NOVEL FUNCTIONS OF IL-27 IN INNATE IMMUNITY:
CHARACTERIZATION OF IL-27-INDUCED INFLAMMATORY
RESPONSES IN HUMAN MONOCYTES AND IMPACT OF HIV
INFECTION ON IL-27 EXPRESSION AND FUNCTION

by

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A thesis submitted to the Graduate Program in Microbiology and Immunology in
the Department of Biomedical and Molecular Sciences
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Abstract

Interleukins, cytokines secreted by leukocytes, are predominant messengers modulating immune responses. Interleukin-27 (IL-27), a key immunomodulatory cytokine, functions to induce both pro- and anti-inflammatory effects in various immune cells. IL-27 is a heterodimeric cytokine, composed of IL-27p28 and Epstein-Bar virus induced gene 3 (EBI3) subunits, and binds to a receptor composed of IL-27Rα (WSX-1) and gp130. Initial studies focused on describing IL-27 functions in skewing T helper cell development to a Th1 response, with few reports on functions in monocytes. Thus, in this thesis, I aimed to characterize novel functions of IL-27 in innate immune responses of monocytes. I initially established that IL-27 induced a pro-inflammatory cytokine profile (IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α) mediated via STAT1/3 and NF-κB signaling pathways. Further investigation led to the discovery that IL-27 could enhance LPS responses via upregulation of TLR4 expression and NF-κB signaling. Together, these studies described novel signaling mechanisms (NF-κB and JAK/STAT crosstalk) and gene targets (cytokines and TLR4) of IL-27 that drive inflammatory responses. In continuing the quest for novel IL-27 functions in innate immunity, I reported IL-27 can upregulate expression of the IFN-responsive, antiviral protein called BST-2. My results showing IL-27-induced expression of BST-2 mRNA and cell surface protein were supported by previous studies reporting IL-27-induced expression of other antiviral molecules. Furthermore, previous studies showed IL-27 could inhibit HIV replication via antiviral gene induction, pointing to potential for IL-27 immunotherapies. In light of the posited role for IL-27 in therapeutics, it became inherently critical to describe how IL-27 functions in the setting of HIV infection. Thus, in my final thesis chapters, I described the effect of HIV infection on IL-27 expression and functions, addressing a substantial void in the literature. Interestingly, a trend of decreased IL-27 expression and significant
Impairment of IL-27-induced gene expression was observed in HIV infection. Therefore, decreased circulating IL-27 and decreased IL-27 responsiveness may collectively dysregulate IL-27 function in HIV. This thesis describes novel, IL-27-driven, proinflammatory responses, and highlights impairment of IL-27 function in HIV infection. This work bridged a gap in knowledge of IL-27 functions in monocytes and highlighted multifaceted mechanisms underlying immunoregulation by IL-27.
Co-Authorship


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# Table of Contents

Abstract ............................................................................................................................... ii
Co-Authorship ...................................................................................................................... iv
Acknowledgements ........................................................................................................... vi
Table of Contents ............................................................................................................... ix
List of Figures ...................................................................................................................... xv
List of Tables ....................................................................................................................... xvii
List of Abbreviations ......................................................................................................... xviii

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

1.1 Monocyte/Macrophage Development and Activation ................................................... 2
1.2 Toll-like Receptors (TLRs) ............................................................................................ 3
1.2.1 TLR expression and function ................................................................................. 3
1.2.2 Toll-like receptor 4 (TLR4) .................................................................................. 3
1.3 Overview of Cytokines and Signaling ......................................................................... 6
1.3.1 Cytokines and Signaling via JAK/STAT .................................................................. 6
1.3.2 General NF-κB signaling ....................................................................................... 10
1.4 IL-27 Biology ................................................................................................................. 13
1.4.1 The IL-27 cytokine and receptor ............................................................................ 13
1.4.2 IL-27-induced signaling ......................................................................................... 15
1.5 IL-27 Functions .............................................................................................................. 18
1.5.1 IL-27 inflammatory functions ................................................................................. 18
1.5.2 IL-27 functions on Th cell differentiation ............................................................... 20
1.6 HIV Infection ............................................................................................................... 20
1.6.1 HIV infection and host restriction factors ............................................................. 20
1.6.2 HIV and monocyte functions ............................................................................... 22
2.4.3 Inhibition of NF-κB DNA binding activity blocks IL-27-induced cytokine expression .......................................................... 55
2.5 Discussion .................................................................................................................. 59
2.6 References .................................................................................................................. 65

Chapter 3: IL-27 enhances LPS-induced proinflammatory cytokine production via upregulation of TLR4 expression and signaling in human monocytes ......................... 68
3.1 Abstract ...................................................................................................................... 69
3.2 Introduction ............................................................................................................... 69
3.3 Materials and Methods .......................................................................................... 72
  3.3.1 Monocyte Isolation ............................................................................................ 72
  3.3.2 Cell lines, Cell Culture and Reagents .............................................................. 72
  3.3.3 ELISA ................................................................................................................ 72
  3.3.4 RNA Isolation and Reverse Transcriptase-PCR (RT-PCR) ......................... 73
  3.3.5 Flow Cytometry ............................................................................................... 73
  3.3.6 Western Blotting .............................................................................................. 74
  3.3.7 Electrophoretic Mobility Shift Assay (EMSA) ................................................ 74
  3.3.8 Indirect Immunofluorescence Microscopy .................................................... 74
  3.3.9 NF-κB Transcription Factor Assay ................................................................. 75
3.4 Results ...................................................................................................................... 75
  3.4.1 IL-27 pre-treatment enhances LPS-induced proinflammatory cytokine expression ............................................................................................................ 75
  3.4.2 IL-27 induces TLR4 expression on CD14+ monocytic cells .............................. 76
  3.4.3 IL-27-induced TLR4 expression is mediated by JAK2 and STAT3 signaling . 79
  3.4.4 IL-27-induced TLR4 expression is mediated via NF-κB activation and IL-27-induced NF-κB activation is dependent on JAK/STAT signaling ................................... 83
3.4.5 IL-27 does not increase LPS binding to monocytes ........................................87
3.4.6 IL-27 induces enhanced TLR4 membrane expression and increased co-localization of CD14 and TLR4 ................................................................. 89
3.4.7 IL-27 priming augments LPS-induced NF-κB activation ....................................92
3.5 Discussion ...........................................................................................................96
3.6 References ........................................................................................................102

Chapter 4 : IL-27 regulates BST-2 expression independently of type-I IFN on human monocytes and T cells ................................................................. 106
4.1 Abstract ...........................................................................................................107
4.2 Introduction .....................................................................................................108
4.3 Methods ..........................................................................................................110
  4.3.1 Cell culture and reagents ...........................................................................110
  4.3.2 Flow Cytometry .......................................................................................110
  4.3.3 Western Blot ............................................................................................111
  4.3.4 RNA isolation and RT-PCR ......................................................................111
  4.3.5 Indirect Immunofluorescence Microscopy ...............................................112
  4.3.6 Statistical Analysis ..................................................................................112
4.4 Results .............................................................................................................113
  4.4.1 IL-27 induces BST-2 cell surface expression on human monocytes and T cells ...................................................................................................................113
  4.4.2 Despite similar signaling pathways to IL-27, IL-6 does not induce BST-2 on monocytes or T cells ......................................................................................114
  4.4.3 IL-27 enhances de novo synthesis of BST-2 ................................................114
  4.4.4 IL-27 induces BST-2 mRNA expression ....................................................118
4.4.5 IL-27 induces BST-2 expression independently of a type I IFN intermediate.
........................................................................................................................................122

4.5 Discussion .....................................................................................................................127

4.6 References ..................................................................................................................131

Chapter 5: *Impact of HIV infection, highly active anti-retroviral therapy, and hepatitis C co-infection on serum interleukin-27* ........................................................................................................134

5.1 Summary .....................................................................................................................135

5.2 Introduction ................................................................................................................135

5.3 Methods ......................................................................................................................135

5.3.1 Serum Collection ..................................................................................................135

5.3.2 IL-27 Enzyme-Linked Immunosorbent Assay (ELISA) ......................................136

5.3.3 Statistical Analysis ...............................................................................................136

5.4 Results .......................................................................................................................136

5.4.1 IL-27 and HIV viral load are negatively correlated..............................................139

5.4.2 HIV-HCV co-infection is associated with lower IL-27 .......................................139

5.4.3 IL-27 varies with CD4 T cell counts ....................................................................139

5.5 Discussion ................................................................................................................140

5.6 References .................................................................................................................142

Chapter 6: *IL-27-induced gene expression is downregulated in HIV-infected subjects* 144

6.1 Abstract .....................................................................................................................145

6.2 Introduction ................................................................................................................146

6.3 Methods ......................................................................................................................147

6.3.1 Study Participants ................................................................................................147

6.3.2 Cell Culture and Monocyte Isolation .................................................................148

6.3.3 Western Blotting .................................................................................................149
6.3.4 RNA Isolation and Real time-PCR .................................................................149
6.3.5 Flow Cytometry .........................................................................................150
6.3.6 ELISA ..........................................................................................................150
6.3.7 Statistical Analysis .....................................................................................150
6.4 Results ...........................................................................................................151
  6.4.1 IL-27-induced STAT1 and STAT3 activation is intact in HIV infection ....151
  6.4.2 IL-27 induces expression of gp130, but not the counterpart receptor subunit, WSX-1, in HIV-negative monocytes ..................................................................................................................151
  6.4.3 IL-27-induced cell surface expression of gp130 is impaired in HIV infection 153
  6.4.4 IL-27-induced mRNA expression of gp130 is impaired in HIV infection ....156
  6.4.5 IL-27-induced cytokine production is downregulated in HIV infection ....158
6.5 Discussion .......................................................................................................158
6.6 References ......................................................................................................158

Chapter 7: General Discussion and Future Perspectives .........................................169
  7.1 General Discussion .......................................................................................170
  7.2 Future Perspectives ......................................................................................179
  7.3 References ....................................................................................................184
Appendix A: Research ethics board (REB) approval .............................................188
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The LPS-TLR4 signaling pathway</td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>General cytokine signaling via the JAK/STAT pathway</td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>General NF-κB signaling</td>
<td>11</td>
</tr>
<tr>
<td>1.4</td>
<td>IL-27 is part of the IL-12 family of heterodimeric cytokines and IL-6 family of heterodimeric cytokine receptors</td>
<td>14</td>
</tr>
<tr>
<td>1.5</td>
<td>The JAK/STAT signaling molecules activated by IL-27</td>
<td>16</td>
</tr>
<tr>
<td>2.1</td>
<td>IL-27 induces expression pro-inflammatory cytokines and chemokines in primary human monocytes</td>
<td>46</td>
</tr>
<tr>
<td>2.2</td>
<td>IL-27 induced expression of IL-6, IP-10, MIP-1-α, MIP-1-β, and TNF-α in primary human monocytes</td>
<td>49</td>
</tr>
<tr>
<td>2.3</td>
<td>IL-27 induces expression of IL-6, IP-10, MIP-1-α, MIP-1-β, and TNF-α in THP-1 cells.</td>
<td>52</td>
</tr>
<tr>
<td>2.4</td>
<td>IL-27-induced cytokine production is mediated by STAT1 and STAT3 activation.</td>
<td>53</td>
</tr>
<tr>
<td>2.5</td>
<td>IL-27-induced cytokine production is dependent on NF-κB activation.</td>
<td>57</td>
</tr>
<tr>
<td>3.1</td>
<td>IL-27 pre-treatment enhances LPS-induced IL-6, TNF-α, MIP-1α, and MIP-1β.</td>
<td>77</td>
</tr>
<tr>
<td>3.2</td>
<td>IL-27 induces TLR4 expression on CD14+ monocyctic cells.</td>
<td>78</td>
</tr>
<tr>
<td>3.3</td>
<td>JAK2 and STAT3 signaling mediates IL-27-induced TLR4 expression.</td>
<td>81</td>
</tr>
<tr>
<td>3.4</td>
<td>NF-κB activation mediates IL-27–induced TLR4 expression</td>
<td>86</td>
</tr>
<tr>
<td>3.5</td>
<td>IL-27 priming does not enhance LPS binding to monocytes</td>
<td>88</td>
</tr>
<tr>
<td>3.6</td>
<td>IL-27 induces enhanced TLR4 membrane expression and increased co-localization of CD14 and TLR4</td>
<td>91</td>
</tr>
</tbody>
</table>
Figure 3.7: IL-27 priming augments LPS-induced NF-κB signaling........................................95

Figure 4.1: IL-27- and IFN-induced BST-2 cell surface expression on monocytes and T cells.................................................................116

Figure 4.2: Despite similar signaling pathways to IL-27, IL-6 does not induce BST-2 on monocytes or T cells. ........................................117

Figure 4.3: IL-27 enhances intracellular BST-2 expression.........................................................120

Figure 4.4: IL-27 enhances mRNA expression of BST-2.............................................................121

Figure 4.5: IL-27-induced intracellular BST-2 is found within the secretory pathway....123

Figure 4.6: IL-27 induces BST-2 expression independently of a type-I IFN intermediate. ......................................................................................126

Figure 5.1: The effect of HIV viral load, HCV co-infection, and CD4 T cell counts on IL-27 expression in human serum. .................................................................138

Figure 6.1: IL-27-induced STAT1 and STAT3 activation is not altered in HIV infection. ......................................................................................................................152

Figure 6.2: IL-27 induces expression gp130 in monocytes from HIV negative individuals, a function that is impaired in monocytes from HIV positive individuals. ............155

Figure 6.3: IL-27-induced mRNA expression of gp130 is impaired in HIV infection......157

Figure 6.4: IL-27-induced cytokine production is downregulated in HIV infection........160

xvi
List of Tables

Table 2.1: Cytokine primers.................................................................40
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Virus</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>APOBEC</td>
<td>Apolipoprotein B mRNA-editing, Enzyme-Catalytic, Polypeptide-like</td>
</tr>
<tr>
<td>ART</td>
<td>Anti-retroviral Therapy</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>BST-2</td>
<td>Bone Marrow Stromal Antigen - 2</td>
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<tr>
<td>CAPE</td>
<td>Caffeic Acid Phenethyl Ester</td>
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<tr>
<td>CC</td>
<td>Cold Competitor</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>cDNA</td>
<td>Complimentary Deoxyribonucleic Acid</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>ECD</td>
<td>Electron Coupled Dye</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>EMSA</td>
<td>Electrophoretic Mobility Shift Assay</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescin-isothiocyanate</td>
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<tr>
<td>GAS</td>
<td>Gamma-Interferon Activated Sites</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage- Colony Stimulating Factor</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GRR</td>
<td>Gamma-Interferon Response Region</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>HAART</td>
<td>Highly Active Anti-retroviral Therapy</td>
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<td>HEK</td>
<td>Human Embryonic Kidney Cell Line</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>IKK</td>
<td>IκB Kinase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
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<tr>
<td>IP-10</td>
<td>Interferon Gamma-induced Protein-10</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon Stimulated Response Elements</td>
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<tr>
<td>IκB</td>
<td>Inhibitor Kappa B</td>
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<tr>
<td>JAK</td>
<td>Janus Kinase</td>
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<tr>
<td>LBP</td>
<td>LPS Binding Protein</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>M-CSF</td>
<td>Macrophage – Colony Stimulating Factor</td>
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<td>MD-2</td>
<td>Myeloid Differentiation Protein-2</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MTA</td>
<td>5’-deoxy-5’(methylthio) adenosine</td>
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<tr>
<td>NES</td>
<td>Nuclear Export Signal</td>
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<td>NF-κB</td>
<td>Nuclear Factor Kappa B</td>
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<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PeCy5</td>
<td>Phycoerythrin-Cyanine 5</td>
</tr>
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<tr>
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<td>Polyvinylidene Fluoride</td>
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<tr>
<td>ROI</td>
<td>Reactive Oxygen Intermediate</td>
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<td>RORyt</td>
<td>RAR-related Orphan Receptor gamma</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
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<tr>
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<td>Reverse Transcriptase – Polymerase Chain Reaction</td>
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<tr>
<td>SD-1029</td>
<td>Jak2 Inhibitor</td>
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<tr>
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<td>Suppressor of Cytokine Signaling</td>
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<td>SS</td>
<td>Supershift</td>
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<td>ST3 VII</td>
<td>STAT3 Inhibitor</td>
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<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
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<td>TNF</td>
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<td>TRIM</td>
<td>Tripartite Motif</td>
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<td>Tyk2</td>
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CHAPTER 1:

INTRODUCTION
1.1 Monocyte/Macrophage Development and Activation

Early in hematopoiesis, a stem cell will commit to differentiate down a lymphoid lineage, giving rise to lymphocytes (B, T and NK cells), or a myeloid/erythroid lineage, giving rise to erythrocytes, leukocytes, and megakaryocytes\(^1\). Later in hematopoiesis in the bone marrow, a granulocyte-monocyte progenitor will differentiate into a promonocyte and migrate into the blood, where it will further differentiate into a monocyte. Monocytes circulate in the blood stream where they function in immune surveillance and coordination of immune responses, via cytokine production\(^2\). All human monocytes express cell surface CD14, and can be further classified into subsets based on CD16 expression\(^3\), with the CD16-negative cells representing the most abundant ‘classical’ monocyte population\(^4\). Within this thesis I have worked with CD14+ monocytes, and did not specifically deplete CD16+ populations in my experiments. Being in circulation, monocytes can respond to chemokine gradients induced homeostatically or in response to infection, resulting in activation and homing to specific tissues where they can replenish macrophage/dendritic cell populations and aid in clearance of infection\(^2\).

Monocyte activation includes the differentiation into a macrophage, resulting in enhanced cellular characteristics, such as: increased number and complexity of intracellular organelles, increased phagocytic capability, increased production of hydrolytic enzymes, and increased cytokine production\(^5\). Indeed, monocytes/macrophages are fundamental to pathogen clearance and inflammation, representing key players in innate immunity, while also harboring the ability to initiate adaptive immune responses via antigen presentation and T cell activation. The bipolar functions of monocytes/macrophages on innate and adaptive immunity result from their dominant role in secreting cytokines and chemokines to coordinate responses of various
immune cells. Furthermore, monocyte/macrophage function itself is also regulated by cytokines present in the cell environment\textsuperscript{6,7}. Since monocytes play such a dominant role in coordinating immune responses via cytokine activities, the focus of my thesis has been to elucidate the effect of IL-27 on monocyte functions.

1.2 Toll-like Receptors (TLRs)

1.2.1 TLR expression and function

TLRs play a critical role in early innate immunity to invading pathogens by sensing microorganisms. These evolutionary conserved receptors recognize highly conserved structural motifs only expressed by microbial pathogens, called pathogen-associated molecular patterns (PAMPs). PAMPs include various bacterial cell wall components such as lipopolysaccharide (LPS), peptidoglycans and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA\textsuperscript{8}. Upon recognition of PAMPs, intracellular signaling cascades activate transcription factors like NF-κB, and subsequent expression of proinflammatory cytokines helps resolve infection, while at the same time, induction of effector cytokines directs adaptive immune responses\textsuperscript{9}. To date, 10 different human TLRs have been characterized\textsuperscript{10-14}, and cytokine-mediated regulation of TLR expression remains poorly understood. TLR expression analysis in primary human leukocytes showed professional phagocytes express the most varied TLR profile, with CD14+ mononuclear cells expressing the greatest amount of TLR2, 4, and 8\textsuperscript{15}.

1.2.2 Toll-like receptor 4 (TLR4)

In monocytes, the expression of TLR4 is important in mediating pro-inflammatory cytokine production in response to bacterial infection. Lipopolysaccharide (LPS), a
component of gram-negative bacterial cell membranes, is the primary ligand characterized to bind TLR4 and induce inflammation\textsuperscript{16, 17}. However, effective TLR4 signaling requires other binding partners. Indeed, LPS recognition occurs via the coordinated sequence of binding events between both soluble and cell membrane proteins, including LPS-binding protein (LBP), myeloid differentiation protein-2 (MD-2) and CD14\textsuperscript{18} (Figure 1.1). LPS recognition first occurs via the soluble LBP protein, which facilitates the interaction of LPS with CD14\textsuperscript{19}. CD14, anchored in the cell membrane or in soluble form, is a key TLR4 co-receptor, pivotal in the transfer of LPS to the MD2/TLR4 complex to initiate signal cascades\textsuperscript{20,21}. MD-2, another soluble protein, is responsible for recognition of LPS in MD-2/TLR4 complex, and has been shown to bind LPS in the absence of TLR4\textsuperscript{22}. Upon LPS binding, TLR4 oligomerizes, and initiates intracellular signaling cascades via protein-protein interactions of its own Toll-interleukin-1 receptor (TIR) domains with various adaptor molecules which can drive signal transduction, including: MyD88 (myeloid differentiation primary response gene 88), TIRAP/Mal (MyD88-adaptor-like), TRIF (TIR domain-containing adaptor inducing IFN-β), and TRAM (TRIF-related adaptor molecule)\textsuperscript{23}. Although different TLRs utilize specific sets of these adaptor proteins to mediate downstream signaling, TLR4 is unique in that it utilizes all of these adaptor molecules, resulting in a broad range of pro-inflammatory effects\textsuperscript{23}. Thus, the sequential binding events of soluble, cell membrane and cytoplasmic components is required to elicit TLR4 responses (Figure 1.1). Since CD14 plays a complimentary role in LPS recognition and subsequent responses, the expression and signal transduction of TLR4 and CD14 in response to IL-27 is examined in chapter 3 of this thesis.
Figure 1.1: The LPS-TLR4 signaling pathway. LPS is first recognized by soluble LBP, which facilitates interaction with CD14. CD14 subsequently transfers LPS to the MD-2/TLR4 complex, which initiates signaling cascades via adaptor proteins (Mal, MyD88, TRAM, and TRIF) recruited to the TIR domains of TLR4. Adaptor proteins can drive MyD88-dependent signal cascades, leading to proinflammatory cytokine production, or MyD88-independent cascades, leading to Type I interferon gene expression. NF-κB is a common transcription factor activated by both pathways, driving pro-inflammatory gene expression (adapted from Lu Y.-C. et al. 2008).
1.3 Overview of Cytokines and Signaling

1.3.1 Cytokines and Signaling via JAK/STAT

Cytokines are paramount to the generation of timely, effective, and appropriate immune responses. These small molecular weight, secreted proteins drive a wide variety of cellular processes by transmitting signals between cells, effectively acting as ‘messengers’ of the immune system. In general, cytokine signaling mechanisms are quite similar in their initiation. As depicted in Figure 1.2, cytokines first bind to their low affinity receptor alpha chains that are cytokine specific. The alpha unit then associates non-covalently with a common signal-transducing unit, the β receptor chain. The resulting dimeric receptor is imparted with an improved affinity for the cytokine bound, and can now transduce signals into the cell\textsuperscript{24}. Cytokine receptor families exist whereby groups of cytokines utilize the same β receptor subunits. In such situations, various cytokines with broad functions can induce similar activation signals, resulting in functional redundancy\textsuperscript{24,25}. For example, IL-6 receptor family members utilize the common β subunit, gp130, shared with many cytokines, including IL-27, IL-6, oncostatin M (OSM), IL-11, leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), ciliary neurotrophic factor (CNTF), and neuropoietin (NP)\textsuperscript{26}. Thus, numerous cytokines can share receptor subunits and signaling pathways, resulting in redundancy of biological effects. This redundancy of cytokine signaling provides many parallel avenues of immune regulation to ensure appropriate and effective immune responses. On a similar note to that of cytokine receptor families, structurally related cytokines are also grouped into families. Pertinent to this thesis is the IL-12 cytokine family, a unique group of cytokines that are heterodimeric, including IL-12, IL-23, IL-27,
Figure 1.2: General cytokine signaling via the JAK/STAT pathway. In a basal state, JAKs and STATs are unphosphorylated (left side), with JAKs bound to cytokine receptors and STATs resting in the cytoplasm. Cytokines binding occurs via initial recognition by receptor alpha (α) chains, followed by dimerization with receptor beta (β) chains to initiate signal transduction. Upon receptor dimerization, JAKs are autophosphorylated, then phosphorylate tyrosine residues on the cytoplasmic tails of cytokine receptors to serve as docking sites for recruitment of STATs. Encircled 'p' denotes phosphate group. Upon phosphorylation of STATs, dimerization occurs to form functional transcription factors which translocate to the nucleus and induce gene expression.
and IL-35. Individual family members are composed of distinctly different combinations of cytokine and receptor subunits, imparting a unique biological response to each cytokine, dependent on binding partners. In particular, IL-12-family cytokines have unique STAT signaling signatures and effects on Th cell differentiation and maintenance, processes that will be further discussed in relation to IL-27 functions later in this introduction.

The janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is the main mechanism of cytokine signaling and represents a relatively rapid membrane-to-nucleus signaling mechanism. To date, four JAK family members have been described, JAK1-3 and Tyk2, and seven STAT members, STAT1-6, 5a and 5b. In a resting state, JAKs are inactive and associated with the cytoplasmic domains of cytokine receptors via binding of repeated JAK homology domains (JH3-JH7) located at the amino termini of JAK proteins. On the other hand, the C terminus contains the JH1 domain responsible for the kinase activity of JAKs that drives subsequent signal transduction. Adjacent JH2 domains are structurally similar to JH1 domains, although they lack enzymatic activity, and thus, they are regarded as ‘pseudokinase’ domains. JH2/pseudokinase domains have been previously shown to play a role in regulating kinase activity of JH1 domains, thus representing a mechanism by which JAKs remain inactive in a resting state. Therefore, JAKs are found inactive and pre-associated with cytoplasmic tails of cytokine receptors enabling a rapid response upon receptor engagement. As seen in Figure 1.2, upon cytokine binding, the receptor dimerizes (α and β subunits) and receptor-associated JAKs become autophosphorylated. Activated JAKs phosphorylate specific residues on cytoplasmic domains of the receptor, creating a
docking site for STATs\textsuperscript{34}. STATs recognize the phosphorylated receptor via their Src (sarcoma) Homology 2 (SH2) domains which are specific to unique cytokine receptor chains\textsuperscript{35}. Additionally, STAT proteins themselves have conserved tyrosine residues near their SH2 domain which can act as substrates for phosphorylation\textsuperscript{35}. Upon phosphorylation, STATs translocate from the receptors and dimerize to form functional transcription factors. The possibility for STAT homo- and hetero-dimers offers one mechanism of signal specificity and gene regulation, unique to each cytokine. Dimerized STATs translocate to the nucleus to induce targeted gene expression via binding to conserved enhancer elements, most commonly interferon stimulated response elements (ISRE), gamma-interferon activated sites (GAS), and gamma-interferon response regions (GRR)\textsuperscript{34}.

Since one of the defining features of JAK/STAT signaling is the relatively fast signal cascade to gene expression, there is also a quick shut-off of these signaling pathways. Indeed, STATs can be inactivated by dephosphorylation, degradation, nuclear export, negative feedback inhibition by proteins such as SOCS-family proteins (suppressor of cytokine signal), and competitors for STAT binding sites\textsuperscript{30,36}. Briefly, the classical negative regulatory SOCS proteins are rapidly expressed upon cytokine stimulation and inhibit cytokine signaling pathways by a variety of mechanisms, including inactivation of JAKs, blocking access of STATs to binding sites, and ubiquitination of these signaling proteins\textsuperscript{37,38}. Due to the importance of phosphorylation cascades in JAK/STAT signaling, the negative regulation via phosphatase activity has also been described. For example, phosphatases can dephosphorylate JAK proteins and cytokine receptors\textsuperscript{39,40} or act directly upon STAT proteins to return them to their basal, unphosphorylated state\textsuperscript{41,42}. 

9
Although many different inhibitory mechanisms have been proposed, in general, the negative regulation of JAK/STAT pathways is continuing to broaden in scope. Overall, cytokine signaling via JAK/STAT pathways is intricately regulated with many opportunities for regulation of gene expression, including differences in DNA-binding specificities of STAT homo- versus heterodimers, redundancy of STATs in regulating similar genes, and most interestingly, the crosstalk of JAK/STAT with other signaling pathways, like NF-κB.

1.3.2 General NF-κB signaling

Since cytokines can activate various other highly conserved signaling proteins, in addition to JAK/STAT molecules, an understanding of the cross talk between the various signaling pathways is important in determining cytokine specificity. Previous studies, including my own, have demonstrated a role for JAK/STAT signaling in the activation of NF-κB, and a strong link has been shown between STAT3 and NF-κB activation in various cancer model systems and in general cytokine signaling. NF-κB transcription factors have an evolutionarily conserved role in eliciting innate and adaptive immune responses, driven by the dimerization of different combinations of family members, which include: p50, p52, p65 (Rel A), c-Rel, and RelB. The cascade of events leading to activation of these transcription factors involves the coordinated interactions of activators and repressors. As seen in Figure 1.3, NF-κB dimers are basally found in a resting state, bound by repressive IκB proteins. Upon cytokine receptor binding, there is recruitment of adaptor proteins, and consequently, IκB kinases (IKKs) become activated. IKKs phosphorylate the repressive IκB proteins, leading to ubiquitination and targeted degradation of IκBs. This renders NF-κB dimers free to
Figure 1.3: General NF-κB signaling. NF-κB dimers are found in a non-active state in the cytoplasm, bound by repressiveIkB proteins (left side). Upon cytokine receptor binding, IkB kinases (IKKs) become activated, which subsequently phosphorylate IkB proteins, resulting in ubiquitination and proteasomal degradation of IkB. The removal of IkB renders NF-κB dimers free to translocate to the nucleus to regulate gene expression. The precise point of JAK/STAT involvement in the NF-κB signaling cascade has yet to be elucidated.
translocate to the nucleus and bind to κB sites in enhancer/promoter sequences, where they can recruit co-activators or co-repressors to modulate gene expression\textsuperscript{49}. For the most part, the NF-κB family members hetero- or homo-dimerize to activate transcription. More specifically, heterodimers of p50 and p65 are most often attributed to upregulated gene expression and inflammation\textsuperscript{51}. However, to a lesser extent, homo-dimerization of the specific p50 or p52 members can repress gene expression\textsuperscript{52-55}.

The shut-off of NF-κB signaling events remains less well described in comparison to the negative regulation of JAK/STAT signaling. Studies have shown the ability of IκB proteins to shuttle DNA-bound NF-κB complexes back out of the nucleus via nuclear-export sequences (NES)\textsuperscript{56, 57}, however NES sequences have not been found in all IκB proteins\textsuperscript{58, 59}, underlying the presence of additional negative regulatory mechanisms. Additionally, there is controversy on how IκB proteins enter the nucleus. Indeed, studies have shown nuclear-localization sequences (NLS) on IκB proteins\textsuperscript{60}, and a ‘piggy-back’ proposal has been made, whereby IκB proteins can shuttle into the nucleus in association with other NLS-containing proteins\textsuperscript{61}. More recent evidence suggests nuclear import of NF-κB-IκB complexes might occur via incomplete masking of the NF-κB NLS by the IκB proteins\textsuperscript{59}. Furthermore, a few studies reviewed in Ghosh and Karin (2002) described a model in flux, whereby shuttling into and out of the nucleus was continuous\textsuperscript{50}. However, based on the availability and exposure of NES and NLS sequences, the majority of NF-κB complexes are found resting in the cytoplasm. Relating these events back to JAK/STAT signaling, reports have shown that SOCS1, in addition to repressing JAK/STAT, can also decrease NF-κB transcriptional activity, although the precise
mechanisms are not well defined\textsuperscript{62,63}. Thus, much is left to be described on the negative regulation of NF-κB signaling.

Although I have provided some insight on the mechanisms of cytokine signaling, it must be recognized that transcriptional regulation of genes results from the coordination of a myriad of signaling factors, whereby STATs and NF-κB represent only two key players in the overall game of gene activation. With the above understanding of the main signaling mechanisms used by cytokines in general, a shift in focus to the distinct structure of the IL-27 cytokine and receptor, along with IL-27-specific signaling pathways and biological effects will now be made.

1.4 IL-27 Biology

1.4.1 The IL-27 cytokine and receptor

IL-27 was first identified in 2002 by Pflanz \textit{et al.} and is a heterodimeric cytokine composed of an IL-27p28 (p28) subunit and Epstein-Bar virus induced gene 3 (EBI3) subunit\textsuperscript{64}. As depicted in Figure 1.4, the IL-27p28 subunit, like IL-12p35, is a four-helical cytokine subunit, whereas EBI3 structurally resembles the p40 subunit of IL-12 and shares homology with IL-6Rα\textsuperscript{64-66}. In humans, IL-27p28 and EBI3 are predominantly co-expressed by antigen presenting cells (APCs) such as monocytes/macrophages and dendritic cells (DCs)\textsuperscript{64}. IL-27, induced by various bacterial, parasitic, and host-derived inflammatory stimuli, modulates the delicate balance of infection-induced immune responses\textsuperscript{67}. Indeed, TLR activation drives expression of IL-27, with TLR ligands LPS and Poly(I:C) shown to induce expression of both cytokine subunits\textsuperscript{68,69}. Further evidence on the role of TLRs in IL-27 expression was the report that expression of the
Figure 1.4: IL-27 is part of the IL-12 family of heterodimeric cytokines and IL-6 family of heterodimeric cytokine receptors. The IL-12 receptor, which has homology to gp130, consists of IL-12Rβ1 and IL-12Rβ2. IL-12 is a heterodimeric cytokine, composed of a light chain (IL-12p35) and a heavy chain (IL-12p40), linked by a disulphide bond, as shown. IL-6 has a single subunit cytokine which can bind to its receptor composed of two gp130 subunits, shared with the IL-27 receptor, and a unique IL-6Rα subunit. IL-6 can also bind to soluble IL-6Rα (sIL-6Rα), and elicit signaling on broader cell types which do not endogenously express IL-6Rα. The IL-27 heterodimeric cytokine is composed of EBI3 (Epstein–Barr virus induced gene 3) and IL-27p28 subunits, and binds to a receptor composed of gp130 (β chain) and WSX-1 (IL-27Rα chain). Figure adapted from Gee et al. 2009\textsuperscript{27}. 
EBI3 subunit was significantly reduced in cells lacking TLR2, TLR4, TLR9, and MyD88 (a TLR-induced signaling molecule)\textsuperscript{70}. Taken together, expression of IL-27 is driven by pro-inflammatory responses of APCs.

The IL-27 receptor (IL-27R) is heterodimeric, composed of the IL-27Rα (WSX-1) subunit, unique for the binding of IL-27, and the β receptor subunit, gp130, shared with the receptor for IL-6 and numerous other cytokines (Figure 1.4)\textsuperscript{26,64,71}. IL-27 is unique amongst cytokines because the IL-27R complex is used only by IL-27 and currently no other cytokines have been shown to signal through this receptor. The WSX-1 chain was identified as a result of sequence homology with the gp130 chain and as such is a characteristic type I cytokine receptor\textsuperscript{72,73}. Although IL-27 can bind with low affinity to WSX-1 in the absence of gp130\textsuperscript{71,74}, for effective signal transduction both IL-27R subunits must be co-expressed\textsuperscript{71}. A wide variety of cells can respond to IL-27, as the co-expression of IL-27R subunits has been found on endothelial cells, mast cells, B cells, monocytes, Langerhan’s cells, dendritic cells, and Th cells\textsuperscript{71,74-77}. IL-27-mediated functions are varied within these different cell types, and the pleiotropic effects of IL-27 are often underpinned by differential signaling pathways activated, as discussed in more detail below.

1.4.2 IL-27-induced signaling

Many receptor alpha chains have short or absent cytoplasmic domains resulting in the dependency on beta chains to generate signal transduction. IL-27Rα (WSX-1) has a short cytoplasmic domain compared to gp130, but does have conserved tyrosine residues that impart the ability to activate JAK/STAT proteins\textsuperscript{72}. As seen in Figure 1.5
Figure 1.5: The JAK/STAT signaling molecules activated by IL-27. IL-27 receptor chains are associated with JAK1, JAK2, and Tyk2, which can subsequently activate STAT1, STAT3, STAT4 or STAT5. IL-27 predominantly signals through STAT1 (shown enlarged), and to a lesser extent, STAT3 (adapted from Gee et al. 2009).
WSX-1 can physically associate with JAK1 and upon phosphorylation of tyrosine residues can activate STAT1\textsuperscript{73,78}. Furthermore, CD4+ T cell stimulation with IL-27 leads to activation of JAK2, Tyk2, STAT3, STAT5, and weakly STAT4\textsuperscript{76,79,80}. However, in monocytes IL-27 only activates STAT1 and STAT3\textsuperscript{71}. Overall, in terms of IL-27 signaling, the WSX-1 subunit contributes predominantly to STAT1 phosphorylation and gp130 to STAT3 phosphorylation\textsuperscript{71}. Furthermore, it is evident that IL-27-induced STAT signals can be modulated across different cell types and activation states. For example, in early activated CD4 T cells (anti-CD3/CD28 for 24hrs) IL-27 induced STAT1/3 activation, while in fully activated CD4+ T cells (anti-CD3/CD28 for 7 days), IL-27 stimulation resulted in only STAT3 activation, with STAT1 phosphorylation markedly reduced relative to levels observed in early activated CD4+ T cells\textsuperscript{81}. The biological significance of this differential activation has yet to be described. However, this observation indicates that IL-27 signaling is highly regulated in immune responses.

The negative regulation of IL-27-induced JAK/STAT signaling is not fully described, however, it is known that IL-27 can induce SOCS3 expression in CD4+ T cells and macrophages, and to a lesser extent, SOCS1 expression\textsuperscript{82-84}. More specifically, SOCS3-mediated inhibition of the gp130 receptor chain has been described as SOCS3 binding to gp130, which thereby inhibits JAK activation and consequent inhibition of downstream STAT3 activation\textsuperscript{85,86}. An additional study has also shown the ability of SOCS3 to directly inhibit JAK2 activation\textsuperscript{87}. Most recently, Zeitvogel et al. (2012) highlighted the vital importance of IL-27-induced SOCS3 in regulating inflammation in keratinocytes, and proposed that a deficiency in IL-27-induced SOCS3 expression may contribute to chronic skin inflammation\textsuperscript{83}. 
In chapter 3 of this thesis, my work identified crosstalk of IL-27-induced JAK/STAT and NF-κB signaling pathways. Indeed, two of my studies indicated the ability of IL-27 to signal via NF-κB in primary monocytes and the THP-1 monocytic cell line. Although the crosstalk of JAK/STAT and NF-κB have been characterized by a few limited studies, the specific ability of IL-27 to activate NF-κB in monocytes had not been previously reported. However, IL-27-induced NF-κB activation in human keratinocytes had been reported. Thus, mechanisms of how IL-27 mediates NF-κB activation are poorly understood. In terms of IL-27-induced gene expression, it is likely that STATs and NF-κB work in concert at promoters to drive efficient expression of IL-27-responsive genes. Indeed, the cooperation of STAT3 and NF-κB in the regulation of gene expression has previously been described, and includes cooperative binding in adjacent regions of promoter sequences and physical interactions between the two transcription factors.

1.5 IL-27 Functions

1.5.1 IL-27 inflammatory functions

The primary function of IL-27, like many cytokines, is difficult to classify, as there is no single functional silo that encompasses all of the reported IL-27 effects. Indeed, numerous pro- and anti-inflammatory activities have been attributed to IL-27, and the outcome of the IL-27 response is highly dependent on cell type, activation state and infection status of the model system used. For example, in the setting of septic peritonitis, IL-27 was rapidly produced and resulted in suppression of neutrophil
migration and activation\textsuperscript{74}. Other studies in the setting of \textit{Mycobacterium tuberculosis}-mediated or LPS-mediated immune activation showed similar repressive functions of IL-27\textsuperscript{77,92,93}. In LPS-activated neutrophils and monocytes/macrophages, IL-27 stimulation suppressed reactive oxygen intermediates (ROI) production, thereby suppressing bactericidal activity of these cells in response to TLR ligation\textsuperscript{74}. Taken together, these studies support a regulatory role of IL-27 in activated model systems, whereby prevention of excessive inflammatory responses is observed upon IL-27 treatment.

On the other hand, we and others have also described the ability of IL-27 to induce pro-inflammatory responses in non-activated immune cells, and in particular, in resting/non-activated monocytic cells. Early studies around the time of IL-27 discovery demonstrated that IL-27-stimulated human monocytes had increased mRNA transcription of the inflammatory cytokines: IL-1\textbeta, TNF-\textalpha, IL-12p35, and IL-18\textsuperscript{71}. Accordingly, Chapter 2 of this thesis is in agreement with these findings, as it more robustly characterized IL-27 as a strong inducer of pro-inflammatory cytokine expression (IL-6, IP-10, MIP-1\textalpha, MIP-1\beta, and TNF-\textalpha) at the transcript and protein level in human monocytes\textsuperscript{88}. Furthermore, a more recent study indicated that pre-treatment with IL-27 could enhance proinflammatory cytokine production in murine monocytes exposed to TLR ligands\textsuperscript{94}. On a similar note, my work in Chapter 3 characterized IL-27 priming of LPS responses, whereby enhanced pro-inflammatory cytokine expression and NF-\kappaB activation was observed in monocytes pre-treated with IL-27 prior to LPS stimulation\textsuperscript{47}. Thus, we and others have demonstrated a strong pro-inflammatory role for IL-27 in resting monocytes.
1.5.2 IL-27 functions on Th cell differentiation

Initial studies into the functions of IL-27 were performed on T cells and described a dominant role for IL-27 in the skewing of T helper cell differentiation to a Th1 response, versus Th2 or Th17\(^{75,95}\). Indeed, IL-27, produced early in APC activation, upregulated the Th1 transcription factor, T-bet, and the T-bet responsive gene, IL-12Rβ2, thereby sensitizing naïve CD4 T cells to the potent effects of IL-12, and resulting in enhanced IFN-γ production\(^{76,78,79}\). Thus, IL-27 is an early driver of Th1 responses in initial Th cell differentiation. At the same time, IL-27 can also inhibit the Th2 transcription factor, GATA-3, thereby suppressing Th2 differentiation\(^{76}\). Further roles for IL-27 in skewing towards Th1 differentiation also include suppression of IL-17 production and the Th17-specific transcription factor, RORγt, resulting in inhibition of Th17 development\(^{81,96,97}\).

Therefore, the role of IL-27 in Th polarization appears to be early skewing to Th1 differentiation, and repression of Th2 and Th17 lineages. The function of IL-27 on T cells was not the focus of this thesis, and therefore, discussion will be limited in this area.

1.6 HIV Infection

1.6.1 HIV infection and host restriction factors

HIV first attaches to target cells via the CD4 cell surface receptor, which can be found on T helper cells (CD4 T cells) and monocytes/macrophages. A secondary interaction of HIV with a specific chemokine receptor on host cells, namely, CCR5 or CXCR4, mediates fusion of the viral and host membranes, allowing entry of the viral capsid into the host cell cytoplasm. From here, the viral capsid uncoats to release the viral RNA genome and accessory proteins, and can establish infection. Briefly, as reviewed in Friedrich et al (2011), viral RNA is reverse transcribed (via the virally encoded reverse
transcriptase enzyme) to DNA, which can then shuttle into the nucleus to integrate into the host genome\textsuperscript{98}. Once integrated, the virus effectively establishes life-long infection. At this point, proviral DNA can be transcribed like any other cellular genes to give rise to progeny virions, or can remain ‘latent’ or transcriptionally silent. Although the primary targets of HIV infection include CD4 T cells, monocytes, and macrophages\textsuperscript{99}, HIV also induces deleterious ‘bystander effects’ on a broad range of host cells, via various mechanisms which can be elicited by virtually all viral proteins (gp120, gp160, Tat, Nef, Vpr, Vpu, and Vif)\textsuperscript{100}. Since HIV only encodes a total of 15 different proteins, the ability of the virus to wreak broad immune havoc depends on interaction with hundreds of host proteins\textsuperscript{98}. Despite the myriad of deleterious effects HIV can elicit, great success has been achieved in treating the infection, with more than 25 different anti-retroviral drugs available for use, which when used in combination (highly active anti-retroviral therapy/HAART), can restore immunologic function and prolong life significantly\textsuperscript{101}. However, several inherent problems with HAART remain, including poor patient compliance stemming from high levels of toxicities, and the ongoing risk of creating selective pressure for emergence of drug-resistant strains. Interestingly, recent discoveries of host-derived anti-HIV factors have created new hope that these conserved restriction factors seem to be quite effective against HIV and the broader family of retroviruses. For example, APOBEC3G, an endogenous cytidine deaminase, has well-described efficacy at inhibiting viral replication\textsuperscript{102}, and disruption of HIV uncoating mechanisms by the host-derived TRIM5α protein has also been described, although the mechanisms are less well characterized\textsuperscript{103}. More recently and pertinent to this thesis, is the characterization of a widely expressed host restriction factor called BST-2/tetherin, which functions to ‘tether’ budding virions to the surface of host cells, reducing infectivity and ongoing viral
replication\textsuperscript{104,105}. Thus, it is interesting to speculate on the therapeutic potential of bolstering innate viral defense mechanisms such as those of APOBEC, TRIM, and BST-2 proteins.

1.6.2 HIV and monocyte functions

Characteristic of HIV infection is a chronic state of immune activation\textsuperscript{106-108}, a key viral pathogenic mechanism that is mediated, in part, by dysfunctional cytokine production and function\textsuperscript{109-111}. The repercussions of chronic immune activation on immune cell function are not well defined, but could alter the pathogenesis of HIV, opportunistic infections, and HIV-specific immunity. HIV infection can impair various monocyte/macrophage functions, including phagocytosis\textsuperscript{112}, antigen uptake and major histocompatibility (MHC) class II expression\textsuperscript{113}, and cytokine production and function\textsuperscript{112,114-117}. Interestingly, HIV-infected monocytes/macrophages do not succumb to the same cytopathic effect HIV has on CD4 T cells, rendering monocytes important long-term viral reservoirs that can disseminate virus to various tissues throughout the body during the course of infection\textsuperscript{112,118,119}. HIV can remain latent inside monocytic cells, integrated in the host genome where, upon an inducing stimulus, production of infectious virions can resume. This results in viral evasion of the host immune response, and persistence of HIV infection despite successful anti-retroviral therapy\textsuperscript{118,120}. A better understanding of how HIV infection impairs monocytic function will lead to novel strategies on how to restore immunologic function and ameliorate disease progression.
1.6.3 HIV and IL-27

In the setting of HIV infection, limited studies describe IL-27 functions in monocytes. However, earlier work on the related cytokine, IL-12, has been promising. In HIV infection, levels of circulating IL-12 decrease, resulting in diminished IL-12 functions\textsuperscript{121}-\textsuperscript{123}. Interestingly, several studies have looked at administering IL-12 as an HIV therapy, and have proven effective\textsuperscript{124,125}. However, although much is known on how HIV affects IL-12 functions, how HIV affects IL-27 function has not been described. Recent exciting studies have shown that IL-27 can significantly inhibit HIV replication in CD4 T cells and monocytes/macrophages via induction of antiviral genes\textsuperscript{126,127}. Thus, since IL-27 has such anti-HIV properties, it could potentially be an effective adjunct to current anti-HIV therapy. However, before the potential for IL-27 administration can be explored further, it is paramount to delineate what effects HIV infection has on IL-27 functions, something that is currently poorly described in the literature. In Chapters 5 and 6 of this thesis I have addressed these unknowns.

1.7 Hypotheses and Objectives

1.7.1 Overview

When I first began my PhD studies, there was a void in the literature on the functions of IL-27 in monocytes, as most initial studies were performed in T cells. Thus, I initiated studies to characterize the IL-27-induced cytokine profile in monocytes, along with characterization of the signaling pathways mediating these responses (Chapter 2). My results from these studies prompted me to further investigate the pro-inflammatory functions of IL-27, resulting in the discovery that IL-27 can enhance LPS responses via upregulation of TLR4 expression and signaling (Chapter 3). In the meantime, IL-27 was
described by others as an anti-HIV cytokine, and shown to induce expression of the anti-viral APOBEC family of proteins. Therefore, in Chapter 4 of this thesis, I investigated if IL-27 could affect expression of another well-known, host-derived anti-HIV protein, BST-2/tetherin. Lastly, since IL-27 represented a potential therapeutic target for boosting HIV-specific immune responses, I finished my thesis by describing the effects of HIV-infection on IL-27 expression and IL-27 functions (Chapter 5 and 6).

1.7.2 Hypotheses

Herein, this thesis was guided by three main hypotheses:

- **HYPOTHESIS 1:** IL-27-mediated immunoregulation includes prevention of excessive inflammation in the setting of infected or activated monocytes. On the contrary, in the setting of non-activated or ‘resting’ monocytes within this thesis, it is hypothesized that complimentary mechanisms of IL-27 immunoregulation will promote inflammatory responses, observed as IL-27-induced pro-inflammatory cytokine production and enhancement of LPS responses (Chapters 2, 3).

- **HYPOTHESIS 2:** Since IL-27 was previously shown to induce type I IFN-responsive genes, it is hypothesized that IL-27 induces expression of BST-2/tetherin, an IFN-responsive, host-derived anti-HIV protein (Chapter 4).

- **HYPOTHESIS 3:** In the setting of HIV infection, previous reports have shown dysfunctional cytokine production and function, along with broad activation of immune cells. Therefore, it is expected that IL-27 expression will be decreased and IL-27 pro-inflammatory functions will be downregulated in HIV-infected subjects (Chapters 5, 6).
1.7.3 Specific objectives

- **CHAPTER 2:** To characterize the cytokine profile and signaling pathways induced by IL-27 in non-activated (resting) human monocytes.

- **CHAPTER 3:** To delineate the molecular mechanisms by which IL-27 can enhance LPS responses in human monocytes.

- **CHAPTER 4:** To determine if IL-27 induces the anti-HIV protein, tetherin.

- **CHAPTER 5:** To describe the effect of HIV infection on expression levels of IL-27.

- **CHAPTER 6:** To describe the effect of HIV infection on IL-27-induced signaling and gene expression.
1.8 References


27. Gee K, Guzzo C, Che Mat NF, Ma W, Kumar A. The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. Inflamm Allergy Drug Targets. 2009;8(1):40-52.


94. Kalliolias GD, Ivashkiv LB. IL-27 activates human monocytes via STAT1 and suppresses IL-10 production but the inflammatory functions of IL-27 are abrogated by TLRs and p38. *J Immunol.* 2008;180(9):6325-33.


CHAPTER 2:

INTERLEUKIN-27 INDUCES A STAT1/3 AND NF-KB DEPENDENT PROINFLAMMATORY CYTOKINE PROFILE IN HUMAN MONOCYTES
2.1 Abstract

IL-27 is a heterodimeric cytokine bridging innate and adaptive immunity by playing a role in the activation of naive T cells and in development of Th1 cells. Additionally, recent evidence supports a role for IL-27 in the activation of monocyctic cells. Both pro-inflammatory and anti-inflammatory activities have been attributed to IL-27; however, the role played by IL-27 in the activation of human monocyctic cells in terms of cytokine production has not been well described. Our results show that IL-27 is a strong inducer of proinflammatory cytokine and chemokine expression, including enhancement of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression in human primary monocytes. Furthermore, we observed that IL-27-induced cytokine and chemokine production was mediated by STAT1, STAT3, and NF-κB activation. Understanding how IL-27 exerts its effects on monocyctic cells will identify important molecular mechanisms in the regulation of immune responses, particularly in the modulation of monocyte activation.

2.2 Introduction

IL-27 was identified in 2002 by Pflanz et al. and is a heterodimeric cytokine composed of an IL-27p28 (p28) subunit and Epstein-Barr virus induced gene 3 (EBI3) subunit. The heterodimeric IL-27 receptor (IL-27R) is composed of the IL-27Rα (WSX-1/TCCR) subunit, unique for the binding of IL-27, and the gp130 subunit which is shared with the receptor for IL-6.

IL-27Rα has a short cytoplasmic domain compared to gp130 and has conserved tyrosines which impart the ability to bind STATs through SH2 domains and JAKs via a membrane proximal box 1 motif. IL-27Rα has been shown to directly associate with
JAK1 as well as STAT1\textsuperscript{7,8}. In naive CD4 T cells, IL-27 induced the activation of both STAT1 and STAT3, but in fully activated T cells, IL-27-induced activation of STAT1 was lost and IL-27-induced STAT3 activity was retained\textsuperscript{9}. This suggests that IL-27 may illicit differential signaling events dependent on cell type and activation state.

IL-27 is an immunoregulatory cytokine that has a role in initiation of the T cell response; however, several recent reports suggest that IL-27 also plays a key role in the regulation of monocytic cell function. Primary human monocytes express the IL-27 receptor chains, and respond to IL-27 by the induction of STAT1 and STAT3 phosphorylation\textsuperscript{5}. In a mouse model of experimental tuberculosis, IL-27 inhibited the production of TNF and IL-12 in activated peritoneal macrophages\textsuperscript{10}. In addition, IL-27 can affect macrophage activation in a similar manner as IL-10 and IL-4 by inhibiting IL-12p40 expression in activated bone marrow-derived macrophages\textsuperscript{11}. The same study showed that alternatively activated macrophages exhibit upregulated WSX-1 expression and as a result, enhanced IL-27-induced signals. These results strongly suggest a role for IL-27 in the regulation of inflammatory responses directed by macrophages. Additionally, IL-27 induces a similar subset of IFN-inducible genes as IFN-\textgreek{a} stimulation in human macrophages\textsuperscript{12}. Recently IL-27 was shown to enhance MHC class I and II expression in the human monocytic cell line, THP-1\textsuperscript{13}. Another report indicates that IL-27 also induces IL-1\textgreek{a}, IL-1\textgreek{b}, IL-18, and TNF-\textgreek{a} mRNA in mast cells, and IL-1\textgreek{b}, TNF-\textgreek{a}, IL-12p35 and IL-18 mRNA in monocytes\textsuperscript{5}. However the molecular mechanisms utilized by IL-27 to induce cytokine production in human monocytic cells have not been well characterized.
In this study we describe a novel IL-27-induced cytokine profile in human monocytic cells. Our results show that IL-27 is a strong inducer of pro-inflammatory cytokine expression (IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α). We demonstrate for the first time that IL-27-induced cytokine expression is dependent on the activation of STAT1, STAT3, and NF-κB in human monocytic cells.

2.3 Experimental Procedures

2.3.1 Cell Culture and Reagents

THP-1 cells (pro-monocytic leukemic cells), were obtained from ATCC. Cells were cultured in Iscove’s Modified Dulbecco’s Medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco). Recombinant IL-27 (IL-27) was used at a concentration of 120ng/mL. Caffeic acid phenethyl ester (CAPE) was used as a potent and specific inhibitor of NF-κB binding activity.

2.3.2 Isolation of Primary Monocytes

Purified monocytes were isolated from whole blood obtained from healthy donors in agreement with the Queen’s University Research Ethics Board approval. Whole blood samples were diluted with an equal volume of PBS-EDTA (1mM) + 2% FBS and layered over Lympholyte (Cedarlane Laboratories) in a 4:3 ratio of diluted blood to lympholyte. Overlayed tubes were centrifuged for 20 minutes at 1200xg, room temperature, with the brake off. The fraction of peripheral blood mononuclear cells (PBMCs) was extracted and primary monocytes were isolated by magnetic negative selection using the Human Monocyte Enrichment Kit (StemCell Technologies).
2.3.3 Cytokine Array

The Human Cytokine Array Panel A kit was used to determine relative levels of cytokines released from primary human monocytes (R&D Systems), as per the manufacturer’s instructions. Briefly, primary monocytes were cultured in medium alone or in the presence of IL-27 for 24 hours. After blocking the array membranes, culture supernatants were mixed with Cytokine Array Panel A Antibody Cocktail and incubated for 1 hour at room temperature. Supernatant-antibody mixture was then added to the membrane for an overnight incubation at 4°C. After incubation membranes were and incubated with streptavidin-HRP for 30 minutes at room temperature. Membranes were then washed and incubated with chemiluminescent reagent. Chemiluminescent detection was performed using the Alphalnnotech H2D Imager (Alphalnnotech). Each cytokine is present in duplicate on the array and the average of both spots was taken for expression analysis of each cytokine. Fold increase in cytokine production was calculated over background using densitometry as measured by the analysis software on the HD2 imager.

2.3.4 ELISA

Primary monocyte and THP-1 cell culture supernatants were used in ELISA analysis to quantify expression of IL-27-induced cytokines as per the manufacturer's (BioSource) instructions: IL-6 (CHC1263), IP-10 (CHC2363), MIP-1α (CHC2203), MIP-1β (CHC2293), and TNF-α (CHC1753). Briefly, 96-well microplates were coated overnight at 4°C with capture antibodies (IL-6: 1µg/mL, IP-10: 2µg/mL, MIP-1α: 2µg/mL, MIP-1β: 2µg/mL, or TNF-α: 2µg/mL). Plates were washed and blocked for 1 hour at room temperature, followed by incubation with standards or sample (undiluted culture
 supernatants). Samples were added in duplicate. Immediately following standard and sample addition, detection antibody was added to microplates (IL-6: 0.16µg/mL, IP-10: 0.2µg/mL, MIP-1α: 0.16µg/mL, MIP-1β: 0.16µg/mL, or TNF-α: 0.32µg/mL) and incubated for 2 hours at room temperature with continual shaking. Wells were washed, incubated with streptavidin-HRP, washed again, and incubated with TMB Stabilized Chromogen (BioSource). Reactions were stopped with the addition of 1.8N H2SO4 and absorbencies were immediately measured on the BioTek ELx800 Absorbance Microplate Reader. ELISA results are shown as an average of the duplicate wells ± standard deviation.

2.3.5 RNA Isolation and RT-PCR

Total RNA was extracted from cell pellets using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich). RNA (0.5µg) was reverse transcribed using the Moloney Murine Leukemia Virus Reverse Transcriptase Enzyme (Invitrogen). Equal aliquots of cDNA (2µL) were used for IL-6, IP-10, MIP-1α, MIP-1β, TNF-α, and 18SrRNA amplification using specific primers (Table 2.1) with 5X Taq Polymerase Master Mix (New England Biolabs) containing dNTPs, MgCl2, KCl, and stabilizers. PCR cycles were performed on the Px2 Thermal Cycler (Thermo Electron Corporation). The following annealing temperatures were used: IL-6, MIP-1β, RANTES, and 18S rRNA at 55ºC; MIP-1α and TNF-α at 65ºC; and IP-10 at 56ºC. The amplified products were resolved by electrophoresis on 1.2% agarose gels and visualized by UV detection of ethidium bromide intercalation on the Alphalnnotech HD2 Imager (Alphalnnotech).
Table 2.1: Cytokine primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>5'-GAA CTC CTT CTC CAC AAG CG-3'</td>
<td>5'-GAA TCC AGA TTG GAA GCA TCC-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A-3'</td>
<td>5'-GGC AAT GAT GAT CCC AAA GTA GAC CTG CCC AGA CT-3'</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>5'-CCT TGC TGT CCT CCT CTG CA-3'</td>
<td>5'-CAC TCA GCT CTA GGT CGC TG-3'</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>5'-TGT CTC TCC TCA TGC TAG TA-3'</td>
<td>5'-GTA CTC CTG GAC CCA GGA T-3'</td>
</tr>
<tr>
<td>IP-10</td>
<td>5'TGA AAA AGA AGG GTG AGA AGA G-3'</td>
<td>5'-GGG AGA TGG GAA AGGG TGA GG-3'</td>
</tr>
<tr>
<td>RANTES</td>
<td>5'-TTC CTG CAG AGG ATC AAG ACA GCA-3'</td>
<td>5'-TGT GGT AGA ATC TGG GCC CTT CAA-3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5'-TTC GGA ACT GAG GCC ATG AT-3'</td>
<td>5'-CGA ACC TCC GAC TTT CGT TT-3'</td>
</tr>
</tbody>
</table>
2.3.6 Western Blotting

Levels of phosphorylated STAT1, STAT3 and NF-κB p50 were measured by Western Blot analysis. Cell pellets were lysed in a buffer (1M HEPES, 0.5M NaF, 0.5M EGTA, 2.5M NaCl, 1M MgCl2, glycerol, Triton-X-100, and dH2O) containing a protease inhibitor cocktail (Pierce). Protein concentrations were obtained using the Bradford Assay and absorbencies were measured using a Varioskan Rev. 2.0 (Thermo Electron Corporation) 96-well plate reader. Proteins were subjected to an 8% polyacrylamide SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Millipore). The membranes were probed with monoclonal mouse anti-phospho-STAT1 (Santa Cruz), monoclonal mouse anti-phospho-STAT3 (Santa Cruz), or polyclonal rabbit anti-phospho-NF-κB p50 (Santa Cruz) followed by HRP-conjugated goat anti-mouse or goat anti-rabbit polyclonal antibody (Santa Cruz). The membranes were stripped (1M Tris-HCl (pH 6.8), β-Mercaptoethanol, 2% SDS, and 0.7 mM DTT) at 50°C with gentle agitation and washed with TBST buffer (150 mM Tris-HCl, 1 M NaCl, and 1% Tween 20) seven times followed by re-probing with polyclonal rabbit anti-human-STAT1 or STAT3 (Cell Signalling) or polyclonal rabbit anti-human NF-κBp50 (Santa Cruz) to confirm equal loading. Monoclonal mouse anti-human β-actin (Sigma) was used as an additional loading control to pan-p50 for the NF-κB phospho-p50 immunoblots. All immunoblots were visualized by Enhanced Chemiluminescence (ECL) (Amersham Biosciences) on the AlphaInnotech H2D Imager (AlphaInnotech).

2.3.7 Electrophoretic Mobility Shift Assay (EMSA)

EMSAAs were performed to determine activation of STAT1, STAT3, and NF-κB DNA binding. Briefly, cells were stimulated with IL-27 and nuclear and cytoplasmic proteins
were extracted from cell pellets using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce), as per the manufacturer’s instructions. Nuclear protein concentration was measured by the Bradford method.

EMSAs were performed using biotin-labeled probes specific for the STAT1, STAT3 and NF-κB binding sites, with the respective sequences as follows: STAT1: 5’-CAT GTT ATG CAT ATT CCT GTA AGT G-3’, STAT3: 5’-GAT CCT TCT GGG AAT TCC TAG ATC-3’, NF-κB: 5’AGT TGA GGG GAC TTT CCC AGG C-3’. Binding reactions were performed using the Lightshift Chemiluminescent EMSA Kit (Pierce) as per the manufacturer’s instructions. Briefly, all binding reactions included 5μg of nuclear extract, 10X Binding Buffer (100mM Tris, 500mM KCl, 10mM DTT), 50% glycerol, 100mM MgCl2, Poly dIdC, 1% NP-40, and nuclear extract. Supershift reactions were performed for 1 hour at room temperature using 2μg of polyclonal rabbit anti-STAT1 and anti-STAT3 (Cell Signaling) or 2μg of polyclonal rabbit anti-NF-κB p50 and anti-NF-κB p65 (Santa Cruz). To determine the specificity of the proteins for the probe sequence, nuclear proteins were incubated for 30 minutes at room temperature with an excess (200-fold) of annealed, unlabelled, cold competitor probe. DNA-protein complexes were then resolved on a 5% non-denaturing polyacrylamide gel, transferred to nylon membranes (Pierce) and cross-linked (Spectroline UV Crosslinker, Fisher Scientific). Chemiluminescent detection was performed using the Lightshift Chemiluminescent EMSA Kit (Pierce), as per the manufacturer’s instructions, and visualized on the AlphaInnotech H2D Imager (AlphaInnotech).
2.3.8 siRNA Transfections

THP-1 cells (0.1x10⁶) were transfected either with STAT1, STAT3, or control siRNA (Santa Cruz Biotechnology). Briefly, siRNA were incubated for 45 minutes at room temperature with transfection reagent (Santa Cruz) and serum free IMDM media. The siRNA-transfection reagent mixtures were subsequently added to cell cultures and incubated at 37°C for 5 hours. Serum free media was then replaced with IMDM-10% FBS media and cells were cultured in the presence of IL-27 for 4 hours to detect cytokine expression by RT-PCR.

2.3.9 Flow Cytometry Staining and Analysis

IL-27-induced intracellular cytokine production was measured using flow cytometry. Cells were cultured in 96-well plates in the presence or absence of IL-27 for 24 hours at 37°C. Brefeldin A (BFA) was added to cell cultures 12hrs prior to harvesting for analysis. Cells were pelleted, washed two times with PBS+ 0.1% sodium azide, and fixed in 1% paraformaldehyde for 20 minutes. Pellets were then washed PBS/ 0.1% sodium azide and re-suspended in 0.1% saponin. Cells were stained with PE-conjugated IL-6 (eBioscience), PE-conjugated IP-10 (R&D Systems), PE-conjugated MIP-1α (eBioscience), FITC-conjugated MIP-1β (R&D Systems), or PE-conjugated TNF-α (eBioscience). Data were acquired with the Epics XLMCL flow cytometer (Beckman Coulter, Miami, FL) and analyzed using the WinMDI version 2.9 software package (J. Trotter, Scripps Institute, San Diego).
2.4 Results

2.4.1 IL-27 induces mRNA and protein expression of pro-inflammatory cytokines in human monocytes

To date, the IL-27-induced cytokine profile in human monocytes has not been well characterized, however, a role for IL-27 in modulating inflammatory responses via induction of cytokines has been demonstrated\(^5,10\). To more fully elucidate the IL-27-induced cytokine profile in primary human monocytes, we performed a cytokine array measuring expression levels of 36 different cytokines in response to IL-27. Monocytes from a healthy donor were either cultured in medium alone or in the presence of IL-27 for 24 hr and cell culture supernatants were analyzed for cytokine expression.

For analysis, the cytokines were grouped as follows: (i) chemokines, (ii) Th1 cytokines, (iii) anti-inflammatory cytokines, (iv) cytokines involved in inflammation and cell differentiation, (v) IL-12 and IL-17 family cytokines, and (vi) Th2 cytokines (Figure 2.1A). IL-27-mediated induction of cytokine expression was observed in the chemokine (MIP-1-\(\alpha\), MIP-1-\(\beta\), IP-10, GRO-\(\alpha\)), proinflammatory (TNF-\(\alpha\), IL-1\(\beta\)), and IL-12 family (IL-6) groups of cytokines. Interestingly, decreases in cytokine levels in response to IL-27 treatment were also observed (Figure 2.1A, Panel i: serpinE, IL-8, Panel ii: IL-16, and Panel iv: MIF).

Due to the evidence that IL-27 is involved in mediating inflammatory processes\(^5,10,14,15\), we were interested in delineating the molecular mechanism used by IL-27 to induce expression of proinflammatory cytokines. Therefore, we focused on the chemokines
Figure 2.1: IL-27 induces expression pro-inflammatory cytokines and chemokines in primary human monocytes. Primary human monocytes were isolated by negative selection (StemCell Technologies). Monocytes were cultured in either media alone or in the presence of IL-27 for 24 hr. (A) Cell supernatants were harvested and subjected to cytokine array analysis of 36 different cytokines. Cytokines were classified into 6 subsets with similar functions such as: (i) chemokines, (ii) Th1 cytokines, (iii) anti-inflammatory cytokines, (iv) cytokines involved in inflammation and cell differentiation, (v) IL-12 and IL-17 family cytokines, and (vi) Th2 cytokines. Densitometry analysis of the cytokine arrays showed IL-27 significantly induced IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α by a greater than 2-fold increase in chemiluminescence detection. * indicates a greater than 2 fold increase in expression levels. ND, not detected. (B) The same cell culture supernatants used for the cytokine array in panel A were analyzed for IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression by ELISA.
and cytokines having a greater than two-fold increase in response to IL-27 stimulation: IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α (Figure 2.1A, asterisks). To quantitate levels of these cytokines induced by IL-27, we performed ELISAs for IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α using the same culture supernatant as used for the cytokine array. Induction of all five cytokines was observed in response to IL-27 (Figure 2.1B).

To further confirm these observations, we performed ELISAs to examine IL-27-induced cytokine kinetics (Figure 2.2A) and intracellular cytokine expression by flow cytometry (Figure 2.2B) on five separate primary monocyte donors (representative data from an individual donor is shown). The ELISAs demonstrated that IL-27 induced rapid secretion of IL-6, MIP-1α, MIP-1β, and TNF-α after 4 hr IL-27 treatment and expression of these cytokines peaked between 8 and 24 hr (Figure 2.2A). We detected a similar increase in IP-10 expression after 4 hr IL-27 treatment, however expression of IP-10 further increased dramatically at 48 hours. Additionally, we examined intracellular expression of the cytokines by a flow cytometry in BFA-treated cells that were stimulated with IL-27 for 24 hr. From these experiments, we detected IL-27-mediated enhancement of intracellular cytokines IL-6, IP-10, MIP1α, MIP1β, and TNF-α (Figure 2.2B). We then performed RT-PCR analysis on primary human monocytes treated with IL-27 for 2-8 hours to determine whether IL-27 mediates cytokine production at transcriptional levels. Cytokine induction was observed after 2 hours of IL-27 treatment for IL-6, IP-10, MIP-1β, and TNF-α, and MIP-1α induction was seen after 4 hours (Figure 2.2C).
Figure 2.2: IL-27 induced expression of IL-6, IP-10, MIP-1-α, MIP-1-β, and TNF-α in primary human monocytes. Primary human monocytes were isolated by negative selection and were cultured in either media alone or IL-27 for the following conditions: (A) Cells were treated with IL-27 for 0 to 48 hours and cell supernatants were analyzed for IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression by ELISA. (B) Cells were treated with IL-27 for 24 hours and IL-6, IP-10, MIP-1-α, MIP-1-β, and TNF-α expression were detected by intracellular cytokine staining by flow cytometry. Representative histograms are shown with cells cultured in medium alone (shaded histogram) overlayed with the histogram for IL-27-treated cells (dark line). (C) Cells were treated with IL-27 for times ranging from 2 to 8 hours. Levels of mRNA for IL-6, IP-10, MIP-1-α, MIP-1-β, and TNF-α expression were measured by RT-PCR. 18S rRNA was used as a loading control. Results shown are from one donor and representative of five different donors.
To define the molecular mechanisms used by IL-27 to induce these cytokines, we used the human pro-monocytic cell line, THP-1 as our model system. An increase in protein expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α in response to IL-27 treatment was observed with ELISA (Figure 2.3A) and flow cytometry (Figure 2.3B). Similarly, in THP-1 cells treated with IL-27 for 2 to 16 hr, mRNA levels of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α were upregulated after 2 hr of IL-27 treatment for all cytokines with the exception of IL-6, which was upregulated after 4 hr of treatment (Figure 2.3C).

2.4.2 IL-27-induced STAT1 and STAT3 are required for cytokine induction

Primary human monocytes show tyrosine phosphorylation of STAT1 and -3 in response to IL-27 stimulation. We hypothesized that STAT1 and/or STAT3 may be involved in IL-27-mediated cytokine induction. We confirmed the ability of IL-27 to induce STAT1 and STAT3 activation in THP-1 cells, detecting phosphorylation of STAT1 and -3 within 5 to 15 minutes of IL-27 treatment (Figure 2.4A).

We also assessed whether IL-27 induced the activation of these transcription factors using gel shift assays. We observed an enhanced binding of STAT1 and STAT3 within 5 minutes of IL-27 stimulation (Figure 2.4B). Cold competitor probes (CC) abrogated the STAT-DNA complexes. Supershift assays were also performed using anti-STAT1 or anti-STAT3 specific antibodies. Incubation with supershift antibodies resulted in disruption of the protein/DNA complexes. Interestingly, disruption of the STAT-DNA complexes was found with either STAT1 or STAT3 specific antibodies, indicating the formation of STAT1/STAT3 heterodimers.
IL-6 (pg/mL)  
MIP-1α (pg/mL)  
MIP-1β (pg/mL)  
TNF-α (pg/mL)  

IL-6 Expression  
IP-10 Expression  
MIP-1α Expression  
MIP-1β Expression  
TNF-α Expression  

Relative Cell Number  

(A)  

(B)  

(C)  

18S rRNA  
IL-6  
IP-10  
MIP-1α  
MIP-1β  
TNF-α  

--- IL-27 (hr) ---
Figure 2.3: IL-27 induces expression of IL-6, IP-10, MIP-1-α, MIP-1-β, and TNF-α in THP-1 cells. THP-1 cells were cultured in either media alone or IL-27 for the following conditions: (A) Cells were treated with IL-27 for 0 to 48 hours and cell culture supernatants were analyzed for IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression by ELISA. (B) Cells were treated with IL-27 for 24 hours and IL-6, IP-10, MIP-1-α, MIP-1-β, and TNF-α expression were detected by intracellular cytokine staining by flow cytometry. Representative histograms are shown with cells cultured in medium alone (shaded histogram) overlayed with the histogram for IL-27-treated cells (dark line). (C) Cells were treated with IL-27 for times ranging from 2 to 16 hours. Levels of mRNA for IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression were measured by RT-PCR. 18S rRNA was used as a loading control. Results shown in this figure are representative of five separate experiments.
Figure 2.4: IL-27-induced cytokine production is mediated by STAT1 and STAT3 activation. THP-1 cells were either cultured in media or IL-27 under the following conditions: (A) Cells were treated with IL-27 for times ranging from 5 to 30 minutes. Cell pellets were harvested and whole cell lysates were separated by PAGE. Membranes were probed with anti-phospho-STAT1 (p-STAT1) or anti-phospho-STAT3 (p-STAT3) antibodies. Membranes were stripped and re-probed with pan STAT1 or pan STAT3 antibodies as indicated. (B) Cells were treated with IL-27 for times ranging from 5 to 30 minutes. Cell pellets were harvested and nuclear protein extracts were subjected to EMSA to measure DNA binding activity of STAT1 and STAT3. Cold competitor (CC) lanes contained 200X excess of unlabelled probes compared to biotin-labeled probes. Supershift (SS) antibodies specific for STAT1 or STAT3 were able to interfere with complex formation. Results shown are representative of five separate experiments. (C) THP-1 cells cultured in medium alone were transfected with control, STAT1, or STAT3 siRNA, followed by RT-PCR analysis with primers specific for either STAT1 or STAT3 expression. 18SrRNA primers were used as a control. (D) THP-1 cells were transfected with control, STAT1, or STAT3 siRNA and were either cultured in medium alone or in the presence of IL-27 for 4 hours. RT-PCR analysis for IL-6, IP-10, MIP-1-α, MIP-1-β, and TNF-α expression was performed using cytokine-specific primers. Detection of 18SrRNA was used as a loading control. Results shown in this figure are representative of five separate experiments.
To elucidate the role of STAT1 and STAT3 in IL-27-induced cytokine expression, we transfected THP-1 cells with control (scrambled), STAT1, or STAT3 siRNA. To confirm specificity of the siRNA knockdowns, RT-PCR analysis of STAT1 and STAT3 expression showed that transfection of STAT1 inhibited STAT1 mRNA expression, but did not affect STAT3 expression. Similarly, transfection of STAT3 siRNA inhibited STAT3 mRNA expression but did not affect STAT1 expression. Transfection of control sequences did not affect either STAT1 or STAT3 (Figure 2.4C).

To investigate the requirement of either STAT1 or STAT3 in IL-27-induced cytokine expression, we performed RT-PCR analysis on siRNA-transfected THP-1 cells stimulated with IL-27 for 4 hours (Figure 2.4D). As expected, in cells transfected with control siRNA, IL-27 treatment induced the upregulation of all five cytokine mRNA levels. For IL-6 in the presence of STAT1 or STAT3 siRNA, IL-27 treatment resulted in a weaker induction of cytokine expression. In the case of IP-10, STAT1 siRNA transfection resulted in a stronger inhibition of mRNA expression compared to that observed for STAT3 siRNA knockdown. Transfection of STAT1 and STAT3 siRNA equally inhibited MIP-1α expression and MIP-1β expression. Additionally, for TNF-α expression, transfection of either STAT1 or STAT3 siRNA resulted in a decrease of IL-27-induced mRNA expression. Taken together, these results show that IL-27-mediated STAT1 and STAT3 activation is involved in IL-27-induced IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α in human monocytic cells.
2.4.3 Inhibition of NF-κB DNA binding activity blocks IL-27-induced cytokine expression

In addition to the role played by STAT1 and STAT3, we investigated whether NF-κB, a transcription factor commonly associated with cytokine regulation\textsuperscript{17-21}, was induced by IL-27 in monocytic cells. THP-1 cells were treated with IL-27 for times ranging from 5-120 minutes, followed by western blot analysis for phosphorylated NF-κB p50 subunit (p-NF-κB p50) (Figure 2.5A). We observed IL-27-induced phosphorylation of NF-κBp50 (lower band) as well as the p50 precursor p105 (upper band) within 5 minutes of IL-27 stimulation that was sustained up until 60 minutes, and then downregulated at 120 minutes. To confirm that IL-27 induced NF-κB DNA binding, we also performed EMSA analysis (Figure 2.5B). The results demonstrate that IL-27 induced NF-κB binding after 5 min of IL-27 treatment, and peaked between 15 to 30 minutes of IL-27 treatment. This binding is specific to NF-κB as shown by inhibition by cold competitor probes (CC) as well as performing supershift analysis with antibodies to NF-κB p50 and p65 subunits (Figure 2.5B).

In order to determine the role of NF-κB in IL-27-induced proinflammatory cytokine production, we initially attempted to knock down NF-κB expression using siRNA, however, due to the high levels of these proteins we were unable to achieve sufficient knock-down of NF-κBp50 or p65. Therefore, in order to block NF-κB activity we used the NF-κB-specific inhibitor CAPE. As previously described\textsuperscript{22}, 2 hour pre-incubations with CAPE (dose ranges of 5-50μg/mL) were performed to inhibit NF-κB activity prior to IL-27
Figure 2.5: IL-27-induced cytokine production is dependent on NF-κB activation. THP-1 cells were either cultured in media or IL-27 under the following conditions: (A) Cells were treated with IL-27 for times ranging from 5 to 120 minutes. Cell pellets were harvested and whole cell lysates were separated by PAGE. Membranes were probed with anti-phospho-NF-κB p50 (p-NF-κB p50) antibody. Membranes were stripped and re-probed with anti-pan-NF-κB p50 (NF-κB p50) and anti-β-actin antibodies as indicated. (B) Cells were treated with IL-27 for times ranging from 5 to 60 minutes. Cell pellets were harvested and nuclear protein extracts were subjected to EMSA to measure DNA binding activity of NF-κB. Cold competitor (CC) lanes contained 200X excess of unlabelled probes compared to biotin-labeled probes. Supershift antibodies specific for NF-κB p50 (SSp50) or NF-κB p60 (SSp65) were able to interfere with complex formation. Results shown are representative of five separate experiments. (C) THP-1 cells cultured in medium alone were pretreated with the NF-κB inhibitor CAPE for the concentrations indicated. Nuclear extracts were subjected to EMSA analysis for NF-κB DNA binding. (D) THP-1 cells were pretreated with NF-κB inhibitor, CAPE, and were either cultured in medium alone or in the presence of IL-27 for 4 hours. RT-PCR analysis for IL-6, IP-10, MIP-1-α, MIP-1-β, TNF-α, and RANTES expression was performed using cytokine-specific primers. Detection of 18SrRNA was used as a loading control. (E) Primary monocytes were pretreated with CAPE followed by culture in either medium alone or in the presence of IL-27 for 4 hours. RT-PCR analysis for IL-6, IP-10, MIP-1-α, MIP-1-β, TNF-α, and RANTES expression was performed using cytokine-specific primers. Detection of 18SrRNA was used as a loading control. Results shown in this figure are representative of five separate experiments.
stimulation. In agreement with others, the concentrations of CAPE and incubation times used did not affect cell viability as tested by trypan blue staining (data not shown)\textsuperscript{22}. We pretreated THP-1 cells with CAPE, followed by stimulation with IL-27 for 15 minutes. Cell pellets were harvested and nuclear proteins were subjected EMSA to confirm the inhibitory action of CAPE on NF-κB DNA binding activity. CAPE inhibition was evident at concentrations from 15\(\mu\)g/mL to 50\(\mu\)g/mL (Figure 2.5C). To confirm that CAPE did not affect STAT1 or STAT3 activation, EMSA analysis was performed for STAT1 and STAT3 DNA binding following CAPE inhibition and no effect was observed (data not shown). Following CAPE pre-treatment, THP-1 cells were stimulated with IL-27 for 4 hours to investigate cytokine expression. RT-PCR analysis showed that CAPE blocked IL-27-induced mRNA expression of IL-6, IP-10, MIP-1\(\alpha\), MIP-1\(\beta\), and TNF-\(\alpha\) (Figure 2.5D). IL-6 expression was abolished at 25\(\mu\)g/mL whereas TNF-\(\alpha\) expression was abolished at 50\(\mu\)g/mL. MIP-1\(\alpha\) expression was abolished at 15\(\mu\)g/mL. IP-10 and MIP-1\(\beta\) expression showed partial inhibition at 15\(\mu\)g/mL and were abolished at 50\(\mu\)g/mL. To control for potential non-specific effects of the CAPE inhibitor in THP-1 cells, we examined expression levels of RANTES, a cytokine not significantly induced by IL-27 treatment as observed by the cytokine array (Figure 2.1A). Levels of RANTES mRNA did not change with IL-27 stimulation and were not significantly affected by CAPE treatment (Figure 2.5D). This indicates that CAPE treatment specifically inhibits expression of IL-27-induced IL-6, IP-10, MIP-1\(\alpha\), MIP-1\(\beta\), and TNF-\(\alpha\) and furthermore that CAPE does not affect cytokine mRNA levels unaffected by IL-27 stimulation.
To further confirm a role for NF-κB in IL-27-induced of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α expression, we examined the effect of CAPE treatment on IL-27-treated primary monocytes. As in the THP-1 cells, primary monocytes were pretreated with CAPE followed by stimulation with IL-27 for 4 hours. RT-PCR analysis showed that CAPE pretreatment blocked IL-27-induced mRNA expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α (Figure 2.5E). With the exception of IP-10, expression of IL-27-induced cytokines was completely inhibited at 10μg/mL of CAPE. Expression of IP-10 was partially inhibited at 10μg/mL and completely abolished by 25μg/mL. Interestingly, primary monocytes were more sensitive to the CAPE treatment as cytokine expression was blocked at lower doses compared to THP-1 cells. In contrast to THP-1 cells, mRNA expression levels of RANTES were slightly upregulated by IL-27 treatment and partially inhibited by CAPE (15-50μg/mL) in primary monocytes. However, at 10μg/mL CAPE, RANTES levels remained consistent whereas levels of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α were significantly decreased. In stark contrast to IL-27-induced mRNA expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α, CAPE treatment at any dose did not fully abolish RANTES mRNA expression in primary monocytes. Taken together, these results indicate a role for NF-κB in IL-27-mediated proinflammatory cytokine expression.

2.5 Discussion
In this study we identified a novel cytokine profile secreted in response to IL-27 in primary monocytes and THP-1 cells. Our results show that IL-27 is a strong inducer of pro-inflammatory cytokine expression (IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α).
Furthermore, we observed that IL-27-mediated cytokine production required STAT1, STAT3, and NF-κB activation.

We initially measured IL-27-mediated cytokine production using an array for detection of 36 cytokines. Results from this experiment showed a strong induction of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α. Additional data from the cytokine array also indicated that IL-27 induced the expression of other cytokines and chemokines (GRO-α and IL-1β), while inhibiting basal expression of others (serpinE, IL-8, IL-16, and MIF). Pflanz et al. (2004) demonstrated IL-27-induced increased mRNA expression of the inflammatory cytokines: IL-1β, TNF-α, IL-12p35, and IL-18 in primary human monocytes. Our study is in agreement with this work as we showed IL-27-induced TNF-α and IL-1β expression. The array did not include IL-18 detection antibodies. Although the array included detection spots for IL-12p70, we did not observe any induction for this cytokine. However, the production of IL-12p70 does not correlate with IL-12p35 induction due to the requirement for IL-12p40 expression.

The initial findings observed using the cytokine array were confirmed by ELISA, intracellular cytokine detection using flow cytometry, and RT-PCR analysis in primary human monocytes and THP-1 cells. Differences in kinetics observed in ELISA time courses between primary monocytes (Figure 2.2A) and THP-1 cells (Figure 2.3A) could be attributed to differences in developmental stages of the cells. Additionally THP-1 cells are transformed cells and as a result of this may exhibit different sensitivities to
activation. However, the concentration of the cytokines induced by IL-27 is similar between the two cells types (Figure 2.2A and Figure 2.3A).

Several studies have characterized IL-27 as a negative regulator of macrophage function\textsuperscript{10,11}. However, these studies examined the effects of IL-27 on activated macrophages. Our experiments were performed on freshly isolated monocytes. Differences in the activation states between macrophages and monocytes may thus account for this apparent dichotomy in IL-27 function. Interestingly, a recent study by Kalliolias \textit{et al} (2008) indicated that IL-27 can prime murine monocytes in a STAT1-dependent manner resulting in enhanced TLR-mediated proinflammatory cytokine expression\textsuperscript{16}.

IL-27 is produced by monocytes and dendritic cells in response to bacterial antigens\textsuperscript{3,25-30}. In this study we provide supporting evidence for the autocrine action of IL-27 as suggested by others\textsuperscript{5,10,13}. IL-27 may play a critical role in modulating the inflammatory response against bacterial infection via inducing proinflammatory cytokines and chemokines. IL-6 and TNF-\(\alpha\) are key regulators of the inflammatory response and the production of MIP-1\(\alpha\) and MIP-1\(\beta\) may serve to recruit cells to sites of infection. In addition, the expression of IP-10, previously shown to be induced by IL-27 in HUVECs\textsuperscript{31}, may serve to recruit activated Th1 cells and NK cells to sites of infection\textsuperscript{32,33}.

Although many studies indicate a role for IL-27 in inducing cytokine production, few indicate the signalling pathways used by IL-27 to modulate cytokine expression.
Previous studies have shown IL-27-mediated effects on monocytic cells resulting from the activation of STAT1 and STAT3. IL-27Rα physically associates with JAK1 and upon phosphorylation of tyrosine residues can activate STAT1 and STAT3. In monocytes, we and others have demonstrated that IL-27 activates JAK/STAT signalling cascades via STAT1 and STAT3. The role of JAK activation in monocytic cells in IL-27-mediated production of cytokines has yet to be determined.

Interestingly, we demonstrate IL-27-induced formation of STAT1/STAT3 heterodimers by EMSA as demonstrated via disruption of complex formation with both STAT1 and STAT3 antibodies. This is in agreement with other studies showing STAT1/STAT3 heterodimer formation with EMSA analysis. This phenomenon explains the dependence of both STAT1 and STAT3 for IL-27-induced cytokine expression.

Additionally, we show for the first time the ability of IL-27 to activate NF-κB. Taken together, our results demonstrated that STAT1, STAT3, and NF-κB are required for IL-27-induced expression of IL-6, IP-10, MIP-1α, MIP-1β, and TNF-α. In THP-1 cells transfected with siRNA for either STAT1 or STAT3, we observed inhibition of IL-27-induced cytokine and chemokine expression. We observed inhibition of IL-27-induced cytokine and chemokine levels in the presence of siRNA for STAT1 or STAT3, but induction of mRNA levels was never completely abolished. Additionally, our results clearly demonstrated a differential requirement for STAT1 or STAT3 for IL-27 induced cytokine and chemokine expression. These observations led us to investigate the role of NF-κB, as we hypothesized that STAT1 and STAT3 were not the sole requirements for
IL-27-mediated cytokine expression. Upon CAPE-mediated inhibition of NF-κB activity, we observed abrogation of IL-27-induced proinflammatory cytokine mRNA in both THP-1 cells and primary human monocytes. Interestingly, CAPE treatment of primary monocytes resulted in inhibition of cytokine and chemokine production at lower doses compared to that observed in THP-1 cells. The difference in sensitivity between these cell types may be due to inherent differences between primary monocytes due to the tumorogenic nature of the THP-1 cell line. These findings indicate a role for NF-κB in mediating IL-27-induced cytokine production in addition to that played by STAT1 and STAT3. Additionally, the role of other transcription factors required for cytokine expression cannot be ruled out at this point.

It is important to note that in our study, EMSA analysis was performed using consensus sequences for STAT1, STAT3, and NF-κB binding. It is likely that the exact sequences to which STAT1, STAT3 and NF-κB bind in the promoter regions of the individual cytokine and chemokine genes may differ and may require additional protein interactions. This may explain the differential requirement observed for each of these transcription factors in IL-27 induced cytokine expression.

Understanding how IL-27 exerts its effects on monocytic cells will identify key signalling mechanisms in the regulation of immune responses, particularly in the development of the Th1 and inflammatory responses. Our study supports the idea that IL-27 can activate the inflammatory response in resting monocytic cells. We demonstrate for the first time that IL-27 treatment of primary human monocytes elicits the induction of pro-
inflammatory cytokines and chemokines that is mediated by STAT1, STAT3, and NF-κB signaling cascades.
2.6 References


CHAPTER 3:

IL-27 ENHANCES LPS-INDUCED PROINFLAMMATORY CYTOKINE PRODUCTION VIA UPREGULATION OF TLR4 EXPRESSION AND SIGNALING IN HUMAN MONOCYTES
3.1 Abstract
Interleukin-27 (IL-27), produced by activated antigen presenting cells, bridges innate and adaptive immunity by regulating the development of T helper cells. Recent evidence supports a role for IL-27 in the activation of monocytic cells in terms of inflammatory responses. Indeed, proinflammatory and anti-inflammatory activities are attributed to IL-27, and IL-27 production itself is modulated by inflammatory agents such as lipopolysaccharide (LPS). IL-27 primes LPS responses in monocytes; however, the molecular mechanism behind this phenomenon is not understood. Herein, we demonstrate that IL-27 priming results in enhanced LPS-induced IL-6, TNF-α, MIP-1α, and MIP-1β expression in human primary monocytes. To elucidate the molecular mechanisms responsible for IL-27 priming, we measured levels of CD14 and Toll-like receptor (TLR)-4, required for LPS binding. We determined that IL-27 upregulates TLR4 in a STAT3- and NF-κB-dependent manner. Immunofluorescence microscopy revealed enhanced membrane expression of TLR4 and more distinct co-localization of CD14 and TLR4 upon IL-27 priming. Furthermore, IL-27 priming enhanced LPS-induced activation of NF-κB family members. This is the first study to show a role for IL-27 in regulating TLR4 expression and function. This work is significant as it reveals new mechanisms by which IL-27 can enhance proinflammatory responses that can occur during bacterial infections.

3.2 Introduction
Interleukin-27 (IL-27) is a heterodimeric cytokine bridging innate and adaptive immunity. First identified in 2002, IL-27 is composed of an IL-27p28 (p28) subunit and Epstein-Barr virus induced gene 3 (EBI3) subunit. The heterodimeric IL-27 receptor (IL-27R) is
composed of the IL-27Rα (WSX-1/TCCR) subunit, unique for binding of IL-27, and the gp130 subunit which is shared with the IL-6 receptor\(^2\). It has been shown that IL-27 signals through activation of STAT1 and STAT3, as well as NF-κB in human monocyctic cells\(^2,3\). IL-27 functions to promote differentiation of naïve T helper cells into Th1 cells\(^1,4\). Additionally, recent evidence supports a role for IL-27 in the activation of monocyctic cells which includes upregulation of proinflammatory cytokine and chemokine production\(^3,5\). Inflammatory agents such as lipopolysaccharide (LPS) induce the production of IL-27\(^1,6\) and both subunits of IL-27, IL-27p28 and EBI3, are predominantly expressed by monocytes/macrophages and dendritic cells\(^1\).

Monocytes are activated by microbial components that are recognized by Toll-like receptors (TLRs). Upon ligand binding, TLRs induce cytokine production and, ultimately, clearance of infection. To date, 10 different human TLRs have been characterized\(^7-11\), and cytokine-mediated regulation of TLR expression remains poorly understood. TLR expression analysis in primary human leukocytes showed professional phagocytes express the most varied TLR profile, with CD14+ mononuclear cells expressing the greatest amount TLR2, 4, and 8\(^12\). In monocytes, the expression of TLR4 is important in mediating inflammatory cytokine production in response to bacterial infection, with lipopolysaccharide (LPS) as the main ligand binding to TLR4 to induce the inflammation\(^13,14\). However, TLR4 signaling requires other binding partners. Indeed, LPS binding occurs via the coordinated sequence of binding events between both soluble and cell membrane proteins, including LPS-binding protein (LBP), myeloid differentiation protein-2 (MD-2) and CD14\(^15\). CD14 is a key LPS co-receptor, pivotal in the initial
binding of LPS and transfer of LPS to the MD2/TLR4 complex to initiate signal cascades\textsuperscript{16}. Since CD14 plays a complimentary role in LPS recognition and subsequent responses, the expression of CD14 is also examined in this paper.

In this study, we show a novel mechanism by which IL-27 primes the LPS response in primary human monocytes. Herein we observed enhanced IL-6, TNF-\(\alpha\), MIP1-\(\alpha\), and MIP1-\(\beta\) expression in response to LPS stimulation of IL-27-primed monocytes. We attribute this enhanced LPS response to an IL-27 time- and dose-dependent increase in mRNA and cell surface expression of TLR4 in human monocytes. Furthermore, we show that IL-27-induced TLR4 expression is mediated via JAK/STAT and NF-\(\kappa\)B signaling pathways. We observed a dependency on JAK2, STAT3, and NF-\(\kappa\)B activation for IL-27-mediated TLR4 expression, but no requirement for STAT1. IL-27 priming did not significantly change total LPS binding to monocytes, nor did it alter expression levels of CD14. Immunofluorescence microscopy revealed enhanced membrane expression of TLR4 and more pronounced co-localization of CD14 and TLR4 upon IL-27 pre-treatment, therefore, priming the cell for enhanced LPS responses. Furthermore, in the presence of IL-27 priming, we observed enhanced and prolonged LPS-induced activation of NF-\(\kappa\)B family members, p50 and p65. Our data delineates a mechanism by which IL-27 can regulate inflammation via upregulation of TLR4 expression and signaling.
3.3 Materials and Methods

3.3.1 Monocyte Isolation

Enriched monocytes were isolated from whole blood of healthy donors obtained under Queen's University Research Ethics Board approval. Whole blood samples were processed as previously described using magnetic negative selection with the EasySep Human Monocyte Enrichment Kit (StemCell Technologies).

3.3.2 Cell lines, Cell Culture and Reagents

THP-1 cells (pro-monocytic leukemic cells), were obtained from ATCC. THP-1 cells transfected with CD14-expressing cDNA plasmids (CD14-THP-1 cells) were kindly provided by Dr. R Ulevitch (The Scripps Research Institute, La Jolla, CA). Cells were cultured in Iscove’s Modified Dulbecco's Medium (Invitrogen) supplemented with 10% fetal bovine serum (ThermoScientific). Cell cultures were pre-treated for 1.5 hours at 37°C with the following inhibitors: SD1029 (Calbiochem), 5'-Deoxy-5'-(Methylthio) adenosine (MTA) (Sigma), STAT3 VII (ST3 VII) (Calbiochem), and Caffeic acid phenethyl ester (CAPE) (Cedarlane Laboratories Ltd.). IL-27 was purchased from R&D Systems and exclusion of contaminating endotoxin in the recombinant IL-27 was confirmed by use of the Limulus Amebocyte Lysate (LAL) assay (Cat # QCL-1000 Lonza). The IL-27 preparation used in this study was confirmed to be <0.1 EU per 1μg cytokine, as per the LAL method.

3.3.3 ELISA

Culture supernatants were used to quantify cytokine expression as described and according to manufacturer (BioSource) instructions: IL-6 (CHC1263), TNF-α (CHC1753),
MIP-1α (CHC2203), and MIP-1β (CHC2293). Absorbencies were measured with the BioTek ELx800 Microplate Reader (Fisher Scientific). Data are representative of the average of duplicate wells ± S.D.

3.3.4 RNA Isolation and Reverse Transcriptase-PCR (RT-PCR)

Total RNA was extracted using the TRI Reagent method (Sigma-Aldrich). RNA (1μg) was reverse-transcribed using the Moloney Murine Leukemia Virus reverse transcriptase enzyme (Invitrogen). cDNA was used for TLR4 and 18SrRNA amplification using 5X Taq Polymerase Master Mix (New England Biolabs) and specific primers as follows: TLR4 fwd: 5’-CAA AAT CCC CGA CAA CCT CC–3’, TLR4 rev: 5’-TGT AGA ACC CGC AAG TCT GTG C–3’, 18SrRNA fwd: 5’-TTG GGA ACT GAG GCC ATG AT–3’, 18SrRNA rev: 5’-CGA ACC TCC GAC TTT CGT TT–3’. PCR cycling was performed on the Px2 Thermal Cycler (Thermo Electron Corporation), with annealing temperatures of 55°C. Products were resolved on 1.2% agarose gels containing ethidium bromide and visualized with the Alphalnnotech HD2 Imager (Fisher Scientific).

3.3.5 Flow Cytometry

Cells were washed in PBS-0.1% azide and stained with fluorochrome-conjugated antibodies: TLR4-FITC (Santa Cruz) and CD14-PE (Beckman Coulter). A fluorescent conjugate of lipopolysaccharide (LPS), LPS-Alexa Fluor 488 (Molecular Probes, Inc), was incubated with cells in culture medium for 1hr at 37°C to facilitate LPS binding. After staining, cells were washed with PBS-azide, and data were acquired with the Epics XL-MCL flow cytometer and analyzed using the WinMDI version 2.9 software package (J.
Trotter, Scripps Institute, San Diego). Live cells were gated and analyzed for TLR4 expression and LPS binding.

3.3.6 Western Blotting

Cell pellets were lysed and protein concentrations measured by Bradford Assay. Samples (50 μg protein) were subjected to 10% polyacrylamide SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Millipore). Membranes were probed with anti-phospho-STAT3 (Cell Signaling), anti-pan-STAT3, anti-phospho-JAK2, anti-pan-JAK2, anti-phospho-NF-κB p50, and anti-HSP90 as a loading control (Santa Cruz Biotechnology). Immunoblots were visualized by Enhanced Chemiluminescence (Amersham Biosciences) on the Alphalnnotech HD2 Imager.

3.3.7 Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed to measure STAT1 and NF-κB activation. Nuclear proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce). Protein concentrations were quantified with the Bradford method to ensure equal loading of protein. EMSAs were performed using biotin-labelled probes and 10 μg of protein samples resolved on 5% non-denaturing polyacrylamide gels, as previously described.

3.3.8 Indirect Immunofluorescence Microscopy

Cells were cultured (16hrs) in the presence or absence of IL-27 in 12-well plates. Cells were washed and re-suspended in PBS-0.01% azide, followed by TLR-FITC (Santa Cruz) and CD14-eFluor 605NC (eBioscience) staining, as recommended by the manufacturer. Cells were washed and plated on glass bottom dishes (MatTek, Ashland, MA) for microscopy. Images were captured using an Olympus FV1000 laser scanning
confocal microscope and Fluoview 1.7.3.0 software, using a 60X (1.42 NA) oil immersion objective, or a 40X (0.95 NA) objective. Composites of representative images were prepared using Adobe Photoshop CS3 software.

3.3.9 NF-κB Transcription Factor Assay

The TransAM NF-κB transcription factor family assay (Active Motif, Carlsbad, CA) was performed according to manufacturer instructions. This assay, similar to an ELISA, permits a more sensitive and quantitative read-out of NF-κB activation compared to EMSA, while also identifying specific NF-κB family members involved. Briefly, cells were lysed using the Nuclear Extract Kit provided (Active Motif) to obtain nuclear proteins. 96-well plates coated with NF-κB consensus oligonucleotide binding site were incubated with 10μg of nuclear proteins. Individual wells were incubated with primary antibodies to NF-κB subunits p65, p50, p52, or RelB. An HRP-conjugated secondary antibody incubation was performed, followed by chromogenic substrate, stop solution, and spectrophotometry. Absorbencies were measured with the BioTek ELx800 Absorbance Microplate Reader. Data are representative of average optical density of duplicate wells, as suggested by the manufacturer.

3.4 Results

3.4.1 IL-27 pre-treatment enhances LPS-induced proinflammatory cytokine expression

Previously it was reported that IL-27 augmented inflammatory cytokine (IL-6 and TNF-α) production in response to TLR2 and TLR4 ligands in M-CSF-cultured human monocytes. We sought to confirm this in our model system of freshly isolated human
primary monocytes. Monocytes were pre-incubated (primed) with IL-27 (100ng/mL) for 16 hours, culture medium was replaced, and cells were subsequently treated with the TLR4 ligand, LPS (10ng/mL), for 6 hours. We performed ELISA for IL-6, TNF-α, MIP-1α, and MIP-1β expression as a readout for LPS responsiveness (Figure 3.1). With all cytokines assayed we observed a significant increase in LPS-induced expression following IL-27 pre-treatment, compared to expression levels with IL-27 or LPS treatment alone. These results revealed a striking priming ability of IL-27 on LPS responses in primary human monocytes.

3.4.2 IL-27 induces TLR4 expression on CD14+ monocyctic cells

Cytokines such as IL-6, IL-2, TNF-α, and IFN-γ were previously demonstrated to induce TLR4 expression in monocytic cells\textsuperscript{12,18-21}. Therefore, we asked if IL-27-mediated enhancement of LPS responses could be consequent to IL-27 upregulating TLR4 expression. Accordingly, we initially examined cell surface expression of TLR4 in response to a range of IL-27 doses from 50 to 200ng/mL (Figure 3.2A top). The percent of TLR4 positive cells increased with increasing doses of IL-27, from 26.28% in untreated cells to a maximum of 85.07% in cells treated with 100ng/ml IL-27. Cells reached a maximal effect at 100 ng/mL, therefore this concentration was used for all further experiments. We next investigated the kinetics of IL-27-induced TLR4 expression in freshly isolated monocytes. Cell surface expression of TLR4 increased at 16 hours and was sustained up to 48 hours (Figure 3.2A bottom). Additionally, we observed IL-27-induced TLR4 mRNA expression as early as 2 hours, with sustained expression up to 8 hours after IL-27 stimulation (Figure 3.2B). Taken together, these results show IL-27

76
Figure 3.1: IL-27 pre-treatment enhances LPS-induced IL-6, TNF-α, MIP-1α, and MIP-1β. Freshly isolated monocytes were incubated in medium alone or in the presence of IL-27 (100 ng/mL) for 16 hours. After 16 hours, cells were washed and re-suspended in fresh medium containing LPS for an additional 6 hours. Data shown is representative of five different donors, replicate wells were averaged and standard deviation is shown.
Figure 3.2: IL-27 induces TLR4 expression on CD14+ monocytic cells. (A, top panel) Primary human monocytes were cultured for 16 hours in medium alone or with increasing doses of IL-27 (50 ng/mL to 200ng/mL). Cells were harvested after 16 hours and co-stained for CD14 and TLR4 surface expression. TLR4 expression was measured on live CD14 gated cells by flow cytometry. (A, bottom panel) Primary monocytes were cultured in a time course of IL-27 (100ng/mL), ranging from 0 to 48 hours, cells were harvested, co-stained for CD14 and TLR4, and surface expression was measured by flow cytometry. (B) RT-PCR analysis was performed with TLR4-specific on RNA harvested from cells treated with IL-27 for the times indicated. Amplification of 18SrRNA was performed as a loading control. Data shown is representative of five different blood monocyte donors.
plays a direct role in inducing TLR4 expression at the mRNA and protein level in primary human monocytes. Differences in surface expression of TLR4 and CD14 in the top and bottom panels (Figure 3.2A) are attributed to donor variability. Despite this variability, IL-27-induced TLR4 expression was reproducible in all five donors tested (data not shown). Interestingly, we observed no significant change in CD14 expression in response to IL-27 treatment after 16 hours (Figure 3.2A top and bottom). However, levels of CD14 expression increased slightly after 24 and 48 hours of IL-27 treatment (Figure 3.2A bottom). Since we are priming cells for 16 hours, these results indicate that changes in CD14 expression are not part of the priming mechanism of IL-27, rather, enhanced TLR4 expression may be playing a role in IL-27-mediated enhanced LPS responsiveness.

3.4.3 IL-27-induced TLR4 expression is mediated by JAK2 and STAT3 signaling

We and others have demonstrated that IL-27 signals via the JAK/STAT pathway in primary human monocytes. To decipher the molecular mechanism behind the observed IL-27-induced TLR4 expression we inhibited JAK/STAT signaling and assayed for IL-27-induced TLR4 expression. Firstly, we inhibited JAK2 activation with the JAK2-specific inhibitor, SD1029, previously shown to inhibit JAK2 phosphorylation. To confirm SD1029 blocked IL-27-induced JAK2 phosphorylation, monocytes were pre-treated with SD1029 at doses ranging from 2.5-10 μM, prior to IL-27 stimulation for 15 minutes. Results showed SD1029 inhibits IL-27-induced JAK2 phosphorylation (Figure 3.3A left). We then pre-incubated freshly isolated monocytes with SD1029 at doses ranging from 2.5-10 μM and followed with IL-27 stimulation for 16 hours. TLR4 surface expression decreased in a dose-dependent manner in the presence of SD1029
Figure 3.3: JAK2 and STAT3 signaling mediates IL-27-induced TLR4 expression. (A) Monocytes were cultured in the presence of the JAK2 inhibitor, SD1029, for 1.5 hours with doses ranging from 2.5μM to 10μM. Following the pre-incubation with inhibitor, cells were stimulated with IL-27 for 15 minutes. Activation of JAK2 was measured by Western blotting with anti-phospho-JAK2 (p-JAK2) antibodies (A, left panel). As a loading control, the blot was stripped and re-probed for pan JAK2. Numbers below blot indicate fold increase over untreated cells relative to pan-JAK2 expression. Following the pre-incubation with inhibitor, cells were stimulated with IL-27 for 16 hours and surface stained for TLR4 expression for flow cytometry (A, right panel). (B) Monocytes were cultured in the presence of the STAT1 Inhibitor, MTA, for 1.5 hours with doses ranging from 0.1mM to 1mM. Following inhibitor pre-incubation cells were stimulated with IL-27 for 15 minutes, and nuclear proteins were extracted for electrophoretic mobility shift assay (EMSA) (B, left panel). Cold competitor contained 200X excess of unlabelled probes compared to biotin-labelled probes. Supershift antibodies specific for STAT1 (S1) were able to interfere with complex formation, indicating specificity of STAT1 binding. Monocytes pre-incubated with MTA were followed with IL-27 16hr treatment hours and surface stained for TLR4 expression for flow cytometry (B, right panel). (C) Monocytes were cultured in the presence of the STAT3 Inhibitor, STAT3 VII, for 1.5 hours with doses ranging from 0.25μM to 1μM. Following inhibitor pre-incubation cells were stimulated with IL-27 for 15 minutes, and whole cell lysates were used in Western Blot analysis to determine the efficiency of STAT3 VII in inhibiting STAT3 activation (C, left panel). Membranes were blotted for phosphorylated-STAT3 (p-STAT3) to determine activation, and pan-STAT3 as a loading control. Numbers below blot indicate fold increase over untreated cells relative to pan-STAT3 expression. Monocytes pre-incubated with STAT3 VII were followed with IL-27 16hr treatment hours and surface stained for TLR4 expression for flow cytometry (C, right panel). Cell surface TLR4 expression was measured on CD14-positive cells by flow cytometry. For all flow cytometry, the grey histogram represents cells incubated in medium alone, the bold line represents cells treated with IL-27 alone, and light lines represent cells pre-treated with inhibitor at the doses indicated by arrows. Data shown is representative of five different blood monocyte donors.
(Figure 3.3A right). Furthermore, to control for any non-specific effects of SD1029, we examined the effects of the SD1029 dose range, in the absence of IL-27, on cell surface expression of TLR4 and found no change (data not shown). These results indicate a role for JAK2 signaling in IL-27-induced TLR4 expression.

Since JAK2 phosphorylates and activates STAT1 and STAT3\textsuperscript{23-25}, we investigated the specific roles of STAT1 and STAT3 in IL-27-mediated TLR4 induction using the STAT1-specific inhibitor, MTA, and the STAT3-specific inhibitor, ST3 VI. We first confirmed the inhibitory effect of MTA on STAT1 activation. Monocytes were pre-treated with MTA with doses ranging from 0.1-1mM followed by a 15 minute IL-27 stimulation. MTA is well characterized to specifically inhibit STAT1 nuclear translocation at the doses that we have used, as shown by others\textsuperscript{26,27} and in our previous work\textsuperscript{28}. In this study, STAT1 DNA binding activity was measured by EMSA analysis. Results indicated increasing doses of MTA blocked STAT1 DNA binding (Figure 3.3B left), with the highest dose of MTA pre-treatment (1 mM) showing a return to baseline activation levels. Cold competitor probes abrogated the STAT-DNA complexes and supershift antibodies (S1) specific to STAT1 resulted in disruption of the protein-DNA complexes, observed as the absence of bands. Interestingly, when monocyte cultures were pre-treated with MTA prior to IL-27 stimulation, we observed no inhibition of IL-27-induced surface expression of TLR4 (Figure 3.3B right). These results indicate that, although IL-27 induces the activation of STAT1, STAT1 is not involved in IL-27-induced TLR4 expression on monocytes.
In order to determine whether STAT3 activation plays a role in IL-27-induced TLR4 expression, we employed the use of the STAT3 inhibitor ST3 VII, previously shown to inhibit phosphorylation of STAT3\textsuperscript{29}. We confirmed the inhibitory effects of ST3 VII by pre-treatment of monocytes with ST3 VII, followed by IL-27 stimulation for 15 minutes, and western blot analysis for phosphorylation of STAT3 (Figure 3.3C left). We observed a dose-dependent decreases in STAT3 phosphorylation with increasing doses of ST3 VII pre-treatment, ranging from 0.25-1\textmu M. To determine the role of STAT3 in IL-27-induced TLR4 surface expression, we pre-treated freshly isolated monocytes with the same range of ST3 VII doses, followed with a 16 hour IL-27 stimulation, and surface stained the cells for TLR4 expression by flow cytometry. Results clearly show a dose-dependent reduction in IL-27-induced TLR4 surface expression in cells pre-treated with ST3 VII (Figure 3.3C right). To control for non-specific effects of the inhibitor on TLR4 surface expression, we treated cells in the absence of IL-27 stimulation with the same doses of ST3 VII, and observed no change in TLR4 expression (data not shown). Taken together, these results indicate a role for JAK2 and STAT3 in IL-27-induced TLR4 expression.

3.4.4 IL-27-induced TLR4 expression is mediated via NF-\kappa B activation and IL-27-induced NF-\kappa B activation is dependent on JAK/STAT signaling

In addition to the role played by JAK/STAT signaling, we investigated whether NF-\kappa B, previously shown to be involved in TLR4 expression\textsuperscript{30,31}, mediated IL-27-induced TLR4 expression in our model system. Recently, we demonstrated that IL-27 can activate NF-\kappa B in monocytic cells\textsuperscript{3}. To examine the role of NF-\kappa B in IL-27-induced TLR4 expression, we used the NF-\kappa B-specific inhibitor CAPE\textsuperscript{3,32}. To confirm CAPE inhibited NF-\kappa B activation in primary monocytes, cells were pre-treated with increasing CAPE doses...
ranging from 10-25 μg/mL, followed by IL-27 stimulation for 15 minutes. Nuclear extracts were subjected to EMSA analysis, and showed progressive decreases in NF-κB DNA-binding upon increasing doses of CAPE (Figure 3.4A top). Cold competitor probes abrogated the NF-κB-DNA complexes and supershift antibodies specific to the NF-κB subunits p50 and p65 (S50 and S65) resulted in disruption of the protein-DNA complexes, observed as the absence of bands. To determine the role of NF-κB in IL-27-induced TLR4 surface expression, we pre-treated freshly isolated monocytes with the same range of CAPE doses, followed by a 16 hour IL-27 stimulation, and subsequently measured TLR4 expression on the cell surface. As with SD1029 (JAK2) and ST3VII (STAT3) inhibitors, CAPE pre-treatment inhibited IL-27-induced surface expression of TLR4 on the monocytes in a dose-dependent manner (Figure 3.4A bottom). These results indicate that NF-κB, together with JAK2 and STAT3, mediates IL-27-induced enhancement of TLR4 surface expression.

Previous studies have demonstrated a role for JAK/STAT signalling in the activation of NF-κB, and a strong link has been shown between STAT3 and NF-κB activation in various cancer model systems\textsuperscript{33-35}. Since we observed IL-27-induced expression of TLR4 required JAK2, STAT3, and NF-κB, we tested whether activation of NF-κB required IL-27-induced activation of JAK2 and STAT3. Therefore, we first treated monocytes with SD1029 (JAK2 inhibitor) or ST3 VII (STAT3 inhibitor), and then treated cells with IL-27 for 15 minutes. To delineate the role of JAK/STAT in the activation of NF-κB, we initially measured phosphorylated NF-κB p50 by immunoblotting of whole cell lysates, as previously shown\textsuperscript{3}. Interestingly, we did not observe inhibition of NF-κB p50
Figure 3.4 NF-κB activation mediates IL-27–induced TLR4 expression (A) Primary monocytes were cultured in the presence of the NF-κB inhibitor, CAPE, for 1.5 hours with doses ranging from 10mg/mL to 25mg/mL. Following inhibitor pre-incubation cells were stimulated with IL-27 for 15 minutes, and nuclear proteins were extracted for electrophoretic mobility shift assay (EMSA) (A, top panel). Cold competitor contained 200X excess of unlabelled probes compared to biotin-labelled probes. Supershift antibodies specific for NF-κB p50 (S50) and NF-κB p65 (S65) proteins were able to interfere with complex formation, indicating presence of p50/p65 heterodimers in the visualized bands of interest. Monocytes pre-incubated with CAPE were followed with IL-27 16hr treatment. Cell surface TLR4 expression was measured by flow cytometry (A, bottom panel). The grey histogram represents cells incubated in medium alone, the bold line represents cells treated with IL-27 alone, and light lines represent cells pre-treated with inhibitor at the doses indicated by arrows. (B) Primary monocytes were cultured in the presence of the JAK2 Inhibitor, SD1029, for 1.5 hours with doses ranging from 2.5μM to 10μM. Following the pre-incubation with inhibitor, cells were stimulated with IL-27 for 15 minutes and activation of NF-κB was measured two ways: detection of phosphorylated NF-κB p50 subunit via Western Blot analysis (left panel) or transcription factor ELISA for p50/p65 DNA-binding (right panel). (C) Monocytes were cultured in the presence of the STAT3 Inhibitor, STAT3 VII, for 1.5 hours with doses ranging from 0.25μM to 1μM. Following inhibitor pre-incubation cells were stimulated with IL-27 for 15 minutes and activation of NF-κB was measured two ways: detection of phosphorylated NF-κB p50 subunit via Western Blot analysis (left panel) or transcription factor ELISA for p50/p65 DNA-binding (right panel).
phosphorylation in the presence of JAK2 or STAT3 inhibitors (Figure 3.4B,C left panels). Although we observed no inhibition of p50 phosphorylation in the presence of JAK2 and STAT3 inhibitors by immunoblotting, this is not necessarily indicative of the impact of JAK/STAT inhibition on NF-κB binding activity. Therefore, in order to test the impact of JAK/STAT inhibition on NF-κB DNA binding activity, we performed transcription factor ELISAs for the p50 as well as p65 subunits. These ELISAs were performed in nuclear lysates of THP-1 cells due to inherent difficulties in obtaining sufficient concentrations of primary cell lysates needed for the Active Motif ELISA protocol. These data indicated that upon pre-treatment with SD1029 or ST3 VII (JAK2 and STAT3 inhibitors respectively), IL-27-induced NF-κB binding activity is inhibited.

3.4.5 IL-27 does not increase LPS binding to monocytes

Since we observed enhancement of the LPS response (proinflammatory cytokine production) with IL-27 priming, and we have established that IL-27 can upregulate TLR4 expression on monocytes, we sought to determine if the enhanced LPS response observed was due to increased LPS binding. To do this, we quantified surface binding of a fluorochrome-conjugated LPS (LPS-Alexa) to primary monocytes by flow cytometry. As seen in Figure 3.5, the majority of non-primed (Medium) CD14+ monocytes (87.2%) were bound by LPS, and no significant change in LPS binding was observed in IL-27-primed cells (81.7%). This was expected, as TLR4-LPS binding is also mediated by co-receptors, like CD14. Our data suggests that LPS-TLR4 binding correlates with CD14 expression, both of which do not change in response to IL-27.
Figure 3.5: IL-27 priming does not enhance LPS binding to monocytes. Primary monocytes were cultured in medium alone (Medium) or primed with IL-27 for 16 hours (IL-27). LPS-Alexa (1 μg/mL) was added to all cultures for 1 hour at 37°C to facilitate binding. Cells were harvested and stained for CD14 expression, and data acquired with the Epics XL-MCL flow cytometer. AUTO represents a control for autofluorescence of unstained cells.
3.4.6 IL-27 induces enhanced TLR4 membrane expression and increased co-localization of CD14 and TLR4

The data so far indicate that IL-27 priming for 16 hours can induce TLR4 expression, while not affecting CD14 expression. CD14 transfers LPS to TLR4\(^{36}\), which then triggers TLR4 signaling cascades. Therefore, we hypothesized that IL-27 treatment may increase co-localization of CD14 and TLR4, priming the cells for enhanced LPS responsiveness. To examine the impact of IL-27 priming on CD14 and TLR4 localization we used the THP-1 human pro-monocytic cell line as an initial model system. Immunofluorescence microscopy showed increased TLR4 expression at the cell surface of THP-1 cells primed with IL-27 (Figure 3.6B), compared to cells cultured in medium alone for 16 hours (Figure 3.6A). We observed a population of cells staining intensely for TLR4 in primed cells (Figure 3.6B) which were absent in non-primed cells (Figure 3.6A). As THP-1 cells express relatively low levels of CD14, we utilized THP-1 cells stably transfected with CD14 (CD14-THP-1). These cells constitutively express higher levels of CD14 than untransfected THP-1 cells\(^{17}\). Similarly, in CD14-THP-1 cells we observed the same trend, however, an even more striking upregulation of cell surface expression of TLR4 in primed cells (Figure 3.6C) was seen compared to resting cells (Figure 3.6D). It should be noted that no difference was observed in CD14 expression between primed/non-primed conditions of either cell type. Additionally, we observed a shift from diffuse TLR4 staining in non-primed cells (Figure 3.6A,C) to more distinct membrane TLR4 localization in the primed cells (Figure 3.6B,D). To investigate whether IL-27 priming affected co-localization of TLR4 and CD14, we captured images of CD14-THP-1 cells at a higher magnification (Figure 3.6E, F) to more accurately examine the co-
Figure 3.6 IL-27 induces enhanced TLR4 membrane expression and increased co-localization of CD14 and TLR4. THP-1 cells (A-B), CD14-THP-1 cells (C-F), and primary monocytes (G-H) were cultured for 16 hours in the presence/absence (+/-) of IL-27. Cells were harvested in PBS-0.01% azide, and stained for TLR4-FITC (green fluorescence) and CD14-eFluor 605 (red fluorescence). Images were captured using an Olympus FV1000 laser scanning confocal microscope and Fluoview 1.7.3.0 software, using a 60X (1.42 NA) (E-H) and 40X (0.95 NA) (A-D) oil immersion objective. Increased expression of TLR4 was seen in IL-27-primed THP-1 cells (B) and CD14-THP-1 cells (D), compared to corresponding non-primed THP-1 cells (A) and CD14-THP-1 cells (C). CD14 expression (red) did not change with IL-27 priming. Images for A-D were captured using the 40X objective. To address TLR4/CD14 co-localization, images were captured using the 60X objective on CD14-THP-1 cells cultured in medium alone (E) and primed with IL-27 (F). Arrowheads point to distinct differences in co-localization between non-primed and primed images. Primary monocytes also showed distinct areas of increased TLR4/CD14 co-localization in IL-27-primed cells (H) compared to non-primed (G), as indicated by arrowheads. Images for E-H were captured using the 60X objective. Scale bars represent 50µm (A), 10µm (E), and 20µm (G). Data shown are representative of three primary monocyte donors and four cell line experiments.
localization of these proteins. Non-primed cells exhibited distinctly different localization of TLR4 and CD14, with TLR4 localized distinctly to the plasma membrane and CD14 localized more diffusely (Figure 3.6E arrowhead). However, in cells primed with IL-27 we observed increased co-localization of TLR4 and CD14 at the membrane (Figure 3.6F arrowhead). We confirmed these results in primary monocytes, where we observed differential localization of these proteins in non-primed cells (Figure 3.6G arrowhead) and distinct cellular clusters of co-stained TLR4 and CD14 in primed cells (Figure 3.6H arrowhead). Taken together these results indicate that IL-27 priming induces increased expression and membrane localization of TLR4, without affecting CD14 expression. Furthermore, the data support the notion that part of the IL-27 priming mechanism may include enhanced co-localization of TLR4 and CD14, rendering the cell more responsive to LPS being transferred to TLR4 to initiate signaling cascades.

3.4.7 IL-27 priming augments LPS-induced NF-κB activation

To further characterize IL-27 priming of LPS responses, we sought to investigate if IL-27 pre-treatment followed by LPS stimulation would result in enhanced intracellular signaling. To obtain the relatively high amount of nuclear lysate required for this assay, we used THP-1 cells as our model system. We previously showed that THP-1 cells are representative of the IL-27-primary monocyte response\(^3\). Additionally, THP-1 cells exhibited enhanced TLR4 expression in response to IL-27 stimulation as shown in Figure 3.6A and B, as well as by flow cytometry analysis (data not shown). THP-1 cells were pre-treated with IL-27 for 16hrs, followed with an LPS time course (30–120 minutes), or cultured without IL-27 pre-treatment and LPS time course alone. We first measured NF-κB activation by EMSA analysis, and observed qualitative differences in
non-primed versus primed cells (Figure 3.7A). Multiple NF-κB-DNA complexes were detected, indicating different dimers of NF-κB transcription factors binding to the consensus oligonucleotide. Overall, we observed enhanced and prolonged NF-κB DNA binding activity in IL-27-primed cells compared to cells not primed. Interestingly, at 30 minutes we observed striking differences in the NF-κB subunit complexes activated. To quantify the contribution of individual NF-κB transcription factors to IL-27 priming, we performed TransAM NF-κB transcription factor assays. In Figure 3.7B we compared the activation kinetics of NF-κB transcription factors p65, p50, p52, and RelB. In primed cells (black bars) compared to non-primed cells (grey bars), we observed a strikingly prolonged and enhanced activation of NF-κB p65 after 60 and 120 minutes of LPS stimulation (Figure 3.7B top left). We also observed significantly enhanced activation of NF-κB p50 at all time points in primed cells (Figure 3.7B top right). We examined other NF-κB family members and observed a moderate increase in NF-kB p52 activation at 30 minutes of LPS treatment in primed cells (Figure 3.7B bottom left) and decreased activation of RelB (Figure 3.7B bottom right). Results indicated that IL-27 priming results in enhanced and prolonged activation of NF-kB p65 and p50 in response to LPS stimulation. Taken together our data showed that IL-27 upregulated TLR4 expression, resulting in increased localization with CD14 and enhanced NF-κB activation.
**Figure 3.7: IL-27 priming augments LPS-induced NF-κB signaling.** THP-1 cells were cultured in medium alone or in the presence of IL-27 for 16hrs (primed) then stimulated with an LPS timecourse of 0-120 minutes. (A) Nuclear lysates (10μg) were subjected to EMSA and visualized bands represent activated NF-κB binding to the consensus sequence oligonucleotide probe (biotin labelled). To confirm specificity of the visualized bands for binding to the NF-κB consensus sequence a cold competitor (*CC*) was performed. Results shown were reproducible in 5 replicate experiments. (B) Nuclear lysates (10μg) were used in the TransAM NF-κB transcription factor family assay to quantify NF-κB family member activation in nonprimed cells (grey bars) and IL-27-primed cells (black bars). Graphs represent the activation of individual NF-κB transcription factors: p65 (top left), p50 (top right), p52 (bottom left), and RelB (bottom right). Data shown represent the average of duplicate wells.
3.5 Discussion

We discovered novel mechanisms by which IL-27 can prime the proinflammatory LPS response in primary human monocytes. We demonstrated an IL-27 time- and dose-dependent increase in mRNA and cell surface expression of TLR4 in human monocytes. Furthermore, we showed that IL-27-induced TLR4 expression is mediated via JAK/STAT and NF-κB signalling pathways. We observed a dependency on JAK2, STAT3, and NF-κB activation for IL-27-mediated TLR4 expression. To our knowledge this is the first study to show a role for IL-27 in the induction of TLR expression. We further characterized IL-27 priming by showing enhanced membrane localization of TLR4 upon IL-27 pre-treatment of LPS responses, and more distinct co-localization of TLR4 and CD14 on the surface of monocytes. Additionally, IL-27 augmented LPS-TLR4 intracellular signaling, observed as prolonged and enhanced DNA binding activity of key NF-κB transcription factor subunits p65 and p50, respectively. This work adds to the proinflammatory functions of IL-27 and reveals new mechanisms by which IL-27 can modulate innate immune responses.

We first sought to confirm in our own model system the extent to which IL-27 can prime the LPS response, as this phenomenon was previously demonstrated in M-CSF-treated monocytic cells\(^5\). We used freshly isolated monocytes cultured without addition of recombinant cytokine in our model system. Accordingly, we reported significant enhancement of the LPS response upon priming (pre-treatment) with IL-27, as demonstrated by increased proinflammatory cytokine production. We validated our hypothesis that the IL-27 priming effect on the LPS response correlated with IL-27-induced upregulated TLR4 expression. Other studies have shown that cytokines can
modulate TLR4 expression in monocytic cells to influence innate immune responses\textsuperscript{19-21}. Tamandl et al showed a balance between two inflammatory cytokines, whereby TNF-\(\alpha\) induced hypo-responsiveness to LPS via TLR4 downregulation, while IL-6 induced hyper-responsiveness to LPS via TLR4 upregulation\textsuperscript{19}. This evidence compliments our observation that IL-27-induced TLR4 expression is STAT3-dependent, as both IL-6 and IL-27 signal through gp130 to induce STAT3 activation. Recently, a murine model of STAT3 hyper-activation demonstrated that IL-6-induced STAT3 activation plays a role in regulating LPS-TLR4 responses\textsuperscript{37}. Other cytokines involved in the Th1 response, such as IL-2 and IFN-\(\gamma\), have been shown to mediate upregulation of TLR4 expression on monocytes\textsuperscript{20, 21}. Thus, we hypothesized that IL-27, also an inflammatory cytokine involved in Th1 differentiation, would likely have a role in TLR4 expression. Accordingly, we found that IL-27 induction of TLR4 expression was dose- and time-dependent. Furthermore, to determine if IL-27-mediated induction of TLR4 expression was direct, we performed time courses of IL-27 stimulation on monocytes and measured TLR4 mRNA and protein expression. We found enhanced mRNA expression of TLR4 as early as 2 hours after IL-27 stimulation, and upregulated surface expression within 16 hours, indicating a direct effect of IL-27 on TLR4 expression.

Through the use of specific inhibitors to JAK2, STAT1, STAT3, and NF-\(\kappa\)B, we sought to determine the role of each of these signaling molecules in mediating IL-27-induced TLR4 expression. We found a dependence on JAK2, STAT3, and NF-\(\kappa\)B for IL-27-induced enhancement of TLR4 expression. Little is known about the signaling cascade leading to NF-\(\kappa\)B activation from the IL-27 receptor binding event. Since we observed a
dependency on JAK2, STAT3, and NF-κB for IL-27-mediated induction of TLR4 expression, we hypothesized that JAK2 and STAT3 may act upstream of NF-κB activation. To determine if NF-κB activation was dependent on crosstalk with JAK2 or STAT3, we inhibited the activation of JAK2 and STAT3, followed with IL-27 stimulation, and measured activation of NF-κB. Initially, when performing immunoblotting on whole cell lysates, we did not observe a decrease in p50 phosphorylation in the presence of JAK2/STAT3 inhibitors. Indeed, we observed maintenance of p50 phosphorylation in these conditions (Figure 3.4B, C left panels). Phosphorylation of p50 is not necessarily indicative of NF-κB activation as p50 homodimers can repress NF-κB transcriptional activity\(^{38-40}\). Interestingly, when we performed transcription factor ELISAs on nuclear lysates for both p50 and p65 subunits, we observed that pre-treatment with JAK2/STAT3 inhibitors reduced the DNA-binding activity of NF-κB p50 and p65. This points to a role of JAK/STAT upstream of NF-κB activation. Indeed, our findings are supported in the literature, whereby others have shown the ability of JAK2 to phosphorylate the inhibitor of NF-κB, IκB, rendering NF-κB free to translocate to the nucleus to induce gene expression\(^{33}\). Additionally, in a tumour model system, constitutive activation of NF-κB was dependent on STAT3, due to STAT3-mediated acetylation of the NF-κB RelA subunit, resulting in nuclear retention of the transcription factor and constitutive signaling\(^{35}\).

In terms of IL-27-induced TLR4 expression, it is likely that STAT3 and NF-κB are both required at the promoter to drive efficient expression of TLR4 in response to IL-27. The cooperation of STAT3 and NF-κB in the regulation of gene expression has previously
been described, and includes cooperative binding in adjacent regions of promoter sequences, and physical interactions between the two transcription factors\textsuperscript{34}. Recent work supporting this idea has shown STAT3 and NF-$\kappa$B involvement in the induction of TLR4 expression\textsuperscript{30,31}. Soliman et al described a potential role for STAT3 and NF-$\kappa$B in TLR4 expression in intestinal epithelial cells, with proposed STAT3 and NF-$\kappa$B binding sites in the TLR4 promoter region\textsuperscript{30}.

Since we observed IL-27 upregulated TLR4 expression on monocyte cell surfaces, we thought that a potential priming mechanism of IL-27 may include enhanced LPS binding to cell surfaces, resulting in enhanced LPS signaling. However, LPS binding to monocytes did not appear significantly different between cells cultured in medium compared to those primed with IL-27. Additionally, our results demonstrated that CD14 expression was not affected by IL-27 priming. Taken together, these results indicated that although we observed more surface TLR4 expression in IL-27 primed cells, LPS binding was not further increased as the available CD14 may not be sufficient to facilitate more LPS binding. Additionally, the potential roles of other endotoxin binding proteins, like LBP and MD-2, in this process have yet to be determined. However, we did observe changes in the localization of TLR4 and CD14 on monocytes. Immunofluorescence microscopy showed disperse staining of CD14 in monocytes cultured in medium alone, however, in cells primed with IL-27 we observed re-localization of CD14 to TLR4. We propose that this may prime cells for more efficient transfer of LPS from CD14 to TLR4, resulting in enhanced induction of TLR4 signaling. Indeed, previously it was shown that IL-6-primed PBMC exhibited enhanced LPS-
-induced NF-κB nuclear translocation (activation) by gel shift analysis. Similarly, herein we observed augmented NF-κB activation qualitatively and quantitatively in IL-27-primed cells compared to non-primed cells. In particular, NF-κB p50 and p65 exhibited enhanced and prolonged activation in response to LPS stimulation in IL-27-primed cells. Indeed, heterodimers of NF-κB p50 and p65 signal through the well-described ‘canonical’ signaling pathway, and are most often attributed to upregulated gene expression and inflammation.

The regulation of monocyte/macrophage activation has previously been described as a delicate balance between cytokine-mediated feedforward and feedback mechanisms, resulting in efficient clearance of infection while preventing overactivation of inflammatory responses. Herein, we describe a feedforward mechanism of IL-27 in which this cytokine promotes activation of monocytes in an initial, infection-induced, immune response. Results from this study support a role for IL-27 in a positive feedback loop under inflammatory conditions, as LPS can induce IL-27 expression and the magnitude of LPS responses correlates with total TLR4 expression levels. Therefore, during a gram negative bacterial infection, it is possible that the initial response to LPS induces IL-27 expression, which can then act in an autocrine manner to upregulate TLR4 expression on the same cell to render it more responsive to LPS.

IL-27 is a key player in modulating the delicate balance of infection-induced immune responses, as earlier studies have shown that production of IL-27 itself is induced by various bacterial, parasitic, and host-derived inflammatory stimuli. Numerous pro-
anti-inflammatory activities have been attributed to IL-27, and the outcome of the IL-27 response is highly dependent on cell type, activation state and infection status of the model system used. Indeed, in the setting of septic peritonitis IL-27 was rapidly produced and resulted in suppression of neutrophil migration and activation. Other studies in the setting of Mycobacterium tuberculosis-induced immune activation in macrophages or in LPS-mediated immune activation in macrophages and dendritic cells show similar repressive functions of IL-27. Taken together these studies support a regulatory role of IL-27 in activated model systems, whereby prevention of excessive inflammatory responses is observed upon IL-27 treatment. On the other hand, our model system in this study employs the use of freshly isolated monocytes, which are not cultured in the presence of activating stimuli prior to IL-27 treatment. Therefore, in this setting, IL-27 promotes inflammatory responses to facilitate clearance of infection.

Herein we described the novel finding that IL-27 enhances LPS responses by upregulating TLR4 expression in primary human monocytes. We demonstrated a requirement for STAT3 and NF-κB signaling in IL-27-induced TLR4 expression. Additionally, we showed that IL-27 priming resulted in increased co-localization of TLR4 and CD14, as well as enhanced and prolonged LPS-induced NF-κB activation. Taken together, our work has characterized the molecular mechanisms utilized by IL-27 to promote TLR4 expression in resting primary monocytes and enhance LPS responsiveness. This previously undefined function of IL-27 highlights the ability of this cytokine to significantly bolster innate immune responses.
3.6 References


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CHAPTER 4:

IL-27 REGULATES BST-2 EXPRESSION INDEPENDENTLY OF TYPE I IFN ON HUMAN MONOCYTES AND T CELLS
4.1 Abstract

IL-27 modulates inflammatory responses by influencing monocyte cytokine secretion and CD4 T cell differentiation. Recently, IL-27 was demonstrated to inhibit HIV replication by inducing expression of type I interferon (IFN) and subsequent IFN-dependent apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like (APOBEC)-3, an intracellular antiviral gene. To further characterize other anti-viral properties modulated by IL-27, we examined another IFN-responsive gene: tetherin/bone marrow stromal cell antigen 2 (BST-2). BST-2 retains budding virions at the cell surface, restricting release of enveloped viruses such as HIV. Our study shows that IL-27 can directly induce BST-2 expression, independently of an intermediary type I IFN response. Using flow cytometry to quantify total cellular BST-2 expression, 8h IL-27 treatment resulted in a greater than 2-fold increase in BST-2 on both primary monocytes and T cells. Universal type I IFN, a positive control for BST-2 induction, resulted in a greater than 3-fold increase in BST-2 expression on primary monocytes and T cells. RT-PCR analysis demonstrated IL-27-induced BST-2 mRNA expression as early as 2h after exposure of cells to IL-27 suggesting that IL-27 directly stimulates BST-2 expression. In the presence of the type I IFN-neutralizing protein, B18R, IL-27-induced BST-2 expression was maintained, demonstrating that IFN is not an intermediary in IL-27-induced BST-2. Taken together, our findings identify a novel function of IL-27 as a direct stimulator of BST-2 expression.
4.2 Introduction

Interleukin-27 (IL-27) is an immunoregulatory cytokine that drives innate immune responses and adaptive immunity. IL-27 is a member of the IL-12 family of cytokines, comprised of molecules sharing subunits and receptor chain components. A wide variety of cell types respond to IL-27, as co-expression of the receptor subunits, IL-27Rα (WSX-1/TCCR) and gp130, has been reported in endothelial cells, mast cells, B cells, monocytes, Langerhan’s cells, dendritic cells, and T cells. Previous work demonstrated the ability of IL-27 to induce a similar profile of anti-viral genes to that of IFN-α. Furthermore, the anti-viral gene profile induced by IL-27 inhibited the replication of HIV in both CD4 T cells and monocytes/macrophages. This anti-HIV function of IL-27 was attributed to induction of the antiviral family of APOBEC cytidine deaminase proteins, via an intermediate induction of type I IFN. Our study identifies BST-2 (also known as CD317/tetherin) as an IL-27-inducible protein in HIV target cells: monocytes and T cells.

BST-2 is an interferon (IFN)-responsive host restriction factor expressed in various cell types. Type I IFNs, IFN-α and IFN-β, play a key role in host antiviral defenses by upregulating expression of antiviral genes, including that of BST-2 which inhibits dissemination of virus. BST-2 physically ‘tethers’ or retains budding virions at the cell surface, restricting virus release and ongoing infection. Indeed, two independent studies showed that BST-2 prevents the release of HIV, and that the viral accessory protein Vpu, could counteract this activity. Additionally, BST-2 prevents the release of a broad spectrum of enveloped viruses, including other retroviruses, filoviruses, arenaviruses, paramyxovirus, gamma-herpesviruses, and rhabdoviruses. The
ability of BST-2 to tether this broad group of viruses is driven by common virus features, including lipid envelopes and budding through cholesterol-rich domains of the plasma membrane where BST-2 is concentrated\(^\text{20}\). Thus, expression of BST-2 can have an important influence on virus-host cell membrane interactions. Regulation of BST-2 expression is not well defined, and differences in expression levels on monocytes and T cells have been reported\(^\text{8,21-23}\). Furthermore, although BST-2 is widely recognized as an IFN-responsive gene, evidence exists to support the role for novel stimuli and signaling cascades leading to BST-2 expression\(^\text{24-26}\).

It has been shown that IL-27 can induce type I IFN-responsive genes in human macrophages that are dependent on intermediary IFN-\(\alpha/\beta\) production\(^7\). Since viruses have mechanisms to block type I IFN expression and intracellular signaling pathways, the existence of other mechanisms regulating typical IFN-responsive genes is critical to anti-viral responses. Previous studies have challenged the notion that BST-2 is strictly a type I IFN-responsive gene. One study demonstrated that activated intracellular signalling proteins IRF-3 and IRF-7 can induce BST-2 expression in virus-infected cells, independently of IFN expression\(^\text{24}\). Analysis of the BST-2 promoter indicated binding sites for STAT3, in addition to IFN-responsive elements, pointing to a potential role for STAT3-inducing cytokines in BST-2 regulation\(^\text{25}\). Our data provides the first evidence that a cytokine can induce expression of BST-2, independently of type I IFN intermediates. We show the immunoregulatory cytokine IL-27 can directly upregulate intracellular and cell surface expression of BST-2 on human monocytes and T cells.
4.3 Methods

4.3.1 Cell culture and reagents

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood donations obtained in agreement with the Queen’s University Research Ethics Board approval. Briefly, whole blood was diluted with an equal volume of PBS-EDTA (1mM)/ 2% FBS and layered over Lympholyte (Cedarlane Laboratories, Burlington, ON, Canada) and subjected to density centrifugation. The PBMC fraction was extracted, washed twice in PBS-EDTA, and re-suspended in media for stimulations or further processed by magnetic cell separation to isolate CD4+ T cells or monocytes (StemCell Technologies, Vancouver, BC, Canada). Both cell lines, THP-1 (pro-monocytic leukemic cells) and Jurkat (T cells), were obtained from ATCC. All cells were cultured in Iscove’s Modified Dulbecco’s Medium (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (ThermoScientific, Ottawa, ON, Canada). Recombinant cytokines (IL-6 and IL-27) were purchased from R&D Systems. All IFN stimulations were performed at 1000U/mL with the Universal Type-I Interferon, an alpha IFN hybrid (PBL Interferon Source, Piscataway, NJ, USA). The vaccinia virus-encoded type I IFN receptor, B18R, was used to neutralize type-I IFN intermediates, as previously described (eBioscience, San Diego, CA, USA).

4.3.2 Flow Cytometry

Cells were washed in PBS-azide-FBS (PBS-0.1% azide,1% FBS) and stained with fluorochrome-conjugated antibodies for surface markers as follows: CD14-PE (Beckman Coulter, Mississauga, ON, Canada) and CD3-ECD (Beckman Coulter). BST-2 staining was performed for 30 min at room temperature using rabbit anti-human BST-2 (1:2000)
obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Anti-Bst-2 (cat# 11722) from Drs. Klaus Strebel and Amy Andrews. Subsequent staining with a fluorochrome labelled goat-anti-rabbit IgG-Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR, USA), permitted detection of BST-2 expression. For intracellular staining, cells were first fixed in 4% paraformaldehyde and then surface stained with CD14 and CD3. This was followed by permeabilization with 0.1% saponin and intracellular staining with anti-BST-2, and subsequently stained with secondary antibody (anti-rabbit-Alexa Fluor 488). Data were acquired with the Epics XL-MCL flow cytometer. Analysis was performed using the WinMDI version 2.9 software package (J. Trotter, Scripps Institute, San Diego). PBMC were gated on CD14-positive cells for monocyte analysis, or CD3-positive cells for T cell analysis.

4.3.3 Western Blot

PBMC were lysed and protein concentrations measured by the Bradford Assay. Samples (50μg protein) were subjected to 10% polyacrylamide SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were probed with anti-phospho-STAT3 and anti-pan-STAT3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:500 dilution in 2.5% bovine serum albumin. Immunoblots were visualized by Enhanced Chemiluminescence (Amersham Biosciences, Baie d’Urfe, QC, Canada) on the Alphalnnotech HD2 Imager.

4.3.4 RNA isolation and RT-PCR

RNA extraction and RT-PCR reaction set-up was performed as previously described. Primers specific to BST-2 expression were as follows: fwd: 5’-ACC-CCA-GCT-CCC-

4.3.5 Indirect Immunofluorescence Microscopy

Cells were fixed in 4% paraformaldehyde for 10 minutes at room temperature, washed three times in PBS-1% FBS, and permeabilized in 0.1% saponin. Cells were subsequently stained with rabbit-anti-human BST-2 (1:500) (see ‘Flow Cytometry’ methods), washed and stained with secondary fluorochrome-conjugated antibody, goat-anti-rabbit-Alexa Flour 568 (1:500) (Molecular Probes Inc., Eugene, OR, USA). Cells were washed once and then Hoechst staining to visualize nuclei was performed as previously described28. Cells were washed three times and plated on poly-lysine-coated glass bottom dishes (MatTek, Ashland, MA, USA) 1 hour prior to microscopy to allow settling of cells on dishes for optimal imaging. Images were captured using an Olympus FV1000 laser scanning confocal microscope and Fluoview 1.7.3.0 software, using a 60X (1.42 NA) oil immersion objective. Composites of representative images were prepared using Adobe Photoshop CS3 software.

4.3.6 Statistical Analysis

To assess significance of IL-27-induced BST-2 expression the two-tailed paired Student’s t-test was used, with p<0.05 considered significant.
4.4 Results

4.4.1 IL-27 induces BST-2 cell surface expression on human monocytes and T cells

IL-27 was previously characterized to induce a similar profile of anti-viral gene expression to that of IFN-α in monocyte-derived macrophages and CD4 T cells. Furthermore, Greenwell-Wild et al. showed IL-27 could induce expression of the type I IFN-responsive, anti-viral APOBEC family of cytidine deaminases. Therefore, we reasoned that other anti-viral proteins may be modulated by IL-27 stimulation. Since BST-2 is a type I IFN-responsive protein, we investigated the impact of IL-27 on surface BST-2 expression. PBMC (CD14+ monocytes: Figure 4.1A and CD3+ T cells: Figure 4.1B), THP-1 cells (Figure 4.1C) and Jurkat cells (Figure 4.1D) were either left untreated or were stimulated with IL-27 for times ranging from 8 to 24h. As a positive control, cells were also treated with universal type I IFN, well-characterized to induce BST-2 expression. BST-2 expression was measured on CD14+ monocytes and CD3+ T cells. We observed highest basal expression of BST-2 in cells of the monocytic lineage (CD14+ primary monocytes and THP-1 cells, Figure 4.1A and C), while lower basal expression of BST-2 was observed in primary T cells (Figure 4.1B), with the lowest expression of BST-2 on Jurkat cells (Figure 4.1D). We observed statistically significant induction of BST-2 surface expression in as early as 8h after IL-27 treatment. Additionally, IL-27 and IFN showed similar kinetics of BST-2 induction in both primary monocytes and T cells (Figure 4.1A and B). A similar increase of IL-27-induced BST-2 in primary monocytes was observed in the THP-1 cell line (Figure 4.1C). However, a relatively small increase in IL-27-induced BST-2 was observed in the Jurkat T cell line.
(Figure 4.1D) compared to primary T cells (Figure 4.1B). In contrast to previous studies\textsuperscript{23}, we observed induction of BST-2 expression on Jurkat cells in response to IFN stimulation. Taken together, our results show that basal expression of BST-2 is higher on monocytes compared to T cells, and that IL-27 can significantly enhance surface BST-2 expression on both primary monocytes and T cells.

4.4.2 Despite similar signaling pathways to IL-27, IL-6 does not induce BST-2 on monocytes or T cells

Previous analyses indicated the presence of STAT-responsive elements in the BST-2 promoter\textsuperscript{24,25}, implicating STAT transcription factors as potential drivers of BST-2 expression. IL-27 and the related cytokine family member, IL-6, signal through the common receptor signalling chain gp130 to activate STAT\textsuperscript{3}. Since a previous report identified the presence of a STAT3 binding sequence in the BST-2 promoter\textsuperscript{25}, and IL-27 and IL-6 both induce STAT3 activation\textsuperscript{3}, we reasoned that, like IL-27, IL-6 might enhance BST-2 expression. No increase in surface expression of BST-2 was observed in primary monocytes or T cells treated with IL-6 (Figure 4.2A and B). The bioactivity of the recombinant IL-6 preparation was confirmed by IL-6-induced STAT3 phosphorylation in PBMC (Figure 4.2C). Despite significant STAT3 phosphorylation in response to IL-6, our data indicated that BST-2 is not an IL-6-responsive gene.

4.4.3 IL-27 enhances de novo synthesis of BST-2

Since we observed early upregulation of surface BST-2 in response to IL-27 (8h treatment), and a similar pattern of IL-27-induced BST-2 kinetics to that of IFN-induced
Figure 4.1: IL-27- and IFN-induced BST-2 cell surface expression on monocytes and T cells. PBMC were cultured in the presence of IL-27 (120ng/mL) or IFN (1000U/mL) for time courses indicated, followed by surface staining for BST-2 and cell surface markers CD14 (monocytes, panel A) or CD3 (T cells, panel B). Acquired cells were first gated on live cells, followed by cell type specific gating on surface markers CD14 or CD3. Bars represent the average MFIs from 4 different subjects. Statistical significance of the IL-27/IFN treatment compared to untreated (0) is denoted with * when p<0.05. (C) THP-1 and (D) Jurkat cells were cultured with a similar time course of IL-27 (top panel) or IFN (bottom panel), and MFIs are denoted in brackets. Grey shaded histograms represent background autofluorescence of unstained cells, bold line histograms denoted 'Med' represent cells cultured in medium alone (untreated).
Figure 4.2: Despite similar signaling pathways to IL-27, IL-6 does not induce BST-2 on monocytes or T cells. PBMC were cultured with IL-27 for 16h or with increasing doses of IL-6 for 16h, followed by surface staining for BST-2 and surface markers CD14 or CD3. Live cells were gated, followed by (A) CD14-positive cell (CD14+) gating or (B) CD3+ gating. (C) Western blot analysis for phospho-STAT3 (P-STAT3) and total STAT3 (pan-STAT3) expression in PBMC lysates.
BST-2, we decided to measure intracellular BST-2 expression to further confirm IL-27 mediates induction of BST-2 expression. Accordingly, we stimulated PBMC with IL-27 or IFN for 8h and stained for either cell surface BST-2 or were permeabilized for quantification of total cellular BST-2 expression. Subsequent gating on CD14+ monocytes (Figure 4.3A) and CD3+ T cells (Figure 4.3B) permitted a comparison of BST-2 induction only on the cell surface (non-permeabilized BST-2 staining, Figure 4.3A and B, top panels) versus total cellular levels of BST-2 (permeabilized BST-2 staining, Figure 4.3A and B, bottom panels). In CD14+ monocytes (Figure 4.3A) and CD3+ T cells (Figure 4.3B), we observed a striking increase in IL-27-mediated and IFN-mediated BST-2 upregulation, with greater than 2-fold increases over basal levels in IL-27-induced staining in permeabilized cells. Furthermore we observed a stronger IL-27-mediated increase of BST-2 in permeabilized cells compared to surface stained cells. When we performed a similar staining comparison in THP-1 and Jurkat cell lines we observed relatively smaller increases in BST-2 expression upon permeabilization of the cells (Figure 4.3C and D). The relatively fast kinetics of IL-27-induced BST-2 expression, suggests that IL-27 directly induces de novo BST-2 expression.

4.4.4 IL-27 induces BST-2 mRNA expression

To further delineate if IL-27 induces de novo synthesis of BST-2, we isolated monocytes and CD4+ T cells from PBMC fractions and performed a short time course of IL-27 treatment (2-4h). Subsequent RT-PCR analysis indicated IL-27-mediated direct induction of BST-2 mRNA expression in primary monocytes and T cells, as we observed an increase in BST-2 transcripts as early as 2h post exposure to IL-27 (Figure 4.4, top). Similar IL-27 treatments were also performed using THP-1 and Jurkat cell lines. RT-PCR
**Figure 4.3: IL-27 enhances intracellular BST-2 expression.** PBMC were stimulated with IL-27 or IFN for 8h, then in parallel, surface stained or permeabilized and intracellularly stained for BST-2 expression. (A) Co-staining for BST-2 and CD14 on non-permeabilized PBMC (top panel, ‘surface’) and permeabilized PBMC (bottom panel, ‘perm’). Mean fluorescence intensities (MFIs) for CD14+ monocytes are denoted in upper right quadrants of dot plots. (B) Co-staining for BST-2 and CD3 on non-permeabilized PBMC (top panel) and permeabilized PBMC (bottom panel). MFIs for CD3+ T cells are denoted in upper right quadrants of dot plots. Histograms represent similar stimulation and staining (non-permeabilized versus permeabilized) procedures performed in THP-1 (C) and Jurkat (D) cell lines. Grey shaded histograms represent background autofluorescence of unstained cells, bold line histograms denoted ‘Med’ represent cells culture in medium alone (untreated).
Figure 4.4: IL-27 enhances mRNA expression of BST-2. RT-PCR analysis was performed on purified monocytes and T cells from PBMC fractions, as well as THP-1 and Jurkat cell lines. Cells were treated with a short time course of IL-27 treatment (2-4h), followed by amplification of BST-2 transcript expression. 18SrRNA served as a loading control.
analysis revealed induction, although to a lesser magnitude, of BST-2 mRNA in response to IL-27 in these cell lines (Figure 4.4, bottom). These results confirmed IL-27-induced BST-2 expression and indicate that IL-27-induced BST-2 expression is a direct mechanism, occurring independently of intermediates.

4.4.5 IL-27 induces BST-2 expression independently of a type I IFN intermediate.

IL-27 was previously shown to induce antiviral gene expression by two mechanisms: one, an indirect effect via inducing type I IFN production⁷, and the other, a direct mechanism whereby IL-27 induces an IFN-like gene expression profile, in the presence of IFN-neutralizing antibodies⁵. To address whether the effect of IL-27 on BST-2 expression is direct, we first examined the intracellular distribution of BST-2 in response to IL-27. BST-2 had been previously shown to localize intracellularly within the Golgi network and to the plasma membrane via lipid raft association⁸,²⁰. We examined BST-2 localization by confocal immunofluorescence microscopy in THP-1 cells (Figure 4.5, top 2 rows) and PBMC (Figure 4.5, bottom 2 rows). Since IL-27 had relatively small effects on BST-2 expression in Jurkat cells, this cell line was omitted from these analyses. Hoechst staining revealed that the enhanced expression of BST-2 was located outside of the nucleus, localized to both the cytoplasm and plasma membrane in THP-1 and PBMC treated with IL-27 for 8h (Figure 4.5, rows 2 and 4). Our data suggested that in IL-27-stimulated PBMC and THP-1 cells, BST-2 localized to secretory vesicles and to a perinuclear vesicular compartment reminiscent of the Golgi apparatus (Figure 4.5, arrows). These data further support the notion that IL-27 can directly induce expression of BST-2 in THP-1 cells and PBMCs following short (8h) stimulation with IL-27.
Figure 4.5: IL-27-induced intracellular BST-2 is found within the secretory pathway. THP-1 cells (top 2 rows) and PBMC (bottom 2 rows) were cultured in the presence/absence (+/-) of IL-27 for 8h. Cells were harvested and Hoechst-stained for nuclei (blue fluorescence) or intracellularly stained for BST-2 (red fluorescence). Upon IL-27 stimulation (+ panels), increased surface and intracellular expression of BST-2 was observed. Arrows point to distinct regions cells where IL-27 induced enhanced BST-2 within secretory vesicles. Data shown are representative of two primary monocyte donors and four cell line biological replicates.
To investigate whether type-I IFN is responsible for IL-27-induced BST-2 expression, we employed a soluble vaccinia virus-encoded type I-IFN receptor (B18R), previously shown to have potent IFN neutralizing effects\(^{24,29}\). PBMC and THP-1 cells were treated with B18R and universal type I IFN followed by analysis of surface expression of BST-2 by flow cytometry. In the presence of B18R (0.1μg/mL), cells treated with IFN for 16h showed complete abrogation of BST-2 induction (Figure 4.6A). As an additional control, cells treated with IFN for 8h showed a similar potent blockade of the IFN response in the presence of B18R (data not shown). In contrast, IL-27-mediated induction of BST-2 expression was not inhibited in the presence of B18R. As seen in Figure 4.6B (top 3 rows), primary monocytes, T cells and THP-1 cells maintained IL-27-induced BST-2 expression after 8h IL-27 stimulation. Similarly, in cells treated for 16h with IL-27, in the presence of B18R we did not observe significant decreases in IL-27-induced BST-2 expression (Figure 4.6B, bottom 3 rows). Taken together, the data demonstrate that IL-27 induces BST-2 expression independently of type I IFN.
Figure 4.6: IL-27 induces BST-2 expression independently of a type-I IFN intermediate. (A) To confirm potency of B18R for IFN inhibition, cells were cultured in medium alone ('Med'), in the presence of 1000U/mL of IFN for 16h ('IFN 16h'), or the presence of 1μg/mL of B18R + IFN ('B18R + IFN 16h'). CD14+ cells gated from PBMC are shown in column 1, CD3+ cells gated from PBMC in column 2, and THP-1 cells in column 3. Histograms represent live gated cells denoted with mean fluorescence intensity of BST-2 staining in top left corners. (B) To observe the role of IFN in IL-27-induced BST-2 expression, cells were cultured in medium alone, in the presence of IL-27 for 8h, or in the presence of B18R + IL-27 8h (top 3 rows). To observe a later time point of induction, cells were also assayed in medium for 16h, in the presence of IL-27 for 16h, or in the presence of B18R + IL-27 16h (bottom 3 rows). CD14+ cells gated from PBMC are shown in column 1, CD3+ cells gated from PBMC in column 2, and THP-1 cells in column 3. Histograms represent live gated cells denoted with mean fluorescence intensity of BST-2 staining in top left corners. Data are representative of 5 separate PBMC donors.
4.5 Discussion

We define a novel role for IL-27 as a regulator of BST-2 expression, as evidenced by significant upregulation of BST-2 expression in response to IL-27 on human monocytes and T cells. The data support the notion that IL-27-induced BST-2 expression is a direct effect, as we observed upregulated mRNA expression of BST-2 as early as 2h after IL-27 stimulation and increased intracellular BST-2 staining after 8h of IL-27 stimulation. We confirmed that IL-27-induced BST-2 expression occurs independently of type I IFN, as in the presence of an IFN-neutralizing protein (B18R) IL-27-induced BST-2 expression was maintained. This study is the first to identify IL-27 as an inducer of BST-2 expression, which represents a new anti-viral function for IL-27.

Consistent with the findings of others, we report greater steady-state levels of cell-surface BST-2 on monocytes versus T cells. However, these results contrast previous studies which reported undetectable basal expression of BST-2 in primary CD3+ T cells and undetectable basal and IFN-inducible BST-2 in Jurkat cells. Our work shows that type I IFN enhances BST-2 expression on primary T cells and the Jurkat T cell line, contrasting previous reports of weak IFN-induced BST-2 on lymphocytes from tonsil tissues. These differences in BST-2 basal and inducible expression may be attributed to culture conditions and varying sensitivities of detection. Accordingly, we examined both surface and intracellular BST-2 induction by flow cytometry together with mRNA expression levels and, collectively, our data show that IL-27 induces comparatively lower BST-2 expression on T cells compared to that of type I IFN. Furthermore, we report that IL-27 could induce BST-2 expression on both primary human monocytes and THP-1 cells.
Upon identifying the ability of IL-27 to induce BST-2 expression, we decided to investigate whether IL-6 could induce BST-2 in the same way as IL-27, since both cytokines signal via gp130 to activate STAT3. We observed no IL-6-mediated induction of BST-2 on monocytes or T cells. Our finding was in accordance with previous results which observed no changes in induction of cell-surface BST-2 on PBMCs in response to IL-6. The BST-2 promoter region encodes putative binding sites for STAT3 transcription factors, in addition to the IFN-response elements IRF-1/2, ISGF3, and GAS. Furthermore, sequence alignment of human, mouse, and rhesus BST-2 promoters showed STAT binding sites to be conserved, indicating a likely dependency on STAT3 for effective BST-2 gene expression. Since our data indicates that IL-27, but not IL-6, induces BST-2, it is likely that gp130-mediated STAT3 induction alone may not be sufficient for BST-2 upregulation. Indeed, virus-mediated induction of IRF-3 and IRF-7 were demonstrated to be sufficient for BST-2 upregulation in the absence of type-I IFN. IL-27 has also been shown to induce the activation of NF-κB, for which a putative binding site has been identified in the BST-2 promoter. However, stimulation of PBMC with TNF-α, an inducer of NF-κB activity, did not enhance BST-2 expression, while a dependency on STAT1 for IFN-induced BST-2 induction was demonstrated. Since we and others have shown that IL-27 induces activation of STAT1 in both monocytes and T cells, it is possible that this transcription factor may be involved. Therefore a combination IL-27-mediated activation of STAT1/3 in addition to that of NF-κB may cooperate with other factors to influence BST-2 expression.
To extend our findings of upregulated surface BST-2 in response to IL-27, we performed analysis on total cellular content of BST-2 in permeabilized cells. Interestingly, we observed greater increases in IL-27-mediated upregulation of BST-2 at 8h in permeabilized versus non-permeabilized cells showing that total cellular levels of BST-2 are increased in IL-27-stimulated compared to basal levels. This indicates that IL-27 induces de novo synthesis of BST-2, and rules out the possibility that IL-27 modulates BST-2 cell surface expression by enhanced delivery of pre-formed BST-2 to the plasma membrane. The increased magnitude of IL-27-induced BST-2 expression was observed in both permeabilized primary monocytes and T cells, however, this trend was not as significant in the cell lines, THP-1 and Jurkat. It is possible that mechanisms of gene regulation are different in cell lines compared to primary cells, as a consequence of the immortal characteristics of these cells and unique differentiation lineages. It is possible that the BST-2 expression levels in Jurkat cells may not change significantly in response to IL-27, while instead, IL-27 may impact the BST-2 membrane recycling in these cells. Indeed, inducible BST-2 expression on T cells (both primary and cell line) has been previously reported to be inconclusive in the literature, and despite dedicated study to more precise mechanisms governing BST-2 expression specific to T cells, the events still remain unclear\(^{21,22}\).

To investigate the ability of IL-27 to directly induce BST-2 expression, we initially performed RT-PCR analysis on cells treated with IL-27. In these experiments we observed increased BST-2 gene expression in as early as 2h after IL-27 stimulation. To further support the notion that IL-27 can induce de novo synthesis of BST-2, we assessed the subcellular localization of BST-2 following 8h IL-27 stimulation. We
observed IL-27-stimulated cells to have enhanced intracellular expression of BST-2 that localized predominantly to a perinuclear vesicular compartment reminiscent of the Golgi (Fig.5, arrows). This degree of BST-2 expression was not observed in untreated cells. Furthermore, we observed an increase of BST-2 staining in punctate cytoplasmic structures, presumably representing newly synthesized BST-2 in secretory vesicles targeted for the plasma membrane (Fig.5, arrows). Taken together, our microscopy and PCR data indicate that IL-27 directly induces BST-2 expression.

Previous work detected trace levels of IFN-α secretion (~50pg/mL) in human macrophage supernatants following 7h IL-27 stimulation. Since we observed a significant increase in BST-2 expression at 8h following IL-27 stimulation, we reasoned that IL-27-induced type-I IFN was not responsible for mediating the upregulation of BST-2 expression. We confirmed this through the use of B18R to neutralize type I IFN. B18R-treated cells maintained IL-27-induced BST-2 expression indicating that type-I IFN does not play an intermediary role in IL-27-induced BST-2 expression.

In this study we confirm expression levels of BST-2 on monocytes and T cells, and for the first time, report inducible BST-2 expression on monocytes and T cells in response to IL-27 stimulation. Expression of BST-2, a viral restriction factor, on these specific cell types is particularly interesting, as these cell types are targets for infection by HIV. Since BST-2 can inhibit HIV infection, and IL-27 can inhibit HIV replication, it is likely that the influence of IL-27 on BST-2 expression represents a newly defined anti-viral function for IL-27.
4.6 References


CHAPTER 5:

IMPACT OF HIV INFECTION, HIGHLY ACTIVE ANTI-RETROVIRAL THERAPY, AND HEPATITIS C CO-INFECTION ON SERUM INTERLEUKIN-27
5.1 Summary
A newly described cytokine, IL-27, that activates naive CD4 T cells, has recently been shown to be an anti-HIV cytokine. However, the effect of HIV infection on IL-27 expression has not been characterized. We found clinical characteristics including HIV viral load, HCV co-infection, and CD4 T cell counts were associated with changes in serum IL-27. Overall, our results suggest circulating HIV may suppress IL-27, a critical concept in treatment development with this cytokine.

5.2 Introduction
HIV infection causes dysregulated cytokine production\(^1\)-\(^5\), resulting in impaired immunity characteristic of HIV/AIDS. Interleukin-27 (IL-27) is a newly described IL-12 family cytokine critical in development of Th1 responses\(^6\)\(^-\)\(^8\). IL-27 also regulates inflammatory responses in monocytes/macrophages\(^9\),\(^10\) and CD4 T cells\(^7\),\(^11\), targets of HIV. The effect of HIV on IL-27 has not been investigated. Interestingly, studies have demonstrated that IL-27 can inhibit HIV replication\(^12\)\(^-\)\(^14\), leading to the possibility of IL-27 administration as anti-HIV therapy. This study characterizes for the first time how HIV affects IL-27 expression.

5.3 Methods

5.3.1 Serum Collection
In accordance with Queen’s University Research Ethics Board, informed consent was obtained from HIV-positive (n=32) and negative (n=11) individuals. Group 1 (n=6) was naive to highly active anti-retroviral therapy (HAART) with a median viral load (VL) of 24,927 copies/mL, Group 2 (n=10) was receiving HAART with low VL (<500 copies/mL), Group 3 (n=9) was receiving HAART with low VL and co-infected with hepatitis C virus.
(HCV), and Group 4 (n=7) was receiving HAART with high VL (>500 copies/ml, median of 3,003 copies/mL). Of the Group 4 patients, 3 were tolerant/non-adherent to HAART, 2 were two-class resistant (nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), and 1 was one-class resistant (NRTI). Ten patients (n=2, 4, 3, and 1 in groups 1, 2, 3, and 4 respectively) had samples drawn several months apart and therefore are included twice to enhance statistical power. CD4 T cell counts and VLs were obtained during routine clinic visits (Clinical Immunology Outpatient Clinic, Hotel Dieu Hospital, Canada).

5.3.2 IL-27 Enzyme-Linked Immunosorbent Assay (ELISA)

IL-27 ELISA (R&D Systems) was performed as per manufacturer’s instructions. Individual patient sera were assayed in duplicate and averaged for statistical analysis.

5.3.3 Statistical Analysis

Due to small sample size and non-normal distribution of key variables, non-parametric tests (Spearman correlations, Mann-Whitney U, Kruskal-Wallis) were used for analysis, using SPSS Version 17.0 for Windows. P<0.15 was considered a trend of clinical relevance, while p<0.05 was used to define statistical significance.

5.4 Results

Heterodimeric IL-27 ELISAs showed considerable variability in IL-27 expression levels among HIV-negative and HIV-positive patient groups (Figure 5.1a). Interestingly, Group 4 exhibited strikingly low IL-27 expression with minimal variability. Further analysis
Figure 5.1: The effect of HIV viral load, HCV co-infection, and CD4 T cell counts on IL-27 expression in human serum. Serum samples were assayed in duplicate by IL-27 ELISA to detect expression levels of heterodimeric IL-27. (a) The distribution of IL-27 expression in healthy (control) versus HIV-infected subjects is shown. Means and SEMs were calculated as the average of serum IL-27 expression for each group. (b) Spearman’s rho correlation test between viral load and IL-27 expression (pg/mL) is shown amongst all study participants (Overall, top), in Group 1 (middle), and in Group 4 patients (bottom). (c) The Mann-Whitney test for IL-27 expression shown by mean rank, was performed for patients naive to HAART treatment (No HAART, N= 9) and compared to those receiving HAART (HAART, N = 33). This non-parametric test showed a mean rank of IL-27 expression very similar between the two groups resulting in an insignificant p value, indicating no significant difference in distribution of IL-27 expression based on HAART treatment. (d) The Mann-Whitney test in IL-27 expression shown by mean rank in patients co-infected with Hepatitis C Virus (HCV, N= 19) compared to those not infected with HCV (No HCV, N=23) is shown. (e) The Kruskal-Wallis test was used to detect the trend in IL-27 expression as shown by mean rank within CD4 groups. This test was employed amongst all study participants (Overall), which included healthy controls, and for each of the HIV-infected groups independently.
determined that the overall data were not normally distributed (data not shown) therefore we performed non-parametric tests based on medians and ranks.

5.4.1 IL-27 and HIV viral load are negatively correlated

We observed an overall moderate negative correlation between IL-27 and VL using Spearman’s rho (rho=−0.201, p=0.149), (Figure 5.1, top). Results were similar (rho=−0.299) in Group 1 (Figure 5.1b, middle), but the drop in sample size (from 53 to 8) resulted in a substantial loss of power (p=0.47). Interestingly, in Group 4 (Figure 5.1b, bottom) no association was found. Groups 2 and 3 present no detectable viral load, therefore were not included. When HIV-infected patients were grouped as either naive to or on HAART (Figure 5.1c), the Mann-Whitney test found no difference in the mean rank of IL-27 between the two groups, indicating no association of HAART and HIV viral load.

5.4.2 HIV-HCV co-infection is associated with lower IL-27

We investigated whether HCV co-infection might influence IL-27 and observed a decrease in IL-27 in patients co-infected HCV compared to those mono-infected with HIV (Figure 5.1d) which fell just short of statistical significance (p=0.093).

5.4.3 IL-27 varies with CD4 T cell counts

The Kruskal-Wallis test was employed amongst all study participants (Overall) and for each of the HIV-infected Groups (Figure 5.1e). The Overall group indicated a noteworthy trend of IL-27 expression within CD4 groups (p=0.153); IL-27 peaked within the moderate CD4 T cell count group (200-350) and decreased in the low CD4 count group (<200). This was a consistent trend of IL-27 within CD4 groups, with moderate CD4 counts (200-350) showing highest IL-27 in 4 out of 5 groups.
5.5 Discussion

Recent studies identify IL-27 as an anti-HIV cytokine\textsuperscript{12-14} indicating a potential role for IL-27 in control of HIV replication. Herein we describe for the first time how IL-27 levels are modulated in HIV infection. We observed a negative correlation between VL and IL-27; high VL may suppress IL-27, a pathogenic mechanism used by HIV to downregulate immune responses. This is supported by studies showing low expression of the related cytokine, IL-12, in HIV infection\textsuperscript{15,16}. The negative correlation also substantiates previous findings showing IL-27-mediated inhibition of viral replication\textsuperscript{12-14}. The loss of the negative correlation in Group 4 could be attributed to widespread loss of normal immunologic function and/or development of tolerance/resistance to HAART. Whether suppression of IL-27 is mediated via host response or directly by HIV remains to be investigated.

We observed no significant difference in IL-27 among patients naive to HAART versus those receiving HAART, consistent with a recent study showing no effect of HAART on the IL-27-related cytokine, IL-12, and its overproduction observed in acute infection\textsuperscript{1}. Our finding that HCV co-infection was associated with a significant decrease in IL-27 indicates that HCV may suppress IL-27, paralleling previous studies showing HCV core protein inhibits IL-12\textsuperscript{17-19}. On the contrary, studies have shown enhanced circulating cytokines in HCV infection\textsuperscript{20,21}, including IL-12, whereby HCV genotype 1 was associated with a significant increase in IL-12 expression\textsuperscript{20}. Further work investigating HCV viral loads and genotypes in co-infected participants might provide insight on IL-27 suppression.
We observed CD4 T cell count categories to significantly differ in IL-27 expression. In 4 out of 5 groups, a boost in IL-27 from high CD4 T cell counts (>350) to moderate (200-350), followed by a decline in IL-27 in low CD4 counts (<200) was observed. We hypothesize the initial boost in IL-27 to indicate host response to viral insult. IL-27 can inhibit HIV replication\textsuperscript{12-14,21}, therefore upregulating IL-27 could be a protective response to decreasing CD4 T cells and increasing VL. As disease weakens immunity, the virus may then suppress IL-27.

Our findings indicate HIV VL, HCV co-infection, and CD4 T cell counts are strongly associated with changes in serum IL-27. This study is the first to report how HIV infection influences IL-27 expression. Understanding how HIV affects IL-27 is a critical step in determining the potential of IL-27 as a therapeutic adjunct to HIV treatments.


CHAPTER 6:

**IL-27-INDUCED GENE EXPRESSION IS DOWNREGULATED IN HIV-INFECTED SUBJECTS**
6.1 Abstract

During HIV infection, cytokine expression and function become deregulated. Interleukin-27 (IL-27), a key immunoregulatory cytokine, is an important modulator of inflammatory responses. Interestingly, IL-27 can inhibit HIV replication in T cells and monocytes, implicating IL-27 as a potential adjunct to anti-viral treatment. Our previous work demonstrated that circulating HIV may suppress IL-27 expression, therefore, this study, in continuation of our previous work, aimed to understand how HIV affects expression levels of the IL-27 receptor and downstream functions of IL-27. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of HIV-viremic or HIV-negative individuals to assess IL-27-induced gene expression by flow cytometry and ELISA. PBMC were also processed for monocyte enrichment to assess IL-27-induced STAT1/3 activation and gp130 mRNA expression by western blot and real-time PCR, respectively. HIV-viremic individuals demonstrated intact monocyte STAT1/3 signaling in response to IL-27. Levels of the IL-27 receptor chain, gp130 were upregulated in response to IL-27 in HIV-negative individuals, however, in HIV-viremic individuals, this response to IL-27 was lost. Furthermore, we observed significant downregulation of IL-27-induced IL-6, TNF-α, and IL-10 expression in HIV-viremic subjects. In HIV-viremic individuals, observations that IL-27-induced STAT1/3 activation is preserved while IL-27-induced gene expression was impaired indicate HIV-mediated dysregulation of IL-27 functions downstream of IL-27 induced signaling. This provides evidence for a new viral pathogenic mechanism that contributes to the widespread impairment of immune responses observed in the course of infection.
6.2 Introduction

In order for the immune system to clear viral infections, immune cells must be able to produce and respond to cytokines. During HIV infection, cytokine expression and functions become deregulated, contributing to broad immune dysfunction and disease progression. Interleukin-27 (IL-27), can function as a pro- or anti-inflammatory cytokine depending on cell type and activation status\(^1\). IL-27 is a member of the IL-12 family of cytokines, comprised of molecules sharing subunits and receptor chain components\(^2\). The IL-27 receptor (IL-27R) is heterodimeric, composed of the IL-27Ra (WSX-1/TCCR) subunit which is unique for the binding of IL-27, and a \(\beta\) receptor subunit, called gp130\(^3\). The gp130 receptor chain is a commonly shared signaling receptor subunit for numerous cytokines, including IL-6, oncostatin M (OSM), IL-11, leukemia inhibitory factor (LIF), cadiotrophin-1 (CT-1), cadiotrophin-like cytokine (CLC), ciliary neurotrophic factor (CNTF), and neuropoietin (NP)\(^4\). The WSX-1 receptor chain was identified as a result of sequence homology with the gp130 chain and, as such, is a characteristic type I cytokine receptor\(^5,6\). Although IL-27 can bind with low affinity to WSX-1 in the absence of gp130, for effective signal transduction both IL-27R subunits must be expressed\(^3,7\). The IL-27R subunits are co-expressed in endothelial cells, mast cells, activated B cells, monocytes, Langerhan’s cells, activated DCs, and T cells\(^3,7-10\).

The IL-27 intracellular signaling pathway is well defined. Initiated by binding the IL-27R complex, the main signaling components for IL-27 include the JAK/STAT pathway. WSX-1 has a short cytoplasmic domain compared to gp130, which can directly associate with JAK1 to activate STAT1 and STAT3 in naïve CD4 T cells\(^11,12\), while in fully activated T cells only IL-27-induced STAT3 activation was reported\(^13\). Thus, IL-27
may induce differential signal transduction events depending on cell type and activation status. Additionally, we and others have previously described IL-27 as an important regulator of monocytc cell function and have shown IL-27 can induce STAT1/3 activation in human monocytes. More specifically, our previous work characterized novel functions of IL-27 in driving the activation of monocytes, including upregulation of inflammatory responses characterized by pro-inflammatory cytokines expression.

IL-27 is a cytokine that is critical to the initiation of innate immune responses directed by monocytc cells and bridges to adaptive immunity by its influence on T cell differentiation. Thus, IL-27 can play a role in regulating inflammatory responses in monocytes/macrophages and CD4 T cells, both of which are primary targets of HIV infection. Interestingly, IL-27 can inhibit HIV replication in T cells and monocytes/macrophages, implicating IL-27 as a potent anti-HIV cytokine. Previously, we found that clinical characteristics, including HIV viral load, hepatitis C virus coinfection, and CD4 T cell counts were associated with changes in serum IL-27. Herein, we identify how IL-27 functions in the setting of HIV infection, including characterization of IL-27 receptor expression, and downstream functions of IL-27 on induction of pro- and anti-inflammatory gene expression.

6.3 Methods

6.3.1 Study Participants

In accordance with Queen’s University Research Ethics Board approval, informed consent was obtained from 12 HIV-negative (controls) and 13 HIV-positive, viremic, blood donors from the Clinical Immunology Outpatient Clinic (CIOC) at Hotel Dieu
Hospital, Kingston, Ontario, Canada. Three HIV-positive patients had samples drawn several (5 or more) months apart and, therefore, were included twice in some experiments to enhance statistical power. Viral load (copies/mL) and CD4+ T cell counts (cells/μL) were obtained during routine clinic visits at the CIQC. All subjects included in the HIV-positive group were viremic, with a mean plasma viral load of 19086 (330-74912) copies/mL and CD4 T cells counts were 344 (30-771) cells/μL. Six HIV-positive study subjects presented with high viral loads, despite being currently on HAART. However, of these patients, 3 were tolerant/non-adherent to HAART, 2 were two-class resistant (nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), and 1 was one-class resistant (NRTI).

6.3.2 Cell Culture and Monocyte Isolation

THP-1 cells (pro-monocytic leukemic cells), were obtained from ATCC. Cells were cultured in Iscove’s Modified Dulbecco’s Medium (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco). A HEK-293 T cell line constitutively expressing WSX-1/TCCR (HEK-WSX-1) and a control cell line expressing a control cytokine receptor (HEK-control) were gifts from Dr. Nathalie Arbour (McGill University, Montreal, Canada), and were used as a positive control in the WSX-1 flow cytometry staining, as previously described\textsuperscript{19}. Peripheral blood mononuclear cells (PBMC) were isolated by blood overlays on Lympholyte (Cedarlane Laboratories) and density centrifugation. The PBMC fraction was isolated and processed for monocyte enrichment (Stemcell Technologies cat #19058) or cryopreserved in fetal calf serum-dimethyl sulfoxide (10%) at -80°C until future use. Enriched monocytes were stimulated with recombinant IL-27 (120ng/mL, R&D Systems) for 15 mins or 4h, then subsequently processed for Western Blot or real-
time PCR, respectively. Frozen PBMC of all study participants were thawed simultaneously in IMDM + 20% FCS for 24h in 24-well tissue culture plates. This was followed by removal and replacement of media with IMDM + 10% FCS, and subsequent treatment with IL-27 for 24h. Cells were harvested, then PBMC used in flow cytometry and culture supernatants in ELISA.

6.3.3 Western Blotting

Cell pellets were lysed and protein concentrations measured by Bradford Assay. Samples (50μg protein) were subjected to 10% polyacrylamide SDS-PAGE followed by transfer onto polyvinylidene difluoride membranes (Millipore). Membranes were probed with anti-phospho-STAT1, anti-pan-STAT1, anti-phospho-STAT3, anti-pan-STAT3, and anti-HSP90, as a loading control (Santa Cruz Biotechnology). Immunoblots were visualized with the ECL Advance Western Blotting Detection kit (GE Healthcare) on an AlphaInnotech HD2 Imager.

6.3.4 RNA Isolation and Real time-PCR

Total RNA was extracted from monocytes using TRI-Reagent. RNA quantification was performed using the ND-1000 (NanoDrop Technologies) spectrophotometer. RNA (1mg) was reverse transcribed using the Moloney Murine Leukemia Virus reverse transcriptase enzyme (Invitrogen). Template cDNA was used to measure gp130 expression (normalized to 18SrRNA) in cells treated with IL-27 for 4h, relative to expression levels in untreated cells. Amplification was performed with gp130-specific primers, as previously described: 5'-TCTGGGAGTGCTGTTCTGCTTT-3' and 5'-TGTGCCTTGGAGGAGTGTA-3'20, and 18SrRNA primers: 5'-ACT-CAA-CAC-GGG-
AAA-CCT-CAC-C-3', 5'-CCA-GAC-AAA-TCG-CTC-CAC-CAA-C-3' (IDT DNA Technology). Real-time PCR cycling was performed using the SsoFast EvaGreen Supermix (Bio-Rad) on the Rotor-Gene 6000 (Corbett Life Sciences).

6.3.5 Flow Cytometry

PBMC were harvested and washed in PBS (0.1% azide, 1% BSA) and stained with fluorochrome-conjugated antibodies: gp130-FITC and TCCR/WSX-1-PE (R&D Systems), CD14-PE (Beckman Coulter), and CD4-PECy5 (eBioscience). After staining, cells were washed with PBS-azide, and data were acquired with the Epics XL-MCL flow cytometer. Data were analyzed using the WinMDI version 2.9 software package (J. Trotter, Scripps Institute, San Diego). Live cells were gated in analyses.

6.3.6 ELISA

Culture supernatants were used to quantify cytokine expression as previously described and according to manufacturer instructions: IL-6 (Biosource), TNF-α (Biosource), and IL-10 (eBioscience). Absorbencies were measured with the BioTek ELx800 Microplate Reader (Fisher Scientific). Individual patient sera were assayed in duplicate and the averages are reported in this study.

6.3.7 Statistical Analysis

The Medical Research Centre at Queen’s University provided expertise in analyzing patient data. Comparisons of IL-27-induced mRNA expression of gp130 in HIV-negative versus HIV-positive subjects were performed using the independent samples t-test. For flow cytometry and ELISA data statistical analyses performed were similar, and as follows: to assess significant responses to IL-27 within groups (HIV-negative or HIV-
positive) alone, the paired samples t-test was used. Subsequently, change scores were calculated within each group and then compared to assess significant differences in responses to IL-27 between patients groups using the independent samples t-test.

6.4 Results

6.4.1 IL-27-induced STAT1 and STAT3 activation is intact in HIV infection

Since we and others have previously described IL-27-induced STAT1/3 activation as a primary signaling pathway leading to downstream functions of IL-27 in human monocytes, we decided to use STAT1/3 activation as a first indicator of how HIV-infection might affect IL-27 function. We investigated phosphorylation of STAT1 and STAT3 in response to 15 mins of IL-27 treatment on blood monocytes. We observed IL-27-induced phosphorylation of STAT1 and STAT3 (phospho-STAT1/3) in the HIV negative group, with similar responses in the HIV positive group (Figure 6.1). Total (pan) STAT1/3 expression served as a loading control, and to further control for equal loading, blots were stripped and re-probed for HSP90 expression. These observations indicated that IL-27-induced STAT1 and STAT3 activation is preserved in the setting of HIV infection.

6.4.2 IL-27 induces expression of gp130, but not the counterpart receptor subunit, WSX-1, in HIV-negative monocytes

Since we observed no changes in STAT1 or STAT3 activation in monocytes from HIV-positive subjects, we decided to investigate if there might be alterations in expression of the IL-27 receptor chains, gp130 and WSX-1 (TCCR). Interestingly, we observed that a time course of IL-27 treatment resulted in induction of gp130 surface expression on the
Figure 6.1: IL-27-induced STAT1 and STAT3 activation is not altered in HIV infection. Blood monocytes were stimulated with IL-27 for 15 minutes, followed by Western Blot analysis for phosphorylated-STAT1 (phospho-STAT1) and -STAT3 (phospho-STAT3), and total/pan STAT1 or STAT3 (pan STAT1 or pan STAT3). HSP90 expression was a loading control within each subject sampled. Three representative subjects are shown in the HIV negative group (numbered 1-3), and five representative subjects are shown from the HIV positive group (numbered 1-5). Data shown is representative of responses observed from all study subjects within each group, and results shown have been reproducible in triplicate experiments.
specific monocyte population within PBMC (Figure 6.2a). More specifically, we observed two populations of CD4+ stained cells (T cells and monocytes), with only one population responding to IL-27 via upregulation of gp130 (Figure 6.2a, top panel). Furthermore, we confirmed the respective CD4+ stained population responding to IL-27 was monocytes (not CD4+ T cells), as subsequent staining showed the CD14+ population upregulating gp130 in response to IL-27 (Figure 6.2a, bottom panel). Since we identified that IL-27 could induce gp130 expression, we next examined whether IL-27 could upregulate WSX-1 expression. Primary monocytes and the THP-1 monocytic cell line were treated with a time course of IL-27 and co-stained with gp130 and WSX-1 (Figure 6.2b). As expected, in both cell types we observed a significant upregulation of gp130 expression from IL-27 treatment: in monocytes an increase from 32% at baseline (Medium) to 60% of gp130 positive cells after 48h of IL-27 treatment, and from 45% to 60% in THP-1 cells. In parallel, no increases in WSX-1 expression were observed. As a positive control for WSX-1 detection, we performed WSX-1 staining on a cell line constitutively expressing WSX-1, 293-WSX-1 (Figure 6.2c). These results identified gp130 as a novel IL-27 gene target in human monocytes.

6.4.3 IL-27-induced cell surface expression of gp130 is impaired in HIV infection

Since we identified gp130 as a novel IL-27-responsive gene, we decided to investigate if the ability of IL-27 to induce gp130 was altered in the setting of HIV-infection. PBMC from HIV-negative (HIV -) and HIV-positive (HIV +) subjects were stimulated with IL-27 for 24 hours, a time point which showed significant induction of gp130 expression in response to IL-27 in Figure 6.2a. Following IL-27 stimulation cells were surface-stained
Figure 6.2: IL-27 induces expression gp130 in monocytes from HIV negative individuals, a function that is impaired in monocytes from HIV positive individuals. PBMC from HIV-negative subjects were treated with a time course of IL-27 from 16 – 48h. (a) PBMC were co-stained with gp130 and CD4+ (top panel) or CD14+ (bottom panel), and analyzed by flow cytometry. In the bottom panel, the CD14+ cells (monocytes) were the only cells in PBMC fractions that responded to IL-27 by upregulating gp130 expression. (b) Co-staining with gp130 and WSX-1 was performed on freshly isolated (resting) monocytes (top panel) and THP-1 cells (bottom panel). Dot plots shown are annotated with percent of positive cells within each quadrant, and are representative of responses from 3 different blood donors and experiments. (c) Surface staining for WSX-1 was performed on two different transfected HEK-293 T cell lines: cells transfected with WSX-1 (293-WSX-1) and cells transfected with a control cytokine receptor (293-control). Flow cytometry histogram overlays confirmed the reliability of our antibody for detection of WSX-1 expression, as the majority of cells stained positive for WSX-1 in the 293-WSX-1 cell line, while the 293-control cells showed no WSX-1 staining. Grey histogram represents autofluorescence of unstained cells. (d) PBMC from HIV-negative (HIV -) and HIV-positive (HIV +) subjects were stimulated with IL-27 for 24h, then surface stained for gp130 expression. Values shown represent mean fluorescence intensity of surface gp130 in unstimulated (Medium) versus IL-27–stimulated (IL-27 24h) PBMC within each group, with each joined data point representing one subject. For statistical analysis, within-group induction of gp130 expression was assessed separately using the paired samples t-test. To assess differences is IL-27-responsiveness of the HIV – versus the HIV + group, change scores were calculated, followed by between-group changes compared using the independent samples t-test.
for gp130 expression (Figure 6.2d). We observed a significant increase in gp130 expression, as measured by mean channel fluorescence, in HIV-negative subjects (p=0.003), while no induction was observed in HIV-negative subjects (p=0.99). Additionally, when comparing the magnitude of the IL-27 response (fold change in gp130 expression) within the HIV – group to that of the HIV + group, we observed a significant difference (p<0.001) in responses of the two groups to IL-27, pointing to an impaired IL-27 response in HIV infection. Furthermore, we performed statistical analysis to compare basal levels of gp130 expression between the two groups, as it appeared the HIV + group might express higher basal levels of gp130. However, no statistically significant differences could be reported.

6.4.4 IL-27-induced mRNA expression of gp130 is impaired in HIV infection

To follow our findings at the cell surface with gp130, we investigated IL-27-induced gp130 expression at the mRNA level. We initially performed a short time course (4-16h) of IL-27 treatment on primary monocytes and THP-1 cells to characterize the kinetics of IL-27-induced gp130 mRNA expression by real-time PCR. We observed gp130 induction at 4h of IL-27 treatment, in both primary monocytes (Figure 6.3a) and THP-1 cells (Figure 6.3b). To observe the effect of HIV infection on IL-27-induced mRNA expression of gp130, we stimulated monocytes from HIV-negative and HIV-positive individuals for 4h with IL-27, and subsequently compared the induction of gp130 mRNA expression (Figure 6.3c). Results shown represent fold increase in IL-27-induced gp130 expression from 4h of IL-27 treatment compared to untreated cells from the same subject, normalized to 18SrRNA expression. We observed a significant decrease in IL-27-
Figure 6.3: IL-27-induced mRNA expression of gp130 is impaired in HIV infection. (a) Primary blood monocytes and (b) THP-1 monocytic cells were stimulated with a short time course of IL-27, then analyzed by real-time PCR for mRNA expression of gp130. (c) Monocytes from HIV-negative and HIV-positive subjects were stimulated for 4h with IL-27, then analyzed by real-time PCR for gp130 mRNA expression. Comparison of gp130 expression between the two groups was performed with an independent samples t-test. Data shown have been normalized to 18SrRNA expression, and represent induction of gp130 expression relative to levels of expression in unstimulated cells (Medium).
induced mRNA expression of gp130 in the HIV-positive group compared to the HIV-negative group. Values for the two groups were compared using an independent samples t-test, with p<0.001.

6.4.5 IL-27-induced cytokine production is downregulated in HIV infection

To further identify the effect of HIV infection on IL-27 function, we performed cytokine ELISAs on PBMC supernatants treated for 24h with IL-27. We assayed for expression of previously described IL-27-responsive cytokines, specifically, pro-inflammatory cytokines IL-6 and TNF-α, and the anti-inflammatory cytokine IL-10 (Figure 6.4a-c)\textsuperscript{14,21}. We observed significant differences in IL-27-induced cytokine production between groups, with the HIV-positive group showing consistently decreased IL-27-mediated cytokine induction compared to the HIV-negative group. Furthermore, within the HIV-positive group itself, no significant induction of IL-6 or IL-10 was observed in response to IL-27 stimulation, indicating an impairment of IL-27 function. Interestingly, a significant induction of TNF-α was observed within the HIV-positive group in response to IL-27 (p=0.002), however, the magnitude of induction was still significantly less than the magnitude of IL-27-induced TNF-α in the HIV-negative group. Thus, overall, we observed that IL-27-induced production of IL-6, TNF-α, and IL-10 is downregulated in HIV infection.

6.5 Discussion

In this study we report the effect of HIV infection on IL-27 functions in PBMC and monocytic cells. More specifically, we observed intact IL-27-induced STAT1/3 activation in human monocytes. However, we did observe impairment of IL-27-induced gene
Figure 6.4: IL-27-induced cytokine production is downregulated in HIV infection. Supernatants from PBMC cultured with IL-27 for 24h (1x10^6 cells/mL) were used in ELISA to quantify IL-27-induced cytokine production. (a) IL-6, (b) TNF-α, and (c) IL-10 expression in pg/mL are shown, with each set of connected data points representing one subject before and after IL-27 stimulation. Sample sizes are annotated under each group, as subjects that elicited no response had data points superimposed at 0 pg/mL. Statistical analyses of within-group and between-group cytokine induction were performed as described in Figure 6.2.
expression, including downregulation of IL-27-induced gp130 mRNA and surface protein expression, and downregulation of IL-27-induced cytokine production. This study is the first to identify dysregulation of IL-27 functions in the setting of HIV infection, representing additional mechanisms that may contribute to the widespread immunologic dysfunction in HIV infection.

On a minor note, to increase our study sample size, our HIV positive subject group was based on the inclusion criteria of having detectable viral load. Within this group, 6 out of 13 patients reported taking ART, and of these patients, 3 were tolerant/non-adherent to ART and 3 were infected with drug-resistant strains of HIV. Thus, although our HIV positive subject group included some patients on ART, it is likely that drug therapy is not fully effective in these patients, given that all patients exhibited a relatively high viral load.

Previous work demonstrated that HIV can impair cytokine-induced signaling pathways in monocytes and T cells, and in particular, studies showed HIV-mediated dysregulation of JAK-STAT signaling pathways, although the precise mechanisms underlying these observations are unclear. In some cases HIV induces hyperactivation of STAT molecules, inhibits STAT signaling, or can have no effect on STAT signaling. A recent study compared differences in cytokine-dependent STAT signaling in HIV-infected monocyctic cells from patients on/off anti-retroviral therapy (ART) to HIV-negative patients. This study examined STAT-1 activation from stimulation with recombinant cytokines such as: IFN-α, IFN-γ, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), and IL-4. They found no change in STAT1 activation in response to
most cytokines tested (IFN-α, IL-10, GM-CSF, and IL-4), but did report IFN-γ-induced STAT1 to be upregulated\(^\text{24}\). Furthermore, this study went on to investigate IFN-γ-regulated gene expression, but found no correlation between differences in gene expression observed and STAT activation. Similarly, in our study, we observed no significant changes in STAT1 or STAT3 activation in response to IL-27 treatment of monocytes, indicating that IL-27-induced STAT1/3 activation may be preserved in the setting of HIV infection. However, we and others have previously demonstrated that IL-27 can also signal via NF-κB\(^\text{14,15,25}\). Since we did not examine IL-27-induced NF-κB activation in this study, we cannot conclude that all IL-27 signaling pathways remain unaffected in the setting of HIV infection. Indeed, dysregulation of other IL-27-induced signaling pathways may partly explain our observations of impaired downstream gene expression in response to IL-27 in HIV-infected subjects.

Deregulation of cytokine receptor expression during HIV infection has been demonstrated in lymphoid and myeloid cells\(^\text{26-28}\). Currently, no studies have investigated how HIV affects expression of the unique IL-27 receptor subunit, WSX-1. However, some studies have shown dysregulated expression of receptor subunits similar to and shared with the IL-27 receptor (IL-27R). For example, dysregulated IL-12 receptor (homologous to IL-27R) expression has been detected in the setting of HIV infection\(^\text{26, 27}\) and common variable immunodeficiency (CVID)\(^\text{29}\), whereas upregulated gp130 expression has been detected in lymphoid tissues of HIV-infected patients at different stages of disease progression\(^\text{28}\). Interestingly, our study identified the IL-27 receptor chain, gp130, as a novel IL-27-responsive gene in human monocytes. Furthermore, we observed downregulation of IL-27-induced gp130 expression at both the mRNA and
surface protein level in HIV-positive compared to HIV-negative subjects. These findings revealed a defect in IL-27-induced transcriptional responses, resulting in impaired inducible receptor expression on the cell surface. Indeed, further study is required to dissect the precise deficiencies in IL-27-signaling that result in downregulated gene expression, as a disconnect exists where we observed preserved STAT1/3 signalling but impaired gene expression. In line with our findings, previous work in HIV subjects showed a similar disconnect between cytokine signalling and gene expression, and more specifically, the observed differences in STAT1-dependent gene expression did not parallel differences in STAT1 phosphorylation observed in\textsuperscript{24}. Thus, recognizing that gene regulation is a complex process, involving the coordination of a myriad of transcription factors and regulatory proteins, particularly in the setting of HIV infection, regulation of gene expression is likely further compounded by the presence of viral proteins and aberrant host responses.

It is well established that HIV infection causes dysregulated cytokine production, resulting in impaired immune responses and characteristic of HIV infection and progression to acquired immunodeficiency syndrome (AIDS). More specifically, in the setting of HIV-infection, monocytes/macrophages have exhibited enhanced pro-inflammatory cytokine production, with increased basal levels of IL-1, IL-6, IL-8, and TNF-\(\alpha\)\textsuperscript{30-32}. Interestingly, contradictory results on how HIV-infection affects pro-inflammatory cytokine production have also been reported\textsuperscript{33}. A previous study investigated the expression levels of MIP-1\(\alpha\), RANTES, IL-8, IL-1\(\alpha\), IL-3, IL-6, GM-CSF, G-CSF, TNF-\(\alpha\), TGF-\(\beta\)1 in the setting of \textit{in vitro} infected macrophages, and found only MIP-1\(\alpha\) production to be enhanced. Herein, in addition to assessing basal cytokine
production, we also observed IL-27-induced cytokine production, as a measure of IL-27 function. Accordingly, levels of IL-27-induced IL-6, TNF-α, and IL-10 were significantly downregulated in the HIV positive subject group compared to the HIV negative group, indicating impairment of IL-27-induced cytokine expression in the setting of HIV infection.

Pertinent to this study, decreased cytokine production in HIV-viremic patients has previously been observed. Tilton et al. (2006) reported diminished proinflammatory cytokine production in patients with high level viremia experiencing an interruption of ART, and showed an inverse relationship between cytokine production and type I IFN (IFN)-stimulated gene activation. This observation suggests elevated IFN-stimulated gene expression occurs in HIV-viremic subjects off ART, resulting in activated immune cells that are impaired in their ability to upregulate proinflammatory cytokine expression. Furthermore, this study proposed that diminished cytokine production was occurring at a post-transcriptional stage of gene expression, as levels of cytokine mRNAs did not change, but cytokine protein levels were diminished, while at the same time, genes regulating ubiquitination and proteasomal degradation were increased. Taken together, this study showed the presence of IFN in patients with unrestricted viremia contributes to diminished proinflammatory cytokine production, and increases in proinflammatory cytokines were observed upon initiation of ART. Interestingly, previous studies have reported high levels of IFN in HIV-infected patients with advanced infection, and furthermore, it has also been reported that IL-27 can induce IFN expression. Therefore, levels of IFN may be further increased in PBMC from HIV-viremic subjects that have been treated with IL-27, perhaps explaining our observation of downregulated
proinflammatory cytokine expression. Thus, further study on the effect of initiating ART in HIV positive subjects would be useful in delineating mechanisms of downregulated IL-27-induced cytokine production.

This is the first study to characterize the effect of HIV infection on IL-27 function, in terms of STAT signaling and cytokine expression. The fact that we observed preservation of IL-27-induced STAT signaling but impairment of IL-27-induced gene expression indicates dysregulation may be occurring at additional points of IL-27 signaling cascades and gene expression pathways not explored in this study. Indeed, gene regulation during HIV infection is a complex event of interactions between host and viral transcription factors and regulatory proteins. Therefore, a more in-depth analysis of these events is required to fully elucidate how HIV mediates dysregulation of IL-27 functions. This study was a first step in identifying how HIV affects IL-27 functions, and future studies employing an HIV-infection model \textit{in vitro} would provide a more direct observation of how HIV affects IL-27 function. Recent studies have shown IL-27 is an anti-HIV cytokine and indicate the potential role of IL-27 in novel strategies aimed at immunologic control of HIV replication or therapeutic interventions. This study is of value as a critical first step in determining the potential for IL-27 as a therapeutic adjunct.
6.6 References


CHAPTER 7:

GENERAL DISCUSSION AND FUTURE PERSPECTIVES
7.1 General Discussion

In 2007, when I initiated my PhD studies, IL-27 was an emerging cytokine with a well-defined role in adaptive immunity yet no distinct role in innate immunity. Indeed, the first identification of IL-27 as a heterodimeric cytokine was in 2002, while the complete heterodimeric receptor was only elucidated in 2004. Thus, my journey began three years into the discovery of this cytokine/receptor complex, where much ground was yet to be trodden. I set out with the overall goal to characterize novel functions of IL-27 in innate immune responses of human monocytes, a cell type that had been neglected in terms of describing IL-27 activities in the early stages of discovery. At the inception of my work, it was known that IL-27 was predominantly produced by APCs, such as monocytes, and that these cell types could also respond to IL-27, as they expressed the IL-27R. However, little focus was placed on these cell types in terms of characterizing IL-27 function, because early, albeit exciting, reports initially described IL-27 to have multifaceted roles in regulating Th cell differentiation. Furthermore, of the few studies that described IL-27 functions in monocytes, the vast majority of them were performed on 'activated' monocytes, that is, monocytes in the setting of bacterial/parasitic infection or in the presence of activating stimuli, such as M-CSF (macrophage colony-stimulating factor) and LPS. In these studies, monocytes were already mounting inflammatory responses or differentiated into macrophages. Thus, there existed a void in the literature of IL-27 functions in 'non-activated' monocytes, namely, monocytes in the absence of infection or activating stimuli. Therefore, I set forth to address this gap in the literature and characterize novel functions of IL-27 in innate immune responses by examining how monocytes respond to IL-27.
In Chapter 2 of my thesis, I focused on characterizing the cytokine profile induced by IL-27 in monocytes, with intent of getting a broad perspective of IL-27 function in this cell type. This was the first evidence supporting IL-27-driven inflammation and activation of resting monocytes, and laid groundwork for subsequent experiments to extend these observations and identify the molecular mechanisms involved. This work was also the first evidence of IL-27-induced NF-κB activation in monocytes, and, more significantly, an indication of cooperation between JAK/STAT and NF-κB signaling pathways in mediating IL-27 responses. Thus, my results shifted the paradigm of IL-27-induced signal transduction, indicating a novel and significant role of NF-κB in IL-27-mediated responses within monocytes, and unveiling, for the first time, crosstalk between JAK/STAT and NF-κB downstream of the IL-27 receptor.

Upon characterizing an IL-27-mediated pro-inflammatory cytokine profile in monocytes, I also questioned what other pro-inflammatory functions IL-27 could influence in these cells. Thus, in Chapter 3 of this thesis, I further investigated the pro-inflammatory functions of IL-27 and identified a role for IL-27 in a positive feedback loop of inflammation. In the search for molecular mechanisms underlying these observations, I hypothesized that IL-27 might induce expression of TLR4, thus, rendering monocytes more responsive to LPS. Particularly interesting in this work was the observation that JAK2, STAT3, and NF-κB signaling molecules were all required for IL-27-induced TLR4 expression. The precise molecular interactions between JAK/STAT and NF-κB downstream of IL-27R engagement should be the focus of future studies, as this may translate to discovery of novel signaling cascades amongst other related cytokines. Furthermore, future work including detailed TLR4 promoter analysis would enhance our
understanding of cytokine-mediated regulation of TLR4 expression, as previous reports have shown that other cytokines (IL-6, TNF-α, IFN-γ) can also influence TLR4 expression. Investigating the role of other transcription factors, such as PU.1, may also aid in deeper characterization of the molecular mechanisms of IL-27-induced TLR4 expression. Indeed, PU.1 is a myeloid- and B cell-specific transcription factor that functions in macrophage-specific gene expression. It is likely that PU.1 may interact with STATs and NF-κB at the TLR4 promoter, as previous studies have shown strong involvement of PU.1 in TLR4 expression and cooperative binding of PU.1 and STAT proteins has been demonstrated in promoter regions. On a similar note, overexpression of PU.1 resulted in enhanced IL-6 production in response to LPS, further implicating PU.1 in LPS/TLR4 responses. To the best of my knowledge, no studies have examined the effect of IL-27 on PU.1 activation; therefore, these experiments could be an exciting and informative exercise.

In Chapter 4 of this thesis, I continued the quest in describing novel functions of IL-27 in innate immunity, and demonstrated that IL-27 could directly upregulate expression of BST-2, a well-characterized innate, anti-viral protein, originally described as an IFN-responsive gene. Midway through my PhD studies, two independent research groups identified a close relationship between IL-27 and IFN, indicating similar anti-viral gene expression amongst these cytokines. Interestingly, both groups differed on the molecular mechanisms underlying these observations, debating whether or not IL-27 required signaling through an IFN-intermediate to induce anti-viral gene expression. With this in mind, I sought to determine if IL-27 could directly induce expression of the IFN-responsive anti-viral gene called BST-2, which had not been examined in previous IL-27
studies. In my work I observed IL-27-mediated induction of cell surface and intracellular BST-2 expression, with confocal microscopy showing IL-27-induced expression in compartments adjacent to the nucleus, reminiscent of localization in the Golgi apparatus. A similar pattern of BST-2 localization in the secretory pathway (Golgi apparatus and trans-Golgi network) was previously reported via confocal immunofluorescence microscopy\textsuperscript{\ref{18}}. Interestingly, this study suggested that since BST-2 is localizing to sites of protein sorting, it may play a role in chaperoning the transport of other newly synthesized proteins\textsuperscript{\ref{18}}. Indeed, BST-2 exhibits sequence homology to a chaperone protein called BAP31, involved in intracellular transport of membrane proteins. On the same note, BST-2 contains numerous motifs that have been characterized as transport signals\textsuperscript{\ref{19}}. Additionally, it is well characterized that BST-2 localizes to lipid-rich microdomains\textsuperscript{\ref{20}}, indicating the potential for BST-2 to sort proteins via lipid rafts. Thus, Blasius et al. postulated that BST-2 residing intracellularly within the Golgi network may support its function to aid in trafficking/secrection of newly synthesized cytokines\textsuperscript{\ref{18}}. This proposed function of BST-2 is pertinent to this thesis as a whole, as the IL-27-mediated induction of BST-2 may impact expression of other IL-27 target genes. For example, IL-27-induced BST-2 may function to chaperone the transport of other IL-27-induced proteins, such as TLR4 and pro-inflammatory cytokines. Future studies aimed at delineating the broader functional outcomes of IL-27-induced BST-2 expression might investigate IL-27-induced protein production in BST-2 knockout models or in the presence of siRNA knockdown of BST-2. Thus, my description of IL-27-induced BST-2 expression signifies a new potential mechanism by which IL-27 can influence protein expression.
Final experiments performed in Chapter 4 with a potent IFN inhibitor (B18R) ruled out the involvement of an IFN intermediate in IL-27-induced BST-2 expression within 8h of IL-27 treatment. Although my experiments discounted the role of IFN in mediating early/initial induction of BST-2 in response to IL-27, it is likely that IFN is an intermediary in IL-27-induced BST-2 at later time points. Indeed, it is known that IL-27 can induce IFN expression (type I and II), therefore, there is a high likelihood that IL-27-induced IFN may synergize with the direct effects of IL-27, and mediate enhanced BST-2 expression at later time points. Within this thesis, experiments were performed in cells treated for 8h with IL-27, conceivably too early for an IFN intermediate to be expressed, secreted, and fed back in an autocrine manner to induce BST-2 gene expression and protein production. To extend these findings, an ELISA performed on culture supernatants from a broad-range IL-27 time course (0, 2, 4, 6, 8, 12 and 24 hours) would show at what precise time point IFN is secreted in response to IL-27, giving an indication of when IFN may be feeding back to synergize with IL-27-specific induction. Additionally, previous studies have reported intact expression of a variety of IL-27-mediated anti-viral genes in the presence of neutralizing antibodies to IFN\textsuperscript{17,21,22}, while one study had reported abolishment of IL-27-mediated antiviral gene expression in the presence of neutralizing antibodies\textsuperscript{16}. Based on the discrepancy of these results, culture conditions and timing of the neutralizations produce different results, indicating that IFN intermediates may be involved only at specific time points of IL-27 stimulation. Within this thesis I have provided evidence for BST-2 as a novel and direct gene target of 8h IL-27 treatments, with further study recommended on the involvement of IFN intermediates at later time points of IL-27 stimulation.
In Chapter 5 and 6 of this thesis, I investigated the effect of HIV-infection on IL-27 expression and function. At the onset of my investigations no literature on the interactions of HIV and IL-27 had been reported. Still to date, to the best of my knowledge, the only work showing the effect of HIV on IL-27 has been these two chapters of my thesis. My initial work in Chapter 5 showed clinical characteristics, including HIV viral load, hepatitis C virus co-infection, and CD4 T cell counts, were associated with changes in serum IL-27. These results suggested circulating HIV might suppress levels of circulating IL-27. This study was key to my next chapter (Chapter 6), because it was the first line of evidence showing that impaired monocytic function in HIV infection may be due, in part, to loss of IL-27 function. However, amidst my own work characterizing how HIV affects IL-27, two studies reported on the reciprocal interaction, that is, how IL-27 can impact HIV. Both studies showed that IL-27 could significantly inhibit HIV replication in CD4 T cells and monocytes/macrophages via induction of antiviral genes. Thus, since IL-27 had demonstrable anti-HIV properties, the immunotherapeutic potential of this cytokine was apparent. However, before the potential for IL-27 administration could be explored further, it was critical to determine how IL-27 functions in the setting of HIV infection.

The overall aim of Chapter 6 was to describe the effect of HIV infection on IL-27 functions. I sought to characterize IL-27 receptor expression, signal transduction and downstream gene expression in HIV infected subjects compared to uninfected subjects. Since my results showed intact IL-27-mediated STAT activation, but impairment of IL-27-mediated gene expression, it is probable that other aspects of IL-27 signaling are dysregulated in HIV infection, likely the NF-κB pathway and other transcriptional
regulatory proteins, which were not examined in my study. Furthermore, my experimental techniques were not sensitive enough to detect WSX-1 expression on monocytes and PBMC. However, positive controls indicated my experimental protocols were successful and affirmed that basal expression of WSX-1 is very low. Thus, it seems that minimal expression of WSX-1 is required to transduce IL-27 signals, which is expected, as this is the α chain of the receptor, primarily responsible for recognition of the cytokine, with minor roles in initiating signaling. Expression levels of WSX-1 are likely just enough to bind or ‘capture’ IL-27, and then dimerize with gp130, the β receptor chain, to drive signal transduction. Since WSX-1 was so weakly detected, I was not able to analyze the effect of HIV-infection on this receptor chain. Thus, it is possible that HIV infection may impact WSX-1 expression, and the prospect of HIV-mediated suppression of WSX-1 expression could contribute to the impairment of IL-27-induced gene expression observed in this thesis.

Of particular interest the observed impairment of IL-27-induced gene expression in the setting of HIV infection, despite intact signaling mechanisms. At this time we cannot conclude that all IL-27-induced gene expression is impaired in HIV infection, indeed, it could be that there is just selective and regulated suppression of some IL-27-induced genes, perhaps only those that impact HIV replication. For example, we observed downregulation of IL-27-induced IL-6, IL-10, and TNF-α expression, and all 3 of these cytokines have previously been shown to modulate various aspects of the HIV life cycle, with well-defined roles for IL-6 and TNF-α in mediating enhanced HIV replication\textsuperscript{25}. Thus, perhaps in the setting of viral infection, the downregulation of IL-27-induced proinflammatory gene expression is an advantage. At the same time, it is possible that
IL-27 might retain functions in inducing anti-viral gene expression. Therefore, future work should investigate IL-27-induced antiviral gene expression (APOBEC, BST-2, etc.) in the setting of HIV infection, to determine if IL-27 maintains its antiviral effects despite losing its proinflammatory effects. Indeed, virus and host are constantly counteracting each other, and within Chapters 5 and 6 of my thesis, it seems that virus (HIV) is downregulating host defenses (IL-27 expression and functions), a viral pathogenic mechanism geared towards overcoming host defenses to establish and maintain infection. Further delineation of the precise IL-27-specific molecular mechanisms affected by HIV will help determine how to effectively capitalize on the innate anti-viral functions of IL-27, and could inform novel therapeutic design. A more in-depth perspective on future directions of this work follows in ‘Future Perspectives’.

Taken together, the work of this thesis contributes to a model in which IL-27 can modulate a delicate balance of infection-induced immune responses. This thesis provides evidence by which IL-27 can drive initial, innate immune responses, which can then bridge to induction of long-term, adaptive immune responses, as evidenced by others. Indeed, IL-27 is one of the earliest secreted cytokines induced in response to inflammatory stimuli, with gram-negative infection and LPS as potent inducers of IL-27 expression, while TLR2 and TLR9 agonists also showed the ability to induce IL-27 expression. Additionally, this thesis provided novel evidence that early HIV infection may induce IL-27 expression, perhaps indicating that TLR3 and TLR7/8 agonists might also active IL-27 expression. Thus, in the initial stages of bacterial and viral infection, IL-27 secreted by activated APCs can act in an autocrine and paracrine manner to enhance the activation of APCs via mechanisms that include upregulated pro-inflammatory.
cytokine/chemokine secretion and TLR expression\textsuperscript{29,30}, as shown in this thesis, and via other mechanisms that include enhanced co-stimulatory molecule and class I and II MHC expression\textsuperscript{31}. Upon activation, APCs can present antigen via antigen-MHC complexes to naïve CD4+ and CD8+ T cells, and depending on the cytokines present in the environment, highly specific T cell responses can be elicited, influencing the outcome and clearance of infection. Thus, IL-27, a cytokine present early in infection, not only affects early innate responses of APCs, but can also significantly influence initial T cell responses. For example, in the presence of IL-27, Th0 cells are polarized to a Th1 lineage commitment and skewed away from Th2 and Th17 development, thereby maximizing killing efficiency of APCs and CD8+ T cells which resolve infection\textsuperscript{4-6}. Indeed, previous worked has shown IL-27 can increase CD8+ T cell-mediated cytotoxicity via upregulation of IFN-γ and granzyme B production\textsuperscript{32}. Therefore, it is possible that IL-27 participates in a positive feedback loop of early infection-induced inflammatory responses, commencing with an initial response to infectious stimuli that induces IL-27 expression from monocytic cells as a result of TLR triggering. IL-27 can then act in an autocrine manner to upregulate pro-inflammatory gene expression (cytokines, chemokines and TLR4) to propagate clearance of infection.

In the setting of HIV infection, it appears that IL-27 expression is decreased during the progression of HIV disease (inverse correlation to viral load), indicating that IL-27 may not play a role in anti-HIV immunity beyond initial infection stages, however this cannot be ruled out at this time. However, at later stages of bacterial and parasitic infection, IL-27 demonstrates remarkable immunoregulatory functions and acts in a contradictory manner, namely to limit excessive inflammatory responses. Indeed, in the setting of
septic peritonitis IL-27 was rapidly produced and resulted in suppression of neutrophil migration and activation\(^8\). Other studies in the setting of *Mycobacterium tuberculosis*-induced immune activation in macrophages or in LPS-mediated immune activation in macrophages and dendritic cells show similar repressive functions of IL-27\(^{33-35}\). Taken together these studies support a regulatory role of IL-27 in activated model systems, whereby prevention of excessive inflammatory responses is observed upon IL-27 treatment. On the other hand, the work within this thesis employs the use of freshly isolated monocytes, which are not cultured in the presence of activating stimuli prior to IL-27 treatment. Therefore, in this setting, I have reported that IL-27 promotes inflammatory responses to facilitate initial immune activation and clearance of infection. New experimental directions to determine the role of IL-27 in regulating inflammation and infection can be found in the ‘Future Perspectives’ section that follows.

### 7.2 Future Perspectives

This thesis has laid the groundwork for numerous avenues of continued study, and the prospect of investigating the novel functions of IL-27 described herein in different model systems is a direction that would both confirm and extend my conclusions. Firstly, it would be useful to investigate novel IL-27 functions characterized in this thesis in *in vivo* model systems. Currently, two mouse lines with mutations in the IL-27R\(\alpha\) chain have been created, the TCCR\(-/-\) strain from the Ghilardi lab\(^3\) and the WSX-1\(-/-\) from the Yoshida lab\(^36\). Both strains result in the loss of signaling via WSX-1, the receptor subunit imparting specificity to binding of IL-27. Thus, these knockout mice exemplify immune responses that would occur in the absence of IL-27 functions, providing a means to identify the requirements for IL-27 in innate immune responses. For example, it would be
informative to compare knockout and wild type LPS responses and downstream pro-inflammatory gene expression. These experiments would delineate whether IL-27 priming simply enhances LPS responses or whether there is an earlier dependency on IL-27 for effective LPS-TLR signaling. It is very likely that additional, still uncharacterized, roles for IL-27 in driving inflammatory responses exist, and IL-27R knockout models may be the key to describing these functions. Indeed, ‘inflammasomes’, multi-protein complexes that drive inflammatory cascades in myeloid cells, have become the recent subject of intense interest in innate immunity and inflammation research. Interestingly, very recent experiments from our lab utilizing TCCR-/- mice (which I have participated in), point to a significant role for IL-27 in driving inflammasome formation. Thus, it is very likely that the future of IL-27 in inflammatory responses will include new and exciting discoveries on IL-27 in inflammasome formation.

As a compliment to the receptor knockout studies, the utilization of an EBI3 knockout model system, which results in mice unable to form the functional heterodimeric IL-27 cytokine, could be useful as a comparable model system. However, results obtained with EBI3/- can be confounded by the fact that the other cytokine subunit, IL-27p28, might be capable of eliciting a response in the absence of EBI3.

Additionally, future work with different in vitro model systems could help elucidate novel functions of IL-27 in activated cell types. Indeed, previous studies have shown IL-27 to be a typical ‘immunoregulatory’ cytokine, in the sense that, depending on the activation status of the cell, IL-27 can be pro- or anti-inflammatory, that is, driving inflammation in a non-activated state, while preventing excessive inflammation in an activated state. Thus, since my observations in this thesis pointed to a predominant pro-inflammatory role of
IL-27 in non-activated monocytes, it would be interesting to see if in the setting of activated monocytes/macrophages (i.e. LPS, PMA, or M-CSF treated cells), IL-27 functions change to repression of inflammation. These studies would be particularly informative on the role of IL-27 if performed in the TCCR-/- and EBI3-/- model systems. Along the same lines, extending these studies into dendritic cells would also compliment this thesis and broaden the characterization of IL-27 functions in innate immune responses. Furthermore, microarray studies comparing IL-27-induced signaling and gene activation across APCs would characterize broader transcriptional profiles of IL-27 in innate immunity, and offer a more global characterization of IL-27-driven inflammation. More specifically, investigating the precise roles for STAT1/3 and NF-κB in mediating IL-27-induced gene activation within macrophages and DCs, would also compliment this thesis well. Chromatin immunoprecipitation assays on IL-27-treated cells would extend my conclusions herein on the requirements for STAT1/3 and NF-κB in expression of IL-27-responsive genes, such as, pro-inflammatory cytokines, TLR4, and BST-2. Indeed, detection of bound STAT3 and NF-κB in the promoters of the IL-27-target genes would offer a direct approach to identifying the role of these transcription factors in IL-27 responses, and might help to explain cell-type differences in IL-27 responses.

Working with patient samples is confounded by immense variability between study subjects, including factors such as HCV co-infection, drug treatments, drug compliance, variability in HIV strains, and even genetic polymorphisms within study subjects themselves. Thus, performing in vitro HIV infections on a well-controlled cell line would facilitate a more clear-cut delineation of how HIV mediates impairment of IL-27 functions. Indeed, in vitro infections would permit deeper experimentation on molecular
mechanisms, as culture conditions and cell numbers obtained would not be limited as when using patient samples. Furthermore, future studies investigating the impact of individual viral proteins on expression and function of IL-27 would also offer a more controlled model system. For example, it is known that monocytes are highly resistant to HIV infection, with only small percentages of total monocytes in circulation becoming infected with HIV. Despite this, normal functions of monocytes are severely impaired in HIV infection due to bystander effects of viral proteins like Nef and gp120. These proteins can be secreted from infected cells\textsuperscript{40,41} and exert effects on neighboring uninfected (bystander) cells\textsuperscript{42,43}. Indeed, Nef is well recognized for its ability to alter cell signaling pathways by physically interacting with cellular proteins and other host cell factors to deregulate the normal transcriptional program within monocytes/macrophages\textsuperscript{43-50}. Similar to Nef, studies have shown immunomodulatory effects of the viral protein gp120 on monocyte/macrophage functions, including cytokine release and signal transduction\textsuperscript{51-55}. Additionally, the role of gp120 in dysregulation of APC function has also been documented, showing effects of gp120 on cytokine/chemokine production, phagocytosis, and antigen presentation\textsuperscript{53}. Taken together, it is apparent that Nef and gp120 are key culprits of the observed AIDS pathogenesis in monocyte/macrophages, as they can interfere with normal cell signaling and disrupt cross-talk pathways coordinating immune responses. Thus, use of recombinant Nef and gp120 \textit{in vitro}, prior to IL-27 stimulation, may provide insight on molecular mechanisms underpinning the dysregulation of IL-27 function observed in HIV-infection.
In totality, this thesis opens many doors for future discovery of IL-27 pleiotropy in immune responses. It has become apparent through this thesis that IL-27 can drive pro-inflammatory responses from many different angles, including upregulation of inflammatory signaling, cytokine production, TLR expression, and LPS responses. I believe that the multifaceted nature of IL-27 described herein is just the tip of the iceberg, with many unknown functions beneath the surface waiting to be described.
7.3 References


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APPENDIX A: RESEARCH ETHICS BOARD (REB) APPROVAL

QUEEN'S UNIVERSITY HEALTH SCIENCES AND AFFILIATED TEACHING HOSPITALS
ANNUAL RENEWAL

Queen's University, in accordance with the "Tri-Council Policy Statement, 1998" prepared by the Medical Research Council, Natural Sciences and Engineering Research Council of Canada and Social Sciences and Humanities Research Council of Canada requires that research projects involving human subjects be reviewed annually to determine their acceptability on ethical grounds.

A Research Ethics Board composed of:

Dr. A. F. Clark, Emeritus Professor, Department of Biochemistry, Faculty of Health Sciences, Queen's University (Chair)
Dr. H. Abdollahi, Professor, Department of Medicine, Queen's University
Dr. R. Brison, Professor, Department of Emergency Medicine, Queen's University
Dr. M. Evans, Community Member
Dr. S. Horgan, Manager, Program Evaluation & Health Services Development, Geriatric Psychiatry Service, Providence Care, Mental Health Services Assistant Professor, Department of Psychiatry
Ms. J. Hudacin, Community Member
Ms. D. Morales, Community Member
Ms. P. Newman, Pharmacist, Clinical Care Specialist and Clinical Lead, Quality and Safety, Pharmacy Services, Kingston General Hospital
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Ms. S. Rohland, Privacy Officer, ICES Queen's Health Services Research Facility, Research Associate, Division of Cancer Care and Epidemiology, Queen's Cancer Research Institute
Dr. B. Simchison, Assistant Professor, Department of Anaesthesiology, Queen's University
Dr. A. N. Singh, WHO Professor in Psychosomatic Medicine and Psychopharmacology Professor of Psychiatry and Pharmacology Chair and Head, Division of Psychopharmacology, Queen's University Director & Chief of Psychiatry, Academic Unit, Quinte Health Care, Belleville General Hospital
Dr. E. Tsai, Associate Professor, Department of Paediatrics and Office of Bioethics, Queen's University

has reviewed the request for renewal of Research Ethics Board approval for the project Regulation of Cytokine Function in Human Immune System Cells as proposed by Dr. K. Gee of the Department of Biomedical and Molecular Sciences, at Queen's University. The approval is renewed for one year, effective February 01, 2012. If there are any further amendments or changes to the protocol affecting the participants in this study, it is the responsibility of the principal investigator to notify the Research Ethics Board. Any unexpected serious adverse event occurring locally must be reported within 2 working days or earlier if required by the study sponsor. All other adverse events must be reported within 15 days after becoming aware of the information.

Date: January 18, 2012
Chair, Research Ethics Board

Renewal [ ] Renewal 2 [ ] Extension [X] Code# MICR-014-08 Romeo file# 6004445

188