during allergen exposure via presenting processed allergen peptides and stimulating Th2 cells [64]. In asthmatic airways, Th2 cells secrete cytokines IL-4 and IL-13, which mediate isotype class switching in B cells to produce allergen specific IgE. The binding of allergen-IgE complexes to Fc receptors, FcεRI and FcεRII, on mast cells, basophils and other inflammatory cells results in their activation, further driving allergic inflammation [65]. IL-5 secreted by Th2 cells supports eosinophil differentiation and thus promotes increased eosinophil numbers in asthma [66], and IL-9
promotes differentiation and recruitment of mast cells to the airway [67]. All of the above discussed mechanisms can contribute to the immunopathology of asthma however, the contribution of specific pathways depends on the particular asthma phenotype(s). The role and magnitude of each immunological response varies between patients and is associated with the severity of disease.

Epithelial cells lining the airways are the site of RV infection and allergen exposure, and play a central role in the pathogenesis of asthma via secretion of chemokines and cytokines that potentiate inflammatory responses. IL-33 is secreted by asthmatic epithelial cells and promotes differentiation and chemotaxis of Th2 cells, via binding the ST2 receptor specifically expressed on these cells [68]. Thymic stromal lymphopoitin (TSLP) is secreted by both asthmatic epithelial cells and mast cells, and induces secretion of the chemokines TARC/CCL17 and MDC/CCL22 that also promote recruitment of Th2 cells, via binding CCR4 also specifically expressed on Th2 cells [69].

Epithelial-derived IL-25 has been shown to significantly drive the pathogenesis of asthma via activation of IL-25R+ lymphoid cells, which in turn significantly enhance Th2-mediated inflammatory responses. Nuocytes, also termed innate helper cells or innate helper type 2 cells, are CD25R- lineage-negative lymphoid cells [70]. In response to IL-25 and IL-33, nuocytes are thought to be principal producers of IL-4, IL-5 and IL-13 which promote the deleterious Th2 responses in asthmatic lungs. Thus, it is now emerging that nuocytes are a central effector cell type in asthma [71]. Figure 1.1 summarises the cellular and humoral immune pathways in asthma.
1.2.5. Therapy

Currently, no cure for asthma exists and treatment acts to achieve better long-term control of asthma. Therapy administered depends on the severity of asthma and is classified based on short-term quick relief or long-term control. For treatment of mild asthma, occasional use of quick acting bronchodilators e.g. short-acting β-adrenergic agonists (SABA) are sufficient to rapidly reverse airflow obstruction [48]. For persistent asthma, better long-term control is achieved by inhaled corticosteroids (ICS) that act to suppress airway inflammation and is the mainstay for asthma therapy; benefits include fewer symptoms, fewer exacerbations and increased lung function and overall quality of life [48]. Airway biopsy specimens from patients treated by ICS for a long term demonstrate fewer mast cells, eosinophils, T cells and DCs in the mucosa and submucosa of the airway and reduced vascularity, goblet cell hyperplasia and destruction of the epithelium [72, 73]. Long-acting β-
agonists can be used in combination with ICS to target bronchoconstriction and airway inflammation in patients with severe persistent asthma [48]. Other drugs to control mild-persistent asthma include leukotriene-receptor antagonists that block the effects of leukotrienes at the type I cysteinyl leukotriene receptor [74]; in addition to bronchodilation, blood eosinophil levels decreases in response to this treatment [75]. Furthermore, a novel therapy using an anti-IgE blocking monoclonal antibody (omalizumab) significantly reduces circulating IgE and consequentially results in the downregulation of FcεR1 on immunomodulatory cells thus, reducing activation of mast cells and basophils that mediate allergic inflammation in the airway [76].

1.3. Asthma Exacerbations

1.3.1. Introduction

In asthma, acute exacerbations can be triggered by a number of stimuli including respiratory virus infections, allergens, cold/humid air and/or exercise to name a few. During exacerbations the intensity of airway inflammation increases and can lead to life-threatening narrowing and even closure of the airways. Respiratory virus infections including RVs, RSV and influenza are the main precipitants of severe exacerbations, accounting for 80-85% of exacerbations in school age children [77], with RV infections being the major culprit accounting for ~50% and 80% of exacerbations in children and adults respectively [78-80]. Exacerbations contribute significantly to morbidity and medical expenditure in asthma. Additionally, death rates from asthma are higher in winter coinciding with the rise of respiratory virus infections [81]. Despite use of prophylactic and symptom relieving medications, asthma mortality rates remain high, indicating the need for development of new therapies to target specific pathogenic cellular and molecular mechanisms involved in virus-induced exacerbations.

The immunopathogenesis of acute exacerbations is different to steady state asthma, demonstrated by increased airway inflammation, particularly of the lower airway, including increased cellular infiltration with changes in its composition e.g. more eosinophils, neutrophils and activated T cells, higher concentration of inflammatory mediators, increased mucus production resulting in mucus plugging of the airway and enhanced AHR [81]. The inflammatory profile differs depending on the specific stimulus of exacerbation e.g. allergen vs virus.
There are currently few treatment options for acute asthma exacerbations and their efficacy is limited, thus prevention is the main principle employed for asthma exacerbation therapy. Treatment options include inhaled bronchodilators and systemic/oral steroids, though these are only partially effective and have significant side effects [77]. The dose of inhaled glucocorticoids that induces maximum benefit in persistent asthma only reduces the frequency of severe exacerbations by 40% [82] and the same dose does not reduce exacerbation frequency in school aged children [83]. These striking statistics illustrate the urgent need for further research into the pathways of virus-induced asthma exacerbations, as drugs used for treatment of stable asthma are largely ineffective for treatment of exacerbations, suggesting that different pathogenic mechanisms are at play.

1.3.2. Interaction Between Infection & Allergic Asthma
Numerous studies have identified an interaction between virus and allergen exposure and increased risk of enhanced airway inflammation and pathogenesis of virus-induced asthma exacerbations in individuals with pre-existing allergic sensitisation [84-88]. Children with increased nasal eosinophilia and serum IgE demonstrated increased risk of wheezing during colds [89] and, experimental RV infection studies in humans showed enhanced inflammation and physiological effects in those with allergic sensitisation characterised by higher IgE levels [27, 87, 88, 90]. The largest increases in asthma-related hospital admissions were seen with virus infected allergen-sensitized asthmatic subjects that were concomitantly exposed to allergen [84]. Allergens predominantly stimulate Th2 cells that secrete IL-5, resulting in eosinophil mediated allergic airways inflammation however, virus infection induces IL-8/CXCL8 secretion by epithelial cells and macrophages promoting neutrophil recruitment. It is possible that the interaction between allergen and virus challenge could significantly enhance airway inflammation contributing to development of exacerbations.

1.3.3. Mechanisms of RV-Induced Asthma Exacerbations
The association between RV infection and asthma exacerbations poses the question of how these viruses induce or potentiate acute exacerbations, and why asthmatics are more predisposed to the effects of respiratory infection. The mechanisms resulting in an exaggerated response during exacerbations are not fully understood, but a number of pathways have been identified through in vitro work using bronchial epithelial cells (BECs) and both mouse and human experimental RV infection studies. Human studies provide
extremely valuable opportunities to investigate the roles of RV in induction of asthma exacerbations.

1.3.3.1. RV-Modulated Epithelial ICAM-1 Expression
Major group RVs utilise ICAM-1 on epithelial cells for attachment and entry. RV infection has been shown to upregulate ICAM-1 expression in BECs in vivo and in vitro, with enhanced expression observed in asthmatic compared to non-asthmatic BECs [91, 92]. This effect could potentially contribute to exacerbations via promoting increased adherence and recruitment of polymorphonuclear cells and lymphocytes, thereby intensifying cellular inflammation and promoting virus spread [81].

1.3.3.2. RV-Induced Epithelial Expression of Proinflammatory Mediators
Epithelial cells are the primary site of RV infection and replication in the airway, and play an important role in the innate and adaptive immune responses to infection. RV infection of BECs in vitro results in secretion of a range of proinflammatory cytokines and chemokines including IL-1, IL-6, IL-8/CXCL8, GM-CSF, RANTES/CCL5 and IP-10/CXCL10 [25, 37, 93]. Expression of these mediators have been confirmed by analyses of human clinical samples (BAL, nasalpharyngeal aspirate and sputum) from experimental infection studies, and was associated with increased numbers of neutrophils (via IL-8/CXCL8), eosinophils (via RANTES/CCL5) and T cells (via IP-10/CXCL10) in asthmatic airways, which in turn correlated with increased AHR and prolonged and enhanced airway narrowing characterised by a reduction in FEV₁ [31, 94, 95]. Induced sputum from experimentally infected volunteers demonstrated increased numbers of eosinophils and neutrophils in asthmatics compared to non-asthmatics [96, 97], and enhanced cellular inflammation of the bronchi of asthmatics was due to eosinophil, CD4+ T cell and CD8+ T cell infiltration [94]. Mast cell activation was indicated by increased levels of leukotrienes and prostaglandins in asthmatic airways [98]. In contrast to the pathological consequences of enhanced cellular responses in asthmatics, RV-induced nitric oxide production was shown to mediate protection, indicating a potential target for therapy [86]. Despite the potential harmful consequences of the proinflammatory mediators expressed by BECS, they also play a role in antiviral immunity, thus a fine balance between protection and pathology exists and further investigation is required to define this balance.

Expression of the above proinflammatory mediators requires NF-κB, and increased levels of activated NF-κB p65 and p50 subunits are observed in bronchial biopsies, sputum cells and
cultured BECs from asthmatics compared to non-asthmatics [99, 100]. Higher levels of NF-κB p65 protein is also detected in PBMCs from individuals with moderate and severe asthma compared to non-asthmatics at baseline [101, 102]. These data suggest that NF-κB could be a good therapeutic target to inhibit excessive proinflammatory responses during virus infection in asthmatics, although the beneficial role of this transcription factor for defence against pathogens, particularly for IFN expression, needs to be fully assessed in asthmatics.

1.3.3.3. RV Infection of the Lower Airways in Asthmatics
RVs replicate optimally at 33°C and thus normally infect the upper airways however, bronchoscopy has demonstrated the presence of RVs in BECs of the lower airways of asthmatics, which may be involved in potentiating lower airways disease [37, 103]. Studies have confirmed no difference in infectivity or virus yields upon infection of upper and lower airway epithelial cells in vitro [104]. Therefore, it is possible that the damaged epithelium in asthmatics increases susceptibility to infection in the lower airways, not normally seen in healthy individuals [105]. Assessment of lactate dehydrogenase (LDH) levels in sputum, a marker for virus-induced lower airway cellular damage, from virus infected patients admitted to hospital due to exacerbations demonstrated a strong correlation between increased LDH, severity of exacerbation and length of hospital stay; this suggests that virus-induced lower airways damage is a major precipitant of more severe exacerbations [106]. Although asthmatics are not more susceptible to RV infections per se, they often display more severe and prolonged symptoms [87, 107], strongly suggesting inherent differences in responses to respiratory viral infections between asthmatics and non-asthmatics.

1.3.3.4. Deficient type I and III Interferon Responses in Asthmatic Airway Epithelial Cells
Such differences include deficient innate antiviral immune responses detected in atopic asthmatic BECs cultured ex vivo and infected with RV-16. In these cells, IFN-β production was impaired after RV infection compared to cells from non-asthmatic volunteers, which correlated with increased virus replication, impaired induction of apoptosis and increased cell lysis; BECs from non-asthmatics underwent early apoptosis, thus limiting virus replication [108]. These pathogenic responses detected in asthmatic BECs were reversed upon exogenous administration of IFN-β in vitro, thereby identifying IFN-β as a novel potential therapeutic option to treat or prevent RV-induced asthma exacerbations via augmenting antiviral immunity. Expression of type III IFNs by asthmatic BECs and macrophages upon RV infection in vitro was also found to be deficient, which correlated
significantly with severity of RV-induced asthma exacerbations and virus load in experimentally infected individuals [109]. It is possible that excessive TGF-β expression in asthmatic airways acts to suppress type I IFN responses, thereby mediating enhanced RV replication [2]. Further work is needed to confirm type I and III IFN deficiencies in vivo during RV infection of asthmatics.

1.3.3.5. Imbalanced Th1 and Th2 Responses to RV Infection in Asthmatics

Adaptive immune responses are also key for antiviral immunity, although the interaction between virus and allergen-induced responses are unclear. Virus infections induce a Th1 response characterised by IFN-γ expression and recruitment of CD8+ T cells, which is opposite to that seen in asthmatics where a Th2 response dominates with expression of IL-4, IL-5 and IL-13. The balance between Th1 and Th2 responses is pertinent for viral control. In asthmatics, a lower IFN-γ:IL-5 ratio detected in sputum after RV infection correlated with more severe cold symptoms and deficient clearance of virus in vivo, compared to non-asthmatics [30]. This study implies that deficient virus-mediated Th1 responses in asthmatics are associated with increased disease due to poor virus control. In vitro RV infection of PBMCs obtained from asthmatics and non-asthmatics resulted in secretion of IFN-γ, IL-12, IL-10 and IL-13 in both groups however, significantly lower levels of IFN-γ and IL-12, and expression of IL-4 was detected with asthmatic PBMCs [110]. Deficient induction of Th1 cytokines and IL-10 and augmented Th2 cytokine expression in asthmatics was observed during experimental RV-16 infection; Th1 and IL-10 responses were associated with protection from exacerbation, and increased disease severity was associated with augmented Th2 responses [27]. These observations suggest that the imbalance between Th1/IL-10 and Th2 responses in asthmatics compared to healthy individuals contributes to the pathogenic mechanisms of RV-induced asthma exacerbations.

1.4. Interleukin-15

1.4.1. Structure, Expression & Regulation

IL-15 is a pleiotropic cytokine that is a member of the four α-helix bundle family of cytokines. IL-15 was discovered in 1994 by two independent groups whom isolated a 14-15kDa lymphokine that was able to mimic IL-2-dependant proliferation of CTLL-2 cells and stimulate activation of large granulocytes [111, 112]. IL-15 is structurally similar to IL-2 and shares the IL-2 receptor β and common γ chains (IL-2Rβ and IL-2Rγ/cγ), thus shares some immunomodulatory properties with IL-2 [113] and other cytokines that use the cγ chain.
including IL4, IL-7, IL-9 and IL-21. Binding to its more specific, high affinity receptor α subunit (IL-15Rα) confers its unique functions.

IL-15 mRNA has been detected in both immune and non-immune tissues including macrophages, DCs, keratinocytes, epidermal skin cells, fibroblasts and nerve cells to name a few [112], reflecting its wider spectrum of physiological functions unlike IL-2, that is almost exclusively produced by and modulates T cells. The main producers of IL-15 are monocytes, macrophages and DCs and expression is induced by a number of stimuli including various proinflammatory cytokines, type I IFNs, dsRNA, LPS and bacteria such as  S. pneumoniae and viruses including herpesvirus 6 and 7, influenza and vaccinia virus [114-118]. In addition to these stimuli, cellular production of functional IL-15 protein requires passing stringent control mechanisms during transcription, translation, translocation and intracellular trafficking [119].

The IL-15 gene is composed of nine exons and eight introns however, the mature 114-amino acid protein is encoded by only four exons (5-8) and 3 introns [120]. Transcription occurs after binding of various transcription factors to the promoter region, which contains consensus sequences for NF-κB, IRF-E, γIRE, NF-IL-6, myb, αIFN-2, AP-1 and GCF [121], many of which are important for activating various arms of the immune system [122]. As specific factors able to initiate transcription in one cell type may not do so in another, it is suggested that there is a cell-specific control over gene expression, adding further complexity into the control of IL-15 mRNA expression.

There are two alternatively spliced variants of IL-15 that differ only in the length of the signal peptide: a 48-amino acid long signal peptide isoform (IL-15 LSP) and a 21-amino acid short signal peptide isoform (IL-15 SSP), of which the latter is expressed at lower levels [123]. IL-15 LSP is stored in the cytoplasm and has been shown to co-localise with IL-15Rα in the nucleus, perhaps to act as an autocrine regulatory mechanism to downregulate IL-15 transcription [123]. IL-15 LSP is trafficked to the plasma membrane for cell signalling [124].

Although IL-15 mRNA is detected in many tissues types, protein detection has been limited to a few cell types including monocytes, macrophages, DCs and epithelial cells [118, 125, 126]. Transfection studies demonstrated that equal expression of IL-2 and IL-15 mRNA yielded far more IL-2 protein compared to IL-15 [127], and many studies report it difficult to detect released soluble IL-15 in cell culture supernatants and biological fluids despite mRNA
expression. These discrepancies result from multiple regulatory barriers during translation and intracellular trafficking of IL-15, which is an indication of the potency of IL-15 signalling, as demonstrated by the damage caused by excessive IL-15 activity in proinflammatory disorders [128] such as rheumatoid arthritis [129], inflammatory bowel disease [130] and systemic lupus erythematosus [131].

Translational barriers include the presence of multiple translation initiation start sites (AUGs) in the 5’-UTR of IL-15 mRNA (12 in humans and 5 in mice), removal of which enhanced protein production 10-15-fold [127, 132]. In addition, the signal peptide is not optimal for translation as replacement with that of CD33 resulted in significantly greater IL-15 protein production. Deletion of the molecular hurdles present in IL-15mRNA increased protein production more than 250-fold [127].

IL-15Rα is a type I transmembrane protein and binds IL-15 with an extremely high affinity (Kₐ ≥10¹¹ M⁻¹) [133]. IL-15Rα mRNA is expressed by most haematopoietic and parenchymal cells including B and T cells, macrophages, thymic and bone marrow stromal cells, endothelial cells and in lung, spleen, liver, heart and skeletal muscle tissues [134]. Intracellular trafficking, secretion and signalling of IL-15 is dependant on IL-15Rα expression; within the endoplasmic reticulum, IL-15 protein is stabilised upon association with IL-15Rα, preventing its degradation by the proteasome [135] and the complex is then transported to the cell surface via the secretory pathway [136].

1.4.2. Signalling
IL-15 signals by a unique mechanism called trans-presentation, which depends on cell to cell contact and has only been characterised recently, consistent with the difficulty in protein detection reported by previous studies. As Figure 1.2 illustrates, IL-15 remains attached to the cell surface in complex with IL-15Rα of the producing cell and signals to adjacent IL-15 responsive cells via the IL-2Rβ/cγ dimeric or IL-15Rα/IL-2Rβ/cγ trimeric receptor, with an intermediate affinity to the former [135, 137, 138]. However, signalling may occur by more than one mechanism; cleavage of membrane bound IL-15Rα by metalloproteinases such as TACE and ADAM17 has been shown to produce a soluble form of the receptor (sIL-15Rα) [139, 140], and soluble IL-15/IL-15Rα complexes have also been detected and shown to induce expansion of NK and T cell populations in vivo [135, 138]. Although trans-presentation is widely accepted as the predominant mechanism at play, further work is
needed to fully characterise the ways in which IL-15 signals to mediate specific responses in vivo.

IL-15 shares the Janus Kinase (JAK) – Signal Transducer and Activator of Transcription (STAT) signalling pathway with many cytokines for the generation of inflammatory and adaptive immune responses [141]. Upon binding to the receptor complex, IL-15 signals through the IL-2R β and cy chains; JAK1 is recruited by the β chain bringing it into close proximity with JAK3, that is constitutively associated with the cy chain, resulting in their cross-phosphorylation; activation of these kinases results in phosphorylation of STAT3 and STAT5, which translocate to the nucleus as homo- and heterodimers to initiate expression of target genes [113]. IL-15 can also activate other signalling cascades through recruitment of other non-receptor tyrosine kinases including Src, Lck, Lyn, Fyn, and Syk and can cross-talk with proteins in the PI3K and MAPK pathways [134].

Structural analysis demonstrated no obvious docking sites or signalling motifs in the short cytoplasmic tail of the IL-15Rα subunit. However, studies have observed some signalling mediated through this chain via recruitment of TRAF2, culminating in NF-κB activation in TNF-α activated fibroblasts [142]. IL-15 mediated anti-apoptotic effects in a B cell line, in addition to enhanced phagocytosis in neutrophils, was found to be mediated through recruitment of Syk to the IL-15Rα chain [143, 144]. These observations suggest that IL-15Rα is able to signal independently of the IL-2Rβ/cy complex.
Figure 1.2 IL-15 trans-presentation.

(a) IL-15Rα binds and stabilises IL-15 protein in the endoplasmic reticulum, and the complex is transported via the secretory pathway to the cell surface. (b) The IL-15/IL-15Rα complex is presented in ‘trans’ to adjacent cells expressing the dimeric IL-2Rβ/γc receptor complex. [Diagram from Jakobisiak et al. (145)].
1.4.3. Role of IL-15 in the Immune System

IL-15 is a proinflammatory cytokine produced early after infection and exhibits a number of antiviral functions via stimulating immune cells (summarised in Table 1.2). Macrophages produce and respond to IL-15, which is important for their functional maturation as antigen presenting cells, and for expression of iNOS [146] and IL-12, of which the latter in turn stimulates IFN-γ production in multiple cell types as a positive feedback mechanism [147]; nitric oxide, IL-12 and IFN-γ production are severely impaired in IL-15 knockout mice. IL-15 signalling in human monocytes induces IL-8/CXCL8 and monocyte chemotactic protein-1 (MCP-1/CCL2) production, attracting neutrophils and monocytes to the site of infection [148]. A high level of IL-15 signalling in macrophages results in increased production of proinflammatory cytokines IL-1, IL-6 and TNF-α compared to low-level signalling, which favours IL-10 expression [149].

IL-15 expression by DCs is induced by LPS, dsRNA, type I IFNs and CD40 ligation stimulation [118], and DC mediated IL-15 signalling is crucial for NK cell development, activation and cytotoxicity (further discussed in Section 1.6.5). Stimulation of DCs by IL-15 results in their functional maturation and activation, including upregulation of IFN-γ expression and co-stimulatory molecules CD40, CD86 and MHC class II [147]. Epithelial-derived IL-15 in the airway has been shown to stimulate monocyte differentiation into DCs [150]. In human follicular DCs, IL-15 signalling enhances proliferation and chemokine secretion [151]. Moreover, IL-15 is a survival factor for DCs, as numbers in blood and spleen are significantly reduced due to increased DC apoptosis in IL-15 and IL-15Rα knockout mice, and exogenous administration of IL-15 partially restored numbers [152].

Neutrophils are amongst the first innate cells to arrive at sites of inflammation and act to remove and kill pathogens via phagocytosis and release of granules containing anti-microbial proteins. They express all 3 components of the IL-15 receptor complex and responses to IL-15 signalling include cytoskeletal rearrangement, increased phagocytosis [153], delayed apoptosis, IL-8 production – thereby amplifying the neutrophilic response [154, 155] – and induction of MHC class II expression allowing antigen presentation by neutrophils [156].

IL-15 also influences the adaptive immune response, though T cells do not express IL-15. Most significantly, IL-15 is critical for the basal homeostatic proliferation required for maintenance of CD44^{high} CD8+ memory T cells, as IL-15Rα knockout mice have a reduced pool of these cells [157, 158], and transfer of IL-15 expressing DCs from wild type to IL-15...
knockout mice resulted in the appearance of this population [152]. IL-15 promotes survival of CD44\textsuperscript{high} CD8+ memory T cells via inducing expression of anti-apoptotic molecules Bcl-2 and Bcl\textsuperscript{XL} [159]. IL-15 also induces Bcl-2 expression in CD44\textsuperscript{lo} CD8+ T cells, supporting survival of thymic and peripheral CD8+ T cells [160]; however, their lower expression levels of IL-2Rβ reflect their lower dependency on IL-15 for survival compared to memory CD8+ T cells. IL-2 mediated activation induced T cell death is inhibited by excessive IL-15 signalling, further exemplifying the role of IL-15 in T cell survival [161].

CD4+ T cell survival is not dependant on IL-15 as normal levels of CD4+ T cells are detected in IL-15 and IL-15Rα knockout mice. Although, in the presence of TCR engagement, IL-15 can induce significant proliferation of CD4+ T cells, in addition to expression of CD40 ligand and the co-stimulatory molecule CD28, promoting more effective interactions with antigen presenting cells [162].

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Functional Effects of IL-15 Signalling</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>Induces functional maturation and activation required for antigen presenting functions; and induces iNOS and IL-12 production.</td>
<td>[146, 147]</td>
</tr>
<tr>
<td>Dendritic Cells</td>
<td>Induces functional maturation and activation required for antigen presenting functions; and promotes DC survival.</td>
<td>[147, 152]</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>Stimulates iNOS production; enhances phagocytosis; and inhibits apoptosis.</td>
<td>[144]</td>
</tr>
<tr>
<td>NK Cells</td>
<td>Supports development, differentiation, homeostasis, proliferation and activation.</td>
<td>[157, 163-165]</td>
</tr>
<tr>
<td>CD8+ T Cells</td>
<td>Supports homeostasis and proliferation of memory CD8+ T cells; and supports survival of CD8+ T cells at all developmental stages.</td>
<td>[158, 160]</td>
</tr>
<tr>
<td>CD4+ T Cells</td>
<td>Promotes proliferation.</td>
<td>[166, 167]</td>
</tr>
<tr>
<td>B Cells</td>
<td>Inhibits apoptosis; supports proliferation and differentiation; and promotes production of IgA, IgG1 and IgM.</td>
<td>[168-170]</td>
</tr>
</tbody>
</table>

Table 1.2 Functional effects of IL-15 signalling on immune cells.
1.5. Interferons

1.5.1. Type I Interferons

Interferons (IFNs) were discovered 50 years ago as molecules produced by cells upon stimulation with inactivated virus, which then protected the cells against subsequent infection with live virus. Thus, IFNs were first described as molecules that could ‘interfere’ with virus replication [171]. Type I IFNs are a family of cytokines produced early after viral infection by host cells and are crucial for antiviral defence. The type I IFN family is composed of several members: IFN-α, -β, -κ, -ε and -ω, of which IFN-α and IFN-β are the best characterised. There are thirteen and seven IFN-α subtypes in mice and humans respectively and only one IFN-β in both species [172]. Mice deficient in IFN-β or subunits of the type I IFN receptor are more susceptible to viral infections [173, 174].

1.5.2. Expression of Type I Interferons

Cells possess a number of molecular sensors that can detect viral infection and stimulate redundant pathways to induce expression of type I IFNs. The Toll-like receptor (TLR) family of transmembrane proteins recognises conserved pathogen-associated molecular patterns (PAMPs) and were the first receptors demonstrated to sense viral nucleic acids [175]. TLR3 recognises double-stranded RNA (dsRNA), a common viral PAMP not normally produced by host cells but produced by both DNA and RNA viruses as a by-product of viral replication and transcription [176]. TLR7 and TLR8 recognise specific viral PAMPs on single-stranded RNA (ssRNA) such as the genomic RNA of influenza virus and HIV [177], and TLR9 recognises DNA viral PAMPs (e.g. unmethylated CpG motifs) such as the genomic DNA of herpesviruses [178].

These TLRs are located in endosomes, thus enhancing viral nucleic acid detection as endosomes are part of the replication cycle of many RNA viruses; for example, during endosome-mediated internalisation of virions or during the budding stage of viral particles allowing detection by TLRs 7/8/9, or from endocytosis of viral replication products from lysed or apoptotic virus-infected cells allowing detection by TLR3 [178]. In addition, endosomal compartmentalisation of the sensors facilitates differentiation between self and foreign nucleic acids. TLR3 has been shown to be expressed on the surface of macrophages and fibroblasts for detection of extracellular dsRNA, likely released upon virus-mediated lysis of cells [175].
The complex signalling networks triggered by these TLRs results in activation of the neural factor κB (NF-κB), activating protein 1 (AP-1) and interferon regulatory factor 3 (IRF3) (via TLR3) or IRF7 (via TLR7/8/9) to stimulate expression of IFN-α and IFN-β [178]. Plasmacytoid DCs in particular are specialised to produce copious amounts of type I IFNs in the early antiviral response, facilitated by their constitutive expression of TLR7/8/9 and IRF7.

Secondly, type I IFN expression also results from recognition of viruses via stimulation of cytoplasmic pattern recognition receptors that detect viral RNA, resulting in elimination of replicating viruses. Two of these sensors include the RNA helicases retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated gene 5 (MDA-5) that is also known as a RIG-I-like receptor [179]. RIG-I is currently the best characterised of the two molecules, and interaction of the helicase domain with dsRNA ultimately activates NF-κB, AP-1 and IRF-3 for type I IFN production [180]. In BECs, TLR3, RIG-1 and MDA-5 all coordinately recognise RV infection, resulting in the initiation of innate anti-viral defences [181].

1.5.3. Type I Interferon Signalling & Expression of Interferon Stimulated Genes
The type I IFNs share a common ubiquitous heterodimeric receptor composed of the IFN-α receptor 1 and 2 subunits (IFNAR1 and IFNAR2). Ligand binding results in activation of JAK1 and tyrosine kinase 2 (TYK2), which are constitutively associated with the cytoplasmic tails of the receptor subunits. These in turn phosphorylate STAT1 and STAT2, resulting in formation of a trimeric complex with IRF9, known as the IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates into the nucleus and binds to promoter sequences containing IFN-stimulated response elements (ISREs), resulting in expression interferon stimulated genes (ISGs) [178]. Type I IFNs also stimulate other intracellular signalling pathways that result in expression of ISGs [182].

Since their discovery over 25 years ago, more than 300 ISGs have been identified, and the resultant effector molecules act to generate an ‘anti-viral’ state within cells and mediate the immunomodulatory functions associated with IFNs. However, the function of many ISGs remains uncharacterised [183]. Type I IFN signalling also acts in an autocrine and paracrine fashion to further enhance type I IFN production, for example early IFN-β expression results in induction of IRF-7 to stimulate expression of IFN-α [184].
Of the many ISGs, serine/threonine protein kinase (PKR) and 2’-5’ oligoadenylate synthetase (OAS) are the best characterised. PKR is activated upon binding dsRNA (e.g. viral replicative intermediates) and acts to block protein synthesis via phosphorylation of a number of substrates, including the protein synthesis initiation factor eIF2α, thereby limiting viral replication [185]. PKR is also involved in dsRNA-induced signal transduction pathways that upregulate other antiviral mechanisms, including activation of transcription factors NF-κB, STAT1, IRF-1 and p53, and induced expression of FAS for initiation of apoptosis [186]. OAS is also activated by dsRNA and subsequently generates 2’-5’ oligoadenylates that bind to RNaseL, leading to its dimerisation and activation; this activated endoribonuclease cleaves viral and cellular ssRNA including 28S ribosomal RNA, and thereby inhibits protein synthesis and has been shown to prevent replication of encephalomyocarditis virus, Coxsackie virus B4, West Nile virus, HCV and some retroviruses [187]. In addition, the cleaved RNAs can activate RIG-I and MDA-5, resulting in a positive feedback loop amplifying IFN-β production [188]. Similar to PKR, the OAS/RNaseL system is implicated in signalling pathways that promote apoptosis of infected cells, further indicated by the damage to host cell machinery as a result of the mechanism of action. IFN-induced apoptosis of infected cells can also result via induction of TNF-related apoptosis-inducing ligand (TRAIL).

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible) is an ISG that interferes with the assembly and maturation stage of new virus particles including influenza [189], thereby limiting virus replication. Viperin acts through a number of different mechanisms which are still largely under investigation [190]. Type I IFN signalling also induces expression of immunomodulating cytokines, including IL-12, IL-15 and IFN-γ, which act on immune cells to direct activation and development of the appropriate downstream innate and adaptive immune responses.

1.5.4. Type II Interferon
Type II IFN only comprises IFN-γ [191] and is expressed during viral infections. In vitro stimulation of T and NK cells with phytohemagglutinin and phorbol esters or ionophores induces IFN-γ expression. In contrast to type I IFNs, IFN-γ is produced by activated macrophages, DCs, NK cells and T cells and not by virus infected cells. Stimulation of NK cells by type I IFN and IL-12 can induce IFN-γ expression during viral infections [192, 193]. IFN-γ expressing cells can respond in an autocrine/paracrine fashion to IFN-γ, resulting in enhanced activation of macrophages and NK cell cytotoxic activities. IFN-γ promotes
development of Th1 adaptive responses via inducing upregulation of MHC class II molecules on DCs for presentation of antigens and activation of T cells, expression of Th1 cytokines by CD4+ T cells and development of cytotoxic CD8+ T cells [194, 195]. Expression of chemokines MIG/CXCL9 (monokine induced by IFN-γ), IP-10/CXCL10 (IFN-γ 10kD inducible protein) and I-TAC/CXCL11 (IFN-inducible T-cell α-chemoattractant) are stimulated by IFN-γ to mediate leukocyte recruitment to the site of infection [187]. Thus, IFN-γ primarily stimulates cell-mediated responses that protect the host from intracellular pathogens.

IFN-γ signals via the heterodimeric receptor composed of IFNGR1 and IFNGR2 subunits, which are constitutively associated with JAK1 and JAK2 respectively [191]. IFN-γ binding results in cross-phosphorylation of the JAK proteins, which in turn phosphorylate STAT1, forming transcriptional activator complexes of STAT1 homodimers also called IFN-γ activated factor (GAF). GAF translocates to the nucleus and binds DNA regulatory sequences containing IFN-γ activated sequences (GAS), promoting expression of specific ISGs [196]. Type I IFNs can also activate this signalling pathway, but not as strongly as IFN-γ [182].

1.5.5. Type III Interferons

The type III IFN family is composed of IFN-λ1, -λ2 and -λ3 in humans, also known as IL-29, IL-28A and IL-28B respectively, and IFN-λ1 is non-functional in mice [197, 198]. IFN-λ has been shown to inhibit hepatitis B virus (HBV) replication in a mouse hepatocyte cell line [199], HCV replication in the human Huh7 hepatoma cell line [199] and over-expression of IFN-λ in a vaccinia virus construct significantly reduced viral replication in mouse models of several virus infections [200].

Type III IFNs can be induced by the same stimuli as type I IFNs, including a number of viruses and TLR agonists [201, 202], and expression of type I and III IFNs are similarly regulated by transcription factors NF-κB and AP-1 [203] thus, both type I and III IFNs are usually co-expressed in virus infected cells. Signalling by type III IFNs via a receptor complex of IFN-λR1/L-28Ra and IL-10R2 chains stimulates the same JAK-STAT pathway as type I IFNs, inducing a similar repertoire of genes (including OAS, PKR and upregulation of MHC class I molecules) and resultant antiviral activity in cells [197, 204, 205]. Despite the similar expression pattern and resultant biological activities upon signalling, the relative magnitude of gene expression induced by type I IFNs is generally greater than that by type III IFNs [203]. Differences in signalling strength by the different receptor complexes, receptor density on
the surface of cells and receptor distribution between cell and tissue types contributes to the specific functions of these cytokines. Epithelial cells express significant levels of IFN-λR1, and appear to be the main targets of IFN-λ signalling [206]. Studies using IFN-λR1 knockout mice show that IFN-λ signalling is particularly important for the immune response against viral infections that preferentially infect lung and/or gastrointestinal tract epithelial cells [207].

1.6. Natural Killer Cells

1.6.1. Introduction
NK cells are large granular cytotoxic lymphocytes that are derived in the bone marrow and participate in early defence as part of the innate immune system. They possess some overlapping functions with CD8+ T cells but do not express the antigen-specific T cell receptor nor CD3. NK cells were initially identified in 1975 by their ability to spontaneously lyse certain susceptible tumour cell lines [208-210]. In humans, NK cells comprise ~10-15% of peripheral blood lymphocytes (PBMCs), and are also found in peripheral tissues such as the lungs and spleen [210, 211]. Surface expression of NK1.1 is used as an NK cell marker in some rodent species however, NKP46 is now the common NK cell marker across all mammalian species [212]. Human NK cells are usually defined as CD3- CD56+ [213]. The importance of NK cells is best characterised during protection against viruses and tumour immunesurveillance [214, 215].

1.6.2. NK Cell Activating & Inhibitory Surface Receptors
In the last two decades, much work has identified cell surface receptors involved in the functional activities of NK cells, and their activation is governed by the balance of activating and inhibitory signals received from these germ-line encoded receptors. Activating receptors (ARs) include those that bind soluble ligands (e.g. cytokines and chemokines) and those that interact with cell surface molecules on target cells; some of these receptors include cytokine receptors for IL-12, IL-15, IL-18, IL-21, type I IFNs and IFN-γ; CD16, the FcyRIIIA cell surface receptor that mediates antibody-dependant cell cytotoxicity (ADCC); NKG2D, which binds stress ligands expressed on the surface of infected or transformed cells including MICA, MICB and ULBP molecules; the natural cytotoxicity receptors NKP46 and NKP44, which bind specific virus-derived molecules such as hemagglutinins from influenza and parainfluenza viruses [216]; and TLRs. Most ARs are coupled to and signal via intracytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs).
Alternatively, inhibitory receptors (IRs) such as killer cell immunoglobulin-like receptors (KIRs) in humans, CD94/NKG2A heterodimers in both humans and mice and lectin-like Ly49 dimers in mice, recognise MHC class I molecules [217, 218] that are constitutively expressed on most healthy nucleated cells, and are particularly important for the ‘missing self’ hypothesis [219]. IRs on NK cells signal via intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs) [220] and act to spare healthy cells from NK cell cytotoxicity. Certain viral infections and cellular transformations downregulate MHC class I molecules on the surface of cells to evade CD8+ T cell immunity; however, this removes/reduces the overriding inhibitory signals received by NK cells, and the decision to selectively kill the distressed cell is tipped by the weight of signals from ARs, otherwise summarised as the ‘dynamic equilibrium concept’.

1.6.3. NK Cell Cytotoxicity & Expression of Soluble Mediators

The role of NK cells in anti-viral immunity was initially recognised upon observation of their enhanced cytotoxicity in response to virus-induced type I IFNs [221] however, NK cell mediated early defence is important against some and not all viruses [222]. NK cell cytotoxicity against virus-infected cells occurs via several direct and indirect pathways. Formation of an immunological synapse between the NK cell and target cell allows directed secretion of lytic granules at the interface, whilst minimising damage to healthy surrounding cells [223]. Unlike cytotoxic CD8+ T cells, lytic granules are preformed in resting human NK cells, emphasising the rapid nature of the response [224]. Lytic granules are lysosomes with specialised secretory machinery, containing membrane-perturbing proteins granulysin and perforin, serine proteases granzyme A, B and C, and CD95/FAS ligand, which act together to induce caspase-dependant apoptosis and perforin-mediated lysis of target cells [225, 226]. Activated NK cells are described to have a serial killing ability, generating new lytic granules and ARs after detaching from killed cells, to kill other susceptible cells [227]. Expression of FAS ligands, TNF-α and TNF-related apoptosis-inducing ligands (TRAIL) by activated NK cells induces apoptosis via engagement of death receptors (TNF receptor family) on target cells [228]. IFN-β inducible expression of TRAIL (TNF-related apoptosis-inducing ligand) by mouse NK cells is ISGF3 mediated and linked to anti-EMCV activity [229].

Activated NK cells also secrete cytokines that play important anti-viral and immunomodulatory roles to help shape the appropriate innate and adaptive immune
responses. In murine models of virus infection, particularly with cytomegalovirus (CMV), cytokines released by activated macrophages, DCs and infected tissue cells, such as IL-1, IL-10, IL-12, IL-15 and IL-18 stimulate NK cells [230, 231]; in response and depending on the specific receptor-ligand interactions such as engagement of ARs NGK2D, 2B4, LFA-1 and/or CD16 during target cell recognition, NK cells can relay and amplify these signals via producing proinflammatory cytokines and chemokines including IFN-γ, TNF-α, MIP-1α/CCL3, MIP-1β/CCL4, IL-8/CXCL8, IL-12 and RANTES/CCL5 [231].

NK cells are major producers of IFN-γ during viral infections particularly during the early immune response, and along with TNF-α stimulates a number of functions key for viral clearance (Figure 1.3). Upon pathogen-mediated activation, DCs and macrophages produce a number of soluble mediators such as type I IFNs, IL-12 and IL-15 that stimulate NK cell IFN-γ production, which feeds back to enhance activation of macrophages and DCs to secrete more proinflammatory cytokines and chemokines into the microenvironment, including MIP-1α/CCL3, IP-10/CXCL10 and MIG/CXCL9 to regulate trafficking of NK cells and other immune cells [232]. IFN-γ and TNF-α act to sensitize target cells to NK cell cytotoxicity via NF-κB-depndant ICAM-1 upregulation, promoting adhesion and formation of lytic synapses [233]. NK cell produced IFN-γ also mediates inhibition of viral replication in infected cells [234], and also acts on potential target cells to increase surface expression of MHC class I and/or II molecules, promoting development of a Th1 adaptive immune response, which is critical for efficient antiviral immunity.
Figure 1.3 NK cell mediated immune functions.

Macrophages and DCs activated upon microbial challenge secrete soluble factors, including type I IFNs, IL-15, IL-12 and IL-18, which prime NK cell cytotoxicity and cytokine/chemokine secreting functions. Primed NK cells secrete IFN-γ and TNF-α (red arrows), which boosts maturation and activation of macrophages and DCs, allowing more effective antigen presentation to T cells; and IFN-γ stimulation of T cells promotes development of a Th1 phenotype, thus helping shape the adaptive immune response. Moreover, NK cells exhibit regulatory functions (blue arrows) such as the ability to kill immature DCs, hyperactivated macrophages and activated CD4+ T cells, though these activities are controlled by inhibitory receptors on NK cells. (Diagram taken from Vivier et al., 2008 Nat Immunol [235]).

1.6.4. Importance of NK Cell Mediated Antiviral Immunity

In mice, many virus infections including herpes simplex viruses (HSV), influenza and coxsackie virus can be controlled by NK cells. Some of the most convincing evidence for the importance of NK cells in early virus control resulted from studies showing increased susceptibility to murine CMV (MCMV) after NK cell depletion, and alternatively increased resistance upon adoptive transfer of NK cells in NK cell deficient mice [236, 237]. Defects in NK cell cytotoxicity or IFN-γ production alone also increases susceptibility to MCMV infection in mice [235, 238]. Lessons learnt in mouse studies holds true in humans for specific viruses, as demonstrated by severe recurrent or chronic disseminating herpes group virus infections, including HSV, Epstein-Barr virus (EBV) and human CMV documented in the few cases of natural selective NK cell deficiencies [239, 240]. Thus, there are compelling correlations between reduced or no NK cell function and susceptibility to viruses, indicating a clear role
of NK cell defence against viruses. The role of NK cells during RV infections in humans (asthmatic and non-asthmatics) is yet to be characterised.

1.6.5. IL-15 Mediated Regulation of NK Cells

IL-15 and IL-15Rα are both critical for the generation and maintenance of NK cells, as demonstrated by the severe lack of NK cells in both IL-15 and IL-15Rα knockout mice [157, 158]. IL-15 expression on bone marrow stromal cells supports development of NK cell precursors and aids differentiation of immature NK cells [241]. In the periphery, IL-15 promotes survival/homeostasis of NK cells via inhibiting expression of the pro-apoptotic molecule Bim and upregulating expression of survival promoting Bcl-2 [242].

Many studies have demonstrated that regulation of NK cell activities is the most prominent antiviral function of IL-15. For example, resting NK cells exhibit high levels of perforin and granzyme B mRNA but low protein levels, and stimulation with IL-15 removes this translational block resulting in significant increases in protein levels; thus, IL-15 supports the conversion of resting NK cells into cytolytic effectors [243, 244]. Stimulation of human NK cells in vitro with IL-15 in combination with IL-12 induces production of IFN-γ, TNF-α and MIP-1α/CCL3 [245].

DCs co-localise with NK cells in lymphoid organs and peripheral tissues to mediate trans-presentation of IL-15 [138, 246]. Lucas et al. [247] demonstrated that priming of NK cell effector functions in vivo in response to viral and bacterial pathogens requires IL-15 production and trans-presentation by CD11c<sup>high</sup> DCs, expression of which was induced upon type I IFN stimulation of CD11c<sup>high</sup> DCs; this was the first study to demonstrate the type I IFN, DC, IL-15 and NK cell link in vivo, necessary for the activation of resting NK cells during infections. Activation of NK cell cytolytic functions is critical for clearance of a number of viral infections, particularly of the herpes group [164, 165, 248, 249]. Infection of human PBMCs with herpes viruses resulted in induction of IL-15, which in turn was responsible for increased NK cell cytolytic activity [116, 250]. Human herpes virus-6 infection of PBMCs in vitro resulted in rapid upregulation of NK cell cytotoxicity, which was inhibited by the presence of monoclonal antibodies against IL-15 [251].

In mice, survival/accumulation of proliferating NK cells during MCMV infection necessary for virus clearance was dependant on IFN-α/β-mediated IL-15 induction [193]. A mouse model
of genital HSV-2 infection showed that protection was critically dependant on the presence of IL-15, NK cell and NK-T cells [252]. Similarly, control of HSV-1 infection was shown to be due to IL-15-mediated enhancement of NK cell activity that suppressed viral replication [250]. During vaccinia virus infection in mice, viral clearance was accelerated upon infection with an IL-15 expressing recombinant vaccinia virus compared to wild type virus, and the effect was attributed to IL-15 promoted survival/accumulation and increased cytolytic activity of NK cells [117, 253]. However, IL-15 driven NK cell responses can also contribute to immunopathology and mediate disease progression, as observed in patients with active Hepatitis B or C virus infection [254]. The role of IL-15 on NK cell responses during RV infection and RV-induced asthma exacerbations, whether beneficial or not, are unknown; therefore, it is the aim of this study to investigate these responses, in addition to their regulation by type I IFNs.

1.7. Mouse Models of RV Infection and RV-Induced Asthma Exacerbation

A mouse model of RV infection was published by Bartlett et al. [255] whom demonstrated minor group RV infection of BALB/c mice and major group RV infection of transgenic BALB/c mice expressing a mouse-human ICAM-1 chimera. Airway neutrophilia, expression of proinflammatory cytokines and chemokines and virus replication was demonstrated in these mice, similar to that observed in humans. A mouse model of RV-induced exacerbation of allergic airway inflammation (asthma) was also demonstrated, which allows investigation of RV infection in an asthma mouse model with OVA as the inhaled allergen. In this study, we used the above models to investigate the role of IL-15 in the immune response to RV infection and RV-induced exacerbation of allergic airways. RV-mediated lower airways disease is prominent in asthmatics and our mouse models demonstrate lower airway infection, thus appropriately modelling the situation in humans.

1.8. Study Rationale, Hypotheses, Aims & Thesis Outline

1.8.1. Rationale

Deficient expression of IFN-β by bronchial epithelial cells from asthmatics infected with RV ex vivo has been demonstrated, which was associated with increased viral replication and impaired induction of apoptosis [108]. Deficient expression of IL-15 by BAL macrophages from asthmatics infected with RV ex vivo and lower levels of BAL IL-15 protein in asthmatics compared to non-asthmatics was also demonstrated, which was inversely correlated with lower airway symptom severity, AHR and viral load during subsequent in vivo experimental RV infection [256]. Type I IFN stimulation of DCs in vivo results in upregulation and trans-
presentation of IL-15 to NK cells, which is necessary for activation of NK cell effector/antiviral functions during infections [247]. These studies imply that IL-15 expression is deficient in asthma, which is associated with deficient IFN-β expression, and may play a role in the pathogenesis of RV-induced asthma exacerbations. The role of IL-15 and NK cells during RV infections has not been previously investigated. In this study, we aimed to further examine the link between type I IFNs, IL-15 expression and the NK cell responses in the aforementioned mouse models, to more specifically define the importance of this pathway during RV infections in allergic (asthma) and non-allergic airways.

1.8.2. **Hypotheses**

i. IL-15 expression is induced in the airways and lungs during RV infection.

ii. RV-induced IL-15 is necessary for the NK cell responses to RV infection.

iii. RV-induced IL-15 and the NK cell responses affect the lung viral load.

iv. RV-induced IL-15 is dependant on type I IFN signalling.

v. NK cells are a major source of IFN-γ production early after RV infection, and the absence of NK cell IFN-γ production is associated with development of a Th2 response.

vi. RV-induced IL-15 and the associated NK cell responses are deficient during RV infection in allergic airways (asthma).

1.8.3. **Aims**

i. To investigate the magnitude, kinetics and cellular source(s) of IL-15 and IL-15Rα expression in the airways and lung tissue in response to RV infection.

ii. To investigate whether the NK cell responses to RV infection is dependant on IL-15 signalling using two models of deficient IL-15 signalling: (i) administration of an IL-15 neutralising antibody intranasally at the time of infection to transiently and locally block IL-15 in the airways/lungs and (ii) IL-15Rα knockout mice which have a constitutive and systemic block to IL-15 signalling.

iii. To investigate the role of type I IFN signalling on RV-induced IL-15 and the NK cell responses using IFNAR1 knockout mice.

iv. To investigate expression of Th2 mediators in response to RV infection in the aforementioned models of deficient IL-15 signalling and type I IFN signalling.

v. To investigate RV-induced IL-15 and the NK cell responses in a mouse model of RV-induced asthma exacerbation.
1.8.4. Thesis Outline

Chapter 1 is the introduction which explains the background of the topics covered within this thesis, including RVs, asthma, asthma exacerbations, type I IFNs, IL-15 and NK cells. Chapter 2 details the materials and methods used within this study. This is followed by the results chapters: Chapter 3 investigates RV-induced IL-15 expression and the NK cell responses in a mouse model of RV infection; Chapter 4 investigates IL-15 dependancy of the NK cell responses to RV infection using 2 different models of deficient IL-15 signalling; Chapter 5 investigates the importance of type I IFN signalling for RV-induced IL-15 expression and NK cell responses using IFNAR1 knockout mice; and Chapter 6 investigates IL-15 expression and the NK cell responses in a mouse model of RV-induced asthma exacerbation. Lastly, Chapter 7 entails a discussion of the results presented in this thesis, the conclusions and plans for future work.
2. Chapter 2: Materials and Methods

2.1. Virological Techniques

2.1.1. Virus Propagation

RV-1B was obtained from the American Type Tissue Culture Collection (ATCC VR-1366) and was passaged 7 times in Ohio HeLa cells to produce working stocks. Ohio HeLa cells are grown in HeLa cell growth medium at 37°C with 5% CO₂, in 25x175 cm² flasks. When flasks were 90% confluent, the cells were washed twice with phosphate buffered saline (PBS), prior to inoculation with 10ml working stock RV-1B in HeLa infection medium for 1 hour (1h) at room temperature (RT) with gentle agitation. An additional 10ml of HeLa infection medium was added followed by incubation at 37°C for 24h or until 100% cytopathic effect (CPE) is observed. The cells and medium were harvested, freeze-thawed twice at -80°C to release the virus into the supernatant, which was then centrifuged and filtered via 0.2μm syringe filter. This RV-1B inoculum stock was then used to infect H1 HeLa cells (ATCC CRL-1958) to produce experimental RV-1B working stocks for in vivo work.

H1 HeLa cells were grown in the same conditions as Ohio HeLa cells, however the infection protocol is modified. At 90% confluence, the growth medium was replaced with 20ml of infection medium containing working stock RV-1B. Flasks were gently agitated at RT for 1h prior to incubation at 37°C for approximately 24h or until CPE is observed throughout. The cells are collected and washed with PBS during 4 rounds of centrifugation, prior to freezing the cell pellet in 36ml of PBS at -80°C.

2.1.2. Virus Purification and Concentration

The cell pellet/PBS mix was freeze-thawed twice to lyse the cells, followed by centrifugation to remove cellular debris. Virus was precipitated out by dissolving 2.8g of Polyethylene Glycol-6000 (Fluka Germany or Sigma) in the supernatant containing 4ml of 5M (final concentration) sodium chloride, followed by incubation at 4°C for 1h. The precipitate was recovered by centrifugation, re-dissolved in 15ml of PBS, and further centrifugation removed any insoluble matter. The supernatant was then filter-sterilized via a 0.2μm syringe filter into 15ml Amicon Ultra centrifugal filtration devices (Millipore, USA), and the virus washed twice with 15ml PBS before concentration to ~2.5ml. RV-1B stocks were stored at -80°C and were routinely neutralised with serotype-specific antibodies (ATCC) to confirm identity.
2.1.3. **Virus Titration**

Ohio HeLa cells were seeded at 7.4x10⁵ cells/ml in 96-well plates in 150μl HeLa virus titration medium. RV-1B stocks were serially diluted to give concentrations from 10⁻¹ to 10⁻⁸ and 50μl of each dilution was added to 8 replicate wells of HeLa cells. Control wells contained no virus. Plates were incubated at 37°C (5% CO₂) for 5 days, after which CPE was assessed by light microscopy and scored to calculate the 50% tissue culture infective dose (TCID₅₀) via the Spearman-Karber formula.

2.1.4. **Virus Inactivation**

UV-inactivated virus (UV-RV-1B) was used as a control for the inoculum. Concentrated virus was exposed to 1200μJ/cm² ultraviolet (UV) light for 30min.

2.2. **Mice**

6-8 week old female BALB/c mice were purchased from Charles River UK and were housed in specific pathogen-free conditions. Type I IFN receptor1 knockout mice (IFNAR1⁻/⁻) were a gift from Dr Cecilia Johansson, and 6-8 week female control C57BL/6 mice were purchased from Harlan UK. IL-15Rα knockout mice and control B6.129 mice were purchased from The Jackson Laboratory USA, and bred under specific pathogen-free conditions. All animals were housed at the CBS facility at St. Mary’s Campus, Imperial College London.

2.3. **Mouse Models**

2.3.1. **RV Infection Model**

Prior to intranasal (i.n.) challenge, mice were lightly anaesthetised with vaporized isoflurane (‘Isoflurane-Vet’, Merial, UK). On day 0 (d0), BALB/c, C57BL/6, B6.129, IFNAR1 ko and/or IL-15Rα ko mice were treated i.n. with 50μl RV-1B (5.0x10⁶ TCID₅₀), UV-inactivated RV-1B (UV-RV-1B) or PBS (mock-infected). Terminal anaesthesia was performed at the indicated times post-infection (p.i.) for end-point analyses via intraperitoneal (i.p.) injection of 200μl of pentobarbitone sodium solution (‘Pentoject’, AnimalCare Ltd, UK).
2.3.2. RV Infection Model with Neutralisation of Airway/Lung IL-15

BALB/c mice were treated i.n. with 50μl RV-1B (5.0x10⁶ TCID₅₀) or PBS (mock-infected). Immediately after infection (0h) and 24h later, mice were treated i.n. with 20μg of an IL-15 neutralising antibody (M96), which was a gift from Amgen Inc., or mouse IgG2a isotype control (R&D Systems). Terminal anaesthesia was administered at 24h and 48h p.i. for endpoint analyses.

![Figure 2.1 RV infection model with antibody-mediated neutralisation of airway/lung IL-15](image)

**Table 2.1** RV infection model with neutralisation of airway/lung IL-15, treatment group nomenclature.
2.3.3. **RV-Induced Asthma Exacerbation Model**

All BALB/c mice were sensitized i.p. with 200μl of PBS containing 50μg chicken egg ovalbumin (OVA) (Calbiochem, UK) and 2mg aluminium hydroxide (Alum) (Sigma, USA) on d-13. Mice were then challenged i.n. with 30μl PBS containing 40μg OVA or PBS alone on d-2, -1 and 0. Immediately following the third OVA challenge on d0, mice were also infected i.n. with 50μl RV-1B (2.5x10⁶ TCID₅₀) or UV-RV-1B. Lung function was assessed at 24h p.i. (section 2.3.3.1). Terminal anaesthesia was administered at the indicated times p.i. for end-point analyses.

![Figure 2.2 RV-induced asthma exacerbation model.](image)

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>I.P. Sensitization (d-13)</th>
<th>I.N. Challenges (d-2, -1 &amp; 0)</th>
<th>I.N. Infection (d0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV-OVA</td>
<td>OVA-ALUM</td>
<td>OVA</td>
<td>RV-1B</td>
</tr>
<tr>
<td>UV-OVA</td>
<td>OVA-ALUM</td>
<td>OVA</td>
<td>UV-RV-1B</td>
</tr>
<tr>
<td>RV-PBS</td>
<td>OVA-ALUM</td>
<td>PBS</td>
<td>RV-1B</td>
</tr>
<tr>
<td>UV-PBS</td>
<td>OVA-ALUM</td>
<td>PBS</td>
<td>UV-RV-1B</td>
</tr>
</tbody>
</table>

Table 2.2 RV-induced asthma exacerbation model, treatment group nomenclature.
2.3.3.1. Lung Function: Assessment of Airway Hyperreactivity (AHR)

Airway hyperreactivity was assessed using a whole body plethysmography system (Electromedsystems, UK) to determine enhanced pause (Penh) area under the curve (AUC). Mice were challenged for 1min to increasing doses (0, 3, 10, 30 and 100mg/ml) of aerosolized acetyl-β-methyl-choline chloride (methacholine) (Sigma, USA) in H₂O, and Penh was assessed over 5min periods. A skilled technician (Peter Sowinski) performed this work and the analysis was carried out by myself. Data was acquired and analysed using eDacq v1.8 software (Electromedsystems, UK), and is presented as Penh AUC for the 5min log period after methacholine challenge. The equation for calculation of Penh is as follows:

\[
\text{Penh} = \left( \frac{\text{PEF}}{\text{PIF}} \right) \times \left( \frac{\text{TE}}{\text{Rt}} - 1 \right)
\]

PEF = Peak Expiratory Flow (ml/s)  
PIF = Peak Inspiratory Flow (ml/s)  
Te = Time of Expiration (total)  
Rt = Relaxation Time (time taken for expiration of 65% total expiratory volume)

2.4. Sample Harvesting & Processing

2.4.1. Bronchoalveolar Lavage (BAL)

Lungs of mice were lavaged from the trachea with 1.5ml of BAL fluid. Cells were separated from the supernatant/BAL fluid via centrifugation and the latter stored at -80°C for protein analysis. BAL cells were treated with 1ml ACK buffer for 1min to lyse the red blood cells, prior to resuspension in 1ml of BAL/lung culture medium. The total live BAL cell counts were performed by trypan blue exclusion.

2.4.2. Cytospins

Cytospin3 system (Shandon, USA) was used to spin 100μl of the BAL cell suspension onto Shandon Cytoslides. Slides were air dried, followed by fixation with 90% methanol and staining with xanthene and thiazine (REASTAIN Quick-Diff kit, Thermo Shandon, PA, USA). Slides were air dried again, blinded to experimental conditions and counted using light microscopy (400x magnification); at least 300 cells were counted per slide to determine differential cell counts.
2.4.3. **Lung Tissue Processing for Protein Analysis**

The right azygous, cardiac and diaphragmic lung lobes were excised post BAL, rinsed in PBS and stored at -80°C until further processing. Lungs were homogenised using a rotor-stator for 30 seconds in 600μl homogenising buffer. The homogenate was centrifuged and the clarified supernatant removed and stored at -80°C for protein analysis by ELISA (Section 2.9).

2.4.4. **BAL Cell and Lung Tissue Processing for RNA Extraction**

BAL cell suspensions were centrifuged and resuspended in 350μl of RLT cell lysis buffer (Qiagen, USA) and stored at -80°C until further use. The right apical lung lobe was excised following BAL, rinsed in PBS and stored at -80°C in 600μl of RNAlater (Qiagen, USA).

2.4.5. **Isolating Lung Cells for Flow Cytometry**

The left lung lobe was excised post BAL for preparation of a single-cell suspension. Lung tissue was crudely dissociated in gentle MACS C-tubes containing 5ml of digestion buffer using the gentle MACS Dissociator system (Miltenyi Biotech, Germany) before incubation for 45mins at 37°C (5% CO₂). A second round of mechanical dissociation completed the digestion protocol before clarification by centrifugation. Red blood cells were lysed in 5ml of ACK buffer for 5min, which was neutralised by lung culture medium. Cells are filtered through a 100μm cell strainer and washed with PBS, before re-suspending in 2ml lung culture medium. The total live lung cell counts were performed by light microscopy with trypan blue exclusion and expressed as total number of cells of the left lung lobe.

2.5. **Flow Cytometry**

2.5.1. **Surface Staining of BAL and Lung Leukocytes**

1x10⁵ BAL cells or 1x10⁶ lung cells were incubated with 5μg/50μl of Fc Block (anti-mouse CD16/CD32, BD Biosciences) for 15mins at 4°C. Subsequently, 50μl of fluorochrome-conjugated monoclonal antibodies specific for surface markers (Table 2.3) diluted in FACS buffer was added to the cells/Fc Block and incubated for 30min at 4°C in the dark. Cells were then washed with PBS and incubated with 100 μl of a live/dead cell marker (Aqua, LIVE/DEAD Fixable Dead Cell Stain Kit, Invitrogen) for 30min at 4°C in the dark. After further washes in PBS, cells were fixed and permeabilised during incubation with BD Cytofix/Cytoperm buffer (BD Biosciences) for 20min at 4°C in the dark. Cells were then washed, resuspended in FACS buffer and analysed immediately.
2.5.2. Intracellular Cytokine Staining (ICS) of BAL and Lung Leukocytes

Unsorted lung and BAL cells were stimulated ex vivo prior to staining protocols to detect IFN-γ and IL-4 production. 1x10^6 cells were incubated in 1 ml of lung cell culture medium containing 50ng/ml phorbolmyristate acetate (PMA) (Sigma USA), 500ng/ml ionomycin (Sigma, USA) and 1μl/ml BD GolgiPlug or BD GolgiStop (BD Biosciences) for 3h at 37°C (5% CO₂). Following stimulation, cells were stained and fixed/permeabilised as detailed in Section 2.5.1. Subsequently, cells were washed in BD Perm/Wash (BD Biosciences), and incubated with 100μl of fluorochrome-conjugated monoclonal antibodies specific for intracellular cytokines or isotype control antibodies (Table 2.3) diluted in BD Perm/Wash, for 30min at 4°C in the dark. Cells were then washed with BD Perm/Wash, resuspended in FACS buffer and analysed immediately.

2.5.3. Flow Cytometry Data Acquisition and Analysis

Stained cells were acquired using a BD LSR II digital flow cytometer (BD Biosciences, San Jose, CA) and BD FACS Diva software. Data was analysed using FlowJo 9.3.1.2 software. Representative gating strategies and control stains for both surface and intracellular staining of BAL and lung cells are illustrated in Figures 2.3-2.7. All flow cytometry data are presented as percentage of cell type of the lymphocyte gate or total number of cell type (calculated using flow cytometry percentage of cells and total lung or BAL leukocyte counts).
<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone</th>
<th>Conjugate</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat anti-mouse CD3e</td>
<td>500A2</td>
<td>Alexa® 700</td>
<td>1:200 – 2.5μg/ml</td>
</tr>
<tr>
<td>Hamster anti-mouse CD4</td>
<td>RM4-5</td>
<td>V450</td>
<td>1:800 – 0.63μg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse CD8a</td>
<td>53-6.7</td>
<td>PE-Cy7</td>
<td>1:400 – 1.25μg/ml</td>
</tr>
<tr>
<td>Hamster anti-mouse CD69</td>
<td>H1.2F3</td>
<td>FITC</td>
<td>1:100 – 5μg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse Nkp46</td>
<td>29A1.4</td>
<td>PerCP-Cy5.5</td>
<td>1:100 – 5μg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse NK1.1</td>
<td>PK136</td>
<td>PE</td>
<td>1:100 – 5μg/ml</td>
</tr>
<tr>
<td>Mouse anti-human GranzymeB</td>
<td>GB12</td>
<td>APC</td>
<td>1:200 – 2.5μg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse IFN-γ</td>
<td>XMG1.2</td>
<td>PE</td>
<td>1:100 – 5μg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse IFN-γ</td>
<td>XMG1.2</td>
<td>FITC</td>
<td>1:100 – 5μg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse IL-4</td>
<td>11B11</td>
<td>PE</td>
<td>1:100 – 5μg/ml</td>
</tr>
<tr>
<td>Rat anti-mouse CD16/CD32</td>
<td>2.4G2</td>
<td>None (purified)</td>
<td>1:100 – 5μg/ml</td>
</tr>
<tr>
<td>Isotype control GranzymeB</td>
<td>-</td>
<td>APC</td>
<td>1:200 – 2.5μg/ml</td>
</tr>
<tr>
<td>Isotype control IFN-γ</td>
<td>-</td>
<td>PE</td>
<td>1:100 – 5μg/ml</td>
</tr>
<tr>
<td>Isotype control IFN-γ</td>
<td>-</td>
<td>FITC</td>
<td>1:100 – 5μg/ml</td>
</tr>
<tr>
<td>Isotype control IL-4</td>
<td>-</td>
<td>PE</td>
<td>1:100 – 5μg/ml</td>
</tr>
</tbody>
</table>

Table 2.3 Monoclonal antibodies used for flow cytometry (BD Biosciences).

Abbreviations: PE – Phycoerythrin, FITC – Fluorescein isothiocyanate, PerCP – Peridinin chlorophyll proteins, Cy – Cyanine and APC – Allophycocyanin.

2.6. **Taqman Quantitative PCR**

2.6.1. **RNA Extraction and Reverse Transcription**

The right apical lung lobe was homogenised in 600μl RLT cell lysis buffer (Qiagen, USA) using rotor-stator for 30 seconds, and RNA was extracted using the Qiagen RNeasy Mini kit (Qiagen, USA) according to manufacturers’ instructions. This kit was also used to extract RNA from lysed BAL cells. During the extraction protocol, contaminating DNA was removed by incubation with DNase I (Qiagen, USA). The eluted RNA (5μg/sample extraction) was converted into cDNA in reactions containing 10μM random hexamers as primers (Promega, USA), 0.5μM dNTPs, RT (reverse transcriptase) buffer and 0.2units/μl Reverse Transcriptase (all Omniscript RT kit, Qiagen, USA); the reaction was performed for 1.5h at 37°C.

2.6.2. **qRT-PCR**

Taqman quantitative real-time PCR (qPCR) was carried out using 1μl of cDNA with Quantitect Probe PCR Mastermix (Qiagen, USA) and primers (Invitrogen, UK) and FAM/TAMRA-labelled probes (Qiagen, UK) specific for the 5’–untranslated region of the
gene of interest, positive sense RV RNA (Cantoli et al., 2006) or 18s ribosomal RNA (Table 2.4). cDNA was diluted 1:100 in dH2O for analysis of 18s ribosomal RNA (rRNA) expression levels. Reactions were performed in duplicate on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA). PCR cycling conditions were as follows: 2min at 50°C, 10min at 95°C and 45 cycles of 15 seconds at 95°C and 1min at 60°C. Data was analysed on SoftMax Pro software, and results are expressed as gene mRNA or viral RNA copies per μl of cDNA sample generated from the RT process (copies/μl). Table 2.4 is an example of the analysis producing the normalised gene copy numbers per sample of a data set/experiment. Gene copy numbers were obtained using a plasmid DNA standard curve run on the same Taqman qPCR plate, which was then normalised by taking into account the differences in 18s rRNA Ct values between each sample in a data set/experiment.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Gene Copy Number (Duplicate1)</th>
<th>Gene Copy Number (Duplicate2)</th>
<th>Mean Gene Copy Number</th>
<th>18s Ct Value (Duplicate1)</th>
<th>18s Ct Value (Duplicate2)</th>
<th>Mean 18s Ct Value</th>
<th>18s Difference (Subtraction of smallest 18s Ct value of data set from sample 18s Ct value)</th>
<th>$2^{18s}$ difference</th>
<th>Normalised Gene Copy Number per μl of sample cDNA (2^18s difference Multiplied by Mean Gene Copy Number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6670</td>
<td>5438</td>
<td>6054</td>
<td>19.000</td>
<td>19.056</td>
<td>19.028</td>
<td>0.482</td>
<td>1.397</td>
<td>8457</td>
</tr>
<tr>
<td>2</td>
<td>3037</td>
<td>2627</td>
<td>2832</td>
<td>18.776</td>
<td>18.672</td>
<td>18.724</td>
<td>0.179</td>
<td>1.132</td>
<td>3206</td>
</tr>
<tr>
<td>3</td>
<td>2245</td>
<td>2433</td>
<td>2339</td>
<td>19.032</td>
<td>18.861</td>
<td>18.947</td>
<td>0.401</td>
<td>1.321</td>
<td>3089</td>
</tr>
<tr>
<td>4</td>
<td>3053</td>
<td>3750</td>
<td>3402</td>
<td>18.820</td>
<td>18.925</td>
<td>18.873</td>
<td>0.327</td>
<td>1.254</td>
<td>4268</td>
</tr>
<tr>
<td>5</td>
<td>2147</td>
<td>2339</td>
<td>2243</td>
<td>19.073</td>
<td>18.971</td>
<td>19.022</td>
<td>0.476</td>
<td>1.391</td>
<td>3121</td>
</tr>
<tr>
<td>6</td>
<td>2316</td>
<td>2274</td>
<td>2295</td>
<td>19.202</td>
<td>19.234</td>
<td>19.218</td>
<td>0.672</td>
<td>1.594</td>
<td>3657</td>
</tr>
<tr>
<td>7</td>
<td>2075</td>
<td>2087</td>
<td>2081</td>
<td>18.969</td>
<td>19.117</td>
<td>19.043</td>
<td>0.497</td>
<td>1.412</td>
<td>2938</td>
</tr>
<tr>
<td>8</td>
<td>2579</td>
<td>2789</td>
<td>2684</td>
<td>18.623</td>
<td>18.468</td>
<td>18.546</td>
<td>0.000</td>
<td>1.000</td>
<td>2684</td>
</tr>
<tr>
<td>9</td>
<td>2601</td>
<td>3105</td>
<td>2853</td>
<td>18.641</td>
<td>18.541</td>
<td>18.591</td>
<td>0.046</td>
<td>1.032</td>
<td>2945</td>
</tr>
<tr>
<td>10</td>
<td>4483</td>
<td>5299</td>
<td>4891</td>
<td>19.036</td>
<td>18.964</td>
<td>19.000</td>
<td>0.454</td>
<td>1.370</td>
<td>6702</td>
</tr>
</tbody>
</table>

Table 2.4 Example of Taqman qPCR Analysis. Calculations to generate gene mRNA or viral rRNA copies per μl of cDNA sample generated from the RT process after RNA extraction from lung tissue or BAL cells, in which 5μg of extracted RNA was used to generate cDNA in each sample. Plasmid DNA standards were run on each Taqman qPCR plate to generate a standard curve, from which gene copy numbers were obtained based on corresponding Ct values. Gene copy numbers were then normalised between samples in an experimental data set based on differences in the quantity of the house keeping gene 18s rRNA.
2.6.3. qPCR Standards

PCR products for specific genes were generated (Section 2.6.2) and separated on a 1% (w/v) agarose, tris-acetate-EDTA (TAE) gel alongside a 100bp-12kB DNA ladder (Invitrogen, USA). The correct sized PCR product was excised from the gel and purified using a QIAquick gel extraction kit (Qiagen, USA), and subsequently ligated into pCR2.1 TOPO TA vector (Invitrogen, USA). Chemically competent TOP10 *E.coli* cells (Invitrogen, USA) were transformed with the vector by heat shock, and cultured overnight at 37°C on Lysogeny broth (LB) agar plates containing 50μg/ml ampicillin (Sigma, USA). Colonies transformed with vectors containing the insert were screened by restriction digest using *EcoR*I restriction endonuclease (Promega, USA) and by qPCR as described. Positive clones were amplified by culturing in batch overnight in LB broth containing 50μg/ml ampicillin at 37°C with shaking. Plasmid DNA was purified using a plasmid ‘Maxi’ or ‘Midi’ kit (Qiagen, USA), quantified by qPCR and diluted in nuclease-free H2O for use as a standard in further qPCR assays.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer/Probe</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-15</td>
<td>Forward</td>
<td>cctgcaagtctctccccaaattct</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>tccctgcagcgcgtcttaag</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – cgccccaaagacctgctgctatct– TAMRA</td>
</tr>
<tr>
<td>IL-15Rα</td>
<td>Forward</td>
<td>tcccccaacgttcccaaatg</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>aacccctgcaccaaccaa</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – cgaaatggccatctctacatcggtctc– TAMRA</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Forward</td>
<td>ccatcatgaaaggtgtaag</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>gagagggctgtgtg/aggaa</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – tccccaagtcggctgctgtg/aggaa– TAMRA</td>
</tr>
<tr>
<td>IFN-λ2/3</td>
<td>Forward</td>
<td>aaagggatcaccatgtgctc</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>tcaagccgctctctctgat</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – tcccccaaaaggtgcagc– TAMRA</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Forward</td>
<td>tccatgtgctatgtg/aggaa</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>tggctctgaggattttctag</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – tccacatctttttgcagtt– TAMRA</td>
</tr>
<tr>
<td>OAS-1a</td>
<td>Forward</td>
<td>tccctggtcagaatctctcctca</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ccccaagggaggtcatctct</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – caagctctgatcccgaatctcatgc cat c – TAMRA</td>
</tr>
<tr>
<td>PKR</td>
<td>Forward</td>
<td>cgggaacacctcctctacggtgc</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ggggaacacattcttttagtcatagac</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – agctgtgggggggctcagtc– TAMRA</td>
</tr>
<tr>
<td>Viperin</td>
<td>Forward</td>
<td>cgaagacatgaatacagatc</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>aattaggagggctgcgggggg</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – ccagccagcagggctcagg– TAMRA</td>
</tr>
<tr>
<td>IL-4</td>
<td>Forward</td>
<td>acaagagaagggacgcat</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>gaagccctcagcgcaggtgctc</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – tccctacagcaacaggaaga– TAMRA</td>
</tr>
<tr>
<td>IL-5</td>
<td>Forward</td>
<td>ccatgagccacagtggtaaa</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>gggcactgcgtggggagtctatc</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – ccgcctcaccggctctgtggcaaga– TAMRA</td>
</tr>
<tr>
<td>IL-13</td>
<td>Forward</td>
<td>gatattgcatggcttctgaacc</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>gggctcctctctctgtggtctag</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – cggccctcctcaggtctcctca – TAMRA</td>
</tr>
<tr>
<td>HRV</td>
<td>Forward</td>
<td>tgaagagccagccttttgct</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>gctacaggtttgaagttaggcc</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – tgaatttgcggccctctgtaag– TAMRA</td>
</tr>
<tr>
<td>18s</td>
<td>Forward</td>
<td>cgccgtagagtgg/aaatct</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>cttcttggc/aaatgcggg</td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>FAM – acggccgcaagcggaccaga – TAMRA</td>
</tr>
</tbody>
</table>

Table 2.5 Taqman quantitative PCR primers and probes.
2.7. ELISA

ELISAs for mouse IL-1β, IL-4, IL-5, IL-6, IL-12, IL-13, IL-15, IFN-λ/IL-28, KC/CXCL1, tumour necrosis factor alpha (TNF-α), Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES/CCL5), macrophage derived chemokine (MDC/CCL22), thymus and activation regulated chemokine (TARC/CCL17), IFN-γ inducible protein 10 (IP-10/CXCL10), IFN-inducible T cell alpha chemoattractant (I-TAC/CXCL11), and Eotaxin/CCL11 were all performed with Duoset ELISA kits (R&D Systems, USA) according to manufacturers’ instructions. All steps were performed at RT. Briefly, 96-well Nunc Maxisorp Immunoplates (Thermo-Fischer, USA) were coated with 100μl of capture antibody in PBS overnight, and subsequently washed thrice with ELISA wash buffer and blocked with 250μl reagent diluent for 1h. Plates were washed and 100μl of appropriately diluted sample (BAL fluid or clarified lung homogenate) was added in duplicate and incubated for 2h. Plates were washed and incubated with a biotinylated detection antibody in reagent diluent for 2h, followed by further washes and incubation with 250ng/ml Streptavidin-horseradish peroxidase (HRP) conjugate in reagent diluent for 20min. After the final washes, 100μl of 3,3′,5′ tetramethylbenzidine (TMB) single solution chromagen substrate (Invitrogen, USA) was added and the reaction subsequently stopped upon addition of 50μl 1M H₂SO₄.

The soluble IL-15/IL-15Rα complex ELISA kit (eBiosciences) differed in that the capture antibody was incubated at 4°C overnight and both detection antibody and HRP were incubated for 1h. IFN-α and IFN-β ELISA kits (R&D Systems/PBL Interferon Source, USA) also differed to the above protocols as follows. Primarily, plates provided were pre-coated with capture antibody. For the IFN-β ELISA, incubation with sample, secondary antibody and HRP were performed for 1h. For the IFN-α ELISA, sample was incubated simultaneously with detection antibody for 1h at RT with shaking (450rpm), followed by overnight incubation at 4°C. Subsequently, plates were washed and incubated with HRP solution for 2h at RT with shaking, prior to washing and reaction development.

Proteins were quantified by comparison to an 8-point standard curve using recombinant protein. Plates were analysed by a Spectramax Plus plate reader, and the optical density of each well determined by the absorbance at 450nm. Data was analysed with Softmax Pro 3.1.2 software (Molecular Devices, USA).
2.8. Immunohistochemistry

Formalin-fixed paraffin/embedded mouse lungs were single stained for immunohistochemistry as previously described [255]. Briefly, lungs sections were deparaffinised and rehydrated to expose immunoreactive epitopes and incubated in a microwave oven (model NN SN00W; Panasonic, Milano, Italy) with the sections immersed in citrate buffer (5mM at pH6.0) for 40min. Slides were then incubated in 3% hydrogen peroxide/PBS to inhibit endogenous peroxidase activity and subsequently washed in PBS. Incubation in 0.1% saponin/PBS permeabilised cell membranes. Non-specific staining was blocked by incubation with coating serum (5% rabbit serum) for 20 min at RT, followed by PBS washes. Sections were then incubated for 1h at RT with goat anti-mouse IL-15 (R&D Systems) at dilution of 1:50. Negative control slides were stained with normal goat non-specific immunoglobulins (Santa Cruz Biotechnology) at the same concentration. After PBS washes, sections were incubated with rabbit anti-goat biotinylated antibody (Vectastain Elite ABC Kit, Vector Laboratories; www.vectorlabs.com) for 30 min at RT, followed by PBS washes. Subsequently, slides were incubated with ABC reagent (Vectastain Elite ABC Kit, Vector Laboratories) for 30min at RT, followed by incubation with chromogen-fast diaminobenzidine (DAB) as a chromogenic substrate. Slides were then counterstained in haematoxylin and mounted on permanent mounting medium. Staining and scoring my light microscopy was performed by Gaetano Caramori (Rome, Italy).

2.9. Statistical Analysis

All graphical data are expressed a means ± SEM of at least 4 mice per experimental condition, and is representative of or including at least 2 independent experiments. Data was analysed by two-way ANOVA, and individual significant differences between specific groups were indicated by Bonferroni’s post-tests. Significant differences are indicated within 95% confidence intervals i.e. p <0.05, and specific p values are indicated in figure legends. Where differences between data are not significant, no statistics are stated. All data was statistically analysed by Prism4 software (Graphpad, USA).
<table>
<thead>
<tr>
<th>Medium/Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa Cell Growth Medium</td>
<td>Dulbecco’s Modified Eagles Medium (DMEM) (PAA Laboratories) containing 2mM L-glutamine, 20μM HEPES buffer, 1% (v/v) sodium bicarbonate and 10% (v/v) foetal bovine serum (FBS) (all Gibco).</td>
</tr>
<tr>
<td>HeLa Cell Infection Medium</td>
<td>Same as ‘Growth Medium’ except 2% (v/v) FBS.</td>
</tr>
<tr>
<td>HeLa Cell Virus Titration Medium</td>
<td>Same as ‘Growth Medium’ except 4% (v/v) FBS.</td>
</tr>
<tr>
<td>Lung/BAL Cell Culture Medium</td>
<td>RPMI 1640 Medium (PAA Laboratories) containing 2mM L-glutamine, 20μM HEPES buffer, 1% (v/v) sodium bicarbonate, 10% (v/v), 100μg/ml streptomycin (Sigma, USA), 100units/ml penicillin (Sigma, USA) and 10% (v/v) foetal bovine serum (FBS).</td>
</tr>
<tr>
<td>BAL Fluid</td>
<td>Phosphate buffered saline (PBS) (Company) containing0.5M disodium EDTA solution (Gibco) and 12mM lidocaine hydrochloride monohydrate (Sigma).</td>
</tr>
<tr>
<td>ACK Lysis Buffer</td>
<td>1 litre dH₂O containing 0.15M Ammonium chloride (Sigma), 1mM potassium bicarbonate (Sigma) and 0.1m disodium EDTA (Gibco). Filter-sterilized through a 0.22μm filter.</td>
</tr>
<tr>
<td>Lung Digestion Buffer</td>
<td>‘Lung Cell Culture Medium’ containing 1mg/ml Collagenase Type XI (Sigma) and 30μg/ml Bovine Pancreatic DNase I Type IV (Sigma).</td>
</tr>
<tr>
<td>Lung Homogenisation Buffer</td>
<td>PBS containing ‘Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets’ (Roche).</td>
</tr>
<tr>
<td>FACS Buffer</td>
<td>PBS containing 1% (w/v) BSA (Sigma) and 0.01% (v/v) sodium azide.</td>
</tr>
<tr>
<td>ELISA Wash Buffer</td>
<td>PBS containing 0.05% (v/v) polyethylene glycol sorbitan monolaurate (‘Tween20’, Sigma)</td>
</tr>
<tr>
<td>ELISA Reagent Diluent</td>
<td>PBS containing 1% (w/v) BSA (Sigma)</td>
</tr>
<tr>
<td>RLT Cell Lysis Buffer</td>
<td>RLT lysis buffer (Qiagen) containing 2-mercaptoethanol (Sigma).</td>
</tr>
<tr>
<td>LB Medium</td>
<td>1 litre of dH₂O with 10g Tryptone powder (Fluka, Germany), 5g yeast extract (Sigma) and 5g sodium chloride (VWR, UK). Autoclave sterilised.</td>
</tr>
<tr>
<td>LB Agar</td>
<td>‘LB Medium’ containing 14g Bacto-Agar (Difco, USA).</td>
</tr>
</tbody>
</table>

Table 2.6 Media and Buffers.
Figure 2.3 Representative flow cytometry gating for live, single lymphocytes.

All flow cytometry data is presented as live lymphocytes as the common denominator. Abbreviations: FSC-A Forward Scatter-Area, FSC-W Forward Scatter-Width, SSC-A Side Scatter-Area, SSC-W Side Scatter-Width.
Figure 2.4 Representative flow cytometry gating for BAL lymphocytes in BALB/c mice and NK cells in C57BL/6 mice. Numbers indicated on plots represent the percentage of events falling within the indicated gate.
Numbers indicated on plots represent the percentage of events falling within the indicated gate.

Figure 2.5 Representative flow cytometry gating for lung lymphocytes in BALB/c mice and NK cells in C57Bl/6 mice.
Figure 2.6 Representative flow cytometry gating for intracellular cytokine staining in lung NK cells.

Numbers indicated on plots represent the percentage of events falling within the indicated gate.
Figure 2.7 Representative flow cytometry gating for intracellular cytokine staining in lung T cells.

Numbers indicated on plots represent the percentage of events falling within the indicated gate.
3. **Chapter 3: Results - IL-15 expression and the NK cell response during RV-1B infection**

3.1. **Introduction**

RV infections are amongst the most frequent infections encountered and are the most common etiological agents of the common cold [3]. RV infections are self limiting, and a healthy host does not usually suffer profoudly. However, RV infections are associated with more severe airways disease in asthmatics as they trigger approximately two thirds of acute exacerbations [77], and this is the major reason for investigating the immune response to RV infections.

IL-15 is a proinflammatory cytokine that exhibits a number of antiviral properties, and plays a significant role in defence against viruses. Stimulation of macrophages and DCs with LPS, dsRNA and IFN-α/β has been demonstrated to induce IL-15 expression [118]. To date, only one study has specifically analysed expression of IL-15 in response to RV infection using cultured macrophages [256]; data of IL-15 expression and its role in the immune response to RV infections in vivo is lacking.

NK cells are key innate antiviral effector cells, which unlike T cells do not require priming for activation. NK cells express a variety of activating and inhibitory receptors that allow quick and early detection of virus infection, resulting in their activation and early control of virus replication and spread. IL-15 is critical for the development and maintenance/homeostasis of NK cells, as demonstrated by their absence in IL-15 and IL-15Rα knockout mice. As discussed in chapter 1, regulation of NK cell responses appears to be the main reported antiviral function of IL-15.

No previous study has provided a thorough analysis of IL-15 and NK cell responses to RV infection in vivo. Analysis of this pathway may provide insight into the early innate control of RV spread, which could be important when assessing potential therapeutic targets for individuals that experience more severe RV infections. A mouse study of RV infection can provide a more thorough analysis of IL-15 and type I IFN expression in addition to the NK cell response than afforded in human studies. A comprehensive timecourse allows us to examine gene expression and protein levels in the airway lumen and lung tissue. Flow cytometry allows real time analysis of the NK cell response in both the airway/mucosal surfaces (in BAL) and lung tissue, via measuring expression of surface and intracellular molecules, some of which represent an activated status.
3.2. Hypotheses & Aims

3.2.1. Hypotheses

i. RV-1B infection increases IL-15 and IL-15Rα expression in the lungs and this is associated with RV-induced type I IFN expression.

ii. RV-induced IL-15 expression is associated with recruitment/expansion and activation of NK cells in the airway and lungs and precedes T cell responses.

3.2.2. Aims

To investigate IL-15 expression and the NK cell response in BALB/c mice during infection with a minor group RV (RV-1B).

i. To determine the virus load in the lungs using Taqman qPCR.

ii. To determine the magnitude, kinetics and cellular source(s) of IL-15 and IL-15Rα in the airway and lungs using Taqman qPCR, ELISAs and immunohistochemistry.

iii. To determine expression of IFNs, ISGs, and proinflammatory cytokines and chemokines using Taqman qPCR and ELISAs.

iv. To determine the magnitude, kinetics and activation status of the NK cell response in the airway and lungs using flow cytometry.

v. To examine the acquired T cell response in the airway and lungs using flow cytometry.
3.3. Results

3.3.1. Virus Load

BALB/c mice were infected i.n. with RV-1B (5.0x10^6 TCID_{50}) or UV-irradiated RV-1B (UV-RV-1B). Virus load in the lungs was determined at various time points post-infection (p.i.), by detecting viral RNA in lung homogenate via qPCR. Here, we demonstrated persistence of the virus in the lungs between d1-2 p.i. in RV-1B treated mice, after which levels declined though virus remained detectable at d7 p.i., consistent with previous findings [255] (Fig 3.1).

![Figure 3.1 Virus load in the lungs during RV-1B infection.](image)

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock). Lungs were harvested at the indicated timepoints p.i., and processed for RNA extraction and cDNA was made by reverse transcription as described. RV-1B viral RNA copies were quantified by qPCR, data was normalised against 18s rRNA expression and presented as RNA copies/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (***p<0.001). Only statistically significant differences are shown.

3.3.2. Differential BAL Cell Counts

Total BAL cell counts demonstrated a significant and rapid influx of cells into the airways by d1 p.i. with RV-1B infection, with numbers peaking at d2 and declining thereafter (Fig 3.2a). The airways cellular inflammatory profile was determined by differential cell counts based on cellular morphology from cytospin slides of BAL cells. As previously reported by Bartlett et al. [255], RV-1B induced a peak of neutrophilia at d1 p.i. (p<0.001 compared to controls), at which neutrophils made up to ~80% of the total BAL cells (Fig 3.2 b&c); neutrophilia was resolved between d4-7. Lymphocyte numbers also increased in the BAL at d2 p.i. with RV-1B and peaked at d4, at which they comprised ~35% of the total BAL cells (p<0.001 compared
to controls) (Fig 3.2 d&e); an elevated percentage of BAL lymphocytes was still detected at d7. These responses were virus-replication dependant as they were not observed in mice treated with UV-RV-1B.

Figure 3.2 Differential BAL cell counts during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock) and BAL was performed at the indicated timepoints p.i. Recovered live BAL cells were counted (a) and processed for cytospin slides. Slides were blinded to experimental conditions and cells differentially counted to give the percentage and absolute number of (b&c) neutrophils and (d&e) lymphocytes in the airway. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B and mock treated mice at the indicated times (**p<0.01, *p<0.05). Only statistically significant differences are shown.
3.3.3. BAL Proinflammatory Mediators

As previously reported by Bartlett et al. [255], RV-1B infection induced production of a range of mediators including the following as detected by ELISAs in the BAL fluid. Coinciding with the peak of neutrophilia, production of KC, a neutrophil chemoattractant, peaked at d1 p.i. infection (p<0.001) and declined thereafter, though levels at d2 were still significantly elevated (p<0.001) (Fig 3.3a). Proinflammatory cytokines IL-6 and IL-1β were both significantly induced at d1 p.i. with RV-1B (p<0.001), and though IL-6 levels rapidly decreased thereafter, IL-1β remained elevated at d2 p.i. (p<0.01) (Fig 3.3 b&c). Lastly, TNF-α was induced at d2-4 p.i. (p<0.01), after which levels declined (Fig 3.3d).

![Graphs showing BAL cytokine levels](a) (b) (c) (d)

Figure 3.3 BAL cytokines and chemokines produced in response to RV-1B infection.

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (d0), and BAL was performed at the indicated timepoints p.i. Protein levels of (a) KC, (b) IL-6, (c) IL-1β and (d) TNF-α were quantified by ELISA in the BAL fluid and expressed as pg/ml. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B treated animals at the indicated times (**p<0.01, *p<0.05). Only statistically significant differences are shown.
3.3.4. Expression of IL-15 and IL-15Rα

3.3.4.1. BAL and Lung IL-15 and IL-15Rα Gene Expression

IL-15 is reported to be expressed by monocytes, macrophages and DCs [254]. qPCR analysis of BAL cells demonstrated a sharp peak of IL-15 and IL-15Rα mRNA expression with RV-1B infection at d1 p.i. (p<0.001 compared to controls), after which levels rapidly declined (Fig 3.4 a&amp;b). Analysis of lung homogenate demonstrated a significant upregulation of IL-15 mRNA by ~3-fold in RV-1B treated mice at d1 p.i. (p<0.001 vs UV-RV-1B; p<0.01 vs mock), and levels declined thereafter though remained elevated over mock controls at d2 (p<0.01) (Fig 3.4c). Expression of IL-15Rα in the lungs during RV-1B infection mirrored that of IL-15, with a 2-fold increase of mRNA levels at d1 p.i. (p<0.001) (Fig 3.4d). Comparison of the two sources demonstrates that BAL cells expressed much higher levels of both genes at d1 p.i. compared to lung tissue, suggesting that they may be a more important source of IL-15/IL-15Rα signalling.

3.3.4.2. Lung IL-15 and IL-15Rα Protein Expression

To detect IL-15 and IL-15Rα protein in lung homogenate that was either intracellular or attached to the cell surface, lungs were homogenised and the clarified supernatant analysed by ELISA. In coordination with mRNA expression, we detected upregulation of both IL-15 protein and soluble IL-15/IL-15Rα complexes (sIL-15/IL-15Rα) at d1 p.i. with RV-1B infection (p<0.001 vs UV-RV-1B; p<0.01 vs mock) (Fig 3.4 e&amp;f). Extremely high levels of protein were detected suggesting a high degree of background associated with this method, though previously reported constitutive expression of IL-15 by lung epithelium [257] explains detection of protein in control groups. To further confirm IL-15 protein expression using a different technique, immunohistochemistry was performed on lung sections to illustrate increased expression of IL-15 protein at d1 and d2 p.i. with RV-1B (Fig 3.5).
Figure 3.4 RV-1B infection induces IL-15 and IL-15Rα expression in BAL cells and lung tissue.

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock), and lungs were harvested after BAL was performed at the indicated timepoints p.i. Lungs and BAL cells were processed for RNA extraction and cDNA made by reverse transcription as described. Gene expression levels of (a & c) IL-15 and (b& d) IL-15Rαwere quantified by qPCR in lung and BAL cells. Data was normalised against 18s rRNA expression and presented as copies of mRNA/μl of sample. Protein expression of (e) IL-15 and (f) soluble IL-15/IL-15Rα complexes were analysed by ELISA using lung homogenate, and expressed as pg/ml. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
Figure 3.5 Immunohistochemistry staining for IL-15 expression in the lungs of BALB/c mice in response to RV-1B infection.

IL-15 expression in (a&b) RV-1B, (c&d) UV-RV-1B and (e&f) mock treated mice at d1 and d2 p.i. respectively. (g) Negative control. Total magnification x400.
3.3.5. **Expression of Type I and III Interferons**

Previous studies have reported that IFN-β is required for IL-15 expression, thus we hypothesised that RV-induced IFN regulates IL-15 expression. IFN-α mRNA was not tested by qPCR as 13 subtypes exist; however, IFN-α protein was detected in the BAL fluid by ELISA at d1 p.i. with RV-1B (p<0.001 vs controls) and levels rapidly declined thereafter (Fig 3.6a). IFN-β expression was analysed by qPCR and ELISA. Very low mRNA levels were detected in lung homogenate (data not shown) but significant expression in BAL cells at 10h and d1 p.i. with RV-1B was observed (p<0.01 and p<0.001 vs controls) (Fig 3.6b). Protein was detected in both the BAL fluid and lung homogenate at d1 p.i. with RV-1B (p<0.001 and p<0.05 vs controls) (Fig 3.6 c&d). Thus, we showed that RV-mediated IL-15 expression was associated with expression of type I IFNs at d1 p.i. and both responses were virus-replication dependent.

Type III IFNs are known to be virally induced and their expression is also an indication of IFN-α/β signalling. In the lungs, IFN-λ2/3 mRNA levels peaked at d1 p.i. with RV-1B (p<0.001 compared to controls) and declined thereafter, though mRNA remained detectable at d2-4 p.i. (ns) (Fig 3.6e). Protein levels peaked in the BAL fluid at d1 p.i. with RV-1B, remained significantly elevated over controls at d2 and decreased thereafter however, protein was still detectable in RV-1B treated mice at d7 (p<0.05) (Fig 3.6f).
Figure 3.6 RV-1B infection induces expression of type I and III IFNs in BAL cells and lung tissue.

BALB/c mice were dosed i.n. with RV-1B, UV-RV-1B or PBS (mock), and lungs were harvested after BAL was performed at the indicated times after infection. Protein levels of (a) IFN-α, (c) IFN-β and (f) IFN-λ in the BAL fluid and (d) IFN-β in lung homogenate were analysed by ELISA and data expressed as pg/ml. Lung and BAL cells were processed for RNA extraction and gene expression levels of (b) IFN-β in BAL cells and (e) IFN-λ2/3 in lung tissue were quantified by qPCR. Data was normalised against 18s rRNA expression and presented as copies of mRNA/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
3.3.6. **NK Cell Response to RV-1B Infection**

Flow cytometry was used to assess the magnitude, kinetics and activation status of the NK cell response to RV-1B infection in BALB/c mice. Recovered BAL and lung cells were stained for surface expression of CD3, NKp46 and CD69, and for intracellular cytokines IFN-γ and GranzymeB.

3.3.6.1. **BAL NK Cells**

NK cells are defined as CD3- and NKp46+ in BALB/c mice, and in the absence of infection there were virtually no NK cells in the BAL. In response to RV-1B infection, there was a large increase in NK cell numbers in the airway at d1 p.i., which peaked at d2 (p<0.001 vs controls) (Fig 3.7b), at which they comprised ~30% of total BAL lymphocytes (Fig 3.7a) and levels declined thereafter. To define the populations of activated NK cells, CD3- live lymphocytes were gated on, from which the percentage and absolute numbers of NKp46+/CD69+, NKp46+/IFN-γ+ and NKp46+/GranzymeB+ cells were analysed. Correspondingly, the percentage and absolute numbers of activated NK cells positive for the above markers, peaked at d2 p.i. in the airway (p<0.001 vs controls), after which levels rapidly declined (Fig 3.7 c-h). Thus, we showed that this RV-mediated influx of activated NK cells into the airway was temporally or in time associated with expression of IL-15 and IL-15Rα at d1 p.i.
Figure 3.7 BAL NK cell response during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock), and BAL was performed at the indicated timepoints p.i. 1x10⁵ BAL cells were stained for surface markers CD3, CD69 and NKp46, intracellular cytokines IFN-γ and GranzymeB and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as percentage and absolute numbers of BAL.
(a&b) CD3-/NKp46+, (c&d) CD3- gated, NKp46+/CD69+, (e&f) NKp46+/IFN-γ+ and (g&h) NKp46+/GranzymeB+ NK cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 3 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.

3.3.6.2. Lung NK Cells

In the lungs, the percentage of CD3-/NKp46+ NK cells was elevated in RV-1B treated mice compared to controls at d1-4 p.i., though significance was not reached (Fig 3.9a). However, upon translation of percentages using total lung cell counts (Fig 3.8), a significant increase in absolute numbers of NK cells was observed at d2-4 p.i. with RV-1B (Fig 3.9b), and numbers returned to baseline levels by d7.

Despite total numbers being significantly elevated only by d2 p.i., NK cells in the lungs had already upregulated expression of CD69, IFN-γ and GranzymeB by d1 p.i. with RV-1B compared to controls (Fig 3.9 c-h). However, the peak of expression of these activation markers on NK cells was on d2 p.i. with RV-1B, demonstrated by both percentage and absolute cell numbers (p<0.001 vs controls). Percentage of IFN-γ+ and GranzymeB+ NK cells peaked at d2 p.i. with RV-1B compared to controls (p<0.001) and levels remained elevated at d4 over controls (Fig 3.9 e&g); this trend was similarly mirrored by the total number of NK cells positive for IFN-γ or Granzyme B (Fig 3.9 f&h). Resolution of the NK cell response in the lungs was observed by d7.

![Figure 3.8 Total lung leukocyte count during RV-1B infection.](image)

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock), and the left lung lobe excised after BAL was performed at the indicated time points p.i. Lungs were digested as described, and the resultant single-cell suspension counted to give the total number of live cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 3 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (***p<0.001). Only statistically significant differences are shown.
Figure 3.9 Lung NK cell response during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock), and the left lung lobe excised after BAL was performed at the indicated timepoints p.i. Lungs were digested as described and 1x10^6 cells were stained for surface markers CD3, CD69 and NKp46, intracellular cytokines IFN-γ and GranzymeB and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as percentage of lymphocytes and absolute numbers of (a & b) CD3- /NKp46+ NK cells, CD3- gated double positive (c & d) NKp46+/CD69+, (e & f) NKp46+/IFN-γ+ and (g & h)
NKp46+/GranzymeB+ NK cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 3 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (**p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.

3.3.7. T Cell Response to RV-1B Infection

3.3.7.1. BAL T Cells

CD4+ T cells were recruited/expanded in the airway from d2 p.i. with RV-1B compared to controls, with the percentage and absolute number peaking at d7, at which point they comprised ~40% of BAL lymphocytes (Fig 3.10 a&b). The percentage of CD69+ and IFN-γ+ CD4+ T cells were consistently elevated in RV-1B treated mice compared to controls at d1-7 p.i. (Fig 3.10 c&e), however absolute numbers increased progressively between d2-7 (Fig 3.10 d&f).

Similarly, the number of CD8+ T cells in the airway incrementally increased from d2 p.i. with RV-1B until d7 (Fig 3.11b), at which point they comprised ~10% of BAL lymphocytes (Fig 3.11a). The percentages of CD69+ and IFN-γ+ CD8+ T cells were elevated by d1 p.i., however significant increases in cell numbers were observed only from d2 until d7 (Fig 3.11 d&f).
Figure 3.10 BAL CD4+ T cell response during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock), and BAL was performed at the indicated timepoints p.i. 1x10^5 BAL cells were stained for surface markers CD3, CD4 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as percentage of BAL lymphocytes and absolute numbers of (a & b) CD3+/CD4+ T cells, and CD3+ gated double positive (c&d) CD4+/CD69+ and (e&f) CD4+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 3 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
Figure 3.11 BAL CD8+ T cell response during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock), and BAL was performed at the indicated timepoints p.i. 1x10^5 BAL cells were stained for surface markers CD3, CD8 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as percentage of BAL lymphocytes and absolute numbers of (a &b) CD3+/CD8+ T cells and CD3+ gated double positive (c&d) CD8+/CD69+ and (e&f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 3 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (**p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
3.3.7.2. Lung T Cells

The percentage of CD4+ T cells in the lungs was elevated in RV-1B treated mice over controls between d4-7 p.i., however we observed the absolute number of CD4+ T cells increase from d2 (Fig 3.12 a&b). Both percentage and number of CD69+ CD4+ T cells increased incrementally in the lungs from d1 p.i. with RV-1B until d7. The percentage of IFN-γ+ CD4+ T cells was elevated in RV-1B treated mice over controls at d4-7 p.i, however the total number of cells had increased significantly from d2 and remained similarly elevated by d7 (Fig 3.12 e&f).

In contrast, the percentage of CD8+ T cells did not significantly change upon RV-1B infection, but a sharp increase in cell number was observed at d2 p.i. with RV-1B, and numbers decreased thereafter (Fig 3.13 a&b). The percentage of CD69+ CD8+ T cells was consistently elevated in RV-1B treated mice from d1-7 p.i., though numbers peaked significantly at d2 and declined thereafter though remaining elevated over controls until d7 (Fig 3.13 c&d). The number of IFN-γ+ CD8+ T cells also peaked at d2 p.i. with RV-1B but was not significantly elevated over controls by d4 (Fig 3.13 e&f).
Figure 3.12 Lung CD4+ T cell response during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock), and the left lung lobe excised after BAL was performed at the indicated timepoints p.i. Lungs were digested as described and 1x10^6 cells were stained for surface markers CD3, CD4 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as percentage of lung lymphocytes and absolute numbers of (a &b) CD3+/CD4+ T cells and CD3+ gated double positive (c&d) CD4+/CD69+ and (e& f) CD4+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 3 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
Figure 3.13 Lung CD8+ T cell response during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B, UV-RV-1B or PBS (mock), and the left lung lobe excised after BAL was performed at the indicated timepoints p.i. Lungs were digested as described and 1x10^6 cells were stained for surface markers CD3, CD8 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as percentage of lung lymphocytes and absolute numbers of (a&b) CD3+/CD8+ T cells and CD3+ gated (c&d) CD8+/CD69+ and (e&f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 3 independent experiments. Statistical significance is for RV-1B compared with UV-RV-1B or PBS treatments at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.
3.4. Chapter Summary

In BALB/c mice, IL-15 was upregulated in BAL cells and lung tissue at days 1-2 after RV-1B infection. Immunohistochemistry analysis of lung sections indicated expression of IL-15 protein on the apical surface of epithelial cells lining the airway, supporting the trans-presentation signalling mechanism. Additionally, the high levels of IL-15 mRNA detected in BAL cells also suggests that they are important sources of IL-15, with macrophages a likely source as demonstrated in humans [256].

We show that IL-15 expression is associated with expression of type I IFNs early after infection; IFN-β mRNA was detected as early at 10h after infection in BAL cells, followed by expression of IL-15 mRNA at day 1 in both BAL cells and lung tissue. Therefore, we suspect that IFN-β plays a role in regulating RV-mediated IL-15 expression. IFN-λ has not been previously reported to induce IL-15 expression, and we found IFN-λ protein levels to persist in the BAL fluid as late as day 7 after infection, whereas IL-15 protein levels decline after day 1.

The airway/lung leukocyte response to RV-1B infection in BALB/c mice initially comprised of airway neutrophilia early after infection, accompanied by an activated NK cell response at days 1-2, which was followed by T cell mediated inflammation at days 2-7 after infection. Thus, IL-15 and type I IFN expression was associated with the recruitment/expansion of NK cells in both the airway and lungs. The NK cells exhibited an activated phenotype due to increased expression of surface CD69 and intracellular GranzymeB and IFN-γ. NK cells are capable of producing TNF-α and detection of elevated levels in the BAL fluid at days 2-4 after infection may be at least in part attributed to the NK cell response to RV-1B infection. Clearance of virus as detected by reduction in viral RNA levels in lung homogenate occurred after the peak of the NK cell response at day 2 after infection.

In the airway and lungs, CD4+ and CD8+ T cells were activated, with respect to CD69 and IFN-γ expression, from day 1 after infection. Recruitment/expansion of BAL CD4+ and CD8+ T cells and lung CD4+ T cells was observed from day 2 after infection, with numbers increasing until day 7, with greater numbers of CD4+ T cells compared to CD8+ T cells. The lung CD8+ T cells response peaked at day 2 after infection and declined thereafter, suggesting the response may be associated with IL-15 signalling.
The data presented constitutes the first *in vivo* evidence of RV-induced IL-15 and its association with type I IFN expression and the NK cell response in a mouse model of infection. These data provide a basis for further investigating the specific roles that IL-15 signalling plays during RV infection, specifically addressing its necessity for the NK cell response.
4. **Chapter 4: Results** - Immune responses to RV infection in the absence of IL-15 signalling *in vivo*.

4.1. **Introduction**

Understanding the roles of IL-15 in RV infections may be important for the development of more targeted therapeutics to promote key pathways of antiviral host defence. The role of IL-15 regulated NK cell responses in antiviral host defence has been well characterised for a number of viruses including the herpes group viruses, hepatitis B and C and HIV; however, *in vivo* evidence implicating the importance of this pathway for respiratory virus infections is lacking. Two models of deficient IL-15 signalling are employed in this chapter to highlight innate and adaptive responses that are affected by the absence of this signalling pathway during RV-1B infection.

We established in the previous chapter that RV-induced IL-15 is expressed at day 1 after infection in mice and is followed by the recruitment/expansion and activation of NK cells in the airway and lung tissue. Therefore, we first aimed to investigate the dependency of the NK cell response to IL-15 signalling, through use of an IL-15 neutralising antibody (M96), which was administered intranasally at the time of infection in addition to 24h after infection, to transiently and locally block IL-15 signalling in the airway/lungs.

Secondly, to further investigate other specific roles that IL-15 plays in the immune response to RV, we infected IL-15Rα ko mice that have a constitutive and systemic block to IL-15 mediated responses. These mice have a severe deficiency in NK cells due to a developmental block in the absence of IL-15 signalling, therefore, this model also indirectly assesses the importance of NK cells for early control of RV-1B infection.
4.2. Hypotheses & Aims

4.2.1. Hypotheses

i. IL-15 signalling in the airway/lungs is required for the recruitment/expansion and activation of NK cells into the lungs during RV infection.

ii. NK cell activation affects RV clearance.

iii. NK cells are the major source of IFN-γ production during RV infection.

iv. Deficient NK cell IFN-γ production during RV infection is associated with development of a Th2 response.

4.2.2. Aims

We aimed to explore these hypotheses using 2 different models of IL-15 deficiency, which differ in transient and local vs constitutive and systemic blocks to IL-15 signalling.

Part 1: To investigate the dependency of the NK cell response during RV-1B infection to IL-15 signalling in the airway/lungs, via co-administration of an IL-15 neutralising antibody (M96) at the time of infection in BALB/c mice to transiently inhibit IL-15.

Part 2: To investigate the immune responses to RV-1B infection in the absence of systemic and constitutive IL-15 signalling, and indirectly assess the importance of NK cells during RV-1B infection using IL-15Rα ko mice.

i. To determine the virus load in the lungs using Taqman qPCR.

ii. To determine the expression of IFNs, ISGs, proinflammatory cytokines and chemokines and Th2-associated mediators in the BAL and lungs using Taqman qPCR and ELISAs.

iii. To examine the NK cell response in the airway and lungs using flow cytometry.

iv. To examine the acquired T cell response in the airway and lungs using flow cytometry.
4.3. **Results – Part 1: Transient and Local Neutralisation of IL-15 During RV Infection**

In this study, BALB/c mice were treated i.n. with 50μl RV-1B (5x10^6 TCID₅₀) at 0h in addition to either 20μg M96 IL-15 neutralising antibody (‘RV-M96’), 20μg mlG2a isotype control (‘RV-ISO’) or PBS (‘RV-PBS’) at 0h and 24h p.i. Mock-infected mice were treated with PBS alone. The nomenclature for the different treatment groups in this model are summarised again in Table 4.1.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>RV-1B Infection 0h – i.n.</th>
<th>M96 0h &amp; 24h – i.n.</th>
<th>Isotype Control 0h &amp; 24h – i.n.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV-PBS</td>
<td>YES</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>RV-M96</td>
<td>YES</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>RV-ISO</td>
<td>YES</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>Mock (PBS)</td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
</tr>
</tbody>
</table>

Table 4.1 Experimental groups of the RV infection model with neutralisation of airway/lung IL-15.

4.3.1. **Virus Load**

To determine if IL-15 neutralisation in the airway/lungs affects the lung viral load, we measured the level of RV vRNA via qPCR in lung homogenate. However, we found that treatment with M96 during RV-1B infection had no significant effect on virus load at 24h and 48h p.i. (Fig 4.1).
BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. Lungs were harvested at 24h and 48h p.i. and processed for RNA extraction, and cDNA made by reverse transcription as described. RV-1B viral RNA copies were quantified by qPCR. Data was normalised against 18s rRNA expression and expressed as viral RNA copies/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001 & *p<0.05). Only statistically significant differences are shown.

**4.3.2. Differential BAL Cell Counts**

To determine if IL-15 signalling affects the inflammatory cell profile in the airway during RV infection we assessed the differential BAL cell counts of cytospins. As shown in chapter 3, RV-1B infection results in an increase in total BAL cell counts, however we found that the presence of M96 did not affect this (Fig 4.2a). Additionally, IL-15 neutralisation did not affect the degree of neutrophilia in the airway during RV-1B infection (Fig 4.2 b&c). We have also previously shown that BAL lymphocyte numbers increase at 48h p.i. with RV-1B; however, here we demonstrate that the presence of M96 significantly reduces the percentage and number of lymphocytes detected at 48h p.i. with RV-1B (Fig 4.2 d&e).
Figure 4.2 Effect of IL-15 neutralisation in the airway/lungs on total and differential BAL cell counts during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. BAL was performed at 24h and 48h p.i. and recovered live BAL cells were counted (a) and processed for cytospin slides. Slides were blinded to experimental conditions and cells differentially counted to give the percentage and total number of (b&c) neutrophils and (d&e) lymphocytes in the airway. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.
4.3.3. Expression of Lung IL-15 and IL-15Rα

Expression of IL-15 and sIL-15/IL-15Rα complexes in response to RV-1B infection was shown in chapter 3. To determine if M96 affects expression of IL-15/IL-15Rα we analysed expression of these molecules by ELISA using lung homogenate. We found that neutralisation with M96 did not affect RV-mediated IL-15 expression (Fig 4.3 a&b).

![Figure 4.3 IL-15 neutralisation in the airway/lungs does not affect RV-induced IL-15 and IL-15Rα.](image)

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. Lungs were harvested at 24h and 48h p.i. and processed for protein detection as described. The level of (a) IL-15 and (b) sIL-15/IL-15Rα complexes were quantified by ELISA and expressed as pg/ml. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.

4.3.4. BAL Proinflammatory Mediators

To assess if neutralisation of IL-15 in the airway/lungs effects production of proinflammatory cytokines and chemokines during RV-1B infection, we analysed the BAL fluid by ELISAs. We found that M96 did not affect production of KC, IL-6, IL-1β or RANTES during RV-1B infection (Fig 4.4 a-d). However, RV-M96 treated mice expressed almost 2-fold more TNF-α than RV-PBS and RV-ISO controls at 24h p.i. (***p<0.001), after which levels returned to baseline (Fig 4.4e).

4.3.5. Expression of Type I IFNs, Type III IFNs and Interferon Stimulated Genes

To investigate if IL-15 signalling in the airway/lungs effects expression of type I and III IFNs and thus ISGs during RV-1B infection, we analysed expression by qPCR and ELISAs. We found
RV-induced IFN-α, IFN-β and IFN-λ expression to be unaltered in the presence of M96, and consequentially the ISGs Viperin, PKR, IP-10 and I-TAC were also unaffected during RV-1B infection (Fig 4.5).

![Graphs showing BAL proinflammatory cytokines and chemokines during RV-1B infection.](image)

Figure 4.4 Effect of IL-15 neutralisation in the airway/lungs on expression of BAL proinflammatory cytokines and chemokines during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. BAL was performed at 24h and 48h p.i., and the concentration of (a) KC, (b) IL-6, (c) IL-1β, (d) RANTES and (e) TNF-α were detected in the BAL fluid by ELISA and expressed as pg/ml. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mIgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. BAL was performed and lungs harvested at 24h and 48h p.i. Concentration of (a) IFN-α, (c) IFN-λ2/3, (f) IP-10 and (g) I-TAC in the BAL fluid were quantified by ELISA and expressed as pg/ml. Lungs were processed for RNA extraction and cDNA made by reverse transcription as described. (b) IFN-β, (d) Viperin and (e) PKR mRNA were quantified by qPCR, data was normalised against 18s rRNA
expression and expressed as mRNA copies/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.

4.3.6. Expression of IFN-γ

IFN-γ expression in the lungs was determined by qPCR (Fig 4.6). As expected we observed upregulation in control virus groups at 24h and 48h p.i. compared to mock-infected mice however, mice treated with both RV-1B and M96 demonstrated no upregulation of IFN-γ at either timepoint. Thus, transient neutralisation of IL-15 in the airway/lungs during RV-1B infection results in deficient IFN-γ expression. Production by specific lymphocyte populations is further explored by flow cytometry in sections 4.3.9 and 4.3.10.

Figure 4.6 IL-15 neutralisation in the airway/lungs results in deficient IFN-γ expression in the lungs during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (IS0) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. Lungs were harvested at 24h and 48h p.i. and processed for RNA extraction and cDNA made by reverse transcription as described. IFN-γ mRNA was quantified by qPCR, data was normalised against 18s rRNA expression and expressed as mRNA copies/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001 & *p<0.05). Only statistically significant differences are shown.

4.3.7. Expression of Th2 Mediators

We hypothesized that in the absence IFN-γ expression, which helps drive development of a Th1 response against virus, a Th2 response may develop. Thus, we assessed expression of
molecules commonly associated with Th2 responses. Chemokines TARC and MDC have been reported to be pivotal in development of Th2-mediated responses \[258\]; analysis of BAL fluid by ELISA demonstrated greater production in RV-M96 compared to RV-PBS and RV-ISO treated mice early after infection at 24h but this was only statistically significant for TARC and not MDC, and by 48h levels had equalized between all virus treated groups (Fig 4.7 a&b). We also assessed expression of the classical Th2 cytokines by qPCR, and though we did not detect IL-4 and IL-5, a small increase in IL-13 mRNA levels in the lungs of RV-M96 treated mice was observed compared to RV-PBS and RV-ISO, but this was not statistically significant and levels were very low (Fig 4.7c). This data provides some indication of a weak Th2 response in the absence of IL-15 and IFN-γ signalling in the airway and lungs during RV-1B infection of BALB/c mice.

**Figure 4.7 IL-15 neutralisation in the airway/lungs result in increased expression of Th2 mediators in the BAL and lungs during RV-1B infection.**

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. BAL was performed and lungs were harvested at 24h and 48h p.i. The concentration of (a) TARC and (b) MDC in BAL fluid was measured by ELISA and expressed as pg/ml. Lungs were processed for RNA extraction and cDNA made by reverse transcription as described. (c) IL-13 mRNA was quantified by qPCR, data was normalised against 18s rRNA expression and presented as mRNA copies/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group, representative of at
least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01 & ***p<0.001). Only statistically significant differences are shown.

4.3.8. Effect of M96 Antibody Treatment on Splenic and Lung Lymphocytes

We used flow cytometry to determine if i.n. administration of M96 antibody affects lymphocytes in the lungs and spleen in the absence of RV-1B infection. We show that splenic and lung CD4+ and CD8+ T cell frequencies are not altered 48h post antibody treatment (Fig 4.8 c-f). However, the frequency of NK cells in the lungs had decreased by ~2.5% at 24h and by ~4% at 48h post antibody treatment compared to mock and isotype control treated mice (Fig 4.8h). Additionally, the frequency of splenic NK cells at 48h post treatment had decreased by ~2% in M96 treated mice compared to controls (Fig 4.8g). Thus, we demonstrate that 20μg of M96 given i.n. results in depletion of NK cells as early as 24h post treatment in the lungs, and the decrease in splenic NK cell frequency implies that the antibody is acting systemically by 48h despite the specific local administration.
Figure 4.8 Intranasal administration of M96 IL-15 neutralising antibody does not affect T cell populations but reduces the frequency of lung and splenic NK cells by 48h after administration.

BALB/c mice were treated i.n. with M96 (IL-15 neutralising antibody), mlgG2a isotype control (ISO) or PBS (Mock) at 0h and 24h. The spleen and left lung lobe was excised after BAL was performed at 24h and 48h post-treatment, and the tissues digested as described. The resultant live single cell suspension was counted to give the total number of (a) spleen and (b) lung cells. 1x10^6 cells were stained for surface markers CD3, CD4, CD8 and NKp46 and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage of (c&d) CD3+/CD4+ T cells, (e&f) CD3+/CD8+ T cells and (g&h) CD3−/NKp46+ NK cells of the lymphocyte gate in the spleen and lung respectively. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.
4.3.9. NK Cell Response

Flow cytometry was employed to assess the dependency of the NK cell response in both the airway and lungs to IL-15 signalling during RV infection. Cells were stained for surface markers CD3, NKp46 and CD69, in addition to intracellular cytokines GranzymeB and IFN-γ. NK cells were defined as CD3-/NKp46+ cells within the lymphocyte population.

4.3.9.1. BAL NK Cells

As established in chapter 3, NK cells entered the airway at 24h and peaked at 48h p.i. with RV-1B infection, which was observed in this study with RV-PBS and RV-ISO treated mice to equal extents. However, we demonstrate that in RV-M96 treated mice, there was a significant block to the proliferation/expansion of NK cells in the airway compared to the virus controls (Fig 4.9a). Consequentially, there was a deficiency in the number of activated CD69+, GranzymeB+ and IFN-γ+ NK cells in RV-M96 compared to RV-PBS and RV-ISO treated mice at both 24h and 48h p.i. (Fig 4.9 b-d). Therefore, this data clearly demonstrates the ability of M96 antibody, via neutralisation of IL-15 signalling, to block the recruitment/expansion of NK cells in the airway during RV-1B infection.
Figure 4.9 IL-15 neutralisation in the airway/lungs results in deficient BAL NK cell responses during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. BAL was performed at 24h and 48h p.i., and 1x10⁵ cells were stained for surface markers CD3, CD69 and Nkp46, intracellular cytokines GranzymeB and IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the absolute number of BAL (a) CD3⁻/Nkp46⁺ NK cells, CD3⁻ gated double positive (b) Nkp46⁺/CD69⁺, (c) Nkp46⁺/GranzymeB⁺ and (c) Nkp46⁺/IFN-γ⁺ NK cells. Data are expressed as means ± SEM of 4-5 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.

4.3.9.2. Lung NK Cells

The total lung cell counts did not differ between the groups at 24h p.i. however, total numbers were increased in RV infected groups compared to mock-treated mice at 48h (Fig 4.10). Additionally, at 48h p.i. there was a slight reduction in the total number of lung cells in RV-M96 compared to RV-PBS and RV-ISO treated mice, but this was not statistically significant.

We found the percentage of lung NK cells to be ~2-fold lower in RV-M96 compared to mock-treated mice at 24h and 48h p.i. (p<0.05) (Fig 4.11a), suggesting depletion of NK cells due to
treatment with M96; however, due to increased total lung cell counts in virus treated groups (regardless of M96 treatment), no significant difference was observed in the absolute number of NK cells between RV-M96 compared to mock treated mice at 24h and 48h p.i. (Fig 4.11b).

Upon comparison of virus treated groups, we show that RV-M96 treated mice had reduced frequencies of NK cells compared to RV-PBS and RV-ISO treated mice at 24h and 48h p.i., which translated into ~2.5-3-fold fewer numbers of NK cells in M96 treated mice at both time points. Additionally, the percentage and absolute numbers of CD69+, GranzymeB+ and IFN-γ+ NK cells at 24h and 48h p.i. was increased in both RV-PBS and RV-ISO groups compared to mock treated mice, but was not in RV-M96 treated mice (Figure 4.11 c-h). Thus, we demonstrate that IL-15 neutralisation in the airway/lungs by M96 antibody resulted in a severely deficient NK cell response to RV-1B infection.

![Figure 4.10](image_url)

**Figure 4.10** Effect of IL-15 neutralisation in the airway/lungs on the total lung leukocyte count during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. The left lung lobe was excised after BAL was performed at 24h and 48h p.i. Lungs were digested as described and the resultant live single-cell suspension counted to give the total number lung cells. Data are expressed as means ± SEM of 4-5 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01, *p<0.05). Only statistically significant differences are shown.
Figure 4.11 IL-15 neutralisation in the airway/lungs results in deficient lung NK cell responses during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mIgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. The left lung lobe was excised after BAL was performed at 24h and 48h p.i. and digested as described. 1x10^6 cells were stained for surface markers CD3, CD69 and NKp46, intracellular cytokines
Granzyme B and IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of lung (a&b) CD3- /Nkp46+ NK cells, CD3- gated double positive (c&d) Nkp46+/CD69+, (e&f) Nkp46+/GranzymeB+ and (g&h) Nkp46+/IFN-γ+ NK cells. Data are expressed as means ± SEM of 4-5 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.001, *p<0.01 & *p<0.05). Only statistically significant differences are shown.

4.3.10. T Cell Response

4.3.10.1. BAL T Cells

As expected, CD4+ T cell numbers rose in the airway at 48h p.i. in virus treated groups however, we observed a small reduction in number in RV-M96 compared to RV-PBS and RV-ISO groups, but this was not found to be significant (Fig 4.12a). Similarly, a decrease in numbers of CD69+ CD4+ T cells in RV-M6 compared to RV-PBS and RV-ISO groups at 48h p.i. was observed (p<0.05) (Fig 4.12c), though the decrease in number of IFN-γ+ CD4+ T cells was not significant (Fig 4.12e).

The reduction in cell number in RV-M6 treated mice was more pronounced with CD8+ T cells, which were ~50% of those in RV-PBS treated mice at 48h p.i. (p<0.01) (Fig 4.12b). Correspondingly, numbers of CD69+ and IFN-γ+ CD8+ T cells were also reduced in RV-M96 compared to RV-PBS and RV-ISO treated groups at 48h p.i. (p<0.01 for CD69+; p<0.05 for IFN-γ+) (Fig 4.12 d&f). This data suggests that neutralisation of IL-15 during RV-1B infection has a significant effect on the recruitment/expansion and activation of T cells in the airways, and this is more pronounced in CD8+ rather than in CD4+ T cells.

4.3.10.2. Lung T Cells

In the lungs, the percentage of CD4+ T cells did not vary between all groups at either timepoints (Fig 4.13a), though translation to cell numbers demonstrated a slightly impaired increase in total number of CD4+ T cells in RV-M96 compared to RV-PBS treated mice at 48h p.i. (p<0.05) (Fig 4.13b). The percentage of CD69+ and IFN-γ+ CD4+ T cells rose equally at 24h and 48p.i. within virus treated groups compared to mock treated mice (Fig 4.13 c&e); however, cell number revealed a small reduction in total number of activated CD4+ T cells in RV-M96 compared to RV-PBS and RV-ISO treated mice at 48h p.i., though this was not statistically significant (Fig 4.13 d&f).

Similar trends were seen for lung CD8+ T cells, though the deficiency in total number of CD8+ T cells in RV-M96 compared to RV-PBS and RV-ISO was slightly greater, but still not
statistically significant for CD69+ and IFN-γ+ CD8+ T cells (Fig 5.14). Thus, similar to the airway, neutralisation of IL-15 during RV-1B infection appears to affect the recruitment/expansion and activation of CD8+ T cells to the lungs, though the effect is not as great as the effect on the NK cell response.

Figure 4.12 Effect of IL-15 neutralisation in the airway/lungs on the BAL T cell response during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. BAL was performed at 24h and 48h p.i. and 1x10^5 cells were stained for surface markers CD3, CD4, CD8 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the absolute number of BAL (a) CD3+/CD4+, (b) CD3+/CD8+, CD3+ gated double positive (c) CD4+/CD69+, (d) CD8+/CD69+ (e) CD4+/IFN-γ+ and (f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-5 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.001, *p<0.01 & *p<0.05).
Figure 4.13 Effect of IL-15 neutralisation in the airway/lungs on the lung CD4+ T cell response during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. The left lung lobe was excised after BAL was performed at 24h and 48h p.i. and digested as described. 1x10^6 cells were stained for surface markers CD3, CD4 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of lung (a&b) CD3+/CD4+ T cells, CD3+ gated double positive (c&d) CD4+/CD69+ and (e&f) CD4+/IFN-γ+T cells. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
Figure 4.14 Effect of IL-15 neutralisation in the airway/lungs on the lung CD8+ T cell response during RV-1B infection.

BALB/c mice were treated i.n. with RV-1B at 0h in addition to PBS, M96 (IL-15 neutralising antibody) or mlgG2a isotype control (ISO) at 0h and 24h p.i., and mock-infected mice were treated with PBS alone. The left lung lobe was excised after BAL was performed at 24h and 48h p.i. and digested as described. 1x10^6 cells were stained for surface markers CD3, CD8 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of lung (a&b) CD3+/CD8+ T cells, CD3+ gated double positive (c&d) CD8+/CD69+ and (e&f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
4.4. Chapter Summary – Part 1

Treatment of mice intranasally at the time of RV-1B infection and 24h later with IL-15 neutralising antibody to block IL-15 signalling in the airway/lungs did not affect airway neutrophilia levels. However, the percentage and total number of BAL lymphocytes was decreased in the presence of M96 during infection. Levels of the proinflammatory cytokines IL-6, IL-1β and RANTES were not changed in the presence of M96, nor was the expression of IL-15, type I IFNs, IFN-λ and ISGs at 24h and 48h after infection. By looking at total IFN-γ mRNA levels in lung homogenate by qPCR, we found IFN-γ upregulation to be deficient in RV-M96 treated mice compared to controls, which was associated with increased expression of Th2-associated mediators TARC, MDC and IL-13.

Intranasal administration of M96 alone without infection, at the same doses used for infection studies resulted in reduced NK cell frequencies in the lungs (from ~7.0% to 4.0%) and spleen (from ~5.5% to 3.5%) compared to PBS treated mice 48h after treatment. Thus, intranasally administered M96 can act systemically and IL-15 is important for survival/homeostasis of NK cells in the periphery. Despite depletion, at 48h post M96 treatment a population of NK cells still exists in the mouse permitting a potential response to virus infection.

RV-M96 treated mice demonstrated a block to recruitment/expansion of NK cells in both the airway and lungs at 24h and 48h after infection compared to controls – at both time points, RV-M96 and mock treated mice exhibited similar total numbers of NK cells. In addition, airway and lung NK cells in RV-M96 treated mice did not increase expression of CD69, GranzymeB and IFN-γ compared to virus controls. Thus, we show that transiently blocking IL-15 in the airway/lungs during RV-1B infection in mice inhibits the recruitment/proliferation and activation of NK cells, even in the presence of type I IFN signalling.

IL-15 neutralisation did not significantly affect the CD4+ T cell response to RV-1B infection in mice. However, we show reduced CD8+ T cell recruitment/expansion and activation (CD69 and IFN-γ expression) in the airway and lung tissue of RV-M96 treated mice compared to controls at 48h after infection. The reduced number of IFN-γ+ CD8+ T cells in M96-RV treated mice was mild compared to the severely deficient expression in NK cells, and expression in CD4+ T cells was intact. Due to the large deficiency in total IFN-γ mRNA levels observed in lung tissue of RV-M96 treated mice compared to virus controls, we suggest that NK cells are the main producers of IFN-γ in BALB/c mice early after RV-1B infection. To
further investigate the roles of IL-15 signalling and the importance of NK cells in the immune response to RV infection, we analysed the response in IL-15Rα ko mice.
4.5. Results – Part 2: RV-1B Infection in IL-15Rα ko Mice

In this study, IL-15Rα ko and control B6.129 mice were infected i.n. with RV-1B (5x10⁶ TCID₅₀) and ‘Mock’ mice (0h) were treated i.n. with PBS only. IL-15Rα ko mice have a constitutive and systemic block to IL-15 signalling and consequentially have a severe deficiency in NK cells.

4.5.1. Virus Load

To determine if the absence of IL-15 signalling and NK cells affects the lung virus load, we measured the level of RV-1B viral RNA in the lungs. We found a higher and more sustained virus load in the lungs at both 8h (ns) and 24h p.i. (p<0.05) compared to B6.129 mice (Fig 4.15), and virus was cleared by both groups by 96h.

![Figure 4.15 IL-15Rα ko mice have an increased lung viral load after RV-1B infection.](image)

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and lungs were harvested at the indicated timepoints p.i. Lungs were processed for RNA extraction and cDNA made by reverse transcription as described. RV-1B viral RNA copies were quantified by qPCR, and data was normalised against 18s rRNA expression and presented as vRNA copies/µL of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (*p<0.05). Only statistically significant differences are shown.

4.5.2. Differential BAL Cell Counts

Similar to infection with IL-15 neutralising antibody, there was no significant difference in the total number of BAL cells, nor in the degree of neutrophilia between IL-15Rα ko and B6.129 mice in response to RV-1B infection (Fig 4.16 a-c). Also, in agreement with the neutralising antibody study, we detected a deficient BAL lymphocyte response in IL-15Rα ko
compared to B6.129 mice, as determined by both percentage and total number at 48h and 96h p.i. (Fig 4.16 d&e).

Figure 4.16 Total and differential BAL cell counts during RV-1B infection in IL-15Rα ko mice.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and BAL was performed at the indicated time points p.i. Recovered live BAL cells were (a) counted and processed for cytospin slides. Slides were blinded to experimental conditions and cells differentially counted to give the percentage and total number of (b&c) neutrophils and (d&e) lymphocytes in the airways. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
4.5.3. BAL Proinflammatory Mediators

Proinflammatory cytokines and chemokines produced in response to RV-1B were measured in the BAL fluid by ELISA. No differences were detected in the expression of KC and RANTES in response to virus in both groups (Fig 4.17 a&e). However, unlike the neutralising antibody study, higher levels of IL-6 at 24h p.i. (p<0.001) and IL-1β at 24h (p<0.01) and 48h (p<0.01) were observed in IL-15Rα ko compared to B6.129 mice (Fig 4.17 b&c). Additionally, levels of TNF-α were slightly elevated in IL-15Rα ko compared to B6.129 mice at 24h and 48h p.i., though the differences were not statistically significant (Fig 4.17d).

4.5.4. Expression of Type I IFNs, Type III IFNs and Interferon Stimulated Genes

Analysis of the BAL fluid by ELISA demonstrated that expression of IFN-α was deficient and short lived in IL-15Rα ko mice; levels peaked at 48h p.i. and had declined to baseline by 72h in IL-15Rα ko compared to B6.129 mice, which peaked at 72h p.i. and with ~2-fold more IFN-α (Fig 4.18a). Lung IFN-β expression was detected by qPCR, and though higher levels of mRNA were detected in B6.129 compared to IL-15Rα ko mice at 24h p.i., the difference was not statistically significant (Fig 4.18b).

Similarly, BAL IFN-λ2/3 was found to be deficient in IL-15Rα ko compared to B6.129 mice. A small peak of production was detected at 24h p.i. in IL-15Rα ko mice, however higher levels were detected in B6.129 mice at 24h, which was sustained at 48h (p<0.01) and declined thereafter (Fig 4.18c). Thus, these data suggests that during RV-1B infection, expression of type I and III IFNs is compromised in the absence of IL-15 signalling.

However, despite detection of less type I and III IFN production in IL-15Rα ko mice, no significant differences were detected between IL-15Rα ko and B6.129 mice in expression of the ISGs PKR, OAS-1a, I-TAC and IP-10 in response to RV-1B infection (Fig 4.18 d-g). This is in agreement with data obtained from the IL-15 neutralising antibody study suggesting that absence of IL-15 signalling during RV-1B infection does not appear affect expression of ISGs.

4.5.5. Expression of IFN-γ

On the other hand, in response to RV-1B infection, expression of IFN-γ was found to be severely deficient in IL-15Rα ko compared to B6.129 mice as detected by qPCR in lung homogenate (p<0.01 at 24h p.i.) (Fig 4.19). Thus, in agreement with the IL-15 neutralising
antibody study, our data suggests that IL-15 signalling during RV-1B infection is necessary for IFN-γ expression.

Figure 4.17 Expression of BAL proinflammatory cytokines and chemokines in response to RV-1B infection in IL-15Rα ko mice.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and BAL was performed at the indicated time points p.i. The concentration of (a) KC, (b) IL-6, (c) IL-β, (d) TNF-α and (e) RANTES in the BAL fluid was determined by ELISA and expressed as pg/ml. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
Figure 4.18 Expression of BAL and lung type I and III IFNs and interferon stimulated genes in response to RV-1B infection in IL-15Rα ko mice.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and lungs were harvested after BAL was performed at the indicated timepoints p.i. (a) IFN-α, (c) IFN-λ2/3, (f) I-TAC and (g) IP-10proteins were detected in the BAL fluid by ELISA and expressed as pg/ml. Lungs were processed for RNA extraction and cDNA made by reverse transcription as described. (b) IFN-β, (d) PKR and (e) OAS-1a mRNA was detected by qPCR, data normalised against 18s rRNA expression and presented as mRNA copies/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is
representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.

**Figure 4.19** IL-15Rα ko mice have a deficient IFN-γ response during RV-1B infection.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and lungs were harvested at the indicated timepoints p.i. Lungs were processed for RNA extraction and cDNA made by reverse transcription as described. IFN-γ mRNA copies/μL of sample were quantified by qPCR and data was normalised against 18s rRNA expression. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (**p<0.01). Only statistically significant differences are shown.

4.5.6. Expression of Th2 Mediators

We were also prompted to look for expression of Th2 mediators in this study based on the deficient expression of IFN-γ. MDC and TARC were detected in BAL fluid by ELISA and found to be induced during infection, and though higher levels were observed in IL-15Rα ko compared to B6.129 mice the differences were not statistically significant (Fig 4.20 a&b). We also investigated expression of classical Th2 cytokines in lung tissue by qPCR. Increased expression of IL-4 mRNA was detected in IL-15Rα ko mice during RV-1B infection, with significantly higher levels at 96h p.i. compared to B6.129 mice (p<0.01) (Fig 4.20c). IL-5 was found to be induced in response to RV-1B infection in IL-15Rα ko but not B6.129 mice; levels peaked at 24h p.i. in IL-15Rα ko mice and declined thereafter however, differences between the groups were not statistically significant (Fig 4.20d). Lastly, expression of IL-13 was also significantly induced in IL-15Rα ko mice but not B6.129 mice; expression was detected by 8h p.i. in IL-15Rα ko mice (p<0.05) and peaked at 24h (ns) (Fig 4.20e). Therefore, in the absence of IL-15 signalling during RV-1B infection, characteristic traits of a Th2 response developed in IL-15Rα ko mice.
Figure 4.20 IL-15Rα ko mice demonstrate increased expression of Th2 cytokines and chemokines in BAL fluid and lung tissue during RV-1B infection.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and lungs were harvested after BAL was performed at the indicated timepoints p.i. (a) MDC and (b) TARC proteins were detected in the BAL fluid by ELISA and expressed as pg/ml. Lungs were processed for RNA extraction and cDNA made by reverse transcription as described. (c) IL-4, (d) IL-5 and (e) IL-13 mRNA were detected by qPCR, data normalised against 18s rRNA expression and presented as mRNA copies/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.
4.5.7. NK Cell Response

As previously stated, IL-15Rα ko mice have a constitutive block to IL-15 signalling and therefore, NK cells are unable to develop. Flow cytometry was used to confirm this and analyse the response in B6.129 mice; BAL and lung cells were stained with monoclonal antibodies specific for CD3, NK1.1, CD69, GranzymeB and IFN-γ. NK cells were defined as CD3+/NK1.1+ cells within the lymphocyte gate, and the representative flow plots in Figure 4.21 illustrate the absence of these cells in the BAL and lung of IL-15Rα ko mice in comparison to control B6.129 mice.

![Flow plots illustrating NK cell response](image)

Figure 4.21 Representative flow plots of BAL and lungs NK cells in B6.129 and IL-15Rα ko mice.

Flow plots illustrate the presence of CD3+/NK1.1+ NK cells within the gate, in the airway and lungs of (a&c) B6.129 mice, and their absence in (b&d) IL-15Rα ko mice. Numbers indicated on plots represent the % of events falling within the indicated gate.
4.5.7.1. BAL NK Cells

Figure 4.22 clearly demonstrates the total absence of an NK cell response to RV-1B infection in the airway of IL-15Rα ko mice. Similar to that seen with BALB/c mice in chapter 3, the total number of BAL NK cells in B6.129 mice peaked at 24h p.i. and remained elevated at 96h (Fig 4.22a), and these cells were shown to be activated based on their expression of CD69, GranzymeB and IFN-γ (Fig 4.22b-d).

![Graphs showing BAL NK cell response to RV-1B infection in IL-15Rα ko mice.](image)

**Figure 4.22** The BAL NK cell response to RV-1B infection in IL-15Rα ko mice is absent.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and BAL was performed at the indicated time points p.i. 1x10⁵ BAL cells were stained for surface markers CD3, CD69 and NK1.1, intracellular cytokines GranzymeB and IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the absolute number of BAL (a) CD3-/NK1.1+ NK cells, CD3- gated double positive (b) NK1.1+/CD69+, (c) NK1.1+/GranzymeB and (d) NK1.1+/IFN-γ+ NK cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (**p<0.01 & ***p<0.001). Only statistically significant differences are shown.
4.5.7.2. Lung NK Cells

The total number of lung cells was observed to increase in response to RV-1B infection in both groups; however, numbers in IL-15Rα ko mice were found to be lower than those in B6.129 mice at both 24h and 48h p.i. with RV-1B infection (p<0.05) (Fig 4.23). Regardless of RV-1B infection, NK cells were absent from the lungs of IL-15Rα ko mice, though an NK cell response was detected during RV-1B infection in B6.129 (Fig 4.24); compared to 0h, the number of NK cells increased and peaked at 24h p.i. with RV-1B and declined back to baseline by 96h, and similarly the numbers of CD69+, GranzymeB+ and IFN-γ+ NK cells also peaked at 24h and declined to baseline (0h) levels by 96h (Fig 4.24 d, f&h).

![Graph showing total lung leukocyte count during RV-1B infection in IL-15Rα ko mice.](image)

**Figure 4.23 Total lung leukocyte count during RV-1B infection in IL-15Rα ko mice.**

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and the left lung lobe excised after BAL was performed at the indicated timepoints p.i. Lungs were digested as described and the resultant live single-cell suspension counted. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (*p<0.05). Only statistically significant differences are shown.
**Figure 4.24** The lung NK cell response to RV-1B infection in IL-15Rα ko mice is absent.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and the left lung lobe excised after BAL was performed at the indicated timepoints p.i. Lungs were digested as described and 1x10⁶ cells were stained for surface markers CD3, CD69 and NK1.1, intracellular cytokines GranzymeB and IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data
are presented as the percentage and absolute number of lung (a&b) CD3−/NK1.1+ NK cells, CD3−
gated double positive (c&d) NK1.1+/CD69+, (e&f) NK1.1+/GranzymeB and (g&h) NK1.1+/IFN-γ+ NK
cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2
independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice
treated with RV-1B, at the indicated times (**p<0.01 & ***p<0.001). Only statistically significant
differences are shown.

4.5.8. T Cell Response

4.5.8.1. BAL T Cells

In the airway, CD4+ T cell numbers increased after infection with RV-1B in both IL-15Rα ko
and B6.129 mice; no differences in numbers were detected between the groups and
numbers were greatest at 96h p.i. (Fig 4.25a). The number of CD69+ T cells increased at 24h
and 48h p.i. equally in both groups however, at 96h p.i. B6.129 mice had ~1.5-fold more
CD69+ CD4+ T cells compared to IL-15Rα ko mice (p<0.01) (Fig 4.25c). However, no
significant differences in the number of IFN-γ+ CD4+ T cells were detected between the
groups during RV-1B infection (Fig 4.25e).

On the other hand, there was a striking deficiency of CD8+ T cells in the airway in IL-15Rα ko
compared to B6.129 mice in response to RV-1B infection. In B6.129 mice, CD8+ T cells were
recruited into the airway by 24h p.i., numbers peaked at 48h (p<0.001) and remained
significantly elevated at 96h (p<0.001) compared to IL-15Rα ko mice (Fig 4.25b). Similarly,
there was a lack of activated CD69+ and IFN-γ+ CD8+ T cells in the airway of IL-15Rα ko mice
compared to B6.129 mice, which demonstrated a significant increase in the number of
activated CD8+ T cells, peaking at 48h p.i. (p<0.001) and declining thereafter (Fig 4.25 d&f).
Figure 4.25 The BAL CD8+ T cell but not CD4+ T cell response during RV-1B infection in IL-15Rα ko mice is deficient.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and BAL was performed at the indicated time points p.i. 1x10^5 BAL cells were stained for surface markers CD3, CD4, CD8, and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the absolute number of BAL (a) CD3+/CD4+ T cells, (b) CD3+/CD8+ T cells, CD3+ gated double positive (c) CD4+/CD69+, (d) CD8+/CD69+, (e) CD4+/IFN-γ+ and (f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (***p<0.001, **p<0.01& *p<0.05). Only statistically significant differences are shown.
4.5.8.2. Lung T Cells

As no differences were detected in percentage of lung CD4+ T cells during RV-1B infection between IL-15Rα and B6.129 mice (Fig 4.26a), the total number was lower in IL-15Rα ko mice at 24h and 48h p.i. compared to B6.129 mice (p<0.01 & p<0.001) (Fig 4.26b) due to the total lung cell count profile (Fig 4.23). However, the percentage of CD69+ and IFN-γ+ CD4+ T cells was higher at 24h-48h and 24h-96h p.i. respectively in IL-15Rα ko compared to B6.129 mice (Fig 4.23 c&e); upon translation into cell number, we observed only slightly lower total numbers of CD69+ and IFN-γ+ CD4+ T cells within the lungs of IL-15Rα ko compared to B6.129 mice at 24h and 48h p.i., and the differences were not statistically significant (Fig 4.23 d&f).

The lung CD8+ T cell response to RV-1B infection in IL-15Rα ko mice mirrors that in the airway. Firstly, the baseline percentage of CD8+ T cells in the lungs is ~2-fold lower in IL-15Rα ko mice, and there was little/no increase in percentage with RV-1B infection compared to B6.129 mice (Fig 4.27a). Thus, no noticeable increase in the total number of lung CD8+ T cells was detected at any time point p.i. compared to B6.129 mice, which demonstrated recruitment/expansion of CD8+ T cells in the lungs at 24h and 48h p.i. (P<0.001), and numbers declined thereafter (Fig 4.27b). Additionally, lung CD8+ T cells in IL-15Rα ko mice demonstrated deficient upregulation of CD69 and IFN-γ in response to RV-1B infection, compared to B6.129 mice, of which the later demonstrated increased numbers of activated lung CD8+ T cells at 24h and 48h p.i. (p<0.001) (Fig 4.27 c-f). Thus, in the absence of IL-15 signalling, IL-15Rα ko mice had significantly fewer lung CD8+ T cells at baseline, which failed to increase in numbers and activate upon RV-1B infection.
Figure 4.26 Lung CD4+ T cell response to RV-1B infection in IL-15Rα ko mice.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and the left lung lobe excised after BAL was performed at the indicated timepoints p.i. Lungs were digested as described and 1x10^6 cells were stained for surface markers CD3, CD4 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of lung (a&b) CD3+/CD4+ T cells, CD3+ gated double positive (c&d) CD4+/CD69+ and (e&f) CD4+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.
Figure 4.27 The lung CD8+ T cell response to RV-1B infection in IL-15Rα ko mice is deficient.

IL-15Rα ko and B6.129 mice were treated i.n. with RV-1B or PBS (0h), and left lung lobe excised after BAL was performed at the indicated timepoints p.i. Lungs were digested as described and 1x10^6 cells were stained for surface markers CD3, CD8 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of lung (a&b) CD3+/CD8+ T cells, CD3+ gated double positive (c&d) CD8+/CD69+ and (e&f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IL-15Rα ko compared with B6.129 mice treated with RV-1B, at the indicated times (**p<0.01, *p<0.05). Only statistically significant differences are shown.
4.6. Chapter Summary – Part 2

IL-15Rα ko mice have a systemic and constitutive block to IL-15 signalling, and in response to RV-1B infection no difference in the degree of neutrophilia was detected but significantly fewer BAL lymphocytes in comparison to wild type mice was observed. Additionally, IL-15Rα ko mice demonstrated increased expression of the proinflammatory cytokines IL-6, IL-1β and TNF-α.

During RV-1B infection in the absence of IL-15 signalling, analysis of lung tissue mRNA showed a severe deficiency in IFN-γ expression. This was associated with increased expression of the Th2-associated chemokines TARC and MDC. Moreover, mRNA expression of the classical Th2 cytokines IL-4, IL-5 and IL-13 was detected in lung tissue of IL-15Rα ko mice after infection. Thus, IL-15 signalling appears to be important for induction of an IFN-γ response, the absence of which is associated with development of a Th2-type response to primary RV infection in mice.

A deficient lymphocytic response to RV-1B infection was observed in IL-15Rα ko mice, which was associated with an increased viral load in lung tissue at 8h and 24h after infection. Unlike wild type mice, due to the severe lack of NK cells there was no activated NK cell response to RV-1B infection in IL-15Rα ko mice. Moreover, IL-15Rα ko mice exhibited a lower frequency and absolute number of CD8+ T cells in the lungs at baseline, and a severely impaired CD8+ T cell response in both the airway and lungs was detected after RV infection. In contrast, the absence of IL-15 signalling did not substantially affect the CD4+ T cell response to RV.

In addition to the necessity for IL-15 signalling for the NK cell response demonstrated in Part 1 of Chapter 4, we show in Part 2 that IL-15 signalling is also important for the generation of an activated CD8+ T cell response to RV-1B infection in mice. These results also highlight the pleiotropic nature of IL-15 during virus infection, and how its absence can influence the Th1 vs Th2 balance of the antiviral immune response.
5. **Chapter 5: Results - IL-15 expression and the NK cell responses in the absence of type I IFN signalling**

5.1. **Introduction**

Most if not all nucleated cells are able to produce type I IFNs early after viral infection, a response which represents an early and central regulator of antiviral immune responses. Secreted type I IFNs bind a common heterodimeric receptor composed of type I IFN-α-receptor subunits 1 and 2 (IFNAR1 and IFNAR2), inducing a series of signalling pathways to mediate an antiviral state in infected and surrounding cells, in addition to activating key innate and adaptive immune response via induction of >300 ISGs. Type I IFNs are capable of inducing direct effects on cells to promote antiviral resistance, such as expression of OAS and PKR, and they also stimulate expression of various cytokines and chemokines that act as immunomodulators to direct the antiviral response.

In chapter 3, we demonstrated expression of IL-15 in BAL cells and lung tissue in response to RV infection, which was associated with expression of type I IFNs. Previous reports have demonstrated induction of IL-15 upon *in vitro* stimulation of macrophages, DCs and epithelial cells with IFN-β [118, 256]. Therefore, in this chapter we employed the use of IFNAR1 ko mice to examine the role of type I IFN signalling on RV-induced IL-15 *in vivo*.

The importance of type I IFNs for activation of NK cell responses has been well documented for viral infections including vaccinia virus, herpes viruses and HIV however, the role of type I IFNs on the NK cell response during RV infection is unknown. Additionally, whether type I IFNs regulate NK cell activity via direct or indirect pathways is unclear. As IL-15 has been shown to be induced by type I IFNs and regulates NK cell activities, we suggest that IL-15 is the main intermediary factor that mediates the effects of type I IFNs on NK cells. Therefore in this chapter, we aim to explore the relationships between type I IFNs, IL-15 and NK cells during the immune response to RV infection, and their importance for control of virus replication/infection.
5.2. Hypotheses & Aims

5.2.1. Hypotheses

i. Type I IFN signalling is necessary for induction of IL-15 in the lungs during RV-1B infection.

ii. Type I IFN signalling is necessary for the recruitment/expansion and activation of NK cells in the airway and lungs during RV-1B infection.

iii. A deficient NK cell response results in deficient IFN-γ production, which is associated with increased viral load and development of a Th2 response to virus.

5.2.2. Aims

To investigate the dependency of IL-15 expression and the NK cell response on type I IFN signalling during RV-1B infection, by using type I IFN receptor knockout mice (IFNAR1 ko).

i. To determine the virus load in the lungs using qPCR.

ii. To determine gene expression levels of IL-15 in the lungs using qPCR.

iii. To determine expression of IFNs, ISGs, proinflammatory cytokines and chemokines and Th2-associated mediators using qPCR and ELISAs.

iv. To examine the magnitude, kinetics and activation status of the NK cell and acquired T cell responses in the airway and lungs using flow cytometry.
5.3. Results

5.3.1. Virus Load

IFNAR1 ko and wild type C57BL/6 mice were infected i.n. with RV-1B (5x10^6 TCID₅₀) or PBS (0h baseline). qPCR analysis of lung homogenate demonstrated a significantly higher virus load in the lungs of IFNAR1 ko mice at 8h p.i. with RV-1B compared to C57BL/6 (p<0.001) (Fig 5.1). vRNA levels declined thereafter in IFNAR1 ko mice and no significant difference was observed between the groups at 24h and 48h p.i. Thus, we found that absence of type I IFN signalling does affect the rate of virus replication and/or clearance from the lungs early after infection.

Figure 5.1 IFNAR1 ko mice demonstrate an increased lung viral load after RV-1B infection.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h), and lungs were harvested at the indicated timepoints p.i. Lungs were processed for RNA extraction and cDNA made by reverse transcription as described. RV-1B viral RNA copies were quantified by qPCR and data was normalised against 18s rRNA expression and presented as viral RNA copies/µl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C57BL/6 mice treated with RV-1B, at the indicated times (***p<0.001). Only statistically significant differences are shown.

5.3.2. Differential BAL Cell Counts

Cytospin assays using BAL cells demonstrated the degree of leukocyte inflammation in the airway in the absence of type I IFN signalling during RV-1B infection. Firstly, no differences in the total number of BAL cells between IFNAR1 ko and C57BL/6 mice were observed during the timecourse (Fig 5.2a). However, neutrophilia was significantly greater in IFNAR1 ko mice, in which neutrophils comprised ~60% and ~55% of total BAL cells at 24h and 48h p.i. respectively, compared to ~40% and ~25% in C57BL/6 mice (Fig 5.2 b&c).
In contrast, the lymphocyte response in the airway was severely deficient in IFNAR1 ko mice. At 48h p.i. the percentage of total BAL lymphocytes in IFNAR1 ko mice was ~5% compared to ~25% in C57BL/6 mice, and this deficiency was similarly translated into lymphocyte numbers (Fig 5.2 d&e). We observed the peak of lymphocytes at 48h p.i., our final timepoint, though it is likely that the percentage/number may increase between d2-7 p.i. as seen with BALB/c mice (chapter 3).

**Figure 5.2 Differential BAL cell counts during RV-1B infection of IFNAR1 ko mice.**

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h) and BAL was performed at the indicated timepoints p.i. Recovered live BAL cells were counted (a) and processed for cytospin slides. Slides were blinded to experimental conditions and cells differentially counted to give the percentage and total number (b&c) neutrophils and (d&e) lymphocytes in the airway. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C67BL/6 mice treated with RV-1B, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
5.3.3. BAL Proinflammatory Mediators

To determine if absent type I IFN signalling during RV-1B infection affected production of proinflammatory mediators, we analysed the BAL fluid by ELISA. Firstly, significantly higher levels of KC were produced at 8h p.i. in IFNAR1 ko compared to C57BL/6 mice (p<0.001), after which levels rapidly decreased in both groups (Fig 5.3a). Equal quantities of IL-6 were produced in both groups at 8h p.i. and though levels declined thereafter, the rate was much quicker in IFNAR1 ko mice (Fig 5.3b). No significant differences in expression of IL-12 was detected between both groups (Fig 5.3c). Conversely, deficient production of RANTES, IL-1β and TNF-α was observed in IFNAR1 ko mice compared to C57BL/6 mice at the peak of expression at 24h p.i. (p<0.001 for RANTES; p<0.05 for IL-1β and TNF-α) (Fig 5.3 d, e &f).

5.3.4. Expression of Type I and III Interferons

Amplification of IFN-α/β production occurs via autocrine and paracrine signalling through the type I IFN receptor, thus we measured expression to confirm deficient signalling. No BAL IFN-α protein was detected in IFNAR1 ko mice in contrast to C57BL/6 mice which demonstrated increasing levels between 8-48h p.i. with RV-1B (Fig 5.4a). Similarly, expression of IFN-β mRNA in the lungs was detected only in C57BL/6 mice at 8h p.i. with RV-1B, followed by a peak of protein in the BAL fluid at 24h (Fig. 5.4 b&c).

Additionally, levels of IFN-λ2/3 mRNA in the lungs were ~2-fold higher in C57BL/6 mice at 8h p.i. with RV-1B compared to IFNAR1 ko mice (p<0.05), and elevated mRNA levels persisted in C57BL/6 mice between 8-48h; levels had diminished rapidly in IFNAR1 ko mice by 24h p.i. (Fig 5.4d). Thus, production of type I and III IFNs in IFNAR1 ko mice was deficient in response to RV-1B infection.
Figure 5.3 BAL cytokines and chemokines produced in response to RV-1B infection in IFNAR1 ko mice.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h) and BAL was performed at the indicated timepoints p.i. Protein levels of (a) KC, (b) IL-6, (c) RANTES, (d) IL-12, (e) IL-1β and (f) TNF-α were analysed by ELISA in BAL fluid and expressed as pg/ml. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C57BL/6 mice treated with RV-1B, at the indicated times (**p<0.01, ***p<0.001, *p<0.05). Only statistically significant differences are shown.
IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h), and BAL was performed and lungs harvested at the indicated timepoints p.i. Protein levels of (a) IFN-α and (c) IFN-β in BAL fluid were analysed by ELISA and expressed as pg/ml. Lung tissue was processed for RNA extraction and cDNA made by reverse transcription as described, and the level of (b) IFN-β and (d) IFN-λ2/3 gene expression was quantified by qPCR. Data was normalised against 18s rRNA expression and presented as copies of mRNA/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C67BL/6 mice treated with RV-1B, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.

Figure 5.4 Expression of type I and III IFNs in response to RV-1B infection in IFNAR1 ko mice is deficient.
5.3.5. Expression of Interferon Stimulated Genes

To further confirm absent type I IFN signalling in IFNAR1 ko mice, we analysed expression of various ISGs. BAL IP-10 protein was observed by ELISA at 24h p.i. with RV-1B in C57BL/6 but not IFNAR1 ko mice (p<0.001) (Fig 5.5a). Likewise, upregulation of OAS-1a, Viperin and PKR mRNA in the lungs was detected by qPCR in C57BL/6 but not IFNAR1 ko mice from 8h p.i. with RV-1B (Fig 5.5 b-d).

![Graphs showing expression of IP-10, OAS, Viperin, and PKR mRNA in IFNAR1 ko and C57BL/6 mice.](image)

Figure 5.5 Expression of interferon stimulated genes in response to RV-1B infection in IFNAR1 ko mice is deficient.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h), and BAL was performed and lungs harvested at the indicated timepoints p.i. Protein levels of (a) IP-10 in BAL fluid was analysed by ELISA and expressed as pg/ml. Lung tissue was processed for RNA extraction and cDNA made by reverse transcription as described, and the level of gene expression of (b) Viperin, (c) OAS and (d) PKR were quantified by qPCR. Data was normalised against 18s rRNA expression and presented as copies of mRNA/μl of sample. Data was expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C67BL/6 mice treated with RV-1B, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
5.3.6. Expression of IL-15 and IL-15Rα

We analysed expression of IL-15 by qPCR to determine if its production is dependent on IFN-α/β signalling during RV-1B infection. Agreeably, we found deficient mRNA expression in IFNAR1 ko mice compared to C57BL/6 mice, which demonstrated a peak of IL-15 mRNA in the lungs at 8h p.i. with RV-1B (Fig 5.6a). IL-15Rα mRNA was upregulated in both groups at 8h p.i., though levels were ~2-fold higher in C57BL/6 compared to IFNAR1 ko mice (Fig 5.6b).

![Figure 5.6](image)

**Figure 5.6 Expression of IL-15 and IL-15Rα in response to RV-1B infection in IFNAR1 ko mice is deficient.**

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h) and lungs harvested at the indicated timepoints p.i. Lung tissue was processed for RNA extraction and cDNA made by reverse transcription as described, and the level of gene expression of (a) IL-15 and (b) IL-15Rα were quantified by qPCR. Data was normalised against 18s rRNA expression and presented as copies of mRNA/μL of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C67BL/6 mice treated with RV-1B, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.

5.3.7. Expression of IFN-γ

We also analysed total IFN-γ mRNA expression in the lungs by qPCR. We found the peak of expression at 8h p.i. with RV-1B in both groups, though the quantity in C57BL/6 mice was ~2-fold higher than in IFNAR1 ko mice (p<0.001) (Fig 5.7). By 24h and 48h p.i., levels had declined though remained higher in C57BL/6 compared to IFNAR1 ko mice (ns). Lymphocyte specific expression of IFN-γ in the airway and lungs is presented in sections 5.3.9 and 5.3.10.
Figure 5.7 Expression of IFN-γ in response to RV-1B infection in IFNAR1 ko mice is deficient.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h) and lungs harvested at the indicated timepoints p.i. Lung tissue was processed for RNA extraction and cDNA made by reverse transcription as described, and the level of IFN-γ gene expression was quantified by qPCR. Data was normalised against 18s rRNA expression and presented as copies of mRNA/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C67BL/6 mice treated with RV-1B, at the indicated times (***p<0.001). Only statistically significant differences are shown.

5.3.8. Expression of Th2 Mediators

Production of Th2 cytokines and chemokines in the airway and lungs is characteristic of allergy and asthma. IFN-γ strongly potentiates development of Th1-mediated responses however, we suggest that its deficiency in IFNAR1 ko mice may allow development of a Th2 response to RV-1B infection. Firstly, MDC and TARC in BAL fluid were measured by ELISA. Both groups of mice upregulated these proteins in response to RV-1B infection, though levels were significantly higher in IFNAR1 ko mice at 8h p.i. for MDC, and at 24h and 48h p.i. for TARC compared to C57BL/6 mice (Fig 5.8 a&b).

Secondly, expression of the classical Th2 cytokines IL-4, IL-5 and IL-13 in the lungs were analysed by qPCR. A sharp peak of IL-4 and IL-5 mRNA expression was detected in IFNAR1 ko mice compared to C57BL/6 mice at 8h p.i. (p<0.001), after which levels rapidly declined (Fig 5.8 c&d); however, mRNA copy numbers detected at the peak of expression were low, particularly for IL-5. No differences in expression of IL-13 were detected between IFNAR1 ko and C57BL/6 mice with RV-1B infection. Overall, in the absence of type I IFN signalling, this data suggests the development of a weak Th2 response to RV-1B infection as observed in IFNAR1 ko mice.
Figure 5.8 IFNAR1 ko mice demonstrate increased expression of Th2-associated mediators in response to RV-1B infection.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h) and BAL was performed and lungs harvested at the indicated timepoints p.i. Protein levels of (a) MDC and (b) TARC in the BAL fluid were analysed by ELISA and expressed as pg/ml. Lung tissue was processed for RNA extraction and cDNA made by reverse transcription as described, and the level of gene expression of (c) IL-4, (d) IL-5 and (e) IL-13 were quantified by qPCR. Data was normalised against 18s rRNA expression and presented as copies of mRNA/μl of sample. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C67BL/6 mice treated with RV-1B, at the indicated times (**p<0.01 & ***p<0.001). Only statistically significant differences are shown.
5.3.9. NK Cell Response

No previous studies have addressed the role of type I IFNs on the NK cell response during RV infection, thus we sought to investigate this in IFNAR1 ko mice. To determine the kinetics of NK cell recruitment/expansion and activation in the airway and lungs, leukocytes were stained with monoclonal antibodies specific for CD3, NK1.1, CD69, GranzymeB and IFN-γ and analysed by flow cytometry; NK cells were defined as CD3-/NK1.1+ within the lymphocyte gate.

5.3.9.1. BAL NK Cells

In response to RV-1B infection, there was a significantly deficient airway NK cell response in IFNAR1 ko mice, as demonstrated by the ~8-fold lower number of NK cells observed compared to C57BL/6 mice at 48h p.i. (p<0.001) (Fig. 5.9a). Consequently, almost no CD69+, GranzymeB+ and IFN-γ+ NK cells were observed in the airway of IFNAR1 ko mice compared to C57BL/6 mice, in which numbers of NK cells expressing these activation markers peaked at 48h p.i. (p<0.001) (Figure 5.9b-d). Figure 5.10 illustrates the deficient BAL NK cell response in IFNAR1 ko mice by way of representative flow plots.

5.3.9.2. Lung NK Cells

No differences were detected in the number of total lung cells between the groups at any time point (Fig 5.10a). Thus, as no significant differences were observed in the percentage of CD3-/NK1.1+ NK cells between the groups at any time point (data not shown), the total number of NK cells detected in the lungs was similar in both IFNAR1 ko and C57BL/6 mice all time points, and with respect to baseline levels (0h) both groups demonstrated an increase in numbers of NK cells at 48h p.i. (Fig 5.10b).

Despite similar numbers of lung NK cells, those observed in IFNAR1 ko mice showed deficient expression of activation markers. IFNAR1 ko mice did not upregulate CD69 significantly, unlike C57BL/6 mice which demonstrated an incremental increase in the number of CD69+ NK cells between 0h-48h p.i. with RV-1B (p<0.001) (Fig 5.11c). Intracellular staining demonstrated deficient numbers of GranzymeB+ NK cells in response to RV-1B infection in IFNAR1 ko mice, as numbers were greater in C57BL/6 compared to IFNAR1 ko mice at both 24h and 48h p.i. (p<0.01) (Fig 5.11d). Similarly, the absolute number of IFN-γ+ NK cells was greater in C57BL/6 compared to IFNAR1 ko mice at 24h p.i. (p<0.01) (Fig 5.11e). Figure 5.12 illustrates these differences between the groups as representative flow plots of lung NK cells at 48h p.i., demonstrating that in IFNAR1 ko compared to C57BL/6 mice, ~<5%
vs ~35% of lung NK cells were CD69+, ~20% vs ~35% were GranzymeB+ and ~20% vs ~35% were IFN-γ+.

Figure 5.9 The BAL NK cell response to RV-1B infection in IFNAR1 ko mice is deficient.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h) and BAL was performed at the indicated timepoints p.i. 1x10^5 BAL cells were stained for surface markers CD3, CD69 and NK1.1, intracellular cytokines IFN-γ and GranzymeB and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data is presented as the absolute number of (a) CD3-/NK1.1+ NK cells and CD3- gated double positive (b) NK1.1+/CD69+, (c) NK1.1+/GranzymeB+ and (d) NK1.1+/IFN-γ+ NK cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C67BL/6 mice treated with RV-1B, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
Figure 5.10 Representative flow plots demonstrating deficient BAL NK cell responses at 48h after RV-1B in IFNAR1 ko mice.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B and BAL was performed at 48h p.i. 1x10^5 BAL cells were stained for surface markers CD3, CD69 and NK1.1, intracellular cytokines IFN-γ and GranzymeB and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data is presented as representative flow plots illustrating (a&b) CD3-/NK1.1+ NK cells and CD3- gated
double positive (c&d) NK1.1+/CD69+, (e&f) NK1.1+/ GranzymeB+ and (g&h) NK1.1+/ IFN-γ+ NK cells from IFNAR1 ko and C57BL/6 mice. Data are representative of at least 2 independent experiments. Numbers indicated on plots represent the % of events falling within the indicated gate.

Figure 5.11 The lung NK cell response to RV-1B infection in IFNAR1 ko mice is deficient.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h) and the left lung lobe excised after BAL was performed at the indicated timepoints p.i. Lungs were digested as described and the resultant single-cell suspension was counted to give (a) the total live cell number. 1x10^6 cells were stained for surface markers CD3, CD69 and NK1.1, intracellular cytokines IFN-γ and GranzymeB and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the absolute number of (b) CD3-/NK1.1+ NK cells, and CD3- gated double positive (c) NK1.1+/CD69+, (d) NK1.1+/GranzymeB+ and (e) NK1.1+/IFN-γ+ NK cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C67BL/6 mice treated with RV-1B, at the indicated times (**p<0.01 & ***p<0.001). Only statistically significant differences are shown.
Figure 5.12 Representative flow plots demonstrating deficient lung NK cell responses at 48h after RV-1B infection in IFNAR1 ko mice.

IFNAR1 ko and control C57BL/6 mice were treated i.n. with RV-1B and the left lung lobe excised after BAL was performed at 48h p.i. Lungs were digested as described. 1x10^6 cells were stained for surface markers CD3, CD69 and NK1.1, intracellular cytokines IFN-γ and GranzymeB and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data is presented as representative flow plots illustrating (a&b) CD3-/NK1.1+ NK cells and CD3- gated double positive (c&d)
NK1.1+/CD69+, (e&f) NK1.1+/GranzymeB+ and (g&h) NK1.1+/IFN-γ+ NK cells from IFNAR1 ko and C57BL/6 mice. Data are representative of at least 2 independent experiments. Numbers indicated on plots represent the % of events falling within the indicated gate. Only statistically significant differences are shown.

5.3.10. T Cell Response

5.3.10.1. BAL T Cells

Both IFNAR1 ko and C57BL/6 mice demonstrated an equal increase of ~5-fold in the total numbers of airway CD4+ T cell at 48h p.i. with RV-1B compared to baseline (0h) levels (Fig 5.13a). However, the recruited/expanded CD4+ T cells in IFNAR1 ko mice were unable to upregulate CD69 and IFN-γ to the same extent as C57BL/6 mice; C57BL/6 mice demonstrated ~3-fold more CD69+ CD4+ T cells (p<0.001) and ~2-fold more IFN-γ+ CD4+ T cells (p<0.001) in the airway at 48h p.i. compared to IFNAR1 ko mice (Fig 5.13 c&e).

On the other hand, during RV-1B infection we observed almost no recruitment/expansion of CD8+ T cells into the airway of IFNAR1 ko compared to C57BL/6 mice, which demonstrated a sharp increase in numbers at 48h p.i. (p<0.001) (Fig 5.13b). Consequently, there was a complete deficiency of CD69+ (p<0.001) and IFN-γ+ (p<0.001) CD8+ T cells in the airway of IFNAR1 ko mice compared to C57BL/6 mice at 48h p.i. (Fig 5.13d&f).

5.3.10.2. Lung T Cells

Similar to the airway, both IFNAR1 ko and C57BL/6 mice demonstrated similar numbers of lung CD4+ T cells at all time points investigated (Fig 5.14a). However, lung CD4+ T cells in IFNAR1 ko mice demonstrated deficient upregulation of CD69 and IFN-γ compared to C57BL/6 mice at 48h p.i. (p<0.01) (Fig 5.14 c&e).

The absolute number of lung CD8+ T cells was similar at 0h and 24h p.i. in both groups however, there was deficient recruitment/expansion of CD8+ T cells in IFNAR1 ko compared to C57BL/6 mice at 48h p.i. (p<0.05) (Fig 5.14b). Furthermore, the CD8+ T cells in the lungs of IFNAR1 ko mice failed to upregulate expression of CD69 and IFN-γ in response to RV-1B, unlike C57BL/6 mice at 48h p.i. (Fig 5.14 d&f). Thus, in both the airway and lungs, in the absence of type I IFN signalling during RV-1B infection, there is deficient activation of CD4+ and CD8+ T cells.
Figure 5.13 BAL CD4 and CD8 T cell response during RV-1B infection of IFNAR1 ko mice.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h) and BAL was performed at the indicated timepoints p.i. 1x10^5 BAL cells were stained for surface markers CD3, CD4, CD8 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data is presented as the absolute number of (a) CD3+/CD4+, CD3+ gated (b) CD4+/CD69+, (c) CD4+/IFN-γ+, (d) CD3+/CD8+, CD3+ gated (e) CD8+/CD69+ and (f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C67BL/6 mice treated with RV-1B, at the indicated times (***p<0.001 & *p<0.05). Only statistically significant differences are shown.
Figure 5.14 Lung CD4 and CD8 T cell response during RV-1B infection of IFNAR1 ko mice.

IFNAR1 ko and C57BL/6 mice were treated i.n. with RV-1B or PBS (0h) and the left lung lobe excised after BAL was performed at the indicated timepoints p.i. Lungs were digested as described and 1x10^6 cells were stained for surface markers CD3, CD4, CD8 and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data is presented as the absolute number of (a) CD3+/CD4+, CD3+ gated (b) CD4+/CD69+, (c) CD4+/IFN-γ+, (d) CD3+/CD8+, CD3+ gated (e) CD8+/CD69+ and (f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4-6 mice per group and is representative of at least 2 independent experiments. Statistical significance is for IFNAR1 ko compared with C57BL/6 mice treated with RV-1B, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.
5.4. **Chapter Summary**

In the absence of type I IFN signalling in IFNAR1 ko mice, we observed an enhanced neutrophilic response to RV-1B infection compared to C57BL/6 mice. IFNAR1 ko mice had increased levels of IL-6, similar levels of IL-12 and reduced levels of RANTES and TNF-α compared to C57BL/6 mice. Moreover, we observed deficient upregulation of IL-15, IL-15Rα and IFN-γ mRNA in lung tissue at 8h p.i. in IFNAR1 ko mice. In association, expression of Th2 associated chemokines and cytokines MDC, TARC, IL-4 and IL-5 were increased in the absence of type I IFN signalling during RV infection. Deficient expression of the ISGs IP-10, PKR, OAS and Viperin observed confirmed the lack of type I IFN signalling in IFNAR1 ko mice.

The BAL NK cell response was absent in IFNAR1 ko mice compared to C57BL/6 mice, and the latter demonstrated accumulation in the airway and activation at 24h and 48h p.i. In the lungs, although IFNAR1 ko mice had similar total numbers of NK cells at the time points investigated, the NK cells failed to upregulate CD69 expression and demonstrated deficient upregulation of intracellular GranzymeB and IFN-γ compared to C57BL/6 after infection. Thus, in the absence of type I IFN signalling, the NK cell response to RV-1B infection is impaired. These results were associated with an increased virus load at 8h p.i. with RV in the absence of type I IFN signalling, suggesting that type I IFNs are important for the early control of virus infection/replication.

In both the airway and lung tissue, CD4+ T cells were recruited to a similar extent in IFNAR1 ko and control mice, but deficient upregulation of CD69 and IFN-γ expression in CD4+ T was observed in IFNAR1 ko mice after infection. In contrast, the CD8+ T cells response was absent in IFNAR1 ko mice compared to C57BL/6 mice, which demonstrated recruitment/accumulation and activation in to the airway and lungs at 48h p.i.

These data show that IL-15 is an ISG in the mouse model of RV infection, and type I IFN signalling is critical for the NK cell and CD8+ T cell response. Association of these deficiencies in the absence of type I IFN signalling with an increased lung viral load suggests that these responses are important components of antiviral defence against RV infection.
Chapter 6: Results – IL-15 expression and the NK cell response during RV-induced asthma exacerbation

6.1. Introduction

Asthma is a disease characterised by chronic airway inflammation with episodes of variable and reversible airway obstruction. Respiratory virus infections are the primary cause of asthma exacerbations, significantly contributing to asthma morbidity, mortality and health care costs. RVs are the most common etiological agents of exacerbations and the mechanisms by which RVs mediate asthma exacerbations are unclear. Therefore, further research is necessary to explore more targeted therapeutic options to prevent and/or treat virus-induced asthma exacerbations.

We have established from the previous chapters using mouse models that RV infection upregulates IL-15 expression in the airway and lungs, which is dependent on type I IFN signalling. We showed that the ensuing NK cell response, which is an important source of early IFN-γ after RV infection, is dependent on IL-15. These responses are associated with the decline of virus load in the lungs. However, the pre-existence of a dominant Th2 environment in the lungs of asthmatics results in a somewhat altered immune response to RV infection compared to healthy individuals [27].

Asthmatic bronchial epithelial cells (BECs) infected with RV ex vivo demonstrated deficient IFN-β production, which was associated with increased viral replication and deficient induction of apoptosis compared to BECs from healthy individuals [108]. In addition, IL-15 levels in the BAL fluid of asthmatics has been shown to be lower than that found in non-asthmatics at baseline, and RV infection of BAL macrophages from asthmatics demonstrated deficient induction of IL-15; these results inversely correlated with AHR and virus load during in vivo human experimental RV infection [256]. Furthermore, immunohistochemistry data from bronchial biopsies has illustrated deficient IL-15 upregulation in asthmatics infected with RV compared to non-asthmatics (data not published – biopsies taken from Message et al. [27] study). Therefore, we suggest that deficient IFN-β expression in response to RV infection in asthmatics impairs the IL-15-NK cell antiviral pathway, resulting in increased virus load and prolonged infection due to impaired innate antiviral immunity. It is possible that this mechanism contributes to the development of RV-induced asthma exacerbations.

In this chapter, we have employed a mouse model of RV-induced allergic airways inflammation to specifically investigate IL-15 expression and the NK cell response, to gain further knowledge of the contribution of this pathway to asthma exacerbation pathogenesis.
6.2. Hypotheses & Aims

6.2.1. Hypotheses

i. RV-induced IL-15 is deficient in OVA sensitized and challenged mice.

ii. Deficient IL-15 signalling results in a deficient NK cell response to RV infection in OVA sensitized and challenged mice, resulting in increased virus load.

6.2.2. Aims

To investigate the interaction between allergic inflammation and RV infection and the effect this has on IL-15 expression and NK cell resposens.

To compare the following responses in allergen challenged and non-challenged mice:

i. To determine virus load in the lungs using qPCR.

ii. To determine expression of IL-15, IL-15Rα, Th2 cytokines, IFN-γ and IP-10 in the airway and lungs using qPCR and ELISAs.

iii. To determine the magnitude, kinetics and activation status of the NK cell response in the airway and lungs using flow cytometry.

vi. To examine the acquired T cell response in the airway and lungs using flow cytometry.
6.3. Results
In this chapter, all mice were treated as described in Section 2.33/Figure 2.2. The nomenclature for the treatment groups for this model are summarised again in Table 6.1 below.

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Sensitization (Day -13) OVA/Alum i.p.</th>
<th>Challenges (Days -2, -1 &amp; 0) OVA or PBS i.n.</th>
<th>RV-1B Infection (Day 0) RV-1B or UV-RV-1B i.n.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV-OVA</td>
<td>YES</td>
<td>OVA</td>
<td>RV-1B</td>
</tr>
<tr>
<td>UV-OVA</td>
<td>YES</td>
<td>OVA</td>
<td>UV-RV-1B</td>
</tr>
<tr>
<td>RV-PBS</td>
<td>YES</td>
<td>PBS</td>
<td>RV-1B</td>
</tr>
<tr>
<td>UV-PBS</td>
<td>YES</td>
<td>PBS</td>
<td>UV-RV-1B</td>
</tr>
</tbody>
</table>

Table 6.1 Experimental groups of the RV-induced asthma exacerbation model, and the breakdown of treatments received by mice in each group.

6.3.1. Virus Load
Increased RV replication has been observed in ex vivo infected asthmatic bronchial epithelial cells in comparison to non-asthmatic cells [109], and it is suggested that deficient type I and III IFN production as well as a skewed Th2 response to virus infection are potential causes [27, 108, 109]. Thus, we analysed the virus load in the lungs by qPCR to determine the effect of allergic airways inflammation on RV replication/infection efficiency in the mouse model. We observed ~2-fold more RV viral RNA in RV-OVA compared to RV-PBS treated mice at d1 p.i. (p<0.001), after which levels rapidly declined in both groups.
### Figure 6.1

RV-1B infected mice with allergic airways inflammation demonstrated an increased virus load compared to non-allergic mice.

<table>
<thead>
<tr>
<th>Days Post Infection</th>
<th>RV-OVA</th>
<th>UV-OVA</th>
<th>RV-PBS</th>
<th>UV-PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.0×10^3</td>
<td>***</td>
<td>1.0×10^3</td>
<td>***</td>
</tr>
<tr>
<td>2</td>
<td>4.0×10^3</td>
<td></td>
<td>2.0×10^3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.0×10^3</td>
<td></td>
<td>3.0×10^3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2.0×10^3</td>
<td></td>
<td>4.0×10^3</td>
<td></td>
</tr>
</tbody>
</table>

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. Lungs were harvested at the indicated time points p.i., processed for RNA extraction and cDNA made by reverse transcription as described. RV-1B viral RNA copies were quantified by qPCR, data was normalised against 18s rRNA expression and presented as viral RNA copies/μl of sample. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001). Only statistically significant differences are shown.

### 6.3.2. Differential BAL Cell Counts

Airway cellular inflammation was initially determined by differential staining of BAL cell cytospins. Firstly, BAL cell counts demonstrated significantly greater numbers of cells in the airway in OVA sensitized and challenged mice regardless of infection at all time points compare to PBS challenged mice (Fig 6.2a). In agreement with published data by Bartlett et al. [255] we found enhanced neutrophilia in RV infected OVA sensitized and challenged mice at d1 p.i. (p<0.001) (Fig 6.2b), at which neutrophils comprised ~60% of the total BAL cells in RV-OVA treated mice, compared to ~30% in RV-PBS treated mice (data not shown). Eosinophilia was seen only in OVA-challenged mice, and no significant differences in numbers were detected between RV-OVA and UV-OVA treated mice (Fig 6.2c). Numbers of lymphocytes were hugely elevated in OVA compared to PBS-challenged mice, demonstrating more than a 30-fold increase in numbers as early as d1 post-challenge, which increased between d2-7 (Fig 6.2d). However, no differences in the number of BAL lymphocytes were seen between RV-OVA and UV-OVA treated mice.
Figure 6.2 RV-1B infected mice with allergic airways inflammation demonstrated enhanced airway neutrophilia compared to non-allergic mice.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. BAL was performed at the indicated time points p.i. and recovered live BAL cells were (a) counted and processed for cytospin slides. Slides were blinded to experimental conditions and cells differentially counted to give the total number of (b) neutrophils, (c) eosinophils and (d) lymphocytes in the airway. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.

6.3.3. Expression of Th2 Mediators

IL-4, IL-5 and IL-13 are the classical Th2 cytokines known to be associated with atopic asthmatic airway inflammation. Secretion of these cytokines into the airway during the RV-induced asthma exacerbation mouse model was previously demonstrated (and confirmed in these studies – data not shown), with enhanced levels of IL-4 and IL-5 detected in the BAL fluid of RV-OVA compared to UV-OVA treated mice at d1 p.i. [255]. Here, we confirmed increased expression of these Th2 cytokines through detection in lung homogenate by ELISA. Higher levels of IL-4, IL-5 and IL-13 were found in the lung tissue of RV-OVA compared to UV-OVA treated mice at d1 p.i. (p<0.001 for IL-4 and IL-5; p<0.01 for IL-13) (Fig6.3 a-c), and though the same trend was observed at d2, it was not statistically significant. Levels of IL-4...
and IL-13 declined after d2 post challenge with OVA however, IL-5 remained detectable in the lungs as late as d7 post challenge.

Figure 6.3 Expression of lung Th2 cytokines during an RV-induced allergic exacerbation model.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. Lungs were harvested after BAL was performed at the indicated time points p.i. Lungs were processed for protein detection as described, and the concentration of (a) IL-4, (b) IL-5 and (c) IL-13 was determined by ELISA and presented as pg/ml. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.

6.3.4. Airway Hyperreactivity

Expression of the above Th2 cytokines has previously been associated with increased airway hyperreactivity (AHR) in both human disease and mouse models of RV-induced asthma exacerbation. In human disease models, exacerbation is measured in terms of decreased lung function (changes in FEV₁ and PEF) in addition to increased bronchial hyperreactivity to methacholine or histamine challenges [27, 95]. Here, we used whole body plethysmography at d1 post challenge/infection to confirm exacerbation; AHR was measured in response to
increasing doses of aerosolised methacholine via detecting changes in PenH and airways resistance. We confirmed published findings [255] demonstrating enhanced PenH in RV-OVA compared to UV-OVA treated mice upon challenge with 10 and 30mg/ml methacholine (p<0.05 & p<0.01 respectively), and PBS challenged mice demonstrated no response to any dose of methacholine tested regardless of infection (Fig 6.4). Thus, RV-1B infection resulted in enhanced AHR in mice with allergic airways inflammation.

Figure 6.4 RV-1B infection in allergic mice enhanced airway hyperreactivity.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. At d1 post-challenge/infection, PenH was measured in response to challenge with increasing doses of aerosolised methacholine. PenH is expressed as area under the curve over a 5 minute log period post challenge. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between RV-OVA and UV-OVA treated mice, at the indicated concentrations of methacholine treatment (***p<0.01 & *p<0.05). Only statistically significant differences are shown.

6.3.5. Expression of IL-15 and IL-15Rα

Deficient upregulation of IL-15 and IL-15Rα in asthmatics has been observed in lung biopsies after experimental RV infection compared to non-asthmatic subjects [256](Laza-Stanca, 2011). Thus, we investigated expression in BAL cells and lung tissue in the mouse model of RV-induced asthma exacerbation via qPCR and ELISA. Contrary to human studies, we found significantly enhanced IL-15 and IL-15Rα mRNA expression in lung tissue at d1 p.i. in RV-OVA compared to UV-OVA, RV-PBS and UV-PBS treated mice (p<0.001 for IL-15; p<0.05 for IL-15Rα) (Fig 6.5 a&b). However, protein detection in lung homogenate demonstrated
enhanced expression of IL-15 and sIL-15/IL-15Rα complexes in both RV-OVA and UV-OVA treated mice compared to PBS challenged mice at d1 and d2 p.i., and though a higher level of protein was detected in RV-OVA compared to UV-OVA treated mice, the differences were not statistically significant (Fig 6.5 e&f). This suggests a degree of OVA-specific induction of IL-15 and IL-15Rα protein in lung tissue.

Chapter 3 demonstrated significant induction of IL-15 expression in BAL cells with RV-1B infection however, here we demonstrate enhanced levels of IL-15 and IL-15Rα mRNA expression in BAL cells in RV-OVA treated mice at 10h and d1 p.i. compared to RV-PBS treated mice (Fig 5.5 c&d). Additionally, no induction was detected in UV-RV-1B infected mice, regardless of OVA challenge.

6.3.6. Expression of IFN-γ

Previous chapters demonstrated the importance of IFN-γ production for the immune response against RV infection and its association with IL-15 signalling and the NK cell response. Thus, we analysed its expression in lung tissue by qPCR to determine if allergic inflammation affects its expression in response to RV-1B infection. We found ~3-fold more IFN-γ mRNA in the lungs of RV-OVA compared to RV-PBS treated mice at d1 p.i. (p<0.001), with levels equalizing between the two groups at d2 (Fig 6.6a). Additionally, UV-OVA treated mice also had elevated levels of IFN-γ mRNA compared to RV-PBS treated mice at d1 p.i. (p<0.05), however the trend had reversed by d2 p.i. as levels increased in RV-PBS treated mice (p<0.05). By d4 p.i., levels had declined to baseline in all groups except RV-PBS treated mice, though this was not significant.

Additionally, IP-10 is an IFN-γ inducible protein and an NK cell activating chemokine, therefore we analysed its secretion in to the airway by ELISA to confirm presence of IFN-γ signalling. Consistent with IFN-γ expression, we observed a sharp peak of IP-10 protein in RV-OVA treated mice at d1 p.i. (p<0.001) that had diminished by d2, at which we then detected a smaller peak of IP-10 in RV-PBS treated mice (Fig 6.6b).
Figure 6.5 Expression of RV-induced IL-15 in BAL cells and lung tissue was enhanced in mice with allergic airways inflammation compared to non-allergic mice.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. Lungs were harvested after BAL was performed at the indicated time points p.i. Lungs and BAL cells were processed for RNA extraction and cDNA made as described. (a&c) IL-15 and (b&d) IL-15Rα gene expression was quantified by qPCR in lung and BAL cells. Lungs were processed for protein detection as described, and the concentration of (e) IL-15 and (f) sIL-15/IL-15Rα complex was determined by ELISA and presented as pg/ml. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.
Figure 6.6 Expression of lung IFN-γ and BAL IP-10 during an RV-induced allergic exacerbation model.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. Lungs were harvested after BAL was performed at the indicated time points p.i. Lungs were processed for RNA extraction and cDNA made as described, and (a) IFN-γ gene expression was quantified by qPCR. Data was normalised against 18s rRNA expression and presented as mRNA copies/µl of sample. The concentration of (b) IP-10 in BAL fluid was determined by ELISA and presented as pg/ml. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.

6.3.7. NK Cell Response

No previous studies have characterised the BAL and lung NK cell response during RV-induced exacerbation in mice, and published data from human experimental RV-induced asthma exacerbations is elementary. Thus, we analysed the responses by flow cytometry after BAL and lung cells were stained for surface markers CD3, CD69 and NKp46, and intracellular cytokines IFN-γ, GranzymeB and IL-4.

6.3.7.1. BAL NK Cells

Contrary to our hypothesis, we observed a greatly enhanced NK cell response in RV-OVA compared to RV-PBS treated mice at d1 and d2 p.i. (p<0.001 ) with respect to both percentage and total NK cell numbers (Fig 6.7 a&b); at d1 NK cells comprised ~25% of the total BAL lymphocytes in RV-OVA treated mice, compared to <5% in RV-PBS treated mice (p<0.001), and levels had declined back to baseline on days 4 and 7. Additionally, elevated percentages and numbers of NK cells was detected in UV-OVA compared to RV-PBS treated mice at d1 and d2 p.i. suggesting the presence of an OVA-specific NK cell response; however, the percentage and number of NK cells in UV-OVA treated mice was still ~2-fold lower than in RV-OVA treated mice at both d1 and d2 p.i. (p<0.001).
Similarly, the percentage and number of CD69+ and GranzymeB+ BAL NK cells were elevated in OVA compared to PBS challenged mice, with significantly higher levels in RV-OVA compared to UV-OVA treated mice at d1 and d2 p.i. (Fig 6.7 c, d, g & h). However, only RV-OVA treated mice demonstrated a significant increase in number of IFN-γ+ BAL NK cells at d1 p.i. (p<0.001) (Fig 6.7). By d2 p.i., RV-OVA and RV-PBS treated mice had upregulated IFN-γ expression to an equal extent with respect to percentage of IFN-γ+ NK cells, though the total number was still significantly higher in RV-OVA treated mice (p<0.001) due to the much higher total BAL cell count (Figure 6.2a).
Figure 6.7 The BAL NK cell response to RV-1B infection was enhanced in mice with allergic airways inflammation compared to non-allergic mice.
BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3\textsuperscript{rd} OVA challenge on d0. BAL was performed at the indicated time points p.i. and 1x10\textsuperscript{5} cells were stained for surface markers CD3, CD69 and NKp46, intracellular cytokines IFN-\(\gamma\) and GranzymeB and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data is presented as the percentage and absolute number of (a&b) CD3-/NKp46+ NK cells, and CD3- gated double positive (c&d) NKp46+/CD69+, (e&f) NKp46+/GranzymeB+ and (g&h) NKp46+/IFN-\(\gamma\)+ NK cells. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.001, *p<0.01 & *p<0.05). Only statistically significant differences are shown.

6.3.7.2. Lung NK Cells

The total number of lung cells was greatest in mice treated with RV-OVA at both d1 and d2, demonstrating ~2-fold more cells than RV-PBS and UV-PBS treated mice at d1 (p<0.001) (Fig 6.8). Total numbers in UV-OVA treated mice was increased over RV-PBS (ns) and UV-PBS (p<0.05) treated groups at d1 p.i. however, numbers were similar between UV-OVA and RV-PBS treated mice at d2 p.i. The total number of lung cells had declined in OVA challenged mice by d4 p.i. resulting in similar numbers detected in all groups at d4 and d7 p.i.

The kinetics of NK cell recruitment/expansion in the lungs was similar to that in the airway. Percentage and number of NK cells was significantly increased in OVA compared to PBS challenged mice at d1 and d2 p.i., with ~2-fold more NK cells in RV-OVA compared to UV-OVA treated mice at d1 p.i. (p<0.001), after which levels declined but remained higher in RV-OVA treated mice at d2 (Fig 6.9 a&b).

Similarly, percentages and numbers of activated CD69+ and GranzymeB+ NK cells were elevated only in OVA challenged groups, with ~3-fold greater numbers in RV-OVA compared to UV-OVA treated mice at d1 and d2 p.i. (p<0.001), after which levels returned to baseline (Fig 6.9 c-f). However, a significant increase in total number of IFN-\(\gamma\)+ NK cells was only detected in RV-OVA treated mice at d1 p.i. (p<0.001), despite elevated percentages in UV-OVA treated mice at d1 and d2 p.i. (Fig 6.9g&h).
Figure 6.8 Total lung leukocyte counts during an RV-induced allergic exacerbation model.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. The left lung lobe was excised after BAL was performed at the indicated time points p.i. Lungs were digested as described, and the resultant single-cell suspension was counted to give the total number of live cells. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.
Figure 6.9 The Lung NK cell response to RV-1B infection was enhanced in mice with allergic airways inflammation compared to non-allergic mice.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0.
The left lung lobe was excised after BAL was performed at the indicated time points p.i., and lungs were digested as described. $1 \times 10^6$ cells were stained for surface markers CD3, CD69 and NKP46, intracellular cytokines IFN-γ and GranzymeB and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of (a&b) CD3−/NKP46+ NK cells, and CD3+ gated double positive (c&d) NKP46+/CD69+, (e&f) NKP46+/GranzymeB+ and (g&h) NKP46+/IFN-γ+ NK cells. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (***p<0.001, **p<0.01 & *p<0.05). Only statistically significant differences are shown.

6.3.7.3. IL-4 Expression in NK Cells

Previous reports have suggested that NK cells can develop a Th2 polarity, thus we investigated expression of IL-4 by BAL and lung NK cells during the RV-induced asthma exacerbation model. Unsorted lung and BAL leukocytes were stimulated with PMA, ionomycin and GolgiStop for 3h or left unstimulated, prior to staining with antibodies specific for CD3, NKP46 and intracellular IL-4 and analysed by flow cytometry.

IL-4+ NK cells were detected only with stimulation, and increases in total number were observed with OVA compared to PBS challenged mice in both BAL and lungs. Despite low numbers detected in the airway, both OVA-challenged groups had increased levels of IL-4+ NK cells at d2-7 p.i. however, significantly more IL-4+ NK cells were detected in RV-OVA compared to UV-OVA treated mice at d2 and d4 p.i. (p<0.01) (Fig 6.10a). In the lungs, only RV-OVA treated mice demonstrated an increase in IL-4+ NK cells at d1 and d2 p.i. (Fig 6.10b).

![Figure 6.10 BAL and lung NK cells expressed IL-4 in response to RV-1B infection in mice with allergic airways inflammation.](image)

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. The left lung lobe was excised after BAL was performed at the indicated time points p.i. $1 \times 10^5$ BAL
cells and 1x10^6 lung cells were stained for surface markers CD3 and NKp46, intracellular cytokine IL-4 and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the absolute number CD3+ gated double positive NKp46+/IL-4+ NK cells in the (a) airway and (b) lungs. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.

6.3.8. T Cell Response

6.3.8.1. BAL T Cells
In the airway, the total number of CD4+ T cells significantly increased in OVA compared to PBS challenged mice from d1 p.i. and peaked between d4-7, and no significant differences were detected between RV-OVA and UV-OVA treated mice at any time point (Fig 6.11b). A similar trend was observed for the number of BAL CD69+ CD4+ T cells (Fig 6.11d). In contrast, the percentage of IFN-γ+ CD4+ T cells was increased and ~2.5-fold greater in RV-PBS compared to RV-OVA and UV-OVA treated mice at d2-7 p.i. (p<0.001) (Fig 6.11c); however, due to the much higher total number of cells in the airway of RV-OVA treated mice (Fig 6.2a), the number of BAL IFN-γ+ CD4+ T cells were equally elevated in RV-PBS and RV-OVA treated mice at d4 p.i., and by d7 numbers had declined in RV-PBS compared to RV-OVA and UV-OVA treated mice (Fig 6.11f). Additionally, only RV-OVA treated mice demonstrated a significant increase in number of IL-4+ CD4+ T cells at d4 and d7 p.i. (p<0.001) (Fig 6.11h).

Likewise, the total number of BAL CD8+ T cells was also elevated in OVA compared to PBS challenged groups, and no significant differences were detected between RV-OVA and UV-OVA treated mice, in which numbers peaked at d2 p.i. and declined thereafter (Fig 6.12b). Numbers of CD69+ CD8+ T cells were significantly increased at d2 p.i. in OVA compared to PBS challenged groups, and in RV-OVA compared to UV-OVA treated mice (p<0.001) (Fig 6.12d). Lastly, the percentage of IFN-γ+ CD8+ T cells was greater in RV-OVA compared to RV-PBS treated mice on d1 p.i. and the relationship was reversed on d2 and d7 p.i.; despite this, the total number of IFN-γ+ CD8+ T cells remained significantly greater in RV-OVA compared to RV-PBS treated mice on days 2-7 due to the much higher total number of cells in the airway (Fig 6.12f).
Figure 6.11 BAL CD4+ T cell response during an RV-induced allergic exacerbation model.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. BAL was performed at the indicated time points p.i., and 1x10^5 cells were stained for surface markers CD3, CD4, and CD69, intracellular cytokines IFN-γ and IL-4 and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of (a&b) CD3+/CD4+ T cells, and CD3+ gated double positive (c&d) CD4+/CD69+,
(e&f) CD4+/IFN-γ+ and (g&h) CD4+/IL-4+ T cells. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.

Figure 6.12 BAL CD8+ T cell response during an RV-induced allergic exacerbation model.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. BAL was performed at the indicated time points p.i., and 1x10^5 cells were stained for surface markers CD3, CD8, and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of (a&b) CD3+/CD8+ T cells, and CD3+ gated double positive (c&d) CD8+/CD69+ and (e&f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01 & *p<0.05). Only statistically significant differences are shown.
6.3.8.2. Lung T Cells

In the lungs, the percentage of CD4+ T cells was consistently and equally elevated in OVA compared to PBS challenged groups at d1-7 p.i. (Fig 6.13a). However, the total number of CD4+ T cells was greater in RV-OVA compared to UV-OVA treated mice, and greater in UV-OVA compared to RV-PBS and UV-PBS treated mice at d1 and d2 p.i. (Fig 6.13b). After d2 numbers declined but were still elevated in OVA compared to PBS challenged groups at d7 p.i. Similarly, the percentage and total number of lung CD69+ CD4+ T cells was significantly elevated in OVA compared to PBS challenged groups, with similar levels observed between RV-OVA and UV-OVA treated mice (Fig 6.13 c&d).

Despite variations in the percentage of IFN-γ+ CD4+ T cells between the groups at each time point, translation into cell numbers demonstrated equally elevated numbers of IFN-γ+ CD4+ T in RV-OVA and UV-OVA treated mice between d1-7 p.i. compared to RV-PBS treated mice, except for d4 at which numbers in RV-PBS treated mice increased to similar levels (Fig 6.13f). Expression of IL-4 in CD4+ T cells was only increased in OVA challenged groups, with no significant differences detected between RV-OVA and UV-OVA treated mice; percentage and numbers increased from d1 p.i., peaked at d4 and were still significantly elevated at d7 p.i. compared to RV-PBS and UV-PBS treated mice (Fig 6.13 g&h).

With respect to CD8+ T cells in the lungs, the percentage was greatest in RV-OVA treated mice at d1 p.i., after which it decreased to similar levels detected in the other groups at days 2-7; however, numbers of CD8+ T cells were significantly elevated in RV-OVA compared to UV-OVA, RV-PBS and UV-PBS treated mice at d1 and d2 p.i., after which levels declined to baseline (Fig 6.14b). Additionally, numbers were slightly elevated in UV-OVA compared to RV-PBS (ns) and UV-PBS treated mice (p<0.05) at d1 and d2 p.i. (Fig 6.14a). The percentage and total number of CD69+ CD8+ T cells was significantly elevated only in OVA compared to PBS challenged mice at d1 and d2 p.i., however the total number was ~2-fold greater in RV-OVA compared to UV-OVA treated mice on both days (p<0.001), after which levels declined (Fig 6.14 c&d).

The percentage of lung IFN-γ+ CD8+ T cells was greater in OVA compared to PBS challenged mice on d1 p.i., and this difference was resolved by d4. However, despite similar percentages detected in RV-OVA and UV-OVA treated mice on d1 and d2 p.i., numbers were significantly greater in RV-OVA treated mice (Fig 6.14f). On d1 p.i. numbers were ~2-fold and ~3-fold greater in RV-OVA compared to UV-OVA and RV-PBS treated mice respectively.
(p<0.001). Additionally, UV-OVA treated mice demonstrated elevated numbers over RV-PBS and UV-PBS treated mice on d1 p.i. (p<0.001) and remained elevated over UV-PBS treated mice on d2 (p<0.05).
Figure 6.13 Lung CD4+ T cell response during an RV-induced allergic exacerbation model.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. The left lung lobe was excised after BAL was performed at the indicated time points p.i., and lungs were digested as described. 1x10⁶ cells were stained for surface markers CD3, CD4, and CD69, intracellular cytokines IFN-γ and IL-4 and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of (a&b) CD3+/CD4+ T cells, and CD3+ gated double positive (c&d) CD4+/CD69+, (e&f) CD4+/IFN-γ+ and (g&h)
CD4+/IL-4+ T cells. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.01, *p<0.05). Only statistically significant differences are shown.

Figure 6.14 Lung CD8+ T cell response during an RV-induced allergic exacerbation model.

BALB/c mice were sensitized i.p. with OVA/Alum at d-13 and challenged i.n. with OVA/PBS at d-2, -1 and 0; mice were immediately infected with RV-1B or UV-RV-1B following the 3rd OVA challenge on d0. The left lung lobe was excised after BAL was performed at the indicated time points p.i., and lungs were digested as described. 1x10^6 cells were stained for surface markers CD3, CD8, and CD69, intracellular cytokine IFN-γ and a dead cell marker; staining was analysed on a BD LSR II flow cytometer as described. Data are presented as the percentage and absolute number of (a&b) CD3+/CD8+ T cells, and CD3+ gated double positive (c&d) CD8+/CD69+ and (e&f) CD8+/IFN-γ+ T cells. Data are expressed as means ± SEM of 4 mice per group and is representative of at least 2 independent experiments. Statistical significance is shown between mice of specific treatment groups, at the indicated times (**p<0.001, *p<0.05). Only statistically significant differences are shown.
6.4. Chapter Summary

In this chapter we have demonstrated that RV infection in airways with allergic inflammation (RV-OVA) exacerbated airway neutrophilia compared to control mice (RV-PBS). Within the OVA sensitized and challenged groups, similar levels of eosinophils and lymphocytes were detected in airway regardless of infection with live or inactivated virus. As previously shown in BAL fluid by Bartlett et al. [255], we demonstrated using lung homogenate that only OVA sensitized and challenged mice expressed IL-4, IL-5 and IL-13, and levels were greater in RV-OVA compared to UV-OVA treated mice at days 1-2 p.i.

In contrast to our hypothesis, we detected a dramatic upregulation of IL-15 mRNA in the lungs of RV-OVA treated mice compared to all other groups at day 1 p.i. Similarly, IL-15 mRNA levels in BAL cells was much higher in RV-OVA treated mice compared to controls at 10h p.i., but similar levels were detected in RV-PBS and RV-OVA treated mice at day 1 p.i. However, enhanced levels of IL-15Rα mRNA in lung homogenate and BAL cells was only detected in RV-OVA treated mice, suggesting increased IL-15 signalling potential specific to this group based on the special trans-presentation mechanism for IL-15 signalling. In association with this, enhanced levels of total IFN-γ mRNA in the lungs of RV-OVA treated mice was detected at day 1 p.i. compared to other treatment groups, but by day 2 p.i. similar levels were observed between RV-OVA and RV-PBS treated mice and levels declined thereafter. IFN-γ induces expression of IP-10, and increased levels of BAL IP-10 protein was detected only in RV-OVA treated mice at day 1 and only in RV-PBS treated mice at day 2 p.i.

As shown by flow cytometry, RV-OVA treated mice demonstrated the greatest increase in total NK cells numbers and CD69+, IFN-γ+ and GranzymeB+ NK cells at day 1-2 p.i. in the airway and lungs. Only a small increase in numbers of BAL IFN-γ+ NK cells was observed in RV-PBS treated mice at day 2 p.i. and levels were still significantly less than that observed in RV-OVA treated mice. These results are also in disagreement with our hypotheses. Furthermore, increased numbers of NK cells positive for intracellular IL-4 were detected in RV-OVA treated mice in the BAL at days 2-4 and in the lungs at days 1-2 p.i. compared to control groups.

Numbers of BAL CD4+ T cells were greater in OVA compared to PBS challenged groups, and similar levels of BAL IFN-γ+ CD4+ T cells were detected in RV-OVA and RV-PBS treated mice at day 4 p.i. Only RV-OVA treated mice demonstrated increased numbers of BAL IL-4+ CD4+ T cells at days 4-7 p.i. Similarly, higher levels of BAL CD8+ T cells were observed in OVA
compared to PBS challenged mice, and numbers of BAL CD69+ and IFN-γ+ CD8+ T cells were greatest in RV-OVA compared to control groups.

In the lungs, numbers of CD4+ T cells were significantly greater in OVA compared to PBS challenged mice, and although there were slightly greater total numbers in RV-OVA treated mice, there were similar levels of CD69+, IFN-γ+ and IL-4+ CD4+ T cells in both OVA challenged groups. However, significantly greater numbers of total and activated lung CD8+ T cells were observed in RV-OVA compared to control groups.

All of these exacerbated immune responses observed in RV-OVA treated mice were associated with enhanced AHR compared to controls, in which no increased airway sensitivity was detected in PBS challenged mice. This was associated with an increased virus load in RV-OVA compared to RV-PBS treated mice at day 1 after infection. Thus, it appears that RV infection in airways with allergic inflammation results in both enhanced Th1 and Th2 immune responses, which contributes to poor early virus control and increased AHR.
Chapter 7: Discussion & Future Work

RV infections are amongst the most common viral infections encountered and the most frequent cause of the common cold; they are self-limiting and a mere inconvenience to a healthy host. In asthma, the effects of RV infection can be very different. RV infections cause significant morbidity and mortality in asthmatics as they trigger approximately two thirds of asthma exacerbations, which often results in hospitalisation. Few treatment options for acute exacerbations exist and their efficacy is limited, highlighting the urgent need for studies into the immune mechanisms of disease, particularly focusing on antiviral immunity. IL-15 expression and NK cell functions are key for defence against a number of viral infections however, their role in the immune response to RV infections and in asthma are unknown.

We have used mouse models to examine RV-mediated IL-15 expression and the NK cell responses. We initially examined these responses in a model of RV infection in BALB/c mice. Following detection of RV-induced IL-15, we used two models of deficient IL-15 signalling to determine the role of IL-15 during infection; we employed an IL-15 neutralising antibody to transiently block IL-15 signalling in the airway/lungs of BALB/c mice and IL-15Rα knockout mice that have a constitutive block to IL-15 signalling. IL-15Rα ko mice have an intrinsic NK cell deficiency, allowing us to indirectly assess the importance of NK cells during RV infection. RV induces type I IFNs and IL-15 is a reported ISG, so we next determined if type I IFN signalling is necessary for RV-induced IL-15 and the NK cell responses using IFNAR1 ko mice. Lastly, given the importance of RV in asthma exacerbations, we used a mouse model of this condition to determine how RV and allergen interaction affects IL-15 expression and the NK cell responses.

7.1. RV-Induced IL-15 Expression

BALB/c mice were infected with a minor group RV (RV-1B), which resulted in airway neutrophilia followed by lymphocyte infiltration, and expression of proinflammatory mediators including KC, IL-1β, IL-6, TNF-α, type I IFNs and IFN-λ. These responses were dependant on virus replication as determined by infection with UV-irradiated RV. In UV-RV-1B treated mice, a small degree of neutrophilia was detected at day 1 after infection (though this was not statistically significant compared to PBS or RV-1B treated mice) and is likely induced by immune recognition of viral proteins. Alternatives to this control are PBS treatment alone, or using HeLa cell lysate (cells used to propagate RV-1B) from uninfected...
cells, prepared using the same purification method for RV-1B. Having confirmed the expected immune responses previously published for this model by Bartlett et al. [255], we then assessed IL-15 and IL-15Rα expression.

IL-15 is a pleiotropic cytokine that is upregulated after a number of viral infections [116], suggesting the cytokine plays a role in generating an effective antiviral immune response, particularly through activation of NK cell responses; IL-15 is critical for NK cell development and functions, and NK cells are well known to be important for the innate antiviral immune response. This study is the first in vivo report of RV-mediated IL-15 upregulation. Expression was analysed in lung tissue to primarily address production in epithelial cells, previously reported to constitutively express IL-15. Expression was also analysed in BAL cells as macrophages are known sources of IL-15. As trans-presentation of IL-15 to other cells requires co-expression of IL-15Rα, we analysed levels of the receptor. We found that both IL-15 and IL-15Rα expression peaked at day 1 after infection in BAL cells (mRNA) and lung tissue (mRNA and protein). IL-15 staining of lung sections by immunohistochemistry was localised to the apical surface of epithelial cells lining the airway; we suggest this is spatially optimised for interaction of effector cells in the airway with virus-infected epithelial cells, to stimulate proliferation and activation of IL-15 responsive cells at the site of infection. Thus, early induction of IL-15 in the airway and lungs after RV infection potentially plays a role in developing the antiviral immune response.

In support of this data, we have previously observed IL-15 upregulation in primary human BECs infected with RV in vitro (data not published), and Laza-Stanca et al. [256] demonstrated RV-induced upregulation in human monocyte derived macrophages in vitro. Fawaz et al. [116] conducted a comparative study infecting PMBCs in vitro with different viruses including influenza, RSV, HSV-1, EBV, reovirus, vesicular stomatitis virus and Sendai virus demonstrating early upregulation of IL-15 mRNA. Upregulation after in vivo infection has been observed with herpes group viruses, HIV, vaccinia virus and influenza [123, 259, 260].

Numerous studies have documented the difficulty in detecting secreted IL-15 protein in biological fluids. Accumulating evidence in the last few years has identified trans-presentation as the primary mechanism for IL-15 signalling, whereby IL-15 is stabilised upon binding IL-15Rα in the ER, and the complex is transported via the secretory pathway for presentation on the cell surface. Thus, we directed our efforts towards detecting cell-
associated IL-15 protein by immunohistochemistry and ELISAs using lung homogenate rather than BAL fluid. The lung homogenate ELISA protocol was also recently used to demonstrate influenza induced expression of IL-15 protein in lung tissue [260]. This study showed via DC depletion experiments that plasmacytoid (pDCs) and CD8α+ DCs are the important sources of pulmonary IL-15 trans-presentation during influenza infection, and the level of IL-15 mRNA expressed in alveolar macrophages was approximately 60% of that detected in pDCs/CD8α+ DCs. In our study, we found that BAL cells expressed higher levels of IL-15 mRNA than lung tissue at day 1 after infection, suggesting they may be a more important source of IL-15 signalling however, our detection methods did not allow us to attribute expression to specific cell types. As neutrophils comprised approximately 80% of BAL cells at day 1 after infection and the remaining cells were macrophages, we suggest that these two cell types are potential important sources of IL-15 during RV infection.

7.2. NK Cell Responses During RV Infection

NK cells are innate large granular lymphocytes that act in the early phase of virus infection to eliminate infected cells whilst sparing uninfected cells, in addition to producing cytokines and chemokines that can affect other arms of the innate and adaptive immune responses [261]. IL-15 is widely reported to be important for development, differentiation, survival and activation of NK cells, thus we were interested in the relationship between IL-15 and NK cells during RV infection. We initially assessed the NK cell response to RV infection in BALB/c mice. To identify the role of IL-15 in the NK cell response during RV infection, IL-15 neutralising antibody was administered intranasally at the time of infection in BALB/c mice to transiently block pulmonary IL-15 signalling. IL-15Rα ko mice were used to assess the role of IL-15 and indirectly assess the importance of NK cells during RV infection as these mice lack NK cells.

Little is known about NK cell responses during RV infection. Levandowski et al. [262] observed a decrease in PBMC levels during the acute symptomatic phase of experimental RV infection in humans, which included leukocytes expressing NK cell surface markers, and an associated increase in mononuclear leukocytes in nasal secretions. Levandowski & Horohov [263] demonstrated augmented NK-like cellular cytotoxicity upon incubation of PMBCs with RV in vitro. Thus, existing evidence on the role of NK cells during RV infection is basic, warranting further investigation to determine the importance of this innate response during RV infections, in addition to their role in individuals with more severe RV infections including those with asthma and COPD. Understanding the roles of IL-15 and NK cells during RV infections could reveal potential therapeutic targets for better disease control.
In the BALB/c mouse model of RV infection, recruitment and/or expansion of NK cells in the airway by day 1 after infection was detected, with numbers peaking at day 2, comprising approximately 30% of the total BAL lymphocyte population. In the lungs, numbers were elevated above controls at days 2-4 after infection. These accumulating NK cell populations exhibited an activated phenotype, described by increased expression of the early activation marker CD69 and intracellular IFN-γ and GranzymeB. These data suggest that NK cells play a role in early defence against RV infection. Although the NK cell response was associated with the decline in virus titre, numerous other cellular responses were also associated with the decline of RV in this model. It is not clear which, if any, are important for clearance. Because virus replication is limited in this model and appears to be ‘passively’ cleared, it is difficult to determine if specific immune responses are involved in clearance. However, in support of the role of NK cells in antiviral responses, RV infection of IL-15Rα ko mice that lack NK cells demonstrated increased virus loads at 8h and 24h after infection compared to wild type mice, implying that NK cells are important for controlling early viral load. In our mouse models, we have not shown RV replication per se as our qPCR assay measures viral load via quantifying the amount of positive sense genomic viral RNA in lung tissue; however, detection of increased viral loads in knockout mice over wild type control mice implicates increased viral replication and/or impaired clearance of virus. To demonstrate increased viral replication directly in the future, in situ hybridisation with a sense RNA probe detecting negative strand replicative viral RNA would have to be performed as done by Bartlett et al. [255]. In addition, as the duration of RV replication in the mouse is short, it would be advantageous to develop a model in which replication lasts for several days as observed in clinical and experimental human RV infections [22, 23].

Many studies have indicated the importance of NK cell responses for clearance of viral infections. The best evidence is described by the few reported cases of reduced or absent NK cell activity in human subjects suffering from multiple, recurrent and often persistent herpes virus infections including HSV, CMV and EBV [239, 264-266]. Numerous in vitro and in vivo murine herpes virus infection models (including HSV-1, HSV-2, HHV-6, HHV-7 and EBV) also support the role of NK cells for control of infection.

Clinical observations have indicated an inverse relationship between an increasing magnitude of NK cell deficiency and more severe RSV-induced bronchiolitis in infants [267-269]. The striking correlation between deficient NK cell numbers and severity of RSV induced
disease clearly indicates a significant role of NK cells in protection during RSV infection. In mice, the NK cell response to RSV is similar to that observed with RV, with recruitment of NK cells to the airway and lungs by 48h after RSV infection, at which time they represent the largest IFN-γ producing population prior to the CD8+ T cell response [270, 271]; depletion of NK cells in this model resulted in an increased viral load at day 5 [272]. TLR4 deficient mice infected with RSV demonstrated an impaired NK cell response, which was associated with impaired virus clearance [273]; it is possible that TLR4-mediated detection of RV may be involved in regulation of the NK cell response in our models. Boosting NK cell responses during RSV infection via co-expression of IL-18 in the lungs significantly attenuated the peak virus load, further supporting the beneficial role of NK cells [274]. The RSV-mediated NK cell response in vivo may be regulated by IL-15, induction of which has been shown in monocytes and respiratory epithelial cells infected with RSV in vitro [125].

Influenza infection also results in accumulation of NK cells at days 1-2 after infection in mice [275], and NK cell depletion via administration of anti-asialo GM1 antibodies significantly increases influenza induced morbidity and mortality [276]. Absence of the NK cell activating receptor Natural Killer Cell Receptor 1 (NCR1) in mice increased susceptibility to influenza infection compared to wild type mice [277]. The notion that NK cells play a critical role in clearance of influenza is further supported by a number of reported NK cell evasion mechanisms employed by influenza, including direct infection of NK cells resulting in their functional impairment, facilitating rapid virus replication in respiratory epithelial cells [278]. These studies with RSV and influenza provide support for our data, indicating an important role for NK cells in the immune response to RV infection that is associated with IL-15 expression.

In contrast, a recent study has shown that NK cells contribute to early lung injury during RSV infection in mice [279]. Conflicting evidence suggesting both pathogenic and beneficial effects of NK cells during RSV infection raises the possibility of NK cell-mediated pathogenicity during RV infection, mediated by NK cell produced IFN-γ, release of cytotoxic granules and recruitment and/or activation of inflammatory cells via NK cell secreted proinflammatory cytokines and chemokines. Absent NK cell responses in our RV infection mouse models of deficient IL-15 signalling did not result in reduced levels of inflammatory responses including neutrophilia and BAL proinflammatory cytokine levels; histological analysis of lung sections would further allow us to address whether NK cells contribute to disease pathogenesis during RV infection in these models. It is more difficult to assess
pathogenicity of immune responses in the mouse models of RV infection, as more significant markers of disease severity such as weight loss or lethality observed with RSV and influenza respectively are not seen with RV infection.

7.3. Regulation of NK Cell Responses by IL-15

IL-15 signals via the constitutively expressed IL-2Rβ/γc (CD122) heterodimeric receptor on NK cells. In BALB/c mice, the peak of IL-15 expression in lung and BAL cells at day 1 after RV infection precedes the NK cell responses in the airway and lungs, suggesting that IL-15 potentially regulates NK cell responses during infection. To address the role of IL-15 on NK cell responses, we administered an IL-15 neutralising antibody intranasally at the time of RV infection to transiently block IL-15 signalling in the airway and lungs. Intranasal delivery was chosen to specifically block IL-15 at the site of infection to investigate the effects of RV-induced IL-15 in the airway/lungs on local NK cell accumulation and activation; systemic delivery results in rapid systemic depletion of NK cells and may affect other immune populations in the periphery such as CD8+ T cells, making results upon infection in the lungs difficult to interpret. In this model, we observed a deficient NK cell response indicating that RV-induced IL-15 is necessary for the recruitment/expansion and activation of NK cells in the airway and lungs during RV infection.

RV-induced IL-15 may act as a chemotactic signal in addition to promoting the survival and proliferation of NK cells, resulting in their accumulation during the early phase of infection. Allavena et al. [280] demonstrated the chemotactic effects of IL-15 on NK cells in vitro, and showed that IL-15 enhances LFA-1-dependant NK cell adhesion to vascular endothelial cells, promoting recruitment into tissues. However, IL-15 mediated NK cell recruitment into the airway and lungs during infection has not yet been investigated.

The constitutive expression of IL-15 on a wide range of cells including respiratory epithelial cells and bone marrow stromal cells, in addition to constitutive IL-2Rβ/γc expression on NK cells suggests that IL-15 plays a role in NK cell survival. Carson et al. [281] demonstrated the survival of human NK cells cultured in the presence of IL-15 alone, at concentrations as low as 0.1ng/ml, for up to 8 days, compared to no survival in serum free media alone; this was attributed to IL-15 regulated expression of the anti-apoptotic molecule Bcl-2 preventing or delaying apoptosis of NK cells. Our results support the role of IL-15 in NK cell survival, as administration of IL-15 neutralising antibody resulted in decreased numbers of lung and splenic NK cells 48h after intranasal administration. Proliferation can also contribute to
increased NK cell numbers, previously shown to be IL-15 dependant [193]. IL-15 over-expression in transgenic mice increases NK cell numbers, which could be attributed to increased survival and/or proliferation. Thus, IL-15 may act by a number of mechanisms to promote the accumulation of NK cells in the airway/lungs during RV infection, although the precise mechanism(s) at play were not examined in our studies. It would be valuable to investigate these pathways to further define the roles of IL-15 on NK cell functions during RV infection, with potential implications for therapeutic targets in individuals experiencing more severe RV-mediated disease.

Activated NK cells can modulate immune responses via production of cytokines, specifically IFN-γ, TNF-α, GM-CSF and MIP-1α/CCL3 [282]. NK cell IFN-γ production is important for activation of host monocytes/macrophages after infection and development of appropriate and effective innate and adaptive immune responses [283]. Our model of deficient IL-15 signalling using an IL-15 neutralising antibody demonstrated IL-15-depedant NK cell IFN-γ production in response to RV infection. In our infection models using IL-15Rα ko and IFNAR1 ko mice, we could not attribute deficient expression of IFN-γ detected by total mRNA levels in lung tissue solely to the absent NK cell response as deficient CD8+ T cell responses were also observed, but the data in these models supports the neutralising antibody study i.e. deficient NK cell response associated with deficient IFN-γ response.

These data are in accord with previous reports of IL-15 induced NK cell IFN-γ production. Carson et al. [284] demonstrated that IL-15 produced by LPS activated monocytes in vitro was necessary for optimal NK cell IFN-γ production; however, neutralisation of IL-15 did not completely abrogate IFN-γ expression suggesting that other monocyte-derived cytokines, including IL-12, are able to induce sub-optimal IFN-γ production by NK cells. Buunsgaard et al. [285] showed that neutralisation of endogenous IL-15 in PBMC cultures from HIV infected patients resulted in reduced IFN-γ production. Gosselin et al. [286] reported IFN-γ secretion by PMBCs upon stimulation with IL-15, which was synergistically enhanced in the presence of EBV or HSV-1. Furthermore, increased NK cell derived IFN-γ production is observed in IL-15 transgenic mice [287]. The importance of NK cell produced IFN-γ for defence against virus infections in vivo has been demonstrated by other studies [230, 234].

IL-15 stimulation of NK cell cytolytic functions for elimination of virus infected cells has been reported [116, 284, 286], but no previous evidence exists documenting the necessity of this pathway for RV clearance. Despite observing upregulation of NK cell GranzymeB during RV
infection, it is unclear how important the cytolytic pathway is as NK cell cytolytic activity was not directly measured. However, in our infection model with neutralising antibody, absence of NK cell responses did not affect the lung virus titre, suggesting that cytolytic activity of NK cells is not important for clearance of virus via destroying RV-infected epithelial cells. This is further supported by the limited cytopathic effect seen with RV infection compared to RSV and influenza [288-290]. It is more likely that NK cells, as an important source of IFN-γ, are key for directing development of Th1 responses to drive the appropriate antiviral immune responses (further discussed in Section 7.7).

7.4. **Type I IFN Signalling is Necessary for RV-induced IL-15 and the NK Cell Response**

Most if not all nucleated host cells are able to express type I IFNs early after viral infection, which stimulate expression of hundreds of ISGs to promote development of an antiviral state in surrounding cells and to directly stimulate innate and adaptive immune cells. Type I IFNs have been demonstrated to induce IL-15 expression [118] and consistent with this, RV-induced IL-15 in BALB/c mice was associated with IFN-β expression; in BAL cells, IFN-β mRNA was elevated by 10h after infection followed by upregulation of IL-15 mRNA at 24h. To investigate if RV-induced IL-15 (and thus the NK cell response) in this model is dependent on type I IFN signalling, we analysed these responses during infection of IFNAR1 ko mice. These mice lack the IFN-α-receptor subunit1 and cannot mediate type I IFN signalling.

We found deficient upregulation of IL-15 in RV infected IFNAR1 ko mice, confirming that IL-15 is an ISG in this model. Our results are supported by Laza-Stanca et al. [256] whom demonstrated *in vitro* using macrophages, dose-dependent IL-15 expression upon IFN-β stimulation and inhibition of RV-induced IL-15 in the presence of an IFN-α-receptor subunit2 (IFNAR2) blocking antibody. Therefore, *in vitro* and *in vivo* RV infection models show that RV-induced IL-15 is dependent on type I IFNs. Consistent with these data, Azimi et al. [121] reported the presence of a virus-inducible enhancer region in the IL-15 promoter, composed of an IFN regulatory factor element (IRF-E) adjacent to an NF-κB binding site. Nguyen et al. [193] demonstrated the necessity for IFN-α/β and STAT1 activity for induction of IL-15 during MCMV infection in mice, and Mattei et al. [118] showed IL-15 and IL-15Rα expression by splenic DCs upon IFN-α/β treatment *in vivo* and *in vitro*. Taken together, these studies confirm the importance of type I IFN signalling for the induction of IL-15 in response to virus infections, and support our data indicating type I IFN-dependant RV-induced IL-15 expression.
Type I IFNs play an important role in upregulation of NK cell cytotoxicity and IFN-γ production during virus infections including LCMV, HSV-2 and vaccinia virus, which enhances antiviral immunity [192, 261, 291-293]. In agreement, we found the NK cell response to RV infection dependant on type I IFN signalling. NK cell accumulation in the airway was completely absent in RV infected IFNAR1 ko mice, suggesting a recruitment block in the absence of type I IFN signalling. In the lungs, similar numbers of NK cells were present in IFNAR1 ko and wild type mice, but lung NK cells in IFNAR1 ko mice failed to upregulate CD69, IFN-γ and GranzymeB expression indicating that type I IFN signalling is necessary for activation of NK cells during RV infection.

The deficient NK cell responses in IFNAR1 ko mice were associated with an increased virus load in the lungs at 8h after infection, at which time we also observed deficient expression of IL-15 and IFN-γ. Although deficient NK cell responses were also observed in our infection models with deficient IL-15 signalling (IL-15 neutralising antibody and IL-15Rα ko mice), expression of ISGs were not affected in these models suggesting that type I IFN signalling was intact; this implies that NK cell responses during RV infections are not achieved by direct action of type I IFNs on NK cells, but is likely mediated through type I IFN-induced IL-15 signalling.

These data are supported by previous reports indicating that type I IFNs stimulate accessory cells to produce IL-15, which acts as the intermediary factor to activate NK cells. Lucas et al. [247] reported DCs as the accessory cell for this pathway in vivo. Boudreau et al. [294] supported these findings via demonstrating the necessity of type I IFN-induced IL-15 by DCs for NK cell activation and tumour protection, and that stimulation of NK cells directly by type I IFNs alone was insufficient for activation. During MCMV infection, NK cell proliferation was mediated by type I IFN-induced IL-15 however, enhanced NK cell cytotoxicity required direct stimulation by type I IFNs in this model [193]. Foong et al. [117] demonstrated reduced virus titres as early as day 1 after infection in mice with an IL-15 expressing recombinant vaccinia virus compared to wild type virus, the effect of which was dependant on the presence of IFNs and NK cells. Furthermore, DC-NK cell interactions are becoming more apparent with imaging studies, illustrating the importance of these brief encounters for activation of NK cells in response to pathogenic stimuli [295]. All of these studies support the necessity of type I IFN signalling for IL-15 induced NK cell responses, as we have shown during RV infection in mice.
Our observations are in contrast to that reported by Martinez et al. [293], whom report the necessity for direct type I IFN stimulation of NK cells for their activation and effector function \textit{in vitro} and \textit{in vivo}, for efficient clearance of vaccinia virus infection. Direct stimulation for elimination of adenovirus vectors \textit{in vivo} was also observed by Zhu et al. [292]. Beuneu et al. [296] demonstrated the necessity of both direct type I IFN stimulation of NK cells and indirect stimulation via accessory cells for IL-15 trans-presentation for optimal stimulation of NK cell effector functions in response to poly I:C stimulation in mice. It is likely that both IL-15 and type I IFNs are required for optimal NK cell responses; type I IFNs have numerous roles either functioning through induction of ISGs that in turn regulate NK cells (such as IL-15) or by directly stimulating NK cells. The relative importance of direct vs indirect (via ISGs) effects of type I IFNs will vary depending on the model, though cooperation of the two pathways is likely to be optimal as deficiency of either impedes the NK cell response.

Despite the well known importance of the above pathways, it is necessary to remember that activation of NK cells during virus infection is very complex, as illustrated by the large variety of activating and inhibitory receptors that govern NK cell responses, which not only respond to cytokine stimulation (type I IFNs, IFN-\(\gamma\), IL-12, IL-15 and IL-18) but also pathogen-derived gene products [297]. Regardless of the mechanism used, consistent with previous findings [117, 291, 293], we show in this study that type I IFN signalling is critical for NK cell responses during RV infection, and to our knowledge, this is the first report of IFN-dependant IL-15 mediated NK cell responses for defence against a respiratory viral infection.

\section*{7.5. IL-15 is Required for CD8\(^+\) T Cell Responses During RV Infection}

IL-15 was first discovered by its ability to stimulate proliferation of an IL-2-dependant CTLL-2 mouse CD8\(^+\) T cell line [112, 133]. T cells respond to IL-15 signalling via the intermediate affinity IL-2R\(\beta/\gamma_c\), which is upregulated after activation [298]. The relationship between IL-15 and CD4\(^+\) T cells is unclear, but most reports indicate that IL-15 is not important for CD4\(^+\) T cell functions. This supports our findings of minimal disruption to CD4\(^+\) T cell responses in our mouse models of deficient IL-15 signalling (IL-15 neutralising antibody and IL-15R\(\alpha\) ko mice) during RV infection. IL-15 is critically important for maintaining memory CD8\(^+\) T cell populations as demonstrated by the severely reduced numbers of CD44\(^{\text{high}}\) CD8\(^+\) T cells in IL-15 and IL-15R\(\alpha\) ko mice [157, 158]. IL-15 supports the survival of CD8\(^+\) T cells at all developmental stages, by stimulating increased expression of Bcl-2 [160]; this explains the
dramatically lower frequency and total number of CD8+ T cells observed in the lungs of naïve IL-15Rα ko compared to wild type mice.

Respiratory viral infections elicit Tc1 CD8+ T cell responses that are generally defined as cytotoxic and IFN-γ producing, which promote clearance of the infection. Tc2 CD8+ T cells producing IL-5 have been described in asthma [299] and, CD8+ T cells are reported to contribute to the pathogenesis of virus-induced asthma exacerbations, particularly with RSV infection [300]. The Th2 environment in asthmatic lungs are thought to alter virus-specific CD8+ T cell responses from IFN-γ to IL-5 production (Tc2), further potentiating allergic inflammation and hampering antiviral immune responses [300]. The role of IL-15 in regulating Tc1 vs Tc2 CD8+ T cell responses during virus infection and/or asthma is unknown.

In BALB/c mice, the number of airway and lung activated (CD69+ and IFN-γ+) CD8+ T cells was elevated by day 2 after RV infection however, the response was reduced in mice treated with IL-15 neutralising antibody and completely deficient in IL-15Rα ko mice. Our data suggests that IL-15 plays a role in the accumulation of CD8+ T cells in the airway/lungs during RV infection, in addition to their activation. These data are supported by findings from Verbist et al. [301] whom demonstrated the role of influenza-induced IL-15 for migration of CD8+ effector T cells to the airway in vivo; intranasal delivery of soluble IL-15/IL-15Rα complexes restored the respiratory CD8+ T cell response to influenza infection in IL-15 ko mice, and continuous administration during the contraction phase of the CD8+ T cell response amplified the resultant memory pool generated in wild type mice. Inflammation induced expression of IL-15 on the basolateral surface of airway epithelial cells revealed by Hocke at al. [257] presents an attractive mechanism for spatially optimised IL-15 signalling to attract CD8+ T cells from the vasculature. However, it is unclear whether IL-15 acts directly or indirectly on T cells to induce chemotaxis, and indirect mechanisms such as IL-15 induced expression of LFA-1 (an adhesion molecule that promotes lung-specific trafficking) [302, 303] and chemokine receptors on T cells [302] may play a role during RV infection.

The deficient CD8+ T cell response in IL-15Rα ko mice could result from a combination of poor recruitment, local proliferation, activation and survival signals due to the absence of epithelial, DC and/or macrophage trans-presentation of IL-15 in the airway [249, 260, 304]. We suggest that the reduced but not absent CD8+ T cell response observed with IL-15 neutralising antibody was because the short transient period of IL-15 deficiency did not have a severe impact on the survival of CD8+ T cells. We do not expect the differences in CD8+ T
cell responses between these models to be attributed to insufficient blocking of pulmonary IL-15 by neutralising antibody, as the dose, timing and route of administration resulted in complete inhibition of airway and lung NK cell responses and early NK cell depletion in the spleen, indicating systemic activity despite intranasal administration. However, the transient block with neutralising antibody may not have occurred fast enough to completely prevent IL-15 mediated indirect mechanisms of T cell chemotaxis after RV infection hence detection of some accumulation of CD8+ T cells in the airway and lungs.

The lack of an activated CD8+ T response in both IL-15Rα ko and IFNAR1 ko mice could be due to absent IL-15 mediated survival signals, possibly from pDCs, which are also key producers of type I IFNs. McGill et al. [260] showed that pDCs and CD8α+ DCs in the airway/lungs are the largest source of IL-15, necessary for survival of influenza-specific CD8+ T cells; the absence of these cells despite the presence of other IL-15 producing cells such as epithelial cells and macrophages did not support the survival and accumulation of CD8+ T cells during influenza infection. McGill et al. [260] administered recombinant mouse IL-15Rα/Fc fusion proteins intranasally to block IL-15 signalling resulting in increased apoptosis and thus reduced numbers of CD8+ T cells during influenza infection. Similar to our results which support the role of IL-15 in regulating the magnitude of lung CD8+ T cells after RV infection, they also detected reduced frequency and total number of lung CD8+ T cells in IL-15 ko mice during influenza infection. In vitro, IL-15 supports the survival of naïve and effector CD8+ T cells in the absence of IL-2 via induction of Bcl-2 and Bcl-xL [159, 305, 306]. IL-15 also inhibits IL-2-mediated activation induced cell death, thus potentially enhancing and prolonging the T cell response, which could be associated with better viral resistance or immune-mediated pathology [161].

In contrast, a study of LCMV infection in mice suggests that IL-15 is not necessary for supporting primary CD8+ T cell responses, as similar responses in IL-15 ko mice, IL-15Rα ko mice and wild type mice were observed [307]. We suggest that the dependency for IL-15 by specific immune responses may depend on the particular infectious agent, and the specific tissue/site of infection could also play a role. The importance of IL-15 for stimulation of T cell responses is clearly illustrated by its emerging popularity for incorporation into vaccines, to utilise its immune enhancing properties as an adjuvant that stimulates generation of both primary and memory responses. Poon et al. [308] showed enhanced humoral and cellular responses against the poorly immunogenic H5N1 influenza antigens with an IL-15 expressing
vaccinia based vaccine. These studies support our observations that IL-15 signalling is important for the CD8+ T cell response to RV infection.

Despite detecting accumulation and activation of CD8+ T cells in response to RV infection, it is unclear how important a cytolytic CD8+ T cell response is during the anti-RV response, as similar to the argument for NK cells, minimal epithelial destruction is observed during RV infection. In contrast to a healthy host, a relationship between increased numbers of CD8+ T cells in asthmatics and severity of virus-induced asthma exacerbation has been reported [300]. It is possible that in a healthy host, CD8+ T cell responses are not critical for antiviral immunity during RV infection, but contributes to disease pathogenesis in asthma.

7.6. **Role of Type I IFNs on T cells During RV Infection**

Type I IFNs exert numerous effects on T cells via direct and indirect mechanisms, and their necessity for development of adaptive immune responses against virus infections has been well documented [309]. Similar levels of accumulating CD4+ T cells in the airway and lungs after RV infection in IFNAR1 ko and wild type mice were observed, indicating that type I IFN signalling during RV infection is not necessary for recruitment, proliferation or survival of CD4+ T cells. However, the recruited CD4+ T cells in IFNAR1 ko mice did not upregulate expression of CD69 and IFN-γ after infection indicating impaired activation in the absence of type I IFN signalling. Shiow et al. [310] demonstrated type I IFNs to be potent inducers of CD69 expression on CD4+ T cells, which in turn regulates T cell migration and production of TNF-α and IFN-γ by CD4+ T cells [311]. Previous *in vitro and in vivo* studies indicate the necessity of type I IFNs for T cell activation, but whether this occurs via direct or indirect mechanisms (e.g. via IL-12, IL-15 and/or IL-18 induction) during viral infections is unclear as conflicting evidence exists; the need for intrinsic type I IFN signalling for T cell activation appears to be specific to the infection model investigated [312-315].

We have shown type I IFNs to be critical for accumulation and activation of airway and lung CD8+ T cells during RV infection, and this is associated with IL-15 upregulation. However, unaffected ISG expression and thus type I IFN signalling in IL-15Rα ko mice, despite deficient CD8+ T cell responses, suggests that type I IFNs regulate CD8+ T cell via indirect mechanisms during RV infection. Type I IFNs induce maturation of DCs, mediating upregulation of MHC class II molecules, co-stimulatory molecules (CD40, CD80 and CD86) and a number of cytokines, chemokines and chemokine receptors [316-318]; it is likely that impaired DC maturation and thus presentation of virus antigens for priming and stimulation of T cells
results in the deficient T cell responses observed in IFNAR1 ko mice during RV infection. Type I IFN stimulated expression of IL-15 by DCs [118] may be the indirect mechanism mediating proliferation and survival of activated T cells (as discussed above), previously attributed to type I IFNs during viral infections [319]. Work presented by Ruckert et al. [320] support the notion that DC-mediated IL-15 signalling regulates CD8+ T cell activation, necessary for development of Th1 immune responses in mice. The influenza-specific CD8+ T cell response was shown to be dependent on IL-15 signalling by Nakamura et al. [321]. In addition to IL-15, chemotaxis of T cells and efficient activation of naïve CD8+ T cells is also mediated by the ISG IP-10 [319], expression of which was deficient in IFNAR1 ko mice after RV infection.

Although our studies suggest that type I IFNs act via indirect mechanisms to mediate T cell responses during RV infection, the necessity for direct stimulation of T cells via the type I IFN receptor was shown for activation of these responses during LCMV infection. Adoptively transferred IFNAR1 deficient T cells specific for LCMV into IFNAR1 sufficient mice resulted in significantly reduced proliferation and generation of memory populations compared to controls [313, 322]. Comparison of various reports suggest that direct action of type I IFNs for development of a functional T cell response is necessary for some but not all virus infections. Our data indicates that type I IFN signalling plays an important role in activation of Th1 effector responses during RV infection, though some mechanisms are likely to be mediated indirectly via IL-15 signalling.

7.7. Deficient IFN-γ Responses & Associated Expression of Th2 Mediators

Immune reactions to virus infections are typically mediated by Th1 responses, and early IFN-γ production helps drive Th1 polarisation. Responses to RV infection in asthmatics is characterised by deficient IFN-γ production and augmented Th2 responses (increased BAL IL-4, IL-5 and IL-13 levels), which are associated with more severe RV-mediated disease, virus load and asthma exacerbation severity [27]. IFN-γ is primarily produced by macrophages and NK cells in the innate response and by CD4+ and CD8+ T cells in the subsequent adaptive immune response. In vivo infection studies with measles, MCMV, vaccinia virus and West Nile Virus illustrate the necessity of IFN-γ production for effective antiviral defence, and deficient IFN-γ responses in these models is associated with increased susceptibility to infection and death [230, 323-326]. These studies indicate the importance of IFN-γ production to mediate a successful antiviral immune response, and deficiency is associated with pathogenic outcomes.
We have shown IFN-γ production early after RV infection, which was dependant on RV-induced IL-15. During RV infection in mice, CD4+ and CD8+ T cells accumulate in the airway and lungs after 48h, therefore we suggest that NK cells are the dominant source of IFN-γ early after infection (prior to 48h). However, in the absence of upregulated IL-15 and thus IFN-γ production during RV infection in IL-15Rα ko and IFNAR1 ko mice, we observed increased expression of Th2-associated mediators TARC/CCL17, MDC/CCL22, IL-4, IL-5 and IL-13 in the lungs.

TARC and MDC are Th2 cell attracting chemokines that bind chemokine receptor CCR4 highly expressed on Th2 cells. Recruitment of Th1 cells to inflammatory sites is mediated via predominant expression of CXCR3 and CCR5 on lymphocytes [327]. TARC and MDC are pivotal for development of Th2-dominated allergic disorders such as asthma and allergic rhinitis [328], via mediating recruitment of Th2 cells. Increased levels of TARC and MDC are expressed by the bronchial epithelium of asthmatics and by naïve T cells from asthmatic patients compared to healthy controls [258, 329]. Expression is also higher in Th2 compared to Th1 cell lines [258]. In a murine model of asthma, Kawasaki et al. [330] demonstrated blockade of TARC resulting in diminished AHR, reduced airway eosinophilia, CD4+ T cell infiltration and BAL Th2 cytokine levels, thus strongly implicating TARC in mediating Th2-associated responses; similar results were observed upon neutralisation of MDC [331]. Other studies in mouse asthma models have reported reduced allergic airways inflammation following CCR4 blockade [332].

Although they promote Th2 responses, expression of TARC and MDC were observed during RV infection in wild type mice that primarily exhibit a Th1 response. Others have also found expression of these chemokines during Th1 inflammatory responses, such as observed in atherosclerotic and Crohn’s disease lesions [333, 334]. In contrast, it would be interesting to measure levels of Mig/CXCL9 (monokine induced by IFN-γ), important for enhancing recruitment and activation of Th1 cells, in our models of RV infection to further clarify the Th1/Th2 balance in the absence of IL-15 and type I IFN signalling. During RV infection, we suggest that absence of IFN-γ – the dominant Th1 polarising signal, likely generated by NK cells early after infection – permits Th2 responses to develop, facilitated by increased expression of TARC and MDC and possibly via activation of Th2 cytokine producing nuocytes (innate helper type 2 cells). This is in association with the well known mechanisms of reciprocally regulated Th1 and Th2 responses.
Increased expression of IL-4, IL-5 and IL-13 in the lungs, not normally associated with virus infections, suggests the accumulation of Th2 cells and/or activation of nuocytes in response to RV infection in the absence of an NK cell and IFN-γ response. Our data strongly complements observations by Kaiko et al. [335] whom demonstrated an RSV-induced Th2 response to primary infection. They found that depletion of NK cells abrogated the primary source of IFN-γ early after RSV infection, which in turn resulted in upregulation of Th2 cytokines, eosinophil infiltration and increased mucous production – hallmark features of allergic inflammation. IL-25 promotes development of Th2 responses in models of allergic asthma and helminth worm infections [189, 336-339] however, Kaiko et al. [335] were the first to demonstrate IL-25 mediated induction of virus-specific Th2 cells; they showed suppression of early IFN-γ production by epithelial-derived IL-25. Depleting NK cells resulting in reduced IFN-γ levels during RSV infection enhanced IL-25 production and lung Th2 cytokine gene expression. Although we did not specifically prove NK cells as the early main source of IFN-γ after RV infection, deficiency of IFN-γ production associated with absent activated NK cells in our models of deficient IL-15 and type I IFN signalling indicates this; although deficient CD8+ T cell responses, which normally contribute to IFN-γ production, were also observed in these models, Kaiko et al. [335] showed that depletion of CD8+ T cells prior to RSV infection did not result in a Th2 response. Therefore, it is likely that NK cell produced IFN-γ-mediated regulation of IL-25 expression in the lungs also occurs during RV infection, as evident by the observed Th2 responses in the absence of activated NK cell responses observed in our studies.

Clinical observations that RV and RSV infections during early life are linked to a greater risk of development of childhood asthma [340-343], and that defects in NK cell responses result in more severe RSV bronchiolitis [267-269], we suggest the above discussed mechanisms play a role in development of allergic asthma. Kaiko et al. [335] also demonstrated a casual relationship between deficient NK cell/IFN-γ responses during RSV infection and development of a Th2 response to an innocuous antigen during concomitant exposure, possibly via a mechanism of ‘collateral priming’. Why allergic sensitization rather than tolerance occurs to innocuous proteins early in life are unclear however, these data suggest that a Th2 polarised environment in the airway during virus infection observed with a deficient IFN-γ response, may be a possible mechanism for development of allergic sensitisation. In agreement, our data suggests that imbalances between Th1 and Th2 responses during RV infection, via deficient IL-15 and/or type I IFN signalling and thus NK cell
activation and IFN-γ production, could possibly contribute to development of allergic asthma in early life. Our data also identify a potential mechanism for RV-induced asthma exacerbations. A number of studies have identified a deficiency in antiviral IFN production in asthma during RV infections [108, 109]. This could lead to deficient IL-15 production, NK cell activation and IFN-γ expression, which in turn could contribute to the increased Th2-driven allergic inflammation observed in the asthmatic lung. Figure 7.1 illustrates the interpretation/conclusions of our results on the role of IL-15 during RV infections in non-allergic airways.
Figure 7.1 Role of IL-15 during RV infections.

In response to RV infection, airway epithelial cells produce IFN-β which acts in an autocrine and paracrine fashion upon signalling via the type I IFN receptor complex on surrounding epithelial cells, neutrophils, DCs, and macrophages. Type I IFN stimulation of these cells induces upregulation of IL-15 production and trans-presentation (surface expression of IL-15/IL-15Rα complexes). Trans-presentation of IL-15 to resting NK cells results in their activation, which includes upregulation of the early activation marker CD69, GranzymeB and IFN-γ production. IL-15 signalling can also promote chemotaxis of NK cells into the airways and lungs, and stimulate proliferation and promote survival of NK cells, resulting in their accumulation after RV infection. NK cells are important producers of early IFN-γ after infection, key for driving the development of appropriate Th1 antiviral immune responses necessary for effective viral clearance, including Th1 priming of macrophages and DCs, which in turn prime Th1 CD4+ T cells and Tc1 CD8+ T cells. In conditions of deficient IL-15 signalling, the NK cell response is impaired resulting in deficient IFN-γ production, which permits development of Th2 responses that are not effective for antiviral defence resulting in increased lung viral loads.
7.8. **RV-Induced IL-15 and the NK Cell Response in Allergic Airways**

Airway inflammation plays a central role in the immunopathology of asthma and is mediated by leukocyte infiltration, increased production of mucus and proinflammatory mediators, epithelial damage and airway remodelling processes. The cellular infiltrate of asthmatic airways is comprised of eosinophils, mast cells, basophils and importantly Th2 cytokine expressing T cells; enhanced neutrophilia is detected during respiratory virus infections, and in mice the degree of cellular infiltration correlates with AHR [81, 344, 345]. Asthma exacerbations are a major cause of asthma morbidity, mortality and high health care costs. Approximately two thirds of asthma exacerbations are associated with RV infections however, the mechanisms underlying this relationship are poorly understood. Having shown that deficient NK cell responses lead to enhanced RV-induced Th2 gene expression, we next investigated the relationship between RV infection and allergic airways inflammation to assess IL-15 expression and the NK cell response within allergic airways to model RV-induced asthma exacerbations [255].

RV infection in allergic mice challenged with OVA (RV-OVA) resulted in enhanced airway neutrophilia, increased expression Th2 cytokines and greater AHR to increasing doses of methacholine compared to non-allergic mice (RV-PBS) and allergic mice infected with UV-RV-1B (UV-OVA), confirming features of the model published by Bartlett et al. [255]. In RV-OVA treated mice, we observed dramatically enhanced IL-15 expression at day 1 after infection, which was associated with a very large increase in number of activated IFN-γ and GranzymeB expressing NK cells in the airway and lungs also at day 1; both responses were much greater than in UV-OVA and RV-PBS treated mice. It is interesting that allergen alone induces NK cell responses, and BAL NK cells from UV-OVA treated mice express CD69 and GranzymeB, whereas RV-PBS treated mice exhibit IFN-γ producing NK cells.

These results are in contrast to our hypothesis, in which we postulated deficient upregulation of IL-15 and thus impaired NK cell responses to RV infection in allergic airways, based on the following evidence from human studies. Deficient upregulation of IL-15 at day 4 after experimental RV infection in asthmatics compared to non-asthmatics was observed in bronchial biopsies stained by IHC (data not published – bronchial biopsies obtained from Message et al. [27] study). In addition, Laza-Stanca et al. [256] demonstrated deficient IL-15 expression in asthmatic BAL macrophages infected with RV *ex vivo* and lower levels of IL-15 protein in the BAL fluid of asthmatics at baseline compared to non-asthmatics, and both results inversely correlated with AHR, virus load and disease severity during subsequent
experimental RV infection *in vivo*. The human data indicates that similar to IFNs, IL-15 induction during RV infection is deficient in asthma. Furthermore, the previously discussed trend between NK cell impairment and severity of RSV-induced bronchiolitis in infants suggests that the degree of severity of RV-induced asthma exacerbations may be linked to a spectrum of deficient IFN-β, IL-15 and NK cell responses, all of which we have demonstrated to be interlinked.

However, a comparative study of IL-15 expression in inflammatory pulmonary diseases by Muro et al. [346] demonstrated, by in situ hybridisation and immunohistochemistry using bronchial biopsies, no difference in the levels of IL-15 between asthmatics and non-asthmatics. Much higher levels of IL-15 expression in bronchial biopsies of subjects with sarcoidosis and tuberculosis were observed, which are primarily Th1 chronic inflammatory diseases, and phenotyping of the IL-15-immunoreactive cells revealed the majority of cells to be neutrophils followed by macrophages. Therefore, we suggest that previous human studies demonstrating reduced or deficient IL-15 expression described above in asthma was due to the removal of many IL-15 expressing cells by method of BAL, and detection of soluble IL-15 in BAL fluid may not reflect the true situation as the majority if not all of IL-15 protein would have been cell-associated. Deficient expression observed in bronchial biopsies at day 4 after infection in asthmatics mentioned above, may be misleading as an exacerbated peak of expression in the airway might have occurred earlier after infection in asthmatics, as seen in our mouse model.

As Muro et al. [346] demonstrated neutrophils to be a significant population of IL-15 positive cells in Th1 pulmonary inflammatory diseases, we suggest that the exacerbated neutrophilia observed at day 1 after infection in RV-OVA compared to RV-PBS treated mice in our study contributes to the much higher levels of IL-15 observed in the airway, which in turn mediated the significantly larger influx of activated NK cells; this is consistent with increased IL-15 gene expression in lung tissue and BAL cells, and IL-15 protein in lung tissue found in RV-OVA treated mice. The dynamics of the NK cell response observed mirrors that of the neutrophil response, suggesting a strong association between the two, and previous studies have demonstrated cross-talk between neutrophils and NK cells resulting in reciprocal regulation of their functions [347]. Increased IL-15 expression in the lung tissue of RV-OVA compared to RV-PBS treated mice could also have contributed to NK cell response.
The increased virus load in RV-OVA compared to RV-PBS treated mice at day 1 after infection is in agreement with the increased virus replication detected during ex vivo RV infection of asthmatic compared to non-asthmatic bronchial epithelial cells [108], and increased disease severity (reduced lung function, increased symptom score) and higher virus loads in asthmatics experimentally infected with RV [27, 109]. This suggests that deficient innate responses, likely to arise from deficient antiviral responses in asthmatic airway epithelial cells, that control early virus replication exists in allergic asthmatic lungs as previously implied by Wark et al. [108]. However, detection of enhanced IL-15 expression and NK cell responses early after infection in allergic mice does not support this, and may be a discrepancy between mouse and human models, although these responses are yet to be characterised at early time points in humans. Despite increased virus replication in allergic mice at day 1 after infection, the virus load was driven down very quickly by day 2, which might at least in part result from enhanced NK cell responses that may act to limit virus replication/infection in mice via killing of virally infected cells and increased production of early IFN-γ. The quick decline in virus load in RV-OVA treated mice correlates with the swift resolution of the NK cell response, but we have no solid evidence proving NK cells to be important for virus clearance in this mouse model. It is also unknown if NK cell responses are deficient in human RV-induced asthma exacerbations.

In contrast, NK cells could play a role in mediating increased AHR in RV-OVA treated mice by significantly contributing to enhanced cellular inflammation. Impairment of lung function and increased AHR in experimentally infected asthmatics compared to non-asthmatics was associated with increased cellular infiltration, primarily mediated by significant eosinophil and neutrophil recruitment to the airway [27]. As induction of IL-8/CXCL8, a potent neutrophil chemoattractant, is induced by IL-15 in T cells and monocytes [148, 302], it is logical to suggest that the increased expression of IL-15 in RV-OVA treated mice might contribute to the enhanced neutrophilia detected in these mice – another potential mechanism by which IL-15 could potentiate airway inflammation during RV-induced asthma exacerbations.

Despite increased IFN-γ expression owing to the large activated NK cell response in RV-OVA treated mice, we observed enhanced expression of Th2 cytokines IL-4, IL-5 and IL-13 compared to UV-OVA treated mice. This data is in contrast to that described by Kaiko et al. [335] of IFN-γ production suppressing Th2 responses. Although there is an NK cell-mediated Th1 signal, we suggest it is not great enough to suppress the allergen induced Th2 signal.
Whilst RV infection induces responses that could suppress Th2 pathways, RV also induces expression of many pro-Th2 molecules including CCL17/TARC, CCL22/MDC, IL-25 and STAT-6 (unpublished data). Thus, the net effect of RV infection with allergen challenge is to exacerbate Th2 responses. We would then hypothesise that depletion of NK cells in RV-OVA treated mice would increase the exacerbation phenotype. It is also likely that a pre-existing allergic environment prior to virus infection outweighed the virus-induced Th2 suppressive effects of IFN-γ in our model. In humans, deficient IFN-γ and augmented IL-4, IL-5 and IL-13 responses were detected during experimental RV infection in asthmatics compared to non-asthmatics [27], though NK cell data to complement these findings are lacking.

Whilst the majority of NK cells in lung and BAL expressed IFN-γ following allergen challenge and RV infection (RV-OVA), a small proportion instead expressed IL-4. To our knowledge, we show for the first time the appearance of IL-4 expressing NK cells in the airway and lungs after RV infection in an asthma model. These IL-4 expressing NK cells may potentially contribute to the enhanced IL-4 expression detected in these mice, further potentiating the allergic Th2 responses that contribute to increased AHR. Walker et al. [348] and Korsegren et al. [349] have also demonstrated contribution of NK cells to Th2 inflammation in mouse models. Walker et al. [348] showed NK cells produce IL-5, which significantly contributed to eosinophil infiltration in a mouse model of allergic inflammation using ragweed antigen. This lead to the identification of NK1 and NK2 cells [350, 351]. Increased NK2 cells have been observed in PBMCs from asthmatic subjects [351]. Thus, our data in conjunction with other studies suggests that NK cells could contribute to the augmentation of Th2 responses in vivo during RV infections in asthmatics, and further work into this mechanism would be very interesting.

In this study, we demonstrated exacerbated IL-15 and NK cell responses after RV infection in mice with allergic airways. This is in contrast to the previously reported deficient IL-15 responses observed in asthmatics and asthmatic cells infected ex vivo. Thus, more careful investigation of these responses, particularly in detection of cell-associated IL-15 rather than soluble IL-15, is necessary in human models of RV-induced asthma exacerbations. It is likely that increasing the ratio of NK1:NK2 would be therapeutically useful. The therapeutic potential of IL-15 to boost NK cell responses in asthmatics during RV infection to drive down virus load and help reduce the longevity of infection or increase IFN-γ production to suppress allergic inflammation exists; this potential can only be fully realised after carefully analysing the NK cell responses in human models. The observation of deficient IFN-β
responses in asthmatic cells has prompted development of inhaled IFN-β as therapy to prevent/treat exacerbations. However, as IL-15 is induced by IFN-β and promotes beneficial antiviral immune responses that help reduce virus load, IL-15 may serve as a more specific therapy for RV-induced asthma exacerbations, whilst avoiding potentially pathological side effects induced by the more global regulator of antiviral defence. From our mouse model of RV-induced asthma exacerbations it is unclear if the exacerbated IL-15 and NK cell responses are beneficial or contribute to immune-mediated pathology thus, further work blocking airway/lung IL-15 or depleting NK cells in this model would allow us to address this question. In addition, further studies characterising IL-15 production and the NK cell response in human models will provide more insight into the therapeutic potential of IL-15.
7.9. **Summary of Conclusions**

- RV infection in mice induces IL-15 and IL-15Rα upregulation in lung tissue (primarily via epithelial cells) and BAL cells.
- RV-induced IL-15 depends on type I IFN signalling, as demonstrated by the absence of induction in IFNAR1 ko mice.
- RV infection results in the recruitment/expansion and activation (as determined by CD69, IFN-γ and GranzymeB expression) of NK cells in both the airway and lung tissue.
- RV-mediated NK cell responses require pulmonary IL-15 signalling in mice, as demonstrated by the deficient NK cell responses in mice treated with IL-15 neutralising antibody.
- Absence of both RV-induced IL-15 upregulation and NK cell responses (observed in IFNAR1 ko and IL-15Rα ko mice) is associated with an increase in virus load, demonstrating the importance of these responses for control of early virus replication/infection.
- Type I IFN and IL-15 signalling are also important for the recruitment/expansion and activation of CD8+ T cells during RV infection in mice.
- IL-15 signalling is not important for CD4+ T cell response during RV infection in mice.
- NK cell responses, potentially via IFN-γ production, are important negative regulators of Th2 immunity to RV infection.
- RV-induced IL-15 and the associated NK cell responses in lung and BAL are significantly increased by RV infection in mice with exacerbated allergic airways inflammation.
- The majority of accumulating NK cells express IFN-γ however, IL-4 expressing NK cells also arise in the airway and lungs in response to RV infection in mice with allergic airways inflammation, suggesting that a Th2 environment can polarise Th1 innate components of the antiviral pathway, further potentiating allergic inflammation.
7.10. Future Work

7.10.1. The Role of IL-15 and NK cells in RV Replication In Vivo
Our BALB/c mouse model of RV infection is a good representation of human disease, as similar responses including airway neutrophilia and expression of Th1 proinflammatory cytokines are observed. This mouse model also demonstrates lower airway infection/replication [255], shown with cells of the lower airways in vitro and via detection of virus in the lower airways of asthmatics. It is clear that RV-1B does undergo some replication in the mouse. We have previously demonstrated RV replication (via detection of negative sense genomic RNA) in the mouse via in situ hybridisation in lung sections [255]. Here we report increased levels of viral RNA in IL-15Rα ko and IFNAR1 ko compared to wild type mice. However, it is apparent that the replicative ability of RV in this model is limited as there is not a significant increase in infectious virus in wild type mice. This makes it difficult to interpret results as the decline in virus load may be due to the poor replicative ability and/or the immune response in question. To better investigate the role of IL-15-induced NK cell activation for limiting RV replication/infection, it would be beneficial to try different serotypes other than RV-1B that may demonstrate improved replication that lasts for several days.

7.10.2. Cellular Sources of IL-15 Protein in the Lungs
Problems detecting IL-15 protein initially hampered efforts to investigate this specific pathway during RV infection. Having developed a method to confirm increased IL-15 protein levels in whole lung homogenate during RV infection, it would be beneficial to develop more sensitive methods to define the specific cellular sources of IL-15 trans-presentation in our mouse models of RV infection. Flow cytometry would be a useful tool to do so, allowing us to analyse which cells are particularly important for activation of NK cell responses during RV infection; a panel including antibodies to detect CD11b, CD11c, F4/80, Ly6, and IL-15 (surface and intracellular) would facilitate identification of IL-15 expression by DCs, macrophages and neutrophils. As neutrophils are reported to express IL-15 in Th1 inflammatory lung diseases [346], it may be likely that the dominant neutrophilic response in the airway early after RV infection is a significant source of IL-15, and cross-talk between neutrophils and NK cells might be critical for efficient antiviral responses.
7.10.3. Importance of IL-12 Expression for RV-induced NK Cell Responses
As IL-12 is an important regulator of NK cells in many but not all viral infections, it would be useful to analyse expression of IL-12 in response to RV infection in mice, to confidently attribute the observed NK responses to IL-15 signalling. In particular, measure IL-12 expression in our RV infection model with IL-15 neutralising antibody, to prove that the deficient NK cell responses were due to blocking IL-15 and not deficient IL-12 expression in this model.

7.10.4. Role of IL-25 in Mediating Th2 Responses to RV infection
Analysis of IL-25 expression in RV infected IL-15Rα ko and IFNAR1 ko mice would enable us to tease out the mechanism of primary RV infection induced Th2 responses observed in these mice, which exhibited deficient NK cell/IFN-γ production – similar to the responses detected by Kaiko et al. [335] with RSV. In addition, analysis of nuocytes in these mice, which respond to IL-25 and secrete Th2 cytokines as an innate response, would be very interesting as expression of Th2 cytokines in these models occurred at very early time points.

7.10.5. Intranasal Administration of rmIL-15 in IFNAR1 ko Mice During RV Infection
Other experiments to compliment our data include exogenous administration of recombinant mouse IL-15 into the airway during RV infection in IFNAR1 ko mice. If supplementation of IL-15 recovers the deficient NK cell response, we would be able to conclude that type I IFN signalling is not directly required on NK cells during RV infection, and likely acts indirectly on accessory cells to induce upregulation of IL-15, which in turn mediates the NK cell response. If IL-15-mediated rescue of NK cell responses in IFNAR1 ko mice results in reduction of virus load compared to IFNAR1 ko mice without exogenously administered IL-15, we could conclude that IL-15-mediated NK cell responses are important for limiting early virus replication/infection in mice. This would warrant further investigation of the IL-15-NK cell pathway in human models of RV-induced asthma exacerbations, in which virus load and prolonged infections correlates with disease severity and are potentially linked to deficient antiviral (IFN) responses [108, 109].

7.10.6. Importance of NK Cell Cytotoxic Activity During RV Infection
Analysis of the cytotoxic potential of the accumulating NK cell populations during RV infection in our mouse models would be helpful to demonstrate increased functional activity
of these cells in our mouse models. This would be done by incubating purified NK cell populations recovered from lungs and BAL with chromium labelled NK cell susceptible YAC-1 cells, and the level of chromium released into the supernatant due to lysis of YAC-1 cells by NK cells reflects the degree of NK cell cytotoxicity. Alternatively, flow cytometry analysis of CD107a/LAMP-1 (lysosome-associated membrane protein) surface expression on NK cells would serve as a marker of cytotoxic activity. CD107a is present on the surface of NK cells after exocytosis of cytotoxic granules that mediate lysis of target cells.

7.10.7. Role of IL-15 in the Mouse Model of RV-Induced Asthma Exacerbation

Our study using a mouse model of RV-induced allergic inflammation demonstrated associations between IL-15 expression, the NK cell response, virus load and AHR thus, manipulation of this system is necessary to assign causative effects. We suggest that administration of IL-15 neutralising antibody during RV infection in mice with allergic airway inflammation will allow us to further analyse the role of enhanced IL-15 expression on the exacerbated NK cell response. If the NK cell response is impaired as a result of blocking IL-15, analysis of lung virus load would provide valuable information as to whether the exacerbated NK cell response is beneficial for antiviral defence or not, in which case does it significantly contribute to AHR. Based on the results from our primary RV infection mouse model with IL-15 neutralising antibody, we suggest that blocking IL-15 in the airway/lungs during RV infection in mice with allergic airway inflammation will prevent the exacerbated NK cell response, and as a consequence early IFN-γ expression will be impaired; this removes the suppressive effects of IFN-γ on Th2 responses, permitting levels of epithelial-derived IL-25 to increase, further driving expression of Th2 cytokines in allergic mice and greatly enhancing Th2 inflammation in the lungs, significantly contributing to greater AHR. This would support the role of NK cells in regulation of Th2 responses.

7.10.8. IL-15 Expression and NK Cell Responses to RV Infection in Asthmatics and Non-Asthmatics

In humans, the role of IL-15 and NK cells in RV-induced asthma exacerbations remains unclear. Therefore, further work to more carefully analyse expression of cell-associated IL-15 in BAL cells and lung tissue is necessary early after experimental RV infection in asthmatics and healthy volunteers. Similarly, characterisation of the NK cell response in the airway (BAL) by flow cytometry would provide valuable detailed information about this response in RV infected asthmatics, though the choice of time points for analysis would need to be
carefully determined to ensure the peak of NK cell responses were measured. Taken together, the potential of IL-15 as a therapeutic agent to prevent/treat RV-induced asthma exacerbations rests on further in-depth analysis of the above responses in human models.
Chapter 8: References


76. Prussin, C., et al.


132. Bamford, R.N., et al., Interleukin (IL) 15/IL-15 Production by the Adult T-Cell Leukemia Cell Line HuT-102 is Associated with a Human T-Cell Lymphotrophic Virus Type I R Region/IL-15 Fusion Message that Lacks Many Upstream AUGs that Normally Attenuate IL-15 mRNA Translation.


272.


