Evolution of Innate Immunity in African Catarrhines

By

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ABSTRACT

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Innate immunity is the first line of host defense against invading pathogens, involves activation of innate immune cells via Toll-like receptors (TLRs) and is a major factor affecting host susceptibility to infectious disease. African catarrhine primates share high genomic identity, yet appear to differ in their susceptibility to bacterial infections (i.e. Gram-negative bacterial sepsis, *Neisseria gonorrhoeae*, *Mycobacterium*). These species are hypothesized to have divergent evolutionary histories of pathogen exposure due to differences in geographic distribution and behaviour. The goals of this research were to 1) clarify if the early innate immune responses of African catarrhine species have functionally diverged, and 2) examine possible associations between these responses and pathogen type, primate evolutionary landscape and disease susceptibility. To examine if African catarrhines have evolved different early innate immune responses to environment-specific pathogens, fresh whole blood from *Homo*, *Pan* and *Papio* was stimulated with TLR2 and TLR4 - detected molecular components from pathogens unevenly distributed across primate evolutionary habitats (i.e. *Mycobacterium, Yersinia pestis*) for 90 minutes. Immune activation was assessed by quantifying expression of genes associated with the early innate immune response by real-time PCR. This study shows that *Homo* and *Pan* blood leukocytes typically mount similar early cytokine and chemokine responses to stimuli, while the more distantly related *Papio* mounts opposing responses. The divergence of human/chimpanzee and baboon cytokine/chemokine induction broadly agrees

with observed differences in susceptibility to bacterial diseases, however no association was found between putative pathogen/primate evolutionary environment and gene induction. While early innate immune responses tend to agree with primate evolutionary relationships, there are some notable exceptions to this pattern, including some cytokine responses that are agonist/pathogen-specific (i.e. IL-1β, TNFα, IL-10, IL-6). Taken together, this data suggests a significant divergence between hominoid and baboon early innate immune responses since these species shared a last common ancestor 23-29 million years ago.

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Dedication

To my parents, Charles and Cheryl Brinkworth for encouraging my interests in science and the arts - letting me watch the Jane Goodall National Geographic special over and over, dig up the backyard and play in the Portage du Fort archaeological back-fill.

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LIST OF ABBREVIATIONS

<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
ACTB	Beta-actin
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen presenting cell
AP1	Activator protein 1
ApoL-I	Apolipoprotein L-I
ATF2/JUN	Activating transcription factor 2/ jun proto-oncogene
B2M	Beta-2 microglobulin
BCR	B cell receptor
BLAST	Basic Local Alignment Search Tool
CCL2	CC motif ligand 2
CCL3	CC motif ligand 3
CCL4	CC motif ligand 4
CCL5	CC motif ligand 5
CCL11	CC motif ligand 11
CCR1	CC motif receptor 1
CCR2	CC motif receptor 2
CCR3	CC motif receptor 3
CCR5	CC motif receptor 5
CD	Cluster of Differentiation
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CD14	Cluster of Differentiation 14
CD36	Cluster of Differentiation 36
CD116	Cluster of Differentiation 116
CD120a	Cluster of Differentiation 120a
CD120b	Cluster of Differentiation 120b
CD121a	Cluster of Differentiation 121a
CD130	Cluster of Differentiation 130
CD212	Cluster of Differentiation 212
CDw119	Cluster of Differentiation w 119
CDw131	Cluster of Differentiation w 131
CEBPA	CCAAT/enhancer-binding protein alpha
CpG	Cytosine – phosphate - guanine
Cq	Quantification cycle/cycle at threshold
CREB1	CAMP responsive element binding protein 1
CSF-1	Colony stimulating factor 1
CSF-2	Colony stimulating factor 2
CTL	Cytotoxic T lymphocyte
CXCL1	CXC motif ligand 1
CXCL2	CXC motif ligand 2
CXCL3	CXC motif ligand 3

CXCL12	CXC motif ligand 12
CXCR1	CXC motif receptor 1
CXCR2	CXC motif receptor 2
dsRNA	Double stranded ribonucleic acid
E	Efficiency
E2F1	E2F transcription factor 1
Eamp	Exponential amplification
Egr1	Early growth response protein 1
ELISA	Enzyme-linked Immunosorbant Assay
ELK1	E twenty-six –like transcription factor 1
ESR1	Estrogen receptor 1
EU	Endotoxin unit
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol anchors
H	*Homo* (for Mann Whitney comparisons)
HCV	Hepatitis C virus
HEK-293	Human embryonic kidney cells, 293 experiment (Graham)
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
Hpr	Haptoglobin-related protein
IACUC	Institutional Animal Care and Use Committees
IFNα	Interferon, alpha
IFNγ	Interferon, gamma
IL	Interleukin
IL-1β	Interleukin 1-Beta
IL-1R	Interleukin 1 receptor
IL-1RN	Interleukin 1 receptor antagonist
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-6Ra/CD126	Interleukin 6 Receptor a/Cluster of Differentiation 126
IL-8/CXCL8	Interleukin 8 /CXC motif ligand 8
IL-10	Interleukin 10
IL-10RA	Interleukin 10 Receptor a
IL-10RB	Interleukin 10 Receptor b
IL-12A	Interleukin 12A
IL-13	Interleukin 13
IκK	Ikappa kinase
IκKβ	Ikappa kinase, beta
IκBKG	Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase of, gamma
IκKi	I-Kappa-B Kinase, epsilon
IRAK1	Interleukin-1 receptor-associated kinase 1
IRAK2	Interleukin-1 receptor-associated kinase 2
IRAK4	Interleukin-1 receptor-associated kinase 4

IRAKM	Interleukin-1 receptor-associated kinase M
IRB	Institutional Review Board
IRF-3	Interferon regulatory factor 3
IRF-7	Interferon regulatory factor 7
JNK	c-Jun N-terminal kinase
KW	Kruskal-Wallis test
LAL gel clot assay	Limulus amebocyte lysate gel clot assay
LBP	Lipopolysaccharide-binding protein
LCA	Last common ancestor
LcrV	V antigen from *Yersina pestis*
LMMS	Lipomannan from *Mycobacterium smegmatis*
LPS	Lipopolysaccharride (from *Escherichia coli*, 0111:B4 here)
LRR	Leucine rich repeat
LY96	Lymphocyte antigen 96 (MD-2)
MALP-2	Macrophage-activating lipopeptide – 2
MAPK	Mitogen-activated protein kinase
mg	milligram
MHC	Major histocompatability complex
ml	millilitre
MW	Mann Whitney test
mya	Million years ago
MYC::MAX	V-myc avian myelocytomatosis viral oncogene homolog :: myc-associated factor X
MyD88	Myeloid differentiation primary response gene, 88
Na-heparin	Sodium heparin
NEMO	Nuclear factor – kappa light chain gene enhancer in B cells, essential modulator
NFIC	Nuclear factor I/C
NF-κB	Nuclear factor of kappa light chain gene enhancer in B cells
NF-κB1	Nuclear factor of kappa light chain gene enhancer in B cells, subunit 1
NFκBIA	Nuclear factor of kappa light chain gene enhancer in B cells inhibitor, alpha
ng	nanogram
NK	Natural killer cells
ORF	Open reading frame
p38	P38 mitogen-activated protein kinases
Pa	*Pan* (for Mann Whitney comparisons)
PA	Protective antigen from *Bacillus anthracis*
Pam3CSK4	N-Palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl- [S]-seryl-[S]-lysyl- [S]-lysyl-[S]-lysyl-[S]-lysine (triacylated lipoprotein mimetic)
PAMP	Pathogen-associated molecular pattern
Pap	*Papio* (for Mann Whitney comparisons)
PBMCs	Peripheral blood mononuclear cells
pDC	Plasmacytoid dendritic cells
PGN	Peptidoglycan
Pham 1.0	*Papio hamadryas* genome draft 1 (Baylor College of Medicine

	November, 2008)
PMN	Polymorphonuclear leukocytes
PPARG	Peroxisome proliferators-activated receptor-gamma
PRR	Pattern recognition receptor
PSM	Phenol-soluble modulin
REL	V-Rel avian reticuloendotheliosis viral oncogene homolog
RELA	V-Rel avian reticuloendotheliosis viral oncogene homolog A
SARS-CoV	Severe acute respiratory syndrome coronavirus
SIV	Simian immunodeficiency virus
SP1	Transcription factor SP1 (Specificity protein 1)
SPI1	Spleen focus forming virus proviral integration oncogene
ssRNA	Single stranded ribonucleic acid
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3
TAB2	TGF-beta activated kinase 1/mitogen-activated protein kinase kinase kinase 7 binding protein 2
TAB3	TGF-beta activated kinase 1/mitogen-activated protein kinase kinase kinase 7 binding protein 3
TAK1	Transforming growth factor β–activated kinase 1
TAP	Transcriptional activation programme
TBP	TATA box-binding protein
TBK1	Tank-binding kinase 1
TBRI	Texas Biomedical Research Institute
TCR	T cell receptor
TFAP2A	Transcription factor AP2-alpha
TFB sites	Transcription factor binding sites
TGFβ1	Transforming growth factor Beta-1
Th1	T helper 1 cells
Th2	T helper 2 cells
TIR domain	Toll-interleukin 1 receptor domain
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TP53	Tumour protein 53
TRAF3	TNF receptor-associated factor 3
TRAF6	TNF receptor-associated factor 6
TRAM	Translocation associated membrane protein
TRED	Transcriptional regulatory element database
TRIF	TIR-domain-containing adapter-inducing interferon-β
ug	microgram
ul	microlitre
uM	micromolar
USF1	Upstream stimulatory factor 1

CHAPTER 1:
INTRODUCTION AND BACKGROUND

1.1 Introduction

Several lines of evidence suggest that pathogens have significantly affected the evolution of mammals, including the rapid evolution of immune system genes and the considerable proportion of the mammalian genome dedicated to the immune response (1-3). Multiple pathogens are thought to have exerted selective pressure on primate immune system genes for millions of years (4-9). Humans (*Homo sapiens*) and common chimpanzees (*Pan troglodytes*) are estimated to share >94% genomic identity with baboons (*Papio* sp.), yet these species appear to be differently susceptible to infectious diseases that are, in *Homo*, marked by dysregulation of the inflammation response (gram-negative bacterial sepsis, mycobacterial diseases, *Neisseria gonorrhea*, Hepatitis C virus) (10-18). Specifically, white blood cell (leukocyte) responsiveness to pathogens during the early hours of infection appears to differ among catarrhine species, which may affect the inflammation response and infectious disease progression (19-21). One possible explanation for disparate immune responses between such closely related species is lineage-specific adaptation of the early innate immune response to various pathogens.

The current understanding of catarrhine immune system function and evolution is mainly the result of research on genes and proteins thought to contribute to inter-species differences in primate-viral interactions (5, 22-29). Available information on catarrhine immune responses to pathogenic bacteria is considerably limited, even though bacterial pathogens are major agents of primate disease, are cited as effecting the primate genome and appear to manifest differently in

catarrhine primates (12, 19, 30, 31). Catarrhine microbiota shows significant inter-genus differences in bacterial composition, suggesting that catarrhine species have adapted to proportions and types of bacterial microbes (32-35). Despite the importance of bacterial pathogens to human health and their potential impact on primate evolution, very little is known about the evolution of early immune responses of primates to such pathogens.

To better understand why some catarrhine species have differing susceptibility to bacterial pathogens, this dissertation examines the early immune response of multiple primate species for functional differences. To test if catarrhines have evolved different immune responses blood leukocytes of closely related species can be stimulated with a broad range of pathogens and the induction levels of genes that initiate the immune response can be assessed. *Homo*, *Pan* and *Papio* are hypothesized to have divergent histories of bacterial pathogen exposure due to differences in their geographic distribution and behaviour (36, 37). Toll-like receptors (TLRs) are innate immune system receptors that assist in initiating a general and highly organized immediate immune response by recognizing "non-self" molecules and triggering a signaling cascade that leads to immune protein release. This dissertation examines early innate immune response to TLR-detected pathogens to determine if innate immunity differs in these three African catarrhines. These data are then used to address the effect of purported primate evolutionary landscape-specific pathogen exposure on these responses. To accomplish this overarching goal, I

1) tested for signs of innate immune function divergence by examining cytokine induction in total leukocytes of *Homo*, *Pan* and *Papio* in response to TLR-detected bacterial ligands

and

2) investigated if species responses can be correlated to putative primate evolutionary landscapes by examining the pattern of gene induction after stimulation with TLR-detected pathogens from particular primate-evolutionary environments

The results of this dissertation will contribute to a better understanding of primate immunity and evolution by describing early innate immune responses of African catarrhine blood leukocytes and clarifying the role of TLR-detected pathogens in the divergence of the *Homo*, *Pan,* and *Papio* lineages.

1.2 Primate disease ecology and evolution

Differences in the early immune response in the *Homo*, *Pan* and *Papio* lineages should be expected given that behaviour and geographic distribution of these primates has significantly diverged over the last 23-29 million years (36, 38). Restriction of the *Pan* lineage to forested regions and increased exploitation by *Homo* and *Papio* lineages of savanna landscapes is strongly connected to changes in diet, geographic distribution, group size/composition, social organization and levels of promiscuity that contribute to novel pathogen exposure (36, 37, 39, 40). Catarrhine primate lineages to appear to have co-evolved with different types bacterial pathogens. Available information indicates that proportion and types of species found in bacterial microbiota of extant catarrhine species can differ considerably (33, 35, 41, 42). Catarrhine TLR amino acid sequence have diverged and show evidence of selection. Regions of the bacterial-detecting TLR1, TLR2 and TLR4 exhibit a complex profile of positive selection in primates and a high proportion of missense mutations in *Homo* specifically (43, 44). Associations have been found between primate behaviour and the

evolution of bacterial-detecting TLRs. Variation in female promiscuity has been associated with signatures of positive selection in primate TLR4. The geographic distribution of *Homo* has been correlated with patterns of selection on TLR1 and 4 as well (43, 45).

1.3 Vertebrate immunity

The immune response of vertebrate animals can be classified into "innate" or "adaptive" responses (**Table 1.1**). Innate immunity is a host's first line of defense and only protection against invading pathogens during the first four hours of infection. It is an ancient, genetically inherited, continuous immune response characterized by inflammation, barriers (i.e. mucous, skin) and phagocytosis (46, 47). When innate immune cells recognize invading pathogens, they initiate the release of reactive signaling proteins, known as cytokines. Cytokines degrade pathogens non-specifically and initiate activation of an epitope/pathogen-specific T and B cell mediated adaptive immune response. T and B cells become activated when T and B cell receptors recognize specific epitopes, presented on phagocytic innate immune cell. Activated T and B cells then clonally replicate in secondary lymphoid tissues, to be released as cytotoxic, phagocytic or antibody producing cells that recognize and attempt to eliminate a specific epitope target. The engagement of the adaptive immune response typically occurs after the 4^{th} hour of infection. The first clonal adaptive immune cells are released ~96 hours from the point of initial T or B cell activation. As an infection is cleared over successive hours, most clonal T and B cells die off. A small percentage, however, remain in circulation as memory T and B cells. Memory cells speed the adaptive response to re-encountered foreign epitopes, and are the basis for immunological memory of past infections (**Figure 1.1**). While innate immunity is the only protection against infection progression in the first hours of infection, it

is also a mediator of the adaptive immune response throughout the entire course of infection. As such, regulation of innate immune cell activity strongly influences host survival [reviewed in (48)].

1.3.1 Early innate immunity

During the first two hours of infection activated TLRs initiate an important and tightly controlled response to invading pathogens through the induction of a universal "alarm response", sometimes referred to as the common transcriptional activation program (TAP). This early alarm response is characterized by the induction of a subset of pro- and anti-inflammatory cytokines and chemokines. It is thought to exert great control over the course of infection and to be evolutionarily conserved across mammals (49, 50). This early innate immune response affects early infection course, innate immune cell activity and may affect downstream innate and adaptive immune function (49, 50) (**Table 1.2**). There is little information on how early innate immune response has evolved and is initiated in primates.

	Innate Immune Response	**Adaptive Immune Response**
Life forms	Eukaryota (multi-cellular life)	Gnathostomata (jawed vertebrates)
Time	Immediate (0 hours, onwards)	Early (hour 96 onwards)
Response specificity	Low. Responds to danger signals and molecular motifs broadly shared by microbes	High. Identifies specific antigens/epitopes and generates a clonal T and B cells response.
Immune mechanisms	Phagocytosis, barriers, inflammation, opsonization, fever	Cytotoxic lymphocytes, antibodies
Primary Cell types	Neutrophils, monocytes/macrophages, natural killer cells, eosinophils/basophils, dendritic cells, epithelial cells	T lymphocytes, B lymphocytes
Immune memory	No. Response is inherited	Yes. Memory B and T cells "remember" prior exposure to pathogens

Table 1.1 Comparison of innate and adaptive immune systems

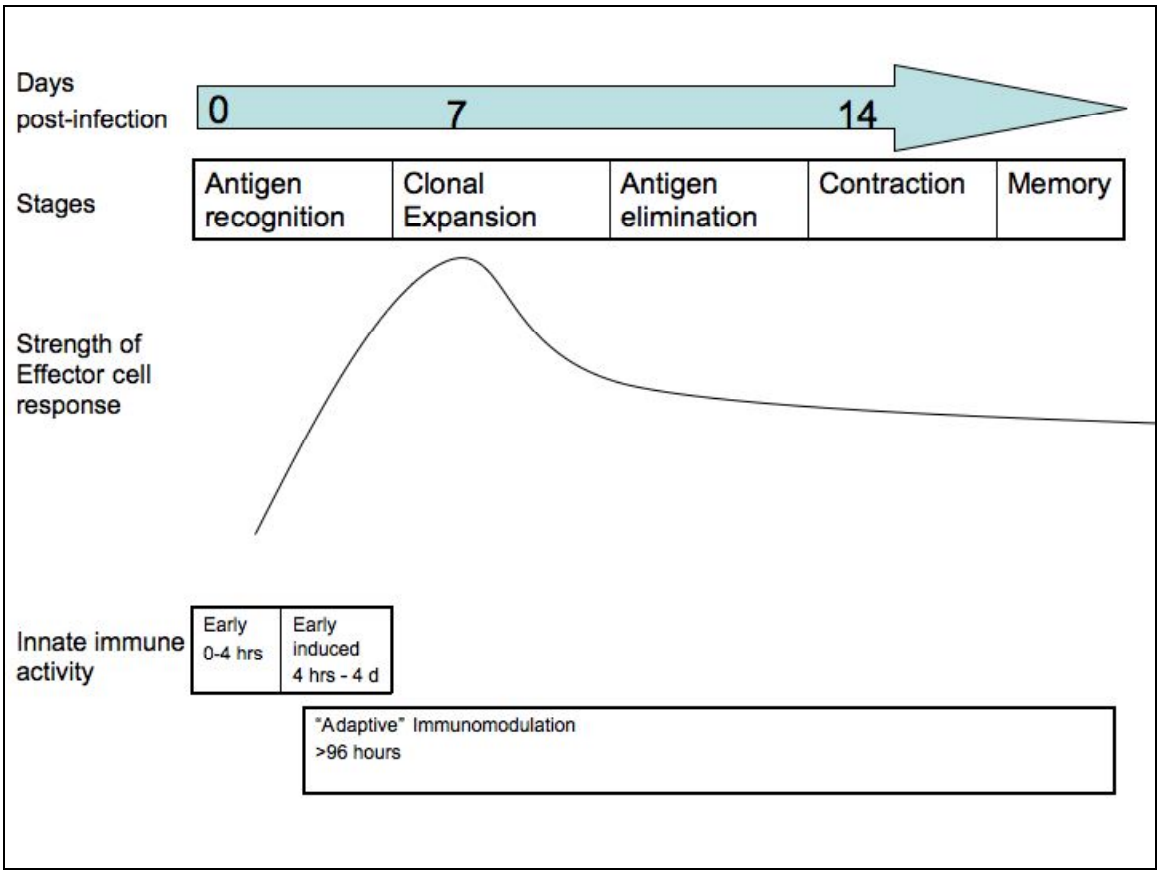


Figure 1.1 Timeline of example innate and adaptive immune response to infection

Phase of Innate Immunity	**Early 0-4 hrs**	**Early induced 4 hrs-4 days**	**"Adaptive" Immune response initiation >96 hrs**
Dominant Innate Immune Activity	Complement	Effector cell recruitment and activation	Antigen transported to lymphoid organs
	Pattern recognition		Modulates adaptive responses
	APC and PMN activation, antigen presentation (Early innate immunity 0-2 hrs)		
	Inflammation		
	Phagocytosis		

Table 1.2 Phases of Innate Immune response

1.3.2 Innate immunity

Blood leukocyte types

A broad range of leukocyte types are involved in innate immunity (**Table 1.3**). Monocytes are pro-inflammatory cytokine producing phagocytes and occasional antigen-presenting cells that can kill invading pathogens and infected host cells by both phagocytosis and cytotoxicity. Monocytes migrate in and out of blood circulation to sites of tissue infection, where they differentiate into tissue-specific macrophages (51). Monocytes represent 3-5% of blood leukocytes in healthy *Homo.* Granulocytes are leukocytes that have cytoplasmic granules. Blood granulocytes include neutrophils, eosinophils and basophils. Neutrophils compose 50-70% of circulating blood leukocytes in *Homo.* Neutrophils are highly motile and phagocytic. Typically, they act as the first innate immune cells at the site of infection, respond to pro- and anti-inflammatory cytokines, and release effector proteins that destabilize microbes when activated [reviewed in (51) (52)]. Neutrophils may also amplify inflammatory responses by releasing cytokines that activate and/or chemoattract other cell types (53). Basophils represent 1% or less of circulating leukocytes in healthy humans (54). They may act as late phase effector cells, but are also known to mediate immediate inflammatory/hypersensitivity responses, stimulate T helper cell type 2 (Th2)-mediated inflammation and may present antigens during helminth infection (55, 56). Eosinophils are cytotoxic effector cells that represent 1-4% of circulating leukocytes in healthy individuals. Eosinophils can detect bacterial, viral and helminth PAMPs directly, phagocytosize pathogens and/or immediately release cationic proteins and damage/remodel host tissues in the process (57). In addition to immediate cytotoxic activities, eosinophils activate naïve T cells and immunomodulate Th2 cell responses in the very early and later stages of infection (58). Natural Killer Cells are cytotoxic lymphocyte cells that release serine proteases known as granzymes that induce apoptosis in viral infected and tumour cells (51).

1.3.3 Adaptive immunity

Blood leukocyte types

The adaptive immune response "adapts" to specific antigens the host has encountered in the past through the development of immunological memory. A limited number of blood leukocyte types are involved in this highly specified response including T lymphocytes and B lymphocytes (59). The specificity of the adaptive immune response is partially mediated through T and B lymphocytes T cell receptors (TCR) and B cell receptors (BCR), which recognize specific antigens (60, 61). Individual T and B cells recognize specific antigens by highly variable TCR and BCR gene rearrangement that affects antigen binding regions during lymphocyte development (60). Blood circulating B cells include plasma B cells and memory B cells that have several functions once activated, including antibody release, clonal expansion/memory and antigen-presentation (61). Broadly, blood circulating T cells can be divided into two classes defined by the expression of CD co-receptors that affect the process of antigen recognition, CD8+ T cells and CD4+ T cells (62). CD8+ T cells are cytotoxic and lyse pathogen-infected host cells. Upon antigen recognition CD4+ T cells, or "helper T cells", release cytokines that regulate the function of other immune cells (61). Neither T cell classes can recognize circulating antigens. T cells can only recognize antigens when they are presented in Major Historcompatability Complexes (MHC) complexes on the cell membranes of antigen-presenting cells (i.e. macrophages)(62).

1.3.4 Cytokines

Immune cell communication is aided by secretion of cytokines, small glycoproteins. Cytokines are potent mediators of cell function and can induce cell death and cell dysfunction in small doses (63). Many cytokines are inducible, while others are constitutively expressed. Cytokines interact in cascades, are capable of inducing one another and, therefore, can regulate their own production in response to environmental stimulation (63). Most cytokines are pleiotropic, their function mediated by binding to cell receptors. Cytokine/receptor binding triggers intracellular signaling cascades that regulate cell activities, including cell activation, cytokine production, apoptosis, cell migration and cell development. As such, cytokines are involved in almost every activity of the innate and adaptive immune systems.

Many cytokines are functionally redundant. Cytokine functional redundancy can mean that initial weak cytokine production can trigger the production of functionally similar cytokines leading to significant signal amplification [reviewed in (63)]. Signal amplification can have important effects on surrounding tissues. Under pathologic conditions, small doses of cytokines can induce significant tissue injury (64-66). Systemic cytokine cascades can lead to widespread tissue and organ injury and host death. As such, cytokine production is highly regulated and with many cytokines having short half lives, sometimes only minutes long (63).

Cell type	Immune Reponse	Role	Cytokines Expressed	Chemokines Expressed
Neutrophils	Innate	Phagocytosis, degranulation, first responders to pro-inflammatory signals,	IL-1β, IL-1RN, TNFα, IFNy, IL-17, IL-6 (67-76)	CCL3 (77), CCL4 (78), IL-8 (71)
Monocytes (Macrophages)	Innate	Immediate inflammation, phagocytosis, antigen presentation, immunomodulation	CSF-2, IL-1RN, IL-6, TNFα, IL-1β IL-10 (49, 79-81)	IL-8/CXCL8, CCL2, CXCL1, CCL3, CCL4, CXCL2, CXCL3, CCL5 (49)
Basophils	Innate	Immediate/allergic inflammation, immunomodulate Th2 responses.	IL-3, IL-4, IL-5, IL-13, (82, 83) IL-6 (54), IL-1β and CSF-2 (82)	CCL3, CCL11, IL-8/CXCL8 (82) (84)
Eosinophils	Innate	Inflammatory reactions,	IL-1β, IL-2, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, IFNγ, CSF-2, TGFβ, TNFα (57),	CCL3, CCL5, CCL8, CCL11, CXCL12 (57),
Lymphocytes	Innate-Adaptive	infected cell lysis/regulate infected/tumour cell lysis (NK cells)	IL-1β, IL-2, IL-6, IL-10, CSF-2, IFNγ, TGFβ TNFα, (85-88),	CCL3, CCL5, CXCL2, IL-8/CXCL8 (85, 89-92)
Lymphocytes	Adaptive	Antigen presentation/antibody secretion (B cells)	CSF2, IL-1β, IL-1RN, IL-2, IL-6, IL-10, IL-12, IFNγ, TGFβ, TNFα (93-97)	CCL2 CCL3, CCL5, IL-8/CXCL8 (98)
		infected cell lysis/regulate immune cells (T cells),	CSF2, IL-1β, IL-2, IL-6, IL-10, TGFβ, TNFα (97, 99-104)	CCL2, CCL3, CCL5, IL-8/CXCL8 (103, 105)

Table 1.3 Blood leukocyte types, activities during acute infection, and select cytokine and chemokine expression in human cell types.

Variation in cytokine levels in response to bacterial infection has been noted between *Homo* and other catarrhine species (65, 106-109). As cytokines are not only signaling molecules, but also cytotoxic proteins, their production may affect the progression of microbial infections. Differences in cytokine production have been linked to host survival. Increased IL-6 and IL-1β expression

during early systemic bacterial infection, for example, is associated with increased organ dysfunction and mortality (65, 107, 110). Particular cytokines affect microbial replication and induce microbial death in a dose dependent manner (111-113). It seems likely that differences in cytokine production between species should affect disease outcomes.

Cytokine function in the immune system has evolved to be highly redundant. There is considerable overlap in blood leukocyte expression of cytokines as well as overlap in cytokine activities (**Table 1.4**). Due to their functional redundancy and diverse structures, cytokine division into subcategories is often debated and revised based on protein structure and function (63, 114). Broadly, cytokine proteins can be grouped into the following functional categories: pro-inflammatory cytokines, chemokines, and anti-inflammatory cytokines.

Pro-inflammatory cytokines are small molecules that induce inflammation. These cytokines include a wide assortment of small molecules including some interleukins, tumour necrosis factors and some growth factors. Interleukins form the largest group of pro-inflammatory cytokines and play an important role in early innate immune responses. Interleukins are molecules involved in immune cell development and differentiation [reviewed in (63). Included within this category are interferons, which restrict viral activity [reviewed in (115)]. Cytokines can be grouped into subgroups based on the cell subtypes they affect. Type 1 cytokines are pro-inflammatory and activate T-helper 1 cells, affecting intracellular parasite and viral infections (i.e. IL-12, TNFα, IL-6, CSF-1). Type II cytokines tend to be anti-inflammatory, stimulate T-helper 2 cells and, therefore, induce antibody responses (i.e. IFN) (116, 117).

Anti-inflammatory cytokines are proteins that inhibit the effects of pro-inflammatory cytokines (i.e. IL-1RN, IL-10). The resolution of pro-inflammatory cytokine activity is very complicated and not well understood. Anti-inflammatory cytokines appear to inhibit pro-inflammatory cytokines by inducing competitive inhibitors, inhibiting pro-inflammatory cytokine receptors, triggering apoptosis and stimulating cell replication and development [reviewed in (63)]. With the possible exception of IL-1RN, many anti-inflammatory cytokines have some pro-inflammatory affects as well (118). IL-6, for example, is often considered as a pro-inflammatory cytokine. The expression of IL-6 can inhibit the production of pro-inflammatory cytokines such as CSF-1 and CXCL2, as well as limit the effect of TNFα and IL-1 cytokines by inducing the production of their soluble receptors (119). Under pathologic conditions anti-inflammatory cytokines do not control pro-inflammatory activities or are over-produced and limit the immune response (120).

Chemokines are cytokines that affect the recruitment and migration of leukocytes to infection sites. In doing so, they affect cell membrane composition, activate leukocytes and stimulate cell differentiation. Chemokines induce chemotaxis, through the induction of cell release from bone marrow (i.e. CSF-1), coordinated development of chemokine gradients, initiation of immune cell adhesion to and rolling along endothelial cell membranes (i.e. IL-8/CXCL8) (121, 122). Importantly, chemokines promote endothelial extracellular matrix proteolysis allowing for cell migration through venular walls (123). Chemokines are typically produced after immune stimulation, though some are active during development (124) (125, 126). Chemokines are very reactive, pro-inflammatory molecules capable of injuring host tissues. As such, their production is usually transient and quickly downregulated [reviewed in (63)].

Ultimately, while cytokine and chemokine induction initiates and modulates many important cellular immune responses, their over-expression is associated with major human infectious diseases. Severe sepsis and septic shock, for example, kill ~375 000 people in the U.S. annually and are characterized by strong and unregulated expression of pro- and anti-inflammatory cytokines (65, 127, 128). Antigen presenting cells secrete IL-10 upon detection of *Mycobacterium tuberculosis*, a response that lowers Th1 responses and thereby enhances susceptibility to infection and reactivation of the disease (129-131). HIV-1 infection is marked by a significant decrease in Th1 cytokines and interferons, and a significant increase in Th2 cytokines, greatly impairing cellular responses, effecting viral load and contributing to disease progression [reviewed in (132)].

Name	Source	Target	Function	Receptor
CCL2	Eosinophils Monocytes (macrophages) Neutrophils Natural killer cells T-cells (133)	Monocytes, memory T cells, basophils,	Chemotaxis, inflammation, T cell activation	CCR2
CCL3	Monocytes (macrophages), natural killer cells, CTL and Th cells	Neutrophils, monocytes (macrophages), T cells, B cells, eosinophils, NK cells, dendritic cells (133-137)	Chemotaxis, inflammation,	CCR5, CCR1
CCL5	T cells, eosinophils (138)	Monocytes, T cells, eosinophils, basophils, dendritic cells (139-141)	Chemotaxis, eosinophil activation, basophil stimulation	CCR5, CCR3, CCR1
CXCL2	Monocytes (macrophages) (134)	Neutrophils (142)	Chemotaxis, inflammation,	CXCR1, CXCR2
CXCL8/IL-8	Monocytes (macrophages) (143)	Neutrophils endothelial cells (122, 144)	Chemotaxis, inflammation, anti-apoptosis	CXCR1, CXCR2
CSF-2	T cells, (macrophages) (100)	Neutrophils, macrophages, monoblasts, stem cells	Promotes half life of neutrophils, anto-apoptosis, cell differentiation and growth (145, 146)	CD116, CDw131
IFNγ	T-cells, Th cells, Natural killer cells (147)	Neutrophils, monocytes, macrophages, T cells (148-152)	Immunomodulatory, anti-viral, inflammation (152)	CDw119
IL-1RN	Monocytes, neutrophils (67, 153)	All IL-1R+ cells (154)	Anti-inflammatory (68)	CD121a
IL-1β	Monocytes (macrophages), neutrophils (69, 155)	All IL-1R+ cells Multiple cell types (154)	Inflammation, activate lymphocytes (155)	CD121a, CDw121b

Table 1.4 Select cytokines and chemokines expressed by blood leukocytes: source cells, target cells and function are listed.

Name	Source	Target	Function	Receptor
IL-6	Monocytes (Macrophages), Th1 cells, B cells (156)	T cells, activated B cells, monocytes (157, 158)	Inflammation, immunomodulatory (159)	IL-6Ra/CD126, CD130 (160)
IL-10	T cells, B cells, activated monocytes (macrophages) (161-163)	Th1, monocytes, activated neutrophils (164)	Anti-inflammatory, CTL chemotaxis, inhibits Th1 responses, enhances natural killer responses (165, 166)	IL-10RA, IL-10RB
IL-12A	Monocytes (macrophages) (167)	T cells, NK cells (167)	Immunomodulatory, stimulates NK and Th1 responses (167)	CD212 (167)
TNFα	Monocytes (macrophages), NK cells, T cells, neutrophils (168-170)	Lymphocytes	Inflammation, apoptosis, lymphocyte chemotaxis, endothelial activation (171, 172)	CD120a, CD120b

Table 1.4 Continued, select cytokines and chemokines expressed by blood leukocytes: source cells, target cells and function are listed.

1.4 Innate immune recognition

An important component of innate immune cell recognition of invading pathogens is the detection of "non-self" molecules by inherited pattern recognition receptors (PRR) and co-receptors on phagocytic cells (173). PRRs recognize molecular motifs that are absent in vertebrate hosts, but conserved and shared across broad groups of microbial organisms. These "pathogen-associated molecular patterns" (PAMPs) include lipopolysaccharide (LPS) from gram-negative bacteria, peptidoglycan (PGN) from gram-positive bacteria, bacterial flagellin and viral single stranded RNA (ssRNA) amongst other motifs. Specific PRRs, sometimes with co-receptors, recognize and bind particular PAMPs. When an innate immune cell PRR detects and binds to a PAMP, an intra-cellular

signaling cascade is triggered, the cell is "activated" and cytokine and co-stimulatory molecule induction initiated (174, 175).

1.4.1 Toll-like Receptors

Toll-like receptors (TLRs) are the most thoroughly studied PRRs to date. Integral to the current understanding of innate immunity, the discovery of TLRs is rooted in studies of developmental biology. The original discovery of the *Drosophila* ortholog Toll in the mid-1980s well preceded a resurgence of academic interest in the long overlooked innate immune system (176, 177). Toll was discovered through an innovative series of genome-wide mutational screens completed in the interests of identifying genes involved in *Drosophila melanogaster* embryonic development. In a search for genes that control embryonic pattern formation, Max-Planck Institute (Tubingen) post-doctoral trainee Christine Nüsslein-Volhard located and recovered multiple mutations of a maternal effect gene associated with dorsal-ventral orientation and development of the embryo (176, 177). This gene is named "Toll", a term that means "weird" or "fantastic" in German. The name allegedly references Nüsslein-Volhard's excited cry of "that's weird" after her review of the identifying mutational screen (178).

In *Drosophila* Toll encodes a type 1 membrane receptor that detects maternal proteins, and triggers developmental pathways in the embryo (179). A decade after its discovery, two major discoveries connected toll to immune function: the first was the 1996 discovery that Toll was required by *Drosophila* to fight fungal infections and the second followed shortly thereafter in 1997 when Medzhitov and Janeway cloned the first mammalian homologue of toll, TLR4, in humans and

found this receptor triggered NF-κB activation in a similar fashion to IL-1R (180, 181). Since that time, 10 functional TLRs have been discovered in primates (182).

Like Toll, TLRs are also type I membrane proteins. They are comprised of a cytoplasmic domain that is highly homologous to the IL-1 receptor cytoplasic signaling domain (Toll-Interleukin-1 Resistance or TIR domain), a transmembrane domain and a "horseshoe" shaped ectodomain comprised of many leucine rich repeats (175, 183). Pathogen recognition occurs when the PAMP binds to the TLR ectodomain and any required co-receptors (**Figure 1.2**). Upon PAMP-TLR binding, the TIR domain recruits adaptor proteins and triggers signaling cascades that initiate transcription factors such as nuclear factor kappa-B (NF-κB), IRF-7, or ATF2/JUN (175, 184). Of these transcription factors, NF-κB is thought to be the predominant regulator of cytokine induction and inflammation (175, 184).

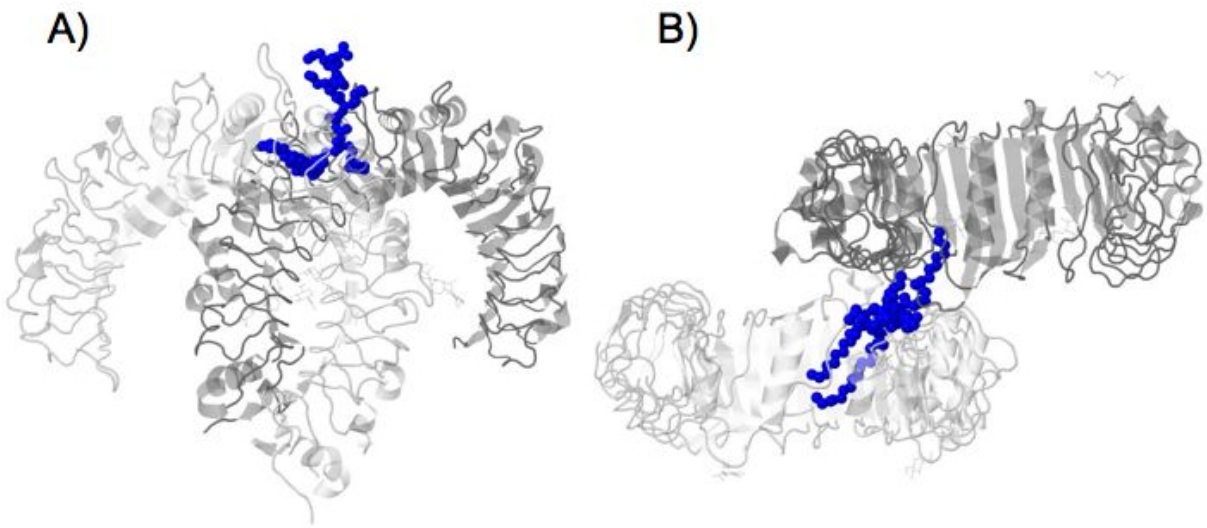


Figure 1.2 TLR2-TLR1 heterodimer bound to PAMP Pam3CSK4
A) Lateral view TLR2 (gray) and TLR1 (white) heterodimerize via PAMP Pam3CSK4TLR2 (blue). B) Pam3CSK4 binding sites are negatively charged pockets in the crown of TLR2 and TLR1 (185)

In mammals, TLR-mediated activation of antigen presenting cells, such as dendritic cells and monocyte/macrophages, plays a key role in the initiation of the innate immune response and modulation of the adaptive immune response (184). The primate TLR family includes 10 functional signaling receptors, which can be divided into cell membrane-bound (TLRs 1,2,4,5,6,10) and intracellular proteins (TLRs 3,7,8,9), and further divided into categories based on the structure of the PAMP they recognize (182) (**Table 1.5**).

The division of TLR labour is associated with the cellular location of these receptors (**Table 1.6**). Cell membrane-bound TLRs (1,2,4,5,6,10), recognize molecular components found on microbial membranes. Intracellular TLRs (3,7,8,9), recognize microbial nucleic acids (184). Furthermore, each TLR recognizes different, broad subsets of microbial organisms via PAMPS. TLR4, for example, recognizes gram-negative bacteria via binding with LPS, while TLR2 recognizes Gram-positive bacteria by binding with PGN (182). Some TLRs may also heterodimerize to recognize a broader range of microbes (i.e. TLR2/1 and TLR2/6). Recent research suggests that differences in TLR-mediated cell signaling and subsequent initiation of cytokine release affect the manifestation of infections in catarrhine hosts (11, 20, 186).

TLR	Cellular location	Co-receptors	Cell types
1	Cell membrane	TLR 2	**monocytes** (macrophages), natural killer cells, **neutrophils**, eosinophils, basophils, B cells, T cells (57, 82, 187-191)
2		TLR1, TLR 6	**monocytes** (macrophages), **neutrophils,** eosinophils, basophils, **natural killer cells**, B cells, T cells (57, 82, 188, 191, 192)
6		TLR 2	**monocytes** (macrophages), B lymphocytes, **neutrophils**, natural killer cells, eosinophils, basophils (57, 82, 188, 191)
4		LY96	**monocytes** (macrophages), **neutrophils**), eosinophils, basophils, natural killer cells, B-cells (57, 82, 187, 190, 193-195)
5			**monocytes** (macrophages), neutrophils, eosinophils, natural killer cells, T cells (57, 187, 190, 191, 196, 197)
10			Monocytes, **neutrophils**, eosinophils, **B cells** (191, 193, 196, 198)
3	Endosome		**natural killer cells** (191)
7			monocytes (macrophages), neutrophils, eosinophils, **B cells** (57, 191, 193, 198)
8			monocytes (macrophages), neutrophils, natural killer cells, B cells (191, 193, 197, 198)
9			monocytes (macrophages), neutrophils, eosinophils, basophils, **B cells** (57, 82, 191, 193, 198)

Table 1.1 Toll-like receptor location, co-receptors and distribution in adult human circulating blood leukocytes.
Cells on which TLRs are strongly expressed are in bold face. Brackets mean expression is regulated by another signal.

TLR	PAMP type	PAMP	Organisms
1/2	Lipoproteins/ Lipopeptides/ GPI anchors/ Lipoglycans	Pam3Cys lipopeptides (199), Lipomannan(200, 201),	Bacteria, Mycobacteria, gram-positive bacteria
2		Peptidoglycan , Glycosylphosphatidylinositol – anchored proteins, lipoteichoic acid, porins, zymosan (202-206)	Gram-positive bacteria, Trypanosomes, yeast
2/6		Pam2CYs lipopeptides, MALP-2, PSM, (207, 208)	Mycobacteria, *Borrelia burgdorferi*, gram positive bacteria
4		Lipopolysaccharide, fungal mannans, heat-shock proteins (209-211)	Gram-negative bacteria
5	Protein	Flagellin (212)	protists
3	Nucleic acids	Double stranded RNA (213)	dsRNA viruses
7		Single stranded RNA, Guanosine analogs (214, 215)	ssRNA viruses
8		Single stranded RNA, imidazoquinolines (216)	ssRNA viruses
9		CpG DNA motifs (217)	Bacterial DNA
10	?	Profilin-like molecule (218)	*Toxoplasma gondii*

Table 1.2 Toll-like receptor-detected pathogen-associated molecular patterns and representative organisms

1.3.2 TLR2 and TLR4 signaling pathways

TLR2 and TLR4 are the most thoroughly researched TLRs and perhaps the best understood innate immune receptors that recognize bacterial microbes. Comparative information on catarrhine responses to TLR2 and TLR4-detected bacterial pathogens is very limited. TLR2 and TLR4 detect several bacterial pathogens that are agents of current human infectious disease, and have been posited in earlier forms to have played a role in the evolution of catarrhine immunity [i.e. *Mycobacterium* (37, 200), *Yersinia* (30, 219), *Leishmania* (36, 220)]. A greater insight into how early immune responses to TLR2 and TLR4-detected pathogens may differ in various catarrhine species is important for a better understanding of the evolution of primate innate immunity and disparities in human and nonhuman primate bacterial disease progression.

TLR2 PAMP recognition

Both TLR2 and TLR4 recognize lipid PAMPs through ectodomain domain-PAMP binding (185, 221). TLR2 is a promiscuous receptor, recognizing a broad range of lipoproteins, lipopeptides and glycosylphosphatidylinositol-anchored proteins (GPI) found on gram-positive bacteria, mycobacteria, fungus and parasites [reviewed in (222)]. This broad detection capability is likely influenced by accessory receptors such as CD14 and CD36, which enhance the detection of triacyl-lipopeptide Pam3CSK4 and lipoteichoic acid respectively (223-225). TLR2 binding promiscuity appears to be strongly linked to the formation of TLR2 heterodimers with TLR1, TLR6, an unidentified TLR ("TLRx", possibly TLR10) and non-TLR proteins (226). TLR2/1 heterodimers can detect triacyl-lipopeptide, while TLR2/6 detects diacyl-lipopeptide (227). TLR hetero- and homodimers appear to form in response to immunostimulation. Crystallographic data available for the structure of synthetic triacylated lipopeptide (Pam3CSK4) bound to partial TLR2 and TLR1 ectodomains indicates that the lipid chains of the PAMP connect the TLRs (185). Specifically, lipid chains are unevenly inserted into hydrophobic channel between TLR1 residues 257-339 and a pocket between TLR2 residues 266-355 in the convex region of the ectodomain where the central and C-terminal domains meet (LRR9 and LRR12). The formation of this complex is further stabilized by protein-protein interactions that occur near the ligand-binding site (**Figures 1.2 and 1.3**). Amino acid substitutions in this region are known to affect TLR2/1 signaling (228). An alignment of catarrhine primate amino acid sequences indicates that there are significant residue substitutions in TLR1 and TLR2 ligand binding regions and at TLR1/2 interaction sites (**Figures 1.3**).

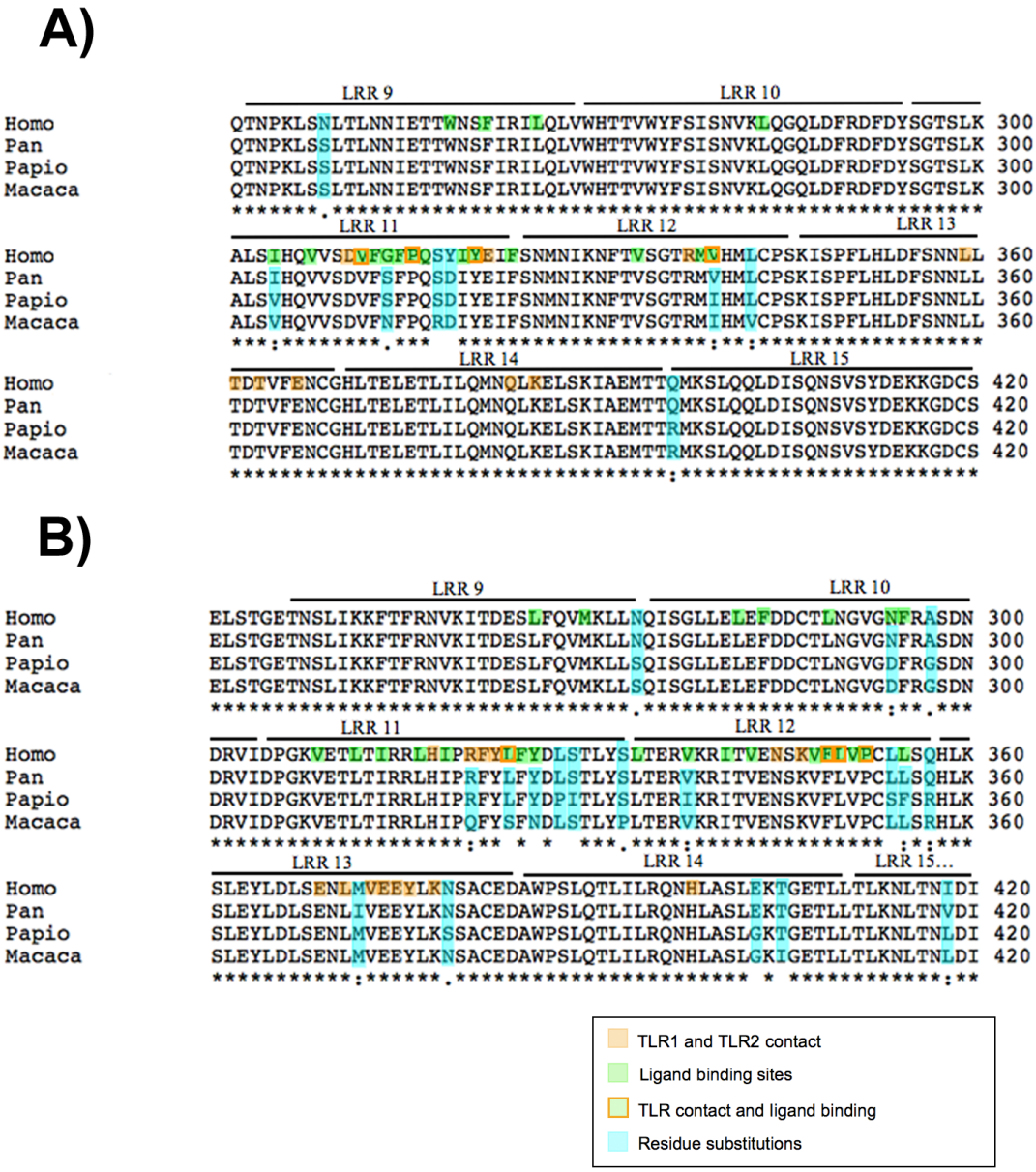


Figure 1.3 Significant inter-species differences in TLR1 and TLR2 contact and ligand binding sites

Published and predicted TLR1 and TLR2 amino acid sequences are aligned for *Homo, Pan, Papio* and *Macaca.* A) TLR 1 alignment shows residue changes at two known ligand binding sites V304I, S313G/S313N, a ligand and TLR2-TLR1 contact site I338V, along with other changes in this important region. B) TLR2 alignment shows important residue changes at ligand binding regions D294V, L355F, N326Y, a ligand binding and TLR2-TLR1 contact site S324L and contact site Q321R. (Ligand and contact sites based on Jin et al., 2007). TLR 1 sequences shown here NP_003254.2, NP_001123937.1, NP_001123896.1, *Papio* acquired and assembled via BLAST of *Papio hamadryas* Pham 1.0 short read archive, Baylor University. TLR2 sequences shown here NP_003255.2 , NP_001123941.1, NP_001123897.1 .

TLR4/LY96 PAMP recognition

Several PAMPS are known to interact directly and indirectly with TLR4 including fusion protein from respiratory syncytial virus (F, RSV), hyaluronic acid and lipopolysaccharide (LPS) (229, 230). Of these, TLR4-LPS interactions are the best understood. LPS is a major component of gram-negative bacterial cell walls and is broadly found in other microbial structures. TLR4 does not bind with LPS directly but interacts with LPS via phosphate charges (221). LPS-mediated cell stimulation occurs through LPS binding to a soluble protein called Lymphocyte-antigen 96 (LY96) and LPS/LY96 complexing with TLR4 (221, 231, 232). TLR4 appears to enhance LY96 – LPS binding (233). LPS binds with cell membrane protein CD14 (which also exists in soluble form) with the assistance of soluble shuttle protein LPS-binding protein (LBP) (234). CD14 transfers LPS to the TLR4/LY96 complex, where it binds to a large hydrophobic pocket within LY96 and one lipid chain creates a bridge between LY96 and TLR4 (221). Mutations to LY96 are known to block LPS-induced dimerization, while *Homo* TLR4 variants are associated with blunted responses to LPS (235-237). An alignment of catarrhine primate amino acid sequences indicates that there are significant residue substitutions in LY96 LPS binding regions and TLR4/LY96 and LPS interaction regions (**Figures 1.4 and 1.5**). Multiple substitutions in the LPS interaction regions of TLR4 and LY96 between suggest differences in LPS binding and the initiation of TLR4/LY96 signaling.

After LPS-induction of the TLR4/LY96 complex, TLR4 oligomerizes and adaptor molecules are recruited to the TLR4 TIR domain (238). TLR TIR domains are highly conserved. TLR4 TIR domain signal transduction is particularly sensitive to amino acid substitutions. TLR4/LY96 complex response to LPS can be severely limited by point mutations to this domain (210). The TIR domains of catarrhine primates differ. An alignment of *Homo, Pan, Papio* and *Macaca*

published amino acid reveals two substitutions in this domain, along with a 13 amino acid deletion at the C-terminal end of the TIR domain (**Figure 1.6)**

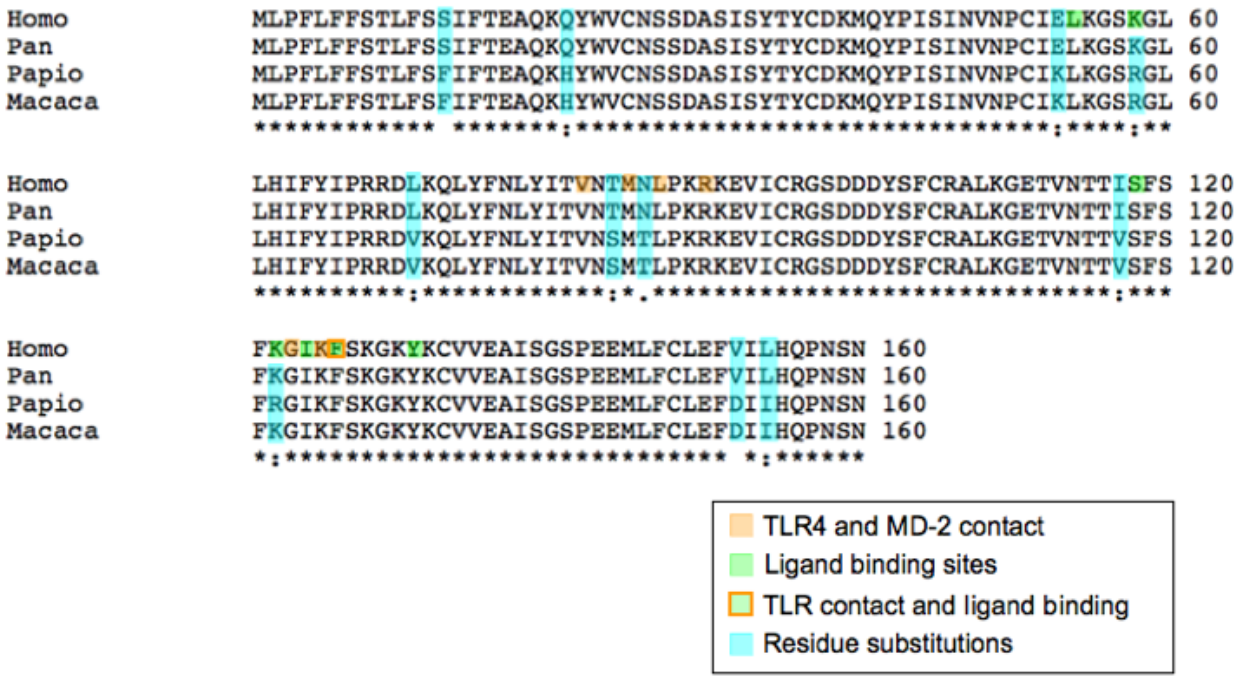


Figure 1.4 Significant inter-species in LY96 at TLR4/LY96 contact and ligand (LPS) binding sites.
Published and predicted LY96 amino acid sequences are aligned for *Homo, Pan, Papio* and *Macaca.* Amino acid differences between species may affect LY96 function as residue changes are known to affect TLR4/LY96 dimerization. There are inter-species residue changes at two known LPS binding sites R58K and K122R, as well as two substitutions within the region of LY96 that associates with TLR4 (V82, M85, L87, R90) S84T and T86N. (Ligand and contact sites based on LPS/MD-2/TLR4 structure PDB 3xfi (221). LY96 sequences shown here, NP_056179.3, NP_001123946.1, NP_001123904.1. *Papio* acquired and assembled via blasting *Macaca* sequence against Nov 2008 *Papio hamadryas* short read archive, Baylor University.

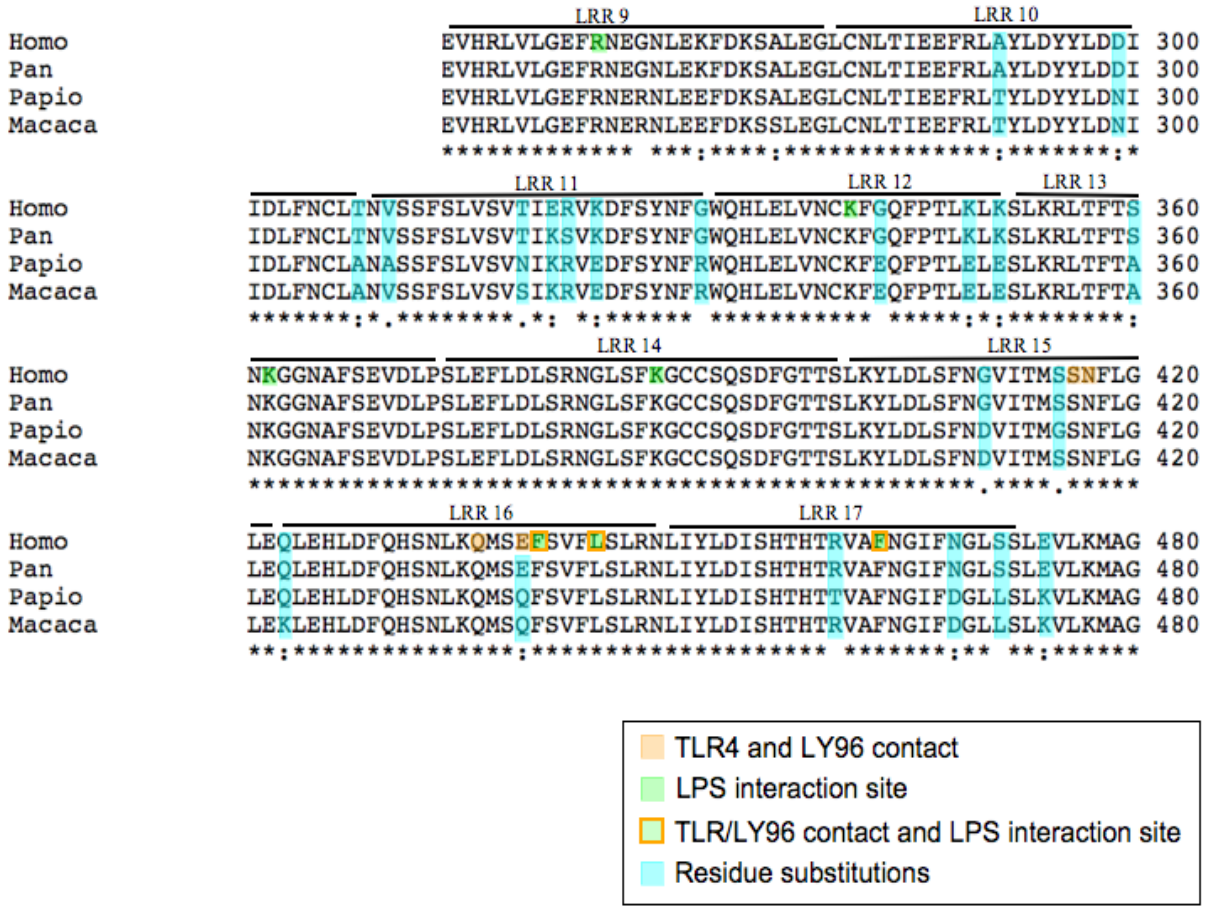


Figure 1.5 TLR4 (LRR9-LRR17) alignment shows important residue changes for *Homo*, *Pan*, *Papio* and *Macaca*.
An important substitution can be found at a LY96 contact site, Q439E, along with other substitutions near TLR4/LY96 and TLR4/LY96/LPS interaction sites. (Ligand and contact sites based on LPS/LY96/TLR4 structure, (221)). TLR4 sequences shown here are NP_612564.1, NP_001138335.1, AAF07059.1, NP_001032169.1 .

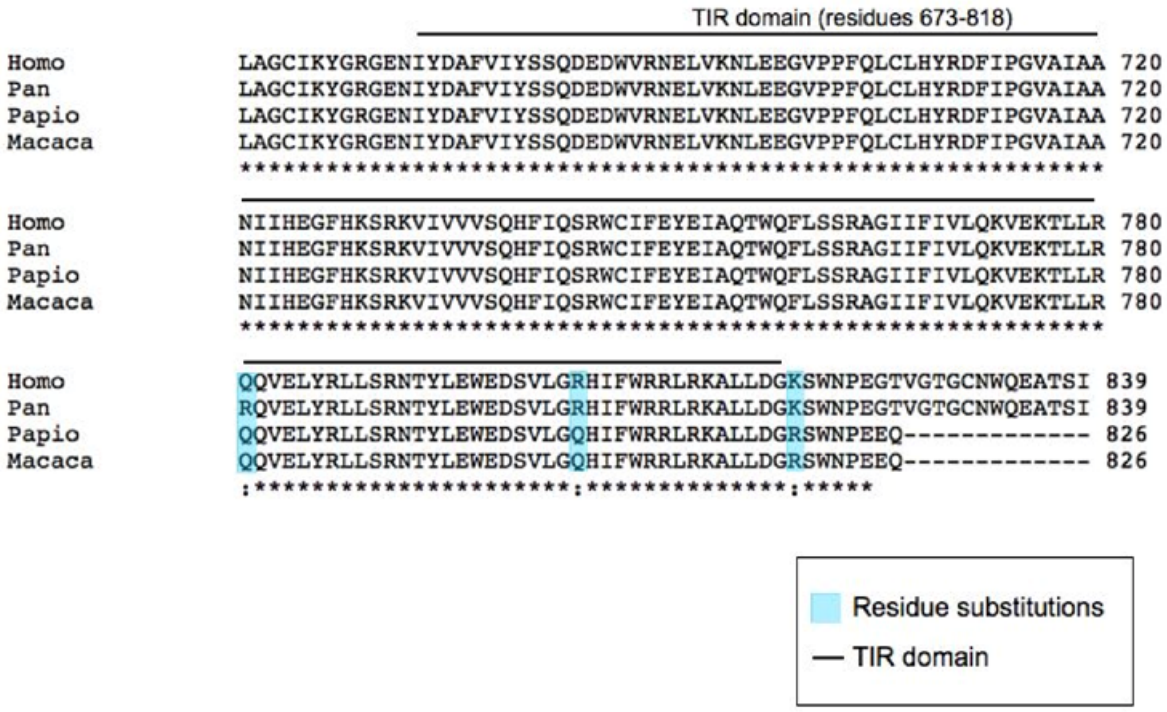


Figure 1.6 TLR4 TIR-domain alignment for *Homo*, *Pan*, *Papio* and *Macaca* shows residue changes and a 13 residue C-terminal end deletion in *Papio* and *Macaca*.
Substitutions in the TLR4 TIR-domain can abolish LPS-based signaling. TIR domain determined using SMART (http://smart.embl-heidelberg.de/ (239)). TLR4 sequences shown here are NP_612564.1, NP_001138335.1, AAF07059.1, NP_001032169.1.

TLR2 and TLR4/LY96 signaling

TLR2 and TLR 4/LY96 can generate different biological responses after PAMP recognition. TLR2 signaling induces the production of mainly pro-inflammatory cytokines, while TLR4/LY96 can induce the production of both pro-inflammatory cytokines and type 1 interferons (184). When TLR4/LY96 and TLR2 gene induction diverges, it appears to be related to the recruitment of specific adaptor molecules to the TIR domain of the receptors. The recruitment of MyD88 to a TLR by sorting adaptor TIRAP, for example, is associated with the activation of NF-κB and mitogen-activated protein kinases (MAPKs) and the induction of pro-inflammatory cytokines (226, 240)

(**Figure 1.7**). The recruitment of TRIF to TLR4/MD-2 by sorting adaptor TRAM, for example, initiates a different signaling cascade which ends with the activation of NF-κB and IRF-3 and the induction of pro-inflammatory cytokines and type 1 interferons. TLR4 signaling may involve recruitment of TRIF and/or MyD88 while TLR2 signaling appears to be MyD88-dependent (241).

MyD88-dependent signaling requires that TIRAP escorts MyD88 to the TLR. When the TLR recognizes a PAMP, IL-1 receptor-associated kinases are recruited to MyD88 (IRAK4, IRAK1, IRAK2, IRAKM). These IRAK molecules are thought to be activated sequentially, leading to the interaction of IRAK1 and TRAF6 and subsequent binding to TAB2 and TAB3 of the TAK1 complex. TAK1 phosphorylates IκKβ, leading to the degradation of the IKK complex and activation of transcription factor NF-kB (242). TAK1 may also activate other transcription factors such as p38 and Jnk through the phosphorylization of MAPKs (243).

TRIF-dependent signaling requires the recruitment of TRIF to the TLR4 complex by TIRAP. TRIF then recruits TRAF6 which activates TAK1 and leads to NF-κB activation or TRAF3 which activates the TBK1/IκKI complex and leads to the nuclear translocation of IRF-3 (184). TRAF3 may also be recruited to MyD88/TLR4, which is associated with the translocation of the entire complex to the endosome, subsequent TAK1 and NF-κB activation (244).

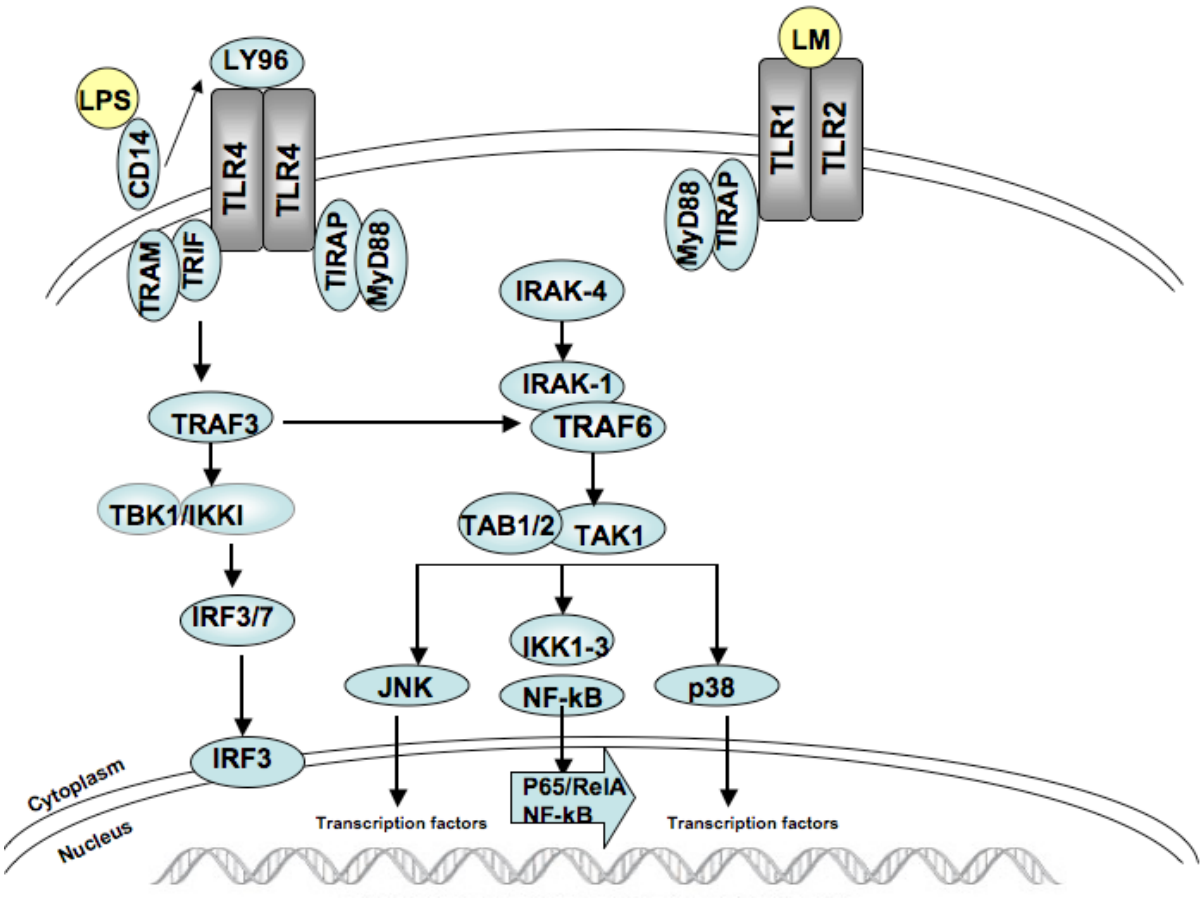


Figure 1.7 Simplified representation of TLR4 and TLR2/1 signaling cascades.
Both MYD88 dependent and independent cascades are shown for TLR4. Based on (238, 240)

1.5 Toll-like receptor variation and host response to infection

Host genetics affect infection susceptibility, level of inflammation response and disease progression (30, 108, 245-247). Several primary immunodeficiency disorders in humans are associated with abnormal expression of genes in TLR pathways (i.e. (IκBKG/NEMO), NFκBIA and IRAK4 deficiency [reviewed in (248, 249). TLR polymorphisms have also been associated with variation in bacterial infectious disease progression with varying consistency.

Multiple TLR2 polymorphisms have been associated with abnormal infectious disease progression. The R753Q polymorphism in the receptor TIR domain has been functionally associated with altered TLR2 signaling during *Borrelia burgdoferi* infection (250). Variation in the number of GT repeats in intron 2, 100 bp upstream from the start codon of the receptor's open reading frame (ORF) has been genetically associated with increased susceptibility to *Mycobacterium tuberculosis* and containment of *Mycobacterium leprae* infection (251, 252). Polymorphism I602S in the transmembrane region of TLR1 has been genetically associated with increased IL-6 production after triacylated lipopeptide stimulation of whole blood and affected lipopeptide induced signaling in HEK-293 cells (253). Similarly, monocytes from individuals homozygous of 602SS were found to produce less TNFα in response to lipopeptide stimulation than individuals heterozygous for 602SI or homozygous for 602II (254)

Several studies have genetically associated TLR4 polymorphisms with variations in infectious disease progression. Single nucleotide polymorphisms TLR4 D299G and T399I have been associated with resistance to *Legionella pneumophila* infections and inconsistently associated with increased susceptibility to gram-negative bacteria induced sepsis, (255-259). Similarly, CD14 C159T transition has been associated with increased risk of septic shock, with conflicting results (260-262). More securely, multiple LY96 polymorphisms have been found to affect cellular responses against bacterial infection. Mutation G56R is located near the LPS-binding region of LY96 and reduces the binding of soluble LY96 to LPS, leading to significantly depressed cellular responses (263). Vasl et. al. (2008) has found that soluble G56R LY96-mediated reduced binding to LPS is rescued by TLR4 co-expression, suggesting that TLR4+/LY96- cells reliant on ectopically expressed LY96 are less sensitive to LPS stimulation (263). A study of 105 individuals with major physical trauma found an association between variations in the LY96 promoter region (-1625G) and increased TNFα

production, as well as increased likelihood of sepsis and organ dysfunction (264). Given that the *Papio hamadryas* genome is a preliminary draft assembly it is more difficult to accurately assemble and compare the promoter region that sits 2-kb upstream from the translational start site than it is to assemble the short LY96 open reading frame (265). The published *Homo*, *Pan*, *Macaca* and predicted *Papio* LY96 open reading frame sequences exhibit glycine at residue 56, but hominoid and Old World monkey sequences diverge at nearby residues 54 and 58 (K54E, R58K).

Catarrhine TLR2, TLR4 and their co-receptors amino acid sequences are known to vary. A comparative examination of TLR2 and TLR4/LY96 – mediated detection of bacterial PAMPS may help explain why catarrhine primates vary in susceptibility to bacterial infections.

1.6 Primate responses to infection

1.6.1 Role of innate immunity

Catarrhine primates exhibit inter-species differences in innate immune responses to a broad range of pathogens that are major agents of human diseases. *Trypansoma brucei* causes fatal sleeping sickness in *Homo* and *Pan*, whereas this pathogen fails to establish infection in *Papio* sp. due to this species' haptoglobin-related protein (Hpr) and apolipoprotein L-I (ApoL-I) – mediated increased trypanolytic activity (266). *Cercocebus atys* plasmacytoid dendritic cells (pDCs) express significantly less IFNα and mount a weaker innate immune response to TLR7/9-detected yellow fever virus 17D vaccine than *Macaca macaca* (267). These observations recapitulate findings of weaker IFNα and innate immune activation in *C. atys* than *M. macaca* in response to SIV *in vivo* and other TLR7/9 ligands *ex vivo* (20). *M. fascicularis* and *Chlorocebus* sp. are noted to express very different chemokines

in the early hours of infection with SARS coronavirus (SARS-CoV). Specifically, *Chlorocebus* sp. exhibits a marked increase in CCL3 production and significantly lower levels of neutrophil chemoattractants (IL-8/CXCL8, CXCL2) than *M. fascicularis*, which corresponds with increased symptom severity in the former species (268). Controlled investigations of differences in primate innate immune responses have mainly focused on primate-viral interactions. As a result, primate-bacterial pathogen interactions are not as well understood. Field observations and the limited number of available controlled studies on infectious disease susceptibility suggest that catarrhine innate immune responses to TLR2 and TLR4/LY96-detected pathogens should differ. An examination into differences in the early innate immune responses of catarrhines to bacterial pathogens is important, as such and examination will shed light on differences in bacterial disease susceptibility and contribute a better understanding of primate immune system evolution.

Catarrhine responses to TLR2 and TLR4/LY96-detected pathogens and ligands

Gram-negative bacteria and LPS

Available comparative veterinary/clinical information on primate responses to TLR2 and TLR4/LY96 detected pathogens is limited but suggests significant divergence between hominoid and cercopithecoid species in disease susceptibility. Most notably, hominoids and cercopithecoids mount divergent immune responses to LPS and gram-negative bacteria (10, 11, 106, 269-271). *Homo* and *Pan* are exceptionally sensitive to stimulation by LPS/endotoxin and gram-negative bacteria during systemic infections, and are, therefore, highly susceptible to gram-negative bacterial-initiated severe sepsis and septic shock (10, 272). Severe sepsis is a systemic and harmful pro-inflammatory innate immune response to bacterial infection that leads to unregulated cytokine release, inflammation, blood coagulation and fibrinolysis [reviewed in (128)]. Severe sepsis in *Homo* is

characterized by high fever, neutropenia and arthralagia, organ dysfunction and blood coagulation and can progress to hypotension and hypoperfusion (septic shock). *Papio,* by contrast*,* appears to be insensitive to LPS and gram-negative bacteria requiring considerably higher doses than *Homo* or *Pan* to develop febrile responses (10, 11, 106, 270). Even when high doses are applied to tissues or intravenously, *Papio* fails to manifest the broader suite of symptoms typical of severe sepsis or septic shock. This trait of insensitivity appears to be shared across several cell types - *Papio* endothelial cells and monocytes are less reactive to LPS from *E. coli* than those of humans (11, 15, 106, 269, 270). A consequence of endotoxin/LPS insensitivity is that it is very difficult to initiate the symptoms of severe sepsis in *Papio*.

Two lines of evidence suggest that there is a difference between the *Homo* and *Papio* TLR4 pathways that leads to blunted febrile response to LPS in *Papio*, including reduced IL-1β expression by monocytes after stimulation (106). *Papio* has been found to develop fevers and produce high levels of IL-1β via monocytes in response to TLR2-detected *Staphylococcus aureus*, but not in response to endotoxin (106). These results suggest that *Papio* insensitivity to LPS is not due to an overall inability to mount a fever, or induce a major cytokine that stimulates that response, but to an unknown difference in a pathway that detects LPS/endotoxin. Additionally, it has been suggested that the divergence of *Papio* and *Homo*/*Pan* responses to gram-negative bacteria and LPS are due to a 13 amino acid deletion in the *Papio* TLR4 TIR domain (273). This hypothesis is mainly supported by the lack of sequence homology between TLR4 TIR domains of other mammals that are insensitive to LPS and *Homo* and has not been directly tested in a functional way (273).

TLR2 detected bacteria, parasites and viruses

While controlled experiments examining catarrhine responses to specific TLR2-detected pathogens are limited, multiple field and laboratory observations suggest a division between hominoids and cercopithecoid innate immune responses to a broad range TLR2-detected pathogens. *Neisseria gonorrhoeae* is detected by TLR2 and can establish urethral infections and attach to the fallopian mucosa of *Homo* and *Pan* but cannot do so in cercopithecoids and other mammals (12, 274, 275). The species-specificity of *N. gonorrhoeae* susceptibility is not well understood, and may be related to increased *Homo* and *Pan* complement binding affinity to *N. gonorrhoeae* porins, some of which have been identified as TLR2 ligands (205, 276). A similar hominoid/cercopithecoid division in susceptibility and early disease manifestation has been reported for TLR2-detected cytomegalovirus, which can cause mild mononucleosis in humans, but does not typically manifest clinically in macaques (277-280). TLR2-pathogen interactions may also affect the divergence of herpes virus manifestations in hominoids and cercopithecoids. Some alphaherpes viruses (i.e. Herpes simplex virus) are thought to enhance TLR2 signaling, leading to increased neuroinflammation. This symptom is typical of the 3-5 day, often fatal human infection with Monkey B virus (a cercopithecoid alphaherpes virus). Neuroinflammation is not found in natural hosts of Monkey B virus, macaques (281-290). Reports that the TLR2-detected pathogen *Shistosoma mansoni* causes diarrhea and liver dysfunction in humans, but appears to less intensively affect the olive baboon (*Papio anubis*) further support a significant divergence in immune response between cercopithecoids and hominoids (291-293).

Reports of *Mycobacterium* sp. infections progressing more rapidly in cercopithecoids than in hominoids are conflicting and may be influenced by the detection bias inherent to infections with long latency periods (294-301). Opportunistic case studies and deliberate inoculations of

cercopithecoid species with *Mycobacterium* are few, making it difficult to compare variability of cercopithecoid infection to the variability of human infection. The available data suggests a complex divergence in catarrhine immune responses to *Mycobacterium*. *Papio* and *Macaca* have been described as exhibiting rapid disease progression when infected with *Mycobacterium bovis* or *M. tuberculosis,* developing advanced lung nodules and pulmonary inflammation within 6 months of the estimated date of infection (295, 296, 299, 300). *Papio* and, less securely, non-human apes have been described as less prone to virulent manifestations of *M. tuberculosis* than macaque species (294, 300). Rhesus macaques (*Macaca mullata*) have been described as highly susceptible to virulent *M. bovis* manifestations, while cynomolgus macaques (*Macaca fascicularis*) are described as less susceptible to infection and more likely to exhibit symptoms similar to humans (300-302).

Taken together, these many observations of differing susceptibility to TLR2 and TLR4/LY96 – detected bacterial pathogens in catarrhines suggest differences in the early innate immune responses of these animals. Specifically, findings of divergent immune responses to such a broad range of TLR2 and TLR4/LY96 pathogens suggest that catarrhine species detect these pathogens and activate TLR signaling pathways differently.

1.7 Catarrhine histories of TLR-detected pathogen exposure seem to differ

Several TLR2-detected bacterial pathogens appear to have been unevenly distributed amongst primate species or emerged in catarrhines at different times in evolutionary history, due to geographic barriers or differences in primate behaviour (i.e. landscape use and diet). These differences in pathogen exposure may provide the basis for pathogen or PAMP-specific immune responses amongst primates. Increased exposure to ungulate faeces in grasslands and increased meat

consumption, for example, have been proposed to have led to the emergence of the progentitor of *Mycobacterium tuberculosis* in hominins 3 million years ago (36, 37). Similarly, it has been proposed that habitual grassland use would routinely expose species such as *Homo* and *Papio* to standing watering pools contaminated with ungulate pathogens, such as the precursor to the TLR2-detected *Bacillus anthracis* (anthrax) (36, 303). *Yersinia pestis* is a gram-negative bacterium that interacts with both TLR2 and TLR4 and the causative agent of plague (219, 304-306). Now a pandemic disease, *Y. pestis* potentially represents one of the strongest agents of pathogen-mediated selection on the species *Homo* in recent centuries. It is thought to have emerged in Asian populations of *Homo* >2600 years ago and spread to Europe and killed nearly 1/3 of the population by 1357 (307, 308). All outbreaks since the 1357 epidemic are reported to have been less virulent. Subsequent outbreaks on other continents are hypothesized to have occurred only recently, suggesting that the *Y. pestis* organism responsible for the black death of the 1300s bypassed African non-human primate populations (307, 309). Uneven exposure of some catarrhine species to virulent TLR2 and TLR4–detected bacterial pathogens may have led to lineage adaptation and the divergence of catarrhine susceptibility to pathogenic bacterial infections.

1.8 Dissertation overview and justification

An important component for understanding disparities in primate infectious disease manifestation is the evolution of the early innate immune response. Toll-like receptors have been shown to regulate infectious disease susceptibility and progression in human and non-human primates [reviewed in (310)]. Specifically, variations in TLR2 and TLR4/LY96 composition and signaling have been associated with alterations in disease progression in humans. Understanding how TLR-mediated gene cytokine/chemokine responses to bacterial pathogens may differ between

species will shed light on why bacterial disease pathogenesis varies greatly between catarrhine species. Specifically, functional analysis of the early hours of infection will contribute to a better understanding of differences in blood leukocyte activation, the first phase of cytokine and chemokine induction during infection, disparities in disease susceptibility/mortality and ultimately the evolution of catarrhine immune systems.

This dissertation examines the evolution of African catarrhine immune responses to TLR2 and TLR4–detected bacterial pathogens. The TLR2 and TLR4 pathways were chosen because African catarrhine species differ considerably in their susceptibility to, and early disease progression of TLR2 and TLR4-detected pathogens. Cytokine and chemokines levels are compared between species because early induction levels of these molecules can be predictive of clinical outcomes of bacterial infection (272, 311). This research tests for signs of early innate immune function divergence between *Homo*, *Pan* and *Papio* by examining cytokine induction in total leukocytes of these species in response to TLR2 and TLR4-detected bacterial PAMPs. PAMPs from pathogens with traditionally broad geographic distribution were used to determine if responses are specific to species or specific to pathogen. Two pathogens that are specific to African catarrhine evolutionary landscapes were also employed in an effort to determine if the early innate immune responses of these primates can be correlated to evolutionary environments. Finally, this dissertation will investigate whether the differences in early innate immune gene induction can be correlated to observations of inter-species differences in disease progression. The results will contribute to a better understanding of primate immunity and evolution by characterizing the early innate immune response of African catarrhines and clarifying the role of TLR-detected pathogens in the divergence of the *Homo*, *Pan,* and *Papio* lineages.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Sample collection

Pan troglodytes and *Papio* sp. (institution identification "*cynocephalus*") fresh blood samples were collected into Na-heparin containing tubes in accordance with institutional IACUC requirements at the Yerkes National Primate Research Center (Atlanta, Georgia) and Texas Biomedical Research Institute, (San Antonio, Texas), respectively (**Appendix A**). Samples were shipped on ice overnight to the City College of New York, New York, NY. Only samples from non-human primates identified as HIV/SIV negative, Hepatitis C virus (HCV) negative and in general good health and appearance were used. Human blood was collected from healthy volunteers in accordance with City College of New York IRB requirements (IRB # 09-0073C) (**Appendix B**). Human samples were collected in Na-heparin containing tubes, stored on ice and rotated to mimic shipping conditions overnight. All subjects were male, save one *Pan* individual. All human subjects were unrelated. Primate facility stud and genetic records indicate that all primate individuals are unrelated.

To test if colony environment affects early immune response a chimpanzee from the Texas Biomedical Institute was included in this study. This animal exhibited similar gene induction to the Yerkes chimpanzees after 90 minutes of stimulation with agonists (**Appendix C**). To test if colony environment affected early innate immune response after stimulus for 90 minutes, a chimpanzee from the Southwest Foundation was included in this study. Gene induction in this animal was found to be very similar to the Yerkes chimpanzees. No difference in blood cell subtype proportions or transcriptional response was found between the Yerkes and Southwest Foundation chimpanzees.

2.2 Whole blood stimulation

Whole blood was stimulated with ten fold serial dilutions of 10 μg/ml – 0.01 μg/ml of TLR2 and TLR4-detected PAMPs representing gram negative bacteria [Ultrapure Lipopolysaccharide from *Escherichia coli* 0111:B4 (LPS) (Invivogen, San Diego, CA) and LcrV from *Yersinia pestis* (LcrV) (Biodefense and Emerging Infections Research Resources Repository, Manassas, VA)], gram positive bacteria [Pam3CSK4 lipopeptide mimetic (Invivogen, San Diego, CA) and Protective antigen from *Bacillus anthracis* (recombinant PA) (Biodefense and Emerging Infections Research Resources Repository, Manassas, VA)] and mycobacteria [Lipomannan from *Mycobacterium smegmatis* (LMMS)], Invivogen, San Diego, CA], at 37°C for 90 minutes. Agonists were added to blood that had been alliquoted into sterile, pyrogen-free microcentrifuge tubes, and the tubes gently inverted 5 times to mix. Endotoxin free, cell culture grade water (Hyclone/ Thermoscientific Cell Culture Grade Water, Logan, Utah) was used as a negative control as well as a medium to suspend and serially dilute PAMPs. All stimulations were completed in duplicate. LcrV and LMMS were deliberately selected to represent pathogens unevenly distributed in primate evolutionary environments. Historical records and a recent minimum spanning tree analysis of 17 *Yersinia pestis* genomes and 256 isolates of diverse global origins representing all biovars suggest *Y. pestis* had a Eurasian distribution from >2600 BP until the last 100 years (307, 309). Recent pairwise comparison of synonymous sites between 6 housekeeping genes in 17 tubercle bacilli strains suggests that a prototypical *Mycobacterium tuberculosis* emerged in hominins ~3.0 million years ago (37). LcrV is shared across all 3 extant pathogenic *Yersinia* species, and Lipomannan (LM) is identical across distantly related extant *Mycobacterium* species and stimulates highly similar immune responses in hosts (200, 201, 312-314).

A stimulation time of 90 minutes was chosen to ensure that changes in gene transcription observed could be attributed to the early innate immune response. To determine which targets could be induced, 10 μg/ml of agonists were applied to blood in the initial experiments, followed by lower doses in later experiments. LMMS and Pam3CSK4 stocks used in this study had minimal endotoxin contamination (0,125 EU/mg by LAL gel clot assay for LMMS and Pam3CSK4, 2.4 EU/ml in LcrV and 0.012 EU/μg in PA) and LPS had less than 3% protein content (bicinchoninic acid protein assay) and did not stimulate TLR2+HEK cells at high doses (10μg/ml dose) according to the manufacturer's (BEI Resources, Manassas, VA; and Invivogen, San Diego, CA) analysis.

2.3 RNA isolation and real time PCR

Whole blood was subjected to hypotonic shock (900 ul endotoxin free water/100 ul whole blood for 20 seconds, 100 ul of 10x endotoxin free PBS to equilibriate) to lyse red blood cells. Total RNA was isolated using Qiagen Qiashredder and RNEasy spin columns as per manufacturers instructions (Qiagen Qiashredder spin columns, Qiagen RNEasy mini-kit, San Diego, CA). To limit genomic DNA contamination, isolated RNA was then incubated with DNAse I for 10 minutes at 20-25°C and re-isolated using Qiagen RNEasy spin columns as per the manufacturer's instructions (Qiagen RNAase-free Dnase I, San Diego, CA). To assess RNA quantity and purity, A260/A280 ratio was measured and calculated using a Nanodrop spectrometer. Samples with a ratio of 1.7 or higher were used in this study. cDNA was synthesized using 0.007-0.01 μg/ml RNA and the Quantitect Reverse Transcriptase kit as per manufacturer's instructions (Qiagen, San Diego, CA). To check for genomic contamination, no-reverse transcriptase controls were synthesized. No product was amplified from no-reverse transcriptase controls as per manufacturer's instruction. Detailed

manufacturer protocols for RNA isolation and cDNA synthesis can be found in **Appendix D**. All cDNA templates were diluted at a 1:10 dilution prior to real-time PCR. All RNA and cDNA samples were stored at -80°C.

Activation of white blood cells after stimulation was quantified by relative real-time PCR. Reactions were performed in 20 ul reaction mixtures, in 96 well optical plates, sealed with optical film on the ABI 7500 real time PCR system (Applied Biosystems, Foster, CA) diluting Roche FastStart SYBR Green Master Mix with Rox (Indianapolis, IN) to 1x concentration using 7 ul of water, 1 ul of each primer (for a final concentration of 0.1 uM of primers) and 1 ul of template (~ 0.01 ng/ul). Reactions were incubated for 2 minutes at 50°C and again at 95°C for 10 minutes, followed by 50 cycles of a two-step amplification procedures including annealing/extension at 59°C for 45 seconds and denaturation at 95°C for 10 seconds. To check that a single amplicon free of genomic contamination was amplified a dissociation cycle was included at the completion of every amplification cycle and included 1 three step cycle of 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds. All Real-time PCR reactions were completed in duplicate and the mean Cq values used to calculate gene induction.

Reaction specificity was assessed by confirming amplicon size on a 1.5% agarose gel with 0.003% ethidium bromide and by DNA sequence. Sequencing reactions were comprised of 10ul of template at ~1ng/ul, and 5 μM of primer suspended in 5ul of water for a total volume 15 ul. Sequencing primers were identical to Real-time PCR primers. Reactions were shipped overnight to the sequencing site for Sanger DNA sequencing (GeneWiz, Cambridge, Massachusetts).

2.3.1 Primer design

Primers were designed in conserved areas of the 3' region of target genes, across different exons and were identical in length and sequence for all three species (Realtimeprimers.com, Elkins Park, PA) (**Appendix E**). Primers were based on mRNA sequences for *Homo, Pan,* and *Papio* genera published in Genbank when available, and predicted genomic sequences when reviewed sequences were not available. The available *Papio* genome (*Papio hamadryas*, Human Genome Sequencing Center, Baylor College of Medicine) is currently only partially assembled. When *Papio* sequences were not available, *Homo* and *Macaca* sequences were used to search the *Papio* genome short read archive for matches using the pairwise alignment program BLAST and assembled gene sequences (PHAM 1.0, Nov. 2008 http://www.hgsc.bcm.tmc.edu/project-species-p-Papio%20hamadryas.hgsc) (315). These assembled sequences were then aligned with all available catarhine reviewed and predicted sequences for a given gene and conserved regions were identified.

2.3.2 Real time PCR calculations

Relative gene induction was calculated by the Pfaffl equation, normalized to the geometric mean Cq of three reference genes GAPDH, ACTB, B2M and corrected for primer efficiency (316, 317). The Pfaffl method of calculating relative gene induction comparatively quantifies mRNA transcription of treated and untreated samples while accounting for differences in the efficiency of PCR amplification. It does so, by calculating the ratio of post-treatment change in the Cq value (measure of fluorescence at threshold) for the target gene to change in the reference gene, normalized to the primer efficiency values of those genes. Inaccurate estimations of primer

efficiency can falsely and exponentially increase or decrease estimations of fold induction. In this study primer efficiency of each primer set was calculated by linear regression of 5, 10 fold serial dilutions of template, where Cq (y) and dilutions (x). The slope (m) of the line was then used to calculate exponential amplification (Eamp) and efficiency (E) (318):

$$\mathbf{Eamp = [10^{-1/m}]-1}$$

$$\mathbf{E = Eamp + 1}.$$

Reactions were repeated using three different individuals, to ensure accurate assessment of PCR efficiency. All efficiency values for the genes amplified in this study were highly similar to each other and between species, ranging between 80-100% (**Appendix F**).

Relative fold gene induction was estimated as follows:

$$\mathbf{Ratio = E_{target}^{\Delta Cq\ target\ (control-treated)} / E_{reference}^{\Delta Cq\ reference\ (control-treated)}}$$

Where E is the primer efficiency, "control" is the mean Cq value of the unstimulated samples "treated" is the mean Cq value of the stimulated samples, "target" is the gene of interest and "reference" is the reference gene.

2.4 White blood cell differential counting

To determine blood leukocyte subtype proportions, blood leukocytes from each individual were fixed and differentially stained using the Wright-Geisma method, according to the

manufacturers instructions (Camco, Fort Lauderdale, FL). Leukocytes were re-suspended in RPMI-1640 media at approximately 0.5 x 10^6 per ml. 100 ul of the cell suspension was immediately applied to the slide cuvette, and centrifuged at 800 rpm for 5 minutes. Slides were dried and then fixed and stained using the Wright-Geisma method. Slides were dried overnight. Subtypes (neutrophils, lymphocytes, monocytes, basophils and eosinophils) were then counted using light microscopy. Cell subtype proportions were determined from a total count of 400 leukocytes. Leukocyte proportions were found to be approximately the same across all species (**Appendix G**)

2.5 Statistics

Gene induction results were log transformed (base 10) and compared pairwise by species using unpaired t test with Welch's correction in GraphPad Prism version 5.0 for Macintosh (GraphPad Software, San Diego, CA). A p value of <0.05 was considered significant.

2.6 Cytokine and chemokine promoter region analysis

To assess if inter-species differences in cytokine and chemokine induction correspond to inter-species differences in the promoter region sequence, 5' regions of genes that exhibited inter-species differences in induction levels were examined for percent shared identity and putative transcription factor binding sites (TFB sites). Nucleotide sequences corresponding to the end site of known gene promoter regions and 1kb upstream from the known transcriptional start sites of each gene that exhibited inter-species differences in induction were collected from the current *Homo sapiens* (GRCh37/hg19, Genome Reference Consortium; February, 2009), *Pan troglodytes* (CHIMP2.1.4, Washington University Genome Center; February, 2011) and *Macaca mulatta*

(MMUL_1, Baylor College of Human Medicine; January, 2006) genomes using the University of California Santa Cruz and Ensembl genome browsers (319, 320) (**Tables 2.1 and 2.2**). As the *Papio hamadryas* genome (Pham 1.0, Baylor College of Human Medicine; November, 2008) is not fully assembled at this time, sequences from the *M. mulatta* MMUL_1 genome were used. The CXCL2 promoter region was not examined as the MMUL_1 genome assembly contains many unknown nucleotides in this region.

As the genetic promoter regions of most non-human primates are poorly defined and promoter regions tend to be highly conserved, the Mammalian Promoter Database (MPromDb) was searched for the chromosomal locations of known promoter regions of human genes (321). These regions were then identified in the non-human primate sequences. The Mammalian Promoter Database annotates the current assemblies of mammalian genomes with promoter regions identified by ChIP-Seq experiments on species-specific cells. IL-1RN promoters are not noted in this database, so their location was derived from Smith et al. 1994 (322). Known transcriptional start sites for each gene were collected from the Mammalian Promoter/Enhancer Database (PEDB) (323).

To assess the inter-species shared identity of promoter sequences, promoter region sequences were aligned pairwise and compared using the alignment program BLAST2SEQ (315). Nucleotide sequences were then submitted to the JASPAR database to search for putative transcription factor binding sites (TFB sites) (324). JASPAR is a database of transcription factor binding site profiles based on position matrices derived from experimentally confirmed nucleotide binding sites in multi-cellular eukaryotes. Putative TFB site locations were predicted using a matrix of human transcription factor binding sites. Only putative sites with a relative profile score of 80% or higher of the maximum score for each respective TFB site were considered. As JASPAR requires

the user select specific TBF sites for the analysis, experimentally confirmed TBF sites for IL-8/CXCL8, CCL3, IL-1β, TNFα, IL-6, IL-1RN and IL-10 listed in the Transcriptional Regulatory Element Database (TRED) (325) (**Table 2.3**). TRED is an automated and hand-curated collection of transcriptional factor binding motifs paired with publications of supporting experimental evidence. Putative TFB sites identified by JASPAR were compared between *Homo, Pan* and *Macaca*. Sites that were not shared across all three species were noted and compared to cytokine and chemokine induction data.

Gene	***Homo sapiens*** GRCh37/hg19	***Pan troglodytes*** CGSC 2.1.3/panTro3 Oct, 2010	***Macaca mulatta*** MGSC Merged 1.0/rheMac2 Jan, 2006
IL8/CXCL8	Chr 4	Chr 4	Chr 5
Gene location	74,606,275 - 74,609,431	56,335,451 - 56,338,601	55,942,644 - 55,945,822
Retrieved sequence	74,605,275 – 74,609,431	56,335,451 – 56,339,601	55,941,644 – 55,945,822
CCL3	Chr 17	Chr 17	Chr 16
Gene location	34,415,605 - 34,417,506	20,858,699 - 20,860,600	31,376,045-31,377,931
Retrieved sequence	34414605 – 34417506	20,857,699 - 20,860, 600	31,376,045 - 31,378,931
IL-1β	Chr 2	Chr 2	Chr 13
Gene location	113,587,337-113,594,356	112,926,002-112,932,999	112,195,088-112,202,103
Retrieved sequence	113,586,337-113,594,356	112,926,002-112,933,999	112,195,088-112,203,103
TNFα	Chr 6	Chr 6	Chr 4
Gene location	31,543,350 - 31,546,110	31,854,897-31,857,659	31,210,434-31,213,207
Retrieved sequence	31542350 - 31546110	31,853,897 -31,857,659	31,209,434 – 31,213,207
IL-6	Chr 7	Chr 7	Chr 3
Gene location	22,766,766-22,771,619	21,347,129-21,351,988	103,512,347-103,517,198
Retrieved sequence	22,765,766-22,771,619	21,346,129-21,351,988	103,512,347-103,518,198
IL-1RN	Chr 2	Chr 2	Chr 13
Gene location	113,885,138-113,891,592	113,154,494-113,160,682	112,497,016-112,503,203
Retrieved sequence	113,884,138-113,891,592	113,153,494-113,160,682	112,496,016-112,503,203
IL-10	Chr 1	Chr 1	Chr 1
Gene location	206,940,948-206,945,839	185,867,407-185,872,309	163,588,070-163,593,055
Retrieved sequence	206,939,948-206,945,839	185,867,407-185,873,309	163,587,070-163,593,055

Table 2 .1 Gene locations and retrieved sequences used in promoter region analysis

Gene	MPromDB ID	Location (*Homo sapiens* GRCh37/hg19)
IL-8 /CXCL8	chr4: 74 825 199	chr4: 74 824 949 - 74 825 449
CCL3	chr17: 31 441 529	chr17: 31 441 279 - 31 441 779
IL-1β	chr2: 113 310 766	chr2: 113 310 516 - 113 311 016
TNFα	chr6: 31 650 914	chr6: 31 650 361 - 31 651 467
IL-6	chr7: 22 733 091	chr7: 22 732 676 – 22 733 507
IL-1RN	N/A Smith et al., 1994	chr2: 113 884 844 -113 885 107
IL-10	chr1: 205 012 736	chr1: 205 012 301-205 013 172

Table 2.2 Known gene promoter region stored in the Mammalian Promoter Database
Essential IL-1RN promoter region provided by Smith et al., 1994.

Transcription Factor	Family	JASPAR Matrix
AP1	Leucine Zipper	MA0099.2
CEBPA	Leucine Zipper	MA0102.2
CREB1	Leucine Zipper	MA0018.2
E2F1	ETS	MA0024.1
Egr1	Beta-Beta-Alpha-Zinc Finger	MA0162.1
ELK1	ETS	MA0028.1
ESR1	Hormone-nuclear Receptor	MA0112.2
MYC::MAX	Helix-Loop-Helix	MA0059.1
NFIC	NF1 CCAAT-binding	MA0161.1
NFKB1	REL	MA0105.1
PPARG	Hormone-nuclear receptor	MA0066.1
REL	Rel	MA0101.1
RELA	Rel	MA0107.1
SP1	Beta-Beta-Alpha-Zinc Finger	MA0079.2
SPI1	Ets	MA0080.2
STAT1	Stat	MA0137.2
STAT3	Stat	MA0144.1
TBP	TATA-binding	MA0108.2
TFAP2A	Helix-Loop-Helix	MA0003.1
TP53	Loop-Sheet-Helix	MA0106.1
USF1	Helix-Loop-Helix	MA0093.1

Table 2.3 Transcription factor binding sites, family and associated matrices selected for JASPAR search of promoter regions.

2.7 Analysis of protein co-evolution

Reciprocal differences in gene induction (i.e. strong induction of gene A in hominoids and low gene B induction in *Papio*) may be associated with the function of two genes co-evolving. As such, genes exhibiting inter-species differences in induction were examined for signature of co-evolution using the Mirrortree server (326). The Mirrortree method assumes that the gene trees of co-evolving proteins will be similar. The program assesses co-evolution of two protein sequences by creating two unrooted phylogenetic trees based on two sets of user-provided orthologous genes and using the neighbour joining method. In the process a matrix of inter-species distances is developed for each gene tree. The program assesses the co-evolution of the two genes by calculating the Pearson's coefficient of corresponding components in the distance matrices. Correlations of 0.95 and higher are considered indicative of co-evolution of the two genes being examined.

Protein sequences for all available primate species were acquired from UNIPROT and NCBI databases, as well as the annotated Ensembl and the UCSC assembled genomic sequences for the TLR1, TLR2, TLR4, CXCL2, IL-8/CXCL8, CCL3, IL-1β, IL-6, TNFα, IL-1RN, IL-10 (**Appendix H**). To test for the identification of false signatures of co-evolution all receptor, cytokine and chemokine protein sequences were compared to three genes found in different chromosome locations and not involved in innate immunity - GAPDH, ACTB and B2M. No combination of immune and control gene tree comparisons produced a correlation coefficient indicative of co-evolution (i.e. 0.95 or above). *Papio hamadryas* and some *Otolemur garnetti, Tarsier syrichta, Microcebus murinus* and *Sairimi bolviensis* sequences were acquired by searching the short read and trace archives of these genomes using the pairwise alignment program BLAST and published homologous human

genetic sequences. Orthologous protein sequences were assembled in FASTA formatted lists and submitted to Mirrortree in list pairs. As the number and type of species that compose the gene trees affect the inter-species distances of the trees, the gene trees to be compared were comprised of the same species (**Appendix I**).

CHAPTER 3: RESULTS

Innate immune function has evolved differently in old world monkeys, apes and humans

3.1 Overview

The early innate immune response is thought to be a conserved, genetically hardwired induction of immune genes during the first two hours after immune stimulation and mediated by innate immune receptors such as TLRs (49, 50, 327). Primate genera *Homo*, *Pan* and *Papio* are close evolutionary relatives that share >94% genetic identity and yet exhibit different degrees of susceptibility to infectious pathogens. To test if the early innate immune response to bacterial microbes has evolved in African catarrhine primates since the divergence of the hominoid and cercopithecoid lineages 23-29 million years ago, blood was stimulated with TLR2 (LcrV *Yersinia pestis*, PA *Bacillus anthracis*), TLR2/1 (Lipomannan *Mycobacterium smegmatis*, Pam3CSK4) and TLR4/LY96 (LPS *Escherchia coli*) PAMPs for 90 minutes. To assess possible correlations between early innate immune response and the uneven historical distribution of some pathogens in primate environments, inter-species responses to LcrV from *Y. pestis*, PA from *Bacillus anthracis,* and LMMS from *M. smegmatis* are compared to responses to ligands representing more broadly distributed pathogens (LPS, Pam3CSK4). Cytokines and chemokines typical of the early innate immune response were determined by relative real time PCR. The examination of catarrhine early innate immune responses to TLR2, TLR 2/1 and TLR4/LY96 detected PAMPS revealed the following:

1) Species responses to bacterial PAMPS generally agree with the phylogenetic relationships of the animals, though some responses are PAMP-specific.
2) *Papio* chemokine responses differ dramatically from *Homo* and *Pan*, irrespective of PAMP

3) Catarrhine pro-inflammatory cytokines in response are sometimes PAMP-specific
4) *Papio* anti-inflammatory responses strongly differ from *Homo* and *Pan*
5) *Homo*, *Pan* and *Papio* early innate immune responses do not appear to correlate with putative historical distribution of pathogens.

Real time PCR rather than ELISA was used to assess relative chemokine and cytokine responses because of the lack of reliable species-specific antibody reagents for non-human primates, particularly baboon. To minimize differences due to variation in amplicon length and base content, a series of PCR primers were designed that were identical across species and amplified highly conserved regions (**See Materials and Methods**). Initial studies assessed the induction of 17 genes, CCL2, CCL3, CCL5, CXCL2, CXCL12, CSF-2, IFNγ, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-1β, IL-1RN, TGFβ1, TNFα, RELA and NFκBIA. Eight of these (CCL3, CXCL2, IL-8/CXCL8, IL-1β, IL-6, TNFα, IL-10, IL-1RN) showed consistent inter-species differences and are described here. Genes up-regulated or down-regulated more than 2 fold over un-stimulated samples were considered induced. All species weakly induced IFNγ and RELA when stimulated, but no significant inter-species differences in induction were observed (**Appendix J**). Though its transcription is triggered by NF-κB transcription, NF-κB inhibitor NFκBIA was not induced above 2 fold. After stimulus CSF-2 was induced in all species. However, CSF-2 was only amplified in un-stimulated controls at 35 cycles or higher making it difficult to accurately calculate fold difference between stimulated and unstimulated samples. IFNγ, RELA and CSF-2 induction is illustrated in **Appendix J** and is not featured in this analysis.

Homo, *Pan* and *Papio* did not generate cytokine and chemokine responses to *Bacillus anthracis* after 90 minutes of stimulation. While all three species generated responses to LcrV from *Yersinia pestis*, these responses were blunted and occurred only at the highest dose. Given that such a pattern of response could be the outcome of contamination, LcrV experimental data is included in (**Appendix K**) for review but will not be included in the larger analysis of TLR2 PAMPS provided here.

White blood cell subtype proportions were calculated by fixing and differentially staining white blood cells from each individual using the Wright-Geisma method (Camco, Fort Lauderdale, FL). Manual counts of leukocyte subtypes (neutrophils, lymphocytes, monocytes, basophils, eosinophils) under light microscopy revealed white blood cell proportions to be approximately the same across all species (**Appendix G**).

3.2 *Papio* chemokine responses differ dramatically from those of *Homo* and *Pan*, irrespective of PAMP.

The induction of three pro-inflammatory chemokines showed inter-species variation in the degree of induction - IL-8/CXCL8, CXCL2 and CCL3. IL-8/CXCL8 and CXCL2 are the primary chemoattractants of neutrophils, while CCL3 is a more generalized chemoattractant that recruits neutrophils, macrophages, natural killer and T cells (89, 122, 328-331). CXCL2 was more strongly induced in *Papio* than in hominoids after stimulation with all three agonists (**Figures 3.1A and Appendix L, M**). This relationship was reproduced, even at low doses (0.01 μg/ml). In contrast, IL-8/CXCL8 was more strongly induced in *Pan* than in *Homo* and *Papio* after stimulation with the three agonists at the highest doses (10 μg/ml) (**Figure 3.1B**). At the highest dose of agonist the IL-

8/CXCL8 response of *Pan* diverged from that of its closer relative, *Homo*. At the lower doses, however, the IL-8/CXCL8 levels of *Homo* and *Pan* were much more similar to each other than to the IL-8/CXCL8 levels of *Papio* (**Appendix L, M)**. The closely related *Homo* and *Pan* display a similar pattern of CXCL2 at all doses of agonist, and IL-8/CXCL8 at low but not high doses of agonist. A similar pattern emerged for CCL3, which was induced in *Homo* and *Pan* more strongly than in baboons at both low and high doses (**Figure 3.1C**). This differs from previous observations of greater CCL3 induction in *Pan* than *Homo*, which was attributed by the authors to higher copy number in *Pan* than in *Homo* (332). Despite a higher CCL3 CNV than *Homo*, *Pan* induction of CCL3 is highly similar to that of *Homo*, suggesting that CNV has little effect on early CCL3 responses to bacterial pathogens. In summary, for *Papio* the primary chemokine response is CXCL2 whereas for hominoids the primary chemokine response is a combination of IL-8/CXCL8 and CCL3.

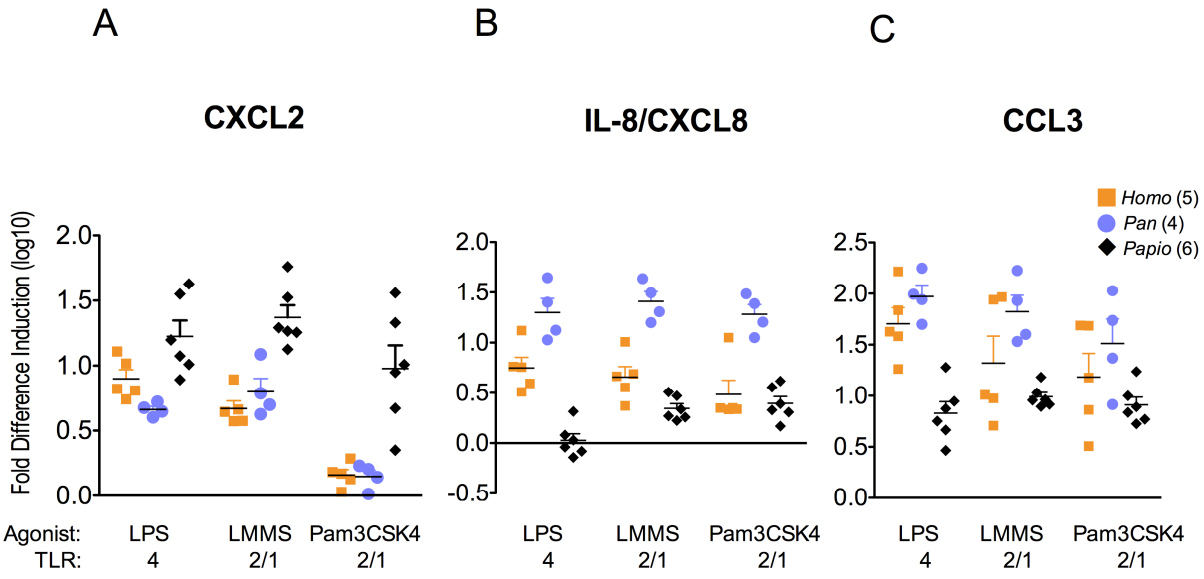


Figure 3.1 *Papio* chemokine responses differ dramatically from *Homo* and *Pan*, irrespective of PAMP.
Homo, *Pan* and *Papio* blood was stimulated with LPS from *E. coli* 0111:B4, LMMS and Pam3CSK4 for 90 minutes and chemokine expression quantified by real-time PCR. 10 μg dose shown here. Coloured bars represent SEM, while black bars represent the mean. Dots in the scatter represent different individuals. Pairwise comparisons of significant difference in gene induction were completed by unpaired t-tests [p value pairwise by PAMP for CXCL2 – LPS: H-Pa = 0.0328, P-Pap = 0.0064, H-Pap non significant (n.s.), LMMS: H-Pa = n.s., Pa-Pap = 0.0045, H-Pap = 0.0002, Pam3CSK4: H-Pa = n.s., Pa-Pap = 0.0065, H-Pap = 0.0064; p value for IL-8/CXCL8 – LPS: H-Pa = 0.0253, Pa-Pap = 0.0012, H-Pap = 0.0011, LMMS: H-Pa = 0.0019, Pa-Pap = 0.0006, H-Pap = 0.0418, Pam3CSK4: H-Pa = 0.0035, Pa-Pap = 0.0007, H-Pap n.s.; p value for CCL3 – LPS: H-Pa = n.s., Pa-Pap = 0.0002, H-Pap = 0.0027, LMMS: H-Pa = n.s., Pa-Pap = 0.0152, H-Pap = n.s., Pam3CSK4 = H-Pa = n.s., Pa-Pap = n.s., H-Pap = n.s.. where H= *Homo*, Pa= *Pan* and Pap= *Papio*].

3.3 African catarrhines display complex patterns of pro-inflammatory responses

Pro-inflammatory cytokine induction (IL-1β, IL-6, TNFα) was examined in all three species in response to all three agonists. In contrast to chemokine responses, the patterns of pro-inflammatory cytokine responses do not always agree with primate evolutionary relationships and often are agonist-specific.

3.3.1 *Homo* exhibits reduced IL-1β induction compared to other primates, irrespective of PAMP.

IL-1β was significantly more strongly induced in *Pan* and *Papio* than *Homo* after stimulation with LMMS and Pam3CSK4 (**Figure 3.2,** $p = <0.05$). A similar IL-1β response was noted after LPS stimulation, though stronger IL-1β induction in *Papio* was not significantly different from *Homo*. This pattern of induction was conserved at both low and high doses of PAMPs and shows *Pan* and *Papio* generally share a stronger IL-1β response than *Homo* (**Appendix L, M**). These results disagree with primate evolutionary relationships and suggest that *Homo* IL-1β responses have evolved to be less overt since *Homo* and *Pan* lineages diverged 6-7 million years ago (333, 334).

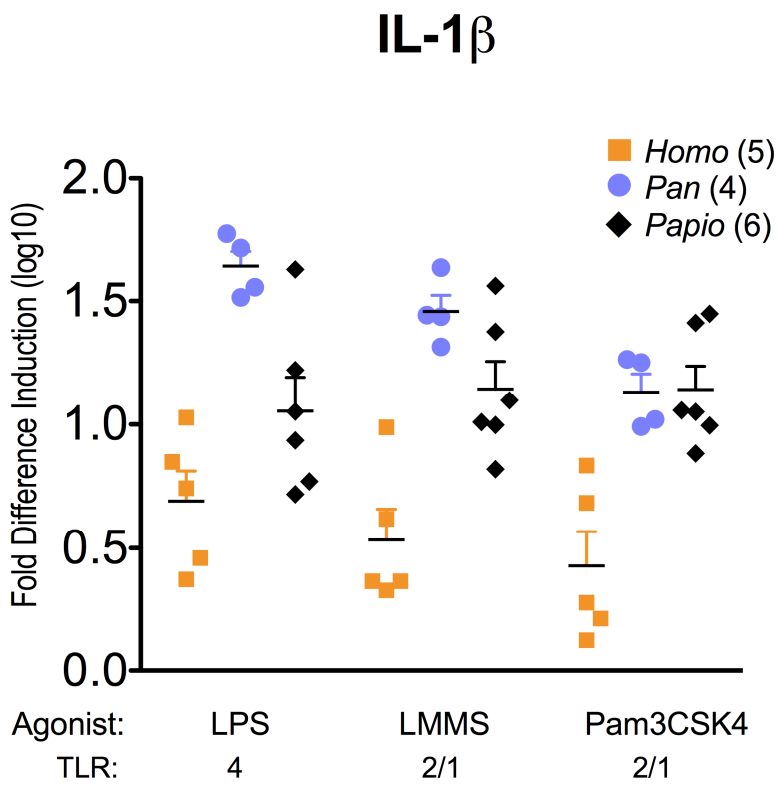


Figure 3.2 *Homo* exhibits reduced IL-1β induction compared to other primates, irrespective of PAMP.
Blood was stimulated with LPS from *E. coli* 0111:B4, LMMS and Pam3CSK4 for 90 minutes and IL-1β expression as determined by real-time PCR. 10 μg dose shown here. 10 μg dose shown here. Coloured bars represent SEM, while black bars represent the mean. Dots in the scatter represent different individuals. Pairwise comparisons of significant difference in gene induction were completed by unpaired t-tests [p value pairwise by PAMP for LPS: H-Pa = 0.0009, Pa-Pap = 0.0080, H-Pap non significant (n.s.), LMMS: H-Pa = 0.0013, Pa-Pap = 0.0466, H-Pap = 0.0066, Pam3CSK4: H-Pa = 0.0066, Pa-Pap = n.s., H-Pap = 0.0039].

3.3.2 TNFα response to LPS is similar among *Homo*, *Pan* and *Papio*, but differs in response to TLR2/1 PAMPs.

In contrast to the species-specific differences observed in the induction of IL-1β, the pattern of induction of TNFα was more complex. *Pan* TNFα induction was significantly stronger than

TNFα induction by the closely related *Homo* and more distantly related *Papio* after stimulation with a high dose of LPS (10μg/ml) (**Figure 3.3**). This difference, however, was not observed at lower doses of LPS (**Appendix L, M**). *Homo* and *Papio* TNFα levels were similar regardless of LPS dose. A very different pattern of TNFα responses emerged after TLR2/1 PAMP stimulation. *Homo* TNFα induction was significantly lower than *Papio* to the two TLR2/1 PAMPs, at both high and low doses (**Figure 3.3, Appendix L, M**). The TNFα responses to TLR2/1 PAMPs agrees with primate phylogeny and mimics the pattern of lowered *Homo* and heightened *Papio* IL-1β expression.

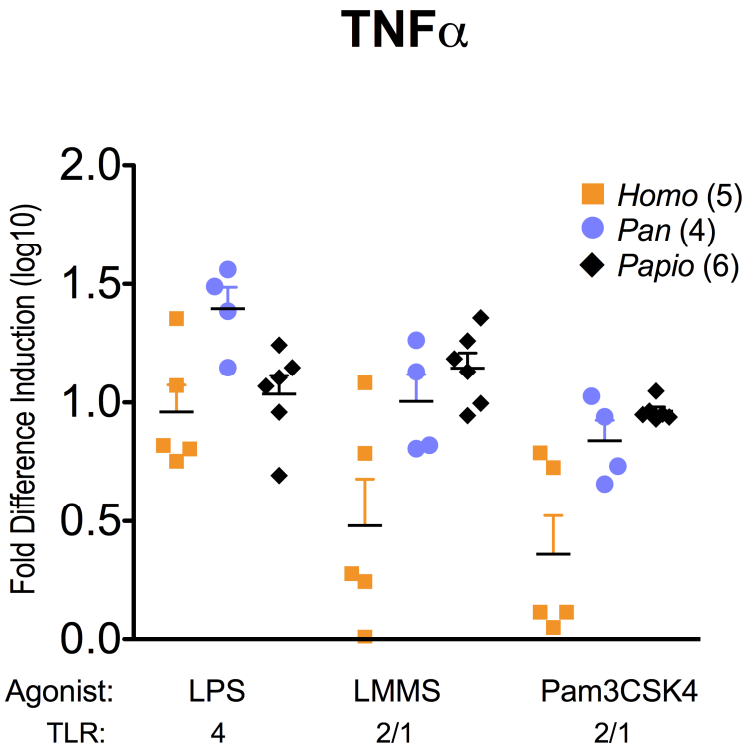


Figure 3.3 Catarrhine TNFα induction is both species- and PAMP-specific.
TNFα expression in *Homo*, *Pan* and *Papio* after blood was stimulated with LPS from *E. coli* 0111:B4, LMMS and Pam3CSK4 for 90 minutes. Relative real-time PCR data for 10 μg/ml shown here. Coloured bars represent SEM, while black bars represent the mean. Dots in the scatter represent different individuals. Pairwise comparisons of significant difference in gene induction were completed by unpaired t-tests [p value pairwise by PAMP for LPS: H-Pa = 0.0238, Pa-Pap = 0.0238, H-Pap = n.s., LMMS: H-Pa = n.s., Pa-Pap = n.s., H-Pap = 0.0324, Pam3CSK4: H-Pa = 0.0484,

Pa-Pap = n.s., H-Pap = 0.0211].

3.3.3 *Papio* exhibits greatly reduced IL-6 induction, irrespective of PAMP

In contrast to observations of chemokine and other proinflammatory cytokine responses, where the relative induction ranged from ~ -0.1-2.2 log (or ~5-50 fold), the relative induction of IL-6 was significantly greater (~1.1-3.1 log, or 15-1200 fold). *Papio* IL-6 induction, however, was strikingly lower than *Homo* IL-6 induction, even at high doses of PAMPs (**Figure 3.4, Appendix L, M**). *Papio* IL-6 induction was also significantly lower than *Pan* IL-6 induction after LPS and LMMS stimulation, though this difference was not observed after Pam3CSK4 stimulation. Taken together this data supports a pattern of greater IL-6 induction in hominoids than *Papio*. A pattern of strong but relatively lesser IL-6 induction in *Papio* agrees with species phylogeny and suggests that primate IL-6 responses diverged in African catarrhines since *Papio* and hominoids last shared a common ancestor 23-29 million years ago.

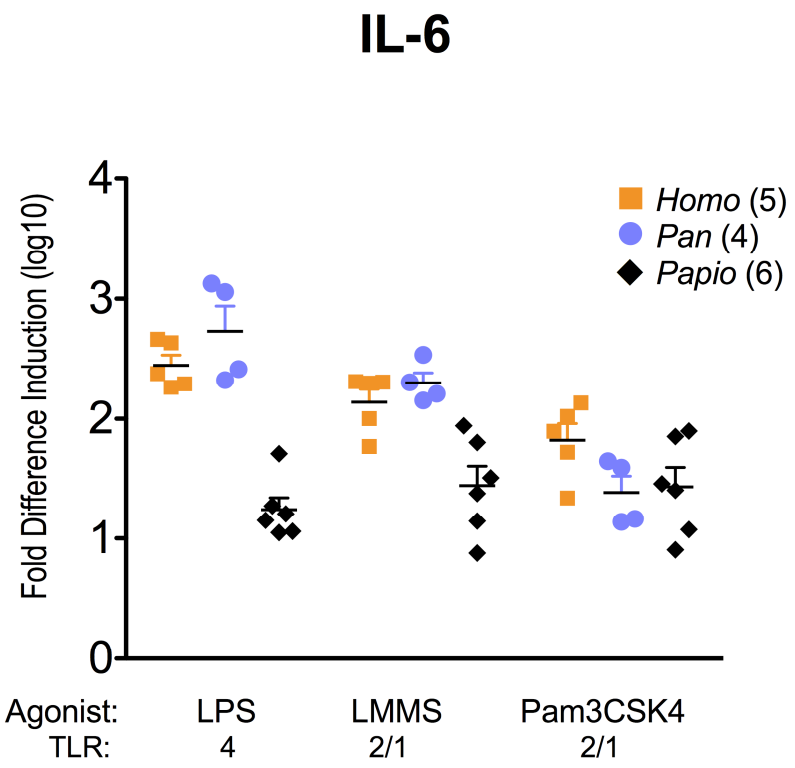


Figure 3.4 *Papio* exhibits reduced IL-6 induction, irrespective of PAMP.
IL-6 response in human, chimpanzee and baboon after stimulation with PAMPs, as determined by relative real time PCR. Coloured bars represent SEM, while black bars represent the mean. Dots in the scatter represent different individuals. Pairwise comparisons of significant difference in gene induction were completed by unpaired t-tests [p value pairwise by PAMP for LPS: H-Pa = n.s., Pa-Pap = 0.0030, H-Pap <0.0001, LMMS: H-Pa = n.s., Pa-Pap = 0.0022, H-Pap = 0.0075, Pam3CSK4: H-Pa = n.s., Pa-Pap = n.s., H-Pap = n.s.].

3.4 *Papio* and hominoid anti-inflammatory cytokine responses are highly divergent

Induction of two anti-inflammatory cytokines (IL-10 and IL-1RN) was examined in all three species in response to all three agonists. Remarkable divergence in *Papio* and hominoid anti-inflammatory responses was observed. Similar to pro-inflammatory responses, the patterns of anti-inflammatory cytokine responses do not always agree with primate evolutionary relationships and are sometimes PAMP-specific.

3.4.1 *Papio* IL-1RN induction is weaker than in hominoids, irrespective of PAMP

Hominoid induction of IL-1RN, the competitive antagonist for IL-1β-IL-1 receptor binding, was significantly stronger than *Papio*, regardless of PAMP ($p < 0.05$) (**Figure 3.5, Appendix L, M)**. While hominoid IL-1RN was induced ~0.5-1.25 log (or 7-16 fold), *Papio* IL-1RN was minimally induced after PAMP stimulation. This observation is surprising since IL-1RN transcription is triggered by the binding of IL-1β/IL-1α to IL-1 receptors (IL-1R), and the level of IL-1RN induction would be expected to be proportional to the level of IL-1β induction (335) (271) (**Figure 3.2**). The ratio of induced IL-1RN to induced IL-1β is significantly lower, not only in *Papio* but also in *Pan* when compared to *Homo* (**Figure 3.6**).

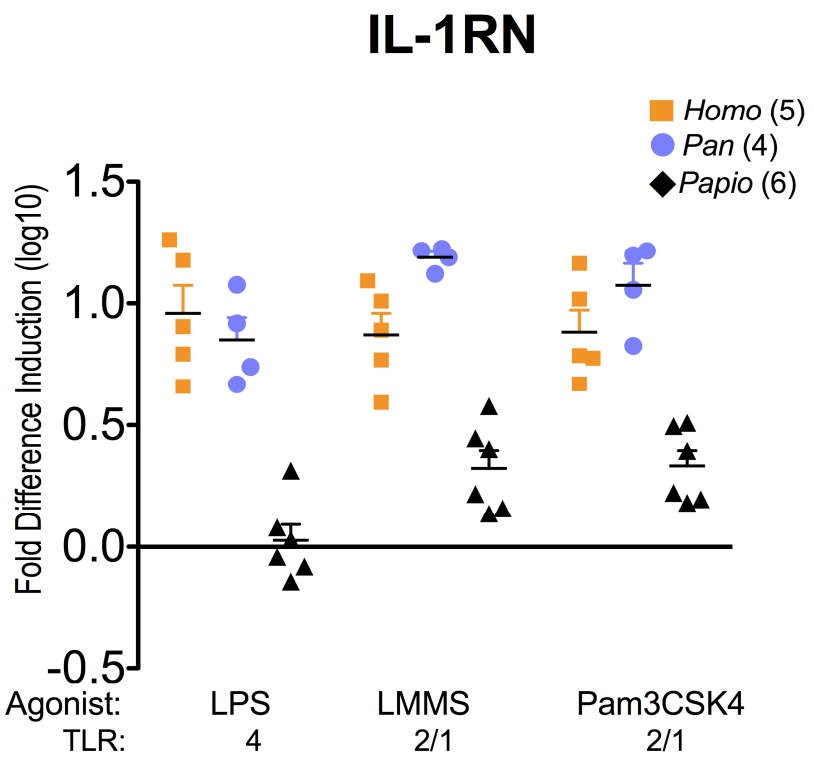


Figure 3.5 *Papio* IL-1RN induction is weaker than in hominoids, irrespective of PAMP. IL-1RN expression levels after 90 minutes of stimulation with LPS from *E. coli* 0111:B4, LMMS and Pam3CSK4, as determined by real-time PCR. 10 μg/ml dose shown here. Coloured bars represent SEM, while black bars represent the mean. Dots in the scatter represent different individuals. Pairwise comparisons of significant difference in gene induction were completed by unpaired t-tests [p value pairwise by PAMP for LPS: H-Pa = n.s., Pa-Pap = 0.0008, H-Pap <0.0004, LMMS: H-Pa = 0.0257, Pa-Pap <0.0001, H-Pap = 0.0014, Pam3CSK4: H-Pa = n.s., Pa-Pap = 0.0011, H-Pap = 0.0015].

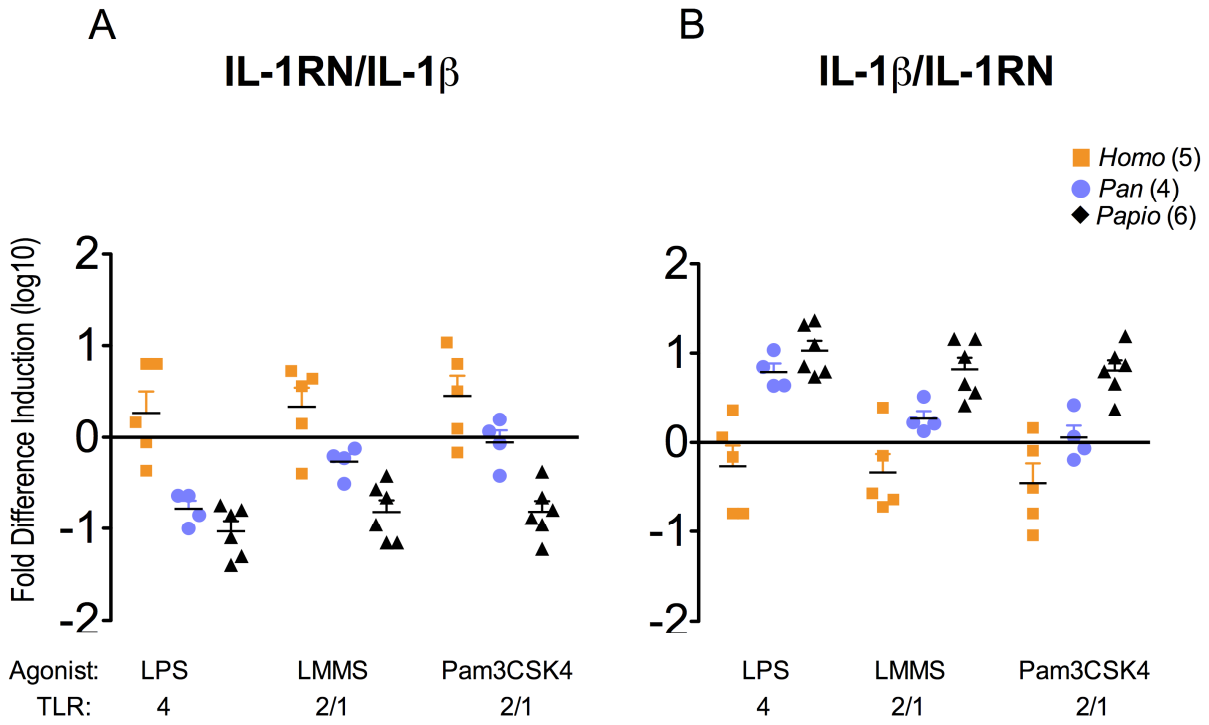


Figure 3.6 *Homo* IL-1RN/IL-1β ratio is higher than *Pan* and *Papio*, irrespective of PAMP. IL-1RN/IL-1β and IL-1β/IL-1RN expression ratios for *Homo*, *Pan* and *Papio* shown here. Gene expression after 90 minutes of stimulation determined by real-time PCR. 10 μg/ml dose shown here. Coloured bars represent SEM, while black bars represent the mean. Dots in the scatter represent different individuals. Pairwise comparisons of significant difference in gene induction were completed by unpaired t-tests [p value pairwise by PAMP for IL-1RN/IL-1β LPS: H-Pa = 0.0084, Pa-Pap = n.s., H-Pap = 0.0040, LMMS: H-Pa = 0.0434, Pa-Pap = 0.0087, H-Pap = 0.0033, Pam3CSK4: H-Pa = n.s., Pa-Pap = 0.0023, H-Pap = 0.0051; IL-1β/IL-1RN LPS: H-Pa = 0.0084, H-Pap = 0.0040, Pa-Pap = n.s., LMMS: H-Pa = 0.0426, Pa-Pap = 0.0050, H-Pap = 0.0023].

3.4.2 *Papio* IL-10 response to TLR2/1 agonists is stronger than in hominoids.

In contrast to IL-1RN, anti-inflammatory cytokine IL-10 was strongly induced in *Papio* and minimally induced in *Homo* and *Pan* after stimulation (**Figure 3.7**). *Papio* IL-10 was induced as much as, or greater (0.29 – 0.81 log, or 4-7 fold), than was observed for *Homo* (0.23-0.52 log, or 2-3 fold) and *Pan* (-0.05 – 0.29 log, 1-2 fold) IL-10 (**Figure 3.7, Appendix L, M)**. Furthermore, *Papio* IL-10

induction was significantly greater relative to *Homo* IL-10 induction for the TLR2/1 PAMPs than it was for TLR4 PAMP, LPS. It is intriguing to note that the pattern of IL1-RN induction appears to be species-specific and independent of PAMP, while the species-specific pattern of IL-10 induction displays some degree of agonist-specificity. In this sense, *Homo* and *Papio* IL-10 induction recapitulates the pattern observed for TNFα. While all responses are low, *Papio* responses to TLR2/1 PAMPs are significantly stronger than *Homo* responses, and species responses to a TLR4 PAMP are very similar.

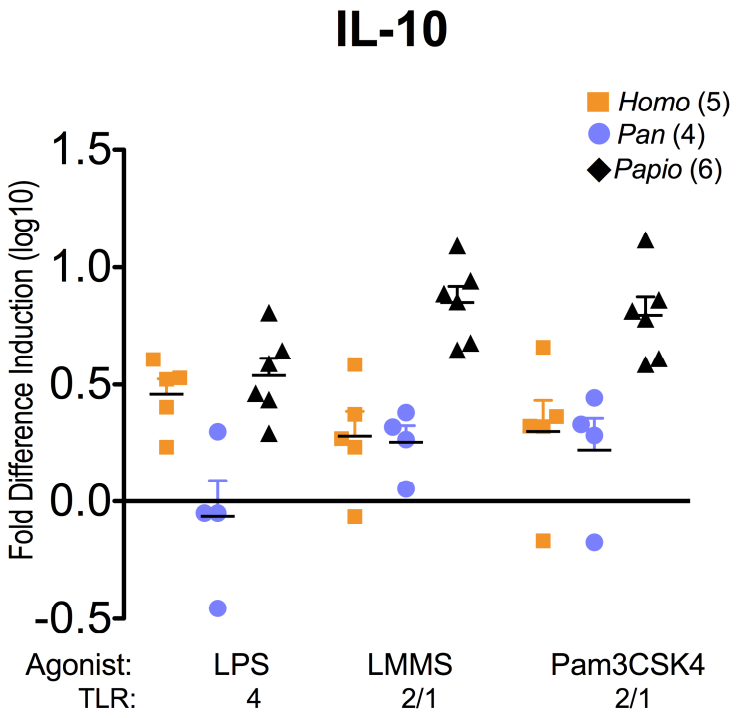


Figure 3.7 *Papio* IL-10 response to TLR2/1 agonists is stronger than in hominoids. IL-10 expression levels after 90 minutes of stimulation with LPS from *E. coli* 0111:B4, LMMS and Pam3CSK4, as determined by real-time PCR. 10 μg/ml dose shown here. Coloured bars represent SEM, while black bars represent the mean. Dots in the scatter represent different individuals. Pairwise comparisons of significant difference in gene induction were completed by unpaired t-tests [p value pairwise by PAMP for IL-10 LPS: H-Pa = 0.0353, Pa-Pap = n.s., H-Pap = 0.0242, LMMS: H-Pa = n.s., Pa-Pap = 0.0005, H-Pap = 0.0026, Pam3CSK4: H-Pa = n.s., Pa-Pap = 0.0144, H-Pap = 0.0183].

3.5 Catarrhine promoter sequence similarity and putative transcription factor binding sites tend to correspond with gene induction patterns

Generally, early cytokine and chemokine induction agrees with catarrhine evolutionary relationships. That is, *Homo* and *Pan* cytokine and chemokine responses were generally more similar to one another than to *Papio* (**Figure 3.8**). An examination of inter-species promoter region sequence identity revealed a pattern of percent shared identity that corresponds to shared patterns of gene induction (**Table 3.1**). *Homo* and *Pan* tend to share highly similar promoter regions, where as the more distantly related cercopithecoid (as the *Papio hamadryas* genome is not fully assembled, close *Papio* relative *Macaca*) tends to have more divergent promoter regions. A search for putative transcription factor binding sites (TFB sites) in promoter regions and 1kb 5' sequences upstream from gene transcriptional start sites revealed a similar pattern. The type and number of TFB sites not shared between all three species tended to be similar between the two most closely related species, *Homo* and *Pan*, and correspond with gene induction patterns (**Table 3.2 and Appendix N**). The importance of the presence or absence of many of putative transcription factor binding sites is difficult to decipher, however it is interesting to note that *Homo* and *Pan* minimally induce IL-10 compared to *Papio*, and are predicted to uniquely share multiple binding sites for a factor that inhibits protein translation *in vitro*, SPI1(336).

A notable exception to a general correspondence between promoter region sequence identity and gene induction can be found in the high shared identity of the IL-1RN promoter region (98%) in all three species, despite hominoids sharing strong IL-1RN induction and *Papio* minimal IL-1RN induction in response to stimulus. Another exception to this pattern is the high shared identity of *Homo* and *Pan* IL-1β (98%) and the comparatively divergent *Macaca* IL-1β promoter regions (91%).

By contrast, IL-1β is strongly induced in *Papio* and *Pan* and minimally in *Homo*. A search for putative TFB sites in the promoter region identifies several *Homo* –specific sites -268 to -214 from the transcriptional start site (**Figure 3.9 and Appendix N**), including putative binding sites for two NF-κB family factors, c-REL/REL (-268 to -259) and RELA (-268 to -259), that do not occur in *Pan* and *Macaca* (-268 to -262). Additionally, *Homo* is predicted to have a unique binding site at locations -221 to -214 for SPI1.

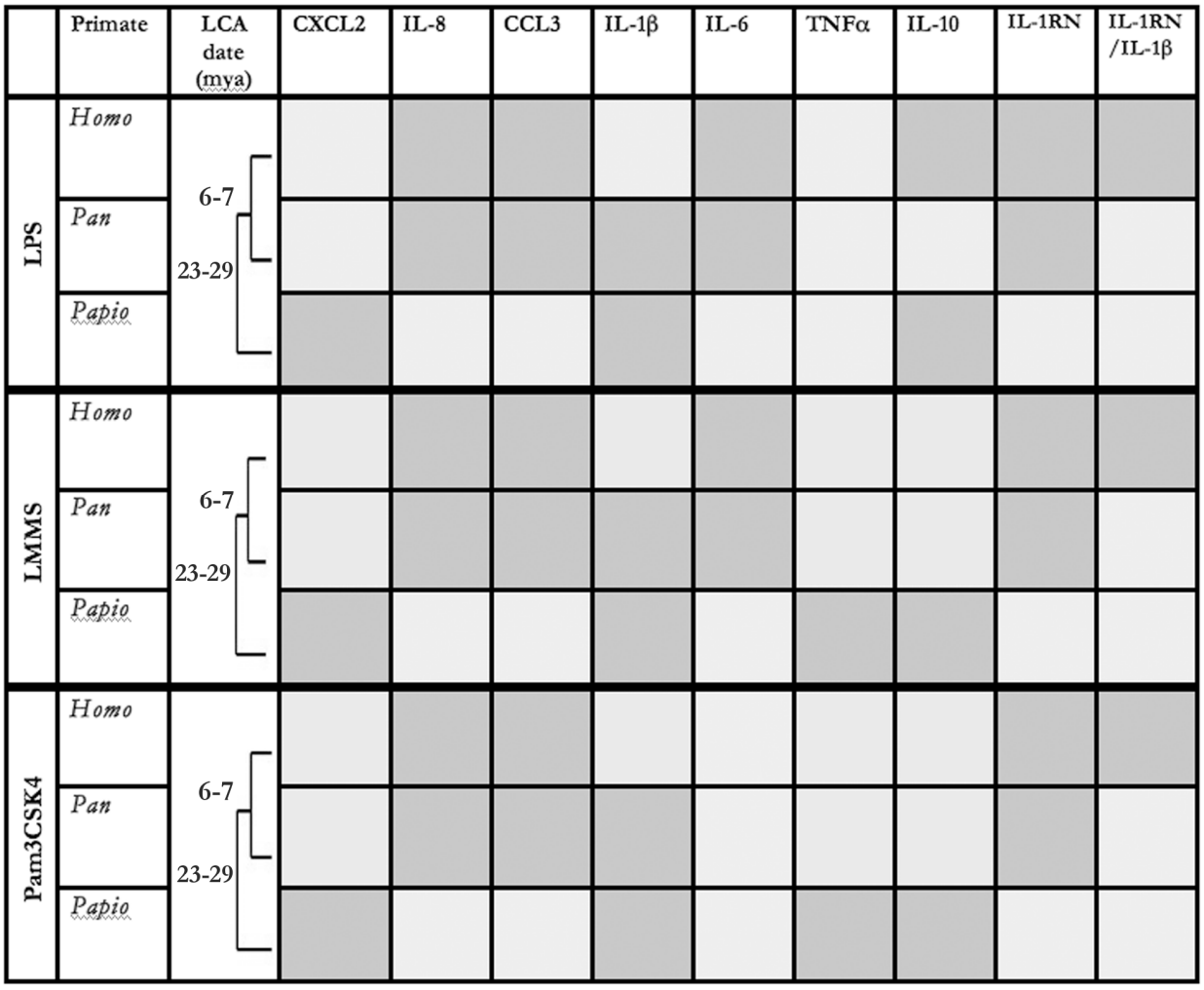


Figure 3.8 African catarrhines exhibit species- and agonist- specific responses to bacterial ligands.
Animals that share dark or light grey symbol for gene expression do not express that particular gene significantly differently from one another. Dark grey: strong induction, light grey: weak induction. LCA (last common ancestor) is listed for *Homo-Pan* and *Pan-Papio* in millions of years (mya)(38).

Subject Promoter	Query Promoter	Percent Shared Identity						
		IL8/ CXCL8	CCL3	TNFα	IL-1β	IL-6	IL-10	IL-1RN
Homo sapiens	*Pan troglodytes*	98%	99%	99%	99%	99%	99%	98%
Homo sapiens	*Macaca mulatta*	96%	93%	94%	91%	95%	96%	98%
Pan troglodytes	*Macaca mulatta*	96%	93%	94%	91%	96%	96%	98%

Table 3.1 Percent shared identity of gene promoter regions for *Homo sapiens*, *Pan troglodytes*, and *Macaca mulatta*.
As the two closest related species, *Homo* and *Pan* tend to share the highest genetic identity in promoter regions. Despite considerable divergence between cercopithecoid and hominoid induction of IL-1RN, all species examined here share 98% genetic identity in this small region. Human promoter region locations were collected from the Mammalian Promoter Database (WISTAR Institute, Philadelphia, Pennsylvania).

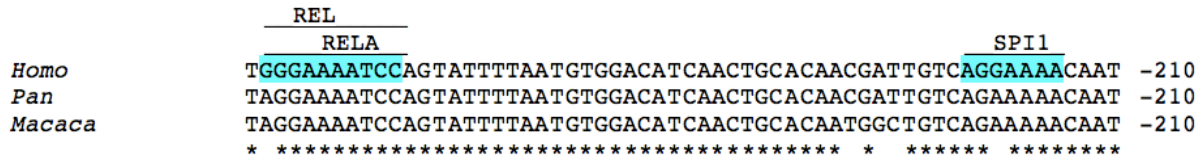


Figure 3.9 Putative unique transcription factor binding sites in the Homo IL-1β promoter region.
Region -269 to -210 shown here. Alignment completed in ClustalW2 (315)

3.6 Summary

In summary, blood from *Homo*, *Pan* and *Papio* stimulated with TLR2 and TLR4 –detected PAMPs for 90 minutes induced the expression of cytokines and chemokines in species-specific and PAMP-specific patterns. *Homo* and *Pan* cytokine and chemokine responses were generally more similar to one another than to *Papio* (**Figure 3.8**). Specifically, PAMPs representing Gram-negative, Gram-positive and mycobacterial agonists stimulated species-specific chemokine and anti-inflammatory responses that agree with the phylogenetic relationships of the three primates. Pro-inflammatory cytokine responses, however, were more complex and were upregulated in species-

specific and PAMP-specific patterns. Specifically, TLR4 agonist LPS from *E. coli* stimulates similar TNFα levels in *Homo* and *Papio*, while TLR2/1 agonists LMMS and Pam3CSK4 induce a significantly stronger TNFα response in *Papio* than *Homo*. *Homo*, *Pan* and *Papio* also generated a very different pattern IL-6 induction after stimulation with LMMS than after stimulation with Pam3CSK4. As expected, the promoter and 1kb 5'regions of these cytokine and chemokine genes tend to be most similar between closely related species. *Homo* IL-1β as well as *Homo* and *Pan* IL-10 promoter regions are predicted to have unique transcription factor binding sites that correspond with noted gene induction patterns.

PAMPs derived from *Bacillus anthracis* (PA) and *Yersinia Pestis* (LcrV) did not reliably stimulate cytokine and chemokine responses during this early phase of the innate immune response. Cytokine and chemokine induction at the 90 minute time point does not appear to correlate with putative historical distribution of pathogens tested here. Rather, *Homo*, *Pan* and *Papio* induce early innate immune responses that are complex combination of PAMP- and species-specific cytokine and chemokine expression.

CHAPTER 4: DISCUSSION

4.1 Summary of research

This study was initiated to help explain why some African catarrhine species appear to have evolved differing susceptibility to bacterial pathogens (200, 205, 232). Most previous studies of non-human primate immune responses have focused on genes and proteins that potentially contribute to inter-species differences in primate-viral interactions on the interests of developing treatments for HIV and HCV (5, 22-29, 337). In contrast, the current understanding of catarrhine immune system responses to bacterial pathogens is considerably limited. Toll-like receptor variants have been associated with differing infectious disease susceptibility and progression in many vertebrate animals, including primates (20, 338-341). Specifically, variations in TLR2 and TLR4/LY96 composition and signaling have been associated with alterations in disease progression in *Homo* (108, 256, 259). Catarrhine TLRs have been hypothesized to have diverged under selection by different bacterial pathogens associated with host geographic distribution and behaviour (43, 45, 254, 337, 342). Recent examinations of TLR1, TLR2, TLR4 genetic sequences, suggest these receptors have been under positive selection in primates and maintain a high proportion of missense mutations in *Homo* specifically (43, 44). To assess if African catarrhines have evolved different early innate immune responses to TLR-detected bacterial pathogens, this study examined the cytokine and chemokine response of *Homo*, *Pan* and *Papio* to TLR2 and TLR4/LY96 –detected bacterial ligands for differences induction and possible associations with putative primate evolutionary environments.

4.2 Early innate immune response is, generally, species-specific

This study illustrates that early (90 min) *Homo* and *Pan* cytokine and chemokine responses are generally more similar to one another than to *Papio*. This pattern of induction agrees with the evolutionary relationships of these animals, as *Homo* and *Pan* lineages diverged 6-7 million years ago and hominoids diverged from the cercopithecoid lineage 23-29 million years ago (38). There are, however, notable exceptions to this pattern. The induction patterns of some cytokines and chemokines are either not congruent with species phylogeny (i.e. IL-1β) or appear pathogen-specific (TNFα, IL-10, IL-6). Taken together the results of this study suggest that African catarrhine early innate immune responses to bacterial pathogens have diverged on phylogenetic lines, with some pro- and anti-inflammatory cytokine responses having evolved to particular pathogens. These findings broadly agree with the available information on *Homo*, *Pan* and *Papio* severe bacterial infection susceptibility (10, 11, 106).

4.2.1 Chemokine induction patterns suggest molecular basis of neutrophil migration differs between hominoids and cercopithecoids.

It is interesting that hominoid and *Papio* chemokine responses strongly diverged regardless of PAMP, as it suggests that hominoids and cercopithecoids have evolved different core chemokine responses to stimulus. As chemokines are immune cell chemoattractants, the strength of their induction has implications for leukocyte migration to sites of infection. IL-8/CXCL8 and CXCL2, for example, are the primary chemoattractants for neutrophils and facilitate the migration of these cells in a dose dependent manner (330, 343)[reviewed in (344)]. Lower IL-8/CXCL8 and higher

CXCL2 and induction by *Papio* compared to *Homo* and *Pan* suggests the coordination of hominoid and cercopithecoid neutrophil responses to infection have diverged in the last 23-29 million years.

Neutrophils are important phagocytes that are amongst the first cells that migrate to infection sites [reviewed in (52)]. Their activities are key for pathogen clearance but can also injure tissues at sites of infection under pathological conditions such as severe sepsis [reviewed in (343, 345)]. Neutrophils migrate to sites of inflammation by rolling along post-capillary venule walls, and then transmigrating endothelial cell barriers [reviewed in (52)]. IL-8/CXCL8 and CXCL2 play two very different roles in this process. IL-8/CXCL8 mediates neutrophil migration to infection sites by binding these cells to the cell membranes of activated and selectin - expressing (E-selectin, P-selectin) endothelial cells. Neutrophil - IL-8/CXCL8 binding arrests cell rolling and initiates neutrophil transmigration of the endothelial cell barrier to the site of infection. Strong hominoid and weak *Papio* IL-8/CXCL8 induction may affect this process as released IL-8/CXCL8 is internalized by endothelial cells, and presented on endothelial cell membranes at the luminal surface (329, 346). Rolling neutrophils bearing CXCR2 are then arrested by IL-8/CXCL8 - CXCR2 binding before transmigration (122, 328, 347). Increased IL-8/CXCL8 concentrations is associated with linear migration of neutrophils *in vitro* (348). Minimal *Papio* IL-8/CXCL8 induction compared to *Homo* and *Pan* suggests lowered IL-8/CXCL8 presentation on *Papio* endothelial cell membranes and fewer neutrophils arrested for endothelial barrier transmigration during the early innate immune response.

While IL-8/CXCL8 can arrest neutrophil rolling and initiate the process of transmigration, CXCL2 appears to induce neutrophil rolling in a dose and P-selectin -dependent manner (330, 349). IL-8/CXCL8 presentation on endothelial cell surfaces and subsequent arrest of neutrophils is E-selection dependent (329, 346). The lack of dependence on E-selectin in CXCL2 mediated

neutrophil migration suggests that CXCL2 expression does not play a role in neutrophil arrest (330). Stronger CXCL2 induction in *Papio* and minimal CXCL2 induction in *Homo* and *Pan* suggests that the rate of neutrophil rolling is higher in *Papio* than hominoids during the early innate immune response.

Strong *Homo* and *Pan* and weak *Papio* induction CCL3 may also affect neutrophil migration and severe infection susceptibility in catarrhines. CCL3 is associated with strong neutrophil endothelial cell adherence and subsequent transmigration of neutrophils (350). Importantly, CCL3 has bee associated with an increase in venular tissue remodeling and microvascular leakage, a symptom of severe sepsis (350). Strong CCL3 induction by *Homo* and *Pan* may lead to inter-species differences in the migration of many other cell types, as CCL3 is a powerful pro-inflammatory activator and chemoattractant of CCR1+ and CCR5+ cells such as monocytes, CD8+ T lymphocytes, natural killer cells and dendritic cells to sites of inflammation (89, 134, 331, 344, 351, 352). It is possible that the cellular response to bacterial infection in *Papio* may be more restricted than in hominoids since CXCL2 attracts primarily neutrophils, whereas CCL3 chemoattracts a broader range of leukocytes.

Interestingly, the pattern of divergent *Papio* and *Homo*/*Pan* chemokine responses corresponds with Gram-negative severe sepsis susceptibility in hominoids. The divergence of *Papio* and hominoid IL-8/CXCL8, CXCL2 and CCL3 induction suggests the number of neutrophils rolling and recruited to infection sites differ and may affect pathogen clearance or tissue injury in severe infections. It is possible that the heightened susceptibility of *Homo* and *Pan* to gram-negative bacterial severe sepsis is, at least in part, mediated by a divergence of core chemokine responses from those of Old World monkeys.

4.2.2 Catarrhine pro-inflammatory cytokine responses can be pathogen-specific

Pro-inflammatory responses generally agreed with the evolutionary relationships of *Homo*, *Pan* and *Papio*. Interesting exceptions to this induction pattern include pro-inflammatory cytokines TNFα, IL-1β and IL-6. TNFα is a powerful pro-inflammatory cytokine that is very quickly expressed after immune stimulation by primarily monocytic cells. It is a major modulator of pro-inflammatory cascades, and an important controller of neutrophil and macrophage activation and recruitment [reviewed in (272) and (353)]. While early TNFα induction is similar across species, it appears to be pathogen-specific in *Homo*. TLR2/1 PAMPs trigger significantly weaker induction in *Homo* than LPS and this response is weaker than the *Papio* TNFα response. Similarly, *Pan* TNFα induction was lower in response to TLR2/1 PAMPs than it was for the same dose of LPS. These findings agree with a previous finding that *Homo* TNFα promoter activity is initiated in a stimulus-specific manner and suggests that *Papio* TNFα production is not PAMP-specific (354). The current study suggests that catarrhine TNFα induction is pathogen-specific and has diverged since hominoids and *Papio* last shared a common ancestor 23-29 million years ago.

That *Homo* and *Papio* TNFα mRNA induction was similar after LPS stimulation is an unexpected finding. Most other studies at similar time points have found that *Homo* immune cells release more stored and newly produced TNFα than *Papio* in response to LPS (11, 106, 269, 355). The disparity in mRNA transcription levels and the amount of TNFα released by these species might represent the evolution of greater TNFα release efficiency in *Homo* (106).

The pattern of IL-1β responses is also unexpected, given the evolution of primates. The more distantly related *Pan* and *Papio* shared strong IL-1β responses to all agonists, while *Homo* IL-1β induction was significantly weaker than *Pan*. This pattern of induction may be explained by putative human-specific REL, RELA and SPI1 binding sites identified in the promoter region of the gene (-268 to -214) (**Figure 3.9, Appendix N**). It is, however, possible that as yet unidentified inter-species differences in very important but more difficult to locate regulatory regions such as enhancers and silencers are responsible for low IL-1β induction in *Homo*. Regardless of regulatory mechanism, low IL-1β induction suggests that *Homo* modulation of IL-β associated responses diverged 6-7 million years ago. IL-1β is the strongest endogenous pyrogen and an important mediator of acute phase, pro-inflammatory and febrile responses (356-359). The consistently low IL-1β response in *Homo* suggests that IL-1β-mediated acute phase and inflammation responses are initiated differently in *Homo* than in *Pan* and *Papio*. Stronger *Papio* than *Homo* IL-1β induction does not agree with previous observations of difficulty stimulating *Papio* febrile responses, however difficulty initiating fever in *Papio* may be explained by weak IL-6 induction in response to LPS compared to hominoids. IL-6 is very pyrogenic, and stimulates apoptosis, acute phase, neutrophil, lymphocyte and complement responses in the minutes following immune stimulation and is weakly induced in *Papio* while very strongly induced in hominoids (360-365).

IL-6 responses to some agonists appear to have diverged since hominoids and *Papio* last shared a common ancestor 23-29 million years ago as *Homo* and *Pan* also mount very strong IL-6 responses to both LPS and LMMS, compared to *Papio*. Specific TLR2/1 -detected pathogens, however, seem to provoke widely different IL-6 responses from these species. In contrast to LMMS, all species induce similar levels of IL-6 after stimulation with another TLR2/1 agonist, Pam3CSK4.

These results suggest that hominoids have evolved IL-6 responses that are specific to mycobacterial and gram-positive pathogens since the hominoid and cercopithecoid lineages diverged.

4.2.3 Anti-inflammatory cytokine responses can be pathogen-specific

Hominoid and *Papio* anti-inflammatory cytokine responses appear to have diverged between 23-29 million years ago. Induction patterns for anti-inflammatory cytokines differ not just between species, but also sometimes between agonists. While IL-1RN appears to be induced in species-specific patterns independent of agonists, *Papio* IL-10 induction exhibits agonist specificity. When stimulated by TLR2/1 agonists, *Papio* mounts a stronger IL-10 response than the hominoids. When stimulated with TLR4/LY96 agonist LPS, however, *Papio* IL-10 responses lower to approximately equivalent to that of *Homo*. These results imply that the strength of the initial *Papio* IL-10 response is pathway specific.

Strong *Papio* and weak hominoid IL-10 responses to TLR2/1 agonists has implications for susceptibility to diseases characterized by dysfunctional pathogen clearance and strong inflammation. IL-10 mainly reduces pro-inflammatory activities by binding to IL-10R1/IL-10R2 receptor complex, triggering the JAK/STAT pathway, subsequently limiting Th1 cytokine production (i.e IFNγ, IL-12, TNFα, CSF2) in cells of myeloid origin (161, 366-369). IL-10 also inhibits NF-κB activation, NF-κB DNA binding activity and consequently most inducible chemokines as well as two pro-inflammatory markers noted to be weakly induced in *Papio,* IL-6 and IL-8/CXCL8 [(370-372)and reviewed in (373)]. The relationship between early IL-10 expression and host survival during bacterial infection is complex. The presence of endogenous or exogenous IL-10

has been associated with increased mortality in *Borrelia burgdorferi*, *Klebsiella pneumonia* and *Mycobacterium* infections (374-376). While IL-10 is also associated with the dissemination of *E. coli* in the host, it attenuates potentially harmful effects of pro-inflammatory proteins and leukocyte migration to sites of infection, thereby lessening the severity of sepsis (377). Strong *Papio* and minimal hominoid IL-10 induction in response to TLR2/1 agonists suggests that hominoid and *Papio* modulation of NF-κB activity, JAK/STAT pathway, Th1 responses have diverged over the last 23-29 million years. This divergence may be rooted in the acquisition of multiple (putative) binding sites for a transcription factor associated with translation inhibition, SPI1, in the *Homo* and *Pan* IL-10 promoter region (6 sites between -913 and 1707) in that time. However, inter-species differences in other regulatory regions (i.e. enhancers and silencers) possibly contribute increased *Papio* IL-10 induction. The similarity of *Papio* and *Homo* IL-10 responses to LPS suggests that initial IL-10 induction does not contribute cercopithecoid resistance to severe gram-negative sepsis.

Hominoid IL-1RN induction is significantly stronger than *Papio* IL-1RN induction, suggesting that the feedback mechanism by which IL-1β/IL1-receptor binding induces the production of competitive inhibitor IL-1RN and the downregulation of IL-1β induced inflammatory responses (i.e. IL-6, IL-8/CXCL8 induction), has diverged in these species [reviewed in (154)]. The molecular basis for both species-specific IL-10 and IL-1RN induction has yet to be determined. Many factors may be responsible for altered anti-inflammatory induction in *Papio* and hominoids. It seems unlikely that minimal IL-1RN induction by *Papio* reflects differences between hominoid and *Papio* in the levels of IL-1RN-inducing IL-1β, as *Papio* induces significantly more IL-1β than *Homo*. This relationship is reflected in the IL-1β/IL-1RN ratio, where *Papio* and *Pan* induce proportionally much more IL-1β than *Homo*. *Papio* minimally induces IL-1RN, which implies that IL-1β responses

are modulated differently in this species and may contribute to the noted difficulty in stimulating fever responses in *Papio* (106, 270). The divergent IL-1RN induction may also reflect a difference in the ability of *Papio* to trigger the IL-1R pathway via IL-1β. Weak IL-1RN induction could be the outcome of several steps leading to IL-1β/IL-1R complex binding, including lowered IL-1β/IL-1R binding affinity or inefficient IL-1β expression (106). Taken together these results suggest that control of the IL-1R pathway has evolved differently in these primates since *Papio* and hominoids diverged.

4.3 *Homo* does not appear to be hyper-reactive to infection, compared to other primates.

In contrast to several studies examining specific blood leukocyte sub-types at later time points, the results of this study are not in strict agreement with findings that *Homo* leukocytes are hyper-reactive and trigger pro-inflammatory and apoptotic genes more readily compared to *Pan* during the early hours of stimulus (4-6 hours) (21) (19). The data presented here indicates that when total blood leukocyte population is considered, *Homo* and *Pan* initial responses to stimulus are typically very similar. Given that previous studies have examined isolated monocytes or lymphocytes, discrepancies in results may be interpreted as the outcome of slightly later time points, isolating and culturing of cells, the examination of specific cell subtypes and different gene targets to measure immune response. It is likely that the comparable *Homo* and *Pan* cytokine and chemokine responses found in this study are the net effect of cytokine/chemokine induction by different cell subtypes. It is possible that hominoid leukocyte subtypes have undergone some evolutionary convergence to produce similar early innate immune response in whole blood. It seems more likely, however, that isolated leukocyte subtypes cultured and stimulated apart for many hours respond to stimulus differently outside of the context of leukocyte-protein and cell-cell interactions found *ex*

vivo and *in vivo*. When examined in an uncultured, whole blood model, *Homo* and *Pan* early cytokine and chemokine induction are very similar to one another.

4.4 Cytokine and chemokine responses suggest hominoid and cercopithecoid phagocyte "behaviour" has diverged

Total blood leukocyte cytokine and chemokine responses to bacterial PAMPs, however, suggest that hominoid and *Papio* early monocyte or neutrophil function has diverged. While monocytes primarily express most of the cytokines and chemokines discussed here, neutrophils express these genes as well and outnumber monocytes in catarrhine blood by approximately 14 fold (**Appendix G**). The combined divergence of three genes primarily expressed by monocytes (i.e. CXCL2, IL-6, IL-10) broadly agrees with hominoid and *Papio* phylogenetic split and suggests that catarrhine monocytes have evolved different early innate immune responses.

4.5 Early immune responses tend to not agree putative primate evolutionary environments

One of the goals of this dissertation was to assess a possible relationship between putative primate evolutionary landscapes and early innate immune responses. It has been posited that primate evolutionary geographic distribution and ecology has had a significant impact on past pathogen exposure and affected the evolution of the primate immune system (36, 40, 45, 378-380). Support for this hypothesis has been found in differences in human and non-human primate susceptibility to pathogens assumed native to particular primate evolutionary environments. *Papio,* for example, is resistant to the savannah-based pathogen the *Trypanosoma*, the causative agent of sleeping sickness. Presumably less exposed to this pathogen*, Homo* and *Pan* are highly susceptible to infection and

experience 90% mortality rate when untreated (266, 381). Approximately 10% of the Eurasian population has the CCR5delta32 allele that confers resistance to HIV-1 infection, and may have been under selection by *Yersinia pestis* several hundred years before the emergence of HIV (140, 382, 383). Multiple studies have tried to connect pathogen prevalence or selection on immune system genes to specific changes in diet, geographic distribution, group size/composition, and sexual promiscuity with varying degrees of success (36, 37, 39, 40, 43, 45). A fundamental difficulty with any examination of how evolutionary past may have affected immune system evolution is that it inevitably rests on assumptions that current host immune responses are representative of ancient host and pathogen biological interactions. With that caveat mind, the basic assumptions of this study were that early innate immune responses could be subject to evolutionary selection by pathogens and that a strong determinate of past exposure to a particular pathogen is evolutionary landscape.

To examine a possible relationship between disease susceptibility and landscape-based pathogen exposure, *Homo*, *Pan* and *Papio* were stimulated with PAMPs representing pathogens broadly distributed in primate evolutionary environments [LPS *E. coli*, Pam3CSK4 (gram-positive bacteria mimetic)] and pathogens thought to have recently emerged in specific environments associated with hominin evolution (LM from *M. smegmatis*: savannah environments, animal domestication; PA from *B. anthracis*: savannah environments, animal domestication; LcrV from *Y. pestis, Homo* environments in Eurasia) (36, 37, 219, 303-309). This study has found that catarrhine early cytokine and chemokine responses to a wide variety of bacterial pathogens tend to agree with the phylogenetic relationships of primate species. In functional studies of complex immune pathways, such as TLR pathways, pathogen-selected differences that diverge on species lines are effectively undetectable from functional differences that are the outcome of drift. This study is

limited to examining differences that disagree with phylogeny or agree with animal ecology to draw conclusions about pathogen-mediated selection on function.

Surprisingly, pathogens specific to putative primate evolutionary environments did not stimulate a pattern of early gene induction that clearly ties putative landscape to early innate immune response. Most early cytokine and chemokine responses of *Homo*, *Pan* and *Papio* were congruent with primate phylogeny. Hominoid responses to PAMPs were generally similar to one another and different from *Papio* responses. *Homo* low induction of IL-1β by LMMS and strong induction of IL-6 by LcrV, compared to *Pan* and *Papio* were the only responses triggered by landscape-specific PAMPs that were not congruent with primate evolutionary relationships. Other responses that do not agree with primate phylogeny appear to be specific to PAMPS from presumably more broadly distributed pathogens (i.e. IL-6 Pam3CSK4/gram-positive – specific bacteria response in hominoids; *Papio* IL-10 and *Homo* TNFα TLR4/LY96 and TLR2/1 – detected pathogen specific responses) as well as a pattern of consistently weak IL-1β induction in *Homo*.

Several authors have suggested that Papionin evolution and ecology are appropriate models for human evolution (384, 385). The broader implication of finding that *Papio* tends to mount highly divergent early cytokine and chemokines responses to stimulus compared to hominoids, is that *Papio* species are not accurate primates models of the evolution of the human early innate immune response. That *Papio* does not recapitulate *Homo* responses to bacterial PAMPs and LMMS, in particular, suggests that a model of Pliocene human and Papionin evolution that assumes similar ecology and pathogen exposure in African environments is inaccurate, or less important than recent lineage divergence when predicting early innate immune response in catarrhines (36, 384, 385).

One factor influencing PAMP-specific responses in catarrhines may be host-microflora co-evolution. Native bacterial microflora may differ considerably between extant catarrhine species (33, 35, 41, 42). Large proportions of gram-positive bacteria, primarily *Lactobacillus* species, for example, characterize the normal vaginal flora of *Homo* and *Pan* (35) [reviewed in (386)]. *Lactobacillus,* however, appears to be virtually absent and proportions of Gram-negative bacteria representative of pathological conditions in *Homo* are present in the normal vaginal flora of cercopithecoid species (33, 41). Such differences in microflora are associated with differences in innate immune mechanisms of these species, such as higher vaginal pH in cercopithecoids (pH 6-8) and than *Homo* (pH<5) (387, 388). As commensal microflora affects immune regulation and infection susceptibility and progression it may influence pathogen-driven selection on mechanisms such as TLRs [reviewed in (389)].

While it appears that catarrhine species have evolved to mount specific cytokine responses to some bacterial ligands, it may not be possible to define responsible factors. Reconstruction of past factors contributing to the evolution of the immune system is complicated not just by genetic drift and problems inherent to reconstructing ancestral pathogens and hosts, but also by the pleiotropic nature of many immune system components. CXC chemokines, for example, play an important role in other biological functions, such as forebrain development [reviewed in (390) and (391)]. Various cytokines have been associated with bone and non-mineralized tissue development [i.e. TGFβ-1: cartilage development (392); CCL2: CNS architectural development (393)]. Some cytokine genes, such as TNFα, are located in close proximity to immune genes under strong selection or subject to frequent rearrangement. TNFα is located in the Major Histocompatiblity Complex region of human chromosome 6. As such, its non-core regulatory regions are highly variable and subject to increased mutation, which may affect transcription (394, 395).

The identification of factors influencing the evolution of cytokine/chemokine induction is further complicated by their possible co-evolution with other proteins. A comparison of the distance matrices of primate TLR and cytokine/chemokine gene trees for signatures of co-evolution using the Mirrortree server suggests that at least one of the cytokines/chemokine genes in this study is coevolving in concert with a TLR (**Table 4.1**). A Pearson correlation coefficient of 0.979 strongly suggests that primate CXCL2 and TLR1 have co-evolved. While this signature of co-evolution may be the outcome of positive selection for particular protein functions, it is possible that CXCL2 and TLR1 have co-evolved due to their proximity in the primate genome. In *Homo*, CXCL2 and TLR1 are located at 4q21 and 4p14, approximately 35.7 Mbp apart and are a similar distance apart in other haplorhine genomes (GRC37:hg19, MMUL_1, WUSC 3.2/calJac3). Given that many chemokine genes are located in clusters on chromosomes it is possible that pathogen-mediated selection for one protein phenotype has led to selection of expression and structural phenotypes of many other cytokines and chemokines due to gene promixity (367, 396, 397). Such genetic linkage can confuse attempts to identify other factors influencing the evolution of gene expression.

	TLR1	TLR2	TLR4
TLR1			
TLR2	0.682		
TLR4	0.269	0.718	
IL-8/ CXCL8	0.879	0.142	0.299
CXCL2	**0.979**	0.326	0.260
CCL3	0.865	0.108	0.239
IL-1β	0.571	0.688	0.669
TNFα	0.547	0.293	0.541
IL-6	0.944	0.522	0.319
IL-1RN	-0.103	-0.124	-0.097
IL-10	0.929	0.419	0.438
GAPDH	0.558	-0.053	0.112
ACTB	-0.103	-0.113	-0.217
B2M	0.119	0.002	0.231

Table 4.1 Mirrotree analysis of TLR and cytokine/chemokine amino acid sequence co-evolution.
Values represent Pearson correlation coefficient of comparisons of gene tree distance matrices. Values over 0.95 indicates strong similarity between trees and suggests a pair of proteins are co-evolving.

4.6 Implications for severe infections in primates

4.6.1 Hominoid early cytokine and chemokine induction may help explain severe sepsis susceptibility

Cercopithecoid resistance to severe gram-negative bacterial sepsis and hominoid high susceptibility is the most important bacterial disease disparity thus far noted between primate species (10, 11, 106, 270). Severe sepsis and septic shock are the 10th overall cause of death in the U.S.,

effecting approximately 700 000 and killing approximately 35 000 people annually (398). Sepsis triggered by gram-negative bacteria represents approximately 50% of total annual sepsis cases (399). *Homo* and *Pan* sensitivity to LPS and gram-negative sepsis appears to be a derived condition, with cercopithecoids and most other tested mammals requiring high doses of LPS or gram-negative bacteria to induce minimal symptoms (10, 11, 15, 106, 269, 270). *Papio*, for example, requires at least 0.1mg/kg LPS to evoke symptoms characteristic of *Homo* sepsis, whereas *Homo* only requires 2-4 ng/kg (10, 11, 106, 400, 401). Severe sepsis and septic shock are the outcomes of overt and unregulated cytokine and chemokine induction in response to stimulus. The systemic cytokine/chemokine response that follows leads to fever, disruption of endothelial tissues, increased vascular permeability, thrombosis and organ dysfunction, which further contribute to cytokine and chemokine induction [reviewed in (128)]. Mortality is tightly correlated with the induction of cytokines and chemokines during the early hours of disease [(64) and reviewed in (128) and (272)]. An important aspect of understanding this potential "evolutionary watershed" between cercopithecoids and hominoids is an examination of the strength of cytokine and chemokine responses to stimulus (12).

The harmful pro-inflammatory response that triggers severe sepsis is associated with the *de novo* production of two cytokines that *Homo* and *Pan* appears to induce differently than *Papio* regardless of agonist, IL-8/CXCL8 and IL-6 [reviewed in (128)]. It is interesting that hominoid induction of these proteins is very high, while *Papio* induction is very low. High levels of these proteins released during early infection are positively correlated with increased organ dysfunction and mortality (65, 66, 110, 402). When IL-8/CXCL8 levels are suppressed, host survival increases (403-405). Strong *Homo* febrile and pro-inflammatory responses after LPS stimulation are not well explained by induction of the IL-1β or TNFα as *Papio* induction of these cyokines tends to be

stronger than or similar to *Homo* (64, 406, 407). Fever in *Homo* may be due to very strong induction of IL-6 compared to the very weak induction of IL-6 in *Papio*. IL-6 is highly pyrogenic and induces activation of JAK family tyrosine kinases, stimulating the induction of other pro-inflammatory cytokines [reviewed in (408). IL-10 is known to have a protective effect in sepsis and downregulates multiple pyrogenic cytokines, including IL-1β, IL-6, TNFα and IL-8/CXCL8 [reviewed in (409) and (410)]. As *Homo* and *Papio* induce similar IL-10 levels in response to LPS it seems unlikely that early IL-10 induction is responsible for gram-negative severe sepsis susceptibility in *Homo* and resistance in *Papio*.

Induction of cytokines and chemokines that regulate neutrophils may affect *Papio* resistance and hominoid susceptibility to gram-negative bacterial sepsis. Under ideal conditions, neutrophils sequester and eliminate bacterial infections. When host immune responses are exuberant or a pathogen is very virulent, neutrophil activities can become deleterious (410). As such, neutrophils are implicated in the initiation of severe sepsis and much of the associated tissue injury (404, 411, 412). Hominoids strongly express IL-8/CXCL8 and CCL3 both of which traffic neutrophils to sites of infection. Along with arresting neutrophils for transmigration to infection sites, increased IL-8/CXCL8 expression has an anti-apoptotic affect on these cells (413-415). In severe sepsis failure of neutrophils to undergo apoptosis contributes to tissue injury (413, 416). Dysregulation of neutrophil apoptosis can be counteracted by the expression of IL-10, a cytokine that *Homo* and *Papio* induce at similar levels during the early immune responses to LPS (417). *Papio* very lowly induces IL-8/CXCL8 and CCL3, while these chemokines are strongly induced in *Homo*. Low *Papio* IL-8/CXCL8 induction suggests less IL-8/CXCL8 is available to arrest neutrophil rolling and a higher ratio of IL-10 to IL-8/CXCL8 is available to counteract the anti-apoptotic effect of IL-8/CXCL8 in this species. As opposed to hominoids, *Papio* strongly induces CXCL2. This suggests that neutrophil

endothelial rolling may be increased in *Papio,* while the potential deleterious affects of increased neutrophil transmigration to infection sites and extended cell life span may be limited.

Whatever the reason for LPS-sensitivity in *Homo* and *Pan*, it seems likely that hominoid sensitivity and cercopithecoid blunted responses to LPS are due to multiple inter-species differences in LPS-detecting pathways. LPS-sensitivity in *Homo* and *Pan* may be related induced TLR expression rather than baseline TLR expression. A previous examination of TLR4 expression by flow cytometry and suggests that though *Papio* and *Homo* baseline expression on endothelial cells does not significantly differ, *Homo* very quickly expresses more TLR4 than *Papio* after LPS stimulation (15). While early pro-inflammatory cytokine and chemokine may affect sepsis susceptibility, it is possible that activation of transcription factors other than NF-κB are contributing factors. NFκBIA is the inhibitor of NF-κB and is immediately and proportionally transcribed in response to NF-κB transcription (418, 419). In the current study NFκBIA induction was found to be very low and not significantly different between species or agonists (**see Chapter 4**). Minimal and consistent NFκBIA induction suggests that differences in cytokine and chemokine induction at the 90 minute timepoint are regulated by transcription factors other than NF-κB. A possible contributing factor to reduced sensitivity to LPS noted in *Papio* is the significant residue changes in TLR4 complex in the cytoplasmic domain as well as near the extracellular MD-2 and LPS binding sites noted in this study and elsewhere (273). The loss of 13 amino acids at carboxyl terminus (distal cytoplasmic domain) of *Papio* TLR4 has been previously highlighted as potentially contributing to decreased LPS-sensitivity in this species.

4.6.2 Molecular basis for differences in TLR2-detected pathogen based diseases between cercopithecoids and hominoids.

There are only a few case studies and field observations of mycobacterial disease in non-human primates, and no comparative studies of gram-positive bacterial diseases in African catarrhines. Without controlled comparative studies it is difficult to interpret differences between *Papio* and hominoid cytokine and chemokine induction in the context of disease. The results of this study, however, suggest interesting research avenues to further explore catarrhine susceptibility to TLR2-detected bacterial pathogens. Strong induction of IL-10 by *Papio*, compared to the minimal induction by hominoids, may enhance mycobacerial infection progression in *Papio*. IL-10 levels play an important role in mycobacterial infection progression, including dampening T cell responses and blocking phagosome maturation in *Mycobacterium* -infected macrophages [reviewed in (409)]. Both of these activities promote the growth of *Mycobacterium* colonies, as the pathogen can escape both adaptive and innate immune strategies to sequester and kill it. Granulomas typical of established mycobacterial infections tend to produce high levels of IL-10, which seems to further contribute to the survival of the pathogen in the host [reviewed in (420)].

The divergence of *Papio* and hominoid chemokine induction also has implications for mycobacterial infections. Low induction of *Papio* IL-8/CXCL8 may increase species susceptibility to infection, as decreased IL-8/CXCL8 expression is associated with increased susceptibility to tuberculosis and intracellular proliferation of *Mycobacterium* (421, 422). IL-8/CXCL8 can reduce *Mycobacterium* survival, in part, by enhancing the killing of the pathogen by neutrophils. Similarly, CXCL2 is strongly induced in *Papio* while *Homo* and *Pan* weakly induce this chemokine. CXCL2 was produced rapidly after *Mycobacterium tuberculosis* infection, however the role of CXCL2 in

Mycobacterium pathogenesis is not well investigated and may be associated with the strength of neutrophil recruitment (423). Taken together, catarrhine early anti-inflammatory cytokine and chemokine responses to TLR2/1 PAMPS suggest a molecular basis for disparate disease outcomes opportunistically noted in hominoid and cercopithecoid mycobacterial infections.

The induction patterns of two pro-inflammatory cytokines, TNFα and IL-1β are difficult to interpret in the context of susceptibility to TLR2/1 -detected pathogen infection. Both over and/or underexpression of these cytokines is associated with increased susceptibility to mycobacterial and gram-positive bacterial infection in *Homo*. Stronger *Papio* TNFα induction may play a role in susceptibility to mycobacterial infections as heightened TNFα expression has been associated with increased *M. tuberculosis* colony growth (424). That low TNFα levels are also associated with susceptibility to *M. tuberculosis* infection makes comparisons between induction level of this cytokine and disease state difficult (425, 426). Similarly, low IL-1β expression is associated with increased susceptibility to *Staphylococcus aureus,* and *Mycobacterium tuberculosis* (427, 428). These results suggest that *Homo*'s low IL-1β induction increases this species susceptibility to both of these infections.

CHAPTER 5:
SUMMARY AND CONCLUSIONS

5.1 Summary

The divergence of primate immune systems is a subject of both evolutionary and clinical importance. The rapid evolution of immune system genes in concert with considerable portion of the mammalian genome dedicated to the immune system suggests that host-pathogen interactions have had a profound affect on the evolution of mammals (4-9). With multiple pathogens suggested to have shaped the immune system and populations of primates, pathogen-mediated selection has been proposed as a major factor influencing the evolution of primate species (1, 2, 36, 37, 39, 40, 42, 45, 378, 381). That African catarrhine species appear to be differently susceptible to major bacterial-mediated infectious diseases that are characterized by dysfunctional inflammation (gram-negative bacterial sepsis, mycobacterial diseases*, Nesseiria gonorrhea*) is congruent with this hypothesis and suggests that innate immunity of some catarrhine species has diverged (10-12, 15, 275, 294, 298). One possible explanation for disparate immune responses between such closely related species is the evolution of the primate early innate immune response with particular pathogens. To better understand why some catarrhine species appear to have evolved differing susceptibility to bacterial pathogens, it is important to examine the early innate immune response of multiple primate species for functional differences. This dissertation tested the early innate immune responses of three African catarrhine species (*Homo*, *Pan* and *Papio*) to a broad range of bacterial pathogens detectable by major mediators of innate immunity, Toll-like receptors (TLRs). This dissertation investigates if 1) African catarrhine early innate immune responses differ and 2) those responses can be correlated to observations of inter-species differences in disease progression and evolutionary landscapes.

Through stimulation of *Homo*, *Pan*, and *Papio* blood with TLR2 and TLR4 – detected PAMPS, this dissertation provides evidence that

1) catarrhine blood leukocyte early cytokine and chemokine responses to bacterial pathogens differ
2) generally, catarrhine early innate immune responses agree with primate phylogeny
3) chemokine responses tend to be species-specific and diverged 23-29 million years ago
4) cytokine responses show some pathogen-specificity
5) early innate immune responses do not appear to correlate with putative historical distribution of pathogens and primate evolutionary environments

In summary, *Homo*, *Pan* and *Papio* generally mount early innate immune responses to bacterial pathogens that agree with species phylogeny, and may have acquired specific adaptations to TLR2/1 and TLR4-detected pathogens. With a few PAMP-specific exceptions (i.e. TNFα, IL-10, IL-6), most cytokine and chemokine responses appear species-specific and suggest that TLR-mediated immunity significantly diverged in catarrhine primates over the last 23-29 million years. An interesting exception to this pattern is the low induction of *Homo* IL-1β, compared to the strong induction in *Pan* and *Papio,* which suggested a divergence of *Homo* IL-1β responses from those of other catarrhines over the last 6-7 million years. IL-1β induction pattern is the only *Homo* – specific pattern that breaks with species phylogeny and is consistent across all stimuli. In total, the complex combination of PAMP- and species-specific cytokine and chemokine induction suggests that TLR-mediated immunity has diverged multiple times in catarrhine primates over the last 23-29 million years.

5.2 Contributions

This is the first evolutionary study of African catarrhine early innate immune responses to gram-negative bacterial, gram-positive bacterial and mycobacterial pathogens. Most studies of primate immune system evolution have tried to address this subject by completing genetic sequence analysis. Examinations of selection and variability can offer some insight into the evolutionary stability of immune gene sequences, but cannot determine if immune system gene function has actually evolved. Most functional studies of primate immunity have focused on responses to viral pathogens. This is, to my knowledge, the first study that has examined *Homo*, *Pan* and *Papio* early innate immune responses to a broad range of bacterial pathogens. Similarly, this study is unique because it examines the role of both TLR-detected pathogens and pathogens specific to putative evolutionary primate environments in the evolution of immune system function. The results presented here suggest that while early innate immune responses tend to agree with species phylogeny and not putative landscape, some responses appear to be pathogen or PAMP-specific. These results suggest that TLR2/1 and TLR4 -detected bacterial pathogens have affected primate evolution.

This dissertation has implications for both disease susceptibility and the evolution of complex immune strategies. The divergence of *Homo*/*Pan* and *Papio* chemokine expression suggests that the molecular basis of neutrophil migration differs between hominoids and cercopithecoids. Cytokine and chemokine induction patterns also suggest that hominoid and cercopithecoid phagocytic cells respond to stimulus differently. The results of this research suggest that early inflammatory responses are modulated differently in hominoids and in cercopithecoids and may shed light on why these animals appear to respond to severe bacterial infections differently. In

particular, divergent *Papio* and *Homo/Pan* chemokine responses may help explain extreme sensitivity of hominoids to gram-negative bacterial severe sepsis and the comparative resistance of cercopithecoids. These results also suggest that hominoids and cercopithecoids initially respond to some TLR2/1 -detected pathogens differently and may provide a molecular basis for further investigation into susceptibility to *Mycobacterium* and gram-positive bacterial species. Ultimately, these results provide new information on cytokine and chemokine induction that can assist in characterizing the evolution of African catarrhine early innate immune responses to infectious pathogens.

5.3 Future directions

The data presented in this dissertation provides several new avenues of study into the evolution of primates and the innate immune system. While this dissertation demonstrates inter-species differences in cytokine and chemokine responses to bacterial pathogens, this data is preliminary. To better understand how primate responses to bacterial pathogens have evolved on phylogenetic lines, more information on the responses of other species is necessary. A logical next step in this study is to expand the number of species under investigation. This is an avenue I intend to pursue. Considerably more commercially produced, high quality PAMPs have become available since this research was completed. There is now an opportunity to include a broader range of landscape-specific pathogens in such a study.

Further investigation into mechanisms regulating different chemokine induction in *Homo*, *Pan* and *Papio* (i.e. promoters, enhancers, transcription factors) may provide insight into why the induction of these genes differs between these species. While this study found that close

evolutionary relatives tend to share highly similar promoter regions, the incomplete nature of the *Papio hamadryas* (Pham 1.0) genome and regions of the *Macaca mulatta* (MMUL_1) genome prevented the prediction and analysis of other important and typically more distant regulatory regions (i.e. enhancers and silencers) which can affect gene transcription. With further refinement of these genomes, initial investigations of inter-species differences in cytokine and chemokine regulatory regions will be eased. In the view of this author, potential inter-species differences in cell trafficking suggest the evolutionary divergence of complex cellular immune strategies. Such differences may also help explain disparate susceptibilities to infectious diseases in African catarrhines. As such, *ex vivo* or model *in vivo* examinations of potential differences in cell migration would be a very interesting research avenue to pursue. Given the expense and ethical considerations for accessing non-human primates for cell migration studies, additional *in vivo* species information relevant to receptor expression or endogenous ligands would provide a native biological context for *ex vivo* model studies.

The study of primate innate immune function and its evolution provides exciting opportunities for better understanding of pathogen-host interactions, disparities in primate disease susceptibility and the affect of pathogens on the evolution of primates. Further investigation of the TLR pathways and cytokine and chemokine induction mechanisms implicated in this study may provide great insight into the evolution of the primate innate immune system and, in particular, the evolution of human susceptibility to severe bacterial infections.

APPENDIX A

Non-human primates used in this study

Full identification information is provided here for all non-human primates included in this study (TBMRI = Texas Biomedical Research Institute, formerly Southwest Foundation).

Pan troglodytes

Research Center	Animal no./Name	Global Stud no.	Sex	Date of Birth	Dam (global stud no.)	Sire (global stud no.)	Collection Date
Yerkes	C0580/Brodie	5005	Female	Jan 10, 1984	389	492	Nov 23, 2009
Yerkes	C0389/Rogger	2363	Male	Jan 1, 1969	N/A	N/A	Mar 31, 2010
Yerkes	C0583/Travis	5570	Male	Feb 8, 1989	Cissie 526	Ripper 457	Apr 5, 2010
TBMRI	4x0526Sean	5824	Male	Mar 16, 1992	N/A	N/A	Sept 2, 2010

***Papio* sp. (*cynocephalus*)**

Research Center	Animal ID	Date of Birth	Sex	Collection Date
TBM	9012	Aug 6, 1989	Male	Oct 14, 2009
TBM	17969	Feb 27, 2002	Male	Feb 17, 2010
TBM	15588	Aug 19, 1999	Male	Mar 17, 2010
TBM	15296	Mar 12, 1999	Male	Apr 20, 2010
TBM	18325	Aug 5, 2002	Male	Aug 3, 2010
TBM	19240	Apr 20, 2003	Male	Sept 30, 2010

APPENDIX B

Internal Review Board approval

Granted by the International Board of the City College of New York and registered with the City University of New York, Graduate School and University Center.

Institutional Review Board
160 Convent Avenue
Shepard Hall, Room S-16
New York, New York 10031
IRB@ccny.cuny.edu

December 22, 2009

Jessica Brinkworth
Sophie Davis/ Microbiology & Immunology

RE: **09-0073C Comparison of the Innate Immune Response of Humans and Non-Human Primates**

Dear Jessica Brinkworth:

The City College IRB has approved the above study involving humans as research subjects. This study was Approved - Expedited Category: 2 - based on 45CFR46.

IRB Number: **09-0073C** This number is a City College IRB number that should be used on all consent forms and correspondence.

Approval Date: **December 21, 2009**
Expiration Date: **December 20, 2010**

THIS APPROVAL IS FOR A PERIOD OF ONE-YEAR OR LESS. YOU SHOULD RECEIVE A COURTESY RENEWAL NOTICE BEFORE THE EXPIRATION OF THIS PROJECT'S APPROVAL. HOWEVER, IT IS YOUR RESPONSIBILITY TO INSURE THAT AN APPLICATION FOR CONTINUING REVIEW APPROVAL HAS BEEN SUBMITTED BEFORE THE EXPIRATION DATE NOTED ABOVE. IF YOU DO NOT RECEIVE APPROVAL BEFORE THE EXPIRATION DATE, ALL STUDY ACTIVITIES MUST STOP UNTIL YOU RECEIVE A NEW APPROVAL LETTER. THERE WILL BE NO EXCEPTIONS. IN ADDITION, YOU ARE REQUIRED TO SUBMIT A FINAL REPORT OF FINDINGS AT THE COMPLETION OF THE PROJECT.

Consent Form: All research subjects must use the approved and stamped consent form. You are responsible for maintaining signed consent forms for each research subject for a period of at least three years after study completion.

Mandatory Reporting to the IRB: The principal investigator must report, within five business days, any serious problem, adverse effect, or outcome that occurs with frequency or degree of severity greater than that anticipated. In addition, the principal investigator must report any event or series of events that prompt the temporary or permanent suspension of a research project involving human subjects or any deviations from the approved protocol.

Amendments/Modifications: All amendments/modifications of protocols involving human subjects must have prior IRB approval, except those involving the prevention of

IRB#: 09-0073C **Consent Version Date: 12/17/10**

Institutional Review Board
The City College of New York

The City University of New York

Shepard Hall, 108B
160 Convent Ave
New York, NY 10031
212-650-7902 (IRB Office)
http://www.ccny.cuny.edu/irb/

Jessica Brinkworth – Principal Investigator

SOPHIE DAVIS SCHOOL OF BIOMEDICAL EDUCATION,
THE CITY COLLEGE OF NEW YORK

CONSENT FORM

Title of study: "Comparison of the innate immune response of humans and nonhuman primates"

Purpose of the Study:

You are invited to be in a research study that will analyze the response of blood cells, removed from normal human subjects and placed in test tubes, to various products of infectious agents, and compare these responses to those of primates. **You were selected as a possible participant because you fulfill the criteria of a normal, healthy subject and you have indicated that you do not have any acute or chronic illnesses, such as long term infections, bleeding disorders or malignancies.** We ask that you read this form and ask any questions you may have before agreeing to be in the study.

This study is being conducted by: Jessica Brinkworth, Graduate School of the City University of New York, Dept. of Anthropology; co-PI, Jack Silver, Ph.D., faculty advisor, Sanna Goyert, Ph.D., Sophie Davis School of Biomedical Education, Department of Microbiology and Immunology.

Background Information

You are being invited to participate in this study because you can serve as a normal subject as described below. Blood cells tak normal healthy adults and placed in test tubes respond to components of bacteria, parasites or viruses and become activated and variety of proteins, some of which are important in immune responses to microbes. Our interest is to determine if there are diffe responses of primates and humans to some of these microbial products. For this study, ten ml (2 teaspoons) of blood will be dr your arm by venipuncture, placed in a test tube, and used to measure the effects of various microbial components on protein production by blood cells. You, personally, will not be exposed to any bacteria, parasites or viruses. Results obtained with human blood cells will be compared to those obtained from blood cells obtained from chimpanzees.

 Subject's Initials: _____ Date: _____

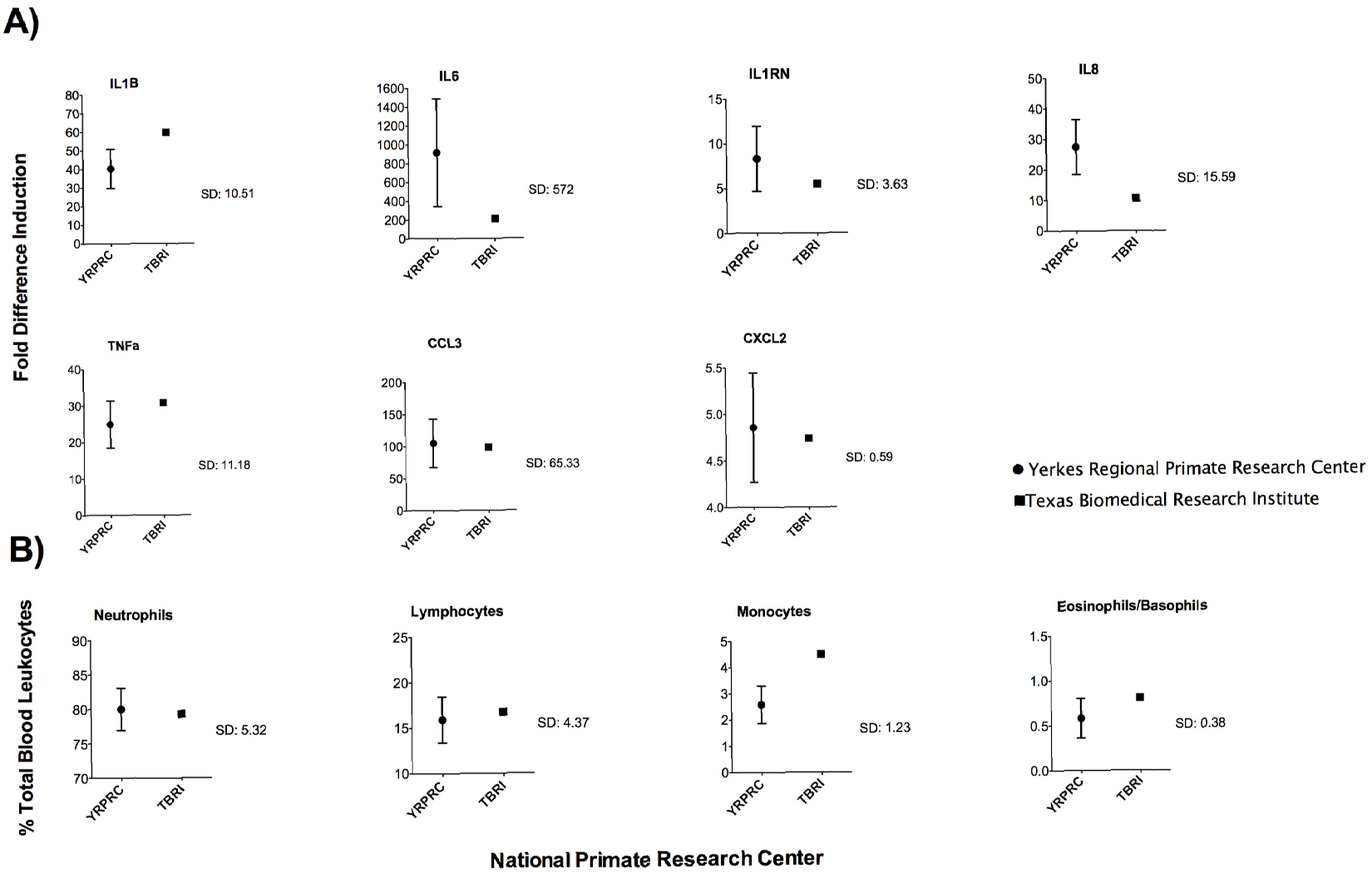
(IRB Official Use Only)
This Consent Document is approved for use by The City College New York's Institutional Review Board (IRB).
Only the IRB-stamped approved form may be used.
Approved: From: 1/09/2011 *To:* 1/08/2012
The study expiration date applies for this form
Template rev. date: 3/23/2011

CCNY IRB APPROVED

APPENDIX C

Comparison of blood leukocyte proportions and response to stimulation for Pan troglodytes individuals housed at different primate research facilities.

To examine if life history might affect early innate immune responses to TLR-detected PAMPs, leukocyte proportions and cytokine and chemokine responses to LPS of *P. troglodytes* individuals from different primate research centers were compared A) Blood leukocyte subtype proportions for *P. troglodytes* housed at different primate research facilities are very similar. Samples were acquired from Yerkes Regional Primate Research Center (YRPRC, n=3) and Texas Biomedical Research Institute (TBRI, n=1). Blood leukocytes were isolated by hypotonic shock and differentially strained using Wright-Geimsa staining protocol. TBRI *P. troglodytes* blood leukocyte subtype proportions fall within the range or within 2 standard deviations of the YRPRC values. B) Transcriptional responses of *P. troglodytes* housed at different primate research facilities are very similar. Samples were acquired from Yerkes Regional Primate Research Center (YRPRC, n=3) and Texas Biomedical Research Institute (TBRI, n=1). TBRI *P. troglodytes* responses fell within the range or within 2 standard deviations of the YRPRC values.


A)
Fold Difference Induction
IL1B
SD: 10.51
IL6
SD: 572
IL1RN
SD: 3.63
IL8
SD: 15.59
TNFa
SD: 11.18
CCL3
SD: 65.33
CXCL2
SD: 0.59
YRPRC
TBRI
Yerkes Regional Primate Research Center
Texas Biomedical Research Institute
B)
% Total Blood Leukocytes
Neutrophils
SD: 5.32
Lymphocytes
SD: 4.37
Monocytes
SD: 1.23
Eosinophils/Basophils
SD: 0.38
National Primate Research Center

APPENDIX D

Red Blood Cell Lysis protocol, Manufacturer RNA isolation and cDNA synthesis protocols

* Please note that many of the reagents supplied in these molecular biology kits are described by Qiagen as proprietary. Therefore, specific composition and concentrations of these reagents are not available, and reagents names are limited to the names Qiagen has supplied.

Red Blood Cell Lysis Protocol

1) aliquot 200 ul of stimulated or fresh blood into a microcentrifuge tube
2) microcentrifuge 5 rpm for 5 minutes
3) discard supernatant (plasma)
4) add 900 ul of cell culture grade, endotoxin-free, sterile water to each tube and mix immediately, pipetting up and down for exactly 20 seconds
5) immediately add 100 ul of 10x endotoxin-free PBS to the tube to make the solution isotonic
6) microcentrifuge at 4 rpm for 5 minutes
7) discard supernatant, keep leukocyte pellet
8) repeat in 200 ul aliquots until all of sample has been processed
9) combine leukocyte pellets
10) immediately proceed to RNA isolation, or slide fixing protocol

* **Qiagen RNeasy Minikit** (Qiagen catalogue # 74106)

Total RNA Isolation protocol

Loosen blood leukocyte pellet by flicking tube

Cell lysis (with GITC-containing buffer)
1) add 350 ul of Buffer RLT (with 10 ul of β-Mercaptoethanol per 1 ml RLT)
2) pipette to mix

Sample homogenization (shear genomic DNA)
3) pipette lysate into Qiashredder spin column (in 2 ml collection tub) (Cat #79656)
4) microcentrifuge at 2 minutes at maximum speed (13 000 rpm)

Bind RNA to column membrane
5) add 350 ul of 70% ethanol to lysate
6) mix well by pipetting
7) apply 700 ul of sample (including precipitate) to RNeasy mini spin column (placed in 2 ml collection tube)
8) microcentrifuge for 15 seconds at 10 000 rpm
9) discard flow through

Wash step (3x)
10) add 700 ul of Buffer RW1 to column
11) microcentrifuge for 15 seconds at 10 000 rpm

12) transfer column to new 2ml collection tube
13) pipette 500 ul Buffer RPE onto column
14) microcentrifuge for 2 minutes at 10 000 rpm (to dry silica membrane)

Elute RNA
15) transfer column to new 1.5 ml collection tube
16) pipet 50 ul of RNAse-free water directly onto the column membrane
17) microcentrifuge for 1 min at 10 000 rpm
18) repeat step 18 using elute in same collection tube
19) proceed to DNAse digestion protocol

DNAse Digestion protocol (Qiagen RNAase-free DNAse I Catalogue # 79254)

DNAse digestion
1) combine 10 ul Buffer RDD to 87.5 ul RNA solution/elute
2) add 2.5 ul DNAse I stock solution
3) incubate for 10 minutes at 20-25 °C

Bind RNA to membrane
4) add 350 ul Buffer RLT. Mix well
5) add 250 ul of 96-100% ethanol to diluted RNA
6) mix well by pipetting
7) apply up to 700 ul of sample (including precipitate) to RNEasy mini spin column (placed in 2ml collection tube)
8) microcentrifuge for 15 seconds at 10 000 rpm
9) discard flow through (do not let column touch flow through)

Wash (3x)
10) add 500 ul Buffer RPE to column
11) microcentrifuge for 15 seconds at 10 000 rpm
12) transfer column to new 2ml collection tube
13) pipette 500 ul Buffer RPE onto column
14) microcentrifuge for 2 minutes at 10 000 rpm (to dry silica membrane)
15) transfer column to new 1.5 ml collection tube

Elute RNA
16) pipet 50 ul of RNAse-free water directly onto the column membrane
17) microcentrifuge for 1 min at 10 000 rpm
18) repeat step 18 using elute in same collection tube
19) proceed to cDNA synthesis or freeze at -80°C

* **Qiagen Quantitect Reverse Transcriptase** (Qiagen catalogue # 205311)

cDNA synthesis protocol
DNAse digestion
1) combine in a microcentrifuge tube on ice

2ul gDNA Wipeout Buffer 7x
11 ul template RNA
1 ul RNAse-free water

2) incubate for 2 mins at 42C
3) immediately place on ice

cDNA synthesis

4) combine in a microcentrifuge tube on ice
   1 ul reverse transcriptase
   4 ul RT Buffer
   14 ul of DNAse reaction
5) flick tube to mix
6) centrifuge for 5 seconds
7) incubate for 15 minutes at 42°C

Inactivation of reverse transcriptase, storage

8) incubate for 3 minutes at 95°C
9) proceed to dilute ten fold, and PCR or store at -80°C

APPENDIX E

Genes, primer sequences and accession numbers

Symbol	Forward Primer	Reverse Primer	NCBI accession numbers	Gene Category
GAPDH *Homo sapiens* *Pan troglodytes* *Papio anubis*	5'-GAGTCAACGGATTTGGTCGT -3'	5'- TTGATTTTGGAGGGATCTCG- 3'	NM_002046.3 XM_001162023.1/XM_001162057.1/XM_001162096.1/XM_508955.2 AY179885.1	Reference
ACTB *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- GGCATCCACGAAACTACCTT -3'	5'- CTTGCTGATCCACATCTGCT -3'	NM_001101.3 NM_001009945.1 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Reference
B2M *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- GCTATCCAGCGTACTCCAAA- 3'	5'- AAGACAAGTCTGAATGCTCC -3'	NM_004048.2 NM_001009066.1 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Reference
IFNγ *Homo sapiens* *Pan trogolodytes* *Papio anubis*	5'- ACTGCCAGGACCCATATGTA - 3'	5'- CCTTGATGGTCTCCACACTC -3'	NM_000619.2 XM_001151968.1 AY234217.1	Interferon
IL-1RN *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- AAGATGTGCCTGTCCTGTGT -3'	5'- GCTCAGGTCAGTGATGTTAA -3'	NM_173842.1/NM_173841.1/ NM_000577.3/ NM_173843.1 XM_001147673.1/XM_001147825.1/ XM_001147954.1/ XM_515698.2 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Cytokine
TNFα *Homo sapiens* *Pan troglodytes* *Papio ursinus*	5'- CAGACCAAGGTCAACCTCCT-3'	5'- AGACTCGGCAAAGTCGAGAT-3'	NM_000594.2 XM_001152827.1 AF019963.1	Cytokine
IL-1β *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- GCTTGGTGATGTCTGGTCCA -3'	5'- GAGGCCCAAGGCCACAGGTA-3'	NM_000576.2 XM_001146864.1/XM_001146939.1/XM_001147075.1/ XM_515697.2 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Cytokine

Genes, primer sequences and accession numbers

Symbol	Forward Primer	Reverse Primer	NCBI accession numbers	Gene Category
IL-6 *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- CTGGCAGAAAACAACCTGAA - 3'	5'- GCAGGAACTGGATCAGGACT – 3'	NM_000600.3 XM_001154396.1/XM_001154511.1/XM_518992.2 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Cytokine
IL-10 *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- CCAAGCCTTGTCTGAGATGA - 3'	5'- GCCTTGCTCTTGTTTTCACA- 3'	NM_000572.2 XM_525040.2 AY796417.1	Cytokine
IL-12A *Homo sapiens* *Pan trogolodytes* *Papio anubis*	5'- GAGTTCAAGACCATGAATGC -3'	5'- TGGCACAGTCTCACTGTTGA -3'	NM_000882.2 XM_001156599.1/ XM_516846.2 NM_001112637.1	Cytokine
CCL2 *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- GCTCATAGCAGCCACCTTCA -3'	5' - GGAATCCTGAACCCACTTCT -3'	NM_002982.3 XM_001174545.1/XM_001174551.1 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Chemokine
CCL3 *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'-TTGCTGTCCTCCTCTGCACC -3'	5'-GCTATGAAATTCTGTGGAAT -3'	NM_002983.2 NM_001034082.1 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Chemokine
CCL5 *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- GCCCTCTGCGCTCCTGCATC -3'	5'- ATACTCCTTGATGTGGGCAC -3'	NM_002985.2 XM_001155572.1 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Chemokine
CXCL2 *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'-TGAACTGCGCTGCCAGTGCT-3'	5'-GACAAGCTTTCTGCCCATTC-3'	NM_002089.3 XM_001155614.1 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Chemokine

Continued, Genes, primer sequences and accession numbers

Symbol	Forward Primer	Reverse Primer	NCBI accession numbers	Gene Category
IL-8/CXCL8 *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- GTTTTGCCAAGGAGTGCTAA -3' …… ……	5'- TGATAAATTTGGGGTGGAAA -3' …… ……	NM_000584.2 XM_001156375.1/ XM_001156432.1/ XM_526587.2 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Chemokine
CSF2 *Homo sapiens* *Pan troglodytes* *Papio anubis*	5'- CAGCCACTACAAGCAGCACT -3' …… ……	5'- GGGGATGACAAGCAGAAAGT -3' …… ……	NM_000758.2 XM_527005.2 NM_001112651.1	Cytokine
TGFB1 *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- CTACTACGCCAAGGAGGTCA -3' …… ……	5'- GCCAGGAATTGTTGCTGTAT-3' …… ……	NM_000660.3 XM_512687.2 *Papio hamadryas* genome Nov. 2008 Pham_1.0 *	Cytokine
NFKB1A *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'- CGCTCCTGCATCTGCCTCC -3' …… ……	5'- ATACTCCTTGATGTGGGCA-3' …… ……	NM_020529.2 XM_522823.2 *Papio hamadryas* genome Nov. 2008 Pham_1.0	Transcription inhibitor
REL/c-Rel *Homo sapiens* *Pan troglodytes* *Papio hamadryas*	5'-CCCACGCTCAGGCAATACA -3' …… ……	5'-TGGTGGGATACCTTGCGAAT-3' ……... ……...	NM_002908.2 XM_001159537.1 XM_525761.2 XM_001159382.1 *Papio hamadryas* genome Nov. 2008 Pham_1.0 (Goff et al., 2000)	Transcription factor

Continued, Genes, primer sequences and accession numbers

APPENDIX F

Primer efficiency values for *Homo*, *Pan* and *Papio*.

Primer efficiency was calculated by collecting Cq data of real time PCR reactions for 5 to 10 fold serially diluted template. All reactions were completed in duplicate with negative controls. Using linear regression, the slope of Cq (y) and dilutions (x) was calculated. The slope was then applied as "m" to the following equations to calculate the exponential amplification (Eamp) and efficiency (E) (318):

$$Eamp = [10^{-1/m}]-1$$

$$E = Eamp + 1.$$

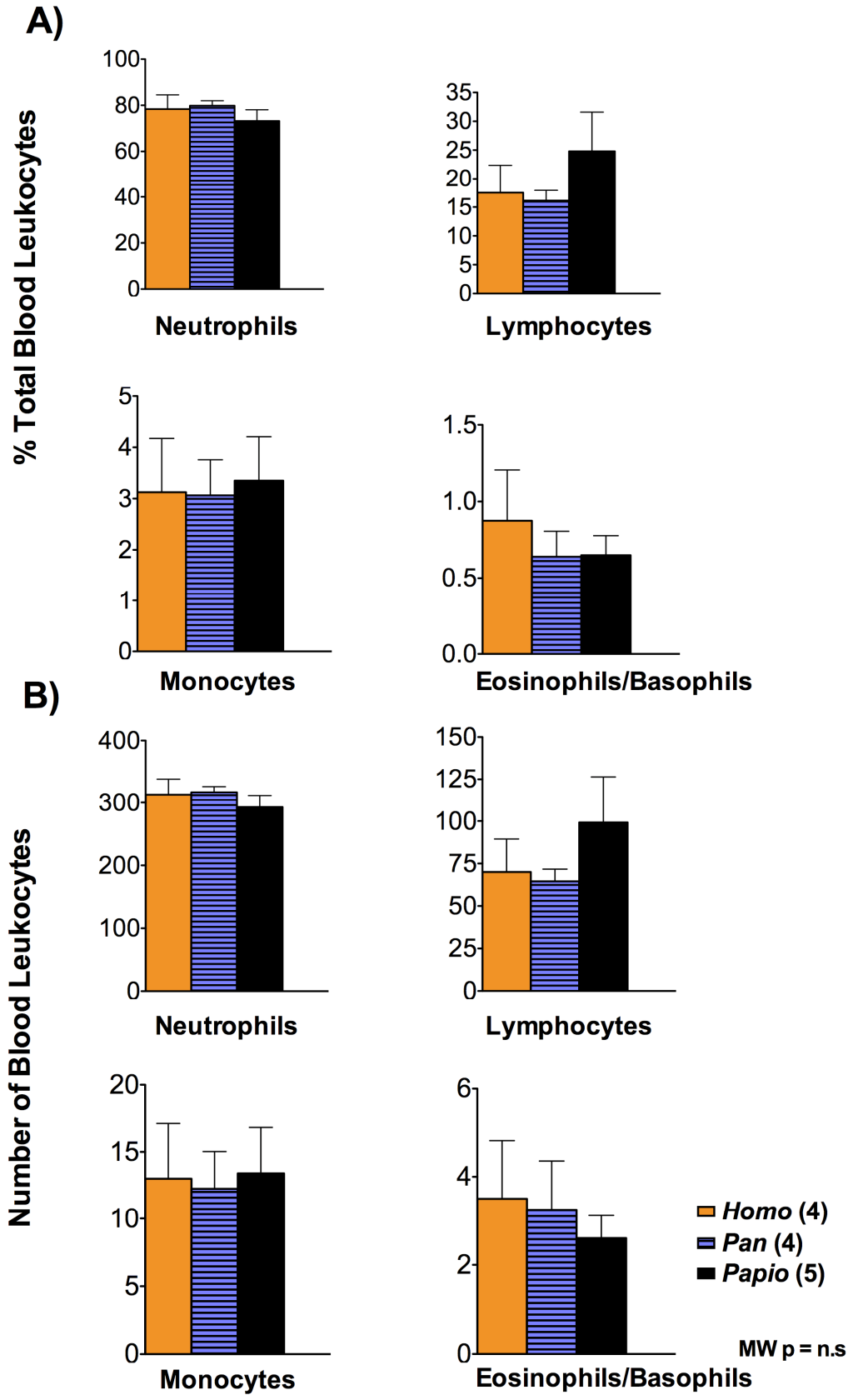
Reactions were repeated using three different individuals, to ensure accurate assessment of PCR efficiency. All efficiency values for the genes amplified in this study were highly similar, falling within 10% of one another (1.8x-2.2x). Efficiency values that could not be attained due to high Cq values/low amplification are marked here as N/A and estimated as 2.0 (none of these targets showed differences in induction either in raw Cq scores or after calculation of fold difference induction).

Gene	*Homo sapiens*	*Pan troglodytes*	*Papio* sp.
GAPDH	1.8	1.9	1.9
ACTB	1.9	1.9	1.9
B2M	1.8	1.9	1.9
CCL2	1.9	2.00	1.9
CCL3	1.8	1.9	1.9
CCL5	1.9	1.9	2.1
CSF2	N/A	N/A	N/A
CXCL2	1.9	2.1	1.9
IFNγ	N/A	N/A	N/A
IL-12A	N/A	N/A	N/A
IL-1RN	2.0	2.0	2.0
IL-1β	1.8	1.8	1.8
IL-6	2.0	2.1	2.0
IL-8/CXCL8	1.9	1.9	1.9
IL-10	1.9	2.0	2.0
NFκBIA	2.1	2.0	2.0
REL	N/A	N/A	N/A
TGFβ-1	2.1	2.0	2.0
TNFα	2.0	1.9	2.0

APPENDIX G

Blood leukocyte subtypes proportions in *Homo*, *Pan* and *Papio*.

Leukocytes isolated by hypotonic shock, stained using Wright-Geimsa staining protocol, and counted under light microscopy (See Materials and Methods). A) Mean percent of cell subtype, by species (neutrophils, lymphocytes, monocytes and eosinophils/basophils) B) mean number of cell subtypes, by species. No significant differences in blood leukocyte proportions were found between species [MW: all comparisons non-significant (n.s.)].


A)
% Total Blood Leukocytes
100
80
60
40
20
0
Neutrophils
35
30
25
20
15
10
5
0
Lymphocytes
5
4
3
2
1
0
Monocytes
1.5
1.0
0.5
0.0
Eosinophils/Basophils
B)
Number of Blood Leukocytes
400
300
200
100
0
Neutrophils
150
125
100
75
50
25
0
Lymphocytes
20
15
10
5
0
Monocytes
6
4
2
0
Eosinophils/Basophils
Homo (4)
Pan (4)
Papio (5)
MW p = n.s

APPENDIX H

Protein sequence source information for Mirrortree analysis.

Amino acid sequences for TLR1, TLR2, TLR4, IL-8/CXCL8, CXCL2, CCL3, IL-1β, TNFα, IL-6, IL-1RN, IL-10, GAPDH, ACTB and B2M for all available primates were collected from NCBI and Uniprot databases as well as the UCSC and Ensembl genome browsers. *Papio hamadryas* and some *Otolemur garnetti*, *Tarsier syrichta*, *Microcebus murinus* and *Sairimi bolviensis* sequences were acquired by searching the short read and trace archives of these genomes using the pairwise alignment program BLAST, and published homologous human genetic sequences.

Species	Source
Homo sapiens	*NCBI Reference Sequence*: NP_003254.2 (TLR1); NP_003255.2 (TLR2); NP_612564.1 (TLR4); NP_000575.1 (IL-8/CXCL8); NP_002080.1 (CXCL2); NP_002974.1 (CCL3); NP_000567.1 (IL-1β); NP_000591.1 (IL-6); NP_000585.2 (TNFα); NP_776213.1 (IL-1RN); NP_000563.1 (IL-10); *Swiss Prot Accession number*: P04406 (GAPDH); P60709 (ACTB); P61769 (B2M)
Pan troglodytes	*NCBI Reference Sequence*: NP_001123937.1 (TLR1); NP_001123941.1 (TLR2); NP_001138335.1 (TLR4); XP_001156432.1 (IL-8/CXCL8); XP_001155614.1 (CXCL2); NP_001029254.1 (CCL3); XP_001147075.1 (IL-1β); XP_518992.2 (IL-6); NP_001038976.1 (TNFα); XP_515698.1 (IL-1RN); NP_001129092.2 (IL-10); XP_508955.1 (GAPDH); ***Swiss Prot*** *Accession number*: Q5R1X3 (ACTB); P61770 (B2M);
Pan paniscus	*GenBank Accession number*: BAG55016.1 (TLR1); BAG55037.1 (TLR4); *Swiss Prot Accession number:* B3Y614.1 (TLR2);
Gorilla gorilla	*GenBank Accession number:* BAG55017.1 (TLR1); BAG55038.1 (TLR4); *Ensemble cds accession number:* ENSGGOP00000025002 (IL-8/CXCL8); ENSGGOP00000026620 (CXCL2); ENSGGOP00000020322 (CCL3); ENSGGOP00000006245 (IL-1β); ENSGGOP00000005226 (IL-6); ENSGGOP00000005887 (TNFα); ENSGGOP00000004479 (IL-1RN); ENSGGOP00000019687 (IL-10); *Swiss Prot Accession number:* B3Y615.1 (TLR2); G3R288 (GAPDH); G3QFP0 (ACTB); P61771 (B2M);
Pongo abelli	*NCBI Reference Sequence:* XP_002814714.1 (TLR1); XP_002815270.1 (TLR2); XP_002814903.1 (IL-8/CXCL8); XP_002814913.1 (CXCL2); XP_002827330.1 (CCL3); XP_002811835.1 (IL-1β); XP_002818205.1 (IL-6); XP_002816766.1 (TNFα); XP_002809587.1 (IL-10); *Swiss Prot Accession number:* Q5RAB4 (GAPDH); Q5R6G0 (ACTB);
Pongo pygmaeus	*GenBank Accession number:* AAM18616.1 (TLR4*); Swiss Prot Accession number:* P16213 (B2M)
Nomascus leucogenys	*NCBI Reference Sequence:* XP_003258626.1 (TLR1); XP_003257902.1 (TLR2); XP_003264105.1 (TLR4); XP_003265784.1 (IL-8/CXCL8); XP_003265790.1 (CXCL2); XP_003281435.1 (CCL3); XP_003277734.1 (IL-1β); XP_003252690.1 (IL-6); XP_003273012.1 (IL-10); *Ensemble cds accession number:* ENSNLEP00000007426 (TNFα); ENSNLEP00000002424 (IL-1RN); *Swiss Prot Accession number*: G1QVR0 (GAPDH); G1R135 (B2M);
Hylobates lar	*GenBank Accession number:* ACC68075.1 (TLR1); ACC68078.1 (TLR4)

Species	Source
Papio anubis	*GenBank Accession number:* AAF07059.1 (TLR4); NP_001167008.1 (IL-8/CXCL8); NP_001185525.1 (CCL3); NP_001167007.1 (IL-6); NP_001106118.1 (TNFα); EY278935.1 (GAPDH); Q6T672 (B2M);
Papio hamadryas	*GenBank Accession number:* AY796417.1 (IL-10); Acquired using BLAT and overlapping segments of full *Homo sapiens* gene sequences (GCRh.18) to search the Pham 1.0 genome. Coding sequences were then assembled using *Homo sapiens* and *Macaca mullatta* refseq sequences listed here. Found Pham 1.0 genome sequences listed here. Contig557578_Contig788793 bp 9581-11935 (TLR2); gnl|ti|1968228868-263980436 bp 724-229, gnl|ti|1925054875-256545668 bp 248-518, gnl|ti|1910994162-252676969 bp 95-270 (CXCL2); Contig432937-Contig658479 12738 to 12778, Contig432937-Contig658479 13345 to 13402, Contig432937-Contig658479 15386 to 15587, Contig432937 - Contig658479 16143 to 16307, Contig432937-Contig658479 17781 to 17911, Contig432937- Contig658479 18701 to 18908 (IL-1β); gnl|ti|1938321275-258111444 bp 1006-207, gnl|ti|2037816640 -276861060 bp 271-875, gnl|ti|1922392904-255631392 bp 181-802, gnl|ti|1939508778-258301804 bp 53-948 (IL-1RN); gnl|ti|1929674439-256951136 bp 243-918, gnl|ti|1934906576-258020087 bp 206-648, gnl|ti|1963914312-257469766 bp 123-564 (ACTB).
Macaca mulatta	*NCBI Reference Sequence*: NP_001123896.1 (TLR1); NP_001123897.1 (TLR2); NP_001032169.1 (TLR4); NP_001028137.1 (IL-8/CXCL8); XP_001092240.1 (CXCL2); NP_001029372.1 (CCL3); NP_001036221.1 (IL-1β); NP_001036198.1 (IL-6); NP_001040614.1 (TNFα); XP_001091833.1 (IL-1RN); NP_001038192.1 (IL-10); NP_001028256.1 (ACTB); *Swiss Prot Accession number*: F6WJJ6 (GAPDH); Q6V7J5 (B2M);
Macaca fasicularis	*GenBank Accession number*: BAG55019.1 (TLR1); BAG55040.1 (TLR4); BAF31886.2 (CXCL2); EHH55842.1 (IL-1β); *Swiss Prot Accession number*: Q95M53.1 (TLR2); P79341.1 (IL-6); P79337.1 (TNFα); P79338.1 (IL-10); Q4R561 (ACTB); Q8SPW0 (B2M);
Cercocebus atys/torquatus	*GenBank Accession number*: ABY64985.1 (TLR1); ABY64986.1 (TLR2); ABY64991.1 (TLR4); ABI18975.1 (TNFα); *Swiss Prot Accession number*: P46653.1 (IL-8/CXCL8); P46648.1 (IL-1β); P46650.1 (IL-6); P46651.1 (IL-10);
Chlorocebus sabaeus	*GenBank Accession number*: ACI28915.1 (IL-8/CXCL8); ACI28917.1 (IL-1β); ACI28913.1 (IL-6); ACI28908.1 (TNFα);
Callithrix jacchus	*NCBI Reference Sequence*: XP_002745960.1 (TLR1); XP_002745427.1 (TLR2); XP_002745779.1 (IL-8/CXCL8); XP_002745777.1 (CXCL2); XM_002748328.1 (CCL3); XP_002757551.1 (IL-1β); XP_002751546.1 (IL-6); XP_002746383.1 (TNFα); XP_002751826.1 (ACTB); *Ensemble cds accession number*: ENSCJAP00000037893 (TLR4); ENSCJAP00000025611 (IL-1RN); XP_002760779.1 (IL-10); *Swiss Prot Accession number*: F7H581 (GAPDH); P63061 (B2M);
Saguinus oedipus	*GenBank Accession number*: ACC68084.1 (TLR1); ACC68085.1 (TLR2); ACC68087.1 (TLR4); AAM76586.1 (TNFα); *Swiss Prot Accession number*: P55079 (B2M);

Species	Source
Saimiri boliviensis	*GenBank Accession number*: AGCE01047613.1, bp 15274-12914 (TLR1); AGCE01050586.1, bp 14466-12112 (TLR2); AGCE01000997.1 bp 13336-12329
Saimiri sciureus	*GenBank Accession number*: AAK92041.1 (IL-1β); AF294757.1 (IL-6); CAD27179.1 (TNFα); *Swiss Prot Accession number*: Q8MKG9.1 (IL-10); Q71UN6 (B2M);
Aotus lemurinus	*GenBank Accession number*: AF097323.1 (IL-6); *Swiss Prot Accession number*: P63063 (B2M);
Tarsius syrichta	*GenBank Accession number*: ABRT010176658.1 bp 2-49, 139-266, 346-429 (CXCL2); ABRT010138398.1, bp 1811-1859, 2546-2663, 3072-3160 (CCL3); ABRT010288519.1 bp 1361-1510, 3540-3700 (IL-6); ABRT010157874.1 bp 5961-6237 (B2M); *Ensemble cds accession number*: ENSTSYT00000005518 (TLR1); ENSTSYP00000010134 (TLR2); ENSTSYP00000003281 (TLR4); ENSTSYP00000003762 (IL-8/CXCL8); ENSTSYP00000002598 (TNFα);
Otolemur garnetti	*GenBank Accession number*: AAQR03072593.1, bp 12383-12463, 12545-12668, 12776-12847 (CXCL2); AAQR03122030.1, bp 38534 – 38541, 58003-57944, 57347-57230, 39265-39375, 23502-23399, 39791-39884, 56845-56752 (CCL3); AAQR03081586.1 bp 19696-20703 (GAPDH); *Ensemble cds accession number*: ENSOGAT00000003281 (TLR1); ENSOGAP00000015591 (TLR2); ENSOGAP00000015330 (TLR4); ENSOGAP00000021370 (IL-8/CXCL8); ENSOGAP00000004815 (IL-1β); ENSOGAP00000003727 (IL-6); ENSOGAP00000009167 (TNFα); ENSOGAP00000008425 (IL-1RN); ENSOGAP00000003633 (IL-10); ENSOGAP00000004212 (B2M); *Acquired by BLAT of Microcebus* ENSMICT00000002791cdna against OtoGar3 genome, OtoGar3:GL873535.1:25761158:25761960 (ACTB);
Microcebus murinus	*GenBank Accession number*: ABDC01306720.1 bp 1208-2215 (GAPDH); *Ensemble cds accession number*: ENSMICP00000002898 (TLR2); ABDC01126428.1, bp 12075-12149, 12844-12961, 13328-13416 (CCL3); ENSMICP00000012151 (IL-1β); ENSMICP00000006110 (IL-6); ENSMICP00000015806 (TNFα); ENSMICP00000003226 (IL-1RN); ENSMICP00000007603 (IL-10); ENSMICP00000002542 (ACTB); ENSMICP00000004591 (B2M);

APPENDIX I

Primate species used in Mirrortree analysis of co-evolution (326)

Genes are listed in the pairs they were analyzed. A "+" indicates inclusion in analysis, while "-" indicates exclusion. Species indicated in short form as follows: H.s.: *Homo sapiens*, P.t.: *Pan troglodytes*, P.p.: *Pan paniscus*, G.g.: *Gorilla gorilla*, P.a.: *Pongo abelli*, N.l.: *Nomascus leucogenes*, H.l.*: Hylobates lar*, P. anu.: *Papio anubis*, P.h.: *Papio hamadryas*, M.m.: *Macaca mulatta*, M.f.: *Macaca fasicularis*, C.a.: *Cercocebus atys/torquatus*, S.b.: *Saimiri boliviensis*, S.s.: *Saimiri sciureus*, S.o.: *Saguinus oedipus*, C.j.: *Callithrix jacchus*, T.s.: *Tarsius syrichta*, O.g.: *Otolemur garnetti*, M.mic: *Microcebus murinus*

Gene Pairs		Species																	
Gene 1	Gene 2	H.s.	P.t.	P.p	G.g	P.a.	N.l.	H.l.	P. anu.	P.h.	M.m.	M.f.	C.a.	S.b.	S.s.	S.o.	C.j.	T.s.	O.g.	M. mic.
TLR1	TLR2	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-
TLR1	TLR4	+	+	+	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-
TLR1	CXCL2	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-
TLR1	IL-8 /CXCL8	+	+	-	+	+	+	-	-	-	+	-	-	-	-	-	+	+	+	-
TLR1	CCL3	+	+	-	+	+	+	-	-	-	+	-	-	-	-	-	+	+	+	-
TLR1	IL-1β	+	+	-	+	+	+	-	-	-	+	+	-	+	-	-	+	-	+	-
TLR1	TNFα	+	+	-	+	+	+	-	-	-	+	+	+	-	+	+	+	+	+	-
TLR1	IL-6	+	+	-	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+	-
TLR1	IL-1RN	+	+	-	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+	-
TLR1	IL-10	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-
TLR1	GAPDH	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-
TLR1	ACTB	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+	-	+	-
TLR1	B2M	+	+	-	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-
TLR2	TLR4	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-
TLR2	CXCL2	+	+	-	+	+	+	-	-	+	+	+	-	-	-	-	+	+	+	-

Primate species used in Mirrortree analysis of co-evolution

Gene Pairs		Species																	
Gene 1	Gene 2	H.s	P.t.	P.p	G.g	P.a.	N.l.	H.l.	P. anu	P.h.	M.m.	M.f.	C.a.	S.b.	S.s.	S.o.	C.j.	T.s.	O.g.	M. mic.
TLR2	IL-8 /CXCL8	+	+	-	+	+	+	-	-	-	+	-	-	-	-	-	+	+	+	-
TLR2	CCL3	+	+	-	+	+	+	-	-	-	+	-	-	-	-	-	+	+	+	+
TLR2	IL-1β	+	+	-	+	+	+	-	+	+	+	+	+	-	+	-	+	-	+	+
TLR2	TNFα	+	+	-	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+
TLR2	IL-6	+	+	-	+	+	+	-	+	-	+	+	+	-	+	-	+	+	+	+
TLR2	IL-1RN	+	+	-	+	+	+	-	+	-	+	+	-	+	-	-	+	+	+	+
TLR2	IL-10	+	+	-	+	+	+	-	-	+	+	+	+	-	+	-	+	+	+	+
TLR2	GAPDH	+	+	-	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+
TLR2	ACTB	+	+	-	+	+	+	-	+	+	+	+	-	-	-	-	+	-	+	+
TLR2	B2M	+	+	-	+	+	+	-	-	-	+	+	-	-	+	+	+	+	+	+
TLR4	CXCL2	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-
TLR4	IL-8 /CXCL8	+	+	-	+	+	+	-	-	-	+	-	+	-	-	-	+	+	+	-
TLR4	CCL3	+	+	-	+	+	+	-	+	-	+	-	-	-	-	-	+	+	+	-
TLR4	IL-1β	+	+	-	+	+	+	-	+	-	+	+	+	-	-	-	+	-	+	-
TLR4	TNFα	+	+	-	+	+	+	+	+	-	+	+	+	-	-	+	+	+	+	-

Primate species used in Mirrortree analysis of co-evolution

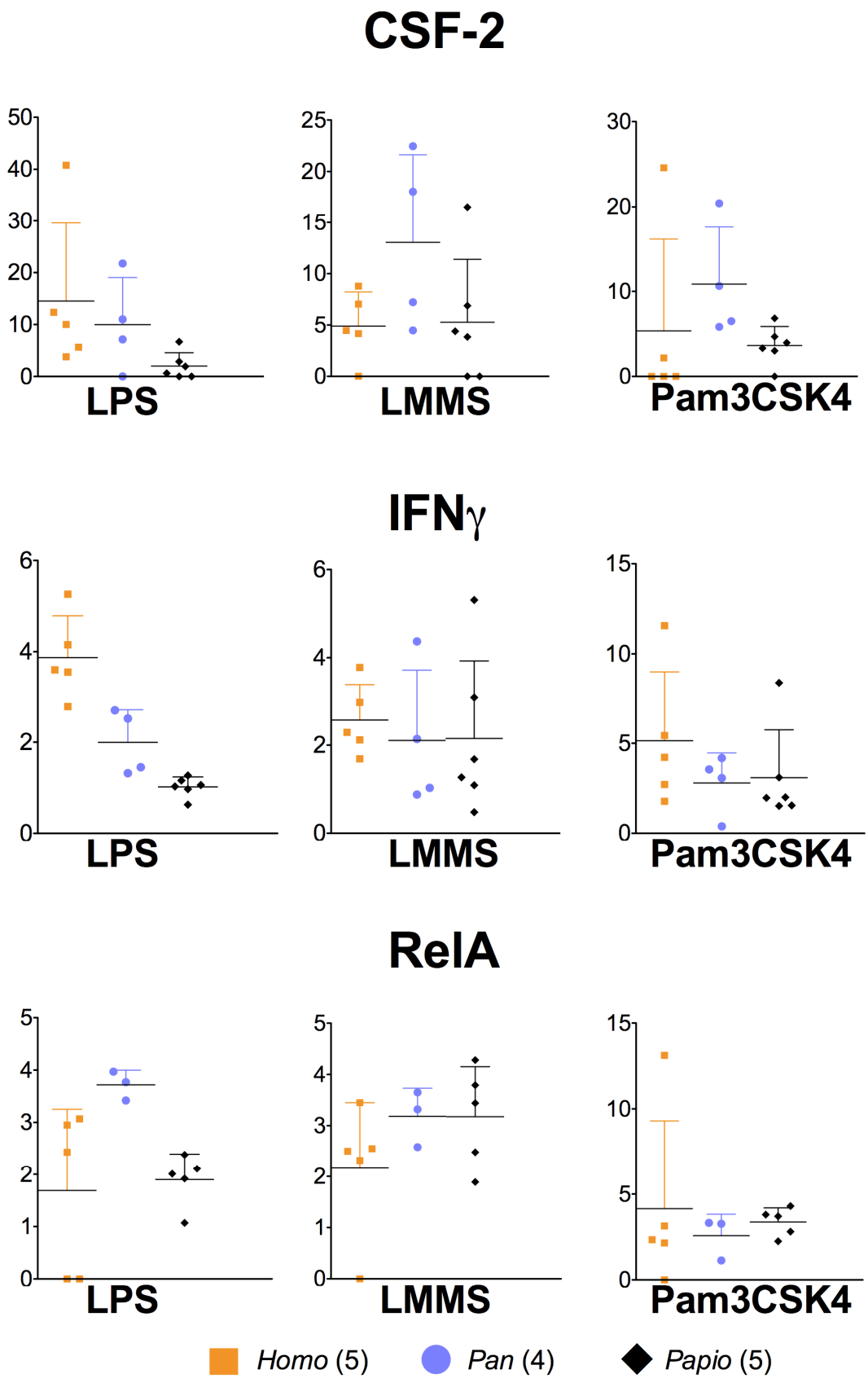
Gene Pairs		Species																	
Gene 1	Gene 2	H.s	P.t.	P.p	G.g	P.a.	N.l.	H.l.	P. anu	P.h.	M.m.	M.f.	C.a.	S.b.	S.s.	S.o.	C.j.	T.s.	O.g.	M. mic.
TLR4	IL-6	+	+	-	+	+	+	-	+	-	+	+	+	-	-	-	+	+	+	-
TLR4	IL-1RN	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-
TLR4	IL-10	+	+	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	+	-
TLR4	GAPDH	+	+	-	+	+	+	-	+	-	+	+	-	-	-	-	+	+	+	-
TLR4	ACTB	+	+	-	+	+	+	-	-	-	+	+	-	-	-	-	+	-	+	-
TLR4	B2M	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	+	+	+	-

Primate species used in Mirrortree analysis of co-evolution

APPENDIX J

Induction profiles of lowly expressed genes

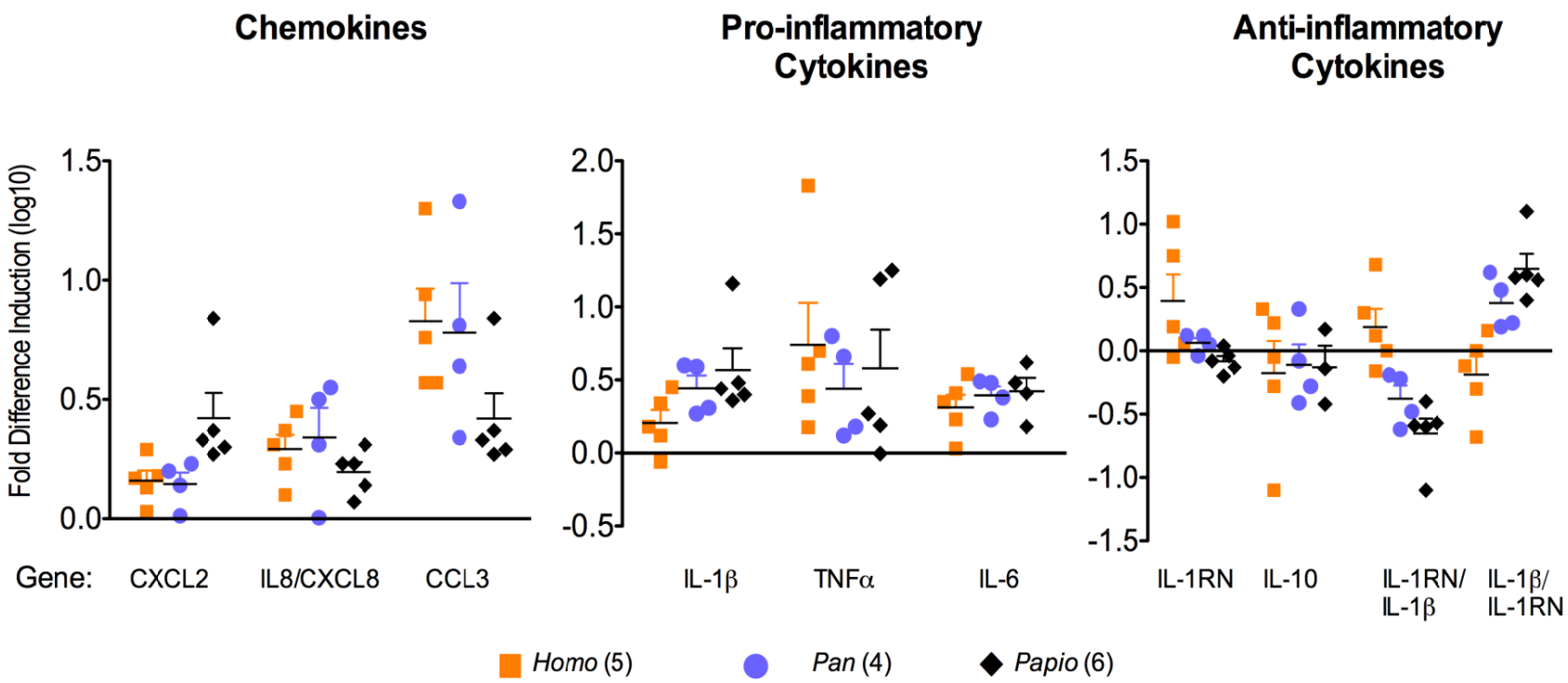
All species weakly induced IFNγ and RELA when stimulated, but no significant inter-species differences in induction were observed. After stimulus CSF2 was induced in all species. However, CSF2 was only amplified in un-stimulated controls at 35 cycles or higher making it difficult to accurately calculate fold difference between stimulated and unstimulated samples.


CSF-2
50
40
30
20
10
0
LPS
25
20
15
10
5
0
LMMS
30
20
10
0
Pam3CSK4
IFNγ
6
4
2
0
LPS
6
4
2
0
LMMS
15
10
5
0
Pam3CSK4
RelA
5
4
3
2
1
0
LPS
5
4
3
2
1
0
LMMS
15
10
5
0
Pam3CSK4
Homo (5)
Pan (4)
Papio (5)

APPENDIX K

Cytokine and chemokine responses to LcrV from *Yersinia pestis*

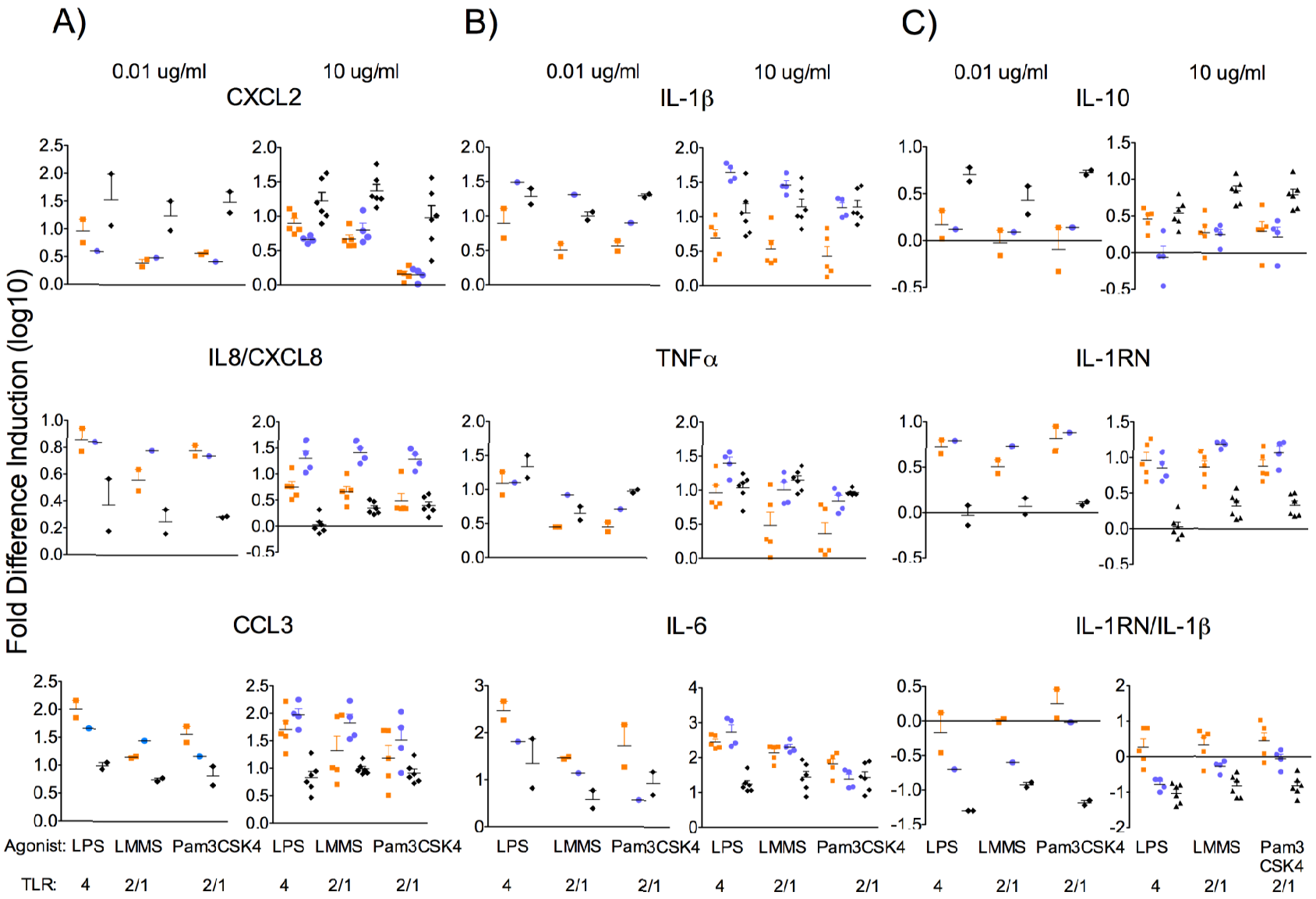
Only the highest dose (10 ug/ml) of LcrV from *Yersinia pestis* stimulated gene induction after 90 minutes. All lower doses (1 ug – 0.01 ug/ml) did not stimulate gene induction, making high dose responses difficult to interpret. The 10 ug/ml dose induced low levels of chemokines, pro-inflammatory cytokines and anti-inflammatory cytokines. Only differences in *Pan* and *Papio* IL-1RN induction were statistically significant. *Pan* IL-1RN induction is significantly stronger than *Papio* ($p < 0.05$). Similar to other PAMPs, LcrV stimulates a ratio of IL-1RN/IL-1β that is higher in *Homo* and IL-1β/IL-1RN that is lower in *Homo* than in non-human primates.


Chemokines
Pro-inflammatory Cytokines
Anti-inflammatory Cytokines
Fold Difference Induction (log10)
1.5
1.0
0.5
0.0
2.0
1.5
1.0
0.5
0.0
-0.5
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Gene:
CXCL2
IL8/CXCL8
CCL3
IL-1β
TNFα
IL-6
IL-1RN
IL-10
IL-1RN/ IL-1β
IL-1β/ IL-1RN
Homo (5)
Pan (4)
Papio (6)

APPENDIX L

Cytokine and chemokine responses at low and high doses of PAMP (0.01 ug - 10 ug/ml)

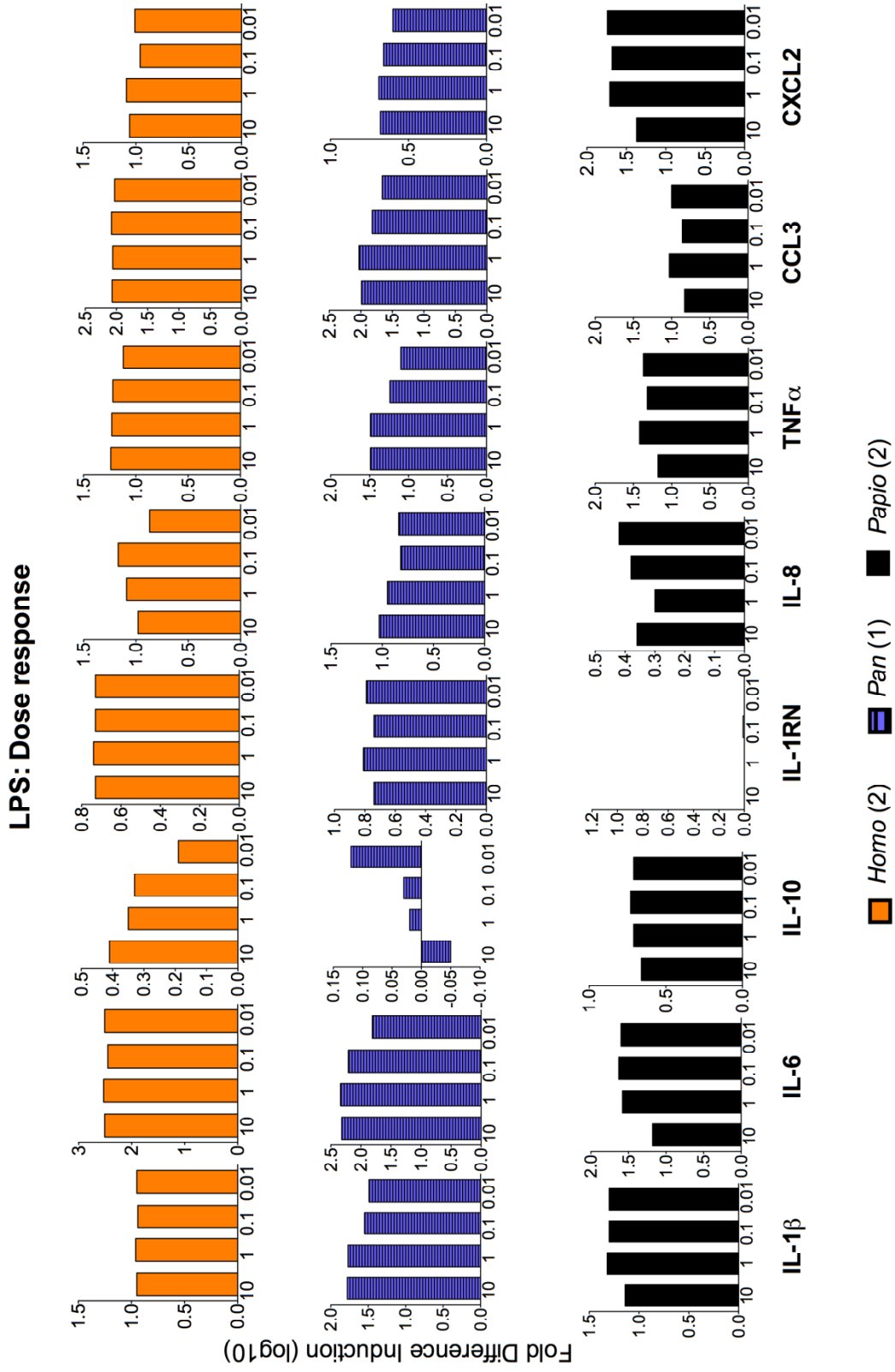
A) Chemokine induction levels. Other than a slight relative increase in *Pan* IL-8/CXCL8 induction at the high dose (10 ug/ml) induction noted at high doses are consistent at low doses. At the lowest dose, *Pan* IL-8/CXCL8 induction is very similar to *Homo*, a pattern that agrees with primate evolutionary relationships. B) Pro-inflammatory cytokine induction levels. The inter-species differences in pro-inflammatory cytokines IL-1β and IL-6 noted at high doses are consistent at low doses. *Pan* induction TNFα is slightly more conserved at low doses of LPS. C) Anti-inflammatory cytokine induction levels. Inter-species differences IL-10 and IL-1RN induction noted at high doses are consistent at low doses. *Papio* IL-10 levels increase and *Homo* IL-10 levels decrease slighty compared to high dose responses after stimulation with 0.01 ug/ml of LPS. At lower doses *Papio* IL-10 response is stronger than *Homo* and *Pan*, a result that agrees with primate phylogeny and responses to TLR2/1 PAMPS. *Papio* IL-10 induction is stronger than *Pan* and *Homo* after LMMS and Pam3CSK4 stimulation. To test the expectation that increased IL-1β transcription would lead to increased IL-1RN transcription, IL-1RN/IL-1β ratios were calculated. Inter-species differences in IL-1RN/IL-1β ratio are consistent at high and low doses of PAMPs.

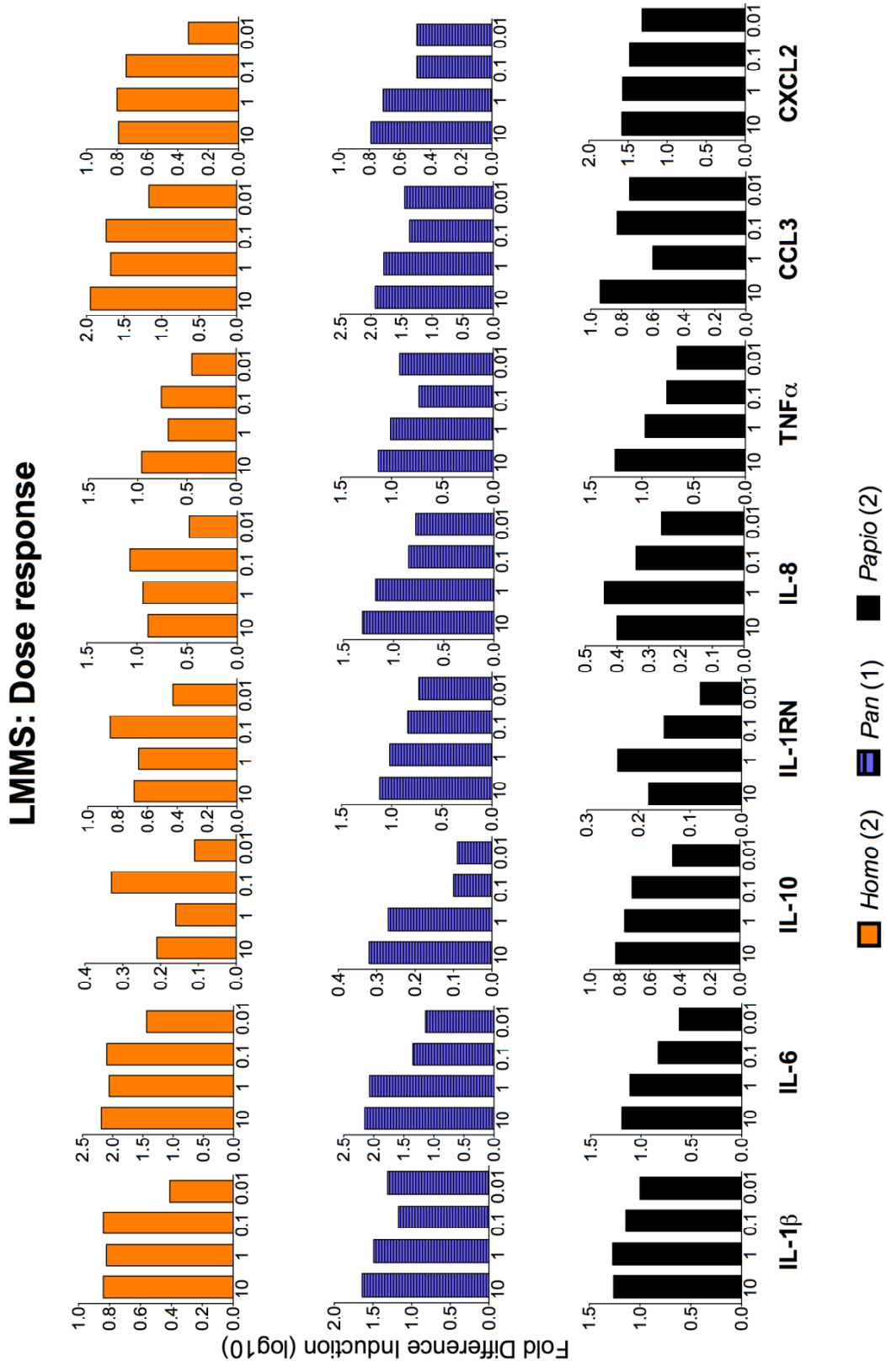

A)
B)
C)
0.01 ug/ml
10 ug/ml
CXCL2
IL-1β
IL-10
IL8/CXCL8
TNFα
IL-1RN
CCL3
IL-6
IL-1RN/IL-1β
Fold Difference Induction (log10)
Agonist: LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4 LPS LMMS Pam3CSK4
TLR: 4 2/1 2/1 4 2/1 2/1 4 2/1 2/1 4 2/1 2/1 4 2/1 2/1 4 2/1 2/1 4 2/1 2/1 4 2/1 2/1

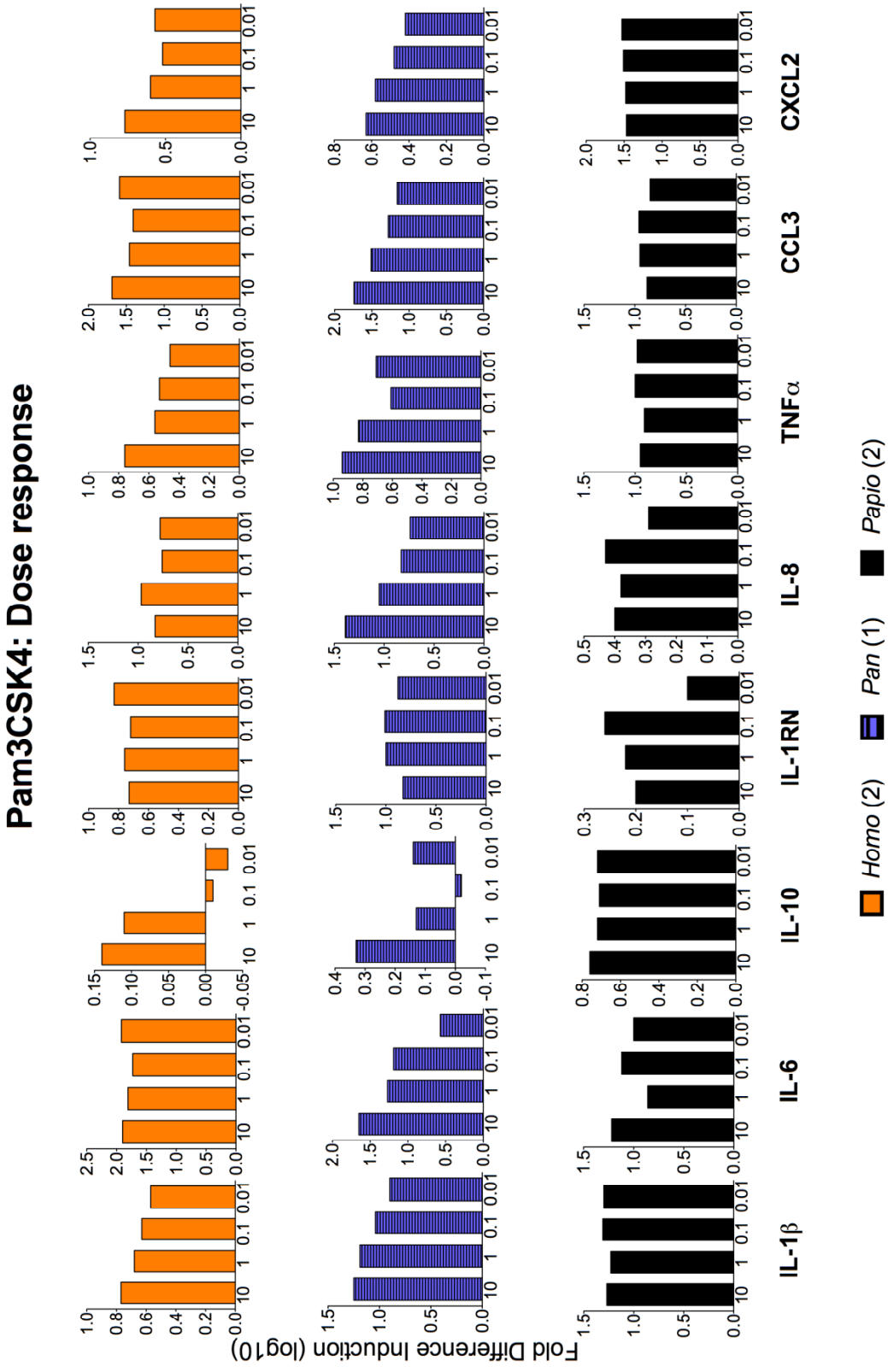
APPENDIX M

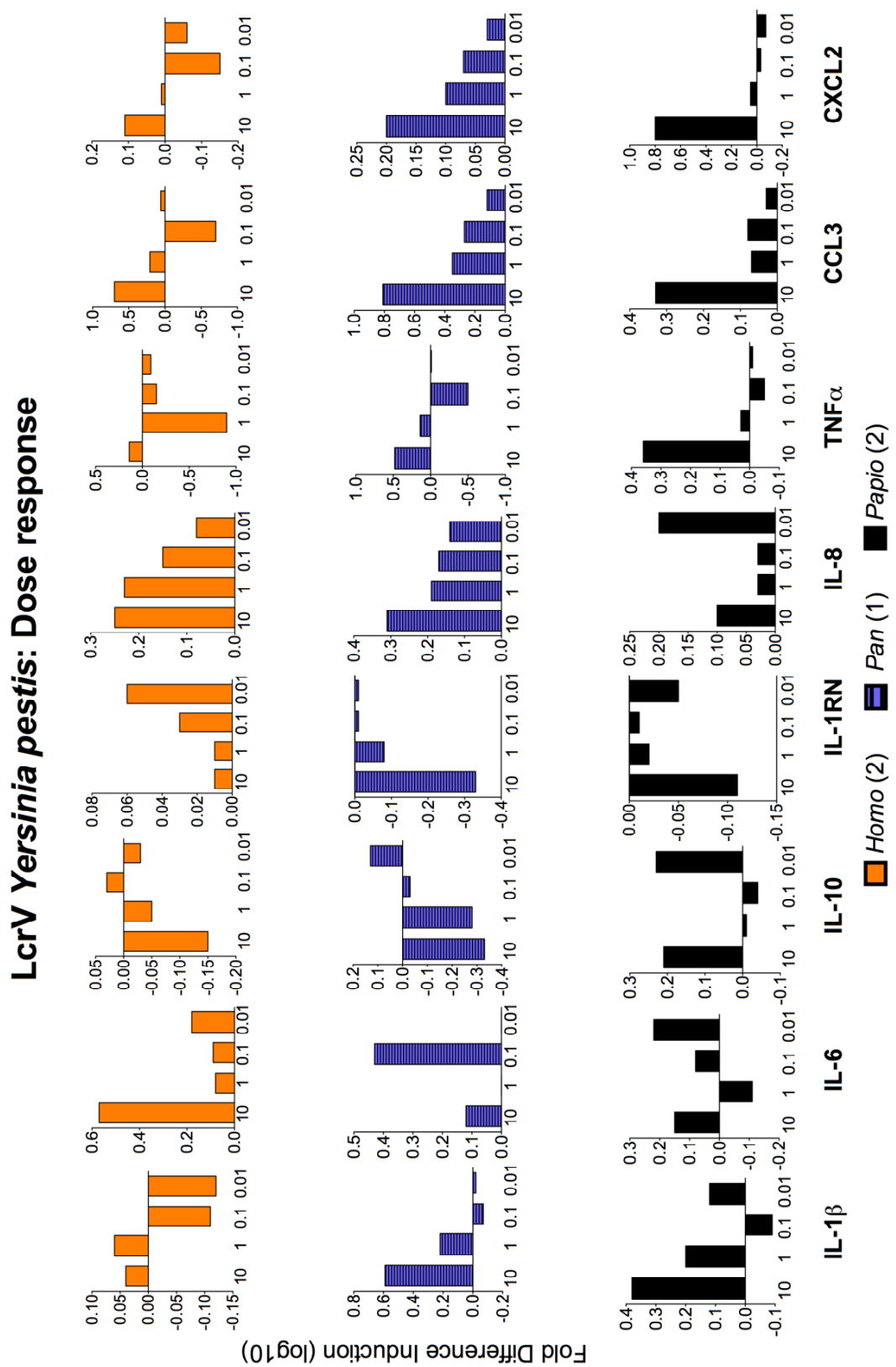
Dose response curves by species

PAMPS were applied to unfractionated blood in 10 fold serial dilutions of 0.01 - 10 ug/ml to *Homo* (2), *Pan* (1) and *Papio* (2) individuals.


LPS: Dose response
Fold Difference Induction (log10)
IL-1β
IL-6
IL-10
IL-1RN
IL-8
TNFα
CCL3
CXCL2
Homo (2)
Pan (1)
Papio (2)


LMMS: Dose response
Fold Difference Induction (log10)
IL-1β
IL-6
IL-10
IL-1RN
IL-8
TNFα
CCL3
CXCL2
Homo (2)
Pan (1)
Papio (2)


Pam3CSK4: Dose response
Fold Difference Induction (log10)
IL-1β
IL-6
IL-10
IL-1RN
IL-8
TNFα
CCL3
CXCL2
Homo (2)
Pan (1)
Papio (2)


LcrV Yersinia pestis: Dose response
Fold Difference Induction (log10)
IL-1β
IL-6
IL-10
IL-1RN
IL-8
TNFα
CCL3
CXCL2
Homo (2)
Pan (1)
Papio (2)

APPENDIX N

Putative transcription factor binding sites in the promoter and 1kb 5' region of cytokine and chemokine genes.

Sequences for *Homo sapiens*, *Pan troglodytes* and *Macaca mulatta* were submitted to JASPAR database for analysis. As the *Papio hamadryas* genome (Pham 1.0) remains only partially assembled, sequences from close relative *Macaca mulatta* (M_MUL 1.0) were used. Sites not shared by all three species are listed here. Sites tend to correspond with primate phylogenetic relationships and gene induction patterns. Unique IL-1β sites in human are noted in blue. Locations from the transcriptional start site and relative score are noted.

Gene	Shared Induction	*Homo sapiens*			*Pan troglodytes*			*Macaca mulatta*		
		Factor	Sites	Score	Factor	Sites	Score	Factor	Sites	Score
CCL3	*Homo* & *Pan*							Egr1	-980 to -970	0.8074
								Egr1	-978 to -968	0.8074
								Egr1	-975 to -965	0.8074
		NFIC	- 616 to -611	0.8403	NFIC	- 616 to -611	0.8403	NFIC	-669 to -664	0.8153
		AP1	- 492 to -486	0.8905	AP1	- 492 to -486	0.8905	CREB1	-596 to -589	0.8861
								USF1	-494 to -488	0.8250
		E2F1	- 445 to -438	0.8133	E2F1	- 445 to -438	0.8133	AP1	-473 to -467	0.8371
								ELK1	-336 to -327	0.8404
								TFAP2A	-334 to -326	0.8855
IL-8/ CXCL8	*Homo* & *Pan*	STAT1	-973 to -964	0.8083	STAT1	-973 to -964	0.8083	AP1	-940 to -934	0.8148
								AP1	-936 to -930	1
		SP1	-938 to -929	0.8269	SP1	-938 to -929	0.827			
		SP1	-934 to -923	0.8671	SP1	-934 to -923	0.8671			
		SP1	-933 to -924	0.8028	SP1	-933 to -924	0.8028	CREB1	-883 to -875	0.8036
					Egr1	-862 to -852	0.8013	STAT1	-809 to -795	0.8083
		AP1	-790 to -783	0.8371	AP1	-790 to -783	0.8371			
		CEBPA	-789 to -781	0.8624	CEBPA	-789 to -781	0.8624			
		TFAP2A	-780 to -772	0.8195	TFAP2A	-780 to -772	0.8388			
		TFAP2A	-766 to -758	0.8270	TFAP2A	-766 to -758	0.8022			
		USF1	-714 to -708	0.8024	USF1	-714 to -708	0.8024			

Putative Transcription Factor Binding Sites

Gene	Shared Induction	*Homo sapiens*			*Pan troglodytes*			*Macaca mulatta*		
		Factor	Sites	Score	Factor	Sites	Score	Factor	Sites	Score
I L-8/ CXCL8	*Homo* & *Pan*	CEBPA	-578 to -570	0.8022	CEBPA	-578 to -570	0.8022			
		USF1	-553 to -547	0.8296	USF1	-553 to -547	0.8296			
		USF1	-551 to -545	0.8172	USF1	-551 to -545	0.8172	AP1	-361 to -355	0.8165
		ELK1	-363 to -354	0.8045	ELK1	-363 to -354	0.8045			
		SPI1	-332 to -326	0.8266	SPI1	-332 to -326	0.8266	CEBPA	-66 to -58	0.8255
		ELK1	73 to 82	0.8308	ELK1	72 to 81	0.8552			
		Stat3	73 to 82	0.8863						
		TFAP2A	134 to 142	0.8156	TFAP2A	134 to 142	0.8156			
		CEBPA	259 to 268	0.8081	CEBPA	257 to 266	0.8081			
		NFIC	261 to 267	0.8365	NFIC	259 to 265	0.8365			
IL-1β	*Pan* & *Papio*							CEBPA	-926 to -918	0.884
								ELK1	-839 to -830	0.8103
								NFIC	-836 to -831	0.809
								ELK1	-574 to -565	0.8078
								SP1	-548 to -539	0.8231
								STAT1	-541 to -527	0.8053
								Stat3	-538 to -529	0.8007
								USF1	-525 to -519	0.8041
								SP1	-453 to -444	0.8207
								SP1	-452 to -443	0.8264
		SPI1	-385 to 379	0.8318	SPI1	-385 to -379	0.8318			
		REL	-268 to -259	0.8567						
		RELA	-268 to -259	0.8261	SPI1	-268 to -262	0.8251	SPI1	-268 to -262	0.8251
		ELK1	-224 to -214	0.8144						
		Stat3	-224 to -214	0.8605						
		SPI1	-221 to -214	0.8251						
								SP1	-159 to -150	0.805
		AP1	-147 to -144	0.8199	AP1	-147 to -144	0.8199	Stat3	-155 to -146	0.8006
		SPI1	-107 to -101	0.8292	SPI1	-107 to -101	0.8292			
		SP1	-32 to -23	0.8407	SP1	-32 to -23	0.8407			
								Stat3	38 to 47	0.8037
					MYC	44 to 54	0.8135			
					USF1	46 to 52	0.8478			

Putative Transcription Factor Binding Sites

Gene	Shared Induction	*Homo sapiens*			*Pan troglodytes*			*Macaca mulatta*		
		Factor	**Sites**	**Score**	**Factor**	**Sites**	**Score**	**Factor**	**Sites**	**Score**
IL-1β	*Pan* & *Papio*							SP1	189 to 198	0.8091
								SP1	191 to 200	0.9435
								SP1	192 to 201	0.9062
								SP1	193 to 202	0.8496
								SP1	196 to 205	0.8054
								SP1	197 to 206	0.8367
								SP1	199 to 207	0.8364
					SP1	206 to 215	0.8666	SP1	200 to 208	0.8995
		SP1	208 to 217	0.8168	SP1	206 to 218	0.9021			
		CEBPA	245 to 253	0.8518	CEBPA	243 to 252	0.8518	SP1	220 to 229	0.8044
		ELK1	281 to 291	0.8187	ELK1	279 to 288	0.8186			
TNFα	*Homo* & *Pan*	USF1	-979 to -973	0.8296	USF1	-979 to -973	0.8296	TFAP2A	-980 to -972	0.852
								NFIC	-970 to -965	0.8086
		NFIC	-894 to -889	0.8090	NFIC	-894 to -889	0.809			
		SPI1	-893 to -887	0.8528	SPI1	-893 to -887	0.8528			
								SP1	-872 to -863	0.8309
		TFAP2A	-773 to -765	0.8017	TFAP2A	-773 to -765	0.8017			
		TFAP2A	-627 to -619	0.811	TFAP2A	-627 to -619	0.811			
		REL	-620 to -611	0.8145	REL	-620 to -611	0.8145			

Putative Transcription Factor Binding Sites

Gene	Shared Induction	*Homo sapiens*			*Pan troglodytes*			*Macaca mulatta*		
		Factor	Sites	Score	Factor	Sites	Score	Factor	Sites	Score
IL-6	*Homo* & *Pan*	REL	-999 to -990	0.8435	REL	-999 to -990	0.8435			
		RELA	-999 to -990	0.8286	RELA	-999 to -990	0.8286			
								TBP	-943 to -929	0.842
								CEBPA	-841 to -833	0.8314
								NFIC	-768 to -763	0.9564
		NFKB1	-701 to -691	0.8297	NFKB1	-701 to -691	0.8298	NFIC	-699 to -694	0.836
		REL	-701 to -690	0.8572	REL	-701 to -690	0.8572			
		NFKB1	-700 to -690	0.8302	NFKB1	-700 to -690	0.8302			
		RELA	-700 to -691	0.8129	RELA	-700 to -691	0.8129			
		NFIC	-680 to -675	0.8360	NFIC	-680 to -675	0.8360			
								AP1	-657 to -651	0.8548
		NFIC	-643 to -638	0.8153	NFIC	-643 to -638	0.8153	SPI1	-643 to -637	0.8133
								SPI1	-642 to -636	0.8333
								NFIC	-637 to -632	0.8481
		MYC	-613 to -603	0.8668	MYC	-613 to -603	0.8668			
		NFIC	-540 to -535	0.8218						
		AP1	-479 to -473	0.8163	AP1	-479 to -473	0.8163			
		NFIC	-374 to -369	0.8857						
		ELK1	-343 to -334	0.8090	ELK1	-343 to -334	0.8090			
		NFIC	-247 to -242	0.8020	NFIC	-247 to -242	0.8020			
		NFIC	-120 to -115	0.8020						
								NFIC	-90 to -85	0.8985
		SP1	-51 to -42	0.8043	SP1	-51 to -42	0.8043			
		SP1	-45 to -36	0.8068	SP1	-45 to -36	0.8068			
								STAT1	68 to 83	0.8169
		ELK1	73 to 82	0.8368	ELK1	73 to 82	0.8368	STAT3	71 to 80	0.8403
								SPI1	75 to 81	0.8251
IL-10	*Homo* & *Pan*	CREB1	-995 to -988	0.8041	CREB1	-995 to -988	0.8041			
		SPI1	-913 to -907	0.8093	SPI1	-913 to -907	0.8093	NFIC	-876 to -871	0.8804
		SPI1	-846 to -840	0.8423	SPI1	-846 to -840				
		SPI1	-718 to -712	0.8292	SPI1	-718 to -712	0.8424			
		SPI1	-707 to -701	0.8463	SPI1	-707 to -701	0.8292			
							0.8463	SPI1	-638 to -632	0.9205
		SPI1	-547 to -541	0.8334	SPI1	-547 to -541		ELK1	-549 to -540	0.8005

Putative Transcription Factor Binding Sites

Gene	Shared Induction	*Homo sapiens*			*Pan troglodytes*			*Macaca mulatta*		
		Factor	Sites	Score	Factor	Sites	Score	Factor	Sites	Score
IL-10	*Homo* & *Pan*	SPI1	-522 to -516	0.8304	SPI1	-522 to -516	0.8304			
					SPI1	-479 to -473	0.8148			
								AP1	-449 to -443	0.8163
		AP1	-62 to -56	0.8148				NFKB1	-419 to -409	0.8071
		REL	675 to 685	0.8043	AP1	-62 to -56	0.8153			
		REL	676 to 685	0.8488	NFIC	674 to 679	0.8188			
		ELK1	752 to 761	0.8014	REL	676 to 685	0.8013			
		TBP	974 to 988	0.8096	ELK1	752 to 761	0.8096			
					TBP	974 to 988	0.8047			
					SPI1	1320 to 1326	0.8491			
								TBP	1324 to 1338	0.8042
		SP1	1381 to 1390	0.8367				NFIC	1373 to 1378	0.8857
		SP1	1387 to 1396	0.8310	SP1	1381 to 1390	0.8367			
					SP1	1387 to 1396	0.8210			
		CREB1	1566 to 1573	0.8132				E2F1	1400 to 1407	0.8133
		SPI1	1707 to 1713	0.8133						
					SPI1	1707 to 1713	0.8133			
								USF1	1792 to 1798	0.8313
								Egr1	1794 to 1804	0.8947
								USF1	1794 to 1800	0.8445
IL-1RN	*Homo* & *Pan*	STAT3	-272 to -268	0.8014						
		SPI1	-268 to -263	0.8078						

Putative Transcription Factor Binding Sites

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