Molecular Signaling Mechanisms and Effector Functions Of the Interleukin-17 Receptor in Human Airway Smooth Muscle Cells and Polymorphonuclear Neutrophils

by

Stéphane Dragon

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Department of Immunology

University of Manitoba

Winnipeg, Manitoba, Canada

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THESIS ABSTRACT

Immunopathological disorders are no longer defined by dysregulated T-helper (Th) type 1/ Th2 responses but account for modulatory cell types such as regulatory and Th17 cells. The newly defined Th17 subset is an effector memory subtype which regulates mucosal host defense responses. A distinctive feature of interleukin (IL)-17 is its ability to invoke neutrophilic responses and to synergize cytokine responses in proximal structural cells. This effect is most evident for proinflammatory cytokines and neutrophil-mobilizing chemokines which are under the regulatory control of the canonical, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. The uniqueness of the IL-17A receptor (IL-17RA) signal transduction pathway however has been a limiting factor in uncovering IL-17-mediated effector functions since the receptor bears little homology to other known receptors and contains a unique cytoplasmic consensus binding motif. Hence, the composition, dynamics and subunit interactions of the IL-17R complex have become an emerging area of research where novel recruitment motifs and adaptor proteins are actively being explored. Our study sought to uncover the signal transduction and molecular mechanisms mediating the initiation and amplification responses induced by IL-17. We hypothesize that *(i)* IL-17 represents a key cytokine which initiates inflammatory responses by acting on proximal structural cells to rapidly release neutrophil-mobilizing chemokines and myeloid growth factors and that *(ii)* IL-17 directly promotes survival responses of immune effector cells. Genomic analysis of stimulated human airway smooth muscle cells support the proinflammatory nature of IL-17 as NF-κB associated genes and chemokines were most significantly upregulated within 2 hours. However, IL-17 induced a modest fold increase in gene expression levels whereby only 4 genes achieved greater than 2 fold increases. This, along with the observation that IL-17 enhanced IL-1β-mediated CXCL8

expression via transcriptional promoter activation levels and post-transcriptional mRNA stabilization mechanisms suggests that IL-17 cooperatively functions with secondary cytokines to mediate inflammatory responses. Despite activating the p38-mitogen-activated protein kinase (MAPK) signaling pathway in peripheral blood neutrophils, IL-17 did not directly affect the apoptotic capacity of these cells but unexpectedly antagonized the survival response mediated by the granulocyte-macrophage colony stimulating factor (GM-CSF). Collectively, our results suggest that IL-17 is a potent synergistic cytokine which signals via the MAPK-NF-κB pathway to indirectly recruit neutrophils via CXC-chemokines produced by non-hematopoietic cells and that IL-17 may potentially dampen inflammatory responses by directly antagonizing inflammatory effector cells.

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TABLE OF CONTENTS

THESIS ABSTRACT II

ACKNOWLEDGEMENTS IV

TABLE OF CONTENTS V

LIST OF TABLES X

LIST OF FIGURES XI

LIST OF ABBREVIATIONS XIII

LIST OF COPYRIGHTED MATERIAL FOR WHICH PERMISSION WAS OBTAINED XXI

1.0. CHAPTER 2

INTRODUCTION 2

1.1.0. DISCOVERY AND CHARACTERIZATION TIMELINE OF IL-17 2

1.2.0. IL-17 ORIGINS 6

1.2.1. Characterizing initial effector functions 7

1.2.2. Granulopoiesis and neutrophilia 8

1.3.0. DESCRIBING IL-17 FAMILY MEMBERS 10

1.3.1. IL-17B and IL-17C 11

1.3.2. IL-17E (IL-25) 12

1.3.3. IL-17F 12

1.3.4. IL-17D 13

1.4.0. IL-17 RECEPTOR (IL-17R) FAMILY MEMBERS 14

1.4.1. IL-17RB (EVI27, IL-17Rh1) 15

1.4.2. IL-17RC (IL-17 receptor-like protein (IL-17RL)) 16

1.4.3. IL-17RD (Similar expression to fibroblast growth factor genes (SEF) 17

1.4.4. IL-17RE 18

1.4.5. Multimeric IL-17R complex 19

1.5.0. IL-17R SIGNAL TRANSDUCTION PATHWAY 21

1.5.1. MAPK pathway 25

1.5.2. mRNA stability 28

1.5.3. Promoters and transcription factor binding-sites 31
1.5.4. JAK-STAT pathway 34
1.5.5. PI3-K and GSK3β pathway 35
1.5.6. Ubiquitin pathway 37
1.6.0. IL-23 AND THE DISCOVERY TH17 CELLS 39
1.6.1. IL-23 receptor 41
1.6.3. IL-21 and Th17 amplification 42
1.6.2. Key lineage-specific transcription factors 43
1.6.4. Negative regulation of Th17 45
1.6.5. Th17 phenotype and lineage-specific markers 46
1.7.0. CELLULAR SOURCES 47
1.7.1. γδ T cells 47
1.7.2. NKT cells 48
1.7.3. LTi-like cells 48
1.8.0. IL-17 AND PROTECTIVE IMMUNITY 49
1.8.1. IL-22 and mucosal host defences 51
1.8.2. IL-17 in health and diseased states 53
1.8.3. Neutrophil homeostasis and innate immune responses 54
1.8.4. Airway neutrophil responses 57
1.8.5. Lung and conducting airway structure 58
1.8.6. Asthma pathogenesis and the role of ASM cells 60
1.9.0. SUMMARY POINTS 61
1.10.0. THESIS OVERVIEW 63
1.10.1. Study Rational 63
1.10.2. General/ Bridging Hypothesis 64
1.10.3. Bridging theme 64
2.0. CHAPTER 2
INTERLEUKIN-17 RECEPTOR SIGNAL TRANSDUCTION AND GENE EXPRESSION PROFILE IN HUMAN AIRWAY SMOOTH MUSCLE CELLS 65
2.1.0. ABSTRACT 66

2.2.0. INTRODUCTION 67

2.3.0. MATERIALS AND METHODS 69

2.3.2. Cell culture and Western blot 70

2.3.3. Gene expression array 71

2.3.4. Quantitative real-time RT-PCR analysis 71

2.4.0. RESULTS 72

2.4.1. IL-17 activates the ERK-RSK pathway 72

2.4.2. IL-17R activates NF-κB regulatory factors 75

2.4.3. IL-17R gene targets are upregulated in the presence of IL-17, IL-1β and IL-22 78

2.4.4. IL-17 induces a dynamic gene expression response 79

2.4.5. IL-17 is a poor activator of the NF-κB regulatory network 82

2.4.6. IL-17 differentially activates specific gene targets in ASM cells of asthmatic individuals 83

2.5.0. DISCUSSION 86

2.6.0. REFERENCES 91

2.7.0. SUPPLEMENTARY DATA 94

3.0. CHAPTER 3

INTERLEUKIN-17 ENHANCES IL-1B-MEDIATED CXCL8 RELEASE FROM HUMAN AIRWAY SMOOTH MUSCLE CELLS 97

3.1.0. ABSTRACT 98

3.2.0. INTRODUCTION 99

3.3.0. MATERIALS AND METHODS 101

3.3.1. Reagents and antibodies 101

3.3.2. Preparation of bronchial human ASM cells 101

3.3.3. Cell stimulation and ELISA assay 101

3.3.4. Real-time RT-PCR analysis 102

3.3.5. CXCL8 mRNA stability 103

3.3.6. CXCL8 promoter luciferase reporter constructs and cell transfection 103

3.3.7. Statistical analysis 104

3.4.0. RESULTS 104

3.4.1. IL-17 enhances IL-1β-mediated CXCL8 protein release from ASM cells 104

3.4.2. IL-17 and IL-1β synergize CXCL8 mRNA induction in ASM cells 105

3.4.3. IL-17 enhances IL-1β mediated CXCL8 promoter activity 107

3.4.4. IL-17 synergizes with IL-1β and requires intact AP-1 and NF-κB response elements 109

3.4.5. IL-17 enhances IL-1β mediated CXCL8 promoter activity through p38, ERK1/2 MAPK and the PI3-K pathway 110

3.5.0. DISCUSSION 113

3.6.0. ACKNOWLEDGMENTS 116

3.7.0. REFERENCES 116

4.0. CHAPTER 4

INTERLEUKIN-17 ATTENUATES THE ANTI-APOPTOTIC EFFECTS OF GRANULOCYTE-MACROPHAGE-COLONY STIMULATING FACTOR IN HUMAN NEUTROPHILS 120

4.1.0. ABSTRACT 121

4.2.0. INTRODUCTION 122

4.3.0. MATERIALS AND METHODS 123

4.3.1. Isolation and cell culture conditions 123

4.3.2. RT-PCR analysis 124

4.3.3. Neutrophil apoptosis 124

4.3.4. Western blotting 125

4.3.5. Flow cytometry analysis of cell surface Fas (CD95) expression 126

4.3.6. Soluble Fas Ligand detection by ELISA 126

4.3.7. Microscopy 126

4.3.8. Caspase-3/7 activity assay 127

4.3.9. Statistics 127

4.4.0. RESULTS 128

4.4.1. Circulating human neutrophils express mRNA and cell surface IL-17A receptors 128

4.4.2. IL-17 attenuates the anti-apoptotic effects of GM-CSF 129

4.4.3. IL-17 does not alter Fas or soluble FasL expression levels 131

4.4.4. IL-17 decreases GM-CSF-induced Mcl-1 protein levels and impairs the ability of GM-CSF to retain Bax in the cytosol 132

5.4.5. IL-17 attenuates GM-CSF-delayed caspase-3/7 activity 135

4.4.6. IL-17 antagonizes GM-CSF-induced p38, ERK1/2 MAPK & STAT5 signaling pathways 136
4.5.0. DISCUSSION 137
4.6.0. ACKNOWLEDGEMENTS 140
4.7.0. REFERENCES 140

5.0. CHAPTER 5

DISCUSSION 144

5.1.0. TISSUE-MEDIATED INFLAMMATORY RESPONSES 144
5.2.0. FUNCTIONAL MODEL OF IL-17 RESPONSES 145
5.3.0. CELL TYPE SPECIFIC-RESPONSES 146
5.4.0. CYTOKINE-MEDIATED AIRWAY INFLAMMATION 147
5.5.0. DIRECT AND INDIRECT FUNCTIONS OF IL-17 ON HEMATOPOIETIC CELLS 149
5.6.0. IL-17 GENE TARGETS AND MICROARRAY STUDIES 150
5.7.0. IL-17-SPECIFIC GENE EXPRESSION PROFILE 153
5.8.0. TIME- AND NUCLEOSOME-DEPENDENT CHROMATIN REMODELING RESPONSES 154
5.9.0. IL-17 AND THE REGULATORY NF-KB NETWORK 155
5.10.0. MICRORNA 156
5.11.0. INDUCIBLE IL-17 GENETIC NETWORK 157

6.0. GENERAL CONCLUSIONS AND SIGNIFICANCE 159

6.1. KNOWLEDGE TRANSLATION 159
6.2. FUTURE DIRECTIONS 160

7.0. REFERENCES 161

List of Tables

Table 1. 1. IL-17 family members ... 14

Table 1. 2. IL-17 gene targets ... 33

Table 1. 3. Neutrophil granule contents in the pathogenesis of lung injury ... 58

Table 2. 1. IL-17R target gene list sorted on probability values ... 76

Table 2. 2. Donor health information ... 83

Table 2. 3. IL-17R target gene list of asthmatic ASM cells sorted on probability values. ... 85

Table 2. 4 (Supplement). IL-17-induced NF-κB-dependent genes ... 94

Table 2. 5 (Supplement). Primer sequences ... 95

Table 2. 6 (Supplement). IL-17R target gene list of healthy ASM cells sorted on probability values ... 96

Table 5. 1. Analysis of IL-17 responses by microarrays ... 153

Table 5. 2. Inducible IL-17 genetic networks ... 158

List of Figures

Figure 1. 1. IL-17/ Th17 characterization timeline 5

Figure 1. 2. Dendrogram (phylogenetic analysis) of IL-17 family members 11

Figure 1. 3. Dendrogram (phylogenetic analysis) of IL-17R family members 19

Figure 1. 4. IL-17 and IL-1 receptor complexes 23

Figure 1. 5. Dendrogram (phylogenetic analysis) of STIR domain proteins 24

Figure 1. 6. Signal transduction of the IL-17R complex 37

Figure 1. 7. Ubiquitin and the IL-17R signaling pathway 39

Figure 1. 8. Th17 development 42

Figure 1. 9. Effector Th17 cell characteristics 53

Figure 2. 1. IL-17RA signal transduction mediates MAPK activation 74

Figure 2. 2. IL-17 gene targets 77

Figure 2. 3. Validation IL-17RA targets by qRT-PCR 79

Figure 2. 4. Dynamic IL-17-induced gene expression profile 81

Figure 2. 5. Activation of the regulatory NF-κB network 82

Figure 2. 6. Differential gene expression profile of IL-17 in healthy and asthmatic ASM cells. 85

Figure 3. 1. IL-17 synergizes with IL-1β to induce CXCL8 protein release 105

Figure 3. 2. IL-17 enhances IL-1β mediated CXCL8 mRNA neosynthesis 107

Figure 3. 3. IL-17 enhances IL-1β mediated CXCL8 promoter activity 108

Figure 3. 4. IL-17 synergy with IL-1β requires functional AP-1 and NF-κB cis-acting response elements 110

Figure 3. 5. p38, ERK1/2 and PI3-K modulate the synergistic activation of the CXCL8 promoter in response to IL-17 and IL-1β 112

Figure 4. 1. Primary human neutrophils express mRNA and surface IL-17RA 129

Figure 4. 2. IL-17 attenuates the anti-apoptotic effects of GM-CSF 131

Figure 4. 3. GM-CSF-induced Mcl-1 protein and cystolic localization of Bax is disrupted by IL-17 133

Figure 4. 4. IL-17 decreases GM-CSF-inhibition of Bax translocation, perinuclear localization and lost of mitochondrial transmembrane potential 134

Figure 4. 5. IL-17 impairs the ability of GM-CSF to inhibit caspase-3/7 activity 135

Figure 4. 6. IL-17 antagonizes GM-CSF-induced MAPK signal transduction pathways......................... 137

Figure 4. 7. IL-17 antagonizes GM-CSF-induced STAT signal transduction pathways 137

Figure 5. 1. Bridging theme 146

List of Abbreviations

3’UTR	3-primed untranslated region
aa	Amino acid
A/CML	Acute/ chronic myeloid leukemia
ACT1	NF-κB activator-1
ActD	Actinomycin-D
AGC kinase	cAMP-dependent protein kinase\ protein kinase G\ protein kinase C
AHR	Airway hyperresponsiveness
ArHR	Aryl hydrocarbon receptor
AICD	Activation-induced cell death
AKT/ PKB	RAC-α serine/threonine-protein kinase/ Protein kinase B
AP-1	Activator protein-1
APAAP	Alkaline phosphatase anti-alkaline phosphatase
APC	Antigen-presenting cell
ARE	AU-rich elements
ARDS	Acute respiratory distress syndrome
ASM	Airway smooth muscle
AU-sequences	Adenylate and uridylate (AUUUA and UUAUUUAUU) sequences
B cell	Bursa of fabricius/ bone marrow-derived cell
BAL	Bronchoalveolar lavage
BCL-2	B-cell CLL/lymphoma-2
BLAST	Basic local alignment search tool
bp	Base pair
BTB	Bric-a-brac, Tramtrack, Broad-complex
C/EBPβ/ -δ	CCAAT/ enhancer binding protein-β/ δ
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CASZ1	Castor zinc finger-1
CBAD	C/EBP-activation domain
CD	Cluster of differentiation

cDNA	Complementary DNA
CRTh2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
CHS	Contact hypersensitivity
CHX	Cycloheximide
CNS	Conserved noncoding sequences
ConA	Concanavalin A
COPD	Chronic obstructive pulmonary disease
COX2	Cyclooxygenase-2
CRE	cAMP response element
CREB	cAMP response element binding protein
CRP	C-reactive protein
CTLA8	Cytotoxic T-lymphocyte antigen-8
CXC	Cysteine I residues separated by one amino acid (X)
DAPI	4',6-diamidino-2-phenylindole
DAVID	Database for annotation visualization and integrated discovery
DC	Dendritic cell
ddCt	Delta-delta cycle threshold
DLN	Draining lymph node
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNFB	Dinitrofluorobenzene
dNTP	Deoxyribonucleotide triphosphate
DTH	Delayed-type hypersensitivity
EBI3	EBV-induced gene-3
EBV	epstein-barr virus
ECD	Extracellular domain
EGF	Epidermal growth factor
eIF-4E	Eukaryotic initiation factor-4E
ELAV	Embryonic lethal abnormal vision
ELK1	Ets-like gene-1

ERK1/2	Extracellular signal-regulated kinases-1/2
ES	Ex-smoker
EST	Expressed sequence tag
Ets	E26 transformation-specific sequence
EVI site	Ecotropic viral integration site
FASL	Fas ligand
FBS	Fetal bovine serum
Fc	Fragment crystallisable region
FEV1	Forced expiratory volume in 1 second
FEV1% Pred	FEV1 percentage of predicted
FGF	Fibroblast growth factor
FICZ	6-formylindolo[3,2-b]carbazole
FITC	Fluorescein isothiocyanate
FN	Fibronectin-III
Foxp3	Forkhead box P3
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA-3	"GATA" sequence binding protein-3
G-CSF	Granulocyte colony-stimulating factor
G-/ C-/ YFP	Green/ cyan/ yellow fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
GO	Gene ontology
GSK3β	Glycogen synthase kinase-3β
HA-tag	Hemagglutinin-tag
hBD2	Human β-defensin
HBE	Human bronchial epithelial cells
HDAC	Histone deacetylases
HDM	House dust mite
HIES	Hyper-IgE syndrome
HL-60	Human promyelocytic leukemia cells

HLA	Human leukocyte antigen
HSP27	27-kDa heat shock protein
HVS	Herpes virus saimiri
IKK	IκB kinase
IFN-γ	Interferon-γ
i.p.	Intra-peritoneal
i.t.	Intra-tracheal
IL	Interleukin
ICD	Intracellular domain
IKK	IκB kinase
iNOS	Inducible NOS
IP	Immunoprecipitation
IPA	Ingenuity pathways analysis
IRAK4	IL-1R-associated kinase-4
iTreg cell	Inducible T regulatory cell
ITS	Insulin, transferrin, selenium
ICAM-1	Intercellular adhesion molecule-1
IgG	Immunoglobulin G
IκB	Inhibitor of κB
IL-TIF/ IL-22	IL-10-related T cell derived inducible factor
IPCR	Inverse- polymerase chain reaction
IRF-4	Interferon regulatory factor-4
JAK	Janus-associated kinase
JNK	c-Jun NH2-terminal kinase
kb	Kilobase
kDa	Kilodalton
Lcn2	Lipocalin-2
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
LTB_4	Leukotriene B4

LTi cells	Lymphoid tissue inducer cells
N-linked glycosylation	Glycans attached to nitrogens of asparagines or arginine side chains
NGF	Nerve growth factor
NKT cell	Natural killer T cells
NO	Nitric oxide
NOS	NO synthase
mAbs	Monoclonal antibodies
MAD	Mammalian homologues of drosophila mothers against decapentaplegic
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MCL-1	Myeloid cell lymphoma-1
MDS	Myelodysplastic syndrome
MEF	Mouse embryonic fibroblast
MEK	MAPK kinase
MEKK1	MAPK kinase kinase-1
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1α / CCL3
MK2/3	MAPK-activated protein kinase-2/3
MKK6/ MAP2K6	Dual specificity MAPK kinase-6
MLN	Mesenteric lymph node
MMP3	Matrix metalloproteinase-3
Mnk1	MAPK signal-integrating kinase-1
MnSOD	Manganese superoxide dismutase
M-PER	Mammalian protein extraction reagent
mRNA	Messenger RNA
MSK	Mitogen and stress-activated protein kinase
MUC5b	Mucin-5b
NCBI	National center for biotechnology information
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells

NIK	NF-κB-inducing kinase
NKT	Natural killer T
NP-40	Nonidet P-40
NS	Non-smoker
OA	Osteoarthritis
ORF13	Open reading frame-13
OVA	Ovalbumin
P/ S	Penicillin/ streptomycin
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PC20	Provocative concentration causing a 20% fall in the FEV1
PCA	Principal component analysis
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
PHA	Phytohemagglutinin
PI	Propidium iodide
PI3-K	Phosphoinositide 3-kinase
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLAD	Preligand assembly domain
PMA	phorbol 12-myristate 13-acetate/ 12-O-tetradecanoylphorbol-13-acetate
PMN	Polymorphonuclear
POZ	Poxvirus zinc-finger
PR genes	Primary response genes
PS	phosphatidylserine
PSI-BLAST	Position-specific iterative BLAST
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride

qRT-PCR	Quantitative real-time reverse-transcription polymerase chain reaction
RA	Rheumatoid arthritis
RA_c	Retinoic acid
RAC	Related to A and C kinases
RANTES	Regulated upon activation, normal T cell expressed and secreted
RAR	Retinoic acid receptor
RefSeq	Reference sequence
RING domain	Really interesting new gene
RNA	Ribonucleic acid
Rnase	Ribonuclease
RORα/γt	RAR-related orphan receptor
RSK	Ribosomal S6 kinase
Runx	Runt-related transcription factor
Sal	Salbutamol
SAA	Serum amyloid A
SAPK/ JNK	Stress-activated protein kinase/ Jun kinase
SCF	Stromal cell factor
SCID	Severe combined immunodeficiency
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEF	Similar expression to fibroblast growth factor genes
SEFIR domain	SEF-IL-17R domain
SEM	Standard error of the mean
siRNA	Small-interfering RNA
SMAD3	MAD and *Caenorhabditis elegans* Sma-3
SR genes	Secondary response genes
ST-WT	Sense target-whole transcript
StpA/C	Saimiri transformation-associated protein of subgroup A/C strain
STAT	Signal transducer and activator of transcription
STIR domain	SEFIR/ TIR domain
T cell	Thymus derived-cell

TAB2/3	TAK-1binding protein-2/3
TAK1	TGF-beta activated kinase11
T-bet	T-box expressed in T cells
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Tcm	Central memory T cells
TEER	Transepithelial electrical resistance
Tem	Effector memory T cells
TCR	T cell receptor
TFBS	Transcription factor binding-site
TGF-β	Transforming growth factor-β
Th	T helper
TILL	TIR-like loop
Tip	Tyrosine kinase interacting protein
TIR domain	TLR/IL-1R domain
TLR	Toll-like receptor
Tn	Neutrophil-regulatory T cell
TNCB	2,4,6-trinitro-1-chlorobenzene
TNF	Tumor necrosis factor-(α)
TPH	Tryptophan hydroxylase
TRAF6	Tumor necrosis factor receptor-associated factor-6
TRIF	TIR domain-containing adapter protein inducing interferon-β
TSS	Transcription start site
Ub	Ubiquitin
UTR	Untranslated Region
WT	Wild-type
ZFP36	Zinc-finger protein-36 (Tristetraprolin)
ZnF	Zinc-finger
$\Delta\Psi_m$	Mitochondrial transmembrane potential

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1.0. CHAPTER 1

INTRODUCTION

1.1.0. DISCOVERY AND CHARACTERIZATION TIMELINE OF IL-17

Cytokines are the communication medium of immune cells and they operate in a localized fashion to regulate innate and adaptive immune responses. Since 1986, two classes of effector CD4$^+$ T cells termed T helper (Th)-1 and Th2 were identified based on their cytokine secretion profiles [1]. Interferon (IFN)-γ and interleukin (IL)-12 positive cells regulated by the signal transducer and activator of transcription (STAT)-4 and T-box expressed in T cells (T-bet) transcription factors, are associated with a polarized Th1 response specializing in intracellular defences against pathogens such as viruses and/or bacteria. In contrast, STAT6 and "GATA" sequence binding protein (GATA)-3 regulates Th2 cell differentiation and are associated with humoral responses mediated by IL-4, IL-5, IL-13 which neutralize metazoan parasitic infections [2]. As illustrated in figure1.1, the first study to report that a IL-17-producing Th population was different from classical Th cells came in the year 2000 when IL-17 was shown to be preferentially expressed in cells primed with *Borrelia Burgdorferi* but not in Th1 polarizing conditions [3]. In 2005, a decade after the discovery of IL-17 [4, 5], two independent groups conclusively demonstrated that IL-17-producing CD4$^+$ effector T cells differentiated into a distinct subset independent of the Th1 or Th2 lineages [6, 7]. The seminal discovery of a third Th subset revised modern concepts of immunity and expanded our knowledge of disorders which did not conform to the Th1/ Th2 paradigm [8, 9]. The investigation of the development and function of the newly identified Th17 subset currently characterized as IL-17A/F [10], IL-21 [11], IL-22 [12] and regulated by the STAT3 [13] and retinoic acid receptor (RAR)-related orphan receptor (ROR)-α

and RORγt+ [14] transcription factors have uncovered novel cell differentiation mechanisms and expanded elusive innate-adaptive immune networks.

IL-17 is a proinflammatory cytokine that mediates host defense responses. Systemic overexpression of IL-17 promotes granulopoiesis via the secretion of stromal cell-derived growth factors resulting in neutrophilia [15-17]. IL-17 also induces cytokines and CXC-chemokines (cysteines (C) residues separated by one amino acid (X)) which recruits neutrophils to sites of inflammation. A crucial role in promoting antimicrobial peptide production was later revealed in IL-17R deficient mice [12, 18-20]. In addition, tissue resident γδ T [21] and natural killer (NK)-T [22] cells were also identified as the major source of IL-17. Importantly, these cells possess invariant T cell receptors (TCR) which can either directly bind microbial glycolipids or interact with cluster of differentiation (CD)-1d to rapidly respond to fungal and extracellular pathogens. This response complements lengthy antigen processing and presentation by major histocompatibility complex (MHC) to form a distinct IL-17/ Th17 response by initiating and amplifying a rapid neutrophilic response in mucosal/ serosal tissues [23, 24].

The IL-17A receptor (IL-17RA) is the prototypical member of the ubiquitous IL-17 receptor family whose signal transduction pathway parallels those of the IL-1R and toll-like receptor (TLR)-4. Elucidating the IL-17R signal transduction was largely hampered by the fact that the cytoplasmic tail of the IL-17RA is distinct and lacked a consensus binding/ recruitment motif [25]. A defining functional feature of IL-17 is its ability to synergize, or exponentially increase cellular responses to tumor necrosis factor-α (TNF), IL-1β and IL-6 via a largely undefined, post-transcriptional mechanism regulating mRNA stability [26]. This effect is most evident for

proinflammatory cytokines and neutrophils-mobilizing chemokines under the control of the canonical nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathway [27]. However, the composition, dynamics and subunit interactions of the IL-17R complex remains an emerging area of research where novel recruitment motifs and adaptor proteins are actively being researched.

Research efforts in the field of IL-17 biology have largely been focused on identifying biomolecular markers associated with lineage commitment and on differentiation factors mediating Th17 cell fate decisions. To date, the exclusive presence of transforming growth factor (TGF)-β principally determines effector Th cell fates as it promotes the reciprocal development programs of inducible T regulatory (iTreg) and Th17 cells, and suppresses the differentiation of Th1 and Th2 subsets [28]. Importantly, Th1 (IFNγ) and Th2 (IL-4) cytokines are potent inhibitors of early Th17 development whereas iTreg-inducing factors, IL-2 and retinoic acid (RA) inhibit the later stages of Th17 development [28]. Antigen presenting cell (APC)-derived IL-23 represents a terminal differentiating factor for Th17 since its function is dependent the expression of the IL-23R induced by TGF-β and IL-6 or IL-21 [29, 30]. Moreover, identification of IL-17/ IFN-γ double positive cells have also revealed a Th1 convergence program of Th17 precursors which has renewed interest in plasticity between Th lineages [31-33]. Thus, microenvironmental cues regulating Th development and plasticity are areas of research which seeks to ultimately manipulate immune responses in order to generate novel and effective approaches for treatment and vaccine development.

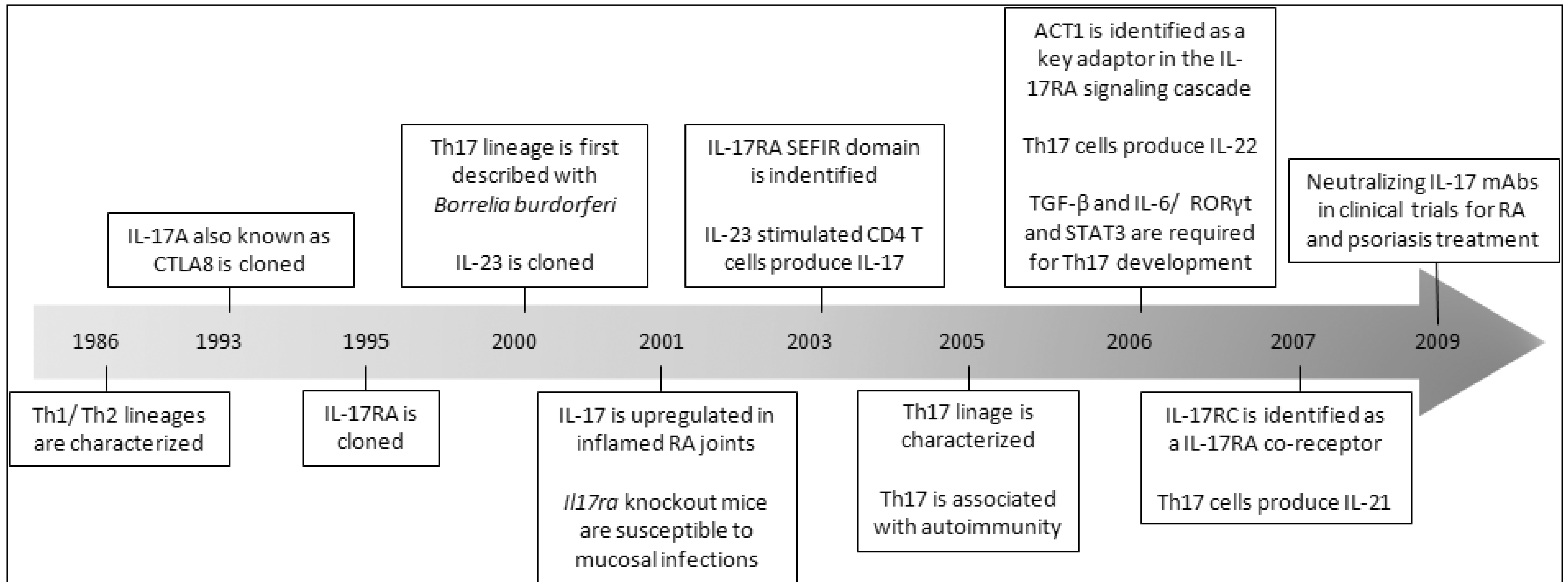

Figure 1. 1. IL-17/ Th17 characterization timeline *(adapted from [34])*
APC: antigen-presenting cell, CIA: collagen-induced arthritis, CTLA8: cytotoxic T lymphocyte antigen 8, EAE: experimental autoimmune encephalomyelitis, HIES: hyper IgE syndrome, IFNγ: interferon-γ, RORγt: retinoic acid receptor-related orphan receptor-γt, SEFIR: SEF/IL-17R, STAT3: signal transducer and activator of transcription 3, TGFβ: transforming growth factor-β.

1.2.0. IL-17 ORIGINS

The sequence of IL-17, formerly known as cytotoxic T-lymphocyte associated antigen (CTLA)-8 was first described in a subtractive, $CD4^+$ T cell murine complimentary (c)DNA library. The primary cDNA library was derived from phorbol myristate acetate (PMA)-ionomycin stimulated T cells which were subtracted from a mixture of unstimulated, photobiotinylated-labeled mRNA derived from mouse lymphoma EL4 (TIB-39) and rat thymoma WFu(C58NT)D (TIB-236) T cells [4]. Four clones which corresponded to the sequences of *ctla8* (*Il17*), *Ccl3* (macrophage inflammatory protein 1α), tryptophan hydroxylase (*Tph*; a rate-limiting enzyme in the biosynthesis of serotonin) and an unreported gene were identified. Interestingly, CTLA-8 was actually derived from the rat thymoma and not from the CTL counterpart as its name inaccurately implies [35]. The protein corresponding to the *ctla8* open reading frame was homologous to the putative protein encoded by the 13th open reading frame (ORF13, formerly KCLF2) of herpes virus saimiri (HVS). Putative aa sequences of human CTLA8 (163aa) shares 63% identity with mouse (147aa) which shares 88% identity with rat (150aa) and 57% aa identity to the ORF13 of HVS (151aa) [35, 36]. HVS, also known as *saimiriine herpesvirus 2* is the classical prototype of the double stranded DNA γ_2-herpesviruses subfamily (rhadinoviruses family) which includes the Kaposi's sarcoma-associated herpesvirus. HVS is a naturally occurring benign pathogen of squirrel monkeys but the T-lymphotropic virus is highly oncogenic in Old and New World primates. Interestingly, rhadinoviruses have the unique ability to pirate cellular genes from host cells and incorporate them into their genomes. As such, HVS harbours in addition to IL-17, a homologous D-type cyclin, the G-protein-coupled receptor CXCR1, the superantigen homologue *Ie14/Vsag* and several inhibitors of the complement and apoptosis pathways. These homologues are hypothesized to mediate the apathogenic persistence in their

natural host as viral null mutants for *Orf13/Il17* retained full replicative, transforming and pathogenic capabilities in infected hosts [37]. The pathogenic effects elicited by the virus are attributed to the saimiri transformation-associated protein of subgroup A strain (stpA), stpC and the tyrosine kinase interacting protein (tip).

1.2.1. Characterizing initial effector functions

The *Ctla8* gene was mapped on mouse chromosome 1A and human chromosome 2q31 by radioactive *in situ* hybridization. Murine CTLA8 corresponds to a transcript of 1.35kb which contains several adenylate and uridylate (AU)-rich repeats at the 3' untranslated region (UTR) [4]. Sorted CD4^{+} peripheral blood T cells stimulated with either CD3 and CD28 monoclonal antibodies (mAb) or phytohemagglutinin (PHA) and ionomycin revealed transcripts of approximately 1.9kb. Predicted aa sequence of hIL-17 shares 72% aa identity with ORF13 HVS and 63% with mCTLA8 [36]. Human (h)IL-17 also contains a single N-linked glycosylation site and can be secreted in both glycosylated (20kDa) and non-glycosylated (15kDa) forms. Human IL-17 also contains 6 cysteine residues and non-reducing polyacrylamide gel electrophoresis (PAGE) revealed the presence of dimers, as immunoprecipitates migrated at 30- and 38kDa bands [38]. Initial functional characterization demonstrated that activated CD4^{+} sorted peripheral blood T lymphocytes secreted pg–ng levels of IL-17 as determined by solid-phase binding assays and upregulated IL-6 and CXCL8 expression from human foreskin fibroblasts. IL-17 also increased surface expression of intercellular adhesion molecule (ICAM)-1 in contrast to human leukocyte antigen (HLA)-ABC, HLA-DR and lymphocyte function-associated antigen (LFA)-1 which were not significantly altered [5, 38]. The addition of murine (m)CTLA-8 to suboptimal concentration of concanavalin A (conA), PHA or immobilized anti-TCR mAb increased T cell

proliferation and addition of soluble, neutralizing IL-17R abolished T cell proliferation by inhibiting IL-2 production [5].

Further characterization of IL-17 gradually uncovered its propensity to induce inflammatory and hematopoietic cytokine production from stromal cells. Whereas no effects on proliferation, cytokine secretion, phenotype and cytotoxicity of peripheral blood mononuclear cells (PBMC) or purified $CD4^+$ and $CD8^+$ T cells were observed, primary cultures of synovial fibroblasts, venous endothelial and lung epithelial cells released granulocyte colony-stimulating factor (G-CSF), IL-6, CXCL8 and prostaglandin E2 (PGE_2). IL-17 was also demonstrated to indirectly sustain and differentiate $CD34^+$ haematopoietic progenitors into the neutrophil lineage, a hallmark of IL-17 function [15]. In addition, IL-17 was observed to synergize the function of TNF and IFN-γ-induced IL-6 and GM-CSF secretion, a second prominent inflammatory feature of this cytokine. Subsequently, IL-17 was demonstrated to markedly enhance G-CSF mRNA stability in murine 3T3 fibroblasts (CCL-92) independently of *de novo* protein synthesis. Addition of actinomycin D (ActD) 4h post-IL-17 stimulation stabilized G-CSF mRNA but the combination of IL-17 with LPS significantly increased the transcript half-life by 2h. Pretreatment with cycloheximide (CHX) suggested that IL-17 stabilizes G-CSF mRNA by downregulating or accelerating the degradation of a negative regulatory protein such as an RNase [39].

1.2.2. Granulopoiesis and neutrophilia

In order to determine the role of IL-17 *in vivo*, transgenic mice overexpressing mIL-17 were generated but failed to be carried to term. As such, an alternative approach relying on a recombinant adenovirus gene transfer expression system was used. This technique was first

described in studies to determine the *in vivo* functions of TNF, where an adenovirus vector engineered to encode a soluble TNF receptor effectively blocked *in vivo* functions of TNF [40]. Adenovirus-mediated gene transfer of mIL-17 cDNA targets hepatocytes to express the transgene which resulted in a transient transgenic phenotype with dramatic effects on granulopoiesis. Within 2-3d, mice developed neutrophilia with a maximum granulopoietic response by the seventh day [16] and serum levels of IL-17 positively correlated to absolute neutrophil counts [41]. Granulocyte-macrophage colony stimulating factor (G-CSF) and stromal cell factor (SCF) released by non-hematopoietic cells of the spleen and bone marrow (BM) were demonstrated to promote the granulopoietic response induced by IL-17 [42]. Additionally, intra-tracheal (i.t.) installation of hIL-17 in rats induced the selective recruitment of neutrophils into the airways, and this effect was impaired by anti-hIL-17 mAb or pretreatment with corticosteroids. The lag time observed for the mobilization of neutrophils into the airways was consistent with an indirect effect mediated by IL-17. Cotreatment of hIL-17 with TNF also highlighted the potentiating role of IL-17 as CXCL8 release from airway epithelial cells was substantially increased and directly observed to mediate the neutrophil chemotactic response *in vivo* [17]. Intra-peritoneal (i.p) administration of IL-17 also resulted in the selective recruitment of neutrophils into the peritoneal cavity via the up-regulation of neutrophil-specific chemokines such as CXCL1 [43].

Characterization of IL-23 uncovered the IL-23/ IL-17/ G-CSF signaling axis which regulates neutrophil homeostasis. IL-23, a heterodimeric cytokine consisting of the IL-12p40 and IL-23p19 subunits is produced by macrophage and dendritic cells (DCs) and regulates the downstream granulopoiesis-inducing function of IL-17A. It has been proposed that peripheral

blood neutrophils continuously transmigrate into tissues where they undergo apoptosis every 24h. The phagocytosis of apoptotic neutrophils by tissue resident macrophages and DCs is an anti-inflammatory mechanism which clears "healthy" cells from the tissues and inhibits cytokine production. Hence, macrophages and DCs which constitutively produce IL-23 will trigger IL-17 production to promote granulopoiesis if low numbers of apoptotic neutrophils reach the tissues until the inhibitory apoptosis-phagocytosis balance is restored [21]. Thus, IL-17 functions as a cross-talk cytokine for the immune and hematopoietic systems promoting the proliferation and differentiation of myeloid progenitors and their recruitment into peripheral tissues.

1.3.0. DESCRIBING IL-17 FAMILY MEMBERS

IL-17A serves as the prototypical family member of the pleiotropic IL-17 family. By 2002, five homologues termed IL-17A-F were identified in genome databases by sequence similarity searches using basic local alignment search tools (BLAST). The IL-17 gene family is dispersed throughout the genome and share conserved C-terminal regions with five highly conserved cysteines which are required for the dimerization via a cystine knot configuration. As seen in table1.1, amino acid (aa) sequence similarity, expression and functional responses classify IL-17 family member into three sub-groups [44]. IL-17F, which shares the highest aa sequence identity with IL-17A at 50% is located on chromosome 6p12 and can form homo- or heterodimers with IL-17A. Both cytokines are secreted from activated T cells and mediate neutrophil responses. In contrast, IL-17B-D express approximately 25%aa identity and are produced by stromal cells. Although the function of IL-17B and IL-17D differ slightly, IL-17C expression and function is relatively unknown. As demonstrated in figure1.2, IL-17E is the most divergent family member and predominately mediates Th2-like, eosinophilic responses.

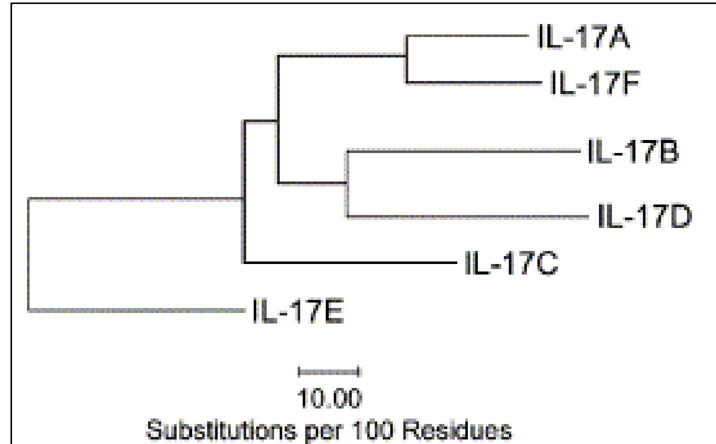

Figure 1. 2. Dendrogram (phylogenetic analysis) of IL-17 family members [44]

1.3.1. IL-17B and IL-17C

IL-17B was the first homologue discovered by two independent groups in the year 2000. IL-17B, a 800bp transcript detected in the pancreas, small intestine and stomach of adult human tissues shares approximately 26-28%aa identity with IL-17A. IL-17C was detected in a human fetal kidney cDNA library although no detectable mRNA signals were initially reported. Recently, IL-17C expression was reported in a broad range of cells including T cells, macrophages and dendritic cells [45]. Whereas IL-17B and IL-17C fail to induce IL-6 in human foreskin fibroblasts, IL-6 expression, in addition to TNF and IL-1β was reported in macrophages of the THP-1 (TIB-202) monocytic cell line [45, 46]. Moreover, IL-17B mRNA was demonstrated to be highly express in spinal cord neurons [47]. The differential activity of IL-17A when compared with IL-17B and IL-17C also suggested that they might bind and activate different cell surface receptors as both IL-17B and IL-17C failed to bind and coprecipitate with the IL-17RA [46, 48].

1.3.2. IL-17E (IL-25)

Within the following year, the remainder of the IL-17 family member were discovered. IL-17E, better known as IL-25 was uncovered by two independent groups. The encoded protein consists of 177aa and is 16–20% identical to IL-17A. IL-25 was initially detected at very low levels in several tissues including brain, kidney, lung, prostate, testis, spinal cord, adrenal gland and trachea by RT-PCR [49] and later detected in Th2-polarized T cells (IL-4/anti-IL-12 mAb/anti-IFN-γ mAb). Daily i.p. injections of IL-25 in mice triggered splenomegaly and in contrast to IL-17A, induced eosinophilia which peaked by the seventh day. These effects were indirectly mediated by increased levels of IL-13 and CCL11 (eotaxin) in tissues and by IL-4 and IL-5 in spleen. Moreover, serum IgE and IgG_1 levels were also indirectly upregulated as IL-4-deficient mice do not produce IgE. Thus, in contrast to the predominant IL-17A-mediated neutrophil response, IL-17E/ IL-25 regulates Th2-associated responses [50, 51].

1.3.3. IL-17F

The gene encoding human IL-17F is located adjacent to IL-17A and encodes a protein of 163aa bearing 50%aa identity to IL-17A. Similar to IL-17A, IL-17F is detected in activated $CD4^+$ T cells and upregulates CXCL8 and G-CSF in primary human fibroblasts. IL-17F also indirectly stimulates progenitor proliferation by inducing the expression of G-CSF and granulocyte macrophage-colony stimulating factor (GM-CSF) by bone marrow accessory cells [52]. A heterodimeric complex consisting of IL-17A/F which functions similarly to the homodimeric counterparts was first observed in co-transfected 293T (CRL-11268) cells and later in mice and humans [10, 53]. Interestingly, the activity of the IL-17F homodimer as demonstrated by

receptor binding affinity and cytokine release assays is approximately 10-fold less potent that IL-17A but is the predominately expressed form in Th17 cells [54].

The crystal structure for IL-17F has also been resolved and has revealed that IL-17F dimerizes in a parallel fashion to the cystine knot protein family. These proteins contain a structural motif in which two disulfide bridges form a loop through which a third disulfide bond passes. Conserved Cys72/ Cys122 and Cys77/ Cys124 of IL-17 adopts a monomer fold typical of nerve growth factor (NGF), although IL-17F lacks the third canonical disulfide bond "tying" the knot structure. Nonetheless, structural modeling identified a 3-dimensional receptor recognition motif shared by IL-17F and NGF [55].

1.3.4. IL-17D

IL-17D was the final member of the IL-17 family to be characterized. IL-17D is located on chromosome 13p11 and is expressed by skeletal muscle, brain, adipose tissue, heart, lung and pancreas. IL-17D is most homologous to IL-17B with 27% identity and at 202aa in length, is the longest IL-17 family member. Treatment of endothelial cells with purified IL-17D protein stimulated the production of IL-6, CXCL8 and GM-CSF but surprisingly, had a suppressive effect on myeloid progenitor cell growth suggesting that IL-17D may signal via a secondary, inhibitory receptor [56].

Taken together, IL-17 functions to stimulate cytokines, chemokines and hematopoietic growth factors which indirectly activate granulocyte (neutrophil)-dependent responses. The limited 16-30%aa identity between IL-17A/F with those of IL-17B-E suggests that distinct subgroups such

as for IL-17E exists within the IL-17 cytokine family. Additionally, the long N-terminal extensions and additional cysteine residue of the IL-17B-E sequence may modify disulfide linkages and its conformation consequently altering receptor binding, signaling and cellular responses.

Ligand	Length	Chromosome	Homology	Source
IL-17A	155aa	6p12	100%	$CD4^{+}$T, $CD8^{+}$T, γδT, & iNKT cells
IL-17F	163aa	6p12	50%	
IL-17B	180aa	5q32	21%	Intestinal, pancreatic & neuronal cells
IL-17C	ND	16q24	27%	Prostate & kidney cells
IL-17D	202aa	13q11	27%	Muscle, brain, heart, lung, pancreas & adipose tissue cells
IL-17E	177aa	14q11	18%	$CD4^{+}$T, NKT, lung epithelial, alveolar macrophages, eosinophils, basophils & mast cells
vIL-17	151aa	ORF13	72%	HVS

Table 1. 1. IL-17 family members [34]

1.4.0. IL-17 RECEPTOR (IL-17R) FAMILY MEMBERS

In 1997, a slide-based autoradiographic screening technique previously used to isolate and clone unidentified ligands was used to identify the IL-17R [57]. Chimeric proteins for HSV13.Fc and CTLA8.Fc were designed and a cDNA library of the murine EL4 thymoma cell line was screened for a single clone that encoded a protein capable of binding the Fc-probes [5]. As seen in figure1.3, the predicted aa sequence of the mIL-17R shares 69% identity with the hIL-17R and exhibit a broad tissue distribution. cDNA encoding the human homologue was thereafter isolated with mIL-17 primers in peripheral blood leukocytes. Located on chromosome 22q11, sequence analysis of the hIL-17R predicted a type I membrane glycoprotein with a 293aa extracellular

domain, 21aa carboxy-proximal transmembrane domain and a particularly long 525aa cytoplasmic tail. The nascent, unglycosylated form has an approximate mass of 105-107kDa whereas endogenous hIL-17R contains seven potential N-linked glycosylation sites and measures 128-132kDa [36]. Engagement of the IL-17R induces NF-κB and mitogen-activated protein kinase (MAPK) activity and IL-6 releases among a subset of inflammatory factors [5, 58, 59]. However, direct binding assays using ^{125}I-labeled hIL-17 transfected in CV-1/ EBMA cells resulted in low affinity values (Ka; $2x10^7$ - $2x10^8$ M^{-1}) suggesting that an additional receptor component was required in order to bind IL-17 at nanogram concentrations, which could elicit biological responses [36].

1.4.1. IL-17RB (EVI27, IL-17Rh1)

The first IL-17R homologue was identified while elucidating proviral integration sites in the BXH2 myeloid leukemia. Ecotropic viral integration site (EVI)-27, a chromosomal region commonly associated with strand breaks in myelodysplastic syndrome (MDS) and in acute and chronic myeloid leukemias (A/CML) was identified by inverse-polymerase chain reaction (IPCR, aka. anchorage-dependent PCR). Two transcripts of 2.7 and 1.9kb were predominantly detected in kidney, brain, liver and testes. A full length 502aa, 56kDa protein which contained a cleavage signal peptide at the N-terminus produced a smaller soluble 288aa, 31kDa protein. Notably, this was the first report of a soluble IL-17R. Moreover, hIL-17RB and hIL-17R were 76% and 69% identical at the aa level compared to their respective murine counterparts suggesting that their function were highly evolutionarily conserved [60].

Similarly, an independent human adult lung expressed sequence tag (EST) database screen for novel IL-17R homologs identified a cDNA clone coding for a 426aa type I transmembrane protein containing an N-terminal cleavage site. Two specific transcripts of 3.5 and 1.4kb were also detected in several endocrine tissues. In this study, ligands for the newly described receptor were investigated by surface plasmon resonance and validated by flow cytometry and immunoprecipitation (IP). Chimeric proteins consisting of the heavy chain constant region IgG1 fused to either the extracellular domains (ECD) of the hIL-17RA or IL-17RB demonstrated that IL-17B but not IL-17A bound the IL-17RB [46].

Finally, a third independent group reported the characterization of a 502aa single transmembrane protein with 26%aa identity to the IL-17RA known as the IL-17R-homogue-1 (IL-17Rh1). In this study, IL-17RB did not bind to a chimeric IL-17A-fc or IL-17C-Fc, but was demonstrated to bind IL-17E-Fc with higher affinity than IL-17B-Fc. Interestingly, IL-17E (IL-25) was also reported to activate NF-κB via a responsive luciferase reporter gene in two human renal cell lines suggesting that NF-κB may regulate IL-17R family downstream signaling [49]. However, since IL-17B and IL-17C did not bind the IL-17R, this suggested that an additional cognate receptor mediated IL-17 cell signaling responses.

1.4.2. IL-17RC (IL-17 receptor-like protein (IL-17RL))

A series of ESTs with homology to the cytoplasmic domain of the IL-17R uncovered a 720aa single pass-transmembrane protein with 22%aa identity to the IL-17RA [61]. Notably, the *Il17rc* gene contains 19 exons which encode over 90 detectable splice variants in prostate, kidney and tracheal tissues. IL-17RC was suggested to function as both a receptor and antagonist since many

isoforms were predicted to encode for a truncated soluble protein lacking the transmembrane and cytosolic domains. However, the relative abundance of IL-17RC transcripts in prostate biopsies revealed that 75% of IL-17RC variants retain the transmembrane domain and that the three most abundant isoforms including the full length receptor accounted for half of all IL-17RC transcripts [62]. Moreover, variation among transcripts predominately occurred at the level of the ECD and IL-17RC could bind to both IL-17A and IL-17F [63, 64]. Interestingly, transfection of hIL-17RA in mIL-17RA deficient fibroblasts does not restore responsiveness to IL-17 indicating that a species specific ancillary component was also required. Cotransfection of hIL-17RC with hIL-17RA however did productively associate to form a multimeric receptor complex able to bind and effectively respond to IL-17A homo- and IL-17A/F heterodimers.

1.4.3. IL-17RD (Similar expression to fibroblast growth factor genes (SEF)

IL-17RD was original identified as the homologue of the similar expression to fibroblast growth factor (FGF) genes (SEF) through an *in situ* hybridization screen of a zebrafish embryo library. SEF encodes a 739aa transmembrane protein which shares 15–20%aa identity to the intracellular region of the hIL-17R [65]. The hSEF or IL-17RD was also independently identified by a genome-wide microarray search for similar intracellular IL-17RA sequences in human umbilical vein endothelial cells (HUVEC) [66]. SEF also contains a soluble splice variant, SEF-s (hSEF-b) and is highly expressed in vascularised tissues such as the kidney, colon, skeletal muscle, heart and the small intestine [67, 68]. Ectopic expression of *Sef* in zebrafish or *Xenopus laevis* embryos initially reported an inhibitory function in limiting FGF signalling during development [65]. In fact, IL-17RD may serve as a feedback regulator of FGF signaling since its expression is positively regulated by epidermal growth factor (EGF) and FGF, and IL-17RD specifically antagonizes

FGF-induced Ras/ extracellular signal-regulated kinases (ERK) signaling. Additionally, IL-17RD was observed to form heteromeric complexes with the FGFR1 and FGFR2 and overexpression of IL-17RD in HUVEC inhibited FGF-responsive reporter genes via an intracellular IL-17R-like domain [69]. Interestingly however, a protein A-agarose pull-down assay failed to detect any hSEF-IgG interactions with IL-17 cytokines suggesting that IL-17RD may serve as a coreceptor for IL-17 [66].

1.4.4. IL-17RE

The final member of the IL-17R family was uncovered in 2006 by a sequence-based homology search in murine genomic databases. IL-17RE encodes a 637aa polypeptide which shares 18%aa sequence identity with mIL-17RC and is largely expressed in lung, kidney, stomach and testis. Alternative splicing of the *Il17re* gene generates up to six isoforms of which two encode for a soluble receptor. Additionally, IL-17RE promotes cellular mitogenic response via the RAS/ MAPK pathway and is thought to either function in opposite fashion to IL-17RD, or to act as a coreceptor for an undefined receptor complex [70].

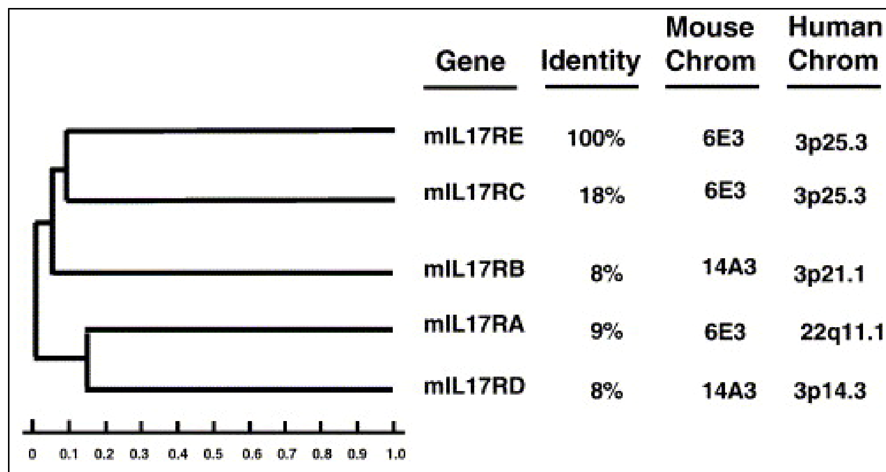

Figure 1. 3. Dendrogram (phylogenetic analysis) of IL-17R family members [70]
Transcripts for IL-17RB is present in endocrine tissues, liver, kidney, pancreas, small intestine and colon, IL-17RC is ubiquitously expressed, being highest in prostate, liver, kidney, muscle and heart, IL-17RD is found in numerous cell types, with high expression in ovary, breast, kidney, heart, skeletal muscle and colon and IL-17RE is expressed in lung, kidney, stomach and testis.

1.4.5. Multimeric IL-17R complex

Type I and II cytokine receptors contain multiple subunits forming a complex generally consisting of a ligand specific α-chain and signal transducing common β- or γ-chain. Although the IL-17R shares little homology with other receptor family, it had long been presumed that the IL-17R consisted of a heteromeric complex. IL-17A was originally observed to bind the IL-17RA with too low of an affinity to correspond to the small experimental doses required to elicit a cellular response indicating that a coreceptor might cooperate with the IL-17R [5]. Furthermore, IL-17RD was also demonstrated to interact with the FGFR1 and FGFR2 [66] and the identification of the IL-17A/F heterodimer suggested that IL-17R family members could form different receptor complexes in order to interact with different ligands [71]. It is also interesting to note that IL-17RB and RD are localized on human chromosome 3p21 whereas IL-17RC and RE are located on chromosome 3p25 and that beside IL-17RA, all of the IL-17R family members contain alternatively spliced isoforms. However the configuration and specific stoichiometry of the IL-17R complex remains under investigation.

The initial report providing evidence that the IL-17R formed a multimeric complex demonstrated that IL-17R-deficient fibroblasts coexpressing a cyan or yellow fluorescent protein (CFP/ YFP) fused to the intra-cellular domain (ICD) of the IL-17RA lost fluorescence resonance energy transfer (FRET) signals 10 minutes after stimulation with IL-17A. FRET signals occurs when fluorophores are 10–100Å apart indicating that the cytoplasmic tails of the

IL-17RA were held in close proximity before stimulation of IL-17A which induced a conformational change causing the cytoplasmic tails to move apart [72]. Since the IL-17RA appears to form a multimer in the absence of ligand, it was proposed that it contains a preligand assembly domain (PLAD) as previously described for the TNFR-1/2 which bind the receptor complex [73]. Two fibronectin-III-like (FN) ECD joined by a non-structural linker region (residues 184–204) were predicted and demonstrated to mediate homotypic interactions between IL-17RA subunits (FN1, residues 69–183; FN2, residues 205–282). The FN1 domain was observed to enhance ligand binding but did not bind or mediate signaling in the absence of the FN2 or linker regions. In contrast, FN2 contains the IL-17 binding site and mediates ligand-independent oligomerization of the IL-17RA. Interestingly, the FN1 domain was proposed to interact with, or recruit a membrane proximal protein upon activation thus rearranging the IL-17RA complex and resulting in the lost of the ICD FRET signal [73].

The IL-17R was subsequently demonstrated to associate with the IL-17RC subunit [63]. When IL-17RA deficient fibroblasts were cotransfected with hIL-17RA and IL-17RC and then treated with either hIL-17A or hIL-17F, CXCL1 production was restored suggesting that a heterodimeric IL-17RA/ RC was required for cell signaling. Moreover, nonreducing sodium dodecyl sulfate (SDS)-PAGE analysis revealed that the IL-17RC migrated as a trimeric complex possibly containing two IL-17RA subunits [74]. Baby hamster kidney (BHK) cells stably transfected with the hIL-17RC were also demonstrated by flow cytometry to bind biotinylated-hIL-17A and hIL-17F [64]. Surface plasmon resonance analysis demonstrated that IL-17A bound the IL-17RA.fc with the fastest on rate (1.4×10^5 M/s) and slowest off rate (2.9×10^{-4}M/s) resulting in the lowest K_d value of 2.2nM. IL-17RA also bound the IL-17A/F heterodimer with an intermediate

on/ off K_d value of 26nM whereas IL-17F had a 100-fold slower on/ off rate resulting in a K_d of 70nM. Unlike the IL-17RA, surface plasmon resonance demonstrated that hIL-17A and hIL-17F bound the hIL-17RC with similar affinities (K_d values of 10-20nM). Importantly, coexpression of hIL-17RA and hIL-17RC did not enhance the ability to respond to hIL-17A or IL-17F, suggesting that the receptors coordinate functional signaling responses rather than mediate a synergistic signaling effect. Moreover, hIL-17RC is highly expressed in non-hematopoietic tissues suggesting that receptor binding affinities may differentiate biological functions of IL-17 family members [71].

Recently, coexpression of the IL-17RA with IL-17RD in HEK293T and in mouse embryonic fibroblast (MEF) was demonstrated to colocalize and precipitate as a multimeric complex. Luciferase assays demonstrated that the ICD of IL-17RD mediated IL-17 signaling by interacting with the TNF receptor-associated factor (TRAF)-6. Of note, IL-17RD also coimmunoprecipitated with IL-17RB suggesting that IL-17R family members may form different receptor complexes in order to respond to different ligands [75]. In support of this notion, the FN1 domain of the IL-17RA may interact with, or recruit specific IL-17RB-E subunits as proposed for IL-17RC upon activation by IL-17A/F [73].

1.5.0. IL-17R SIGNAL TRANSDUCTION PATHWAY

The first crucial mediator identified in the IL-17R signaling cascade was the TNF receptor superfamily and the TLR/IL-1R (TIR) family adaptor protein, TRAF6. IL-17 fails to activate IκB kinases (IKKs) and upregulate IL-6 expression from embryonic fibroblasts derived from TRAF6-deficient mice. Transient transfection of TRAF6 in 293 cells restores IL-17-induced NF-

κB activation by a luciferase reporter assay and reciprocal coimmunoprecipitation demonstrated TRAF6 interacts with the IL-17R. Interestingly, TRAF6 was specifically observed to mediate the IL-17R signal transduction as TRAF2-deficient fibroblasts did not impair effector functions mediated by IL-17A [76, 77].

In 2003, a position-specific iterative BLAST (PSI-BLAST) identified a conserved sequence within the intracellular, membrane-proximal region of the SEF and the IL-17R. As shown in figure1.4, the SEF-IL-17R (SEFIR) domain is highly similar to the TIR domain which mediates homotypic interactions between TLR-4/ IL-1R and the myeloid differentiation primary response gene (MyD)-88 adaptor protein [78]. However, the SEFIR domain contains a homologous TIR-like loop (TILL) which lacks a crucial “BB loop” required for conventional TIR-mediated interactions. As such, TIR domain-containing adapter protein inducing interferon-β (TRIF)-, MyD88 and IL-1R-associated kinase (IRAK)-4-deficient fibroblasts do not impair IL-17-dependent IL-6 production suggesting that the distinctive TILL domain could serve as a specific docking site for SEFIR/ TIR (STIR)-homologues [79]. Moreover, the IL-17RA does not contain a canonical TRAF6-binding motif (P*X*E*XX*Z; *X*, aromatic/acidic residue) implying that additional adaptor proteins mediate the recruitment of TRAF6 to the cytoplasmic tail of the IL-17R.

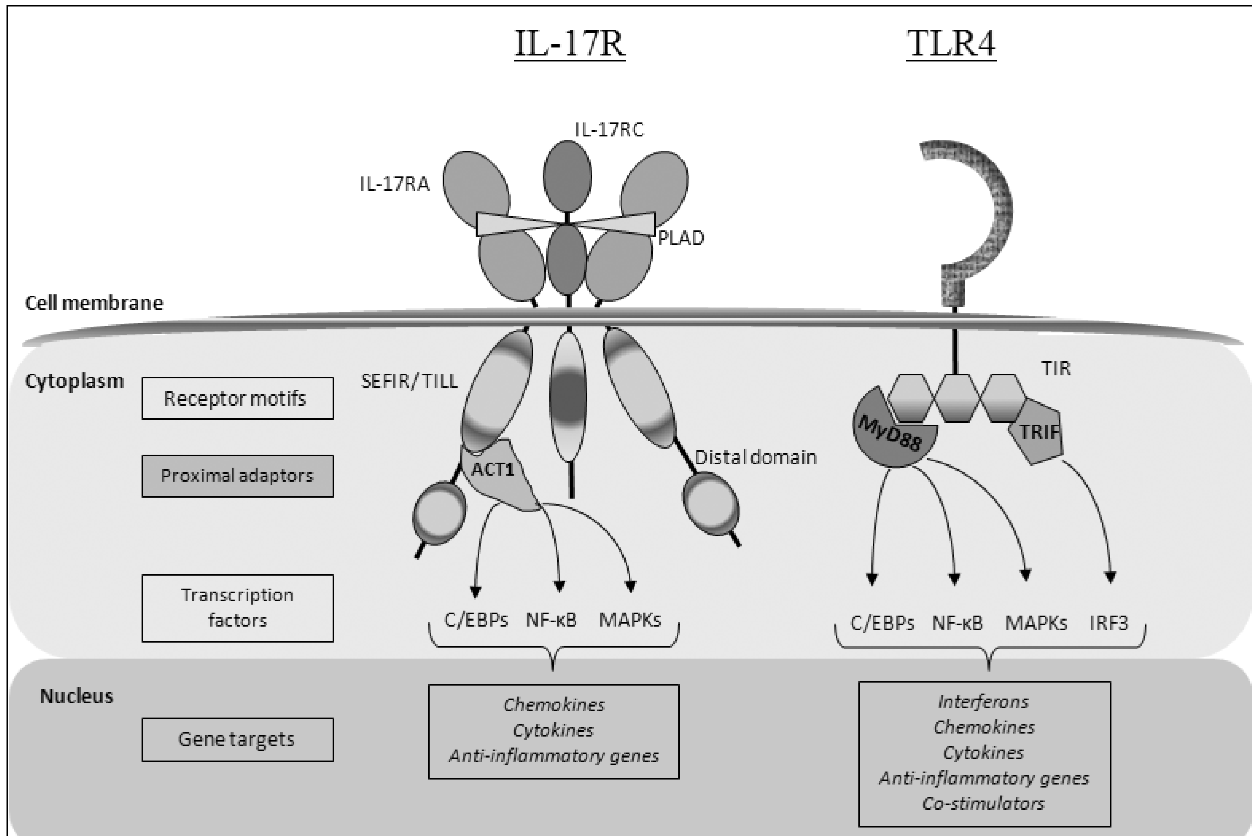

Figure 1. 4. IL-17 and TLR4 receptor complexes *(adapted from [34])*
IL-17R and TLR/ IL-1R signalling pathways use different functional receptor motifs (SEFIR and TILL versus TIR domains) and proximal adaptors (ACT1 versus MYD88 and TRIF), but converge on common pathways (NF-κB, C/EBP and MAPKs) to activate similar, although not identical, gene expression profiles.

An adapter protein bearing two identifiable TRAF binding sites (residues 38–42 and 333–337) in addition to a SEFIR binding sequence in its C-terminal domain was later identified (see figure1.5) [78, 80]. NF-κB activator (ACT)-1, a cytoplasmic protein separately isolated from a constitutively active NF-κB cell line [81] and by a yeast two-hybrid screen using the NF-κB essential modulator (NEMO)/ IKKγ subunit of the IKK signaling complex as bait [82], was observed to directly associate to the IL-17R and to TRAF6 in response to IL-17 [83]. Notably, ACT1-deficient MEF did not impair TNF-, IL-1β- or LPS-inducible gene expression but abrogated those mediated by IL-17A, IL-17F and when in combination with TNF [83].

Hemagglutinin (HA)-tagged ACT1 was also observed to coimmunoprecipitate with FLAG-tagged TRAF3 in 293 cells and negatively regulate CD40-mediated NF-κB activation [84]. TRAF3, an atypical TRAF family member, negatively regulates the NF-κB signaling pathway by targeting the NF-κB-inducing kinase (NIK) for proteasomal degradation and inhibits the NIK-dependent processing of NF-κB2p100 precursor into active p52 subunits [85]. Hence, ACT1 may regulate NF-κB activity depending on whether it interacts with a positive regulator such as TRAF6 to recruit activating signaling complexes or with a negative regulator such as TRAF3 [86]. ACT1 was also demonstrated to interact with the TGF-β activated kinase (TAK)-1 which activates the IKK-NF-κB and stress-activated protein kinase (SAPK)/Jun kinase (JNK) signaling modules [84]. Interestingly, IL-17-induced chemokine expression is decreased in TAK1-deficient MEF indicating that a ACT1-TRAF3/6-TAK1 complex is a critical mediator of the IL-17R signal transduction pathway [87]. In addition, activation of C/EBPβ and -δ via the distal CCAAT-enhancer-binding protein (C/EBP)-activation domain (CBAD) located in the cytoplasmic tail of the IL-17RA is dependent on ACT1 [88].

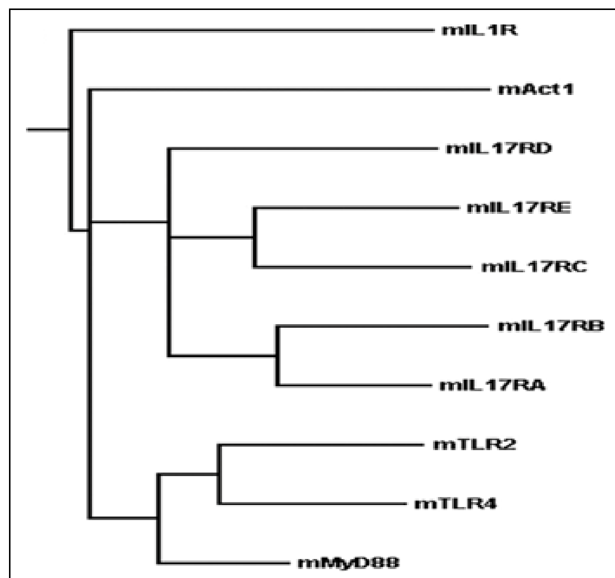

Figure 1. 5. Dendrogram (phylogenetic analysis) of STIR domain proteins [83]

1.5.1. MAPK pathway

Mitogen-activated protein kinases (MAPKs) are a family of conserved protein kinases that phosphorylate specific serine and threonine residues of target proteins to regulate various cellular activities such as proliferation, migration and survival. The MAPK pathway consists of a three-tiered cascade that is activated in response to environmental stress signals such as ionizing radiation, heat or osmotic shock and cytokines. The upstream MAPK kinase-kinase (MAPKKK) phosphorylates downstream MAPKK which relay the signal to MAPKs. Conventional MAPKs consist of three family members; (*i*) the extracellular signal-regulated kinase (ERK), (*ii*) c-Jun NH2-terminal kinase (JNK) and (*iii*) P38 which can translocate to the nucleus and phosphorylate downstream targets such as transcription factors.

Initial analysis of the effector functions mediated by IL-17 revealed it was a "cytokine-inducing cytokine" characterized by a proinflammatory IL-6, CXCL8, PGE_2 and G-CSF response in synovial rheumatoid arthritis (RA) fibroblasts [15]. Interest in the IL-17R signal transduction however was later instigated by a clinical research study which reported that IL-17 upregulated nitric oxide (NO) production *ex vivo* from osteoarthritis (OA) cartilage of patients undergoing knee replacement surgery [89]. Since synovial fluid from OA and RA patients contains significant amounts of NO which exerts detrimental effects on chondrocyte function, the search for new therapeutic targets spurred research into IL-17R signaling pathway. The first study to report the intracellular signaling events mediated by the IL-17R observed a twofold transient increase in free cytoplasmic calcium (Ca) with a time-dependent decrease in cyclic adenosine monophosphate (cAMP) levels in human macrophages. Cell-permeable chemical inhibitors targeting nonspecific tyrosine kinases (genistein) and cAMP protein kinase A (PKA) pathways

abrogated IL-17-stimulatory effects whereas the upstream JNK activator, Ca phospholipid-dependent protein kinase C (PKC) and the ERK upstream activator MAPK-kinase (MEK) suppressed IL-17-induce TNF secretion by 60%. Furthermore, NF-κB DNA-binding activity to sequence-specific probes increased in a time-dependent manner peaking at 4h whereas the binding-activity of cAMP response element (CRE) decreased in a time-dependent fashion [58]. Collectively, early investigation into the IL-17R cell signaling pathways demonstrated that IL-17 activation was associated with, and depended in part on MAPK and NF-κB.

Subsequent reports on the IL-17R focused on identifying IL-17-inducible serine/ threonine-specific protein kinases, transcription factors and IL-17 genes targets. ERK1/2, p38 and JNK were initially observed to mediate IL-17 inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and IL-6 responses in human articular chondrocytes via NF-κB. Interestingly, MEK1 and p38-specific inhibitors did not impair NO responses suggesting that JNK regulated the IL-17R signaling cascade however, as there were no specific inhibitors available for JNK, this was not experimentally determined [59]. *In vitro* immune-complex kinase assays from IL-17-stimulated intestinal epithelial IEC-6 cells demonstrated ERK rapidly (within 10m) phosphorylated the substrate-specific myelin basic protein (MBP) whereas IL-17 induced a gradual increase in p38 and JNK1 activation. IL-17-induced NF-κB activation, NO production and iNOS expression in human OA chondrocytes revealed that IL-17 signaled via ERK1/2, p38 but not via the SAPK/JNK pathway [90]. Although JNK was initially reported to mediate IL-17 responses, ERK activation appears to regulate IL-17R signaling as it is the most consistently reported and rapidly phosphorylated member of the MAPKs [34]. Further work into the IL-17R signal transduction also revealed that TRAF6 mediates MAPK activity as dominant negative expression constructs

inhibit IL-17-induced JNK1/ c-Jun and ERK1/ E26 transformation-specific sequence (Ets)-like gene (ELK)-1 transactivation. TRAF6 was also demonstrated to mediate NF-κB p65/ p50 heterodimer activation by activating downstream NIK and IKK-α kinases [76, 77].

Early studies of the IL-17R signal transduction revealed that IL-17-gene targets differed in cells of stromal and hematopoietic origins including those of the non-myeloid lineage. In response to IL-17, stromal cells such as primary cultures of synovial fibroblasts release IL-6, CXCL8, PGE_2 and G-CSF which was demonstrated to indirectly sustain progenitor $CD34^+$ cell growth and differentiation[15]. In contrast, IL-17 does not directly induce the proliferation or differentiation of hematopoietic progenitors cultured with or without GM-CSF nor does it alter the cytokine profile (IFN-γ IL-4, IL-6, IL-10), phenotype or cytotoxicity of hematopoietic cells such as purified $CD4^+$ and $CD8^+$ T cells activated by PHA, tetanus toxin, IL-2 or of total peripheral blood mononuclear cells (PBMC). Moreover, IL-17 does not affect the proliferation or immunoglobulin (Ig) production of normal tonsil B cells activated by antigen receptor or CD40. However, adherent human monocytes (macrophages) have been reported to produce TNF, IL-1β, IL-6, IL-10, IL-12 and PGE_2 in response to IL-17 [58]. Different stromal cell types also generate different gene profiles; primary cultures of human articular chondrocytes release IL-1β, IL-6, iNOS and COX-2 [59] in response to IL-17 whereas synovial fibroblasts release IL-6, CXCL8, PGE_2 and G-CSF [15].

Cells taken from patients with preexisting inflammatory conditions can also influence the level of responsiveness to IL-17. For example, p38 and MEK1 were demonstrated to regulate iNOS expression and NO release in response to IL-17 in chondrocytes from OA patients [90] whereas

MEK1-inhibitors did not impair NO synthesis in normal human chondrocytes [59]. Discrepancies in CXCL8 release from IL-17-stimulated airway smooth muscle (ASM) cells obtained from patients undergoing surgical resection or lung transplants have also been suggested to depend on the underlying health or epigenetic code of the donors [91]. Furthermore, the signal transduction pathways of IL-17-inducible genes such as for IL-1β and TNF are also differentially regulated. Exposure to CalC (inhibitor of Ca-regulated PKC) decreases both IL-1β and TNF secretion but the nonspecific tyrosine-kinase blocking agent genistein or inhibition of MEK1 suppresses TNF secretion with no detectable effect on IL-1β. Inhibition of extra- (EGTA) or intracellular (thapsigargin) Ca mobilization also significantly suppressed TNF secretion with no effects on IL-1β [58]. Divergent regulation of the human β-defensin (hBD)-2 and CCL20 expression by IL-17 has also been described [92]. Overall, compounded analysis of the IL-17R signaling pathway should be cautiously reviewed as IL-17-gene targets are differentially regulated and can be cell type specific.

1.5.2. mRNA stability

Cellular signaling events mediated by the IL-17R involve the coordinated regulation of both DNA-binding (transcriptional) and RNA-binding (post-transcriptional) factors. IL-17 was initially reported to stabilize lipopolysaccharide (LPS)-induced G-CSF mRNA in synoviocytes which increased G-CSF protein levels in an additive fashion. Pretreatment with CHX, an inhibitor of protein synthesis did not impair but further amplified G-CSF mRNA levels. This suggested that IL-17 and LPS did not require *de novo* protein synthesis in order to generate G-CSF mRNA or to stabilise G-CSF transcript levels [39]. IL-17 was also demonstrated to increase IL-6 mRNA half-life in TNF- and IL-1β-stimulated myofibroblasts without increasing NF-κB

activity suggesting that post-transcriptional mechanisms mediated synergistic responses between IL-17 and proinflammatory mediators [93].

JNK- and p38- MAPK signaling pathways have been observed to regulate AU-rich elements (ARE)-dependent mRNA decay. AREs consist of *cis*-acting pentameric and nonameric AUUUA and UUAUUUAUU sequences found in the 3'UTR of mRNA and mediate the stability or half-life of approximately 2800–9600 genes or 8% of all human transcripts [94]. AREs mediate mRNA stability and were originally identified when the stable β-globin mRNA 3'UTR was replaced with the labile ARE-rich 3'UTR of GM-CSF [95]. Putative *trans*-acting factors such as tristetraprolin and the mammalian homologs of the embryonic lethal abnormal vision (ELAV) proteins AUF1, HuR and He1-N2 were later demonstrated to interact with distinctive ARE domains and mediate stabilizing or destabilizing effects [96]. P38-dependent mRNA stabilization mechanisms were initially demonstrated when constitutively active forms of MAPK-kinase-kinase-1 (MEKK1) and the dual specificity MAPK kinase (MKK)-6 were overexpressed and observed to stabilize a chimeric β-globin mRNA containing the 3'UTR ARE-rich sequences of the CXCL8 transcript. A p38α kinase-inactive mutant was also demonstrated to reverse the protective effects on mRNA stability whereas dominant interfering constructs of SAPK/ JNK and ERK did not [97]. IL-1, LPS and IL-17 were subsequently observed to stabilize mRNAs via p38-dependent mechanisms [98].

IL-17 amplifies responses to secondary stimuli such as IFN-γ, TNF, LPS and IL-1β in stromal cell types [15, 26]. IL-17 enhanced CXCL8 levels in lung fibroblast and epithelial cells and was independent of increased transcriptional activity since the combination of IL-17 with TNF or

LPS marginally increased NF-κB, activator protein (AP)-1 and C/EBP activation. Accordingly, IL-17 was observed to profoundly increase the half-life of CXCL8 and IL-6 mRNA when stimulated with TNF and LPS [99]. IL-17A also significantly amplified CXCL8 mRNA levels in ASM cells via a posttranscriptional p38-dependent mechanism. ASM cells transfected with a constitutively active form of MKK6 was demonstrated to secrete increased levels of CXCL8 and inhibition of the p38 pathway with SB203580 significantly decreased the stability of CXCL8 mRNA in response to TNF and IL-17A [91]. Moreover, MAPK signal-integrating kinase-1 (Mnk1) and the eukaryotic initiation factor 4E (eIF-4E) regulating the formation of the pre-initiation complex were phosphorylated by IL-17 in a p38-dependent manner [98]. Although p38 activity is reported to disrupt tristetraprolin, an RNA-binding protein that promotes decay of mRNAs [100], pharmacological inhibitors and downstream p38 kinase deficient MAPK-activated protein kinase (MK)-2/3 MEFs demonstrated that p38 was dispensable for the increase in mRNA half-life. As such, a tristetraprolin-insensitive sequence in the 3' UTR was reported to regulate IL-17- and IL-1β-mediated stabilization of CXCL1 [101]. IL-17-mediated COX2 mRNA stabilization was also reported to act on distal mRNA sequences as deletion of the 3'UTR-AREs had no effect on COX2 levels suggesting that IL-17 activated a distinct group of ARE-independent, RNA-binding proteins [98].

An alternative pathway mediating IL-6 secretion by IL-17 in combination with TNF was demonstrated to occur at the transcriptional level in the osteoblastic MC-3T3 cell line. NF-κB and C/EBP binding sites in the IL-6 promoter were demonstrated to increase transcripts and gene expression levels independently of mRNA stability mechanisms. C/EBPβ and C/EBPδ are upregulated after IL-17 stimulation and are critical regulators of IL-6 since C/EBPβ and C/EBP-

δ-deficient mice do not express IL-6 [102]. Additionally, NF-κB and IκBζ, a NF-κB coregulator were also reported to stabilize secondary response (SR) genes induced by IL-17 with TNF such as for lipocalin (Lcn)-2 [103]. The synergistic effect mediated by IL-17 have also been observed at the transcriptional and posttranscriptional level and ERK inhibitors as opposed to p38 inhibitors markedly decreased IL-6 [104] and CXCL8 [105] transcript levels in human pancreatic myofibroblasts and ASM cells, respectively.

1.5.3. Promoters and transcription factor binding-sites

To identify potential transcription factors mediating the IL-17 gene profile, comparative analysis of eighteen IL-17 gene targets (Table 1.2) were assessed for enrichments of transcription factor binding-sites (TFBS). Computer-assisted analysis of mammalian proximal promoter sequences 1kb upstream of the transcription start site (TSS) has revealed 4 statistically overrepresented TFBS. Proximal NF-κB and C/EBP sites within 600bp of the TSS were categorized as regulating immuno-regulatory molecules such as cytokines and host defense genes (IL-6, Lcn2, matrix metalloproteinase (MMP)-3, hBD2, G-CSF and COX2). A second group consisting of NF-κB binding sites 300bp of the TSS with variable C/EBP, AP-1 or Oct-1 binding sites exclusively regulated chemokine gene expression (CXCL1, CXCL2, CXCL5, CCL2, CCL20, and CX3CL1). A third group containing C/EBPβ and C/EBPδ genes were observed to be independent of NF-κB binding sites and had little or no conservation in TFBS. Enriched TFBS for Ikaros was also identified in IL-17-inducible gene promoters but was determined to be an artefact of the inputted search matrix sequence due to the similarity of the NF-κB binding sequence [106]. An activating transcription factor (ATF)-2/ CRE binding site was also independently reported to regulate IL-17-induced COX2 promoter activity [98]. Thus, the overlapping TFBS in IL-17-inducible gene

promoters suggests that IL-17 activates a combination of transcription factors in order to regulate the gene expression profile. Accordingly, nuclear import and DNA-binding of NF-κB induced by IL-17 occurs within 15-30 minutes whereas the activation of C/EBP occurs 2-4h post-stimulation implying IL-17 activates a dynamic transcriptional program. IκBζ, an inducible regulator of NF-κB which is expressed 2-4h post-IL-17 stimulation has also been reported to associate with NF-κB p50 homodimers and regulate secondary response genes such as for IL-6, hBD2 and Lcn2 [103, 107, 108]. Collectively, IL-17 has been reported to activate NF-κB, AP-1 (c-Jun, fos, elk1), C/EBPβ/δ, ATF2 and STAT3 transcription factors at varying time points.

Gene Name	Gene description	Other Name
Chemokines		
CXCL1	Chemokine (C-X-C motif) ligand 1	KC, Groα
CXCL2	Chemokine (C-X-C motif) ligand 2	MIP2, Groβ
CXCL5	Chemokine (C-X-C motif) ligand 5	LIX
CXCL6	Chemokine (C-X-C motif) ligand 6	GCP-2
CXCL8	Chemokine (C-X-C motif) ligand 8	IL-8
CXCL9	Chemokine (C-X-C motif) ligand 9	MIG
CXCL10	Chemokine (C-X-C motif) ligand 10	IP10
CXCL11	Chemokine (C-X-C motif) ligand 11	I-TAC
CCL2	Chemokine (C-C motif) ligand 2	MCP-1
CCL5	Chemokine (C-C motif) ligand 5	RANTES
CCL7	Chemokine (C-C motif) ligand 7	MCP-3
CCL11	Chemokine (C-C motif) ligand 11	Eotaxin
CXCL12	Chemokine (C-X-C motif) ligand 12	SDF-1
CCL20	Chemokine (C-C motif) ligand 20	MIP3α
Inflammation		
IL6	Interleukin 6	IL-6
IL-19	Interleukin-19	
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	GM-CSF
CSF3	colony stimulating factor 3 (granulocyte)	G-CSF
ICAM-1	Intracellular adhesion molecule-1	
PTGS2	Prostaglandin-endoperoxide synthase	COX2
NOS2	Nitric oxide synthase 2	iNOS
LCN2	Lipocalin 2	24p3
DEFB4	defensin beta 4	BD2
S100A7	S100 calcium binding protein A7	Psoriasin
S100A8	S100 calcium binding protein A8	Calgranulin A
S100A9	S100 calcium binding protein A9	Calgranulin B
MUC5AC	Mucin 5, subtypes A and C, tracheobronchial/gastric	
MUC5B	Mucin 5, subtype B, tracheobronchial	
EREG	epiregulin	
SOCS3	Suppressor of cytokine signaling-3	
MMP1	Matrix metallopeptidase 1	
MMP3	Matrix metallopeptidase 3	
MMP9	Matrix metallopeptidase 9	
MMP13	Matrix metallopeptidase 13	
TIMP1	Tissue inhibitor of metallopeptidase 1	
Transcription		
CEBPB	CCAAT/enhancer binding protein beta	C/EBPβ, NF-IL-6
CEBPD	CCAAT/enhancer binding protein delta	C/EBPδ, NF-IL-6β
NFKBIZ	nuclear factor of kappa light gene enhancer in B-cells inhibitor zeta	IκBζ, MAIL

Table 1. 2. IL-17 gene targets [25]

1.5.4. JAK-STAT pathway

The janus-associated kinases (JAK)-STAT pathway is the principal signaling mechanism for a wide array of cytokines and growth factors such as interferons, IL-6 and GM-CSF. Following the binding of cytokines to their cognate receptor, STATs are recruited to cytoplasmic tail by one of four tyrosine JAK family members (JAK1-3, TYK2). Once activated, homo- or heterodimers of STATs (STAT1-6) dimerize and translocate to the nucleus to modulate the expression of target genes. JAKs can also cross-talk and activate other signaling pathways such as the PI3-kinase and MAPKs.

Activation of the JAKs and STATs by IL-17 was initially demonstrated in 1999 by immunoblots in human U937 monocytic leukemia cells [109]. IL-17 rapidly stimulated tyrosine phosphorylation of JAK1-3 and Tyk2 and STAT1-4 within 0.5 to 30m. Functional studies in primary HBE showed pretreatment with AG490, a specific JAK2 inhibitor, impaired IL-17-mediated IL-6 and mucin (MUC)-5B responses whereas ERK (U0126 and PD98059) and phosphoinositide 3-kinases (PI3-K) (wortmannin) inhibitors exerted no effects [110]. In contrast, the pan-JAK inhibitor (JAK inhibitor I) but not the JAK2 inhibitor was observed to inhibit IL-17-induced hBD2 expression in HBE [20]. Moreover, the differential regulation of JAKs and MEK inhibitors on the respective expression of hBD2 and CCL20 suggested the presence of a JAK-dependent and -independent NF-κB activation pathway [92]. IL-17 stimulation also led to phosphorylation of JAK1-2, and the pan-JAK inhibitor or a JAK1 small-interfering (si)-RNA, blocked the downstream phospho-inactivation of the glycogen synthase kinase (GSK)-3β at S9 and expression of hBD2, IL-19, CXCL1-3 and CXCL6 in HBE cells. Interestingly, inhibitor of JAKs and PI3-K did not impair the DNA-binding activities of NF-κB suggesting the presence of an

NF-κB-independent JAK/ PI3-K/ GSK3β signaling pathway [111]. IL-17 stimulation was also observed to phosphorylate STAT3 in human ASM cells, and inhibition of JAKs suppressed CCL11 expression also supporting a role for the JAK-STAT pathway in the IL-17R signal transduction [112].

1.5.5. PI3-K and GSK3β pathway

The phosphatidylinositol 3-kinases (PI3-K) constitute a unique and conserved family of intracellular lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositol or phosphoinositides (PtdIns). Three classes of PI3-K (I-III) based on the substrate preference and sequence homologies have been identified. Class I are heterodimeric enzyme consisting of a regulatory (p85, p101) and a catalytic subunit (p110) which convert PI into secondary messengers such as PI(3)P, PI(3,4)P2 and PI(3,4,5)P3. Class II comprises three catalytic isoforms without regulatory subunits whereas class III are similar to class I enzymes but only produce PI(3)P. Secondary messengers function to recruit signalling proteins which contain a pleckstrin homology (PH) domain such as protein kinase B (AKT), to the plasma membrane to activate intracellular signaling pathways and mediate various cellular functions.

The first study to investigate the role of PI3-K in the IL-17R signal transduction demonstrated that inhibition of PI3K by LY294002 had no substantial effect on IL-6 or CXCL8 release in HBE cells [113]. Similarly, inhibition of PI3-K did not impair IL-17F (ML-1)-mediated GM-CSF [114], IL-17A-mediated CCL20 [92] expression in HBE cells or CCL11 and CXCL8 responses in human ASM cells [105, 112]. However, PI3-K inhibitors (LY294002, wortmannin) abolished IL-17-induced phosphorylation of the related to A and C kinases (RAC)-α and the serine/ threonine-

protein kinase/ protein kinase B (AKT/ PKB) and inhibited IL-6, CXCL8 and IL-23p19 release in fibroblast-like synoviocytes of RA patients [115, 116]. IL-17A was also observed to rapidly upregulate phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) levels in HBE cells and immunoblots revealed that downstream PI3-K signaling molecules AKT and GSK3β (S9) were phosphorylated. Pretreatment with LY294002, silencing of the PI3-K-p110α subunit or overexpression of the intrinsic phosphatase and tensin homolog (PTEN) PI3-K inhibitor significantly decreased IL-17A-induced hBD2 promoter activity [111]. Similarly, overexpression of a constitutively active GSK3β mutant resulted in decreased IL-17A-induced hBD2 promoter activity. The negative regulation of GSK3β was also observed in bone-marrow derived ST2 cells where overexpression of wild-type GSK3β phosphorylated and inhibited the transcriptional activity of C/EBPβ and impaired *Lcn2*, *Ccl2*, *Ccl7* and *Cxcl5* expression [88]. Additionally, the pan-JAK and PI3-K inhibitor (LY294002) did not inhibit NF-κB p65/ p50 binding activity indicating that the JAK and PI3-K pathways did not mediate the activation of NF-κB induced by IL-17 [111]. Thus, as shown in figure1.6, two independent pathways were proposed to mediate IL-17 cell signaling responses; (*i*) a canonical ACT1/ TRAF6/ TAK1/ NF-κB pathway regulating the rapid transcription of mainly primary response genes and a (*ii*) complementary JAK1/ PI3-K/ AKT/ GSK3β/ NF-κB-independent signaling cascade which regulates transcriptional co-regulators such as for C/EBP which may regulate secondary response genes.

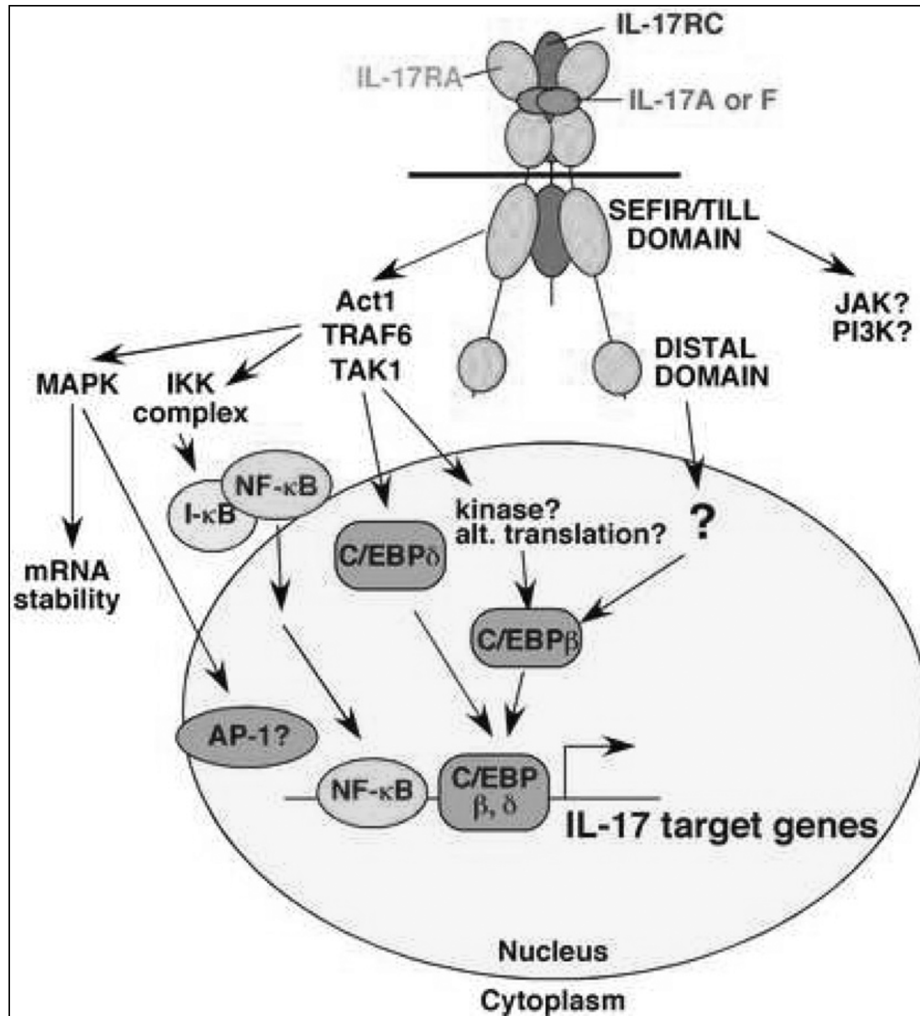

Figure 1. 6. Signal transduction of the IL-17R complex [25]
IL-17A/F triggers signaling mediated via two known motifs in the IL-17RA tail: a SEFIR/TILL domain, which contains elements homologous to TIR domains and a distal domain that activates C/EBPβ. Signal transduction complexes consisting of ACT1, TRAF6 and TAK1 coordinate MAPK, NF-κB and C/EBP activation whereas an NF-κB-independent pathway signals via JAK1, PI3K, GSK3β.

1.5.6. Ubiquitin pathway

Ubiquitination is a post-translational modification which consists of the covalent attachment of one or more ubiquitin monomers to target proteins. In response to IL-17A and IL-17F, polyubiquitination of the IL-17R has been reported in fibroblast-like synoviocytes transfected with HA-tagged ubiquitin. The really interesting new gene (RING) domain at the N-terminal of

TRAF6 functions as an E3 ubiquitin (Ub) ligase which directly mediates lysine (K)-48- and K63-polyubiquitination of the IL-17R. K48-linked polyubiquitin chains are reported to promote 26S proteasome degradation whereas the noncanonical K63-linked polyubiquitin chains function as scaffolding in order to form signaling complexes facilitating protein interactions [117]. Recently, bioinformatic and molecular modeling analysis identified ACT1 as a putative member of the U-box–containing E3 ubiquitin ligase family. The E3 ligase activity of ACT1 was crucial for K63-polyubiquitination of TRAF6 which was observed to mediate the activation of IL-17-induce ERK, JNK, NF-κB and expression of IL-6, CXCL1 and GM-CSF [118]. Notably, TAK1-binding protein (TAB)-2/3 was also reported to bind K63-linked polyubiquitined TRAF6 through a zinc finger domain in order to activate a TAK1-IKK-NF-κB complex as shown in figure1.7 [118, 119]. Therefore, ubiquitin signals mediate signal transduction events of the canonical IL-17R pathway and add another layer of complexity to its regulation.

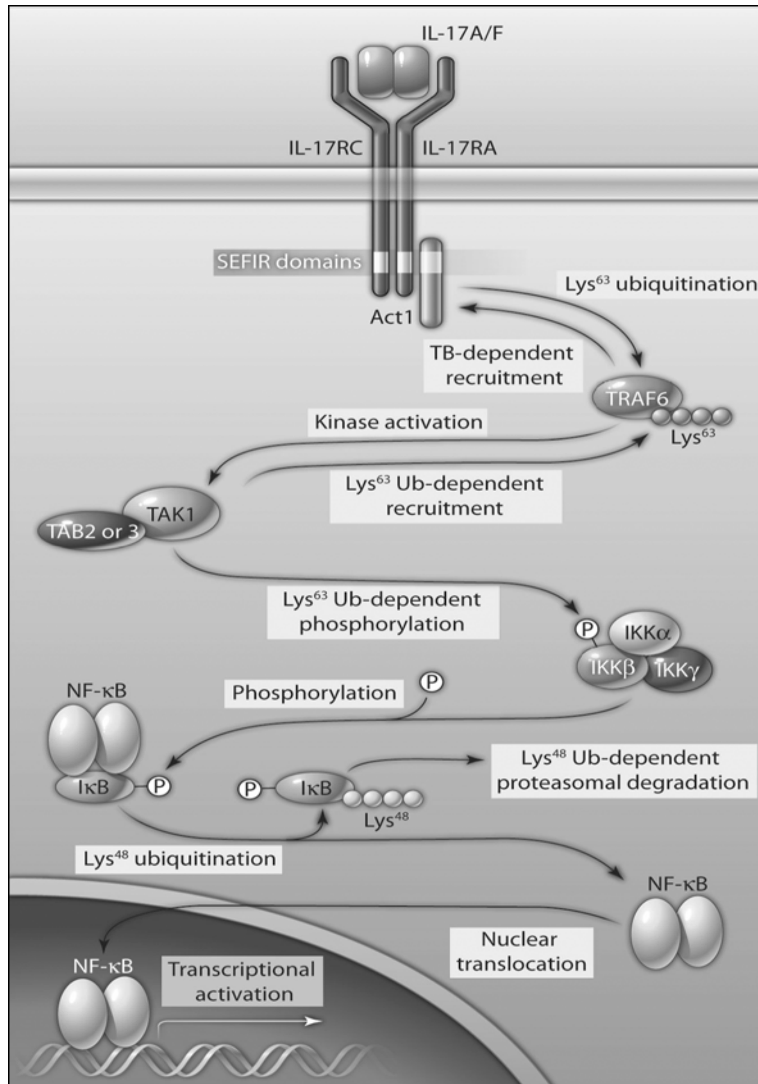

Figure 1. 7. Ubiquitin and the IL-17R signaling pathway [120]
Activation of the IL-17R complex by IL-17A/7F induces the recruitment of ACT1 and the induction of its E3 ubiquitin ligase activity. TRAF6 is recruited to ACT1 via TRAF-binding domains and undergoes K63-mediated ubiquitination. This leads to the K63-ubiquitin-dependent recruitment of TAB2/3 and to the activation of TAK1. TAK1 phosphorylates the IKK complex which then phosphorylates the regulatory IκB proteins. Phosphorylated IκB proteins undergo K48-mediated ubiquitination and become targeted for proteasomal degradation. The dissociation and degradation of the IκB proteins enables the translocation of the NF-κB complex to the nucleus where it directs transcription of a wide range of target genes.

1.6.0. IL-23 AND THE DISCOVERY TH17 CELLS

Identifying cellular sources and upstream factors regulating IL-17 release was key in understanding the biological function and role of IL-17 in protective immunity. The first study to

report that IL-17-producing T cells were distinct from the Th1 and Th2 lineage used TCR-transgenic DO11.10 splenocytes stimulated with the arthritis-lyme disease causing spirochete, *Borrelia Burgdorferi*. Transgenic naive T cells primed in either a Th1-polarizing (ovalbumin (OVA) + IL-12), inflammatory (OVA + IL-6/ IL-18) or infectious condition (OVA + *B.Burgdorferi*-derived lipopeptides) revealed that the latter two conditions contained higher levels of IL-17-producing cells [3]. However, identifying the factors required for the differentiation and expansion of naive cells into Th17 cells would first require the characterization of IL-23. IL-23 is a heterodimeric cytokine consisting of the shared IL-12p40 subunit and the IL-23p19 subunit which was discovered by a sequence database search for IL-6 family homologues [121]. The initial clue that IL-23 promoted Th17 differentiation came from an intracellular cytokine analysis of draining lymph node (DLN) T cells of IL-23p19-deficient mice which contained IFN-γ- but no IL-17-positive cells [122]. Addition of IL-23 to wild-type memory T cells (CD4+ $CD44^{hi}$ $CD62L^{lo}$ $CD45RO^{hi}$) also impaired the growth of Th1 cells and expanded a T cell subset coexpressing IL-17A/F, TNF and IL-6 [123, 124]. Systemic and specific overexpression of IL-23p19 in bone marrow-derived cells resulted in widespread inflammation with a predominant neutrophil response typical of early overexpression studies with IL-17 [125]. Nevertheless, IL-23p19-deficient mice produce IL-17 when stimulated with IL-23, and DLN cells taken from IL-$12p40^{-/-}$ mice which lack both IL-12 and IL-23 can still generate Th1 or Th17 cells when cultured *ex vivo* with either IL-12 or IL-23, respectively. Furthermore, IL-23 cannot differentiate naive $CD4^{+}$ T cells into Th17 cells *in vitro* implying that additional factors mediate the initial commitment to the Th17 lineage. Indeed, although the IL-12Rβ1 subunit is constitutively expressed on naive T cells, the IL-12Rβ2 and IL-23R subunits require T cell activation in order

to upregulate one of the two ligand-specific chains to gain responsiveness to either IL-12 or IL-23, respectively [126].

1.6.1. IL-23 receptor

TLR ligands such as LPS stimulate the production of IL-6 from DCs which renders pathogen-specific T cells refractory to the suppressive activity of $CD4^+$ $CD25^+$ Tregs [127]. Similar studies reported that Tregs cultured in the presence of proinflammatory cytokines increased the production of IL-17 from naive $CD4^+$ T cells [128-130]. TGF-β in combination with IL-6 was reported to mediate the *in vitro* differentiation of naive T cells into IL-17-producing cells and this process was amplified with the addition of IL-1β or TNF [128]. Moreover, IL-1R1-deficient mice were also devoid of antigen-specific Th17 cells [131]. Elucidation into the molecular signaling mechanisms mediated by TGF-β and IL-6 revealed that STAT3 upregulated the expression of the IL-23R [7, 132]. Notably, two independent reports which originally proposed that Th17 cells did not derive from the Th1/ Th2 effector lineage demonstrated that IL-23 could induce IL-17 production if IFN-γ and IL-4 were neutralized [6, 7]. Thus, IL-23 promotes the proliferation of Th17 cells whereas the combination of TGF-β and IL-6 is essential for the initial differentiation of naive cells into the Th17 lineage. Nonetheless, anti-CD25 Treg-depleted $Il6^{-/-}$ mice can still yield Th17 cells indicating that an alternative, IL-6-independent pathway also regulates Th17 differentiation [29]. IL-15 was observed to stimulate IL-17 production from activated PBMCs and purified spleen $CD4^+$ cells however the regulatory mechanism mediating this process has not been elucidated [133, 134]. Hence, the reciprocal and antagonistic actions of TGF-β, IFN-γ and IL-4 on Th17, Th1 and Th2 development provided an early model of divergence between lineage class as seen in figure 1.8 [130].

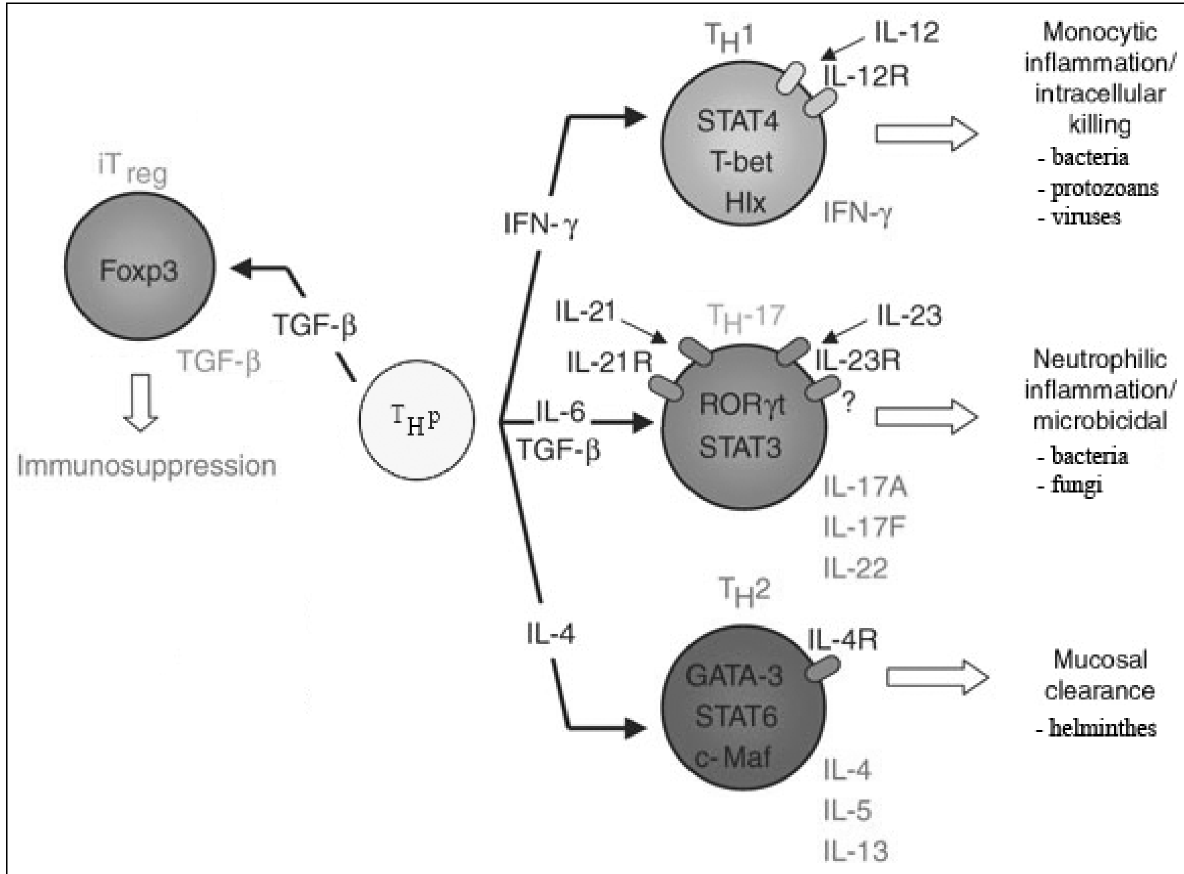

Figure 1. 8. Th17 development [135]
Naive CD4+ T cell precursor cells (Thp) differentiate into four effector subsets (Th1, Th2, Th17 and iTregs). The differentiation program is governed by selective cytokines and transcription factors where each subset accomplishes specialized immune functions.

1.6.3. IL-21 and Th17 amplification

In conditions favouring Th17 cell development [136], IL-21 mRNA levels were observed to be significantly increased and similarly to IL-6, IL-21 was observed to specifically upregulate RORγt and IL-23R expression via STAT3 activation [137]. The combination of IL-21 with TGF-β was also demonstrated to overcome the function of IL-6 as naive *Il6*$^{-/-}$ T cells could differentiate into Th17 cells [29]. As such, the neutralization of IL-21 in Treg-depleted *Il6*$^{-/-}$ mice abrogated the generation of Th17 cells [30] and similarly to IFN-γ/ IL-4-driven Th1/ Th2 cell development, IL-21

was observed to sustain and amplify Th17 cells through an IL-21 autocrine feedback loop [30]. It is also interesting to note that in the absence of TGF-β, IL-1β in combination with IL-6, IL-21 or IL-23 can differentiate naive T cells into Th17 cells [31, 138-140]. This suggests that alternative and/ or complementary mechanisms mediating Th17 lineage commitment have not been fully elucidated.

1.6.2. Key lineage-specific transcription factors

Initially, $CD4^+$ T cells isolated from mice lacking Th1- or Th2-specific transcription factors (STAT1, STAT4, T-bet or STAT6) retained the ability to differentiate into Th17 cells when activated *in vitro* with IL-23 [7]. Th17 cells also lack T-bet, Hlx and GATA-3 expression indicating that a new class of lineage-specific transcription factors mediated the Th17 differentiation program [135]. The initial identification of a Th17-specific transcription factor came from studies investigating the role of the retinoic acid-related orphan nuclear hormone receptor (ROR) family. The ROR-γt isoform was isolated from a cDNA screen for factors that protected T cell hybridomas from TCR-mediated activation-induced cell death (AICD) [141]. RORγt was initially observed to extend the survival of double-positive ($CD4^+CD8^+$) thymocytes during positive selection and was later reported to be critical for the development of intestinal lymphoid tissue inducer (LTi) cells and LTi-like cells [142]. In the latter study, *Rorγt*$^{gfp/+}$ mice which were originally designed in order to identify LTi-like cells uncovered a dim green fluorescence protein (GFP)-positive subpopulation of $CD4^+$ TCR-αβ and -γδ T-cells in the lamina propria. Isolation and stimulation of the GFP-positive LTi-like and $CD4^+$ T cells produced IL-17 whereas mice deficient in RORγt or IL-6 lacked GFP^{dim}/ IL-17-positive cells [14]. The induction of RORγt was

dependent on IL-6/ IL-21-induced STAT3 activation and both transcription factors were observed to cooperatively regulate IL-17 production.

Analysis of the distal and proximal elements of the m*Il17* promoter revealed two conserved noncoding sequences (CNS) sites located -19 and -5kb from the TSS including a runt-related transcription factor (Runx), STAT and ROR response element. STAT3 was demonstrated to directly bind to the *Il17* and *Il21* promoters and conditional STAT3-deficient T cells have impaired IL-17 production [11, 13, 132]. Moreover, the crucial role of STAT3 in mediating RORγt-dependent Th17 development was further substantiated in the hyper-immunoglobulin E syndrome (HIES), a primary immune deficiency which lacks IL-17-positive cells due to missense STAT3 mutations [143, 144]. RORα and RORγt were also reported to be strongly induced by IL-6 or IL-21 in the presence of TGF-β and RORα-deficiency results in decreased IL-17 expression. Coexpression RORα and RORγt synergistically results in greater Th17 differentiation responses and double RORα/ γt-deficient mice abrogate Th17 cell development [145]. In addition to RORγt, IL-1 was also observed to activate the interferon regulatory factor (IRF)-4 which cooperatively enhanced IL-17 production. Notably, *Irf4*$^{-/-}$ T cells produce IFN-γ in Th1 polarizing conditions but fail to generate Th17 cells or produce IL-17 [146]. Finally, Runx1 and RORγt were observed to cooperatively upregulate IL-17 expression and promote Th17 cell differentiation by negatively interacting with Foxp3 [147]. Presently, the RORα/ γt-lineage-specific transcription factors predominately mediate Th17 differentiation commitment and IL-17 gene expression by cooperatively interacting with regulatory transcriptional cofactors. In general, the Th17 differentiation pathway is summarized by three distinct stages; (*i*) IL-6/ IL-21 cooperate with TGF-β to subvert the defaulting TGF-β-Foxp3-Treg pathway to drive the initial

"differentiation" program of naive T cells towards the Th17 lineage. (*ii*) The resulting activation of STAT3 upregulates RORγt which cooperatively upregulate IL-23R and IL-21 expression to "amplify" Th17 proliferation in an autocrine manner. (*iii*) Finally, IL-23 "stabilizes" the Th17 effector phenotype and cytokine expression profile [148].

1.6.4. Negative regulation of Th17

TGF-β is a suppressor of Th1 and Th2 differentiation pathways and drives the conversion of naive T cells into iTregs. Activation of naive T cells by TGF-β in addition to proinflammatory cytokines such as IL-6, IL-21 and IL-22 relieves the inhibition mediated by forkhead/ winged helix transcription factor forkhead box P3 (Foxp3) on RORγt function and promotes Th17 cell development [129, 149]. Regulatory factors such as the vitamin A metabolite retinoic acid (RA_c) however was demonstrated to suppress Th17 differentiation by inhibiting RORγt via the activation of the mammalian homologues of drosophila mothers against decapentaplegic (MAD) and *Caenorhabditis elegans* Sma (SMAD)-3 [150, 151]. Moreover, the dioxin ligand-activated transcription factor aryl hydrocarbon receptor (ArHR) was also observed to regulate iTreg and Th17 cell differentiation in a ligand-dependent manner where 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) promotes iTreg and 6-formylindolo[3,2-b]carbazole (FICZ) promotes Th17 differentiation in mice [152]. Treg- and Th1-associated cytokines IL-2 and IL-27 can respectively impair Th17 development via the activation of STATs. Although RORγt requires STAT3 activation for Th17 development, IL-2-induced STAT5 activation functions to constrain Th17 development as observed in conditional IL-2 and STAT5 Cre-lox knockout mice [33]. Likewise, the IL-27 heterodimer which consists of the epstein-barr virus (EBV)-induced gene 3 (EBI3) and the IL-6/ IL-12 homologue IL-27p28 subunit suppresses Th17 cell development via an undefined

STAT1-dependent process [153]. Thus, unlike the reciprocal differentiation network regulating Th1/ Th2 lineage commitments, the default program induced by TGF-β favours the development of Foxp3-iTregs unless a secondary proinflammatory signal induced by IL-1β, IL-6, IL-21 or IL-22 activates RORγt and the Th17 differentiation program.

1.6.5. Th17 phenotype and lineage-specific markers

Phenotypical characterization of cytokine and chemokine receptors has been instrumental in defining Th subsets and understanding their function. Notably, antigen-specific central memory (Tcm) and effector memory (Tem) cells partake in immune surveillance and mount rapid recall responses to pathogens. $CCR7^+$ Tcm are highly proliferative lymph node–homing cells whereas $CCR7^-$ Tem predominately localize in peripheral, non-lymphoid tissues. Polarized Th1 cells express CXCR3, CCR2 and CCR5 and bind CD62P/E whereas Th2 cells selectively express CCR3, CCR4, CCR8, the prostaglandin D_2 receptor CRTh2 and are selectin ligand negative [154, 155]. Th17 are characterized as Tem ($CD4^+CD45RO^+CCR7^-CCR6^+$) cells which have a low proliferative capacity and selectively coexpress CCR4, CCR6 and CCR2 [156, 157]. CCR4 is a skin-homing marker which binds numerous ligands including CCL2, a shared CCR2 ligand. CCR6 homes to CCL20 expressing cells in skin and mucosa-associated tissues and plays an important role in recruiting Th17 cells to inflamed tissues. CD161, a C-type lectin-like receptor with homology to CD69 was also recently described in circulating and gut-resident Th17 cell populations [158]. Furthermore, the expression of CXCR3 in addition to CCR4 and CCR6 can discriminate between potential Th17 subsets such as the IL-17/ IFN-γ-double positive cells. Interestingly, no IL-4 coexpression has been reported in Th17 cells [31, 159, 160].

1.7.0. CELLULAR SOURCES

IL-17A transcripts were initially described in activated $CD4^+$ and $CD8^+$ $CD45RO^+$ memory T lymphocytes but mounting evidence suggested that cell types other than T cells could produce IL-17 [5, 15, 124, 134, 161, 162]. For instance, mRNA of murine (m)IL-17 was originally detected in a cDNA library generated from αβ-TCR^+ $CD4^-$ $CD8^-$ thymocytes [163] and low levels of IL-17 are detectable in the bronchoalveolar lavage (BAL) fluid of severe combined immunodeficient (SCID) mice [134]. To date, IL-17A mRNA has been detected in blood monocytes, neutrophils [134, 164] and in eosinophils of asthmatic individuals [165] however IL-17 is mainly produced by tissue resident "innate" γδ T (60%), invariant (i)NKT and LTi-like cells (25%) and by circulating CD4/ $CD8^+$ T (15%) cells [23].

1.7.1. γδ T cells

An initial study investigating inflammatory responses induced by i.p. injections of FFL, a Fas ligand (FasL)-expressing erythroleukemia cell line uncovered that SCID mice had decreased IL-17 levels compared to WT mice. Although they were unable to identify the cellular source of IL-17, flow cytometry analysis demonstrated that γδ-TCR ($CD4^-$ $CD8^-$) T cells were the major producers of IL-17 [166]. In a subsequent study, stimulation of resident mesenteric lymph node (MLN) cells revealed the presence of three IL-17-positive cell populations consisting of conventional αβ-TCR $CD4^+$, γδ-TCR ($CD3^{hi}$ $CD4^-$ $CD8^-$ $CD45RB^{lo}$) and NKT-like $V\beta8^+$ ($CD3^{int}$ $CD4^-$ $CD8^-$ $NK1.1^-$) cell types [21]. A subpopulation of αβ- and γδ-TCR-positive cells was also identified in the lamina propria of $Ror\gamma t^{gfp/+}$ mice where 50% of RORγt-GFP cells were γδ-TCR T cells compared to 10% of αβ-TCR T cells [14]. In addition, IL-17-positive γδ T cells were observed to express IL-23R and release IL-21 and IL-22 in response to IL-1β and IL-23 without

TCR engagement [167, 168]. γδ T cells also express TLR1, TLR2 and the β-glucan receptor dectin-1, which enables direct responses to bacterial lipids and fungal polysaccharides [169]. Importantly, not all γδ T cells express IL-17 but two subsets based on the expression of CD122 can discriminate between IL-17-producing CD122^{lo} and IFN-γ-producing CD122^{hi} γδ T cells. Thus, IL-17-producing γδ T cells represent a distinct cell type which consists of a large pool of tissue resident cells rapidly able to respond to cytokines or pathogens with no apparent antigen specificity.

1.7.2. NKT cells

Conventional NK cells respond to IL-12 and secrete IFN-γ whereas mucosal NK cells respond to IL-23 and produce IL-22. Although both cell types do not express IL-17, iNKT cells were reported to express RORγt, IL-23R and produce high amounts of IL-17 in response to *Sphingomonas wittichii* and *Borrelia burgdorferi* derived-glycolipids [170]. iNKT cells lack the NK1.1 cell surface marker but express the nonpolymorphic MHC class I-like molecule CD1d and the invariant, Vα14/ mice or 24Jα18/ human TCR chain. Notably, iNKT cells are predominately located in the lungs as opposed to the liver or spleen suggesting that subsets of NKT cells may reside in specific organs and mediate distinctive immune responses [171].

1.7.3. LTi-like cells

Rag2$^{-/-}$ splenocytes were demonstrated to produce IL-17 in response to IL-23 whereas *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ splenocytes which lack both T and NK cells did not. Although DCs and NK1.1^{+} cells were not observed to express IL-17, RORγt^{+} Lin^{-} CD4^{+} LTi-like cells were positively detected in splenic and intestinal cryptopatch aggregates [172]. LTi and LTi-like cells mediate the development of organized lymphoid structures such as the lymph nodes, Peyer's

patches and other gut-associated lymphoid tissues which do not form in RORγt-deficient mice [173]. LTi-like cells have also recently been proposed to elicit host defense responses via the secretion of IL-17 and IL-22. Interestingly, LTi-like cells share some features with IL-22-producing NK cells and may have analogous developmental pathways since LTi cells can express NK1.1^{+} and differentiate into an NK-like cell [174].

1.8.0. IL-17 AND PROTECTIVE IMMUNITY

The first clue that microbial agents could elicit a Th17 response came from the initial observation that coincubation of ova-specific DO11.10 CD4^{+} T cells with OVA peptides and microbial lipopeptides of *Borrelia burgdorferi* or *Mycobacterium bovis* generated a distinctive IL-17, TNF and GM-CSF cytokine profile [3]. Endotoxin exposure and i.t. administration of IL-17 induced a Th17 response which was characterized by a rapid and strong pulmonary neutrophil response indirectly mediated by CXCL1, CXCL2 and CXCL8 which could be blocked in IL-17-neutralized mice [17, 134, 164, 175]. IL-17-deficient mice had decreased inflammatory cell infiltrates in response to contact hypersensitivity (CHS) reactions and to recall challenges of dinitrofluorobenzene (DNFB) and 2,4,6-trinitro-1-chlorobenzene (TNCB) in the ear epidermis. Interestingly, antigen non-specific cells such as neutrophils have been observed to elicit CHS responses since depletion of granulocytes and CXCL2 prior to secondary challenge inhibits ear-swelling reactions [176, 177]. IL-17 was also observed to mediate delayed-type hypersensitivity (DTH) and AHR responses in allergic mice [178].

However, IL-17's ability to regulate host defence responses in mucosal tissues was not uncovered until an independent research group which was unable to generate viable IL-17

knockout mice, developed IL-17R-deficient mice. In contrast to the viable IL-17$^{-/-}$ mice, IL-17R$^{-/-}$ mice housed in pathogen free facilities developed severe focal alopecia which progressed to sever skin ulcerations. Ulcers in the mucous membranes of the mouth and eyes were colonized by *staphylococcus* species from which the mice ultimately succumbed [179]. This indicated that IL-17R-deficient mice had impaired bacterial host defences. As such, a pulmonary *Klebsiella pneumonia* infection model, which largely affects immunocompromised individuals, was subsequently investigated. IL-17R-deficient mice were observed to be highly susceptible to *K. Pneumonia* infection and succumbed to the infection within 48h. Excessive bacterial growth, perivascular edema and bacterial dissemination in the IL-17R knockout animals were associated with reduced G-CSF and CXCL2 expression in the lungs [18]. Similarly, neutralizing endogenous IL-17 in mice by pretreatment with adenovirus encoding a soluble IL-17R had similar outcomes. In wild-type mice however, IL-17-positive CD4^{+} and CD8^{+} T cells were rapidly detected 6h post-infection and overexpression of IL-17 by adenovirus gene transfer enhanced chemokine production, neutrophil recruitment and survival outcomes [180].

IL-17R-deficient mice infected by *Candida Albicans,* an opportunistic yeast-like fungus, were also observed to succumb to disseminated infection due to impaired neutrophil responses. Overexpression of mIL-17A by adenovirus gene transfer was observed to protect wild-type mice from lethal doses of *C. albicans* by upregulating G-CSF production and the absolute neutrophil cell counts [19]. Likewise, impaired neutrophil migration to infected *Toxoplasma gondii* tissues in IL-17R$^{-/-}$ mice lead to fatal outcomes 3 weeks post-infection [181]. Hence, host defence responses mediated by IL-17 are dependent on generating and recruiting neutrophils for microbial and fungal clearance. In addition, gene expression arrays in human tracheobronchial epithelial (TBE)

cells revealed that in addition to chemokines and growth factors, IL-17 upregulated acute-phase response genes such as hBD2, CCL20 and Lcn2 indicating that IL-17 could directly regulate microbicidal responses [20, 182].

1.8.1. IL-22 and mucosal host defences

The IL-10-related T cell derived inducible factor (IL-TIF) also known as IL-22 was discovered as an IL-9 inducible gene by a subtracted cDNA library screen of the murine BW5147 T cell lymphoma [183]. IL-22 was initially observed to upregulate acute phase proteins such as serum amyloid A (SAA), α1-antichymotrypsin and haptoglobin in hepatocytes and was associated with inflammatory reactions since i.p. injections of LPS increased IL-22 expression [184]. Additionally, IL-22 was observed to mediate wound-healing mechanisms enhancing the clonogenic potential of HBE cells and restoring the barrier integrity as demonstrated by transepithelial electrical resistance (TEER) measurements of injured epithelium [185]. The IL-22R consists of the ubiquitous IL-10R2 chain and the restricted IL-22R1 subunit which is selectively expressed on activated skin keratinocytes and in tissues of the digestive and respiratory tract [186]. Detailed cytokine analysis of activated Th1/ Th2/ Th17 subsets revealed that IL-22 was also abundantly expressed in Th17 cells. IL-23 was also observed to upregulate IL-22 expression in tissue resident NK and IL-17-expressing γδ T cells. Given that Th17 cells coexpressed IL-22 and that the inducible IL-22R is restricted to activated fibroblast and epithelial cells, this suggested that Th17-cytokines could regulate antimicrobial defences in mucosal tissues. Accordingly, IL-17 and IL-22 were observed to cooperatively upregulated host defense genes such as CXCL1, CXCL5, CXCL8, CXCL9, IL-6, IL-19, hBD2, Lcn2, G-CSF and S100A7/ A8/ A9/ A12 [12, 185, 187]. Neutralization of IL-22 prior to *K. pneumoniae* infection decreased IL-6, Lcn2 and CCL3 levels which resulted in

bacterial dissemination that was exacerbated by the absence of IL-17A. Although IL-17 was required for the elimination of extracellular and fungal pathogens such as *K. Pneumoniae, P. Aeruginosa, M. Pulmonis* and *C. Albicans*, its expression was dispensable for the clearance of Th1-dependent intracellular pathogens such as *M. Tuberculosis* and *L. Monocytogenes* [156, 188]. Therefore, IL-17 responds in association with IL-22 (figure 1.9) to mobilize rapid and protective host defence responses in mucosal tissues against selective extracellular and fungal pathogens via neutrophil-activating factors (CXC-chemokines and G-CSF), antimicrobial peptides (defensins, mucins and S100 proteins) and acute phase proteins (IL-6, Lcn2, C-reactive protein (CRP) and SAA) [189].

Module	Polarizing cytokine(s)	Transcription factor	Homing receptor(s)	Effector cytokine(s)	Target cell	Function
T_H1	IL-12, IFN	T-bet	CXCR3	IFN-γ	Macrophages	**Bacteria**
T_H2	IL-4	GATA-3	CCR4/CRTh2	IL-4, IL-5, IL-13	Eosinophils	**Parasites**
T_H17	IL-6,IL-1β,TGF-β	ROR-γt	CCR6 / CCR4	IL-17, IL-22	Neutrophils	**Fungi**
$_i$Treg	TGF-β	FOXP3	CCR7 / CCR6	TGF-β	DC / T cells	**Regulation**

T_H17 Effector molecule	Function
IL-17A, F	Induces local cytokine and chemokine production, resulting in neutrophil infiltration
IL-21	Autocrine feedback loop for TH17 development. Has important and diverse effects on B, T, NK and myeloid cells
IL-22	Keratinocyte proliferation and expression of antimicrobial peptides of the S100 family
GM-CSF	Myeloid progenitor stimulation, phagocyte activation
TNFα	Pleiotropic proinflammatory cytokine
IL-6	B-cell activation, TH17 differentiation, acute phase response, other pleiotropic activities
Integrin α3	Cell adhesion
CXCL1	Neutrophil recruitment
CCL7	Mixed leukocyte recruitment
CCL17	T cell and basophil recruitment
CCL20	B- and T cell recruitment
CCL22	T cell and basophil recruitment
PDGF-β	Blood vessel growth and repair

Figure 1. 9. Effector Th17 cell characteristics [190, 191]
Th subsets involved in immune protection and regulation and the Th17 effector phenotype.

1.8.2. IL-17 in health and diseased states

Tissue resident γδ T, NKT and effector memory Th17 cells play an important role in the early stages of inflammation by inducing strong chemotactic and antimicrobial responses [9]. However, dysregulated IL-17 responses can also lead to proinflammatory and autoimmune disorders. Circumstantial evidence suggests that dysregulated subsets, or effector phenotypes of Th17 cells

may be pathogenic and have been observed to coexpress IL-17/ IL-21 in psoriasis [192], IL-17/ TNF in rheumatoid arthritis [193], IL-17/ IFN-γ in multiple sclerosis [194] and IL-17/ IL-23 in inflammatory bowel disease [195]. The pathogenic effects of Th17-associated effector cytokines have recently been outlined in an extensive review focused on Th17-associated diseases [196]. Neutralizing antibody strategies targeting IL-17A for the treatment of rheumatoid arthritis are currently in phase I clinical trials and have reported favourable outcomes. Beneficial effects in reducing afflicted psoriasis skin areas have been reported in phase II trials when neutralizing either IL-17A or the IL-12/IL-23p40 subunit [34, 196, 197]. Thus far, investigation into Th17-associated cytokines and effector functions have uncovered key mechanisms mediating protective host defences and generated novel therapeutic targets to alleviate inflammatory disorders.

1.8.3. Neutrophil homeostasis and innate immune responses

Distinctively characterized by a segmented nucleus containing two to five lobes, polymorphonuclear neutrophils are the most abundant white blood cell type and serve as a critical line of defense against invading pathogens. Neutrophils share a common hematopoietic origin with monocytes, eosinophils, basophils, thrombocytes and erythrocytes and derive from a pluripotent colony forming unit-granulocyte, erythrocyte, monocyte, macrophage (CFU-GEMM) cell [198]. In response to IL-3, GM-CSF and G-CSF, these pluripotent cells progressively differentiate into CFU-GM, CFU-G and myeloblasts (earliest recognizable cell of the granulocyte lineage). These cells continue to develop into promyelocytes (presence of primary granules), myelocytes (presence of specific granules), metamyelocytes (commencement of nuclear segmentation, terminally differentiated cell type) and finally mature for 6d in the

hematopoietic cords into banded and segmented neutrophils (neutrophil storage pool is also known as the BM reserve) [199]. Although growth factors and cytokines can trigger granulopoiesis in response to bleeding, hypoxia or infection, the BM reserve can immediately immobilize the maturing neutrophil pool to counteract distress signals (clinically referred to as the left shift). Remarkably, the BM generates approximately $1x10^9$ granulocytes/ kg/ day second only to the production of RBC and platelet estimated at $2.5x10^9$ cells/ kg/ day under normal homeostatic circumstances [200].

Egress from the BM is dependent on the CXCL12/ CXCR4 chemokine signaling axis. CXCL12 (SDF-1α) is constitutively expressed at high levels by reticular cells surrounding the sinusoids which act as endogenous retention signal for neutrophils. Downregulation of CXCL12 by G-CSF or the presence of CXCR2-specific chemokines such as CXCL1 or CXCL2 in the blood stream overcomes the retentive CXCL12 signal and triggers the mobilization of neutrophils by endothelial transcellular migration [201, 202]. With an approximate half-life of 6h, circulating neutrophils downregulate CXCR2 and are cleared from the circulation by homing back to $CXCL12^+$ tissue such as the spleen, liver and BM [203]. In response to local infection or injury however, neutrophils are recruited to inflamed tissues by endothelial postcapillary venules which upregulate a series of adhesion and chemokine molecules to mediate the tethering, slow-rolling, firm-adhesion and diapedesis of neutrophils across the endothelium [204, 205]. Neutrophils can directly respond to pathogens via a series of cell surface pattern recognition receptors such as lectin, scavenger, complement and TLRs which recognize invariant and conserved microbial macromolecules. Opsonisation and phagocytosis of microorganisms triggers a respiratory burst response which generates reactive oxygen intermediates (ROIs) via the NADPH oxidase

complex. These include superoxide (O_2^-), singlet oxygen ($^1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), hypohalous acids (HOX) and chloramines (NH_2Cl, R_2NCl), of which the latter destroy pathogens in phagosomes [206].

Alternatively, extravasated neutrophils which do not encounter or capture pathogens within a 15-45m period degranulate and release a cocktail of antimicrobial and proteolytic proteins in the extracellular environment [207]. Since granule proteins are presynthesized during the neutrophil differentiation process, neutrophil responses are rapid and independent of *de novo* protein synthesis. Peroxidase-negative secondary (specific) and tertiary (gelatinase) granules containing lactoferrin, lipocalin, lysozyme and LL37 (cathelicidin) also known as the "gang of L's" and MMP8, MMP9 and MMP25 known as the "gang of M's" are initially released. Lactoferrin and the siderophore neutralizing compound lipocalin are bacteriostatic compounds that sequester free iron from bacteria. MMPs function to degrade ECM proteins such as laminin, collagen, fibronectin and proteoglycans to form viscous and protease rich pus to physically trap pathogens and loosen tissue cohesiveness in order to ease leukocyte chemotaxis [208]. Neutrophils subsequently release peroxidase-positive primary (azurophilic) granules which contain defensins, serprocidins serine proteases (cathepsin G, neutrophil elastase and protease-3) and the hypohalous acid converting enzyme MPO [208, 209]. Importantly, tissue breakdown provokes crucial danger signals which further function to recruit inflammatory cells and trigger tissue repair mechanisms [210]. Finally, neutrophils can also release azurophilic protease-embedded nuclear chromatin to form neutrophil extracellular traps (NETs) in a final attempt to immobilize and stop the spread of microorganisms [211, 212].

1.8.4. Airway neutrophil responses

Although neutrophil are traditionally regarded as a phagocytic cell type which engulf and degrade microorganisms, activated neutrophils can synthesize chemokines and cytokines to recruit and regulate the inflammatory response of macrophages and lymphocytes. In the airways, neutrophils contain a variety of prestored, proinflammatory mediators which can negatively affect lung and bronchial epithelial function (Table 1.3.). Proteases and defensins can infringe the integrity of the epithelial layer by degrading a variety of extracellular matrix proteins including elastin, collagen (type I–IV), fibronectin, laminin and proteoglycans. Moreover, neutrophil proteases and lipid mediators can provoke bronchoconstriction, mucus hypersecretion and decrease the frequency of the ciliary beat [213, 214]. In turn, these deleterious effects induce the activation and recruitment of more inflammatory cells and induce the synthesis of epithelium-derived mediators which may impede the resolution of inflammation or tissue repair mechanisms.

In order to avoid an accumulation of inflammatory cells into the local airways, cell recruitment mechanisms must be balanced by effective means of cell clearance in order to avoid potentially harmful damage to tissues. Transepithelial migration is a physiological mechanism by which tissue granulocytes migrate into the lumen of the airways and are terminally removed by mucociliary clearance [215]. Alternatively, spontaneous apoptosis and subsequent engulfment by macrophages also provide an anti-inflammatory mechanism for the removal of these cells. Apoptosis has two major functions in the resolution of inflammation; incapacitate effector functions by blocking vital cellular components and secondly, to target the clearance of effector cells by exposing phagocytosis markers such as phosphatidylserine. Early repercussions of

apoptosis in neutrophils involve shutdown of calcium influx responses, down-regulation of proinflammatory genes and permeabilization of energized mitochondria by Bcl-2 family members which disables chemotaxis, respiratory burst and degranulation responses [216]. The subsequent removal of these cells by macrophages is equally as important because it prevents damage to healthy tissues that would otherwise occur by necrosis cell lysis [217].

Proteases	matrix metalloproteinases (MMP-9, collagenase) serine proteinases (elastase, cathepsin G, proteinase 3)
Reactive oxygen species	myeloperoxidase (MPO), NADPH oxidase hydrogen peroxide, hydroxyl radical, superoxide anion
Non-enzymatic peptides	α-defensins (NHP 1-4)
Lipid mediators	eicosanoids (leukotriene B4)
Proinflammatory cytokines & chemokines	TNF, IL-1β & IL-6, IL-8

Table 1. 3. Neutrophil granule contents in the pathogenesis of lung injury

1.8.5. Lung and conducting airway structure

The respiratory system is composed of two interdependent conducting and respiratory structures. The conducting portion consists of an upper respiratory tract which primarily functions to warm and moisten inhaled air via highly vascularised nasal and oral cavities, rich in mucous and serous glands. The ciliated respiratory epithelium of the upper airways also functions to clear trapped particulate matter by circulating mucus produced by goblet cells in a cephalad motion [218]. The lower airways consist of the tracheobronchial tree, which begins at the trachea and branches off into primary (main), secondary (lobar) and tertiary (segmental) bronchi and into smaller terminal and respiratory bronchioles. A cross-sectional view of the bronchi reveals a ciliated columnar epithelial cell layer anchored to a permeable basement membrane. Beneath the epithelial cell

layer is the lamina propria, a highly vascularised layer of loose connective/ supportive tissue rich in fibroelastic fibres. Underlying the lamina propria are bands of smooth muscle which course through the conducting airways wrapped around the airway lumen. The smooth muscle layer gets progressively thicker in the lower airways and delineates the submucosa which largely contains seromucinous glands and goblet cells. As the bronchi diminish in diameter, the epithelial cell layer become flattened and is devoid of cilia, the lamina propria decreases in thickness and is completely encircled by smooth muscle. The lack of adventitial hyaline cartilage in the bronchioles is what permits constriction of the airways to the point of closure. Neuroendocrine cells which regulate airway and vascular smooth muscle tone are also present in the bronchioles.

Smooth muscle bundles are composed of elongated, spindle-shaped cells which are bound together in irregular branching fasciculi. In contrast to skeletal muscle fibres, smooth muscle fibres are generally shorter and contain a single nucleus which is centrally located. Moreover, the contractile proteins are not arranged in myofibrils as in skeletal and cardiac muscle and do not appear striated. Agonist of ASM contraction include histamine, ACh, LTD_4, bradykinin (BK) and neurokinin A (NKA) which act on G-linked coupled receptors of the G_q family to activate PLC which induces IP_3-dependent Ca influx responses. Relaxation or bronchodilation is mediated by β-adrenoceptor agonists such as isoproterenol which activate the stimulatory G_s subunit of G-coupled proteins to activate cAMP and downstream kinase PKA to sequester and inhibit the influx of Ca [219].

1.8.6. Asthma pathogenesis and the role of ASM cells

In contrast to pulmonary vascular smooth muscle which dynamically regulates blood flow via contraction/ relaxation responses, the physiological function of ASM is uniquely linked to pathological conditions of excessive airway narrowing [220]. "Like a boa constrictor squeezing its prey, smooth muscle bundles narrow the airway lumen when they contract" [221]. As such, ASM modulate key pathogenic characteristics of asthma such as (reversible) airflow obstruction, bronchial hyperresponsiveness and airway inflammation [222]. Inhaled corticosteroids, a mainstay treatment for asthma, has also revealed a causal link between AHR and airway inflammation suggesting that ASM may play a key role in modulating inflammatory responses [223]. ASM are "biological factories" which release several growth factors, proinflammatory cytokines and chemokines [224]. Notably, neutrophils [225, 226], eosinophils [227], mast cells [228] and $CD4^+$ T cells [229, 230] were observed to adhere to ASM cells and the adhesion of $CD4^+$ T cells enhanced ACh-mediated bronchoconstriction and impaired isoproterenol-mediated bronchodilation responses [231]. TLR agonists such as LPS and polyI:C (dsRNA) were also observed to enhance bradykinin-induced contractions [232] whereas inflammatory cytokines such as TNF, IL-1β, IFN-γ and IL-5 were observed to upregulate cell adhesion molecules such as ICAM-1, VCAM-1 and CD44, enhance bronchoconstriction and impair bronchodilation responses [219]. Additionally, ECM proteins have been observed to alter the phenotype and responsive state of ASM [233]. Monomeric type I collagen and fibronectin increases ASM cell mitogenesis in response to PDGF whereas laminin promotes a mature, non-proliferative contractile phenotype [234, 235]. Hence, inflammation modulated by paracrine/ autocrine cytokine secretion, cellular adhesion molecules, ECM deposition and microbial antigens can modulate ASM responsiveness to contractile/ dilation agonists, exacerbate airway reactivity and narrowing responses.

A structural basis for the clinical symptoms of AHR may also be attributed to the remodeling of the airways [236]. Long term structural and functional changes to the respiratory epithelium, vasculature, mucus glands and smooth muscle contribute to a progressive loss of lung function [237]. A defining feature identified in longitudinal studies of asthmatic airways is an abnormal and large cross-sectional area of smooth muscle [238]. The predominant mechanism modulating the increase in muscle mass is unclear but has been postulated to arise from dysregulated cell proliferation (hyperplasia) [239], reduction in cell turnover (apoptosis) [240], increase in cell size/ contractility (cellular hypertrophy) or via the recruitment and differentiation of mesenchymal cell into the smooth muscle bundles. In support for the latter proposition, myofibroblasts which share an intermediate fibroblast-ASM cell phenotype ($CD34^+$, collagen I^+, α-SMA^+ lacking filamentous staining patterns [241]) could augment smooth muscle mass and/ or contractility [242, 243]. ECM deposition and the degradation of elastin could also alter the rigidity and distensibility of the airway wall and decrease lung compliance [233]. Hence, increased smooth muscle surface and/ or force, thickening of the airway wall internal to the smooth muscle bundle and reduced lung compliance may promote excessive airway narrowing responses and pathophysiological AHR.

1.9.0. Summary points

- IL-17A is the prototypical member of the IL-17 family which includes IL-17A-F and is homologous to the ORF13 of herpes virus saimiri.
- IL-17A/ F homo- and heterodimers are primarily released from tissue resident, γδ T, iNKT, LTi-like and Tem $CD4^+$/ $CD8^+$ cells.

- The IL-17RA is ubiquitously expressed and forms a multimeric receptor complex with IL-17RC to induce similar gene expression patterns as those of LPS/ TLR4 and IL-1β/ IL-1R via two distinct, (ACT1/ TAK1/ TRAF6) NF-κB-dependent and (STAT1/ PI3-K/ AKT/ GSK3β) NF-κB-independent signaling pathways.
- IL-17 is a “cytokine inducing cytokine” which promotes proinflammatory neutrophil-mediated responses via chemokines, growth factors, antimicrobial peptides and acute-phase response proteins.
- IL-17 synergizes with TNF, IL-1, IL-6 and IL-22 by both transcriptional and post-transcriptional mechanisms to enhance proinflammatory responses.
- Monocytic Th1 (INF-γ, IL-12, IL-18) responses targets intracellular pathogens such as bacteria, protozoans and viruses whereas humoral Th2 responses (IL-4, IL-5, IL-13) eliminate parasitic helminth infections via mucosal clearance defences. Microbicidal Th17 (IL-17A/F, IL-22, IL-6) neutrophil-mediated responses clear fungi and extracellular bacteria from mucosal/ serosal tissues.
- TGF-β (or IL-1β) with IL-6/ IL-21 activates STAT3 and RORγt which upregulate IL-21 and the IL-23R expression in naive T cell to promote Th17 differentiation and overcome the iTreg differentiation program induced by TGF-β.
- The IL-23/ IL-17/ G-CSF signaling axis is mediated by tissue resident DCs and macrophages which regulate neutrophil homeostasis via a negative feed-back loop dependent on neutrophil chemotaxis and cell turnover.
- Neutrophils are rapidly recruited to the lungs to clear infection but negatively affect airway function by releasing proteases, ROIs and proinflammatory mediators which affect matrix proteins and activate structural resident airway cells.

- Asthma is characterized by AHR, or excessive airway narrowing responses to nonspecific irritants. Underlying inflammatory and remodeling responses ultimately contribute to the development of AHR.
- ASM cells posses both contractile and immunomodulatory properties. Although ASM regulate bronchiole airway calibre and thus limit air flow, it is unclear whether direct functional links exist between ASM and AHR.

1.10.0. THESIS OVERVIEW

1.10.1. Study Rational

A key feature of pulmonary disease is the activation and accumulation of granulocytes in the airways. Of note, persistence of neutrophilic exudates in the airways of asthmatics patients is associated with the progression of severity and is the most notable difference between chronic moderate and severe asthma [244]. The removal of these cells is an essential process in the normal resolution of inflammation as it prevents damage to otherwise healthy tissues. Elevated levels of IL-17, a proinflammatory cytokine and neutrophil recruiting mediator have been associated with several acute and chronic inflammatory disorders such as bacterial infections, organ allograft rejection, intestinal bowel disease, rheumatoid arthritis and asthma. The ability of IL-17 to initiate inflammatory responses and recruit neutrophils has been underlined by a series of comprehensive reports; however, whether IL-17 can modulate smooth muscle function and/ or directly elicit neutrophil activity to exacerbate inflammatory responses in the airways has not been addressed.

1.10.2. General/ Bridging Hypothesis

IL-17 is a proinflammatory mediator that initiates and amplifies mucosal immune responses by acting on local structural cells in synergy with other inflammatory cytokines to produce a selective gene expression signature encompassing CXC-chemokines and myeloid growth factors which intensifies neutrophil recruitment and exacerbates inflammatory responses.

1.10.3. Bridging theme

IL-17 is a proinflammatory cytokine that bridges the adaptive (Th17) and innate (neutrophil) immune response via the release of chemokines and myeloid growth factors from structural cells (smooth muscle). The effects of IL-17 are potentiated when in the presence of accompanying inflammatory cytokines (IL-1β) and acts directly on both structural and effector cells. Herein, this thesis seeks to interlink 3 aspects of the IL-17/ IL-17RA response as outlined in figure5.1; *(chapter 1)* determine target genes upregulated by IL-17 in structural cells, *(chapter 2)* uncover the molecular mechanism mediating the synergistic response of IL-17 and IL-1β on CXCL8 production and *(chapter 3)* identify functional responses of IL-17 on recruited effecter cells.

2.0. CHAPTER 2

INTERLEUKIN-17 RECEPTOR SIGNAL TRANSDUCTION AND GENE EXPRESSION PROFILE IN HUMAN AIRWAY SMOOTH MUSCLE CELLS

This collaborative work is presented as a manuscript in preparation.

Stéphane Dragon[1], Andrew Halayko[2], Stuart J. Hirst[3], Tak H. Lee[4] and Abdelilah Soussi Gounni[1]

[1]Department of Immunology and the [2]Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada. [3]Department of Physiology, Monash University, Melbourne, Victoria, Australia. [4]Division of Asthma, Allergy and Lung Biology, King's College London, Medical Research Council & Asthma UK Centre in Allergic Mechanisms of Asthma, London, United Kingdom.

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SD drafted and produced 100% of the experiments required for the realization of this manuscript.

2.1.0. Abstract

Recent studies into the pathogenesis of airway disorders such as asthma have revealed a dynamic role for airway smooth muscle (ASM) cells in the perpetuation of airway inflammation via the secretion of cytokines and chemokines. In this study, we evaluated the IL-17RA signal transduction and gene expression profile in primary human ASM cells from mild asthmatic and healthy individuals. Non-transformed, low passage primary human ASM cells were treated with IL-17 [10ng/ml] for 2 hours and were probed by the Affymetrix GeneChip array. IL-17A specific gene targets were thereafter validated by real-time qRT-PCR and confirmed by synergistic responses to IL-1β [10ng/ml] and IL-22 [20ng/ml] at 2 and 6 hours. IL-17 rapidly activated the ERK-RSK signaling pathway and p70S6K, a PI3-K downstream target. Our genetic analysis also supports the proinflammatory nature of IL-17 as multiple NF-κB regulatory factors and chemokines were markedly upregulated. Of note, IL-17 mediated a modest fold increase in gene expression levels whereby only 4 genes achieved greater than 2 fold increases. Transcriptional regulators consisting of primary response genes were over-represented and displayed dynamic expression profiles. IL-22 also upregulated IL-17 gene targets and additively increased expression levels when in combination with IL-17, albeit at lower levels than IL-1β. Finally, ASM cells from mild asthmatic individuals upregulated more genes with greater overall variability in response to IL-17 than from healthy ASM cells. Taken together, our results suggest that IL-17 is a potentiating cytokine which mediates additive or synergistic responses to proinflammatory cytokines, and that inducible primary response genes in cooperation with the NF-κB signaling network regulates the IL-17 gene expression profile in ASM cells.

2.2.0. Introduction

Interleukin (IL)-17 plays an important and protective role in host defence by promoting the expansion, mobilization and activation of neutrophils in response to extracellular pathogens [1]. Effector functions of IL-17 include granulopoiesis, neutrophil recruitment via CXC-chemokines and the production of antimicrobial proteins for the clearance of pathogens [2]. T helper (Th)17-associated cytokine IL-22 is also indispensable for the control of bacterial infections as it acts on the epithelium in synergy with IL-17 to upregulate crucial host defense genes [3]. However, dysregulated IL-17 and Th17 responses can also promote chronic inflammatory and autoimmune disorders such as psoriasis, rheumatoid arthritis and multiple sclerosis [4]. In the airways, increased levels of IL-17A and the presence of neutrophils are associated with chronic inflammatory disorders such as cystic fibrosis, chronic obstructive pulmonary disease and asthma [5].

IL-17A has been demonstrated to orchestrate local airway inflammation by cooperating with and inducing proinflammatory cytokines such as tumor necrosis factor-α (TNF), IL-1β, IL-6, granulocyte colony-stimulating factor (G-CSF) and CXC-chemokines in epithelial, fibroblast, vascular endothelial and smooth muscle cells [6]. Alveolar macrophages which were previously demonstrated to mediate acute pulmonary inflammation by releasing IL-1β in asthmatic airways were also recently identified as a central source of IL-17A in the lung [7, 8]. Furthermore, inducible natural killer (iNKT) cells which coexpress IL-17 and IL-22 were observed to mediate ozone-induced airway hyperresponsiveness [9]. Sputum levels of IL-17A correlate with the severity of airway hypersensitivity [5] and IL-17A immuno-reactive cells have been observed within smooth muscle bundles and in the airway lumen of moderate-to-severe asthmatic patients [10]. Despite

regulating the bronchomotor tone, airway smooth muscle (ASM) cells modulate airway inflammation by secreting cytokines, growth factors, matrix proteins and express cell adhesion and co-stimulatory molecules [11]. Activated $CD4^{+}$ $CD25^{+}$ $CD134^{+}$ T cells have been demonstrated to directly interact with myocytes via CD44, CD54 and CD106 and to promote ASM hyperplasia during the infiltration of the airway wall in an experimental model of allergic asthma [12-14]. Hence, effector functions mediated by the IL-17R on ASM cells may reveal new mechanisms which may contribute or exacerbate airway inflammation.

The signal transduction of the IL-17RA is mediated by the upstream adaptor protein, NF-κB activator-1 (ACT1) which is recruited to the cytoplasmic tail of the receptor via the similar expression to fibroblast growth factor receptor and IL-17R (SEFIR) domain. ACT1 interacts with the TNF-receptor associated factor-6 (TRAF6) and activates the TGF-beta activated kinase-1 (TAK1) via the TAK1-binding protein-2/3 (TAB2/3). This kinase complex activates the mitogen-activated protein kinases (MAPK) pathway and the IκB kinase (IKK) enzyme complex to upregulate a subset of proinflammatory genes under the regulatory control of NF-κB [15]. Notably, the IL-17R signal transduction pathway also mediates synergistic responses to TNF, Toll-like receptors (TLR) ligands, as well as to IL-1β and IL-22 via a largely undefined, post-transcriptional mechanism regulating mRNA stability [16, 17]. In our study, we aimed to further characterize the IL-17RA signal transduction pathway by identifying downstream MAPKs, regulatory NF-κB kinases and the gene expression profile induced by IL-17A in ASM cells.

Upon stimulation with IL-17, we observed the rapid phosphorylation of the extracellular signal-regulated kinases-ribosomal S6 kinase (ERK-RSK) and of the 70-kDa ribosomal protein S6

kinase (p70S6K). While no significant activation markers of the NF-κB network were detected, IL-17 significantly upregulated several NF-κB-dependent genes which are involved in the negative feedback of NF-κB. Additionally, 20% (10/ 48 genes) of all significantly upregulated genes at the 2 hour time point were transcriptional regulators consisting mainly of primary response (PR) genes. IL-22 also upregulated IL-17 gene targets and similarly to IL-1β, combined in an additive manner to increase gene expression. Lastly, IL-17 activated a distinctive gene subset in ASM cells from mild asthmatic individuals including proinflammatory genes such as *Il6* and *Cxcl10*. Taken together, our observations support the potentiating function of IL-17 to mediate additive or synergistic responses with IL-1β and IL-22 and that NF-κB regulatory factors in cooperation with inducible PR genes may modulate the IL-17 gene expression profile in ASM cells.

2.3.0. MATERIALS AND METHODS

2.3.1. Bronchial human ASM cells

In accordance with procedures approved by the Research Ethics Committees of King's College Hospital and the Human Research Ethics Board of the University of Manitoba, bronchial human ASM cells were obtained by deep endobronchial biopsy from the right middle or lower lobe bronchi of three healthy (methacholine PC_{20} >16mg/ml, FEV_1 104±1%, mean age 31±8yr, 2 males, 1 female) and three glucocorticoid-naive mild atopic asthmatics (methacholine PC_{20} 0.52±0.59mg/ml, FEV_1 84±6%, mean age 31±3yr, 3 males) volunteers (table2.2). Smooth muscle bundles were visualized with a dissecting microscope and isolated from surrounding tissue using fine needles. Cells were grown by explant culture from airway smooth muscle bundle fragments in 12cm^2 flasks using methods described previously [18, 19]. Fluorescent

immuno-cytochemistry confirmed that near-confluent, fetal bovine serum (FBS)-deprived cells stained for smooth muscle-specific α-actin, desmin and calponin (>95%). Human ASM cells were grown at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin (GibcoBRL, Grand Island, NY). In all experiments, primary human ASM cells were used between passages 3 and 5. Recombinant human IL-1β was purchased from PeproTech Inc. (Rocky Hill, NJ), IL-17A and IL-22 was purchased from R&D Systems (Minneapolis, MN) and unless stated otherwise, all other reagents were obtained from Sigma Chemical Co. (Oakville, Ontario).

2.3.2. Cell culture and Western blot

Confluent human ASM cells were growth-arrested by FBS-deprivation for 48h in DMEM media containing 5µg/ml of human recombinant insulin, 5µg/ml of human transferrin, 5ng/ml of selenium (ITS) and antibiotics. Cells were then stimulated in fresh FBS-free media with or without recombinant human IL-17 [10ng/ml], IL-1β [10ng/ml] or IL-22 [10ng/ml]. Cell extracts were collected by the Thermo Fisher Scientific (Rockford, IL) mammalian protein extraction reagent (M-PER) supplemented with the Roche Applied Bioscience (Laval, QC) complete protease inhibitor cocktail. Western blotting was performed following standard laboratory procedures. Anti-human phosphorylated and total protein antibodies against p38, ERK, SAPK/ JNK, pIKKα/β, IkBα, IkBβ, IkBε, p65, p50 and secondary HRP-conjugated antibodies were purchased from Cell Signaling (Danvers, MA). Antibodies against IL-17R, ACT-1, TRAF1 and TAK1 were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA) and HRP-conjugated GAPDH was obtained from Abcam Inc. (Cambridge, MA). Human proteome profiler phospho-MAPK antibody array kit was purchased from R&D Systems (Minneapolis, MN).

2.3.3. Gene expression array

IL-17A-induced transcripts were collected 2h post stimulation from primary human ASM cells of 3 healthy normal and 3 mild asthmatic donors. In brief, confluent human ASM cells were growth arrested by FBS-deprivation 48h prior to stimulation with fresh FBS-free DMEM media with or without recombinant human IL-17 [10ng/ml]. Total RNA was then carefully extracted by the Qiagen RNeasy method and RNA purity and integrity was evaluated by electrophoretic trace with the Agilent 2100 Bioanalyzer (Mississauga, Ontario). Technical and platform specific procedures for the genome-wide, DNA-based whole transcript (WT) sense target (ST) Affymetrix HuGene v1.0 GeneChip were contracted to the University of Health Network Microarray Centre (UHNMC) Affymetrix Service (Toronto, Ontario). Quality control metrics were evaluated by the Affymetrix Expression Console software v1.1.1 and expression data was normalized by the Robust Multichip Analysis (RMA) algorithm. Statistical analysis, data visualization and gene annotation searches were performed with the Partek Genomics Suite v6.3 (St. Louis, MO), MultiExperiment Viewer v4.4.1 (Boston, MA), Bioconductor v2.3 and Ingenuity Pathways Analysis v7.6 (Redwood City, CA) software. Functional annotation and gene classification analysis were performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) v2008 web-based program as listed on the Gene Ontology website [20].

2.3.4. Quantitative real-time RT-PCR analysis

Confluent human ASM cells were growth arrested for 48h by FBS-deprivation and were stimulated for 2 and 6h in fresh FBS-free media containing either IL-17 [10ng/ml], IL-1β [10ng/ml], IL-22 [10ng/ml] or without cytokines. Cells were then harvested and total RNA was

purified using the Qiagen RNeasy kit (Mississauga, Ontario). Relative levels of mRNA were assessed with the ABI 7500 Real-Time PCR System (Foster City, CA). Product specificity was determined by melting curve analysis and calculation of the relative amount of each cDNA species was determined by the delta-delta cycle threshold (ddCt) method. The amplification of target genes in stimulated cells was normalized to the respective GAPDH levels and results are presented as fold increases over unstimulated controls. Primers were purchased from TIB Molbiol (Adelphia, NJ) and are listed in suppl. Table 2.5.

2.4.0. RESULTS

2.4.1. IL-17 activates the ERK-RSK pathway

To determine which kinases mediate the signal transduction pathway of the IL-17R, adaptor proteins, MAPKs and downstream targets were probed by Western blotting methods. Confluent FBS-deprived primary human ASM cells from healthy donors were stimulated with IL-17 [10ng/ml] for up to an hour and cell extracts were collected. Total protein levels of IL-17R adaptor proteins were also quantified from 2 independent donors and showed little variation with exception to TAK1 which migrated at a slightly higher molecular weight after IL-1β stimulation (figure2.1a). A phospho-MAPK antibody array and conventional Western blots demonstrated robust and sustained ERK phosphorylation levels similar to those seen with IL-1β [10ng/ml] stimulation. Although lower p38 phosphorylation levels were observed compared with IL-1β, no detectable change was found in the activation of stress-activated mitogen-activated protein kinases c-Jun NH2-terminal kinase (SAPK/JNK) phosphorylation. RSK1, a downstream target of ERK was rapidly phosphorylated within 15 minutes in contrast to the mitogen and stress-activated protein kinase (MSK), a p38-ERK downstream homologue (figure2.1b). Furthermore,

protein kinase B (AKT/ PKB), p70S6K and GSK3β (Ser-9) were rapidly phosphorylated within 15 minutes whereas the 27-kDa heat shock protein (HSP27) phosphorylation levels decreased below baseline suggesting this mediator, like GSK3β, may play a regulatory role in the signal transduction of the IL-17R (figure2.1c).

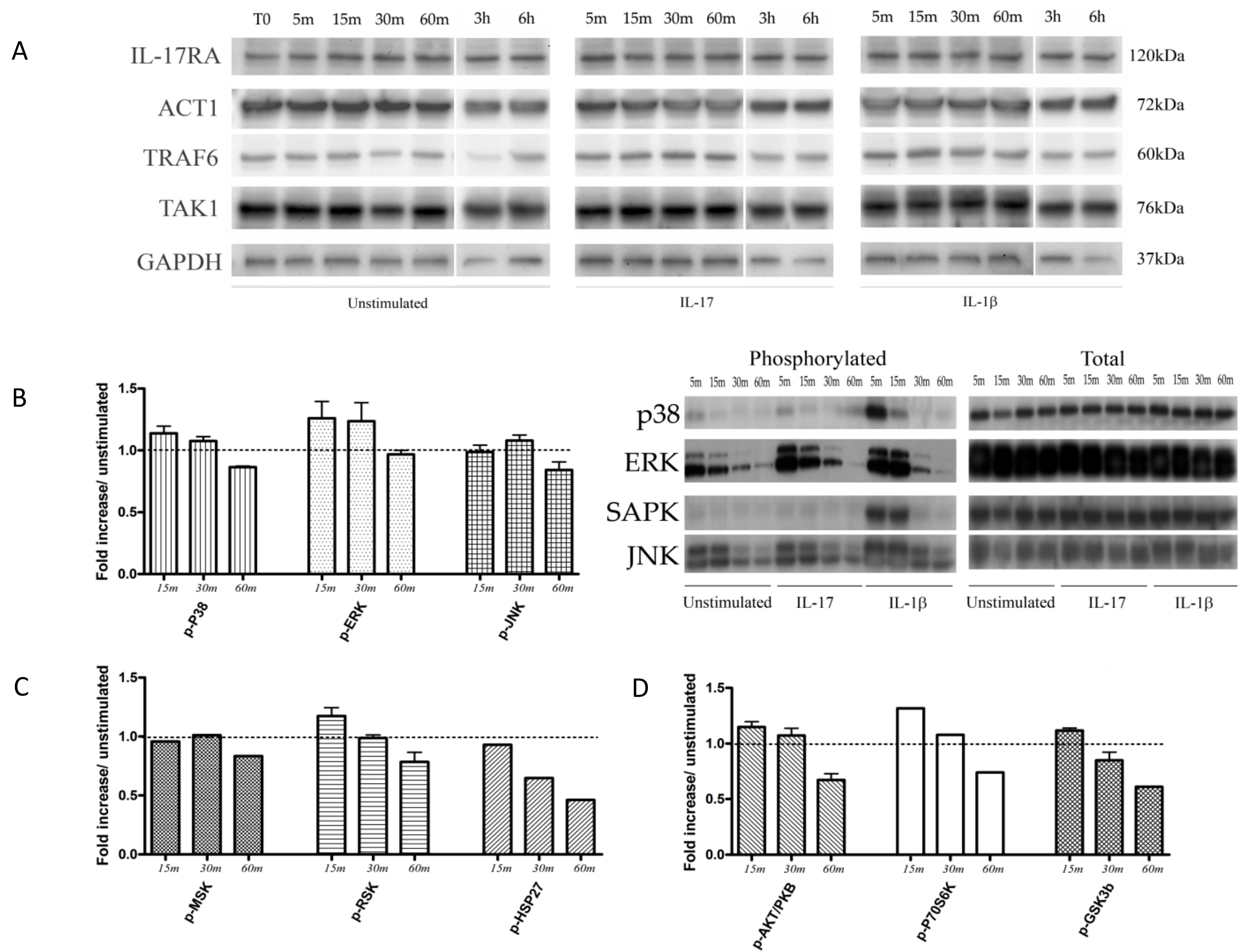


Figure 2. 1. IL-17RA signal transduction mediates MAPK activation
FBS-deprived human ASM cells were stimulated with IL-17 or IL-1β [10ng/ml] for various time points. (A) Cytoplasmic protein levels of known IL-17RA interacting proteins were assessed over a 6h period after IL-17 and IL-1β stimulation. (B) Profiling antibody array (left) and validation by Western blot (right) was used to quantify activated IL-17 target kinases at 15, 30 and 60min post stimulation and corresponds to fold increases of MAPK phosphorylation levels compared to unstimulated controls (mean±SEM, n=2). (C) Downstream kinase targets of p38 and ERK and (D) kinases of associated signal transduction pathways.

2.4.2. IL-17R activates NF-κB regulatory factors

To evaluate IL-17A-specific gene transcripts, primary human ASM cells pooled from 3 healthy and 3 mild asthmatic donors were treated for 2h with IL-17 [10ng/ml] and probed with the Affymetrix GeneChip array. The 2h time point was carefully chosen to identify primary response (PR) gene targets and avoid confounding autocrine mechanisms mediating indirect, or late-phase gene expression responses. Results from 6 patients were pooled and statistical analysis identified 99 gene targets which were significantly up/ down-regulated ($p \leq 0.05$, fold≥ or ≤1.2, figure2.2a) and included several NF-κB regulatory factors, transcriptional regulators and chemokines listed in table 2.1. Of note, IL-17 mediated a modest fold increase in gene expression whereby 4 genes achieved approximately greater than 2 fold increases over unstimulated controls (figure2.2b). Gene ontology analysis revealed that the majority of the upregulated genes largely localised in the cytoplasm and nuclear regions and were categorized as response to stimulus and immune system processes. Transcriptional regulators represented the greatest fraction of gene function whereas chemotaxis and inflammatory response genes represented the greatest fold induction categories (figure2.2.c).

	p Value	Fold	Entrez ID	Name	Description	Location	Type
1	0.000	4.48	64332	***NFKBIZ**	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Nucleus	Transcription regulator
2	0.000	1.98	80149	***ZC3H12A**	zinc finger CCCH-type containing 12A	Unknown	Other
3	0.000	2.08	4792	**NFKBIA**	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Cytoplasm	Other
4	0.001	1.40	5971	**RELB**	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	Nucleus	Transcription regulator
5	0.003	1.55	330	**BIRC3**	baculoviral IAP repeat-containing 3	Cytoplasm	Enzyme
6	0.003	2.55	2919	**CXCL1**	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Extracellular Space	Cytokine
7	0.005	1.21	85463	***ZC3H12C**	zinc finger CCCH-type containing 12C	Unknown	Other
8	0.005	1.72	7128	***TNFAIP3**	tumor necrosis factor, alpha-induced protein 3	Nucleus	Other
9	0.006	1.55	1326	**MAP3K8**	mitogen-activated protein kinase kinase kinase 8	Cytoplasm	Kinase
10	0.006	1.41	2920	**CXCL2**	chemokine (C-X-C motif) ligand 2	Extracellular Space	Cytokine
11	0.006	1.25	54897	**CASZ1**	castor zinc finger 1	Nucleus	Enzyme
12	0.008	1.35	8870	**IER3**	immediate early response 3	Cytoplasm	Other
13	0.011	1.22	79165	**LENG1**	leukocyte receptor cluster (LRC) member 1	Unknown	Other
14	0.012	1.27	6648	**SOD2**	superoxide dismutase 2, mitochondrial	Cytoplasm	Enzyme
15	0.012	1.26	6236	**RRAD**	Ras-related associated with diabetes	Cytoplasm	Enzyme
16	0.012	1.24	4791	**NFKB2**	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	Nucleus	Transcription regulator
17	0.015	1.33	3398	**ID2**	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	Nucleus	Transcription regulator
18	0.024	1.21	83940	**TATDN1**	TatD DNase domain containing 1	Unknown	Other
19	0.029	1.24	874	**CBR3**	carbonyl reductase 3	Cytoplasm	Enzyme
20	0.030	1.22	6348	**CCL3**	chemokine (C-C motif) ligand 3	Extracellular Space	Cytokine
21	0.032	1.27	3383	**ICAM1**	intercellular adhesion molecule 1	Plasma Membrane	Transcription regulator
22	0.035	1.22	146850	**PIK3R6**	phosphoinositide-3-kinase, regulatory subunit 6	Cytoplasm	Other
23	0.039	1.20	4616	**GADD45B**	growth arrest and DNA-damage-inducible, beta	Cytoplasm	Other
24	0.046	1.22	8747	**ADAM21**	ADAM metallopeptidase domain 21	Plasma Membrane	Peptidase
25	0.049	1.23	7597	**ZBTB25**	zinc finger and BTB domain containing 25	Nucleus	Transcription regulator

Table 2. 1. IL-17R target gene list sorted on probability values

Upregulated genes by IL-17 compared to unstimulated controls ($p \leq 0.05$ & $F \geq 1.2$). Gene list excludes any genes not categorized by the NCBI RefSeq accession format as a mature transcript product (prefix NM). * Denotes genes validated by qRT-PCR.

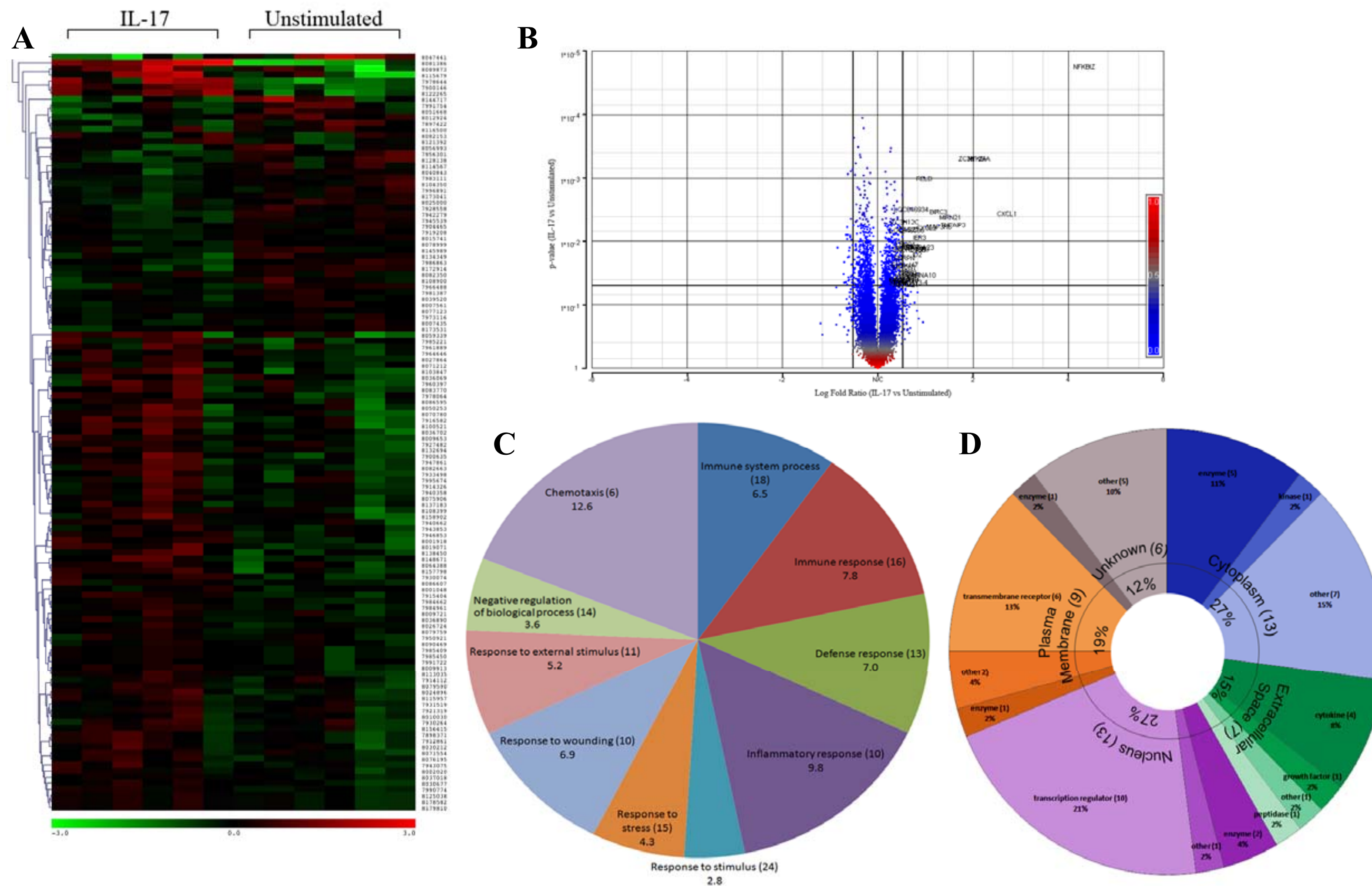


Figure 2. 2. IL-17 gene targets

(A) Heat map of significantly up/down-regulated genes 2h post IL-17 stimulation in primary human ASM cells from 6 independent donors (p≤0.05, fold≥, ≤1.2). (B) Volcano plot demonstrating the distribution of the $\log_2$ fold gene expression vs probability values of IL-17 stimulated cells over unstimulated conditions (p≤0.05, fold≥1.15). (C) Gene ontology pie chart of IL-17-induced biological processes in human ASM cells based on the fold enrichment of each category over unstimulated control conditions (number of genes grouped in each category is indicated in parenthesis). (D) Pie chart illustrating the location and function of 48 genes significantly upregulated by IL-17 (p≤0.05 & F≥1.15). Genes are categorized by location (interior) and are further subgrouped in a color gradient based on function (exterior) as annotated by IPA. The number of genes included in each category is indicated in parenthesis and its representative percentage is indicated below.

2.4.3. IL-17R gene targets are upregulated in the presence of IL-17, IL-1β and IL-22

To validate the results from the GeneChip array, 9 genes mediating various cellular functions including previously reported IL-17 gene targets were selected for qRT-PCR analysis. IL-1β and Th17 cytokine IL-22 were included in the assay to assess whether IL-17 could mediate any specificity or synergy between these two cytokines. Our results demonstrate that after 2h, analysed transcripts were all upregulated by IL-17 and in accordance with our array data, *Nfkbiz* yielded the greatest fold increase (5.3fold±3.1). Of note, additive rather than synergistic increases between IL-17 and IL-1β were observed, conceivably due to the optimal concentrations used and due to the short time frame which may not account for mRNA stability mechanisms [21, 22]. IL-22 had a similar gene expression profile as IL-17 but induced lower levels of *Nfkbiz* (figure2.3). As observed for IL-1β, IL-22 in combination with IL-17 additively increased overall transcript expression and substantially increased *Cxcl8* (*Il8*) levels.

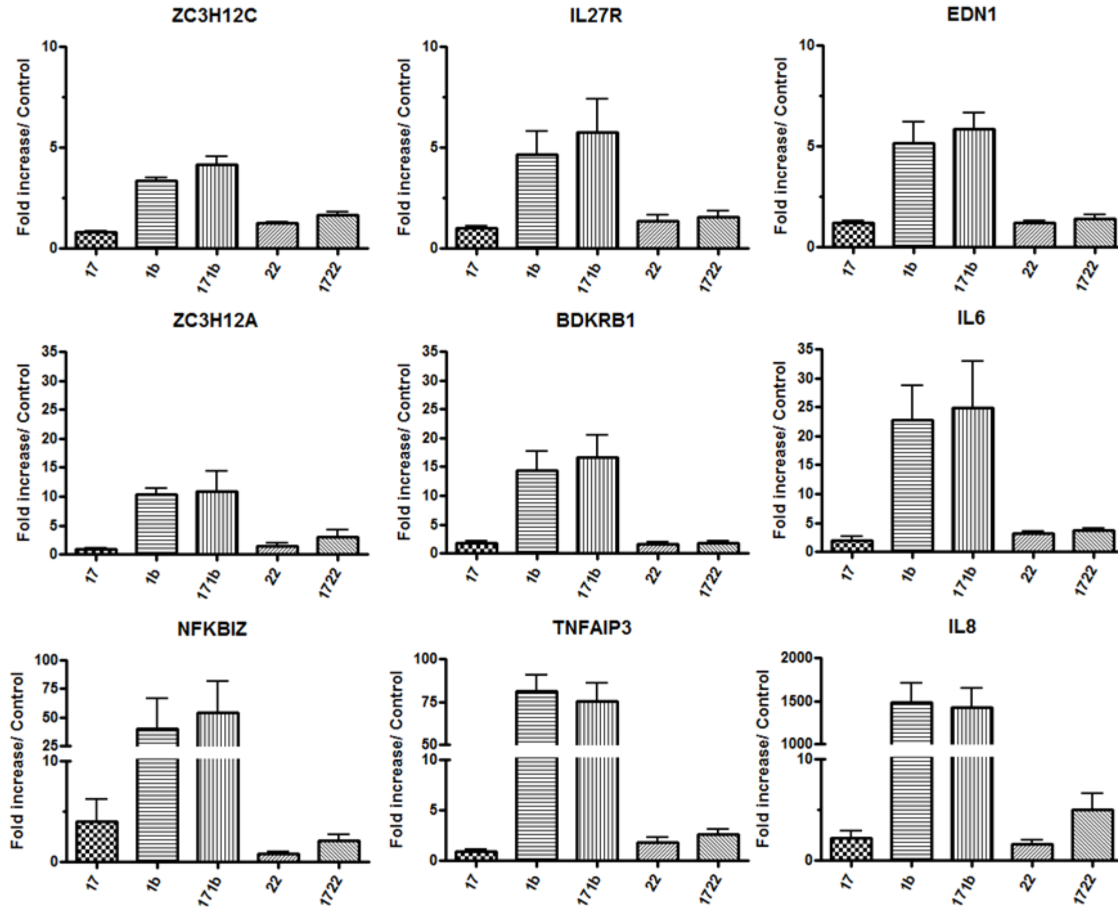


Figure 2. 3. Validation IL-17RA targets by qRT-PCR
Response levels from 9 genes selected from the GeneChip array analysis were confirmed in independent experiments by qRT-PCR. Synergistic responses between IL-17 and IL-1β or IL-22 [10ng/ml] were equally assessed. Results were normalized to the respective GAPDH levels and are presented as the mean fold increase of 6 independent donors over unstimulated controls at 2h post cytokine stimulation.

2.4.4. IL-17 induces a dynamic gene expression response

To assess the dynamic gene expression profile induced by IL-17, we re-assayed transcripts levels 6h after stimulation. As previously reported for activating NF-κB ligands such as TNF, lipopolysaccharide (LPS) and IL-1β, we observed distinct temporal trends where transcripts either had a sustained or up/ down-regulated expression profile. This effect was most notable in IL-1β stimulated conditions where *Zc3h12a* and *Tnfaip3* (A20) had the greatest change in expression between 2 and 6h (3.1±0.4 and 0.5±0.2 fold respectively; figure2.4). Accordingly,

upregulated levels of *Bdkrb1*, *Tnfaip3* and *Nfkbiz* decreased with time whereas *Edn1* and *Il27r* levels were sustained and *Zc3h12c*, *Zc3h12a*, *Il6* and *Cxcl8* levels increased. These results also corroborate with previous reports on NF-κB temporal gene expression patterns where inducible PR genes were suggested to confer specificity to subsequent rounds of transcription [23] [24]. Interestingly, *Zc3h12a* expression levels were trivial in response to IL-22 whereas *Nfkbiz* levels were considerably upregulated at 6h compared to the 2h time point.

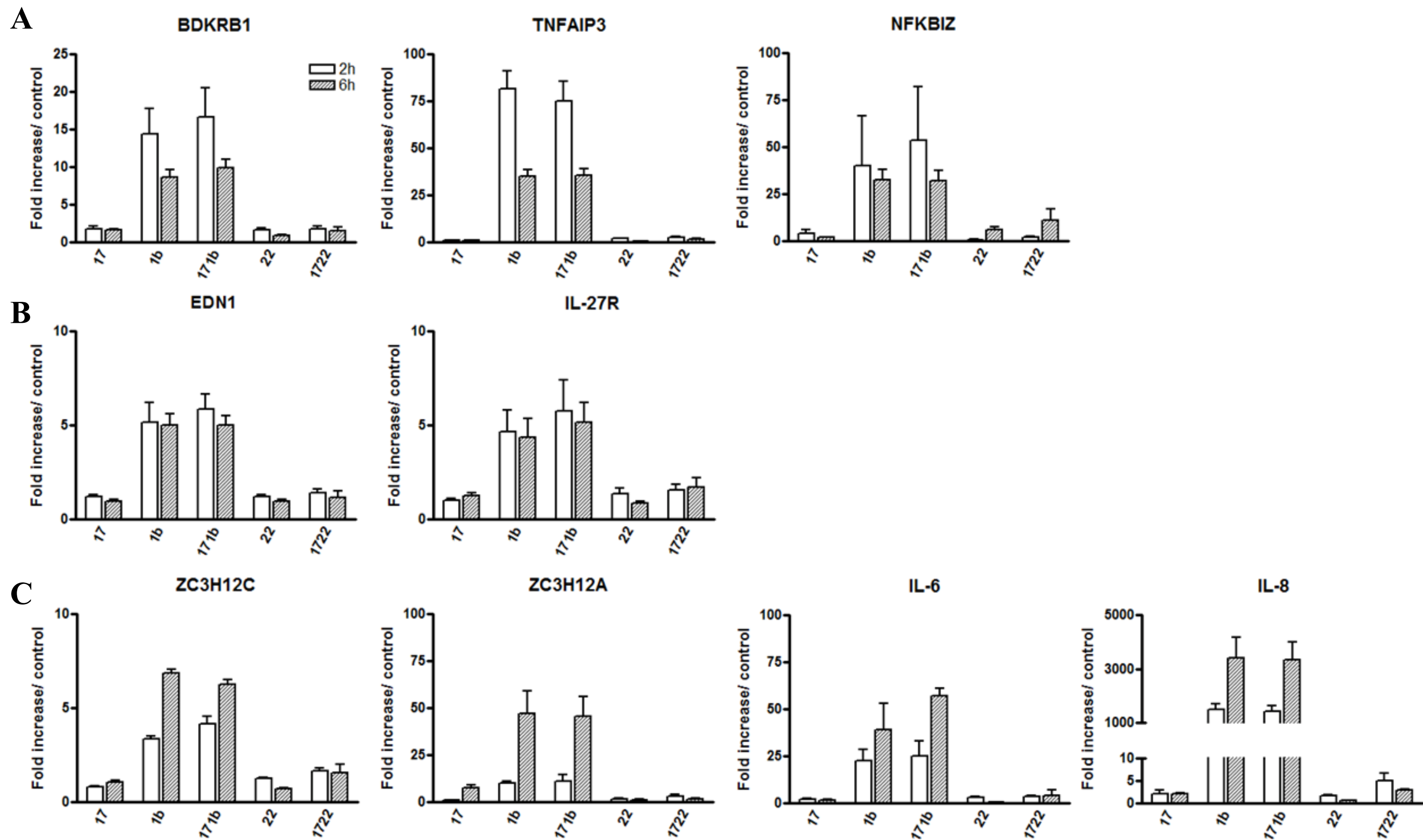


Figure 2. 4. Dynamic IL-17-induced gene expression profile
Comparison analysis of qRT-PCR responses taken at 2h and 6h demonstrated 3 trends. (A) Overall gene expression decreased from the 2h time point. (B) No change in gene expression was observed over 6h. (C) Overall gene expression increased with time. Results were normalized to the respective GAPDH levels and are presented as the mean fold increase of 6 independent donors over unstimulated control cells at 2 and 6h post cytokine stimulation.

2.4.5. IL-17 is a poor activator of the NF-κB regulatory network

Based on our gene array results and previous reports implicating the canonical NF-κB pathway in the IL-17R signal transduction, we investigated which IκB proteins and kinases regulating NF-κB were activated. Although no observable change in total or phosphorylated NF-κB-associated protein levels were detected in response to IL-17 (figure2.5), 16 NF-κB-dependent genes were upregulated by IL-17 which included regulatory members of the IκB family and *Tnfaip3* (Suppl table2.4). In response to IL-1β however, IκBα was rapidly phosphorylated, degraded and re-expressed within 1h and appeared to oscillate as phosphorylation, which precedes degradation could still be detected at the 1, 3 and 6h time points. IκBε, which is responsible for the delayed removal of nuclear NF-κB was also upregulated at the 3h time point whereas protein levels of IκBβ were not altered. Our results suggest that the IL-17R weakly activates NF-κB regulatory factors and sensitive detection methods such as DNA-binding or reporter gene assays may be better suited to detect IL-17-induced NF-κB activity signals.

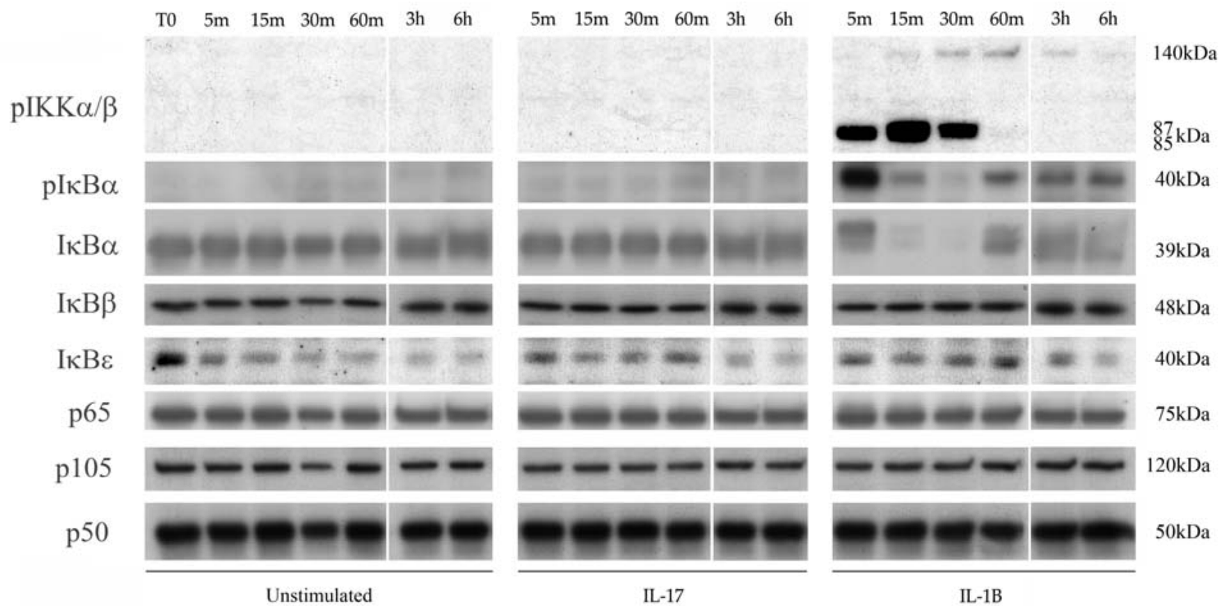


Figure 2. 5. Activation of the regulatory NF-κB network

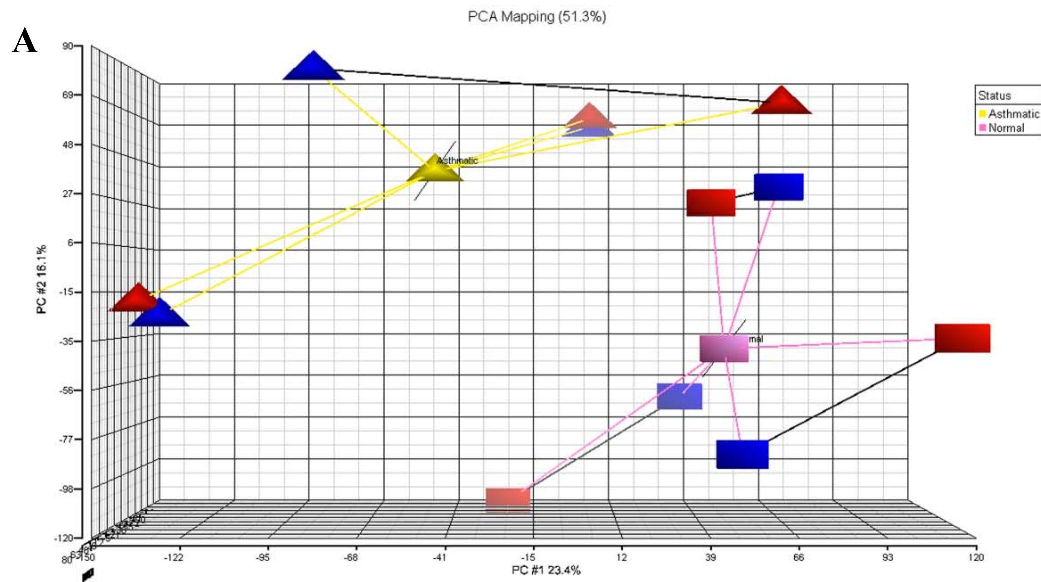
Activation state of cytosolic NF-κB regulatory factors for 6h following stimulation with either IL-17 or IL-1β [10ng/ml].

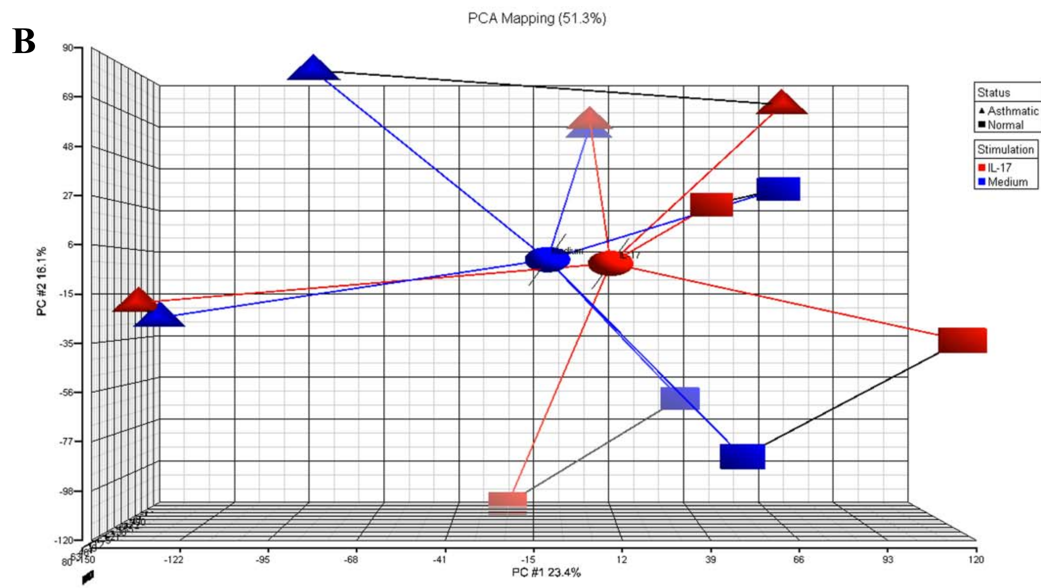
2.4.6. IL-17 differentially activates specific gene targets in ASM cells of asthmatic individuals

To investigate whether IL-17 activated a different gene expression profile in ASM cells of asthmatic individuals, data from patients with a history of mild asthma was stratified from our gene array results. Principal component analysis demonstrated that the mean response to stimulation, based on the gene expression profile from healthy and mild asthmatic individuals was moderately altered but that the status of each group could be clearly differentiated (figure2.6). 24 overlapping genes between the pooled and asthmatic group were largely associated with proinflammatory processes and are partly listed in table2.3. Furthermore, asthmatic ASM cells produced 1.7 times more significantly upregulated genes (162 vs 96 genes) with greater overall variability than healthy ASM cells (1.3±0.3 vs 1.2±0.2 fold). Of note, *Nfkbiz* and *Pttg1* were the only commonly listed transcripts between both groups and *Nfkbiz* had the greatest fold increase over unstimulated controls in both the asthmatic (4.5fold) and healthy (3.2fold) groups. A table of upregulated genes sorted on probability values from healthy ASM cells is also listed in supplementary table2.6.

#	Sex	Age	Smoker	Status	FEV1	FEV1% Pred	PC20	Therapy	Atopy	Allergen
1	M	40	ES	Healthy	5.03	131	>16	- -	N	
2	M	24	NS	Healthy	3.95	82	>16	- -	N	
3	F	31	NS	Healthy	3.69	105	>16	- -	N	
4	M	31	NS	Asthmatic	3.85	85	0.41	Sal	N	
5	M	30	NS	Asthmatic	4.46	89	1.34	Sal	Y	Cat
6	M	33	NS	Asthmatic	3.35	78	0.25	Sal	Y	Cat, HDM

Table 2. 2. Donor health information
Human bronchial biopsies were abstracted from 6 patients between the ages of 25-40. FEV1; Forced expiratory volume in 1 second, FEV1% Pred; percentage of predicted, PC20; provocative concentration causing a 20% fall in the FEV1, ES; ex-smoker, NS; non-smoker, Sal; Salbutamol, HDM; House dust mite.


A
PCA Mapping (51.3%)
Status
Asthmatic
Normal
PC #2 16.1%
PC #1 23.4%


B
PCA Mapping (51.3%)
Status
Asthmatic
Normal
Stimulation
IL-17
Medium
PC #2 16.1%
PC #1 23.4%

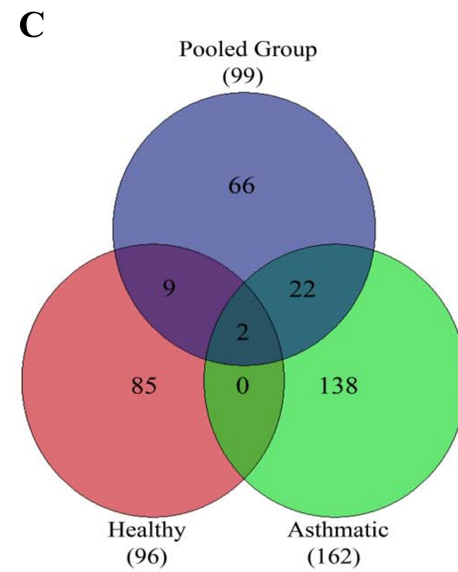

C
Pooled Group
(99)
66
9
22
2
85
0
138
Healthy
(96)
Asthmatic
(162)

Figure 2. 6. Differential gene expression profile of IL-17 in healthy and asthmatic ASM cells.
Principal component analysis (PCA) of IL-17-medaited gene expression responses in ASM cells of healthy and mild asthmatic individuals. (A) The mean stimulus response is characterized by ovals and (B) the mean status response for healthy and asthmatic individuals is indicated in the respective purple and yellow rectangles. (C) Comparative analysis of significantly upregulated genes from the pooled group versus the healthy and asthmatic groups ($p \leq 0.05$, fold≥1.15)

	p Value	Fold	Entrez ID	Name	Description	Location	Type
1	0.001	1.52	1326	**MAP3K8**	mitogen-activated protein kinase kinase kinase 8	Cytoplasm	Kinase
2	0.003	1.22	26873	**OPLAH**	5-oxoprolinase (ATP-hydrolysing)	Unknown	Enzyme
3	0.005	1.31	392376	**OR13C2**	olfactory receptor, family 13, subfamily C, member 2	Unknown	Other
4	0.006	1.54	8870	**IER3**	immediate early response 3	Cytoplasm	Other
5	0.007	1.20	55319	**C4ORF43**	chromosome 4 open reading frame 43	Unknown	Other
6	0.007	1.24	337879	**KRTAP8-1**	keratin associated protein 8-1	Unknown	Other
7	0.008	1.78	80149	***ZC3H12A**	zinc finger CCCH-type containing 12A	Unknown	Other
8	0.008	1.25	85376	**RIMBP3**	RIMS binding protein 3	Unknown	Other
9	0.009	1.42	127069	**OR2T10**	olfactory receptor, family 2, subfamily T, member 10	Unknown	Other
10	0.009	1.24	338328	**GPIHBP1**	glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1	Unknown	Other
11	0.009	1.20	343069	**HNRNPCL1**	heterogeneous nuclear ribonucleoprotein C-like 1	Nucleus	Other
12	0.010	4.52	64332	***NFKBIZ**	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Nucleus	Transcription regulator
13	0.010	1.23	9232	**PTTG1**	pituitary tumor-transforming 1	Nucleus	Transcription regulator
14	0.011	1.35	623	***BDKRB1**	bradykinin receptor B1	Plasma Membrane	G-protein coupled receptor
15	0.012	2.51	2919	**CXCL1**	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Extracellular Space	Cytokine
16	0.014	1.32	2162	**F13A1**	coagulation factor XIII, A1 polypeptide	Extracellular Space	Enzyme
17	0.015	1.34	3569	***IL6**	interleukin 6 (interferon, beta 2)	Extracellular Space	Cytokine
18	0.016	1.26	4057	**LTF**	lactotransferrin	Extracellular Space	Peptidase
19	0.017	1.40	3627	**CXCL10**	chemokine (C-X-C motif) ligand 10	Extracellular Space	Cytokine
20	0.017	1.22	4033	**LRMP**	lymphoid-restricted membrane protein	Cytoplasm	Other
21	0.018	1.33	7100	**TLR5**	toll-like receptor 5	Plasma Membrane	Transcription regulator
22	0.019	1.96	4792	***NFKBIA**	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Cytoplasm	Other
23	0.019	1.89	2920	**CXCL2**	chemokine (C-X-C motif) ligand 2	Extracellular Space	Cytokine
24	0.020	1.40	390637	**LOC390637**	chromosome 15 open reading frame 58	Unknown	Other
25	0.023	1.28	388531	**RGS9BP**	regulator of G protein signaling 9 binding protein	Unknown	Other

Table 2. 3. IL-17R target gene list of mild asthmatic ASM cells sorted on probability values.
Upregulated genes by IL-17 compared to unstimulated controls ($p \leq 0.05$ & F≥1.2). Gene list excludes any genes not categorized by the NCBI RefSeq accession format as a mature transcript product (prefix NM). * Denotes genes validated by qRT-PCR, underlined genes are in common with the pooled group.

2.5.0. Discussion

Activation of p38, ERK and JNK by IL-17 in stromal cells has been previously demonstrated by immunoblotting and functional inhibitor assays [16]. In ASM cells, p38 and ERK are both rapidly activated within 5 minutes but express different activation profiles [25]. p38 phosphorylation is transient and reported to mediate mRNA stabilization mechanisms [22, 26] whereas ERK phosphorylation is sustained and mediates the activation of NF-κB and CCAAT/enhancer binding protein β (C/EBPβ) [27]. Activated ERK was also previously observed in the nucleus when combining IL-17 with TNF which directly, or via downstream kinases such as RSK, regulated transcription factors, co-activators and nucleosomal proteins [28, 29]. RSK may also mediate chromatin remodeling by phosphorylating histone (H)3 on serine (S)10 to recruit histone acetyltransferase complexes and activate transcription of immediate-early genes [30]. Likewise, p38-mediated phosphorylation of MSK1/2 has been reported to enhance the accessibility of selective NF-κB promoters by phosphorylating and/or phospho-acetylating histone H3 [31]. Since RSK- and MSK1- cAMP response element binding protein (CREB) pathways were previously demonstrated to mediate IL-17F signal transduction in normal human bronchial epithelial (HBE) cells [32, 33], we sought to extend these finding in ASM cells. Our observations support a key function for ERK-RSK whereas the low phosphorylation levels of downstream p38 targets suggests this pathway may not play a key role in the immediate signal transduction of the IL-17R. Moreover, p70S6K which is rapidly phosphorylated also shares downstream targets with RSK and promotes the assembly of the translation preinitiation complex [34]. Interestingly, activation of AGC kinases such as AKT/ PKB and p70S6K also suggests that phosphoinositide-dependent protein kinase 1 (PDK1), an upstream and common kinase of p70S6K and RSK may regulate the IL-17R signal transduction pathway. In contrast, GSK3β appears to negatively

regulate IL-17 signaling since it is rapidly phospho-inactivated on S9 prior to returning to baseline levels after 30-60 minutes. As previously reported, over-expression of a constitutive active mutant of GSK3β (S9A) in IL-17-treated HBE cells decreased human beta-defensin-2 (hBD2) promoter activity [35]. Mechanistically, GSK3β is demonstrated to phosphorylate and inhibit the transcriptional activity of C/EBPβ 60 minutes after IL-17 treatment in stromal ST2 cells which results in reduced C/EBP promoter-dependent *Il6* and *Lcn2* expression [36].

The recruitment of NF-κB to target promoters has been demonstrated to occur in two distinct waves. Constitutively bound factors and nucleosome-free regions mediate the rapid induction of PR genes whereas a late (≥4h), nucleosomal modifying and/ or protein synthesis-dependent mechanism mediates the transcription of NF-κB-dependent SR genes [23, 37]. Notably, nucleosome remodeling is required for the efficient recruitment of IκBζ to the transcriptional control regions of SR genes [38]. *Nfkbiz* is a PR gene which localizes to the nucleus and triggers the induction of a subset of TLR- and IL-1-dependent but not TNF-inducible SR genes [39]. Interestingly, *Nfkbiz* is reported to be upregulated in response to IL-17 [40] and to the platelet-derived growth factor (PDGF) [40]. IκBζ has a transcriptional activity domain that is constitutively suppressed by the ankyrin-repeat motif on the carboxy-terminal that is liberated when bound to the NF-κB p50 subunit [41]. As such, NF-κB1 (p50) deficient cells do not express IκBζ-dependent genes such as *Il6* and *Il12b* [39]. Notably, p50-RelA DNA-binding activity observed in IL-17 stimulated HBE cells had sustained p50 DNA-binding activity lasting 3 hours whereas RelA activity returned to baseline within 1 hour [40]. Since *Nfkbiz* expression induced by IL-17 peaks within 2h, IκBζ may bind and perhaps co-activates lingering p50-homodimers to SR promoters. In line with a regulatory role for IκBζ to mediate IL-17-inducible SR genes, knockdown of IκBζ by siRNA

significantly diminishes hBD2 expression in HBE cells [40]. Thus, IκBζ is a selective co-regulator of the NF-κB transcriptome and may contribute to the specificity of the IL-17 response by co-activating SR genes.

IL-17 also induced the expression of *Zbtb25* which contains two distantly spaced C2H2-type zinc-fingers (ZnF) and a BTB/POV domain (Bric-a-brac, Tramtrack, Broad-complex (BTB)/ Poxvirus zinc-finger (POZ)) [42]. POV are associated with general transcriptional repressors which interact with methyl-DNA binding proteins and histone deacetylases (HDACs) to convert chromatin into a repressive state [43]. The Castor zinc finger 1 (CASZ1) also upregulated by IL-17 is a nuclear DNA-binding factor with multiple C2H2-type ZnF and shares homology with the transcription factor IIIA (TFIIIA) class of proteins. CASZ1 can function as a transcriptional activator or repressor depending on interacting cofactors [44, 45]. Thus, these transcriptional regulators in addition to other NF-κB-dependent PR genes such as *Ier3*, *Rrad* and *Sod2* which have been identified in our genomic analysis may play critical roles in mediating or shaping the IL-17 gene expression profile.

ZC3H12 are TNF, TLR- and IL-1β-inducible genes containing a CCCH-type ZnF motif mediating RNase activity [46]. Based on amino acid sequence alignment, ZC3H12 family members share homology with the tandem zinc-finger protein-36 (ZFP36) family suggesting the two groups may share functional features [47]. ZFP36 (tristetraprolin) is a RNA-binding protein that suppresses inflammation by destabilizing and decreasing the half-life of mRNAs. Interestingly, ZFP36 was recently reported to suppress the transcriptional activity of NF-κB-dependent promoters by impairing the nuclear import of the p65 subunit in structural cells [48]. Similarly,

ZC3H12A was reported to negatively regulate NF-κB p65 promoter-binding activity and to destabilize the 3'UTR of IL-6 mRNA, albeit not for *Cxcl1*, *Cxcl10*, *Ccl5* or *Nfkbia* in murine macrophages [46, 49]. However, transfection of *Zc3h12a* in endothelial cells upregulated the expression of several CXC-chemokines and angiogenic factors suggesting that ZC3H12A may regulate the expression of a subset of NF-κB-dependent genes [50]. In line with a regulatory role for ZnF proteins to modulate the IL-17 gene profile, transcripts levels of *Zc3h12a* and *Zc3h12c* which peaked within 6 hours may be delayed in order to regulate SR genes by destabilizing mRNAs of particular NF-κB targets.

NF-κB oscillations were previously proposed as a possible mechanism for IL-17 to mediate specificity in the gene expression profile [40]. Inducible IκBε and IκBβ mediate the delayed removal of nuclear NF-κB and complement the rapid and strong feedback mechanism of IκBα [51]. As such, delayed kinetics and ratios of inducible IκBα/β/ε can mediate nuclear NF-κB translocation dynamics and alter the gene expression profile [52]. IκBδ (p100 homodimers) can also alter prolonged NF-κB (p50-RelA) responsiveness to pathogen-triggered signals mediated by TLRs and alter the expression of SR genes such as for CCL5 [53]. Upregulated levels of *Ikbia*, *Ikbie* in addition to *Nfkb2* (p100) were identified in our gene array although no modifications of IκB family members were observed by blotting methods. Although inhibition by negative feedback of the IκB kinase (IKK) complex is also reported to mediate temporal NF-κB dynamics [54], we were unable to detect IKKα/β activity mediated by IL-17. In fact, our inability to detect any significant degradation products or post-translational modifications of regulatory NF-κB factors induced by IL-17 correlates with its poor ability to activate NF-κB [16, 22]. Alternatively, IL-17 was also previously demonstrated to signal via the atypical NF-κB pathway by activating

NF-κB-inducing kinase (NIK) and IKKα/α which cleaves the p100 subunit into active p52-RelB heterodimers [55]. Notably, cIAP2, a negative regulator of NIK was upregulated in our gene array suggesting that IL-17 could block or terminate atypical NF-κB responses.

The gene expression profile of ASM cells stimulated with IL-17 from mild asthmatic individuals contained significantly more upregulated genes such as *Il6* and *Cxcl10* in comparison to healthy donors. Comparative cluster analysis revealed that 33 genes in the pooled group were mutually upregulated in either the healthy or asthmatic group. Although the remainder of the statistically upregulated genes in the pooled group were not listed in either the healthy or asthmatic groups, doubling the population size to include values from both healthy and asthmatic ASM cells uncovered a subset of common genes which had not achieved significance in either group alone. The poor overlap between groups can be attributable to the fact that IL-17 induces modest gene expression levels at the 2h time point. Owing to the inflammatory nature of asthma, signaling thresholds or regulatory mechanisms may also be altered in these cells allowing for unwarranted or irregular cellular responses to occur. Therefore, IL-17 may mediate unfavourable responses in the airways of patients with preexisting inflammatory disorders such as asthma and COPD by combining with Th17 cytokines or by synergizing the functions of IL-1β or TNF to recruit pulmonary inflammatory cells and exacerbate airway disease [7].

Taken together, our microarray analysis identified primary response genes induced by IL-17 with dynamic expression profiles consisting largely of NF-κB regulatory factors, transcription regulators and chemokines. Importantly, IL-17 mediated a modest fold increase in gene expression but additively combined with IL-22 and IL-1β to upregulate several transcripts. IL-17

also upregulated a distinctive inflammatory gene expression profile in ASM cells of mild asthmatic individuals which may exacerbate airway inflammation when combined with cytokines such as IL-1β. Collectively, our results suggest that IL-17 is a potentiating cytokine able to mediate additive and synergistic responses with proinflammatory cytokines and that the MAPK-NF-κB signaling network may regulate the IL-17 gene signature.

2.6.0. References

1. Louten, J., Boniface, K. & de Waal Malefyt, R. Development and function of TH17 cells in health and disease. *J Allergy Clin Immunol* **123**, 1004-1011 (2009).
2. Ye, P. *et al.* Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* **194**, 519-527 (2001).
3. Liang, S.C. *et al.* Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* **203**, 2271-2279 (2006).
4. Korn, T., Bettelli, E., Oukka, M. & Kuchroo, V.K. IL-17 and Th17 Cells. *Annu Rev Immunol* **27**, 485-517 (2009).
5. Nembrini, C., Marsland, B.J. & Kopf, M. IL-17-producing T cells in lung immunity and inflammation. *J Allergy Clin Immunol* **123**, 986-994; quiz 995-986 (2009).
6. Wang, Y.H. & Liu, Y.J. The IL-17 cytokine family and their role in allergic inflammation. *Curr Opin Immunol* **20**, 697-702 (2008).
7. Hakonarson, H. & Grunstein, M.M. Autocrine regulation of airway smooth muscle responsiveness. *Respir Physiol Neurobiol* **137**, 263-276 (2003).
8. Song, C. *et al.* IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol* **181**, 6117-6124 (2008).
9. Pichavant, M. *et al.* Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J Exp Med* **205**, 385-393 (2008).
10. Al-Ramli, W. *et al.* T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol* **123**, 1185-1187 (2009).
11. Lazaar, A.L. & Panettieri, R.A., Jr. Airway smooth muscle: a modulator of airway remodeling in asthma. *J Allergy Clin Immunol* **116**, 488-495; quiz 496 (2005).
12. Lazaar, A.L. *et al.* T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J Exp Med* **180**, 807-816 (1994).
13. Ramos-Barbon, D., Presley, J.F., Hamid, Q.A., Fixman, E.D. & Martin, J.G. Antigen-specific CD4+ T cells drive airway smooth muscle remodeling in experimental asthma. *J Clin Invest* **115**, 1580-1589 (2005).
14. Hakonarson, H., Kim, C., Whelan, R., Campbell, D. & Grunstein, M.M. Bi-directional activation between human airway smooth muscle cells and T lymphocytes: role in induction of altered airway responsiveness. *J Immunol* **166**, 293-303 (2001).
15. Gaffen, S.L. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* **9**, 556-567 (2009).
16. Shen, F. & Gaffen, S.L. Structure-function relationships in the IL-17 receptor: implications for signal transduction and therapy. *Cytokine* **41**, 92-104 (2008).

17. Zrioual, S. *et al.* Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. *J Immunol* **182**, 3112-3120 (2009).
18. Gueders, M.M. *et al.* MMP-19 Deficiency Promotes Tenascin-C Accumulation and Allergen-induced Airway Inflammation. *Am J Respir Cell Mol Biol* (2009).
19. Chan, V. *et al.* Extracellular matrix regulates enhanced eotaxin expression in asthmatic airway smooth muscle cells. *Am J Respir Crit Care Med* **174**, 379-385 (2006).
20. Dennis, G., Jr. *et al.* DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* **4**, P3 (2003).
21. Dragon, S. *et al.* IL-17 enhances IL-1beta-mediated CXCL-8 release from human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **292**, L1023-1029 (2007).
22. Hartupee, J., Liu, C., Novotny, M., Li, X. & Hamilton, T. IL-17 enhances chemokine gene expression through mRNA stabilization. *J Immunol* **179**, 4135-4141 (2007).
23. Saccani, S., Pantano, S. & Natoli, G. Two waves of nuclear factor kappaB recruitment to target promoters. *J Exp Med* **193**, 1351-1359 (2001).
24. van Essen, D., Engist, B., Natoli, G. & Saccani, S. Two modes of transcriptional activation at native promoters by NF-kappaB p65. *PLoS Biol* **7**, e73 (2009).
25. Rahman, M.S. *et al.* IL-17A induces eotaxin-1/CC chemokine ligand 11 expression in human airway smooth muscle cells: role of MAPK (Erk1/2, JNK, and p38) pathways. *J Immunol* **177**, 4064-4071 (2006).
26. Henness, S. *et al.* IL-17A acts via p38 MAPK to increase stability of TNF-alpha-induced IL-8 mRNA in human ASM. *Am J Physiol Lung Cell Mol Physiol* **290**, L1283-1290 (2006).
27. Patel, D.N. *et al.* Interleukin-17 stimulates C-reactive protein expression in hepatocytes and smooth muscle cells via p38 MAPK and ERK1/2-dependent NF-kappaB and C/EBPbeta activation. *J Biol Chem* **282**, 27229-27238 (2007).
28. Chen, R.H., Sarnecki, C. & Blenis, J. Nuclear localization and regulation of erk- and rsk-encoded protein kinases. *Mol Cell Biol* **12**, 915-927 (1992).
29. Lee, J.W. *et al.* Differential regulation of chemokines by IL-17 in colonic epithelial cells. *J Immunol* **181**, 6536-6545 (2008).
30. Merienne, K., Pannetier, S., Harel-Bellan, A. & Sassone-Corsi, P. Mitogen-regulated RSK2-CBP interaction controls their kinase and acetylase activities. *Mol Cell Biol* **21**, 7089-7096 (2001).
31. Saccani, S., Pantano, S. & Natoli, G. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat Immunol* **3**, 69-75 (2002).
32. Kawaguchi, M. *et al.* The IL-17F signaling pathway is involved in the induction of IFN-gamma-inducible protein 10 in bronchial epithelial cells. *J Allergy Clin Immunol* **119**, 1408-1414 (2007).
33. Kawaguchi, M. *et al.* IL-17F-induced IL-11 release in bronchial epithelial cells via MSK1-CREB pathway. *Am J Physiol Lung Cell Mol Physiol* **296**, L804-810 (2009).
34. Wang, X. *et al.* Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *EMBO J* **20**, 4370-4379 (2001).
35. Huang, F. *et al.* Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF-kappaB activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. *J Immunol* **179**, 6504-6513 (2007).
36. Shen, F. *et al.* IL-17 receptor signaling inhibits C/EBPbeta by sequential phosphorylation of the regulatory 2 domain. *Sci Signal* **2**, ra8 (2009).
37. Tullai, J.W. *et al.* Immediate-early and delayed primary response genes are distinct in function and genomic architecture. *J Biol Chem* **282**, 23981-23995 (2007).
38. Kayama, H. *et al.* Class-specific regulation of pro-inflammatory genes by MyD88 pathways and IkappaBzeta. *J Biol Chem* **283**, 12468-12477 (2008).
39. Yamamoto, M. *et al.* Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature* **430**, 218-222 (2004).

40. Kao, C.Y., Kim, C., Huang, F. & Wu, R. Requirements for two proximal NF-kappaB binding sites and IkappaB-zeta in IL-17A-induced human beta-defensin 2 expression by conducting airway epithelium. *J Biol Chem* **283**, 15309-15318 (2008).
41. Motoyama, M., Yamazaki, S., Eto-Kimura, A., Takeshige, K. & Muta, T. Positive and negative regulation of nuclear factor-kappaB-mediated transcription by IkappaB-zeta, an inducible nuclear protein. *J Biol Chem* **280**, 7444-7451 (2005).
42. Chardin, P., Courtois, G., Mattei, M.G. & Gisselbrecht, S. The KUP gene, located on human chromosome 14, encodes a protein with two distant zinc fingers. *Nucleic Acids Res* **19**, 1431-1436 (1991).
43. Kelly, K.F. & Daniel, J.M. POZ for effect--POZ-ZF transcription factors in cancer and development. *Trends Cell Biol* **16**, 578-587 (2006).
44. Kambadur, R. *et al.* Regulation of POU genes by castor and hunchback establishes layered compartments in the Drosophila CNS. *Genes Dev* **12**, 246-260 (1998).
45. Liu, Z., Yang, X., Tan, F., Cullion, K. & Thiele, C.J. Molecular cloning and characterization of human Castor, a novel human gene upregulated during cell differentiation. *Biochem Biophys Res Commun* **344**, 834-844 (2006).
46. Matsushita, K. *et al.* Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. *Nature* **458**, 1185-1190 (2009).
47. Liang, J., Song, W., Tromp, G., Kolattukudy, P.E. & Fu, M. Genome-wide survey and expression profiling of CCCH-zinc finger family reveals a functional module in macrophage activation. *PLoS One* **3**, e2880 (2008).
48. Schichl, Y.M., Resch, U., Hofer-Warbinek, R. & de Martin, R. Tristetraprolin impairs NF-{kappa}B/p65 nuclear translocation. *J Biol Chem* (2009).
49. Liang, J. *et al.* A novel CCCH-zinc finger protein family regulates proinflammatory activation of macrophages. *J Biol Chem* **283**, 6337-6346 (2008).
50. Niu, J., Azfer, A., Zhelyabovska, O., Fatma, S. & Kolattukudy, P.E. Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). *J Biol Chem* **283**, 14542-14551 (2008).
51. Kearns, J.D., Basak, S., Werner, S.L., Huang, C.S. & Hoffmann, A. IkappaBepsilon provides negative feedback to control NF-kappaB oscillations, signaling dynamics, and inflammatory gene expression. *J Cell Biol* **173**, 659-664 (2006).
52. Ashall, L. *et al.* Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription. *Science* **324**, 242-246 (2009).
53. Shih, V.F. *et al.* Kinetic control of negative feedback regulators of NF-kappaB/RelA determines their pathogen- and cytokine-receptor signaling specificity. *Proc Natl Acad Sci U S A* **106**, 9619-9624 (2009).
54. Cheong, R. *et al.* Transient IkappaB kinase activity mediates temporal NF-kappaB dynamics in response to a wide range of tumor necrosis factor-alpha doses. *J Biol Chem* **281**, 2945-2950 (2006).
55. Awane, M., Andres, P.G., Li, D.J. & Reinecker, H.C. NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. *J Immunol* **162**, 5337-5344 (1999).

2.7.0. Supplementary Data

	p Value	Fold	Entrez ID	Name	Description	Location	Type
1	0.000	4.48	64332	**NFKBIZ**	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Nucleus	Transcription regulator
2	0.000	2.08	4792	**NFKBIA**	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Cytoplasm	Other
3	0.001	1.40	5971	**RELB**	v-rel reticuloendotheliosis viral oncogene homolog B, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (avian)	Nucleus	Transcription regulator
4	0.003	1.55	330	**BIRC3 (cIAP2)**	baculoviral IAP repeat-containing 3	Cytoplasm	Enzyme
5	0.003	2.55	2919	**CXCL1**	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Extracellular Space	Cytokine
6	0.005	1.72	7128	**TNFAIP3 (A20)**	tumor necrosis factor, alpha-induced protein 3	Nucleus	Other
7	0.006	1.55	1326	**MAP3K8**	mitogen-activated protein kinase kinase kinase 8	Cytoplasm	Kinase
8	0.006	1.41	2920	**CXCL2**	chemokine (C-X-C motif) ligand 2	Extracellular Space	Cytokine
9	0.008	1.35	8870	**IER3**	immediate early response 3	Cytoplasm	Other
10	0.011	1.16	4794	**NFKBIE**	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	Nucleus	Transcription regulator
11	0.012	1.27	6648	**SOD2**	superoxide dismutase 2, mitochondrial	Cytoplasm	Enzyme
12	0.012	1.24	4791	**NFKB2**	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	Nucleus	Transcription regulator
13	0.029	1.17	6004	**RGS16**	regulator of G-protein signaling 16	Cytoplasm	Other
14	0.030	1.22	6348	**CCL3**	chemokine (C-C motif) ligand 3	Extracellular Space	Cytokine
15	0.032	1.27	3383	**ICAM1**	intercellular adhesion molecule 1	Plasma Membrane	Transcription regulator
16	0.039	1.20	4616	**GADD45B**	growth arrest and DNA-damage-inducible, beta	Cytoplasm	Other

Table 2. 4 (Supplement). IL-17-induced NF-κB-dependent genes
Upregulated genes by IL-17 compared to unstimulated controls ($p \leq 0.05$ & $F \geq 1.15$). Gene list excludes any genes not categorized by the NCBI RefSeq accession format as a mature transcript product (prefix NM).

Gene	*Entrez GeneID*	*RefSeq*	*Primer Sequences*
il6	3569	NM_000600	*(f)* CTTTTggAgTTTgAggTATACCTAg *(r)* CgCAgAATgAgATgAgTTgTC
cxcl8	3576	NM_000584	*(f)* gCTCTgTgTgAAggTgCAgTT *(r)* AgCTCTCTTCCATCAgAAAgC
bdkrb1	623	NM_000710	*(f)* CAACTACAgTTgTgAACgCC *(r)* TggTCCCAgCAACTCgA
selp	6403	NM_003005	*(f)* TCCAgAATgCCAAgCCAT *(r)* CgATCgAgTACAATCCAATCTTT
end1	1906	NM_001955	*(f)* gTCAACACTCCCgAgCAC *(r)* CCATAATgTCTTCAgCCCTgAg
tnfaip3	7128	NM_006290	*(f)* ACTgCCAgAAgTgTTTCATTg *(r)* CTCTggTTgggATgCTgAC
nfkbiz	64332	NM_031419	*(f)* AgCTTgCAgTATCggTTgAC *(r)* AATggAgCTAATACggTggAg
zc3hiza	80149	NM_025079	*(f)* ggCTATTCATCCACggAgATC *(r)* CACTgCCAgCAggATgC
zc3h12c	85463	NM_033390	*(f)* TgATgACCCTCTTggCAgA *(r)* gAACACTgTTgCTTTTCACCA
cd200r1	131450	NM_170780	*(f)* AgAgCTACTTCCTgTTCCAgg *(r)* TCTCTgTgTAgCTggCATAgg
vsig4	11326	NM_007268	*(f)* TgACATACCCCTTgAAAgCAA *(r)* TggCTgCTTCgTAgACATgC
il27r	9466	NM_004843	*(f)* CAgAgCCAAAAgTACCgTT *(r)* ggCgTTTggCTTCATTT

Table 2. 5 (Supplement). Primer sequences

	p Value	Fold	Entrez ID	Name	Description	Location	Type
1	0.000	1.26	81631	**MAP1LC3B**	microtubule-associated protein 1 light chain 3 beta	Cytoplasm	Other
2	0.001	1.26	131450	**CD200R1**	CD200 receptor 1	Plasma Membrane	Other
3	0.002	1.24	30816	**ERVWE1**	endogenous retroviral family W, env(C7), member 1	Plasma Membrane	Other
4	0.002	3.17	64332	***NFKBIZ**	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Nucleus	Transcription regulator
5	0.003	1.27	255798	**C3orf43**	chromosome 3 open reading frame 43	Unknown	Other
6	0.004	1.20	1906	***EDN1**	endothelin 1	Extracellular Space	Other
7	0.005	1.41	60489	**APOBEC3G**	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	Nucleus	Enzyme
8	0.005	1.33	4276	**MICA**	MHC class I polypeptide-related sequence A	Plasma Membrane	Other
9	0.007	1.22	3352	**HTR1D**	5-hydroxytryptamine (serotonin) receptor 1D	Plasma Membrane	G-protein coupled receptor
10	0.012	1.24	64333	**ARHGAP9**	Rho GTPase activating protein 9	Unknown	Other
11	0.013	1.21	8386	**OR1D5**	olfactory receptor, family 1, subfamily D, member 5	Plasma Membrane	G-protein coupled receptor
12	0.014	1.23	23639	**LRRC6**	leucine rich repeat containing 6	Cytoplasm	Other
13	0.015	1.22	6556	**SLC11A1**	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	Plasma Membrane	Transporter
14	0.016	1.20	51265	**CDKL3**	cyclin-dependent kinase-like 3	Cytoplasm	Kinase
15	0.016	1.34	11326	**VSIG4**	V-set and immunoglobulin domain containing 4	Plasma Membrane	Other
16	0.021	1.27	6754	**SSTR4**	somatostatin receptor 4	Plasma Membrane	G-protein coupled receptor
17	0.022	1.27	154865	**IQUB**	IQ motif and ubiquitin domain containing	Cytoplasm	Other
18	0.022	1.20	23519	**ANP32D**	acidic (leucine-rich) nuclear phosphoprotein 32 family, member D	Unknown	Other
19	0.024	1.27	120126	**UBTFL2**	upstream binding transcription factor, RNA polymerase I-like 2	Unknown	Other
20	0.032	1.21	10321	**CRISP3**	cysteine-rich secretory protein 3	Extracellular Space	Other
21	0.036	1.28	219844	**HYLS1**	hydrolethalus syndrome 1	Unknown	Other
22	0.037	1.27	613211	**DEFB134**	defensin, beta 134	Unknown	Other
23	0.042	1.21	54897	**CASZ1**	castor zinc finger 1	Nucleus	Enzyme
24	0.045	1.25	442184	**OR2B3P**	olfactory receptor, family 2, subfamily B, member 3	Plasma Membrane	Other
25	0.047	1.30	6317	**SERPINB3**	serpin peptidase inhibitor, clade B (ovalbumin), member 3	Extracellular Space	Other

Table 2. 6 (Supplement). IL-17R target gene list of healthy ASM cells sorted on probability values
Upregulated genes by IL-17 compared to unstimulated controls ($p \leq 0.05$ & $F \geq 1.2$). Gene list excludes any genes not categorized by the NCBI RefSeq accession format as a mature transcript product (prefix NM). * Denotes genes validated by qRT-PCR, underlined genes are in common with the pooled group.

3.0. CHAPTER 3

INTERLEUKIN-17 ENHANCES IL-1β-MEDIATED CXCL8 RELEASE FROM HUMAN AIRWAY SMOOTH MUSCLE CELLS

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Stéphane Dragon[1], Muhammad Shahidur Rahman[1], Jie Yang[1], Andrew J. Halayko[2] Helmut Unruh[3] and Abdelilah Soussi Gounni[1]

[1]Departments of Immunology, [2]Physiology and [3]Sections of Respiratory Diseases & Thoracic Surgery, University of Manitoba, Winnipeg, Manitoba, Canada.

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SD and MSR contributed equally to this work. SD contributed to the drafting and to 50% of the data collection & analysis of this manuscript. MSR contributed to the experimental design and data collection.

3.1.0. ABSTRACT

Recent studies into the pathogenesis of airway disorders such as asthma have revealed a dynamic role for airway smooth muscle cells in the perpetuation of airway inflammation via the secretion of cytokines and chemokines. In this study, we evaluated whether IL-17 could enhance the release of CXCL8 induced by IL-1β and investigated the upstream and downstream signaling events regulating CXCL8 gene expression in human airway smooth muscle (ASM) cells. ASM cells were transfected with site mutated AP-1/ NF-κB CXCL8 promoter constructs and were treated with selective p38, MEK1/2 and PI3-K inhibitors to determine the roles of the MAPK and PI3-K signaling pathways. We demonstrate that CXCL8 mRNA and protein levels were upregulated by IL-17 and were further enhanced when combined with IL-1β. The CXCL8 promoter activity induced by IL-17 or IL-1β was dependent on the ERK1/2 and p38 signal transduction pathways although the PI3-K pathway also partially mediated IL-1β activity. The synergistic response mediated by IL-17 and IL-1β was dependent on both the MAPK and PI3-K signal transduction pathways and required the cooperative activation of AP-1 and NF-κB trans-acting elements. Furthermore, post-transcriptional mechanisms regulating mRNA stability were also observed to modulate the synergistic response in ASM cells. Collectively, our observations indicate that the MAPK and PI3-K pathways regulate the synergistic response mediated by IL-17 and IL-1β to enhance the promoter activity, mRNA induction and stability mechanisms for CXCL8 protein synthesis.

3.2.0. INTRODUCTION

Human airway smooth muscle (ASM) cells are key determinants of asthma owing to their ability to contract in response to inflammatory cell products (1). Due to their intrinsic plasticity, airway myocytes also exhibit a capacity for multifunctional behaviour and are actively involved in local inflammation and fibrosis (2). Emerging evidence suggests that ASM cells can also directly contribute to the pathogenesis of asthma by altering the interstitial and extracellular matrix, express cell adhesion and costimulatory molecules and secrete proinflammatory cytokines and chemokines (3, 4). These responses can perpetuate airway inflammation and act on the hyperplastic and hypertrophic growth of ASM cells and contribute to development of airway hyperresponsiveness (AHR) and to the remodeling of the airways (5).

Interleukin (IL)-1β is a proinflammatory mediator released by activated airway macrophages and epithelial cells and is found in elevated levels in the bronchoalveolar lavage (BAL) fluid of asthmatic, chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (ARDS) patients (6-8). *In vivo* and *in vitro* assays have demonstrated that IL-1β can alter airway function by inducing cellular infiltrate, mucus hyperplasia, airway wall thickening, fibrosis and enlarge the distal airspaces (9, 10). Effector mechanisms include the secretion of CXCL8 (IL-8) which has been demonstrated to modulate ASM cell contraction and migration and to mediate airway responsiveness in asthmatic patients (11-13). Notably, CXCL8 negatively affects lung and bronchial epithelial function by mobilizing neutrophils into the airways (14).

Although neutrophil chemotactic factors such as CXCL8, granulocyte macrophage-colony stimulating factor (GM-CSF), leukotriene B4 (LTB_4) and the complement activation product

C5a are increased in BAL fluid from asthmatic individuals (15, 16), the upstream factors regulating neutrophil recruitment are still elusive. IL-17, a pleiotropic T lymphocyte cytokine released from a distinct Th1/ Th2 cell subset (17) is hypothesized to orchestrate the granulocyte influx into the airways via the induction of CXCL8 (18, 19). In humans, elevated levels of IL-17 can be detected in sera, sputum and BAL fluid of asthmatic patients and has been shown to correlate with BAL neutrophilia, myeloperoxidase levels and with AHR (20-24). Recently, the effects mediated by combining IL-17 with tumor necrosis factor-α (TNF) has gained attention for eliciting a synergistic chemokine response from several structural lung cells (25-27). Increases in CXCL8 and IL-6 levels *in vitro* have equally been reported for these cytokines in primary ASM cells (28, 29) however, the modulating effect of IL-17 on IL-1β-mediated CXCL8 induction in ASM cells has not been investigated.

We previously established that primary human ASM cells express the IL-17A receptor and that IL-17 induces CXCL8 expression (30). In this study, we demonstrate a role for IL-17 in synergizing IL-1β-induced CXCL8 release from human ASM cells. The synergistic effect of combining IL-17 with IL-1β is regulated at the transcriptional level and is dependent on the cooperative function of the AP-1 and NF-κB cis-acting elements. ERK1/2, p38 mitogen-activated protein kinases (MAPKs) and the phosphatidylinositol 3-kinase (PI3-K) pathway mediated the upstream signal transduction for the activation of the CXCL8 promoter and post-transcriptional mechanisms regulating mRNA stability were also observed to modulate the synergistic response. Collectively, our observations indicate that IL-17 in combination with IL-1β may amplify the functional recruitment of neutrophils via the synergistic induction of CXCL8 and could potentially exacerbate airway inflammation.

3.3.0. Materials and Methods

3.3.1. Reagents and antibodies

Recombinant human IL-17 and IL-1β were purchased from R&D (Minneapolis, MN). Dulbecco's Modified Eagle Medium (DMEM), Ham's F-12 media, antibiotics (penicillin, streptomycin), deoxyribonucleotide triphosphate (dNTP), superscript reverse transcriptase and Taq polymerase were purchased from (GibcoBRL, Grand Island, NY). The p38 MAPK inhibitor, SB203580 (4-[4-fluorophenyl]-5-[4-pyridyl] 1Himidazole), MEK1/2 extracellular signal-regulated kinase (ERK1/2) inhibitor, U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenyl-thio]butadiene), and the PI3-K inhibitor, wortmannin were purchased from Calbiochem (Mississauga, Ontario). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (Logan, UT) and unless stated otherwise, all other reagents were obtained from Sigma Chemical Co. (Oakville, Ontario).

3.3.2. Preparation of bronchial human ASM cells

Bronchial human ASM cells were macroscopically obtained from healthy segments of the main bronchus in lung resections in accordance with procedures approved by the Human Research Ethics Board of the University of Manitoba. Primary human ASM cells cultures were established as previously described (30). ASM cells were grown at 37°C with 5% CO_2 in DMEM with 10% FBS and antibiotics (100U/ml penicillin and 100μg/ml streptomycin). In all experiments, cells were used in passages 2 to 5.

3.3.3. Cell stimulation and ELISA assay

Confluent ASM cells were growth arrested by FBS-deprivation for 48h in Ham's F-12 media containing 5μg/ml of human recombinant insulin, 5μg/ml of human transferrin, 5ng/ml of

selenium and antibiotics (100U/ml penicillin and 100µg/ml streptomycin). Cells were then stimulated in fresh, FBS-free media with either recombinant human IL-17 (0.1, 1, 10 and 100ng/ml), IL-1β (0.1, 1, 10 and 100ng/ml), in combination or were unstimulated. Supernatants were collected at different time points (24 and 48h), centrifuged for 7min at 4ºC to remove cellular debris and stored at -80°C prior to ELISA. To test the effect of the mRNA synthesis inhibitor actinomycin-D (ActD) on CXCL8 mRNA or protein release, 5µg/ml was added to FBS-deprived ASM cells 30min before stimulation with IL-17 (1ng/ml), IL-1β (1ng/ml) or both (31). Supernatants were recovered at 24 and 48h and processed as described above. Immuno-reactive CXCL8 was quantified by ELISA using matched antibodies from Pierce Endogen (Biosource International, Missisauga, Ontario) according to standard laboratory protocols. The sensitivity limit of CXCL8 was 10pg/ml.

3.3.4. Real-time RT-PCR analysis

Confluent ASM cells were growth arrested by FBS-deprivation and were stimulated in fresh, FBS-free media containing IL-17 (1ng/ml), IL-1β (1ng/ml), in combination or were untreated for 6h. Cells were then harvested and RNA was purified using the guanidinium isothiocyanate method (32). As previously described, relative levels of CXCL8 mRNA were analyzed by quantitative real-time RT-PCR analysis using the Light-Cycler (Roche) (30). Briefly, DNA standards were prepared by PCR using cDNA of cells stimulated with IL-1β (1ng/ml). The amount of extracted DNA was quantified by spectrophotometry and expressed as copy numbers and serial dilutions were used to generate the standard curve. Product specificity was determined by melting curve analysis and visualized on a agarose gel. Calculation of the relative amount of each cDNA species was performed as previously described (33). The amplification of target

genes in stimulated cells was calculated by first normalizing to the respective GAPDH level and is expressed as a fold increase over the respective unstimulated control condition.

3.3.5. CXCL8 mRNA stability

Growth-arrested ASM cells were stimulated with IL-1β (1ng) or in combination with IL-17 (50ng) for 10h prior to the addition of 5μg/ml of ActD. Total cellular RNA was then extracted at the indicated time points post ActD incubation and CXCL8 mRNA expression was quantified by semi-quantitative real-time RT-PCR. CXCL8 mRNA copy numbers were normalized to the respective GAPDH values. Results are presented as the % of mRNA remaining compared to the initial 10h culture time point. As previously described, one phase exponential decay constants (k) were calculated by nonlinear regression of the % of mRNA remaining versus time of the ActD treatment using the GraphPad Prism software (v.4.0) (34).

3.3.6. CXCL8 promoter luciferase reporter constructs and cell transfection

The CXCL8 promoter was amplified with specific primers from human genomic DNA using PCR as described previously (30). Briefly, ASM cells ($4x10^4$) were seeded into 24 well plates in complete media (DMEM/ 10%FBS). At 70% confluency, transfection was performed in triplicates using the ExGen500 method according to the manufacturer's instructions (Fermentas Inc, Mississauga, ON). In each well, 0.8μg of CXCL8 promoter-luciferase DNA construct (wild-type, NF-κB, AP-1 or double mutant) and 0.2μg of the pRL-TK renilla luciferase reporter vector were co-transfected for 24h. Subsequently, the media was changed and cells were pretreated for 1h in the presence or absence of SB203580 (10μM), U0126 (10μM), or wortmannin (100nM) (35) before stimulation with IL-17 (0.1, 1, 10 or 100ng/ml), IL-1β (0.1, 1, 10ng/ml) or in

combination for 12h. Luciferase activity was measured by the dual luciferase reporter assay system (Promega) using a luminometer (model LB9501; Berthold Lumat, Germany. Briefly, 20μl of cell lysates were mixed with 100μl of the luciferase assay reagent II and the firefly luciferase activity was recorded. Subsequently, a 100μl of the stop-and-glo reagent was added and the renilla luciferase activity was measured. All values were normalized to the mock renilla luciferase activity.

3.3.7. Statistical analysis

Data were obtained from experiments performed in triplicates and repeated three times from four different donors. Results were expressed as the mean±SEM and statistical significance was determined using the *Mann-Whitney U* test. p values <0.05 were considered statistically significant.

3.4.0. RESULTS

3.4.1. IL-17 enhances IL-1β-mediated CXCL8 protein release from ASM cells

We initially investigated whether IL-17 could influence IL-1β-mediated CXCL8 release from ASM cells. Primary human ASM cells were treated with IL-17 (0.1, 1, 10 and 100ng/ml), IL-1β (0.1, 1, 10, 100ng/ml) or in combination and the supernatants were harvested 48h later. Suboptimal concentrations of IL-17 in combination with IL-1β (0.1 and 1ng/ml) induced a respective 2.1 and 2.3 fold increase in CXCL8 release compared to the IL-1β-treated condition ($p<0.001$, figure3.1). A synergistic effect between both cytokines was also observed when higher concentrations of IL-17 (10 and 100ng/ml) were added with lower doses of IL-1β (0.1 or 1ng/ml) resulting in a respective 1.3 and 1.9 fold increase compared to the IL-1β condition (data not

shown). Taken together, our data demonstrates that IL-17 can upregulate IL-1β-mediated CXCL8 protein expression in ASM cells.

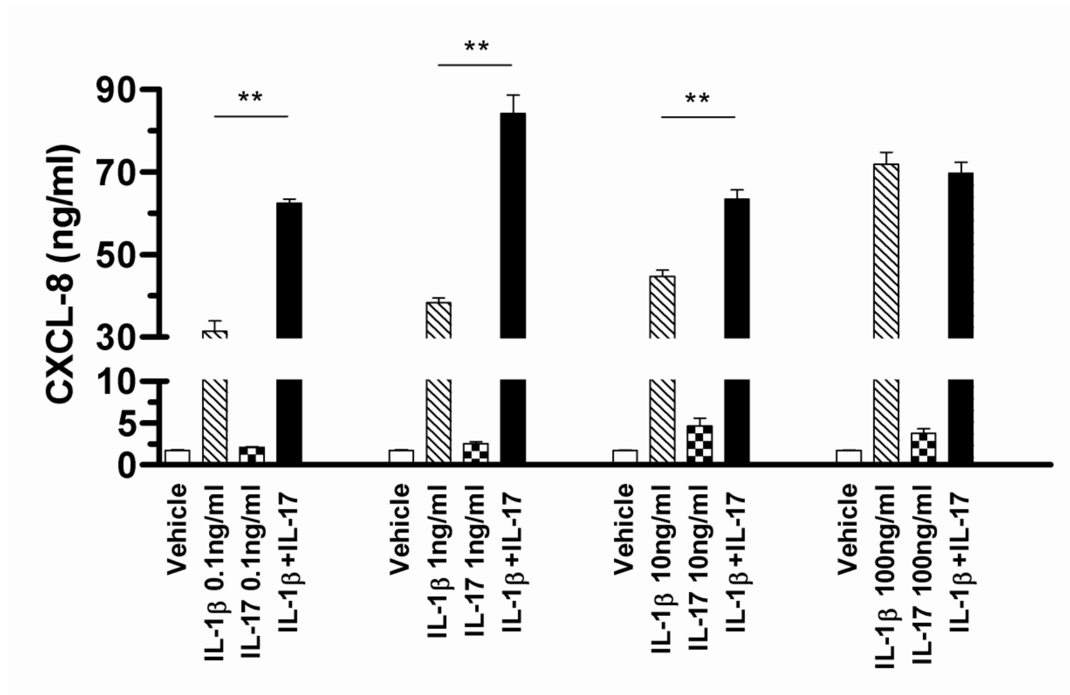

Figure 3. 1. IL-17 synergizes with IL-1β to induce CXCL8 protein release
Supernatants from FBS-deprived ASM cells stimulated with varying concentrations of IL-17, IL-1β and in combination were harvested after 48h. Results from ELISA assays are represented as the mean±SEM from 4 independent assays performed in duplicate. ***p*<0.001 as compared with cells stimulated with IL-1β alone.

3.4.2. IL-17 and IL-1β synergize CXCL8 mRNA induction in ASM cells

To investigate whether IL-17 can modulate IL-1β-induced CXCL8 mRNA, we examined mRNA expression levels by semi-quantitative real-time RT-PCR. Primary human ASM cells were treated with IL-17 (1ng/ml), IL-1β (1ng/ml) or in combination and mRNA was harvested after 6h. IL-17 and IL-1β each induced a 2.7±0.9 and 9.0±1.5 fold increase in CXCL8 mRNA compared to untreated controls (figure3.2a). When used in combination, IL-17 and IL-1β induced a synergistic response upregulating CXCL8 transcripts by 39.2 fold over those induced by IL-1β.

To verify whether the effect of IL-17 on IL-1β-mediated CXCL8 protein expression depended on mRNA neosynthesis, confluent FBS-deprived ASM cells were pretreated with ActD and stimulated with IL-17 (1ng/ml), IL-1β (1ng/ml) or both. After 48h, secreted protein levels from supernatants of the respective IL-17 and IL-1β conditions were increased by 13.0±0.3 and 1382.0±184.0 fold in comparison to unstimulated controls (figure3.2b). Pretreatment with ActD before cytokine stimulation completely abrogated the induction of CXCL8. In addition, CXCL8 mRNA rate of decay was reduced by 2.1±0.1 fold in the combined treated condition compared to IL-1β (figure3.2c, 0.87±0.13 vs 0.42±0.21, $p<0.05$). This effect resulted in a significant increase in CXCL8 transcript levels by 2.0±1.1 fold over the IL-1β treated conditions at the 12h time point. Taken together, our results suggest that the synergy mediated by IL-17 and IL-1β in ASM cells can occur via both transcriptional and post-transcriptional mechanisms.

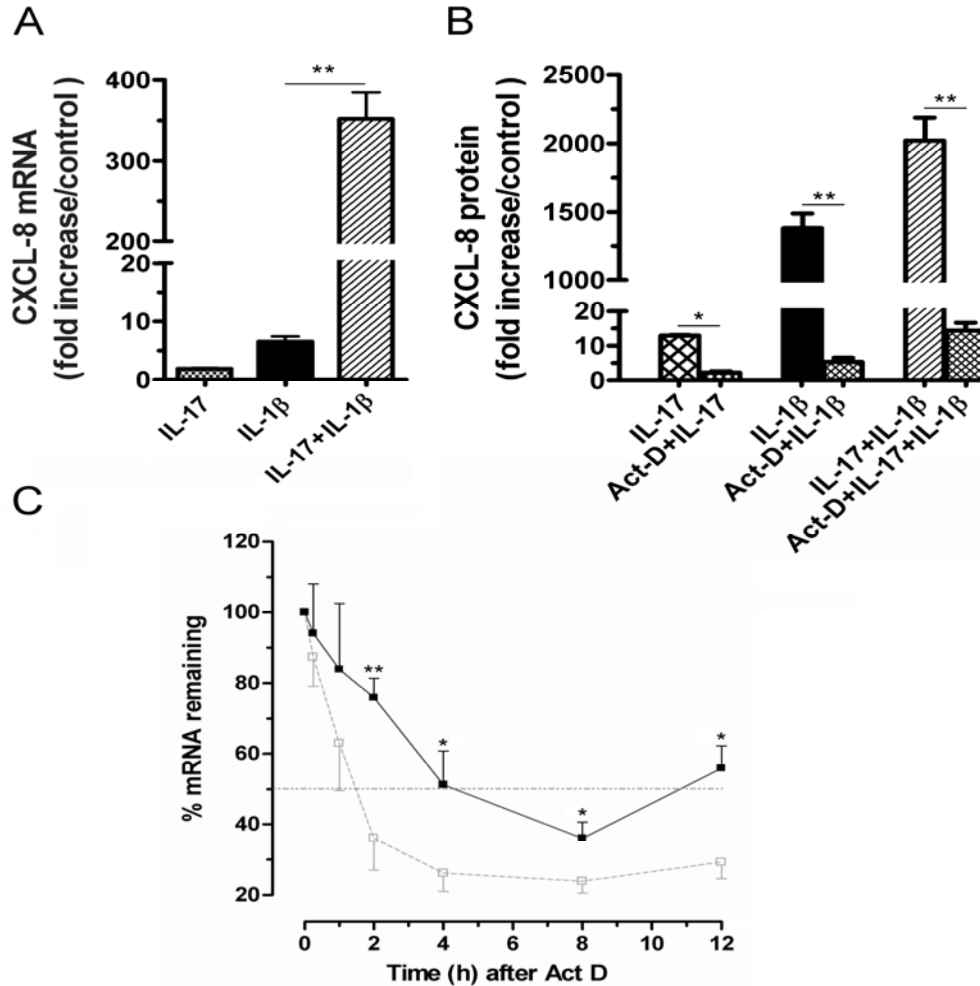

Figure 3. 2. IL-17 enhances IL-1β mediated CXCL8 mRNA neosynthesis
FBS-deprived ASM cells were stimulated with IL-17, IL-1β or both (1ng/ml) and mRNA was harvested after 6h. Real-time RT-PCR was performed as described in materials and methods. Representative data is showed in (A) and corresponds to the fold increase in CXCL8 mRNA to the unstimulated condition (mean±SEM, n=4). (B) Actinomycin D (ActD) abrogates the effect of IL-17 on IL-1β-induced CXCL8 protein release. FBS-deprived ASM cells were pretreated with ActD for 30m before stimulation with IL-17, IL-1β or in combination (mean±SEM, n=4, **p*<0.05, ***p*<0.001). (C) IL-17 combined with IL-1β enhances CXCL8 mRNA stability. ASM cells were cultured with IL-1β (open) or in concert with IL-17 (closed) for 10h before the addition of ActD at various time points (mean±SEM, n=4, **p*<0.05, ***p*<0.01).

3.4.3. IL-17 enhances IL-1β mediated CXCL8 promoter activity

To investigate whether IL-17 can modulate the activity of the CXCL8 promoter, ASM cells were transiently transfected with the proximal CXCL8 promoter fused to a luciferase reporter and

were stimulated with suboptimal concentrations of IL-17, IL-1β or in combination. IL-17 induced a 1.8 and 4.2 fold increase in luciferase activity compared to unstimulated controls at the respective 0.1 and 1ng/ml concentrations (figure3.3). Similarly, IL-1β at 0.1 and 1ng/ml led to a 10.0 and 23.7 fold increase in the CXCL8 reporter gene activity. At the 1ng/ml concentration, combining IL-17 with IL-1β induced a synergistic 1.6 fold increase compared to the IL-1β condition (figure3.3). The combination of both cytokines at different concentrations did not yield better synergistic effects than those shown at 1ng/ml (data not shown). Taken together, these results demonstrate that the synergy observed between IL-17 and IL-1β is mediated in part at the level of the CXCL8 promoter.

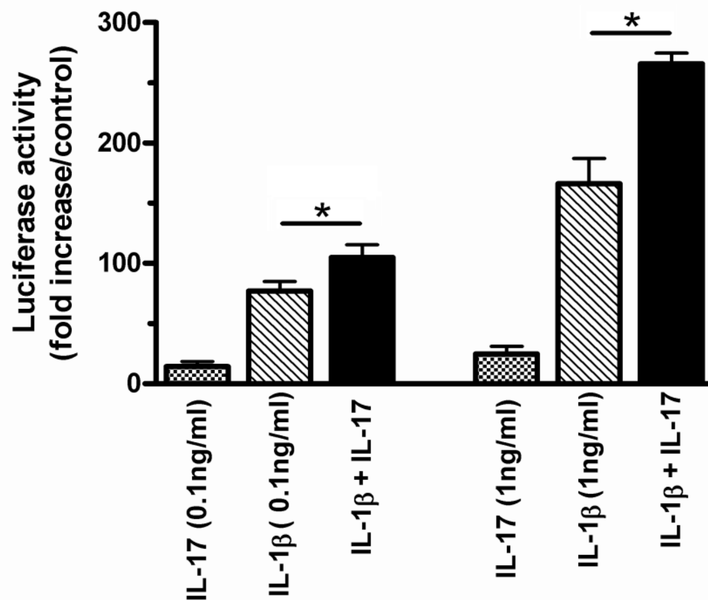

Figure 3. 3. IL-17 enhances IL-1β mediated CXCL8 promoter activity
ASM cells were transiently transfected with the CXCL8 promoter luciferase construct and stimulated with 0.1 or 1ng/ml of IL-17, IL-1β or in combination for 12h. Values are presented as the mean fold increase of luciferase activity normalized to the mock renilla luciferase activity from 4 different donors (*p<0.05).

3.4.4. IL-17 synergizes with IL-1β and requires intact AP-1 and NF-κB response elements

To ascertain which transcription factor binding sites were functionally important in mediating CXCL8 synergistic response between IL-17 and IL-1β, mutated binding sites for AP-1, NF-κB or double AP-1/ NF-κB mutants bearing the CXCL8 promoter luciferase construct were transiently transfected into ASM cells. Following stimulation with 1ng/ml of IL-17, IL-1β or in combination, the luciferase activity for the mutated AP-1 or NF-κB constructs were significantly reduced but were completely abolished in the double mutant (330.3±41.5 fold increase of WT over mock controls, 33.1±2.9 for the AP-1 mutant, 0.8±0.1 for the NF-κB mutant and 0.1±0.1 for the double mutant, figure3.4). These data suggest that the presence of the AP-1 binding site is important for the induction of CXCL8 by IL-17 but that the NF-κB binding site is essential. Deletion of both binding sites led to the abrogation of the CXCL8 promoter activity indicating that the cooperation between AP-1 and NF-κB cis-acting elements is crucial for the induction of the synergistic response.

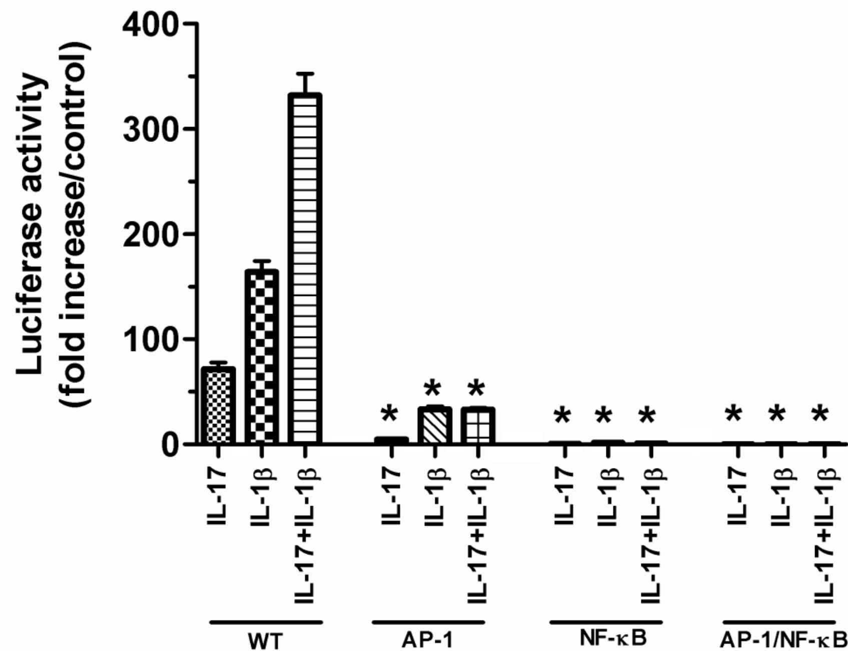

Figure 3. 4. IL-17 synergy with IL-1β requires functional AP-1 and NF-κB cis-acting response elements
ASM cells were transfected with wild-type, AP-1, NF-κB or AP-1/NF-κB double mutant CXCL8 promoter luciferase constructs and stimulated for 12h with 1ng/ml of IL-17, IL-1β or in combination. Values are presented as the mean fold increase of luciferase activity normalized to the mock renilla luciferase activity from 4 different donors (*$p<0.05$).

3.4.5. IL-17 enhances IL-1β mediated CXCL8 promoter activity through p38, ERK1/2 MAPK and the PI3-K pathway

To elucidate the molecular signaling mechanism mediated by IL-17 and IL-1β, CXCL8 promoter activity was assessed in the presence of selective pharmacological inhibitors for p38 (SB203580), MEK-ERK1/2 (U0126) and PI3-K (wortmannin). Pretreatment of ASM cells with SB203580 and U0126 caused a significant inhibition of 40.1±20.7% and 78.6±3.8% in the promoter activity induced by IL-17 compared to the respective control conditions ($p<0.05$, figure3.5a). In contrast, inhibition of PI3-K with wortmannin had no effect on IL-17-induced CXCL8 promoter activity (figure3.5a). Pretreatment with SB203580, U0126 or wortmannin

significantly decreased IL-1β-induced CXCL8 promoter activity by 28.8±14.8%, 77.8±2.9% and 51.8±11.4% respectively (figure3.5b).

The combination or treatment of individual cytokines in SB203580 pretreated cell conditions decreased the CXCL8 promoter activity by 32.5±5.1%. Interestingly, the CXCL8 promoter activity induced by the combination of IL-17 and IL-1β was less sensitive to inhibition by U0126 by approximately 2.6±0.4 fold compared to individual cytokines. This suggests that compensating, signaling pathways were activated by combining IL-17 with IL-1β (figure3.5c). Similarly, wortmannin which decreased CXCL8 promoter activity by 24.2±4.1% in the combined IL-17 and IL-1β condition was 1.6±0.4 fold more effective in suppressing the promoter activity mediated by IL-1β alone. Therefore, in addition to the MAPK and PI3-K pathways, IL-17 and IL-1β synergize to activate alternative signaling pathways mediating CXCL8 promoter responses.

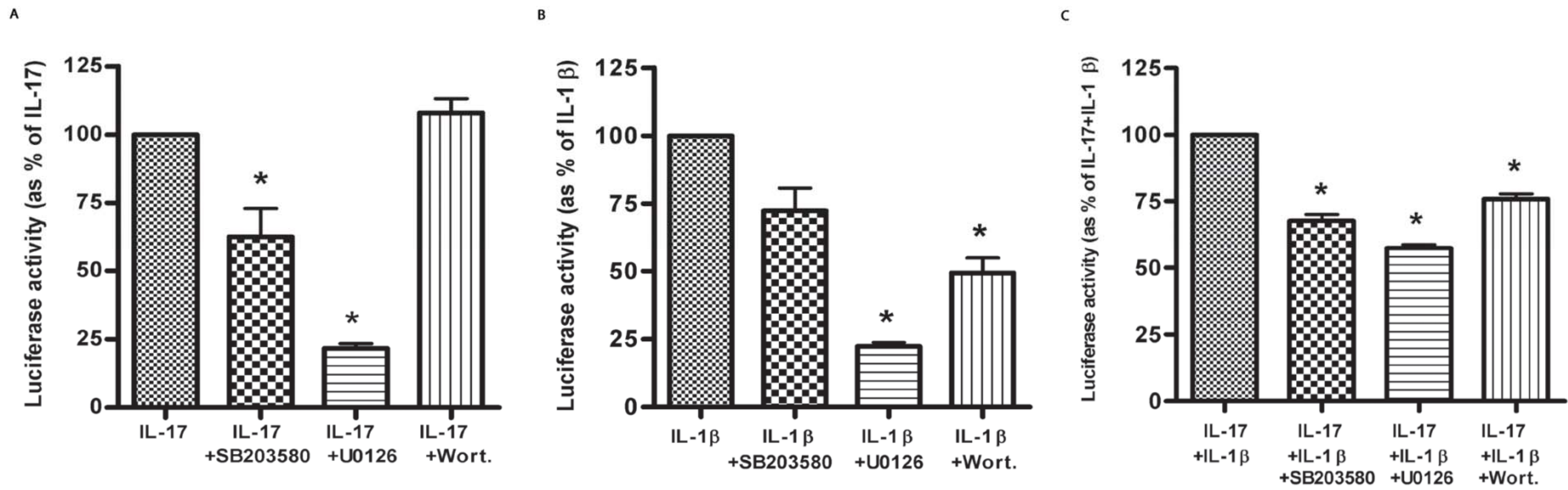

Figure 3. 5. p38, ERK1/2 and PI3-K modulate the synergistic activation of the CXCL8 promoter in response to IL-17 and IL-1β
FBS-deprived ASM cells were pretreated for 1h with SB203580 (10μM), U0126 (10μM), wortmannin (100nM) or vehicle (DMSO) and were stimulated with IL-17A (A), IL-1β (B) or in combination (C) for 12h. Results are expressed as percentages of fold increases over transfected cells stimulated with IL-17, IL-1β or both in the absence of the respective inhibitor (mean±SEM , n=4, *$p<0.05$).

3.5.0. Discussion

Increasing attention has been drawn to the synthetic properties of ASM for promoting the inflammatory component of airway disorders. Besides directing bronchomotor tone, ASM can release a multitude of cytokines which can contribute to the recruitment and activation of inflammatory cells in the airways (4). Of note, CXCL8 a neutrophil attracting chemokine, and IL-17, a T lymphocyte-derived cytokine mediating neutrophilic inflammation, play important roles in the recruitment of neutrophils into the airways (19). Previously, we investigated whether human ASM cells could respond and recruit neutrophils via chemokines by assessing basal mRNA and cell surface expression levels of IL-17RA (30). In this study, we further investigated the upstream signaling mechanisms and the synergistic response mediated by IL-17 and IL-1β which could play a critical role in promoting airway inflammation. We demonstrate that IL-17 enhances IL-1β-mediated CXCL8 mRNA expression and protein levels from primary human ASM cells and that this effect is regulated by the p38, ERK1/2 MAPKs and the PI3-K pathway. We further demonstrate that this response is dependent on the cooperation of the AP-1 and NF-κB cis-acting elements in the proximal CXCL8 promoter and on post-transcriptional mechanisms regulating mRNA stability.

AP-1 and NF-κB cis-acting elements have previously been identified as primary binding sites for the transcription and superinduction of CXCL8 (30, 36, 37). The molecular mechanisms regulating the function of IL-17 in enhancing IL-1β or TNF-induced CXCL8 and IL-6 protein expression have been proposed to occur post-transcriptionally via mRNA stabilization (25, 29). CXCL8 mRNA rate of decay is decreased 4.6 fold when co-treated with IL-17 and TNF which results in higher yields of mRNA levels 12h post-stimulation as compared to the TNF-treated

condition (28). Similarly, when IL-17 was combined with IL-1β, we observed a 2.1 fold increase in CXCL8 mRNA half-life in comparison to that of the IL-1β stimulated condition (figure3.2c). However, identification of common transcriptional regulatory elements in IL-17 gene targets such as 24p3/ lipocalin-2 has also been demonstrated to be directly regulated at the transcriptional level (38). Results from our luciferase activity assay also suggest that IL-17 enhances AP-1 and NF-κB promoter binding sites in a cooperative fashion in order to synergize IL-1β-mediated induction of CXCL8. Therefore, the synergistic effect upheld by IL-17 on IL-1β-mediated CXCL8 production stems from transcriptional and post-transcriptional regulatory mechanisms.

Other groups have investigated inflammatory effects of IL-17 on ASM cells. To date, only CXCL8, eotaxin/ CCL-11 and 8-isoprostane, a biomarker of oxidative stress, have been identified as direct, IL-17-inducible products in ASM cells *in vitro* (30, 35, 39, 40). Although IL-6 superinduction and protein synthesis have been observed in co-treated conditions of IL-17 and TNF, IL-17 alone or in combination with IL-1β fails to enhance IL-6 or granulocyte macrophage-colony stimulating factor (GM-CSF) production after 24h (29). This immuno-regulatory function of ASM cells seems to differ from other lung structural cells such as fibroblasts for which IL-17 has been demonstrated to upregulate pro-fibrotic cytokines (IL-6, IL-11), ELR-chemokines (CXCL8), growth factors (G-CSF) and to mediate synergistic responses with TNF (21, 25, 26, 41). Thus, IL-17 may selectively enhance synthetic functions of ASM cells depending on the nature of the inflammatory stimulus.

MAPKs and NF-κB activation are downstream effector functions of both the IL-1R and IL-17RA signaling pathways (42, 43). We sought to investigate the signal transduction pathways regulating the activity of the NF-κB-dependent CXCL8 promoter. Similarly to Wuyts and colleagues, our results demonstrate a critical function for ERK1/2 since inhibition of upstream MEK1/2 kinase with U0126 inhibited 79 and 78% of CXCL8 promoter activity by IL-17 or IL-1β, respectively (44). Interestingly, when both cytokines were used in combination, CXCL8 promoter activity was increased 1.8 fold suggesting these cytokine cross-talked to activate alternative pathways mediating CXCL8 promoter activity. p38 MAPK pathway is a potential candidate to mediate the synergistic effects of IL-17 and IL-1β since it has been demonstrated to regulate AP-1 transcriptional activity in human vascular smooth muscle cells and to suppress mRNA destabilization mechanisms by targeting AU-rich elements in epithelial cells (45). Our results demonstrate that inhibition of p38 with SB203580 decreased IL-17- and IL-1β-mediated CXCL8 promoter activity by 40 and 21%, but that the combination of both cytokines did not cooperate to further decrease, or increase CXCL8 promoter activity. Taken together, these results suggest that p38 may hold a regulatory function in enhancing CXCL8 promoter activity.

Recently, IL-6 and CXCL8 production from rheumatoid arthritis synovial fibroblasts in response to IL-17 has been shown to signal via NF-κB-dependent and PI3-K/ AKT-dependent pathways (46). Since the PI3-K pathway also activates AP-1 and NF-κB trans-acting elements in response to IL-1 (47), we sought to determine whether this pathway regulated IL-17 and IL-1β synergistic induction of CXCL8. In contrast to IL-1β, inhibition with wortmannin did not affect IL-17-mediated CXCL8 promoter activity in ASM cells suggesting this pathway is not involved for the induction of CXCL8 by IL-17. Interestingly, the combination of both cytokines increased

CXCL8 promoter activity implying that MAPKs amid other signaling pathways play a critical role in regulating the synergistic response.

In conclusion, we demonstrate that IL-17 and IL-1β can individually or in concert, synergize to enhance the promoter activity of CXCL8, mRNA induction, transcript stability and protein synthesis via the cooperative activation of AP-1 and NF-κB in ASM cells. Inhibition of ERK1/2 in either the IL-17 or IL-1β signal transduction pathways led to the greatest decrease in CXCL8 promoter activity whereas p38 appeared to mediate a significant function in modulating the synergistic effect at the transcriptional level. Additionally, the PI3-K pathway activated by IL-1β was also required for the full induction of the synergistic response. Taken together, ASM cells in the presence of IL-17 and IL-1β may amplify the recruitment of neutrophils into the airways and may thus represent a key mechanism for the rapid induction and/ or perpetuation of airway inflammatory responses.

3.6.0. Acknowledgments

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3.7.0. References

1. Amrani, Y., and R. A. Panettieri, Jr. 1998. Cytokines induce airway smooth muscle cell hyperresponsiveness to contractile agonists. *Thorax* 53:713-716.
2. Halayko, A. J., and Y. Amrani. 2003. Mechanisms of inflammation-mediated airway smooth muscle plasticity and airways remodeling in asthma. *Respir Physiol Neurobiol* 137:209-222.
3. Howarth, P. H., A. J. Knox, Y. Amrani, O. Tliba, R. A. Panettieri, Jr., and M. Johnson. 2004. Synthetic responses in airway smooth muscle. *J Allergy Clin Immunol* 114:S32-50.

4. Lazaar, A. L., and R. A. Panettieri, Jr. 2006. Airway smooth muscle as a regulator of immune responses and bronchomotor tone. *Clin Chest Med* 27:53-69, vi.
5. Stewart, A. G. 2001. Airway wall remodelling and hyperresponsiveness: modelling remodelling in vitro and in vivo. *Pulm Pharmacol Ther* 14:255-265.
6. Chung, K. F., and P. J. Barnes. 1999. Cytokines in asthma. *Thorax* 54:825-857.
7. Chung, K. F. 2001. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 34:50s-59s.
8. Bhatia, M., and S. Moochhala. 2004. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. *J Pathol* 202:145-156.
9. Lappalainen, U., J. A. Whitsett, S. E. Wert, J. W. Tichelaar, and K. Bry. 2005. Interleukin-1beta causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung. *Am J Respir Cell Mol Biol* 32:311-318.
10. Johnson, V. J., B. Yucesoy, and M. I. Luster. 2005. Prevention of IL-1 signaling attenuates airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma. *J Allergy Clin Immunol* 116:851-858.
11. Xiao, W., Y. P. Hsu, A. Ishizaka, T. Kirikae, and R. B. Moss. 2005. Sputum cathelicidin, urokinase plasminogen activation system components, and cytokines discriminate cystic fibrosis, COPD, and asthma inflammation. *Chest* 128:2316-2326.
12. Cromwell, O., Q. Hamid, C. J. Corrigan, J. Barkans, Q. Meng, P. D. Collins, and A. B. Kay. 1992. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-1 beta and tumour necrosis factor-alpha. *Immunology* 77:330-337.
13. Govindaraju, V., M. C. Michoud, M. Al-Chalabi, P. Ferraro, W. S. Powell, and J. G. Martin. 2006. Interleukin-8: Novel roles in human airway smooth muscle cell contraction and migration. *Am J Physiol Cell Physiol*.
14. Pease, J. E., and I. Sabroe. 2002. The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. *Am J Respir Med* 1:19-25.
15. Center, D. M., H. Kornfeld, M. J. Wu, M. Falvo, A. C. Theodore, J. Bernardo, J. S. Berman, W. W. Cruikshank, R. Djukanovic, L. Teran, and et al. 1994. Cytokine binding to CD4+ inflammatory cells: implications for asthma. *Am J Respir Crit Care Med* 150:S59-62.
16. Ennis, M. 2003. Neutrophils in asthma pathophysiology. *Curr Allergy Asthma Rep* 3:159-165.
17. Park, H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.
18. Laan, M., Z. H. Cui, H. Hoshino, J. Lotvall, M. Sjostrand, D. C. Gruenert, B. E. Skoogh, and A. Linden. 1999. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* 162:2347-2352.
19. Linden, A. 2001. Role of interleukin-17 and the neutrophil in asthma. *Int Arch Allergy Immunol* 126:179-184.
20. Fossiez, F., O. Djossou, P. Chomarat, L. Flores-Romo, S. Ait-Yahia, C. Maat, J. J. Pin, P. Garrone, E. Garcia, S. Saeland, D. Blanchard, C. Gaillard, B. Das Mahapatra, E. Rouvier, P. Golstein, J. Banchereau, and S. Lebecque. 1996. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* 183:2593-2603.

21. Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, N. Page, R. Olivenstein, J. Elias, and J. Chakir. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 108:430-438.
22. Barczyk, A., W. Pierzchala, and E. Sozanska. 2003. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med* 97:726-733.
23. Prause, O., S. Bozinovski, G. P. Anderson, and A. Linden. 2004. Increased matrix metalloproteinase-9 concentration and activity after stimulation with interleukin-17 in mouse airways. *Thorax* 59:313-317.
24. Hoshino, H., M. Laan, M. Sjostrand, J. Lotvall, B. E. Skoogh, and A. Linden. 2000. Increased elastase and myeloperoxidase activity associated with neutrophil recruitment by IL-17 in airways in vivo. *J Allergy Clin Immunol* 105:143-149.
25. van den Berg, A., M. Kuiper, M. Snoek, W. Timens, D. S. Postma, H. M. Jansen, and R. Lutter. 2005. Interleukin-17 induces hyperresponsive interleukin-8 and interleukin-6 production to tumor necrosis factor-alpha in structural lung cells. *Am J Respir Cell Mol Biol* 33:97-104.
26. Jones, C. E., and K. Chan. 2002. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am J Respir Cell Mol Biol* 26:748-753.
27. Laan, M., O. Prause, M. Miyamoto, M. Sjostrand, A. M. Hytonen, T. Kaneko, J. Lotvall, and A. Linden. 2003. A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-alpha. *Eur Respir J* 21:387-393.
28. Henness, S., E. van Thoor, Q. Ge, C. L. Armour, J. M. Hughes, and A. J. Ammit. 2006. IL-17A acts via p38 MAPK to increase stability of TNF-alpha-induced IL-8 mRNA in human ASM. *Am J Physiol Lung Cell Mol Physiol* 290:L1283-1290.
29. Henness, S., C. K. Johnson, Q. Ge, C. L. Armour, J. M. Hughes, and A. J. Ammit. 2004. IL-17A augments TNF-alpha-induced IL-6 expression in airway smooth muscle by enhancing mRNA stability. *J Allergy Clin Immunol* 114:958-964.
30. Rahman, M. S., J. Yang, L. Y. Shan, H. Unruh, X. Yang, A. J. Halayko, and A. S. Gounni. 2005. IL-17R activation of human airway smooth muscle cells induces CXCL-8 production via a transcriptional-dependent mechanism. *Clin Immunol* 115:268-276.
31. Tran, T., D. J. Fernandes, M. Schuliga, T. Harris, L. Landells, and A. G. Stewart. 2005. Stimulus-dependent glucocorticoid-resistance of GM-CSF production in human cultured airway smooth muscle. *Br J Pharmacol* 145:123-131.
32. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5299.
33. Gounni, A. S., Q. Hamid, S. M. Rahman, J. Hoeck, J. Yang, and L. Shan. 2004. IL-9-mediated induction of eotaxin1/CCL11 in human airway smooth muscle cells. *J Immunol* 173:2771-2779.
34. Wilson, G. M., J. Lu, K. Sutphen, Y. Sun, Y. Huynh, and G. Brewer. 2003. Regulation of A + U-rich element-directed mRNA turnover involving reversible phosphorylation of AUF1. *J Biol Chem* 278:33029-33038.
35. Rahman, M. S., A. Yamasaki, J. Yang, L. Shan, A. J. Halayko, and A. S. Gounni. 2006. IL-17A Induces Eotaxin-1/CC Chemokine Ligand 11 Expression in Human Airway Smooth Muscle Cells: Role of MAPK (Erk1/2, JNK, and p38) Pathways. *J Immunol* 177:4064-4071.

36. Mukaida, N., Y. Mahe, and K. Matsushima. 1990. Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. *J Biol Chem* 265:21128-21133.
37. Roger, T., T. Out, N. Mukaida, K. Matsushima, H. Jansen, and R. Lutter. 1998. Enhanced AP-1 and NF-kappaB activities and stability of interleukin 8 (IL-8) transcripts are implicated in IL-8 mRNA superinduction in lung epithelial H292 cells. *Biochem J* 330 (Pt 1):429-435.
38. Shen, F., Z. Hu, J. Goswami, and S. L. Gaffen. 2006. Identification of common transcriptional regulatory elements in interleukin-17 target genes. *J Biol Chem*.
39. Vanaudenaerde, B. M., W. A. Wuyts, L. J. Dupont, D. E. Van Raemdonck, M. M. Demedts, and G. M. Verleden. 2003. Interleukin-17 stimulates release of interleukin-8 by human airway smooth muscle cells in vitro: a potential role for interleukin-17 and airway smooth muscle cells in bronchiolitis obliterans syndrome. *J Heart Lung Transplant* 22:1280-1283.
40. Wuyts, W. A., B. M. Vanaudenaerde, L. J. Dupont, D. E. Van Raemdonck, M. G. Demedts, and G. M. Verleden. 2004. N-acetylcysteine inhibits interleukin-17-induced interleukin-8 production from human airway smooth muscle cells: a possible role for anti-oxidative treatment in chronic lung rejection? *J Heart Lung Transplant* 23:122-127.
41. Ning, W., A. M. Choi, and C. Li. 2005. Carbon monoxide inhibits IL-17-induced IL-6 production through the MAPK pathway in human pulmonary epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 289:L268-273.
42. Awane, M., P. G. Andres, D. J. Li, and H. C. Reinecker. 1999. NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. *J Immunol* 162:5337-5344.
43. Gerthoffer, W. T., and C. A. Singer. 2003. MAPK regulation of gene expression in airway smooth muscle. *Respir Physiol Neurobiol* 137:237-250.
44. Wuyts, W. A., B. M. Vanaudenaerde, L. J. Dupont, D. E. Van Raemdonck, M. G. Demedts, and G. M. Verleden. 2005. Interleukin-17--induced interleukin-8 release in human airway smooth muscle cells: role for mitogen-activated kinases and nuclear factor-kappaB. *J Heart Lung Transplant* 24:875-881.
45. Winzen, R., M. Kracht, B. Ritter, A. Wilhelm, C. Y. Chen, A. B. Shyu, M. Muller, M. Gaestel, K. Resch, and H. Holtmann. 1999. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *Embo J* 18:4969-4980.
46. Hwang, S. Y., J. Y. Kim, K. W. Kim, M. K. Park, Y. Moon, W. U. Kim, and H. Y. Kim. 2004. IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-kappaB- and PI3-kinase/Akt-dependent pathways. *Arthritis Res Ther* 6:R120-128.
47. Reddy, S. A., J. H. Huang, and W. S. Liao. 1997. Phosphatidylinositol 3-kinase in interleukin 1 signaling. Physical interaction with the interleukin 1 receptor and requirement in NFkappaB and AP-1 activation. *J Biol Chem* 272:29167-29173.

4.0. CHAPTER 4

INTERLEUKIN-17 ATTENUATES THE ANTI-APOPTOTIC EFFECTS OF GRANULOCYTE-MACROPHAGE-COLONY STIMULATING FACTOR IN HUMAN NEUTROPHILS

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Stéphane Dragon, Arash Shoja Saffar, Lianyu Shan, Abdelilah Soussi Gounni

Department of Immunology and the CIHR National Training Program in Allergy and Asthma, University of Manitoba, Winnipeg, Manitoba, Canada.

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SD drafted and produced 100% of the experiments required for the realization of this manuscript.

4.1.0. ABSTRACT

Interleukin (IL)-17A is a pleiotropic, proinflammatory cytokine that is implicated in chronic inflammatory and degenerative disorders. IL-17 has been demonstrated to link activated T lymphocytes to the recruitment of neutrophils in sites of inflammation, however whether IL-17 can mediate neutrophil survival and subsequently affect inflammatory responses has not fully been elucidated. In this study, we demonstrate that human peripheral blood and HL-60 differentiated neutrophils express the cell surface IL-17A receptor (IL-17RA). Additionally, although IL-17A did not affect the rate of spontaneous neutrophil apoptosis *in vitro*, it significantly decreased granulocyte macrophage-colony stimulating factor (GM-CSF)-mediated survival by antagonizing the signal transduction pathways of the p38 and ERK1/2 mitogen-activated protein kinases (MAPKs) and the signal transducer and activator of transcription (STAT) 5B. These events were associated with reduced myeloid cell lymphoma-1 (Mcl-1) protein levels, decreased mitochondrial transmembrane potential and increased translocation and aggregation of Bax to the mitochondria and in an increase of caspase-3/7 activity. Furthermore, these events were independent of increased Fas or soluble Fas ligand expression levels. Taken together, our findings suggest that IL-17 may regulate neutrophil homeostasis and favor the resolution of inflamed tissues by attenuating the delay in neutrophil apoptosis induced by inflammatory cytokines.

4.2.0. INTRODUCTION

Human neutrophils are the most abundant granulocyte accounting for 60% of the total circulating leukocytes found in blood (1, 2). Although shorted lived, polymorphonuclear leukocytes are actively recruited to sites of inflammation by a multitude of chemotactic and infectious agents which can boost their survival. Endothelial transmigration (3), cytokines (4) and bacterial agents such as lipopolysaccharide (LPS) (5) have been identified as neutrophilic survival factors. These factors can contribute to an acute or chronic state of inflammation by prolonging neutrophil activation and delaying the onset of apoptosis. The removal of apoptotic neutrophils is an essential factor in the resolution of the inflammatory response as it prevents damage to healthy tissues that would otherwise occur by necrotic cell lysis (6).

Since neutrophils undergo spontaneous cell death without the need of a positive death signal, anti-apoptotic signals generated by pro-survival factors appear to coordinate the fate of these cells (7). Of note, myeloid cell lymphoma-1 (Mcl-1) retains a principal function in the prolonged survival of these inflammatory cells (8, 9). Cellular levels of Mcl-1 in human neutrophils are collectively associated with increased cell survival and its role as a primary modulator of apoptosis is further accentuated by the absence of B cell lymphoma protein-2 (Bcl-2) and Bcl-X_L (10, 11).

Interleukin (IL)-17 is a pleiotropic, proinflammatory cytokine released by activated $CD4^+$ and $CD8^+$ T lymphocytes predominantly expressing the CD45RO phenotype. IL-17 has been established as a neutrophilic chemoattractant intermediate and links antigen specific T cell with the development and recruitment of neutrophils through the induction of myeloid growth factors

and chemokines from macrophages, fibroblasts, epithelial and endothelial cells (12-16). Elevated levels of IL-17 have also been associated with acute and chronic inflammatory disorders such as inflamed tissue from bacterial infections (17, 18), synovial fluid of arthritic patients (19) and from the sera or bronchoalveolar lavage fluid of asthmatic patients (20, 21). Nevertheless, whether IL-17 has the ability to mediate the activity or the survival capacity of neutrophils and heighten inflammatory responses remains to be fully investigated.

Our present study aims to determine the functional implication of IL-17A in relation to the survival outcomes of human neutrophils. Our results demonstrate that human peripheral blood and differentiated HL-60 neutrophils constitutive expressly cell surface IL-17RA. Stimulation with IL-17A did not induce neutrophil apoptosis however it attenuated the survival effects of GM-CSF when added in combination. Mechanistically, IL-17 antagonized the p38, ERK1/2 (p42/p44) and signal transducer and activator of transcription (STAT) 5B pathways activated by GM-CSF. This effect was associated with reduced Mcl-1 protein levels, decreased mitochondrial transmembrane potential and with an increase in Bax translocation and caspase-3/7 activity. Collectively, our results demonstrate that IL-17 can disrupt GM-CSF anti-apoptotic signaling pathways regulating neutrophil survival responses.

4.3.0. MATERIALS AND METHODS

4.3.1. Isolation and cell culture conditions

This study was approved by the Ethics Committee of the Faculty of Medicine at the University of Manitoba. Blood was collected into sterile heparin tubes from the peripheral vein of healthy volunteers. Neutrophils were separated from whole blood by the dextran, Ficoll-Paque

histopaque sedimentation (Amersham Pharmacia Biotech) and hypotonic lysis method (22). Cytological examination of stained neutrophils by the Wright-Giemsa method (Fisher Scientific) accounted for 95-98% cell purity. Cell viability was greater than 98% as determined by trypan blue dye exclusion method. Human HL-60 promyelotic cell line clone-15 was purchased from the American Type Culture Collection (Manassas, Virginia) and cultured in RPMI 1640 media supplemented with 10% heat-inactivated FBS (Hyclone Laboratories), 100units/ml penicillin and 100μg/ml streptomycin (Gibco BRL) at 37°C in 5% CO_2. Differentiation into neutrophil-like cells was induced by culturing HL-60 cells for 7 days in the presence of 1.25% DMSO v/v.

4.3.2. RT-PCR analysis

Total cellular RNA was extracted from purified peripheral blood neutrophils or from differentiated HL-60 cells by the TRIzol method (Gibco BRL). As previously described, reverse transcription was performed with 2μg of total RNA (22). Oligonucleotide specific primers were synthesized on the basis of the entire coding region of the human IL-17RA gene (GeneBank accession number NM-014339). 35 cycles were carried out for the extracellular domain of the IL-17RA (833bp), forward; 5'-CTAAACTGCACGGTCAAGAAT-3' reverse 5'-ATGAACCAGTACACCCAC-3' and 25 for GAPDH (137bp), forward; 5'-GAAGGTGAAGGTCGGAGT-3' reverse 5'-GAAGATGGTGATGGGATTC-3' (Invitrogen) in a thermal cycler (Mastercycle, Eppendorf, Germany).

4.3.3. Neutrophil apoptosis

As previously described, early apoptotic neutrophils containing intact membranes with externalized phosphatidylserine (PS) residues or late apoptotic neutrophils from which the

membrane integrity has been compromised were detected by mAb annexin-V-FITC and propidium iodide (PI) staining assays (23). Neutrophil apoptosis was also assessed by the staining characteristics of fixed, permeabilized cells exposed to PI, a DNA binding dye and analyzed by flow cytometry (Beckman Coulter, Fullerton, CA) using cell cycle parameters (23). Mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed by two-color density plot analysis of JC-1 (Alexis Biochemicals, San Diego, CA) 610nm/527nm fluorescence staining (24). Cell morphology was assessed by light microscopy for the manifestation of nuclear lobe separation, membrane blebbing and formation of dark, Giemsa-stained pyknotic nuclei (25).

4.3.4. Western blotting

Using standard laboratory protocols, neutrophils were lysed with either a NP-40 (Nonidet P-40, 0.1% SDS in PBS) or with a cystolic/ mitochondrial fractionation buffer supplemented with a protease inhibitor cocktail (Sigma) (26). Protein lysates for each sample were resolved under denaturing conditions by SDS-PAGE and were transferred onto PVDF membranes (Amersham Pharmacia Biotech). Membranes were blocked and incubated with either rabbit anti-human Mcl-1, Bax (DakoCytomation), p-p38, p-ERK1/2 (R&D Systems) or with mAb for p-STAT3 (Santa Cruz Biotechnology) or p-STAT5 (BD Bioscience) for 1h at room temperature. For loading controls, membranes were stripped for 20m using a glycine based stripping buffer (2.2M glycine, 0.5M NaCl pH4.4), blocked and subsequently incubated with either a polyclonal anti-Actin antibody (Sigma), mAb anti-MnSOD antibody (BD Bioscience) or respective antibodies detecting total signaling protein. *Densitometry Analysis* – Band intensity was measured by intergraded density values as determined by the AlphaEase FC software v3.1.2 on a Fluorchem 8800 imager (Alpha Innotech Corp., California).

4.3.5. Flow cytometry analysis of cell surface Fas (CD95) expression

Cell preparations were incubated for 30m on ice with an anti-Fas (DakoCytomation) or respective IgG isotype control mAb (Sigma) before the addition of secondary FITC-conjugated rat-anti mouse IgG (1:200) (Jackson ImmunoResearch Laboratories). Samples were washed between steps and were analyzed by FACScan (Becton Dickenson, Oxnard, CA). Results are presented as the specific mean fluorescence intensity (BD CellQuest Pro software v4.0.2).

4.3.6. Soluble Fas Ligand detection by ELISA

Supernatants were centrifuged at 1200 rpm for 7m at 4°C to remove cellular debris and stored at -80°C. Immunoreactive FasL was measured by ELISA using matched antibodies (R&D Systems) according to standard laboratory protocols. The sensitivity limit for FasL was 31.3pg/ml. Each data point represents readings from 3 independent assays performed in triplicates.

4.3.7. Microscopy

Cytopreparations were fixed with 4% paraformaldehyde for 20m at room temperature on microscope slides after cytospin centrifugation (ThermoShandon, Pittsburgh, PA). Slides were washed with 0.05M Tris-HCl buffered isotonic saline, pH 7.6 (TBS), air dried and stored at -20°C. Thawed cytopreparations were blocked for 20m with a universal blocking solution (DakoCytomation) and were incubated overnight at 4°C with an anti-IL-17RA or isotype control mAb (Jackson ImmunoResearch Laboratories, Sigma). Cytopreparations were then incubated with a rabbit anti-mouse IgG antibody (1:60) (Jackson ImmunoResearch Laboratories) for 45m

followed by alkaline phosphatase anti-alkaline phosphatase (APAAP) for 1h at room temperature. Slides were developed using the Fast-Red substrate followed by hematoxylin counterstaining. For fluorescence microscopy, slides were permeabilized with 0.01% Triton X-100 in TBS for 1m and incubated overnight with mAb anti-MnSOD and polyclonal anti-Bax antibody (10µg/ml) or respective IgG controls. Alexa Fluor 488 $F(ab')_2$ fragment and Alexa Fluor 568 secondary antibodies (10µg/ml) (Molecular Probes) were added sequentially for 1h at room temperature. Slides were extensively washed, counterstained for 5m with DAPI (2µg/ml) (Sigma) and mounted using ProLong anti-fade agent (Molecular Probes). Slides were acquired under oil immersion at 60x magnification by confocal laser scanning microscopy (Olympus IX70 inverted microscope coupled to a Fluoview confocal laser scanning system with Cooke Sensicam) and analyzed with the Fluoview software (Mississauga, Ontario).

4.3.8. Caspase-3/7 activity assay

Cultured neutrophils were washed with PBS and subjected to a lyophilized Caspase-Glo-3/7 substrate solution in combination with a Caspase-Glo-3/7 buffer (Promega). Caspase activity was acquired after 30m incubation by luminometery (model LB9501; Berthold Lumat, MD). Values are expressed in percentages of caspase-3/7 activity relative to GM-CSF treated conditions.

4.3.9. Statistics

Results are expressed as the mean±SEM. Differences between the groups were analyzed using Kruskal Wallis with Dunne test. Differences between pairs were assessed by Mann Whitney U

test using GraphPad Prism v4.0. software. *P* values <0.05 were considered statistically significant.

4.4.0. RESULTS

4.4.1. Circulating human neutrophils express mRNA and cell surface IL-17A receptors

To determine whether human neutrophils expressed the IL-17RA, mRNA and cell surface receptor expression were examined in freshly isolated neutrophils. IL-17RA mRNA was detected in neutrophils from 2 healthy donors (lanes 1 and 2) and in differentiated HL-60 neutrophil-like cells (lane 4) although not in undifferentiated HL-60 promyelocytes (lane 3). Purified CD16$^+$ human neutrophils expressed the cell surface IL-17RA by a geometric mean of 3.5±1.2 compared to isotype control (representative data shown in figure4.1b, n=15). Immunocytochemistry showed specific immunoreactivity to the anti-IL-17RA mAb (figure4.1c, red staining) in all neutrophils compared to the isotype control (figure4.1c, insert). Our results demonstrate that IL-17RA is constitutively expressed on circulating human neutrophils.

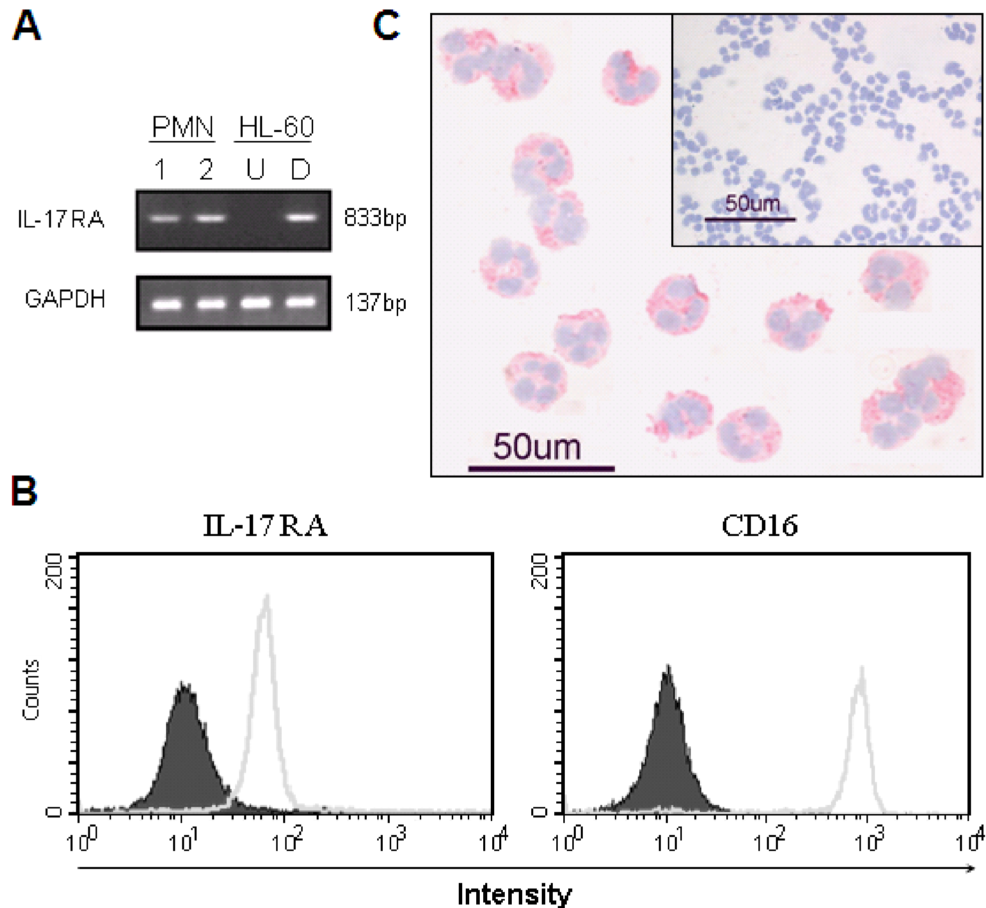

Figure 4. 1. Primary human neutrophils express mRNA and surface IL-17RA
(A) RT-PCR of IL-17RA mRNA expression in peripheral blood neutrophils. Neutrophils from healthy individuals and HL-60 differentiated neutrophils express IL-17RA transcripts (lanes 1, 2 and 4). Undifferentiated HL-60 promyelocytes did not express any detectable transcripts (lane 3). GAPDH served as loading control. (B) IL-17RA (open) had a greater incidence than IgG1 isotype control (solid) in purified $CD16^{+}$ neutrophils (right panel, n=15). (C) Immunocytochemistry of IL-17RA expression in primary human neutrophils. The isotype control is depicted in the upper quadrant.

4.4.2. IL-17 attenuates the anti-apoptotic effects of GM-CSF

To determine whether IL-17 affects neutrophil cell survival responses, spontaneous apoptosis was assessed by annexin-V/ PI staining after 6h cultures. Stimulation with IL-17 alone at 10 or 100ng/ml did not alter the percentage of annexin-V positive neutrophils compared to untreated conditions (figure4.2a, n=4). Conversely, the addition of 10ng/ml of GM-CSF significantly reduced neutrophil apoptosis as previously noted (27). The combination of GM-CSF with 10 or

100ng/ml of IL-17 increased the percentage of annexin-V positive neutrophils by 4.7±2.7% and 8.4±3.3% in comparison to GM-CSF respectively (*P*<0.05). Notably, this effect was not significant when concentrations lower than 10ng/ml of IL-17 was used. Apoptotic cell death determined by DNA fragmentation after 18h cultures also demonstrated no change in the percentage of apoptotic neutrophils treated by IL-17 compared to control conditions (figure4.2b, n=5). The addition of GM-CSF (10ng/ml) significantly reduced the appearance of apoptotic cells by 31.0±5.0% (*P*<0.01) however the combination of GM-CSF with 10 or 100ng/ml of IL-17 increased the percentage of apoptotic neutrophils by 7.2±3.4% (*P*<0.05) and 12.7±3.6% (*P*<0.01) compared to the GM-CSF stimulated condition, respectively. These results demonstrate the ability of IL-17 to reduce the anti-apoptotic effects mediated by GM-CSF on human neutrophils.

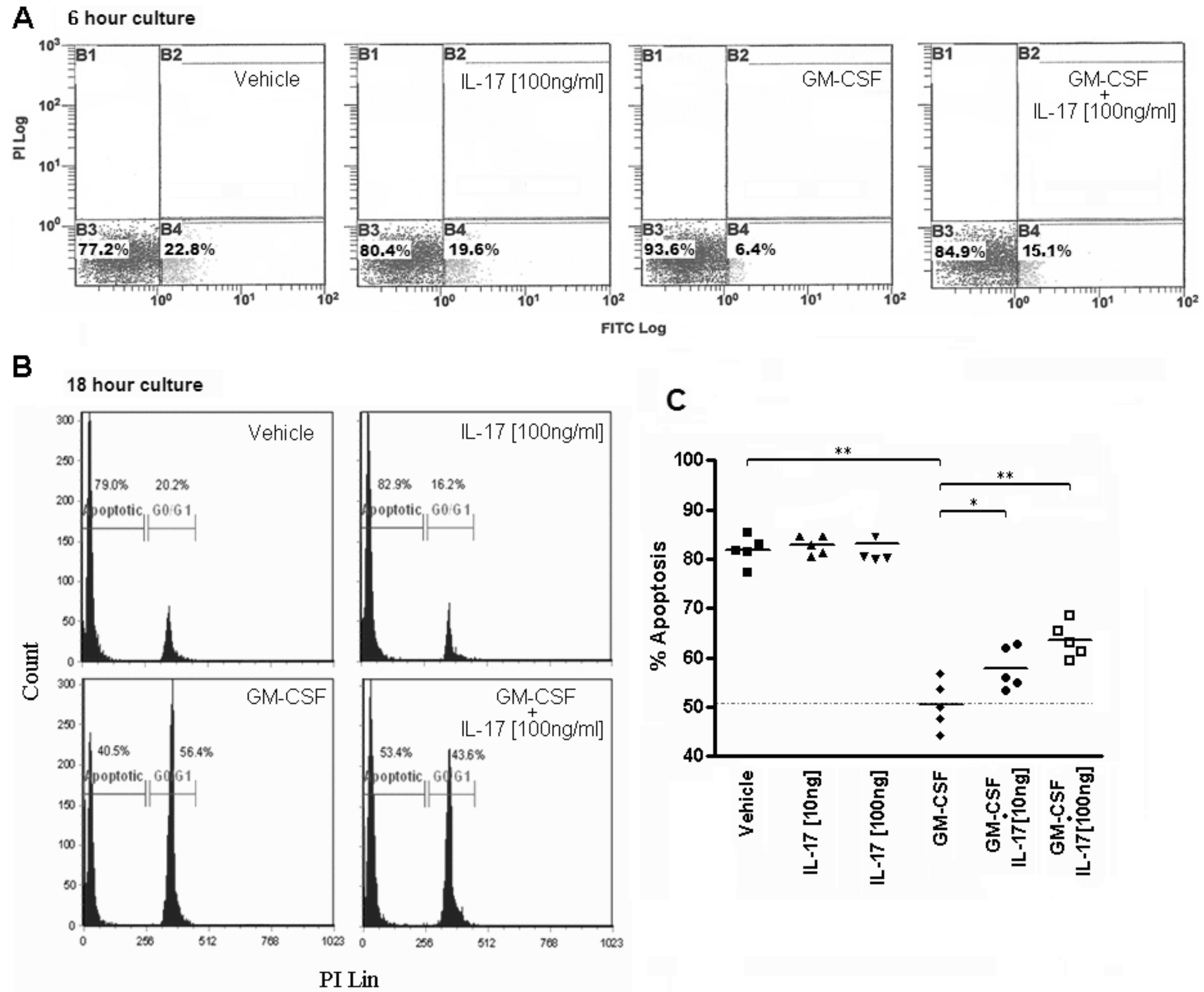

Figure 4. 2. IL-17 attenuates the anti-apoptotic effects of GM-CSF
(A) Peripheral blood neutrophils from 4 healthy donors were stimulated for 6h with 100ng/ml of IL-17 with or without GM-CSF (10ng/ml). Cells were stained with mAb Annexin-V-FITC and PI and analyzed by flow cytometry. (B) Apoptosis was determined by measuring the loss of DNA content stained by PI in neutrophils stimulated for 18h. (C) Represents scatter plot data of neutrophil apoptosis after 18h in five individuals. * $p<0.05$, ** $p<0.01$

4.4.3. IL-17 does not alter Fas or soluble FasL expression levels

To investigate the regulatory mechanisms mediated by IL-17 on GM-CSF-treated human neutrophils, cell surface Fas levels and soluble FasL (sFasL) were examined after 18h cultures. Cell surface expression levels of Fas in GM-CSF combined with IL-17-treated conditions were no different than those of untreated or GM-CSF-treated conditions (MFI: 8.8±1.5 versus 8.5±1.6

or 8.7±1.4 respectively, n=3). Moreover, no detectable differences were observed for sFasL levels by ELISA after 18h (data not shown). These results suggest IL-17 does not impair GM-CSF-induced survival via the extrinsic, FAS/ FASL apoptotic pathway.

4.4.4. IL-17 decreases GM-CSF-induced Mcl-1 protein levels and impairs the ability of GM-CSF to retain Bax in the cytosol

To gain further insight into the molecular mechanism by which IL-17 modulates GM-CSF-induced survival outcomes, Mcl-1 and Bax protein expression were examined by Western blotting. As shown in figure4.3a, treatment with IL-17 did not alter Mcl-1 protein levels compared to untreated conditions. GM-CSF increased Mcl-1 protein levels by 1.8 fold (n=3) compared to unstimulated controls but was reduced by 1.3 fold when 100ng of IL-17 was added in combination. Bax also remained in the cytosol when stimulated with GM-CSF and did not translocate to the mitochondrial fraction. This effect was comparatively reduced when IL-17 was added with GM-CSF which induced the translocation of Bax to the mitochondria. Large Bax aggregates also co-localized with clustered manganese superoxide dismutase (MnSOD) positive mitochondrial regions in apoptotic neutrophils near the outer nuclear membrane (figure4.4b). In contrast, GM-CSF maintained strong MnSOD staining over the nuclear compartment of neutrophils and prevented the formation of Bax aggregates but succumbed when IL-17 was added in combination. Bax translocation also correlated with a decrease in the mitochondrial transmembrane potential. The combination of IL-17 with GM-CSF decreased the JC-1 610/527nm ratio by 1.3 fold compared to GM-CSF-treated neutrophils (figure4.4a). Our data suggest that IL-17 down-regulates GM-CSF-mediated survival signals by disrupting the mitochondrial network and Bcl-2 family members.

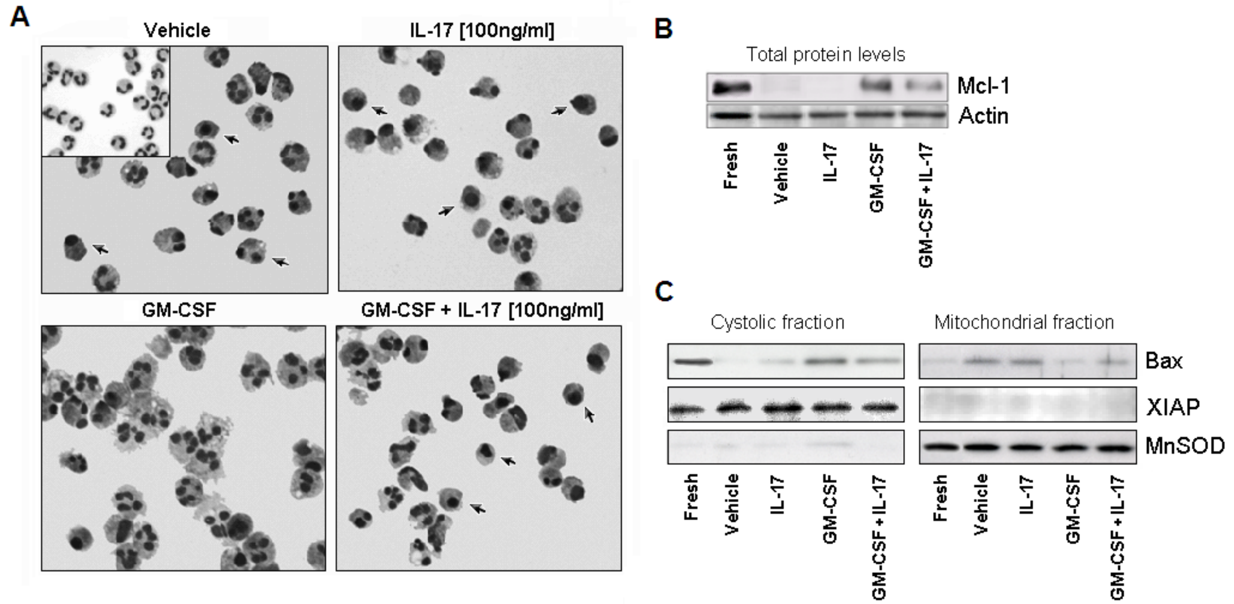

Figure 4. 3. GM-CSF-induced Mcl-1 protein and cystolic localization of Bax is disrupted by IL-17
(A) Cellular morphology of apoptotic human neutrophils (arrow) assessed by the Wright-Giemsa staining method after 18h culture (fresh cells shown in insert). (B) Total Mcl-1 protein levels or (C) translocation of Bax from cystolic to mitochondrial fractions were detected in both fresh and cultured neutrophils for 18h in the presence of 100ng/ml of IL-17 with or without GM-CSF (10ng/ml). Results are representative of 3 individuals.

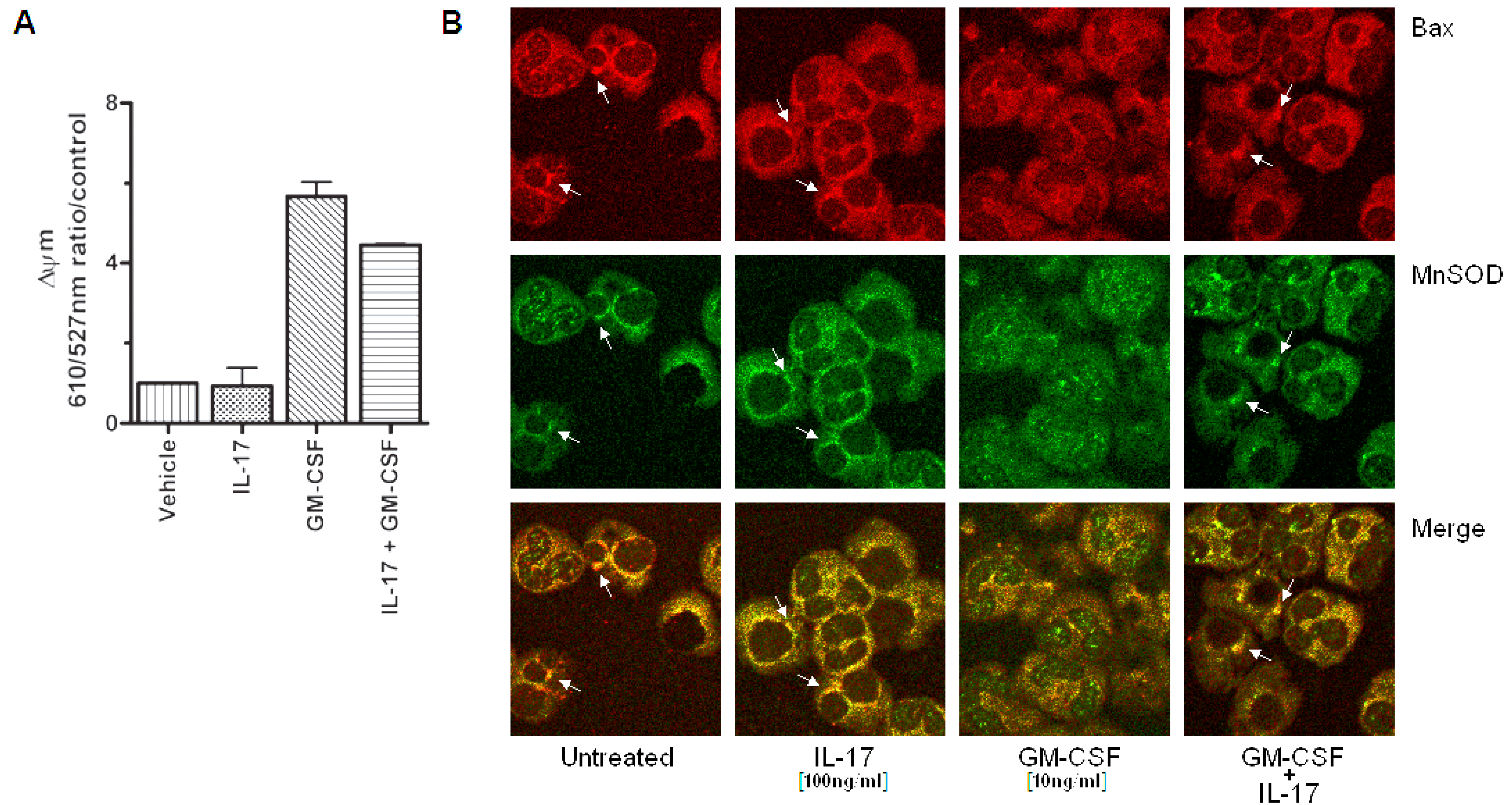

Figure 4. 4. IL-17 decreases GM-CSF-inhibition of Bax translocation, perinuclear localization and lost of mitochondrial transmembrane potential (A) Mitochondrial transmembrane potential ($\Delta\Psi_m$) was assessed after 18h by two-color density plot analysis. Bar graph represent the ratio of 610/527nm positive populations normalized to the vehicle (n=4). (B) Cellular distribution of Bax (red) and mitochondria (green) from cultured neutrophils after 18h. Apoptotic neutrophils contain perinuclear clusters (arrow) of Bax co-localized with mitochondrial MnSOD positive regions. GM-CSF reduced the amount of aggregates and preserved the systemic distribution of Bax and mitochondria. Results are representative of 3 individuals.

4.4.5. IL-17 attenuates GM-CSF-delayed caspase-3/7 activity

To confirm the negative survival effect when combining IL-17 with GM-CSF, executioner caspase-3/7 activity was measured after 18h. IL-17-stimulated conditions induced similar levels of caspase-3/7 activation as those of untreated conditions (figure4.5). Consistent with the observation that GM-CSF decreased the rate of neutrophil apoptosis, GM-CSF considerably decreased caspase-3/7 activity by 2.1±0.8 fold compared to untreated cells ($P<0.01$, n=10). Neutrophils treated with 100ng/ml of IL-17 in combination with GM-CSF expressed greater caspase-3/7 activity than GM-CSF treated cells by 1.3±0.2 fold ($P<0.05$). These results correlate with the ability of IL-17 to diminish GM-CSF-delayed apoptosis by affecting caspase-3/7 activity.

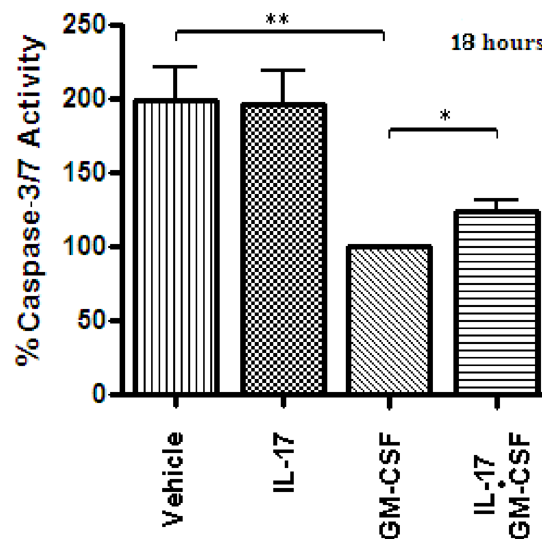

Figure 4. 5. IL-17 impairs the ability of GM-CSF to inhibit caspase-3/7 activity
Caspase-3/7 activity was measured by Caspase-GloTM fluorescence after 18h. Results are expressed as percentages of caspase-3/7 activity compared to those of GM-CSF. Results are representative of 10 individuals. * p<0.05, ** p<0.01

4.4.6. IL-17 antagonizes GM-CSF-induced p38, ERK1/2 MAPK & STAT5 signaling pathways

We next investigated the MAPKs and STATs signaling pathways mediating IL-17 and GM-CSF signal transduction. Freshly isolated neutrophils from 3 healthy donors revealed low baseline levels of phosphorylated p38 activity. GM-CSF induced strong levels of phosphorylated p38, ERK1/2, STAT3 and STAT5B within 10m of stimulation (figure4.6&4.7). Interestingly, the simultaneous combination of both cytokines decreased the intensity and duration of GM-CSF-induced p38, ERK1/2 and STAT5B phosphorylation levels although those of STAT3 were not affected. These results demonstrate IL-17 can partially antagonize the MAPK and STAT signal transduction pathways activated by GM-CSF in human neutrophils.

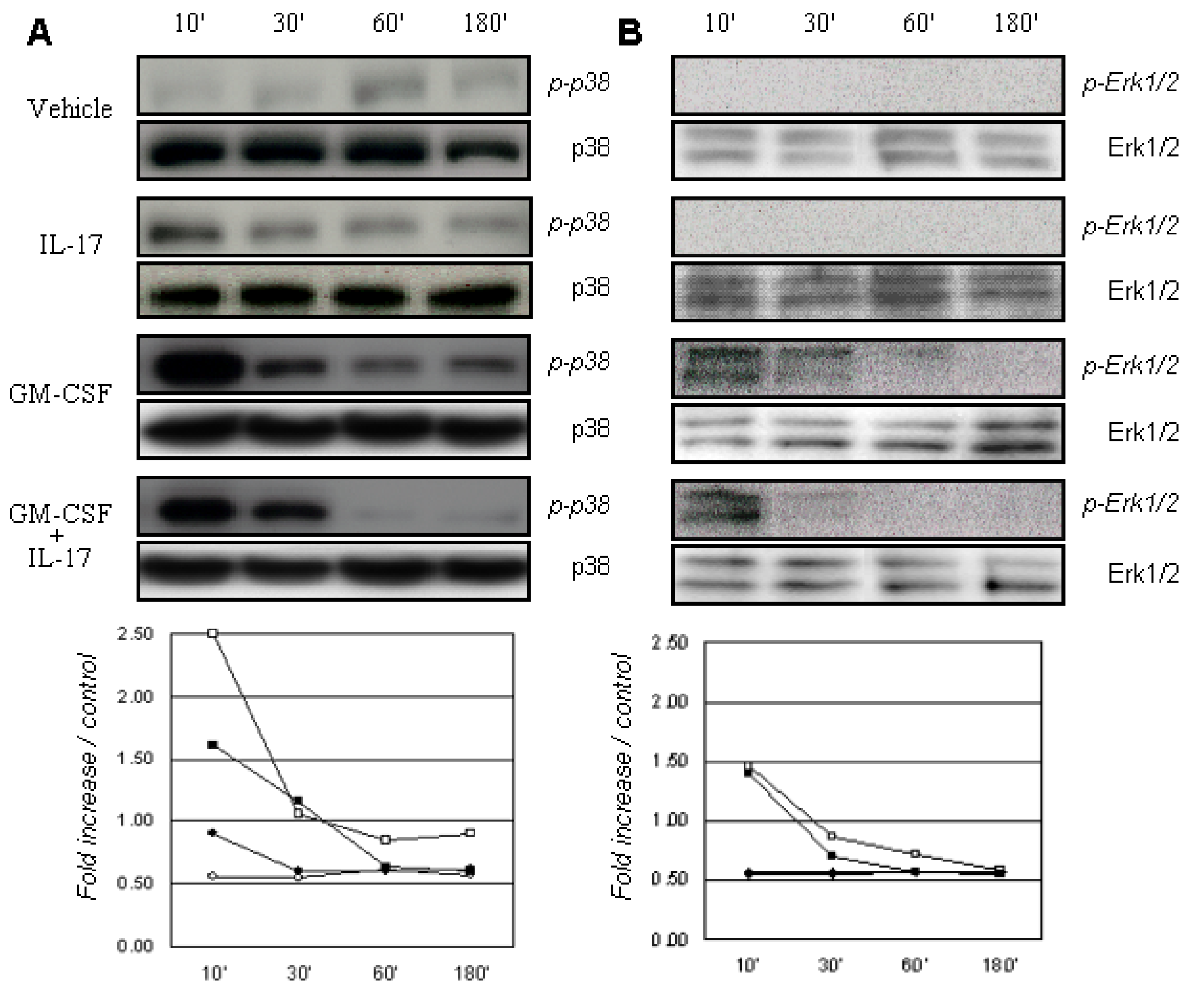

Figure 4. 6. IL-17 antagonizes GM-CSF-induced MAPK signal transduction pathways.
Western blot depicting phosphorylated or total (A) p38 or (B) ERK1/2 protein levels from neutrophils stimulated with GM-CSF or in combination with IL-17 (100ng) and are graphically quantified below. Results are representative of 2 indviduals. ○=Vehicle; ●=IL-17; □=GM-CSF; ■=GM-CSF+IL-17.

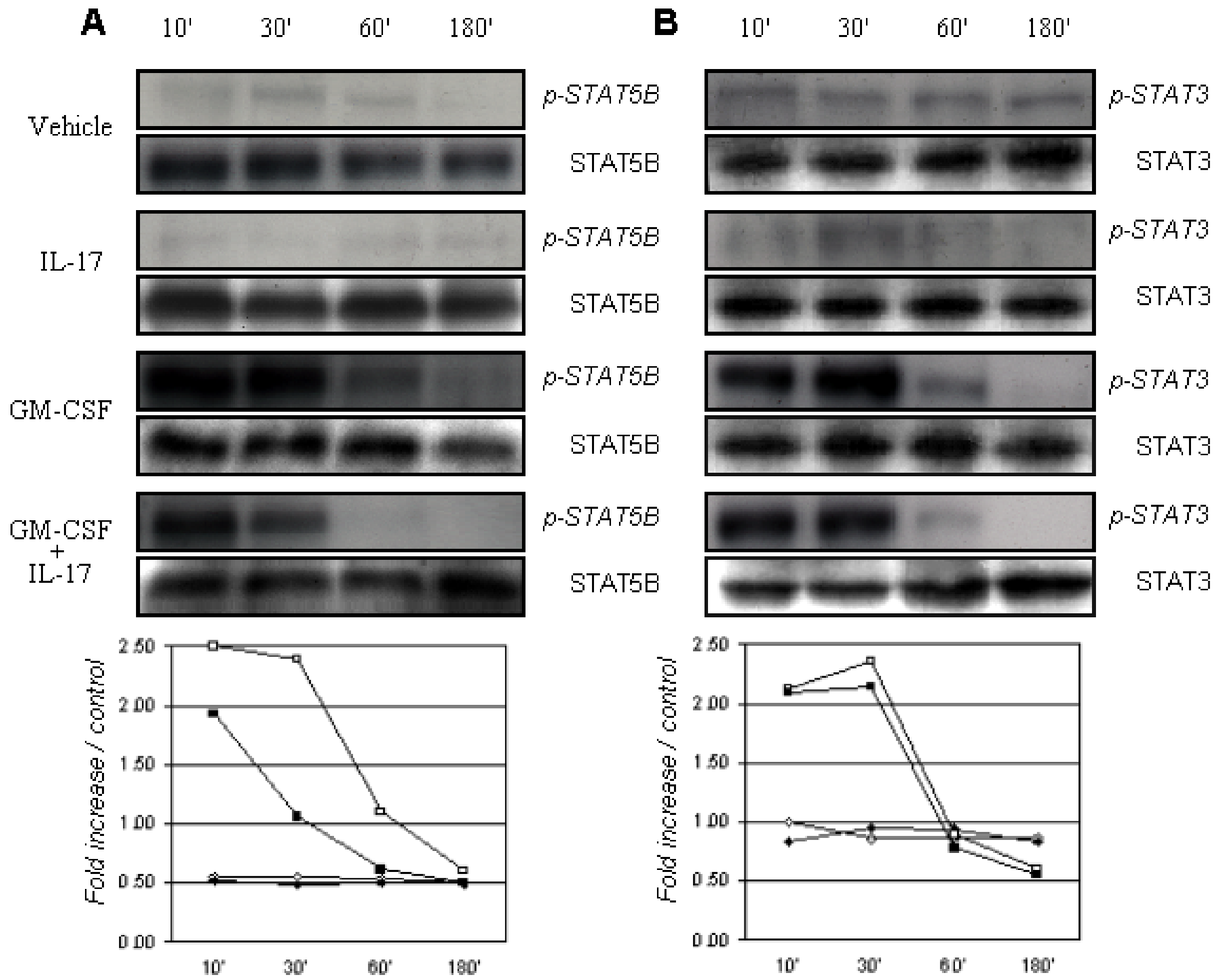

Figure 4. 7. IL-17 antagonizes GM-CSF-induced STAT signal transduction pathways
Western blot depicting phosphorylated or total (A) STAT5B or (B) STAT3 protein levels from neutrophils stimulated with GM-CSF or in combination with IL-17 (100ng) and are graphically quantified below. Results are representative of 2 individuals. ○=Vehicle; ●=IL-17; □=GM-CSF; ■=GM-CSF+IL-17.

4.5.0. Discussion

To date, the focal point of IL-17's function on myeloid cells has been limited to its ability to induce granulopoiesis and indirectly recruit neutrophils. New evidence from our study and others suggest that IL-17 may also possess anti-inflammatory properties. Andoh and colleagues have

demonstrated that IL-17 can specifically inhibit TNF mediated RANTES secretion in human colonic subepithelial myofibroblasts through an auto-regulatory mechanism preventing excessive infiltration of lymphocytes (28). Yang and colleagues identified a novel IL-17R-like protein (hSEF) in human umbilical vein endothelial cells which shared sequence homology with the intracellular domain of the IL-17RA (29). Overexpression of this receptor inhibits the induction of growth factor release in human 293T cells by antagonizing the MEK-ERK signal transduction pathway. Similarly, IL-17D was shown to directly suppress the proliferation of myeloid progenitors in colony formation assays (30). In line with our study, these reports suggest IL-17A may possess a dual role in regulating inflammation; one in the initiation and amplification of the local primary immune response by stimulating structural cells to release chemokines and myeloid growth factors, and a secondary role in moderating effector mechanisms by directly antagonizing inflammatory cells.

As previously reported, IL-17 does not alter the spontaneous rate of neutrophil apoptosis (31) however we demonstrate that IL-17 attenuates the anti-apoptotic activity of GM-CSF. A similar effect for IL-10 in LPS treated human neutrophils has also been demonstrated (32). In this study, IL-10 *per se* had no appreciable impact on the survival rate of neutrophils but significantly inhibited the survival effects of LPS by antagonizing the ERK pathway. GM-CSF has previously been demonstrated to decrease the turnover rate of Mcl-1 via a PI3-K dependent MEK-ERK signaling pathway (33) and exposure of IL-17 with GM-CSF could therefore hinder the synthesis and stability of Mcl-1 by antagonizing this pathway. GM-CSF also activates p38 however its role in mediating neutrophil apoptosis remains largely unclear. Phosphorylation of p38 and ERK1/2 have been reported to prolong neutrophil survival by phosphorylating and inactivating caspases

(34) however phosphorylated-p38 is equally required for the spontaneous induction of neutrophil apoptosis (35). Conversely, STAT3 and STAT5 are associated with the anti-apoptotic activities induced by GM-CSF (36). The inability of IL-17 to impair STAT3 phosphorylation could contribute to the limiting apoptosis-inducing effect when in combination with GM-CSF.

Alternatively, IL-17 may attenuate the anti-apoptotic effect of GM-CSF by phosphorylating and activating pro-apoptotic factors. High levels of pro-apoptotic Bcl-2 family member Bax have been observed in human neutrophils (37). Although total protein levels of Bax do not increase during the onset of apoptosis, translocation and oligomerization of the monomeric protein into the mitochondrial outer wall provokes the release of cytochrome c and of antagonists of inhibitor of apoptosis proteins (IAP) (38). GM-CSF-stimulated primary human neutrophils and PLB-895 differentiated neutrophil-like cells undergo Bax phosphorylation at serine 184 (39) which heterodimerize and becomes neutralized by Mcl-1 in the cytosol. Under the induction of apoptosis however, waning levels of Mcl-1 release Bax from the heterocomplex and allow Bax to translocate to the mitochondria and exercise its pro-apoptotic function (26). IL-17 may initiate the apoptotic process by offsetting the phosphorylation signals mediated by GM-CSF of anti- or pro-apoptotic molecules.

We also demonstrate that IL-17 can impair the ability of GM-CSF to inhibit caspase-3/7 activation. Active caspase-3/7 is an essential effector component of apoptosis which cleaves key cellular proteins and ultimately leads to the oligonucleosomal fragmentation of DNA (40, 41). Substrates from which caspase-3 may impair the signal transduction of GM-CSF include Mcl-1 (42), AKT-1 and the intracellular common β-chain of the IL-3/ IL-5/ GM-CSF receptors (43,

44). Taken together, increased caspase-3/7 activation may down-regulate survival signals transduced by GM-CSF by inactivating crucial elements necessary for these pathways.

In conclusion, we demonstrate that primary human and differentiated HL-60 neutrophils express mRNA and cell surface IL-17RA. The addition of IL-17 did not affect the rate of spontaneous neutrophil apoptosis after 18h but antagonized the MAPK and STAT signal transduction pathways activated by GM-CSF and attenuated associated anti-apoptotic signals. These effects were associated with reduced protein levels of Mcl-1, decreased mitochondrial transmembrane potential and in the increase of mitochondrial translocation of Bax and of caspase-3/7 activity. Collectively, these results suggest that IL-17 may bear a regulatory function in limiting the accumulation and/ or activity of neutrophils in inflammatory sites.

4.6.0. ACKNOWLEDGEMENTS

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4.7.0. REFERENCES

1. Houwen, B. 2001. The Differential Cell Count. *Laboratory Hematology* 7:89-100.
2. Walker, R. I., and R. Willemze. 1980. Neutrophil kinetics and the regulation of granulopoiesis. *Rev Infect Dis* 2:282-292.
3. Watson, R. W., O. D. Rotstein, A. B. Nathens, J. Parodo, and J. C. Marshall. 1997. Neutrophil apoptosis is modulated by endothelial transmigration and adhesion molecule engagement. *J Immunol* 158:945-953.
4. Lee, A., M. K. Whyte, and C. Haslett. 1993. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leukoc Biol* 54:283-288.

5. Hachiya, O., Y. Takeda, H. Miyata, H. Watanabe, T. Yamashita, and F. Sendo. 1995. Inhibition by bacterial lipopolysaccharide of spontaneous and TNF-alpha-induced human neutrophil apoptosis in vitro. *Microbiol Immunol* 39:715-723.
6. Marshall, J. C., and R. W. Watson. 1997. Programmed cell death (apoptosis) and the resolution of systemic inflammation. *Can J Surg* 40:169-174.
7. Edwards, S. W., D. A. Moulding, M. Derouet, and R. J. Moots. 2003. Regulation of neutrophil apoptosis. *Chem Immunol Allergy* 83:204-224.
8. Moulding, D. A., C. Akgul, M. Derouet, M. R. White, and S. W. Edwards. 2001. BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. *J Leukoc Biol* 70:783-792.
9. Edwards, S. W., M. Derouet, M. Howse, and R. J. Moots. 2004. Regulation of neutrophil apoptosis by Mcl-1. *Biochem Soc Trans* 32:489-492.
10. Harter, L., L. Mica, R. Stocker, O. Trentz, and M. Keel. 2003. Mcl-1 correlates with reduced apoptosis in neutrophils from patients with sepsis. *J Am Coll Surg* 197:964-973.
11. Moulding, D. A., J. A. Quayle, C. A. Hart, and S. W. Edwards. 1998. Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival. *Blood* 92:2495-2502.
12. Yao, Z., S. L. Painter, W. C. Fanslow, D. Ulrich, B. M. Macduff, M. K. Spriggs, and R. J. Armitage. 1995. Human IL-17: a novel cytokine derived from T cells. *J Immunol* 155:5483-5486.
13. Shin, H. C., N. Benbernou, S. Esnault, and M. Guenounou. 1999. Expression of IL-17 in human memory CD45RO+ T lymphocytes and its regulation by protein kinase A pathway. *Cytokine* 11:257-266.
14. Fossiez, F., O. Djossou, P. Chomarat, L. Flores-Romo, S. Ait-Yahia, C. Maat, J. J. Pin, P. Garrone, E. Garcia, S. Saeland, D. Blanchard, C. Gaillard, B. Das Mahapatra, E. Rouvier, P. Golstein, J. Banchereau, and S. Lebecque. 1996. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* 183:2593-2603.
15. Aggarwal, S., and A. Gurney. 2002. IL-17: A prototype member of an emerging cytokine family. *J Leukoc Biol* 71:1-8.
16. Jovanovic, D. V., J. A. Di Battista, J. Martel-Pelletier, F. C. Jolicoeur, Y. He, M. Zhang, F. Mineau, and J. P. Pelletier. 1998. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 160:3513-3521.
17. Johnson, R. B., N. Wood, and F. G. Serio. 2004. Interleukin-11 and IL-17 and the pathogenesis of periodontal disease. *J Periodontol* 75:37-43.
18. Luzza, F., T. Parrello, G. Monteleone, L. Sebkova, M. Romano, R. Zarrilli, M. Imeneo, and F. Pallone. 2000. Up-regulation of IL-17 is associated with bioactive IL-8 expression in Helicobacter pylori-infected human gastric mucosa. *J Immunol* 165:5332-5337.
19. Raza, K., F. Falciani, S. J. Curnow, E. J. Ross, C. Y. Lee, A. N. Akbar, J. M. Lord, C. Gordon, C. D. Buckley, and M. Salmon. 2005. Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. *Arthritis Res Ther* 7:R784-795.
20. Wong, C. K., C. Y. Ho, F. W. Ko, C. H. Chan, A. S. Ho, D. S. Hui, and C. W. Lam. 2001. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin Exp Immunol* 125:177-183.
21. Molet, S., Q. Hamid, F. Davoine, E. Nutku, R. Taha, N. Page, R. Olivenstein, J. Elias, and J. Chakir. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 108:430-438.
22. Gounni, A. S., B. Lamkhioued, L. Koussih, C. Ra, P. M. Renzi, and Q. Hamid. 2001. Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. *Faseb J* 15:940-949.
23. Gounni, A. S., B. Gregory, E. Nutku, F. Aris, K. Latifa, E. Minshall, J. North, J. Tavernier, R. Levit, N. Nicolaides, D. Robinson, and Q. Hamid. 2000. Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils. *Blood* 96:2163-2171.

24. Fossati, G., D. A. Moulding, D. G. Spiller, R. J. Moots, M. R. White, and S. W. Edwards. 2003. The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis. *J Immunol* 170:1964-1972.
25. Squier, M. K., A. J. Sehnert, and J. J. Cohen. 1995. Apoptosis in leukocytes. *J Leukoc Biol* 57:2-10.
26. Maianski, N. A., J. Geissler, S. M. Srinivasula, E. S. Alnemri, D. Roos, and T. W. Kuijpers. 2004. Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. *Cell Death Differ* 11:143-153.
27. Brach, M. A., S. deVos, H. J. Gruss, and F. Herrmann. 1992. Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. *Blood* 80:2920-2924.
28. Andoh, A., S. Fujino, S. Bamba, Y. Araki, T. Okuno, T. Bamba, and Y. Fujiyama. 2002. IL-17 selectively down-regulates TNF-alpha-induced RANTES gene expression in human colonic subepithelial myofibroblasts. *J Immunol* 169:1683-1687.
29. Yang, R. B., C. K. Ng, S. M. Wasserman, L. G. Komuves, M. E. Gerritsen, and J. N. Topper. 2003. A novel interleukin-17 receptor-like protein identified in human umbilical vein endothelial cells antagonizes basic fibroblast growth factor-induced signaling. *J Biol Chem* 278:33232-33238.
30. Starnes, T., H. E. Broxmeyer, M. J. Robertson, and R. Hromas. 2002. Cutting edge: IL-17D, a novel member of the IL-17 family, stimulates cytokine production and inhibits hemopoiesis. *J Immunol* 169:642-646.
31. Laan, M., O. Prause, M. Miyamoto, M. Sjostrand, A. M. Hytonen, T. Kaneko, J. Lotvall, and A. Linden. 2003. A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-alpha. *Eur Respir J* 21:387-393.
32. Ward, C., J. Murray, A. Clugston, I. Dransfield, C. Haslett, and A. G. Rossi. 2005. Interleukin-10 inhibits lipopolysaccharide-induced survival and extracellular signal-regulated kinase activation in human neutrophils. *Eur J Immunol* 35:2728-2737.
33. Derouet, M., L. Thomas, A. Cross, R. J. Moots, and S. W. Edwards. 2004. Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis by increasing the stability of Mcl-1. *J Biol Chem* 279:26915-26921.
34. Alvarado-Kristensson, M., F. Melander, K. Leandersson, L. Ronnstrand, C. Wernstedt, and T. Andersson. 2004. p38-MAPK signals survival by phosphorylation of caspase-8 and caspase-3 in human neutrophils. *J Exp Med* 199:449-458.
35. Aoshiba, K., S. Yasui, M. Hayashi, J. Tamaoki, and A. Nagai. 1999. Role of p38-mitogen-activated protein kinase in spontaneous apoptosis of human neutrophils. *J Immunol* 162:1692-1700.
36. Epling-Burnette, P. K., B. Zhong, F. Bai, K. Jiang, R. D. Bailey, R. Garcia, R. Jove, J. Y. Djeu, T. P. Loughran, Jr., and S. Wei. 2001. Cooperative regulation of Mcl-1 by Janus kinase/stat and phosphatidylinositol 3-kinase contribute to granulocyte-macrophage colony-stimulating factor-delayed apoptosis in human neutrophils. *J Immunol* 166:7486-7495.
37. Weinmann, P., P. Gaehtgens, and B. Walzog. 1999. Bcl-Xl- and Bax-alpha-mediated regulation of apoptosis of human neutrophils via caspase-3. *Blood* 93:3106-3115.
38. Maianski, N. A., D. Roos, and T. W. Kuijpers. 2004. Bid truncation, bid/bax targeting to the mitochondria, and caspase activation associated with neutrophil apoptosis are inhibited by granulocyte colony-stimulating factor. *J Immunol* 172:7024-7030.
39. Gardai, S. J., D. A. Hildeman, S. K. Frankel, B. B. Whitlock, S. C. Frasch, N. Borregaard, P. Marrack, D. L. Bratton, and P. M. Henson. 2004. Phosphorylation of Bax Ser184 by Akt regulates its activity and apoptosis in neutrophils. *J Biol Chem* 279:21085-21095.
40. Nicholson, D. W., A. Ali, N. A. Thornberry, J. P. Vaillancourt, C. K. Ding, M. Gallant, Y. Gareau, P. R. Griffin, M. Labelle, Y. A. Lazebnik, and et al. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376:37-43.

41. Talanian, R. V., C. Quinlan, S. Trautz, M. C. Hackett, J. A. Mankovich, D. Banach, T. Ghayur, K. D. Brady, and W. W. Wong. 1997. Substrate specificities of caspase family proteases. *J Biol Chem* 272:9677-9682.
42. Clohessy, J. G., J. Zhuang, and H. J. Brady. 2004. Characterisation of Mcl-1 cleavage during apoptosis of haematopoietic cells. *Br J Haematol* 125:655-665.
43. Yousefi, S., S. Conus, and H. U. Simon. 2003. Cross-talk between death and survival pathways. *Cell Death Differ* 10:861-863.
44. Martinez-Moczygemba, M., and D. P. Huston. 2001. Proteasomal regulation of betac signaling reveals a novel mechanism for cytokine receptor heterotypic desensitization. *J Clin Invest* 108:1797-1806.

5.0. Chapter 5

Discussion

5.1.0. Tissue-mediated inflammatory responses

In response to trauma, infection, post-ischemic or autoimmune injury, inflammation regulated by a complex set of cellular and soluble factors is initiated and results in the classical symptoms of *dolor, rubor, calor, tumor* (pain, redness, heat and swelling) [245]. Mechanistically, neural nociceptors rapidly detect mechanical, thermal and chemical changes in the micro-environment and elicit autonomic responses (pain sensation) which release neuropeptides such as autacoids to trigger vasodilatation (redness and heat) and extravasation of extracellular fluid (swelling). Immunologically, stress or nonphysiological cell death from injured cells act as danger signals for antigen presenting cells (APCs) to trigger immune responses. Necrotic cell death, HSPs, nucleic acids or hydrophobic motifs of hyaluronan degradation and bacterial products such as LPS stimulate pattern-recognition receptors to activate effector Th responses [246]. Although immune responses are generally initiated by tissue-derived signals, these signals may also tailor the effector class of an immune response by modulating the micro-environment of APCs. Such signals, which may mediate organ specific responses to minimize tissue damage could be delivered by innate, tissue resident lymphocytes such as skin-γδ T cells, gut-γδ intraepithelial lymphocytes, liver-NKT cells, lung-alveolar macrophages or peritoneal B-1 B cells [210]. Thus, danger signals may activate tissue and innate cells to initiate specialized cytokine-mediated responses to limit tissue damage and promote repair mechanisms.

5.2.0. Functional model of IL-17 responses

As illustrated in figure5.1, we propose that IL-17 primarily functions as an initiating and amplifier of host defense/ inflammatory responses in mucosal tissues. Tissue resident γδ T, iNTK and LTi-like cells can directly sense and/ or rapidly respond to trauma signals or invading pathogens by releasing IL-17 independently of classical antigen processing and presentation mechanisms. $CD4^+$ and $CD8^+$ memory T cells can also rapidly release IL-17 which cooperates with Th17 cytokines and/ or inflammatory cytokines such as IL-1β and TNF to enhance innate immune responses from proximal stromal cells. Post-transcriptional mechanisms regulating mRNA stability and transcriptional mechanisms induced by IL-17 mediate the additive and synergistic responses to proinflammatory cytokines. Acute phase and antimicrobial factors are directly activated by IL-17 whereas neutrophil are indirectly recruited via the production of CXC-chemokines such as CXCL8. Finally, neutrophils migrate to sites of inflammation where they receive preactivation and survival signals from the inflammatory microenvironment. In cases where the IL-17 signals persist, IL-17 may attenuate the survival capacity and inflammatory potential of neutrophils acting in a negative feedback loop to regulate inflammatory responses. This response may be specific to the airways where IL-1β-producing alveolar macrophages and IL-17-positive γδ and αβ T cells are abundantly present in the tissues.

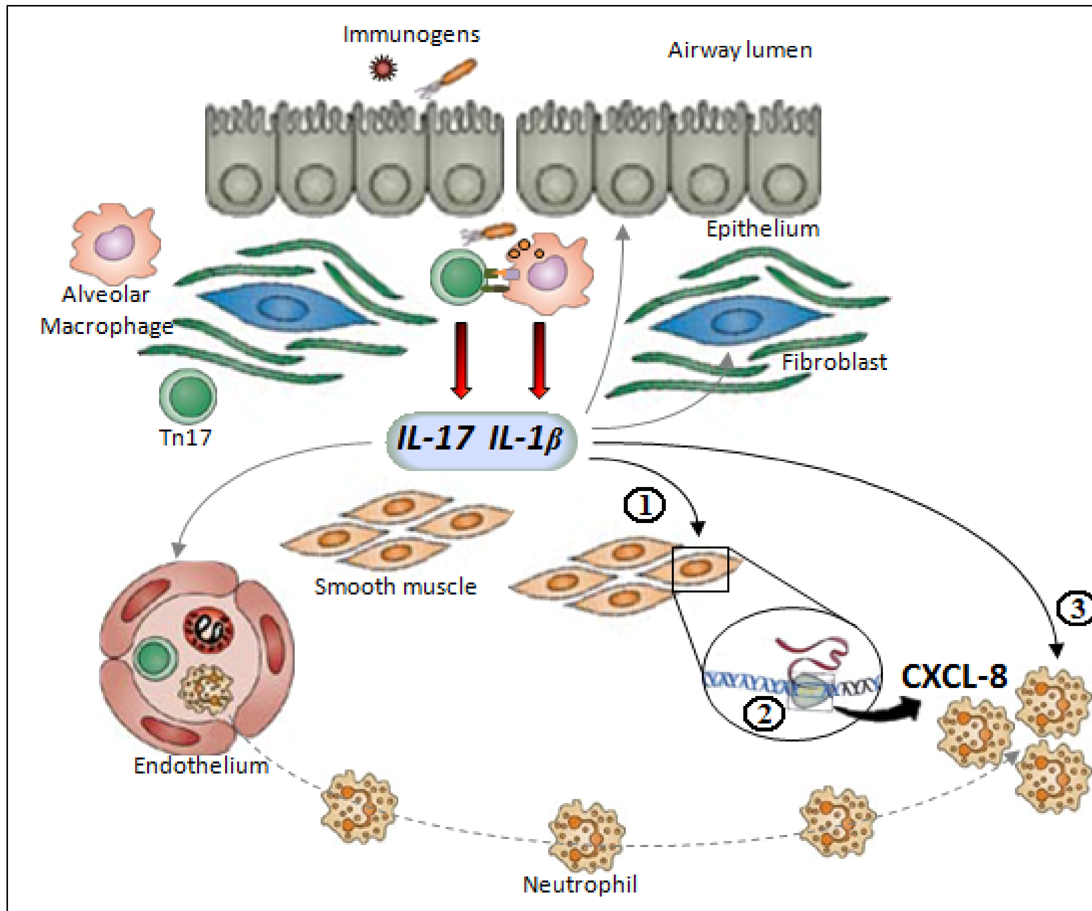

Figure 5. 1. Bridging theme
Activated neutrophil-regulatory T cells (Tn17) and alveolar macrophages indirectly recruit neutrophils to sites of inflammation through the release IL-17 and IL-1β which act in synergy to generate CXC-chemokines from structural cells. (*i*) Chapter 2 seeks to identify IL-17 gene targets from human ASM cells. (*ii*) Chapter 3 investigates the molecular mechanisms mediating the synergistic response between IL-17 and IL-1β on CXCL8 production. (*iii*) Chapter 4 seeks to determine direct effector functions of IL-17 on GM-CSF-primed, human peripheral blood neutrophils.

5.3.0. CELL TYPE SPECIFIC-RESPONSES

The ubiquitous nature of the IL-17R and previously reported gene expression studies suggest that IL-17 can elicit cell-type specific responses. The bone-related receptor activator for NF-κB ligand (RANKL) gene was specifically upregulated in the osteoblastic MC3T3-E1 cell line in response to IL-17 whereas *Muc5ac* and *Muc5b* genes were significantly upregulated in TBE. *Ccl5* expression was also differentially regulated as murine preosteoblasts and MEF upregulated

its expression in response to IL-17 [102] whereas corneal fibroblasts [247], colonic subepithelial myofibroblasts [248] and keratinocytes [249] downregulated its expression. In our array, IL-17 selectively upregulated smooth muscle-specific genes such as the myosin light chain-3 (*Myl3*), myosin binding protein H (*Mybph*), myogenic differentiation-1 (*Myod1*), calponin-1 basic smooth muscle (*Cnn1*) and endothelin-1 (*Edn1*). These genes have not previously been reported as IL-17 gene target supporting the notion that IL-17 may mediate pleiotropic responses by acting in a cell type-specific manner.

5.4.0. Cytokine-mediated airway inflammation

IL-17A can orchestrate local airway inflammation by inducing the release of proinflammatory cytokines and chemokines from epithelial, fibroblast, smooth muscle and endothelial cells. In asthmatic patients, IL-17A expression is increased in the lungs and the severity of airway hypersensitivity correlates with IL-17A levels in the bronchoalveolar lavage (BAL) fluid [250]. However, low levels of gene expression mediated by IL-17 suggest that additional cytokines may drive airway inflammatory responses. Alveolar macrophages, a prominent cell type in the airways can rapidly release cytokines such as IL-1β which can cooperate with IL-17 to amplify gene expression responses [251]. Alveolar macrophages isolated from asthmatic patients were also reported to co-express IL-17 and produced greater levels of IL-1β than from healthy alveolar macrophages *in vitro* [252, 253]. Tissue resident γδ T and iNKT cells can also rapidly release IL-17 which can activate macrophages to release IL-1β and TNF [58, 254]. Although IL-1β and TNF promote pulmonary cell recruitment, they also modulate bronchial hyperreactivity as isolated airways treated with IL-1β or TNF heighten constriction responses mediated by ACh and attenuate relaxation responses induced by isoproterenol, a β-adrenergic agonist [252, 255]. Thus, the

additive and synergistic responses mediated by IL-17 with IL-1β and/ or TNF can exacerbate inflammatory responses and negatively affect airway function [167].

Th17 cytokines can also cooperate with IL-17 to enhance inflammatory responses. IL-22 which is co-expressed by 70% of popliteal CD4^{+} IL-17A/ F Th17 cells functions to maintain the integrity of the bronchial epithelial barrier and stimulates antimicrobial and matrix proteins from myofibroblasts, keratinocytes and epithelial cells [12, 185, 256]. IL-17 and IL-22 cooperatively enhance chemokines (*Cxcl1*, *Cxcl2*, *Cxcl5*, *Cxcl9*) and host defense genes (*Muc1*, *Lcn2*, *Defb4, S100a7*, *S100a12, Pigr, Mmp3*) [12 185] and were observed in our study to additively upregulate *Bdkrb1*, *Tnfaip3*, *Nfkbiz*, *End1*, *Il27r*, *Zc3h12c*, *Zc3h12a*, *Il6* and *Cxcl8* in ASM cells. Similarly, IL-26, a novel IL-10-like cytokine was recently reported to be coexpressed in Th17 cells [138]. IL-26 induces *Tnf* and *Cxcl8* expression in intestinal epithelial cells (IEC) and was upregulated in inflammatory bowel disease (IBD) lesions [257]. Although receptors for IL-17A, IL-17F and IL-22 are expressed on several epithelial and stromal cells, the heterodimeric IL-26R consists of a ubiquitous IL-10R2 and a tissue-restricted IL-20R1 subunit. As such, functional responses to IL-26 have been limited to epithelial colon carcinoma and keratinocyte cell lines [258] however, constitutive IL-20R1 mRNA was detected in lung and skin tissues [259]. Although it is currently unknown whether ASM cells express the IL-26R, the expression of several Th17-cytokine receptors on nonhematopoietic cells strongly suggest that cooperative cytokine responses mediated by IL-17 could characterize a principal method of action.

5.5.0. Direct and indirect functions of IL-17 on hematopoietic cells

Initial investigation of direct effector functions mediated by IL-17 on neutrophil migration revealed no significant chemotactic or additive effect when in combination with CXCL8 [17]. Moreover, IL-17 with or without TNF displayed no substantial effects on the rate of spontaneous neutrophil apoptosis after 48-96h, and IL-17 did not increase MPO activity in isolated rat neutrophils [215, 260]. Although other functional responses such as superoxide production, cell spreading, phagocytosis and mRNA synthesis have not been thoroughly assessed, IL-17 *per se* does not appear to exert any direct effects on granulocytes. However, due to the ubiquitous IL-17RA expression at the cell surface, we sought to investigate whether IL-17 in combination with GM-CSF, a neutrophil priming agent, could elicit functional responses.

Peripheral blood neutrophils are a quiescent cell type that in response to a priming event, gain a heighten state of responsiveness. Extravasation, proinflammatory mediators and growth factors are "dedicated primers" which elicit preactivation signals in neutrophils. Mechanistically, tyrosine kinases phosphorylate non-activating tyrosine-based motifs of target proteins which lowers the activation threshold. For example, the neutrophil cytosol factor (NCF)-1, a subunit of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase complex is tyrosine phosphorylated in response to GM-CSF [261]. Upon an activation signal, Ca-mediated serine/ threonine kinases phosphorylate activating motifs which triggers functional responses such as superoxide production. Although non-primed neutrophils can respond to activating stimulus and generate NADPH-oxidase activity, the activity is much greater in primed cells [262]. Priming also regulates neutrophil responses to cytokines such as IL-2, which requires GM-CSF in order to induce concentration-dependent gene transcription and morphological changes [263].

In our study, we observed that although IL-17 phosphorylated p38, it did not affect cell survival outcomes. However, co-incubation of IL-17 with GM-CSF disrupted MAPK and STAT signaling pathways and decreased survival responses. Although we did not extend our observations to investigate other effector functions, our results suggest that primed neutrophils can directly respond to IL-17. Similar studies have also revealed that freshly isolated blood monocytes are also unresponsive to IL-17 signals unless preactivated by adhesion signals [15, 58]. This appears to be a general regulatory mechanism as LPS-induced TNF and IL-6 responses are greater in *in vitro*-differentiated macrophages compared to freshly seeded monocytes [264]. Hence, leukocytes may require preactivation signals in order to increase their state of responsiveness as they migrate into sites of inflammation and to avoid deleterious systemic inflammatory responses in the general circulation. Indeed, neutrophil-derived serine proteases such as elastase and cathepsin G can cleave the ectodomain of selective cell surface receptors such as CD43 which functions to inhibit neutrophil effector functions [265, 266]. This endopeptidase activity however is inhibited by serum albumin which constitutively binds CD43 in the blood stream. Thus, the physiological function of a primed/ intermediary phenotype may simply represent a regulatory transition state.

5.6.0. IL-17 GENE TARGETS AND MICROARRAY STUDIES

Commercial cDNA microarrays have been available since 1996 and have gained mainstream acceptance since the sequencing of the human genome in 2003. The first large scale investigation of IL-17 gene targets was undertaken in 2001 with a nylon membrane containing 219 genes (207 inflammatory, 9 housekeeping and 3 negative control genes) [267]. As observed in table5.1, multiple gene arrays have since revealed the complex nature of the IL-17 response. Platform-

specific devices, analytical algorithms as well as experimental variables such as concentrations, time points, cell types and organisms have progressively uncovered numerous, often non-overlapping inducible gene targets. Although the gene expression levels can vary greatly between studies, the complex gene profile can be simplified by grouping similar time points to expose a common chemokine gene subset. Of note, subtracted IL-17 gene targets that were identified by combining and comparing IL-17 with TNF were omitted from table5.1 in order to reduce off target, synergy-dependent responses [26, 182]. Remarkably, *Cxcl1* was identified in all studies regardless of the experimental conditions suggesting that *Cxcl1* could serve as a standard for IL-17-depedent responses, in contrast to current readouts of *Il6* or *Lcn2*.

The primary aim of chapter 2 was to uncover IL-17/ IL-17RA-mediated gene targets in non-transformed, low passaged primary human ASM cells. Since IL-17 is associated with both protective immune responses and degenerative chronic inflammatory disorders, we sought to uncover effector functions mediated by IL-17 on normal and mild asthmatic ASM cells. This unique cell type was specifically chosen as it allows us to investigate a wide-range of stromal and inflammatory gene-related responses. FBS-deprived, semi-confluent ASM cells represent a resting cell type responsive to cell growth signals, migration, apoptosis and possess both contractile and immune (synthetic) functions. On a physiological level, ASM cells modulate bronchiole airway calibre and orchestrate inflammation and remodeling events which negatively affect long term airway function. Great precautions were taken in our study to assure a complete clinical history was recorded for each patient before extracting a biopsy from the airway wall of the main bronchus of healthy and mild asthmatic individuals. To determine the IL-17 gene expression profile, whole transcript (WT) sense-target (ST) Affymetrix HuGene v.1.0 gene chips

which interrogate 28,869 annotated genes via 764,885 distinct probes were selected for our study. DNA-based WT ST gene chips offers several advantages over the previous 3'IVT (*in vitro* transcription) RNA-based arrays such as the HG-U133 chip because *(i)* DNA-DNA hybridization are more specific and reduce the occurrence of false positives due to cross-hybridization events, *(ii)* it interrogates the sense-strand as oppose to the uncharacterized anti-sense strand *(iii)* it uses random primers rather than oligo-dT primers which limits amplification to poly(A) transcripts and *(iv)* it eliminates the dye bias via the use of terminal labelling rather than convoluted internal labelling techniques. Hence, the HuGene 1.0ST gene array eliminates technical bias by interrogating amplified, biotinylated sense-strand cDNA.

Authors		2009 Dragon, S	2003 Ruddy, MJ 2005 Ruddy, MJ & Shen, F			2008 Rao, DA	2002 Jones, JE	2005 Parks, H	2008 Zrioual, S 2009 Zrioual, S	2004 Kao, CY 2007 Huang, F
Platform		Affymetrix	Affymetrix			Affymetrix	Clontech	Qiagen (Operon)	Affymetrix	Affymetrix
Array		HuGene 1.0 st.v1	MG-U74v2			HG-U133 Plus 2.0	Custom (nylon filter)	MG-Oligo Set v2.0	HG-U133A	HG-U133A
Genes		28,869	36,000			14,500	207	6,546	33,000	33,000
IL-17 conc.		10ng/ml	200ng/ml			100ng/ml	200ng/ml	500ng/ml (IL-17-Ig)	50ng/ml	10ng/ml
Time point		2h	2h			6h	6h	6h	12h	24h
Organism		Human	Murine			Human	Human	Murine	Human	Human
Cell type		**ASMC**	**MC3T3**	**ST2**	**MEF**	**VSMC**	**HBE**	**MEF**	**RA Syn**	**TBE**
Ccl2	(4)		6.5	92.9	1.8			3.2		
Ccl5	(2)			3.4	1.8					
Ccl7	(2)							2.4	2-10	
Ccl20	(4)					5.5		2.3	548.7	4.5
Cxcl1	(9)	2.6	24.7	122.8	475.6	2.2	8.5	3.9	10-100	3.0
Cxcl2	(3)	1.4							100-500	3.5
Cxcl3	(3)					4.9			100-500	3.5
Cxcl5	(5)		72.8	443.4		4.1			8.6	2.5
Cxcl6	(2)								10-100	2.0
Cxcl8	(4)					3.3	2.5		207.9	2.0
Cx3cl1	**(1)**							**1.8**		
IL1b	**(1)**					**2.6**				
Il6	(3)		5.0			2.8			14.9	
Il11	**(1)**					**3.6**				
Il19	**(1)**									**5.0**
Il23p19	**(1)**								**22.6**	
Csf3	(3)						17.8		9.2	3.5
Lcn2	(3)		2.3	64.0	42.8					
Defb4	**(1)**									**6.0**
Osmr	(2)			1.2	1.6					
Cox2	(2)			2.6	2.0					
Mmp3	**(1)**							**2.9**		
Mmp13	(3)			1.4	2.9			2.9		
Bmp2	**(1)**					**3.9**				
Pscd3	(2)			1.3	1.5					
Ywhag	(2)			2.9	1.2					
Fas	(2)			1.4	7.3					
Ereg	**(1)**				**6.5**					
Cotl1	**(1)**		**1.5**							
Cyp7b1	**(1)**								**2.6**	
Nfkbiz	(3)	4.5		3.0	8.3					
Cebpb	(3)		1.7	6.6	1.7					
Cebpd	(3)		3.4	1.7	2.0					
Sosc3	(2)		1.3	1.2						

Table 5. 1. Analysis of IL-17 responses by microarrays [6, 20, 102, 111, 268-271]
Underlined indicate common genes, **bold** indicates unique genes. ASMC; primary human airway smooth muscle cells (serum-deprived 24h). MC3T3-E1; murine preosteoblasts, ST2; murine stromal bone cells and MEF; mouse embryonic fibroblasts (serum-deprived 16h in 0.3% FBS). VSMC; primary normal human aortic/ coronary artery vascular smooth muscle cells (serum-deprived 48h). HBE; primary normal human bronchial epithelial cells (not serum deprived). MEF; mouse embryonic fibroblasts (serum-deprived 12h). RA Syn; primary human rheumatoid arthritis synoviocytes (not serum deprived). TBE; primary human tracheobronchial epithelial cells (not serum deprived).

5.7.0. IL-17-SPECIFIC GENE EXPRESSION PROFILE

Ten NF-κB-related genes listed in supplementary table2.4 were also identified in a genome-wide analysis of common LPS (50ng/ml) and TNF (10ng/ml) inducible genes at 6h in HUVEC cultures [272, 273]. *Nfkbiz*, a gene initially co-described as an IL-1-inducible nuclear ankyrin-repeat

protein (INAP) [274] and as a molecule possessing ankyrin repeats induced by LPS (MAIL) [275] had the greatest fold increase in response to IL-17 in our array. As previously reported in IκBζ-deficient mice, *Nfkbiz* is a PR gene which mediates IL-1R- and TLR-dependent SR gene expression such as for *Il12b*, *Il6* and *Lcn2* [103, 276, 277]. Interestingly, IκBζ-dependent SR genes are not expressed in NF-κB1 (p50)-deficient cells suggesting an association between both factors [277]. Further investigation of IκBζ uncovered a transactivation domain which is suppressed by ankyrin-repeats located in the carboxy-terminus of the protein. Upon binding the NF-κB p50 subunit, a conformation change liberates and activates the transactivation domain [276]. Hence, p50 homo-dimers which lack transcriptional activity bind to DNA motifs and constitutively repress transcription until IκBζ is expressed and forms a complex with p50 homo-dimers to activate gene transcription [107]. Furthermore, C/EBPβ was also reported to mediate NF-κB-IκBζ transcriptional activation of SR genes in HEK-293 cells [278]. Therefore, we speculate that IκBζ may confer specificity to IL-17 responses since NF-κB, IκBζ and C/EBPβ are activated and regulate IL-17-inducible SR genes such as *Il6* and *Lcn2*.

5.8.0. Time- and nucleosome-dependent chromatin remodeling responses

In our study, the 2h time point was specifically chosen as to avoid confounding autocrine mechanisms mediating indirect, or late-phase gene expression responses. IL-17 was previously reported to upregulate *Muc5b* via an IL-6 autocrine/ paracrine loop in HBE cells [110], and macrophages were also demonstrated to upregulated IL-1β and TNF protein levels by 3h [58]. Hence, our analysis specifically seeks to identify early/ intermediate gene targets directly activated by IL-17. As such, we identified numerous PR genes encoding regulatory factors, transcriptional cofactors and chemokines. PR promoters possess constitutively poised chromatin

structures which constitutively recruit transcription factors to rapidly induce gene transcription. SR promoters however, require a stimulus-dependent modification of the chromatin structure in order to overcome the nucleosomal barrier and expose transcriptional control regions [279, 280]. As such, SR genes require switch/ sucrose nonfermentable (SWI/ SNF) ATP-dependent remodeling complexes to destabilize histone-DNA interactions [281]. Since nucleosome remodelling is required for the recruitment of IκBζ to SR gene promoters, we anticipate that the IL-17RA-mediated ERK-RSK signaling pathway may activate specific nucleosome remodeling (Brg1) and/ or modifying complexes (CREB-binding protein (CBP)-lysine-acetyltransferase (KAT)) to coordinate transcription of SR genes. Moreover, differential qRT-PCR expression levels for seven genes selected in our array between the 2 and 6h time-point suggest the presence of transcriptional regulatory mechanisms. Hence, dynamic expression profiles likely explain why previous arrays investigating time points beyond 2h did not identify *Nfkbiz*, *Cebpb*, *Cepbd* or *Socs3* in their analysis. Moreover, it may also explain why we did not detect SR genes like *Defb4* (hBD2) or neutrophil gelatinase-associated lipocalin (*Ngal*/ *Lcn2*) in our genomic analysis.

5.9.0. IL-17 AND THE REGULATORY NF-κB NETWORK

Transcriptional regulators which mediate positive and negative responses may have been evolutionarily selected for their ability to be rapidly induced. As such, five PR genes (*Nfkbia*, *Ier3*, *Gadd45b*, *Nfkbiz*, *Tnfaip3*) and two delayed-PR genes (*Rrad*, *Sod2*) upregulated by IL-17 in our analysis were also identified in an unrelated, PDGF-treated human T98G glioblastoma cell line [282]. Thus, molecular mechanisms other than NF-κB dimer combinations, abundance/ distribution of inducible cofactors, epigenetic regulation and promoter accessibility must coordinate specific transcriptional responses. In fact, nuclear translocation dynamics has also

been reported to regulate NF-κB gene expression profiles [283 284]. Dynamic interplay between the rapid and strong feedback-mechanism of IκBα with the delayed IκBβ and IκBε responses induce sequential nuclear oscillations of NF-κB with a period of 100m [284-286]. The kinetic delay in gene transcription confers specificity to the secondary wave of transcription by allowing upstream elements and/ or transcription factors to mediate post-translational nucleosome modifications. IκBδ (p100 homo-dimers) and upstream IKK activity were also reported to mediate temporal NF-κB dynamics and alter SR gene expression [287, 288]. Due to the large number of inducible NF-κB regulatory genes in our array, we speculate that IL-17 may selectively modulate temporal NF-κB responses. In fact, NF-κB oscillations were also previously proposed as a possible mechanism for IL-17 to drive differential regulation of downstream SR genes [103]. Thus, investigating NF-κB shuttling responses may unravel a specific regulatory mechanism mediating IL-17 signaling responses.

5.10.0. MicroRNA

MicroRNA (miR) are small noncoding RNAs that regulate gene expression by targeting complementary sequences of protein-coding mRNA for cleavage [289]. miR-124a was recently proposed to fine-tune NF-κB-mediated responses by binding to a partial complementary sequence in the 3'UTR of *Nfkbiz*. However in our study, IL-17 significantly upregulated expression levels of miR-21 by 1.69 fold, $p \leq 0.005$. Upregulated expression levels of miR-21 were originally identified in human glioblastomas and sequence alignment analysis suggest it may have an evolutionary conserved role in regulating gene expression [290, 291]. miR-21 expression is activated by STAT3 via IL-6 stimulation in human myeloma cells (XG-1) [292] and by AP-1 in PMA-stimulated HL-60 promyelocytic cells [293]. Although numerous miR-21

gene sequences have been independently reported, a microarray analysis of miR-21-depleted MCF7 breast cancer cells have identified over 700 genes whose expression levels were differentially modulated [294]. While functional studies investigating miR-21 in normal or non-cancerous cells are relatively limited, it appears that miR-21 likely functions as a regulatory housekeeping gene and may thus not specifically regulate IL-17 gene responses.

5.11.0. INDUCIBLE IL-17 GENETIC NETWORK

Finally, in order to examine the molecular function and genetic network of the IL-17 response, upregulated genes that were statistically significant were inputted into the Ingenuity knowledge database and arranged into a global molecular network. Networks consist of focus genes which were algorithmically generated based on their interaction or connectivity to other known genes. Statistical significance scores based on Fisher's exact test also ranked the networks according to their degree of relevance to IPA's established datasets. Three genetic networks containing more than one focus molecule induced by IL-17 were identified of which the *Gene expression/ Infectious diseases/ Cellular movement* network was the most significant and is shown in table5.2. This network effectively illustrates the key role of NF-κB in regulating IL-17 gene expression responses and highlights the regulatory function of p38, ERK, NFKBIA (IκBα) and AP1 in the signal transduction pathways of the IL-17R.

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	Ap1, **↑APOBEC3G, ↑BIRC3,** C8, **↑CCL3, ↑CXCL1, ↑CXCL2,** ERK, **↑GADD45B, ↑ICAM1, ↑IER3*,** IKK, IL1, IL12, **↑IL27RA,** Interferon alpha, LDL, **↑LTF, ↑MAP3K8,** NF-κB, Nfat, **↑NFKB2,** NfkB-RelA, **↑NFKBIA, ↑NFKBIE (includes EG:4794),** P38 MAPK, PLC, Proteasome, **↑RELB, ↑RGS16,** Sod, **↑SOD2, ↑TNFAIP3,** Ubiquitin, Vegf	41	18	Gene Expression, Infectious Disease, Cellular Movement
2	AEBP1, Akt, Calmodulin, Caspase, **↑CBR3,** Ck2, **↑CYCS (includes EG:54205),** EDA2R, ERRFI1, Hsp70, **↑ID2, ↑IER3*,** IL1F6, Insulin, Jnk, **↑LEFTY2,** LGALS7, **↑LHB, ↑MAFA,** Mapk, MEKKΔ, NFkB, **↑NFKBIZ,** PDGF BB, PI3K, PRDX4, **↑PTMA, ↑PTTG1,** RNF7, **↑RRAD,** SNURF, **↑SPI1,** SPIB, **↑STMN2,** TDPX2	27	13	Cellular Growth and Proliferation, Hematological System Development and Function, Immune and Lymphatic System Development and Function
3	CD1D, **↑HCG 2015956,** HLA-DMA, HLA-DMB, HLA-DOA, **↑HLA-DOB,** HLA-DQB1, **↑HLA-DQB2,** HLA-DQB3, **↑IFNA6,** IFNG, IL2, IL3, IL6, IL20, IL33, IL15RA, IL17F, IL1B, IL1F6, IL20RB, **↑IL22RA1,** iodine, MAPK9, MHC Class II, **↑MIRN21 (includes EG:406991),** MT1F, **↑NFKBIZ,** REL/RELA/RELB, STAT3, TLR8, TNFSF15, **↑VSIG4, ↑ZBTB25, ↑ZC3H12A**	19	10	Cell-To-Cell Signaling and Interaction, Immune Response, Immune and Lymphatic System Development and Function
4	NUFIP1, **↑SNORD13**	2	1	RNA Post-Transcriptional Modification
5	EVPL, **↑PSORS1C2***	2	1	Hair and Skin Development and Function, Organ Morphology, Organ Development
6	TNFSF11, **↑ZC3H12C**	2	1	Cell Death, Cell Morphology, Cell-To-Cell Signaling and Interaction
7	Ggt, **↑GGTLC2**	2	1	
8	PDE3B, PIK3CG, **↑PIK3R6**	2	1	Cell Morphology, Endocrine System Development and Function, Cancer
9	ABI1, ABI2, Arp2/3, BCL2L1, **↑C3ORF10,** C3orf10-Wasf1, CAB39, CYFIP2, DSG1, IL15, KRT78, MOBKL3, NCKAP1, WASF1, WASF2	2	1	Cellular Assembly and Organization, Cellular Development, Hematological System Development and Function

Table 5. 2. Inducible IL-17 genetic networks
The table contains columns with the network number, the names of focus molecules/ upregulated genes identified in our array in **bold** and of associated genes involved in the network. Significance score values (representing the negative log *p* values; 2 = 0.01) and the top function associated to the networks are also listed.

6.0. General Conclusions and Significance

Overall, this study significantly extends our previous knowledge of the IL-17RA-mediated signal transduction and effector functions. The effect of IL-17 on structural cells such as ASM and effector cells such as neutrophils provides further insight into the complex cellular network regulating protective and adverse immune responses. Our results identified a role for MAPKs, NF-κB and AP1 to regulate IL-17-mediated responses in ASM cells and strongly indicate that NF-κB cofactors such as IκBζ, may regulate IL-17-mediated SR gene expression. Our results also suggest that IL-17 may bear a regulatory function in limiting the accumulation and/ or activity of neutrophils in inflammatory sites by directly attenuating their survival responses. Finally, the combination of IL-17 with Th17 or proinflammatory cytokines cooperatively amplifies NF-κB-dependent gene expression responses via transcriptional and post-transcriptional mechanisms where dysregulated responses, may enhance or result in pathological inflammatory disorders.

6.1. Knowledge translation

A pathogenic role in autoimmune and chronic inflammatory disorders has previously been proposed for Th17 cells. As such, understanding effector functions mediated by the IL-17R signal transduction and the gene expression profile may uncover converging or regulatory mechanisms that may be targeted by pharmacological compounds. Moreover, elucidating IL-17's ability to initiate and amplify local immune responses may be crucial for the future development of robust and efficacious vaccination strategies.

6.2. Future directions

Investigation into the structure, function and dynamics of the IL-17R complex has recently uncovered several regulatory factors including subunit interactions, extracellular PLAD- and cytoplasmic SEFIR-, TILL- and CBAD-domains. While substantial research efforts are currently invested in characterizing Th17 differentiation factors, the intracellular signaling events mediating by the IL-17R complex remain largely unknown. Notably, uncovering which factors mediate the specificity of the IL-17 response and regulate the gene expression signature:

- Elucidate the role of inducible NF-κB transcriptional co-factors and of inhibitory IκB family members on the dynamic, IL-17-dependent chromatin remodeling response.
- Identify novel binding motifs in the cytoplasmic tail of the IL-17R and of interacting receptor subunits which may recruit additional adaptor proteins and regulate the signaling cascade.
- Determine and compare the composition of the cell surface IL-17R and of interacting receptor subunits in structural mesenchymal cells versus those of the hematopoietic lineage.

Thus, investigation of the composition, dynamics and downstream factors of the IL-17R has great potential to uncover novel adaptor proteins and signaling complexes which could mediate activation of selective genes or gene subsets. Elucidating the regulatory signaling networks will further our understanding of the role of IL-17/ IL-17RA in human biology and will ultimately allow us to characterize and manipulate inflammatory responses to achieve better health outcomes.

7.0. References

1. Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. & Coffman, R.L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* **136**, 2348-2357 (1986).
2. Chtanova, T. & Mackay, C.R. T cell effector subsets: extending the Th1/Th2 paradigm. *Adv Immunol* **78**, 233-266 (2001).
3. Infante-Duarte, C., Horton, H.F., Byrne, M.C. & Kamradt, T. Microbial lipopeptides induce the production of IL-17 in Th cells. *J Immunol* **165**, 6107-6115 (2000).
4. Rouvier, E., Luciani, M.F., Mattei, M.G., Denizot, F. & Golstein, P. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. *J Immunol* **150**, 5445-5456 (1993).
5. Yao, Z. *et al.* Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* **3**, 811-821 (1995).
6. Park, H. *et al.* A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* **6**, 1133-1141 (2005).
7. Harrington, L.E. *et al.* Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* **6**, 1123-1132 (2005).
8. Steinman, L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* **13**, 139-145 (2007).
9. Ouyang, W., Kolls, J.K. & Zheng, Y. The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* **28**, 454-467 (2008).
10. Chang, S.H. & Dong, C. A novel heterodimeric cytokine consisting of IL-17 and IL-17F regulates inflammatory responses. *Cell Res* **17**, 435-440 (2007).
11. Wei, L., Laurence, A., Elias, K.M. & O'Shea, J.J. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem* **282**, 34605-34610 (2007).
12. Liang, S.C. *et al.* Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med* **203**, 2271-2279 (2006).
13. Chen, Z. *et al.* Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci U S A* **103**, 8137-8142 (2006).
14. Ivanov, II *et al.* The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* **126**, 1121-1133 (2006).
15. Fossiez, F. *et al.* T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med* **183**, 2593-2603 (1996).
16. Schwarzenberger, P. *et al.* IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J Immunol* **161**, 6383-6389 (1998).
17. Laan, M. *et al.* Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* **162**, 2347-2352 (1999).
18. Ye, P. *et al.* Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* **194**, 519-527 (2001).
19. Huang, W., Na, L., Fidel, P.L. & Schwarzenberger, P. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. *J Infect Dis* **190**, 624-631 (2004).
20. Kao, C.Y. *et al.* IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. *J Immunol* **173**, 3482-3491 (2004).
21. Stark, M.A. *et al.* Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* **22**, 285-294 (2005).

22. Pichavant, M. *et al.* Ozone exposure in a mouse model induces airway hyperreactivity that requires the presence of natural killer T cells and IL-17. *J Exp Med* **205**, 385-393 (2008).
23. Ley, K., Smith, E. & Stark, M.A. IL-17A-producing neutrophil-regulatory Tn lymphocytes. *Immunol Res* **34**, 229-242 (2006).
24. Aujla, S.J., Dubin, P.J. & Kolls, J.K. Th17 cells and mucosal host defense. *Semin Immunol* **19**, 377-382 (2007).
25. Shen, F. & Gaffen, S.L. Structure-function relationships in the IL-17 receptor: implications for signal transduction and therapy. *Cytokine* **41**, 92-104 (2008).
26. Hartupee, J., Liu, C., Novotny, M., Li, X. & Hamilton, T. IL-17 enhances chemokine gene expression through mRNA stabilization. *J Immunol* **179**, 4135-4141 (2007).
27. Gaffen, S.L. An overview of IL-17 function and signaling. *Cytokine* **43**, 402-407 (2008).
28. Liang, J., Song, W., Tromp, G., Kolattukudy, P.E. & Fu, M. Genome-wide survey and expression profiling of CCCH-zinc finger family reveals a functional module in macrophage activation. *PLoS One* **3**, e2880 (2008).
29. Korn, T. *et al.* IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* **448**, 484-487 (2007).
30. Zhou, L. *et al.* IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* **8**, 967-974 (2007).
31. Acosta-Rodriguez, E.V., Napolitani, G., Lanzavecchia, A. & Sallusto, F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* **8**, 942-949 (2007).
32. Lee, Y.K., Mukasa, R., Hatton, R.D. & Weaver, C.T. Developmental plasticity of Th17 and Treg cells. *Curr Opin Immunol* **21**, 274-280 (2009).
33. Laurence, A. *et al.* Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity* **26**, 371-381 (2007).
34. Gaffen, S.L. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol* **9**, 556-567 (2009).
35. Yao, Z., Timour, M., Painter, S., Fanslow, W. & Spriggs, M. Complete nucleotide sequence of the mouse CTLA8 gene. *Gene* **168**, 223-225 (1996).
36. Yao, Z. *et al.* Molecular characterization of the human interleukin (IL)-17 receptor. *Cytokine* **9**, 794-800 (1997).
37. Knappe, A. *et al.* The interleukin-17 gene of herpesvirus saimiri. *J Virol* **72**, 5797-5801 (1998).
38. Yao, Z. *et al.* Human IL-17: a novel cytokine derived from T cells. *J Immunol* **155**, 5483-5486 (1995).
39. Cai, X.Y., Gommoll, C.P., Jr., Justice, L., Narula, S.K. & Fine, J.S. Regulation of granulocyte colony-stimulating factor gene expression by interleukin-17. *Immunol Lett* **62**, 51-58 (1998).
40. Kolls, J., Peppel, K., Silva, M. & Beutler, B. Prolonged and effective blockade of tumor necrosis factor activity through adenovirus-mediated gene transfer. *Proc Natl Acad Sci U S A* **91**, 215-219 (1994).
41. Forlow, S.B. *et al.* Increased granulopoiesis through interleukin-17 and granulocyte colony-stimulating factor in leukocyte adhesion molecule-deficient mice. *Blood* **98**, 3309-3314 (2001).
42. Schwarzenberger, P. *et al.* Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. *J Immunol* **164**, 4783-4789 (2000).
43. Witowski, J. *et al.* IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO alpha chemokine from mesothelial cells. *J Immunol* **165**, 5814-5821 (2000).
44. Moseley, T.A., Haudenschild, D.R., Rose, L. & Reddi, A.H. Interleukin-17 family and IL-17 receptors. *Cytokine Growth Factor Rev* **14**, 155-174 (2003).
45. Yamaguchi, Y. *et al.* IL-17B and IL-17C are associated with TNF-alpha production and contribute to the exacerbation of inflammatory arthritis. *J Immunol* **179**, 7128-7136 (2007).

46. Shi, Y. *et al.* A novel cytokine receptor-ligand pair. Identification, molecular characterization, and in vivo immunomodulatory activity. *J Biol Chem* **275**, 19167-19176 (2000).
47. Moore, E.E. *et al.* Expression of IL-17B in neurons and evaluation of its possible role in the chromosome 5q-linked form of Charcot-Marie-Tooth disease. *Neuromuscul Disord* **12**, 141-150 (2002).
48. Li, H. *et al.* Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family. *Proc Natl Acad Sci U S A* **97**, 773-778 (2000).
49. Lee, J. *et al.* IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. *J Biol Chem* **276**, 1660-1664 (2001).
50. Fort, M.M. *et al.* IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* **15**, 985-995 (2001).
51. Kim, M.R. *et al.* Transgenic overexpression of human IL-17E results in eosinophilia, B-lymphocyte hyperplasia, and altered antibody production. *Blood* **100**, 2330-2340 (2002).
52. Starnes, T. *et al.* Cutting edge: IL-17F, a novel cytokine selectively expressed in activated T cells and monocytes, regulates angiogenesis and endothelial cell cytokine production. *J Immunol* **167**, 4137-4140 (2001).
53. Wright, J.F. *et al.* Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells. *J Biol Chem* **282**, 13447-13455 (2007).
54. Liang, S.C. *et al.* An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. *J Immunol* **179**, 7791-7799 (2007).
55. Hymowitz, S.G. *et al.* IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J* **20**, 5332-5341 (2001).
56. Starnes, T., Broxmeyer, H.E., Robertson, M.J. & Hromas, R. Cutting edge: IL-17D, a novel member of the IL-17 family, stimulates cytokine production and inhibits hemopoiesis. *J Immunol* **169**, 642-646 (2002).
57. Goodwin, R.G. *et al.* Molecular and biological characterization of a ligand for CD27 defines a new family of cytokines with homology to tumor necrosis factor. *Cell* **73**, 447-456 (1993).
58. Jovanovic, D.V. *et al.* IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* **160**, 3513-3521 (1998).
59. Shalom-Barak, T., Quach, J. & Lotz, M. Interleukin-17-induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-kappaB. *J Biol Chem* **273**, 27467-27473 (1998).
60. Tian, E. *et al.* Evi27 encodes a novel membrane protein with homology to the IL17 receptor. *Oncogene* **19**, 2098-2109 (2000).
61. Haudenschild, D., Moseley, T., Rose, L. & Reddi, A.H. Soluble and transmembrane isoforms of novel interleukin-17 receptor-like protein by RNA splicing and expression in prostate cancer. *J Biol Chem* **277**, 4309-4316 (2002).
62. Haudenschild, D.R., Curtiss, S.B., Moseley, T.A. & Reddi, A.H. Generation of interleukin-17 receptor-like protein (IL-17RL) in prostate by alternative splicing of RNA. *Prostate* **66**, 1268-1274 (2006).
63. Toy, D. *et al.* Cutting edge: interleukin 17 signals through a heteromeric receptor complex. *J Immunol* **177**, 36-39 (2006).
64. Kuestner, R.E. *et al.* Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. *J Immunol* **179**, 5462-5473 (2007).
65. Tsang, M., Friesel, R., Kudoh, T. & Dawid, I.B. Identification of Sef, a novel modulator of FGF signalling. *Nat Cell Biol* **4**, 165-169 (2002).
66. Yang, R.B. *et al.* A novel interleukin-17 receptor-like protein identified in human umbilical vein endothelial cells antagonizes basic fibroblast growth factor-induced signaling. *J Biol Chem* **278**, 33232-33238 (2003).

67. Xiong, S. *et al.* hSef inhibits PC-12 cell differentiation by interfering with Ras-mitogen-activated protein kinase MAPK signaling. *J Biol Chem* **278**, 50273-50282 (2003).
68. Preger, E. *et al.* Alternative splicing generates an isoform of the human Sef gene with altered subcellular localization and specificity. *Proc Natl Acad Sci U S A* **101**, 1229-1234 (2004).
69. Furthauer, M., Lin, W., Ang, S.L., Thisse, B. & Thisse, C. Sef is a feedback-induced antagonist of Ras/MAPK-mediated FGF signalling. *Nat Cell Biol* **4**, 170-174 (2002).
70. Li, T.S., Li, X.N., Chang, Z.J., Fu, X.Y. & Liu, L. Identification and functional characterization of a novel interleukin 17 receptor: a possible mitogenic activation through ras/mitogen-activated protein kinase signaling pathway. *Cell Signal* **18**, 1287-1298 (2006).
71. Wright, J.F. *et al.* The human IL-17F/IL-17A heterodimeric cytokine signals through the IL-17RA/IL-17RC receptor complex. *J Immunol* **181**, 2799-2805 (2008).
72. Kramer, J.M. *et al.* Evidence for ligand-independent multimerization of the IL-17 receptor. *J Immunol* **176**, 711-715 (2006).
73. Kramer, J.M. *et al.* Cutting edge: identification of a pre-ligand assembly domain (PLAD) and ligand binding site in the IL-17 receptor. *J Immunol* **179**, 6379-6383 (2007).
74. You, Z. *et al.* Interleukin-17 receptor-like gene is a novel antiapoptotic gene highly expressed in androgen-independent prostate cancer. *Cancer Res* **66**, 175-183 (2006).
75. Rong, Z. *et al.* IL-17RD (Sef or IL-17RLM) interacts with IL-17 receptor and mediates IL-17 signaling. *Cell Res* **19**, 208-215 (2009).
76. Awane, M., Andres, P.G., Li, D.J. & Reinecker, H.C. NF-kappa B-inducing kinase is a common mediator of IL-17-, TNF-alpha-, and IL-1 beta-induced chemokine promoter activation in intestinal epithelial cells. *J Immunol* **162**, 5337-5344 (1999).
77. Schwandner, R., Yamaguchi, K. & Cao, Z. Requirement of tumor necrosis factor receptor-associated factor (TRAF)6 in interleukin 17 signal transduction. *J Exp Med* **191**, 1233-1240 (2000).
78. Novatchkova, M., Leibbrandt, A., Werzowa, J., Neubuser, A. & Eisenhaber, F. The STIR-domain superfamily in signal transduction, development and immunity. *Trends Biochem Sci* **28**, 226-229 (2003).
79. Maitra, A. *et al.* Distinct functional motifs within the IL-17 receptor regulate signal transduction and target gene expression. *Proc Natl Acad Sci U S A* **104**, 7506-7511 (2007).
80. Kanamori, M., Kai, C., Hayashizaki, Y. & Suzuki, H. NF-kappaB activator Act1 associates with IL-1/Toll pathway adaptor molecule TRAF6. *FEBS Lett* **532**, 241-246 (2002).
81. Li, X. *et al.* Act1, an NF-kappa B-activating protein. *Proc Natl Acad Sci U S A* **97**, 10489-10493 (2000).
82. Leonardi, A., Chariot, A., Claudio, E., Cunningham, K. & Siebenlist, U. CIKS, a connection to Ikappa B kinase and stress-activated protein kinase. *Proc Natl Acad Sci U S A* **97**, 10494-10499 (2000).
83. Chang, S.H., Park, H. & Dong, C. Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. *J Biol Chem* **281**, 35603-35607 (2006).
84. Qian, Y., Zhao, Z., Jiang, Z. & Li, X. Role of NF kappa B activator Act1 in CD40-mediated signaling in epithelial cells. *Proc Natl Acad Sci U S A* **99**, 9386-9391 (2002).
85. Liao, G., Zhang, M., Harhaj, E.W. & Sun, S.C. Regulation of the NF-kappaB-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. *J Biol Chem* **279**, 26243-26250 (2004).
86. Qian, Y. *et al.* Act1, a negative regulator in CD40- and BAFF-mediated B cell survival. *Immunity* **21**, 575-587 (2004).
87. Qian, Y. *et al.* The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. *Nat Immunol* **8**, 247-256 (2007).
88. Shen, F. *et al.* IL-17 receptor signaling inhibits C/EBPbeta by sequential phosphorylation of the regulatory 2 domain. *Sci Signal* **2**, ra8 (2009).

89. Attur, M.G., Patel, R.N., Abramson, S.B. & Amin, A.R. Interleukin-17 up-regulation of nitric oxide production in human osteoarthritis cartilage. *Arthritis Rheum* **40**, 1050-1053 (1997).
90. Martel-Pelletier, J., Mineau, F., Jovanovic, D., Di Battista, J.A. & Pelletier, J.P. Mitogen-activated protein kinase and nuclear factor kappaB together regulate interleukin-17-induced nitric oxide production in human osteoarthritic chondrocytes: possible role of transactivating factor mitogen-activated protein kinase-activated proten kinase (MAPKAPK). *Arthritis Rheum* **42**, 2399-2409 (1999).
91. Henness, S. *et al.* IL-17A acts via p38 MAPK to increase stability of TNF-alpha-induced IL-8 mRNA in human ASM. *Am J Physiol Lung Cell Mol Physiol* **290**, L1283-1290 (2006).
92. Kao, C.Y. *et al.* Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-kappaB-dependent signaling pathway. *J Immunol* **175**, 6676-6685 (2005).
93. Shimada, M. *et al.* IL-6 secretion by human pancreatic periacinar myofibroblasts in response to inflammatory mediators. *J Immunol* **168**, 861-868 (2002).
94. Bakheet, T., Frevel, M., Williams, B.R., Greer, W. & Khabar, K.S. ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucleic Acids Res* **29**, 246-254 (2001).
95. Shaw, G. & Kamen, R. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**, 659-667 (1986).
96. Winzen, R. *et al.* Distinct domains of AU-rich elements exert different functions in mRNA destabilization and stabilization by p38 mitogen-activated protein kinase or HuR. *Mol Cell Biol* **24**, 4835-4847 (2004).
97. Winzen, R. *et al.* The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J* **18**, 4969-4980 (1999).
98. Faour, W.H., Mancini, A., He, Q.W. & Di Battista, J.A. T-cell-derived interleukin-17 regulates the level and stability of cyclooxygenase-2 (COX-2) mRNA through restricted activation of the p38 mitogen-activated protein kinase cascade: role of distal sequences in the 3'-untranslated region of COX-2 mRNA. *J Biol Chem* **278**, 26897-26907 (2003).
99. van den Berg, A. *et al.* Interleukin-17 induces hyperresponsive interleukin-8 and interleukin-6 production to tumor necrosis factor-alpha in structural lung cells. *Am J Respir Cell Mol Biol* **33**, 97-104 (2005).
100. Sandler, H. & Stoecklin, G. Control of mRNA decay by phosphorylation of tristetraprolin. *Biochem Soc Trans* **36**, 491-496 (2008).
101. Hartupee, J. *et al.* IL-17 signaling for mRNA stabilization does not require TNF receptor-associated factor 6. *J Immunol* **182**, 1660-1666 (2009).
102. Ruddy, M.J. *et al.* Functional cooperation between interleukin-17 and tumor necrosis factor-alpha is mediated by CCAAT/enhancer-binding protein family members. *J Biol Chem* **279**, 2559-2567 (2004).
103. Kao, C.Y., Kim, C., Huang, F. & Wu, R. Requirements for two proximal NF-kappaB binding sites and IkappaB-zeta in IL-17A-induced human beta-defensin 2 expression by conducting airway epithelium. *J Biol Chem* **283**, 15309-15318 (2008).
104. Andoh, A. *et al.* Extracellular signal-regulated kinases 1 and 2 participate in interleukin-17 plus tumor necrosis factor-alpha-induced stabilization of interleukin-6 mRNA in human pancreatic myofibroblasts. *Biochim Biophys Acta* **1591**, 69-74 (2002).
105. Dragon, S. *et al.* IL-17 enhances IL-1beta-mediated CXCL-8 release from human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **292**, L1023-1029 (2007).
106. Shen, F., Hu, Z., Goswami, J. & Gaffen, S.L. Identification of common transcriptional regulatory elements in interleukin-17 target genes. *J Biol Chem* **281**, 24138-24148 (2006).

107. Trinh, D.V., Zhu, N., Farhang, G., Kim, B.J. & Huxford, T. The nuclear I kappaB protein I kappaB zeta specifically binds NF-kappaB p50 homodimers and forms a ternary complex on kappaB DNA. *J Mol Biol* **379**, 122-135 (2008).
108. Cowland, J.B., Muta, T. & Borregaard, N. IL-1beta-specific up-regulation of neutrophil gelatinase-associated lipocalin is controlled by IkappaB-zeta. *J Immunol* **176**, 5559-5566 (2006).
109. Subramaniam, S.V., Cooper, R.S. & Adunyah, S.E. Evidence for the involvement of JAK/STAT pathway in the signaling mechanism of interleukin-17. *Biochem Biophys Res Commun* **262**, 14-19 (1999).
110. Chen, Y. *et al.* Stimulation of airway mucin gene expression by interleukin (IL)-17 through IL-6 paracrine/autocrine loop. *J Biol Chem* **278**, 17036-17043 (2003).
111. Huang, F. *et al.* Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF-kappaB activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. *J Immunol* **179**, 6504-6513 (2007).
112. Rahman, M.S. *et al.* IL-17A induces eotaxin-1/CC chemokine ligand 11 expression in human airway smooth muscle cells: role of MAPK (Erk1/2, JNK, and p38) pathways. *J Immunol* **177**, 4064-4071 (2006).
113. Laan, M., Lotvall, J., Chung, K.F. & Linden, A. IL-17-induced cytokine release in human bronchial epithelial cells in vitro: role of mitogen-activated protein (MAP) kinases. *Br J Pharmacol* **133**, 200-206 (2001).
114. Kawaguchi, M. *et al.* Induction of granulocyte-macrophage colony-stimulating factor by a new cytokine, ML-1 (IL-17F), via Raf I-MEK-ERK pathway. *J Allergy Clin Immunol* **114**, 444-450 (2004).
115. Hwang, S.Y. *et al.* IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-kappaB- and PI3-kinase/Akt-dependent pathways. *Arthritis Res Ther* **6**, R120-128 (2004).
116. Kim, H.R. *et al.* Up-regulation of IL-23p19 expression in rheumatoid arthritis synovial fibroblasts by IL-17 through PI3-kinase-, NF-kappaB- and p38 MAPK-dependent signalling pathways. *Rheumatology (Oxford)* **46**, 57-64 (2007).
117. Rong, Z. *et al.* Interleukin-17F signaling requires ubiquitination of interleukin-17 receptor via TRAF6. *Cell Signal* **19**, 1514-1520 (2007).
118. Liu, C. *et al.* Act1, a U-box E3 Ubiquitin Ligase for IL-17 Signaling. *Sci Signal* **2**, ra63 (2009).
119. Kanayama, A. *et al.* TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Mol Cell* **15**, 535-548 (2004).
120. Levin, S.D. IL-17 receptor signaling: ubiquitin gets in on the act. *Sci Signal* **2**, pe64 (2009).
121. Oppmann, B. *et al.* Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* **13**, 715-725 (2000).
122. Langrish, C.L. *et al.* IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* **201**, 233-240 (2005).
123. Aggarwal, S., Ghilardi, N., Xie, M.H., de Sauvage, F.J. & Gurney, A.L. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* **278**, 1910-1914 (2003).
124. Happel, K.I. *et al.* Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to Klebsiella pneumoniae infection. *J Immunol* **170**, 4432-4436 (2003).
125. Wiekowski, M.T. *et al.* Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J Immunol* **166**, 7563-7570 (2001).
126. Parham, C. *et al.* A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R. *J Immunol* **168**, 5699-5708 (2002).
127. Pasare, C. & Medzhitov, R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science* **299**, 1033-1036 (2003).

128. Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M. & Stockinger, B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* **24**, 179-189 (2006).
129. Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235-238 (2006).
130. Mangan, P.R. *et al.* Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* **441**, 231-234 (2006).
131. Sutton, C., Brereton, C., Keogh, B., Mills, K.H. & Lavelle, E.C. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med* **203**, 1685-1691 (2006).
132. Yang, X.O. *et al.* STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem* **282**, 9358-9363 (2007).
133. Ziolkowska, M. *et al.* High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. *J Immunol* **164**, 2832-2838 (2000).
134. Ferretti, S., Bonneau, O., Dubois, G.R., Jones, C.E. & Trifilieff, A. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *J Immunol* **170**, 2106-2112 (2003).
135. Bettelli, E., Oukka, M. & Kuchroo, V.K. T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* **8**, 345-350 (2007).
136. Chung, K.F. Cytokines in chronic obstructive pulmonary disease. *Eur Respir J Suppl* **34**, 50s-59s (2001).
137. Nurieva, R. *et al.* Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. *Nature* **448**, 480-483 (2007).
138. Wilson, N.J. *et al.* Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol* **8**, 950-957 (2007).
139. Manel, N., Unutmaz, D. & Littman, D.R. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. *Nat Immunol* **9**, 641-649 (2008).
140. Chung, Y. *et al.* Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* **30**, 576-587 (2009).
141. He, Y.W., Deftos, M.L., Ojala, E.W. & Bevan, M.J. RORgamma t, a novel isoform of an orphan receptor, negatively regulates Fas ligand expression and IL-2 production in T cells. *Immunity* **9**, 797-806 (1998).
142. Eberl, G. & Littman, D.R. The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. *Immunol Rev* **195**, 81-90 (2003).
143. Ma, C.S. *et al.* Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J Exp Med* **205**, 1551-1557 (2008).
144. Milner, J.D. *et al.* Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* **452**, 773-776 (2008).
145. Yang, X.O. *et al.* T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* **28**, 29-39 (2008).
146. Brustle, A. *et al.* The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* **8**, 958-966 (2007).
147. Zhang, F., Meng, G. & Strober, W. Interactions among the transcription factors Runx1, RORgammat and Foxp3 regulate the differentiation of interleukin 17-producing T cells. *Nat Immunol* **9**, 1297-1306 (2008).
148. Awasthi, A. & Kuchroo, V.K. Th17 cells: from precursors to players in inflammation and infection. *Int Immunol* **21**, 489-498 (2009).
149. Zhou, L. *et al.* TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. *Nature* **453**, 236-240 (2008).

150. Mucida, D. *et al.* Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* **317**, 256-260 (2007).
151. Xiao, S. *et al.* Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J Immunol* **181**, 2277-2284 (2008).
152. Quintana, F.J. *et al.* Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* **453**, 65-71 (2008).
153. Colgan, J. & Rothman, P. All in the family: IL-27 suppression of T(H)-17 cells. *Nat Immunol* **7**, 899-901 (2006).
154. Campbell, D.J., Kim, C.H. & Butcher, E.C. Chemokines in the systemic organization of immunity. *Immunol Rev* **195**, 58-71 (2003).
155. Appay, V., van Lier, R.A., Sallusto, F. & Roederer, M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* **73**, 975-983 (2008).
156. Acosta-Rodriguez, E.V. *et al.* Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* **8**, 639-646 (2007).
157. Sato, W., Aranami, T. & Yamamura, T. Cutting edge: Human Th17 cells are identified as bearing CCR2+CCR5- phenotype. *J Immunol* **178**, 7525-7529 (2007).
158. Kleinschek, M.A. *et al.* Circulating and gut-resident human Th17 cells express CD161 and promote intestinal inflammation. *J Exp Med* **206**, 525-534 (2009).
159. Liu, H. & Rohowsky-Kochan, C. Regulation of IL-17 in human CCR6+ effector memory T cells. *J Immunol* **180**, 7948-7957 (2008).
160. Annunziato, F. *et al.* Phenotypic and functional features of human Th17 cells. *J Exp Med* **204**, 1849-1861 (2007).
161. Shin, H.C., Benbernou, N., Esnault, S. & Guenounou, M. Expression of IL-17 in human memory CD45RO+ T lymphocytes and its regulation by protein kinase A pathway. *Cytokine* **11**, 257-266 (1999).
162. Liu, S.J. *et al.* Induction of a distinct CD8 Tnc17 subset by transforming growth factor-beta and interleukin-6. *J Leukoc Biol* **82**, 354-360 (2007).
163. Kennedy, J. *et al.* Mouse IL-17: a cytokine preferentially expressed by alpha beta TCR + CD4-CD8-T cells. *J Interferon Cytokine Res* **16**, 611-617 (1996).
164. Miyamoto, M. *et al.* Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways. *J Immunol* **170**, 4665-4672 (2003).
165. Molet, S. *et al.* IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* **108**, 430-438 (2001).
166. Umemura, M. *et al.* Involvement of IL-17 in Fas ligand-induced inflammation. *Int Immunol* **16**, 1099-1108 (2004).
167. Sutton, C.E. *et al.* Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* **31**, 331-341 (2009).
168. Lockhart, E., Green, A.M. & Flynn, J.L. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. *J Immunol* **177**, 4662-4669 (2006).
169. Martin, B., Hirota, K., Cua, D.J., Stockinger, B. & Veldhoen, M. Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. *Immunity* **31**, 321-330 (2009).
170. Michel, M.L. *et al.* Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med* **204**, 995-1001 (2007).
171. Mills, K.H. Induction, function and regulation of IL-17-producing T cells. *Eur J Immunol* **38**, 2636-2649 (2008).
172. Takatori, H. *et al.* Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J Exp Med* **206**, 35-41 (2009).

173. Lugering, A. & Kucharzik, T. Induction of intestinal lymphoid tissue: the role of cryptopatches. *Ann N Y Acad Sci* **1072**, 210-217 (2006).
174. Colonna, M. Interleukin-22-producing natural killer cells and lymphoid tissue inducer-like cells in mucosal immunity. *Immunity* **31**, 15-23 (2009).
175. Hellings, P.W. *et al.* Interleukin-17 orchestrates the granulocyte influx into airways after allergen inhalation in a mouse model of allergic asthma. *Am J Respir Cell Mol Biol* **28**, 42-50 (2003).
176. Dilulio, N.A. *et al.* Groalpha-mediated recruitment of neutrophils is required for elicitation of contact hypersensitivity. *Eur J Immunol* **29**, 3485-3495 (1999).
177. Cattani, F. *et al.* The role of CXCR2 activity in the contact hypersensitivity response in mice. *Eur Cytokine Netw* **17**, 42-48 (2006).
178. Nakae, S. *et al.* Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* **17**, 375-387 (2002).
179. Schwarzenberger, P. & Kolls, J.K. Interleukin 17: an example for gene therapy as a tool to study cytokine mediated regulation of hematopoiesis. *J Cell Biochem Suppl* **38**, 88-95 (2002).
180. Ye, P. *et al.* Interleukin-17 and lung host defense against Klebsiella pneumoniae infection. *Am J Respir Cell Mol Biol* **25**, 335-340 (2001).
181. Kelly, M.N. *et al.* Interleukin-17/interleukin-17 receptor-mediated signaling is important for generation of an optimal polymorphonuclear response against Toxoplasma gondii infection. *Infect Immun* **73**, 617-621 (2005).
182. Shen, F., Ruddy, M.J., Plamondon, P. & Gaffen, S.L. Cytokines link osteoblasts and inflammation: microarray analysis of interleukin-17- and TNF-alpha-induced genes in bone cells. *J Leukoc Biol* **77**, 388-399 (2005).
183. Dumoutier, L., Louahed, J. & Renauld, J.C. Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9. *J Immunol* **164**, 1814-1819 (2000).
184. Dumoutier, L., Van Roost, E., Colau, D. & Renauld, J.C. Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor. *Proc Natl Acad Sci U S A* **97**, 10144-10149 (2000).
185. Aujla, S.J. *et al.* IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* **14**, 275-281 (2008).
186. Kotenko, S.V. *et al.* Identification of the functional interleukin-22 (IL-22) receptor complex: the IL-10R2 chain (IL-10Rbeta) is a common chain of both the IL-10 and IL-22 (IL-10-related T cell-derived inducible factor, IL-TIF) receptor complexes. *J Biol Chem* **276**, 2725-2732 (2001).
187. Wolk, K. *et al.* IL-22 increases the innate immunity of tissues. *Immunity* **21**, 241-254 (2004).
188. Conti, H.R. *et al.* Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J Exp Med* **206**, 299-311 (2009).
189. Dubin, P.J. & Kolls, J.K. Th17 cytokines and mucosal immunity. *Immunol Rev* **226**, 160-171 (2008).
190. Sallusto, F. & Lanzavecchia, A. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. *Eur J Immunol* **39**, 2076-2082 (2009).
191. Ghilardi, N. & Ouyang, W. Targeting the development and effector functions of TH17 cells. *Semin Immunol* **19**, 383-393 (2007).
192. Krueger, G.G. *et al.* A human interleukin-12/23 monoclonal antibody for the treatment of psoriasis. *N Engl J Med* **356**, 580-592 (2007).
193. Kirkham, B.W. *et al.* Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort). *Arthritis Rheum* **54**, 1122-1131 (2006).
194. Matusevicius, D. *et al.* Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult Scler* **5**, 101-104 (1999).

195. Duerr, R.H. *et al.* A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* **314**, 1461-1463 (2006).
196. Korn, T., Bettelli, E., Oukka, M. & Kuchroo, V.K. IL-17 and Th17 Cells. *Annu Rev Immunol* **27**, 485-517 (2009).
197. van den Berg, W.B. & Miossec, P. IL-17 as a future therapeutic target for rheumatoid arthritis. *Nat Rev Rheumatol* **5**, 549-553 (2009).
198. Furze, R.C. & Rankin, S.M. Neutrophil mobilization and clearance in the bone marrow. *Immunology* **125**, 281-288 (2008).
199. Hoffbrand, A.V., Moss, P. A. H., Pettit, J. E. *Essential haematology*, Edn. 5th ed. (Oxford : Blackwell Pub., 2006., Malden, Mass.; 2006).
200. Theilgaard-Monch, K., Porse, B.T. & Borregaard, N. Systems biology of neutrophil differentiation and immune response. *Curr Opin Immunol* **18**, 54-60 (2006).
201. Martin, C. *et al.* Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity* **19**, 583-593 (2003).
202. Burdon, P.C., Martin, C. & Rankin, S.M. Migration across the sinusoidal endothelium regulates neutrophil mobilization in response to ELR + CXC chemokines. *Br J Haematol* **142**, 100-108 (2008).
203. Furze, R.C. & Rankin, S.M. The role of the bone marrow in neutrophil clearance under homeostatic conditions in the mouse. *FASEB J* **22**, 3111-3119 (2008).
204. Simon, S.I. & Green, C.E. Molecular mechanics and dynamics of leukocyte recruitment during inflammation. *Annu Rev Biomed Eng* **7**, 151-185 (2005).
205. Schmid-Schonbein, G.W. Analysis of inflammation. *Annu Rev Biomed Eng* **8**, 93-131 (2006).
206. Robinson, J.M. Phagocytic leukocytes and reactive oxygen species. *Histochem Cell Biol* **131**, 465-469 (2009).
207. Segal, A.W. How neutrophils kill microbes. *Annu Rev Immunol* **23**, 197-223 (2005).
208. Nathan, C. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* **6**, 173-182 (2006).
209. Borregaard, N., Sorensen, O.E. & Theilgaard-Monch, K. Neutrophil granules: a library of innate immunity proteins. *Trends Immunol* **28**, 340-345 (2007).
210. Matzinger, P. Friendly and dangerous signals: is the tissue in control? *Nat Immunol* **8**, 11-13 (2007).
211. Brinkmann, V. *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532-1535 (2004).
212. von Kockritz-Blickwede, M. & Nizet, V. Innate immunity turned inside-out: antimicrobial defense by phagocyte extracellular traps. *J Mol Med* **87**, 775-783 (2009).
213. Hiemstra, P.S., van Wetering, S. & Stolk, J. Neutrophil serine proteinases and defensins in chronic obstructive pulmonary disease: effects on pulmonary epithelium. *Eur Respir J* **12**, 1200-1208 (1998).
214. Borregaard, N. & Cowland, J.B. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**, 3503-3521 (1997).
215. Laan, M. *et al.* A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-alpha. *Eur Respir J* **21**, 387-393 (2003).
216. Ayub, K. & Hallett, M.B. Ca2+ influx shutdown during neutrophil apoptosis: importance and possible mechanism. *Immunology* **111**, 8-12 (2004).
217. Marshall, J.C. & Watson, R.W. Programmed cell death (apoptosis) and the resolution of systemic inflammation. *Can J Surg* **40**, 169-174 (1997).
218. Young, B., Heath, J.W., Wheater, P.R., Lowe, J.S. & Burkitt, H.G. *Wheater's functional histology: a text and colour atlas*, Edn. 4, illustrated. (Churchill Livingstone, 2000, 2006).
219. Shore, S.A. & Moore, P.E. Effects of cytokines on contractile and dilator responses of airway smooth muscle. *Clin Exp Pharmacol Physiol* **29**, 859-866 (2002).

220. Fernandes, D.J., McConville, J.F., Stewart, A.G., Kalinichenko, V. & Solway, J. Can we differentiate between airway and vascular smooth muscle? *Clin Exp Pharmacol Physiol* **31**, 805-810 (2004).
221. Hershenson, M.B., Brown, M., Camoretti-Mercado, B. & Solway, J. Airway smooth muscle in asthma. *Annu Rev Pathol* **3**, 523-555 (2008).
222. Tliba, O. & Panettieri, R.A., Jr. Noncontractile functions of airway smooth muscle cells in asthma. *Annu Rev Physiol* **71**, 509-535 (2009).
223. Hirst, S.J. & Lee, T.H. Airway smooth muscle as a target of glucocorticoid action in the treatment of asthma. *Am J Respir Crit Care Med* **158**, S201-206 (1998).
224. Howarth, P.H. *et al.* Synthetic responses in airway smooth muscle. *J Allergy Clin Immunol* **114**, S32-50 (2004).
225. Lee, C.W. *et al.* Transcriptional regulation of VCAM-1 expression by tumor necrosis factor-alpha in human tracheal smooth muscle cells: involvement of MAPKs, NF-kappaB, p300, and histone acetylation. *J Cell Physiol* **207**, 174-186 (2006).
226. Lee, C.W. *et al.* Tumor necrosis factor-alpha enhances neutrophil adhesiveness: induction of vascular cell adhesion molecule-1 via activation of Akt and CaM kinase II and modifications of histone acetyltransferase and histone deacetylase 4 in human tracheal smooth muscle cells. *Mol Pharmacol* **73**, 1454-1464 (2008).
227. Hughes, J.M. *et al.* Human eosinophil-airway smooth muscle cell interactions. *Mediators Inflamm* **9**, 93-99 (2000).
228. Brightling, C.E. *et al.* Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* **346**, 1699-1705 (2002).
229. Lazaar, A.L. *et al.* T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. *J Exp Med* **180**, 807-816 (1994).
230. Ramos-Barbon, D., Presley, J.F., Hamid, Q.A., Fixman, E.D. & Martin, J.G. Antigen-specific CD4+ T cells drive airway smooth muscle remodeling in experimental asthma. *J Clin Invest* **115**, 1580-1589 (2005).
231. Hakonarson, H., Kim, C., Whelan, R., Campbell, D. & Grunstein, M.M. Bi-directional activation between human airway smooth muscle cells and T lymphocytes: role in induction of altered airway responsiveness. *J Immunol* **166**, 293-303 (2001).
232. Bachar, O., Adner, M., Uddman, R. & Cardell, L.O. Toll-like receptor stimulation induces airway hyper-responsiveness to bradykinin, an effect mediated by JNK and NF-kappa B signaling pathways. *Eur J Immunol* **34**, 1196-1207 (2004).
233. Tran, T. & Halayko, A.J. Extracellular matrix and airway smooth muscle interactions: a target for modulating airway wall remodelling and hyperresponsiveness? *Can J Physiol Pharmacol* **85**, 666-671 (2007).
234. Nguyen, T.T., Ward, J.P. & Hirst, S.J. beta1-Integrins mediate enhancement of airway smooth muscle proliferation by collagen and fibronectin. *Am J Respir Crit Care Med* **171**, 217-223 (2005).
235. Hirst, S.J., Twort, C.H. & Lee, T.H. Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. *Am J Respir Cell Mol Biol* **23**, 335-344 (2000).
236. An, S.S. *et al.* Airway smooth muscle dynamics: a common pathway of airway obstruction in asthma. *Eur Respir J* **29**, 834-860 (2007).
237. Pascual, R.M. & Peters, S.P. Airway remodeling contributes to the progressive loss of lung function in asthma: an overview. *J Allergy Clin Immunol* **116**, 477-486; quiz 487 (2005).
238. Bai, T.R., Cooper, J., Koelmeyer, T., Pare, P.D. & Weir, T.D. The effect of age and duration of disease on airway structure in fatal asthma. *Am J Respir Crit Care Med* **162**, 663-669 (2000).
239. Stewart, A.G. Emigration and immigration of mesenchymal cells: a multicultural airway wall. *Eur Respir J* **24**, 515-517 (2004).

240. Xu, K.F. *et al.* Antigen-induced airway inflammation in the Brown Norway rat results in airway smooth muscle hyperplasia. *J Appl Physiol* **93**, 1833-1840 (2002).
241. Singh, S.R. & Hall, I.P. Airway myofibroblasts and their relationship with airway myocytes and fibroblasts. *Proc Am Thorac Soc* **5**, 127-132 (2008).
242. Schmidt, M., Sun, G., Stacey, M.A., Mori, L. & Mattoli, S. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J Immunol* **171**, 380-389 (2003).
243. Madison, J.M. Migration of airway smooth muscle cells. *Am J Respir Cell Mol Biol* **29**, 8-11 (2003).
244. Caramori, G., Pandit, A. & Papi, A. Is there a difference between chronic airway inflammation in chronic severe asthma and chronic obstructive pulmonary disease? *Curr Opin Allergy Clin Immunol* **5**, 77-83 (2005).
245. Nathan, C. Points of control in inflammation. *Nature* **420**, 846-852 (2002).
246. Matzinger, P. The danger model: a renewed sense of self. *Science* **296**, 301-305 (2002).
247. Maertzdorf, J., Osterhaus, A.D. & Verjans, G.M. IL-17 expression in human herpetic stromal keratitis: modulatory effects on chemokine production by corneal fibroblasts. *J Immunol* **169**, 5897-5903 (2002).
248. Andoh, A. *et al.* IL-17 selectively down-regulates TNF-alpha-induced RANTES gene expression in human colonic subepithelial myofibroblasts. *J Immunol* **169**, 1683-1687 (2002).
249. Albanesi, C., Cavani, A. & Girolomoni, G. IL-17 is produced by nickel-specific T lymphocytes and regulates ICAM-1 expression and chemokine production in human keratinocytes: synergistic or antagonist effects with IFN-gamma and TNF-alpha. *J Immunol* **162**, 494-502 (1999).
250. Wang, Y.H. & Liu, Y.J. The IL-17 cytokine family and their role in allergic inflammation. *Curr Opin Immunol* **20**, 697-702 (2008).
251. de Rochemonteix-Galve, B., Marchat-Amoruso, B., Dayer, J.M. & Rylander, R. Tumor necrosis factor and interleukin-1 activities in free lung cells after single and repeated inhalation of bacterial endotoxin. *Infect Immun* **59**, 3646-3650 (1991).
252. Hakonarson, H. & Grunstein, M.M. Autocrine regulation of airway smooth muscle responsiveness. *Respir Physiol Neurobiol* **137**, 263-276 (2003).
253. Song, C. *et al.* IL-17-producing alveolar macrophages mediate allergic lung inflammation related to asthma. *J Immunol* **181**, 6117-6124 (2008).
254. Bedard, M. *et al.* Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis. *Am J Respir Cell Mol Biol* **9**, 455-462 (1993).
255. Message, S.D. & Johnston, S.L. Host defense function of the airway epithelium in health and disease: clinical background. *J Leukoc Biol* **75**, 5-17 (2004).
256. Andoh, A. *et al.* Interleukin-22, a member of the IL-10 subfamily, induces inflammatory responses in colonic subepithelial myofibroblasts. *Gastroenterology* **129**, 969-984 (2005).
257. Dambacher, J. *et al.* The role of the novel Th17 cytokine IL-26 in intestinal inflammation. *Gut* **58**, 1207-1217 (2009).
258. Hor, S. *et al.* The T-cell lymphokine interleukin-26 targets epithelial cells through the interleukin-20 receptor 1 and interleukin-10 receptor 2 chains. *J Biol Chem* **279**, 33343-33351 (2004).
259. Sheikh, F. *et al.* Cutting edge: IL-26 signals through a novel receptor complex composed of IL-20 receptor 1 and IL-10 receptor 2. *J Immunol* **172**, 2006-2010 (2004).
260. Hoshino, H. *et al.* Increased elastase and myeloperoxidase activity associated with neutrophil recruitment by IL-17 in airways in vivo. *J Allergy Clin Immunol* **105**, 143-149 (2000).
261. Segal, A.W., Heyworth, P.G., Cockcroft, S. & Barrowman, M.M. Stimulated neutrophils from patients with autosomal recessive chronic granulomatous disease fail to phosphorylate a Mr-44,000 protein. *Nature* **316**, 547-549 (1985).
262. Hallett, M.B. & Lloyds, D. Neutrophil priming: the cellular signals that say 'amber' but not 'green'. *Immunol Today* **16**, 264-268 (1995).

263. Girard, D., Boiani, N. & Beaulieu, A.D. Human neutrophils express the interleukin-15 receptor alpha chain (IL-15Ralpha) but not the IL-9Ralpha component. *Clin Immunol Immunopathol* **88**, 232-240 (1998).
264. Gessani, S. *et al.* Enhanced production of LPS-induced cytokines during differentiation of human monocytes to macrophages. Role of LPS receptors. *J Immunol* **151**, 3758-3766 (1993).
265. Nathan, C., Xie, Q.W., Halbwachs-Mecarelli, L. & Jin, W.W. Albumin inhibits neutrophil spreading and hydrogen peroxide release by blocking the shedding of CD43 (sialophorin, leukosialin). *J Cell Biol* **122**, 243-256 (1993).
266. Raptis, S.Z., Shapiro, S.D., Simmons, P.M., Cheng, A.M. & Pham, C.T. Serine protease cathepsin G regulates adhesion-dependent neutrophil effector functions by modulating integrin clustering. *Immunity* **22**, 679-691 (2005).
267. Jones, C.E. & Chan, K. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. *Am J Respir Cell Mol Biol* **26**, 748-753 (2002).
268. Ruddy, M.J., Shen, F., Smith, J.B., Sharma, A. & Gaffen, S.L. Interleukin-17 regulates expression of the CXC chemokine LIX/CXCL5 in osteoblasts: implications for inflammation and neutrophil recruitment. *J Leukoc Biol* **76**, 135-144 (2004).
269. Rao, D.A. *et al.* Interleukin (IL)-1 promotes allogeneic T cell intimal infiltration and IL-17 production in a model of human artery rejection. *J Exp Med* **205**, 3145-3158 (2008).
270. Zrioual, S. *et al.* IL-17RA and IL-17RC receptors are essential for IL-17A-induced ELR+ CXC chemokine expression in synoviocytes and are overexpressed in rheumatoid blood. *J Immunol* **180**, 655-663 (2008).
271. Zrioual, S. *et al.* Genome-wide comparison between IL-17A- and IL-17F-induced effects in human rheumatoid arthritis synoviocytes. *J Immunol* **182**, 3112-3120 (2009).
272. Magder, S., Neculcea, J., Neculcea, V. & Sladek, R. Lipopolysaccharide and TNF-alpha produce very similar changes in gene expression in human endothelial cells. *J Vasc Res* **43**, 447-461 (2006).
273. Shi, G.X., Harrison, K., Han, S.B., Moratz, C. & Kehrl, J.H. Toll-like receptor signaling alters the expression of regulator of G protein signaling proteins in dendritic cells: implications for G protein-coupled receptor signaling. *J Immunol* **172**, 5175-5184 (2004).
274. Haruta, H., Kato, A. & Todokoro, K. Isolation of a novel interleukin-1-inducible nuclear protein bearing ankyrin-repeat motifs. *J Biol Chem* **276**, 12485-12488 (2001).
275. Shiina, T. *et al.* Genomic organization, chromosomal localization, and promoter analysis of the mouse Mail gene. *Immunogenetics* **53**, 649-655 (2001).
276. Motoyama, M., Yamazaki, S., Eto-Kimura, A., Takeshige, K. & Muta, T. Positive and negative regulation of nuclear factor-kappaB-mediated transcription by IkappaB-zeta, an inducible nuclear protein. *J Biol Chem* **280**, 7444-7451 (2005).
277. Yamamoto, M. *et al.* Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature* **430**, 218-222 (2004).
278. Matsuo, S., Yamazaki, S., Takeshige, K. & Muta, T. Crucial roles of binding sites for NF-kappaB and C/EBPs in IkappaB-zeta-mediated transcriptional activation. *Biochem J* **405**, 605-615 (2007).
279. Anjum, R. & Blenis, J. The RSK family of kinases: emerging roles in cellular signalling. *Nat Rev Mol Cell Biol* **9**, 747-758 (2008).
280. Kayama, H. *et al.* Class-specific regulation of pro-inflammatory genes by MyD88 pathways and IkappaBzeta. *J Biol Chem* **283**, 12468-12477 (2008).
281. Ramirez-Carrozzi, V.R. *et al.* A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling. *Cell* **138**, 114-128 (2009).
282. Tullai, J.W. *et al.* Immediate-early and delayed primary response genes are distinct in function and genomic architecture. *J Biol Chem* **282**, 23981-23995 (2007).

283. Ashall, L. *et al.* Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription. *Science* **324**, 242-246 (2009).
284. Hoffmann, A., Levchenko, A., Scott, M.L. & Baltimore, D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science* **298**, 1241-1245 (2002).
285. Nelson, D.E. *et al.* Oscillations in NF-kappaB signaling control the dynamics of gene expression. *Science* **306**, 704-708 (2004).
286. Kearns, J.D., Basak, S., Werner, S.L., Huang, C.S. & Hoffmann, A. IkappaBepsilon provides negative feedback to control NF-kappaB oscillations, signaling dynamics, and inflammatory gene expression. *J Cell Biol* **173**, 659-664 (2006).
287. Shih, V.F. *et al.* Kinetic control of negative feedback regulators of NF-kappaB/RelA determines their pathogen- and cytokine-receptor signaling specificity. *Proc Natl Acad Sci U S A* **106**, 9619-9624 (2009).
288. Cheong, R. *et al.* Transient IkappaB kinase activity mediates temporal NF-kappaB dynamics in response to a wide range of tumor necrosis factor-alpha doses. *J Biol Chem* **281**, 2945-2950 (2006).
289. Lindenblatt, C., Schulze-Osthoff, K. & Totzke, G. IkappaBzeta expression is regulated by miR-124a. *Cell Cycle* **8**, 2019-2023 (2009).
290. Krichevsky, A.M. & Gabriely, G. miR-21: a small multi-faceted RNA. *J Cell Mol Med* **13**, 39-53 (2009).
291. Selcuklu, S.D., Donoghue, M.T. & Spillane, C. miR-21 as a key regulator of oncogenic processes. *Biochem Soc Trans* **37**, 918-925 (2009).
292. Asangani, I.A. *et al.* MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene* **27**, 2128-2136 (2008).
293. Fujita, S. *et al.* miR-21 Gene expression triggered by AP-1 is sustained through a double-negative feedback mechanism. *J Mol Biol* **378**, 492-504 (2008).
294. Frankel, L.B. *et al.* Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J Biol Chem* **283**, 1026-1033 (2008).