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HIV-Induced Dysregulation of IFN-γ Signaling and Programmed Cell Death in Primary Human Monocytes

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HIV-INDUCED DYSREGULATION OF IFN-γ SIGNALING AND PROGRAMMED CELL DEATH IN PRIMARY HUMAN MONOCYTES

By

Abdulkarim Fahad Alhetheel

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the degree of
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Department of Biochemistry, Microbiology, and Immunology
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ABSTRACT

Cytokine responsiveness in monocytes / macrophages (M/M) is critical in their phagocytic, antimicrobial and antigen presenting function. It has been suggested that HIV can impair cell function by disrupting signal transduction induced in response to antigen and cytokines in different immune cell types including CD4+ T cells, CD8+ T cells, and monocytes. A study by Kryworuchko *et al.* showed that Interleukin (IL)-2-induced Signal Transducer and Activator of Transcription (STAT) 5 activation was inhibited in CD8+ T cells from a subset of HIV-infected patient's naïve to therapy, but was restored, at least in part, after antiretroviral therapy (ART). In view of the important biological role of cytokine signaling via the STAT pathway in the regulation of M/M phagocytosis, proliferation, and cell survival, I hypothesized that disruption of the JAK/STAT pathway may lead to dysregulation of M/M effector functions and programmed cell death (PCD) during the course of chronic HIV infection. Therefore, my objectives were first to evaluate cytokine-induced JAK/STAT signaling in monocytes obtained from HIV- individuals and compare it to that found in two groups of HIV+ patients (ART-treated patients for >1 year and patients off therapy for >6 months). Second, I sought to determine the biological impact and the molecular mechanisms responsible for the alterations observed.

Analysis of patient monocytes showed no clear difference in responsiveness among the three groups in the case of Interferon (IFN)-α, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 stimulation. However, in the case of IFN-γ stimulation, the STAT1 transcription factor was significantly upregulated in HIV+ patients off therapy compared to HIV- controls and HIV+ patients on ART. Upregulation of STAT1 activation was not due to alterations in IFN-γ receptor expression but rather the result of increased total STAT1 expression levels.

Treatment of monocytic cells with HIV proteins Gp120 and Vpr was able to induce STAT1 expression in these cells.

Investigations into the significance of the STAT1 hyperactivation in patient monocytes showed that, surprisingly, among the STAT1 responsive genes (HLA-DR, IRF-1, CXCL9, and CXCL10) studied, only chemokine CXCL9 expression was elevated in HIV+ patients on ART compared to the other groups. Since IFN-γ-induced STAT1 activation is associated with PCD and IL-10 has inhibitory effects on IFN-γ-induced signaling and its downstream effects, I evaluated monocyte cell death in response to IFN-γ and IL-10 in all groups. Interestingly, spontaneous and IFN-γ-induced monocyte PCD were elevated in HIV+ patients compared to HIV- controls. Spontaneous PCD was significantly correlated with increased total STAT1 expression but not plasma levels of TRAIL. Interestingly, pretreatment with IL-10 could rescue monocytes from this fate.

Further investigation of how PCD occurs in normal monocytes and how it is regulated by IFN-γ and IL-10, showed that IFN-γ enhanced spontaneous TRAIL secretion and caspase 8 activation. In contrast, IL-10 inhibited spontaneous and IFN-γ-induced TRAIL secretion and caspase 8 activation. Surprisingly and despite this, spontaneous and IFN-γ-induced monocyte PCD appeared to be independent of caspase activation but rather depended on autophagy. LC3-II expression, an autophagy marker, was upregulated spontaneously in cultured monocytes, enhanced further upon IFN-γ stimulation but surprisingly, was also upregulated by IL-10. At the level of the molecular mechanism, I observed that blocking the STAT or Phosphatidyl inositol-3 kinase (PI3K) signaling pathways inhibited PCD in response to IFN-γ and could also thwart the cytoprotective effects of IL-10. Concordantly, blocking STAT

or PI3K activation reduced LC3-II expression in response to IFN-γ or IL-10 stimulation in monocytes.

In conclusion, these results suggest that dysregulation of IFN-γ signaling may contribute to monocyte dysfunction during HIV infection. Furthermore, IL-10 may have a role in enhancing monocyte survival during chronic HIV infection. Thus, understanding the regulation of monocyte PCD via autophagy pathway may have important implications concerning the elimination of this important cellular reservoir for HIV.

DEDICATION

I wish to dedicate this thesis to every person, university, institution, organization who supported me throughout my post-graduate studies, especially, my loving and supportive family and friends.

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LIST OF ABBREVIATIONS

Abs	antibodies
AIDS	Acquired immunodeficiency syndrome
APC	antigen presenting cells
ART	antiretroviral therapy
Atg	autophagy related gene
Bad	Bcl2 associated death promoter
Bax	Bcl2 associated X protein
Bcl2	B cell follicular lymphoma
Bcl-XL	Bcl2-X long form
BSA	bovine serum albumin
Caspase	cysteine aspartate protease
CIS	cytokine-inducible SH2-containing protein
CTLs	cytotoxic T lymphocytes
dsDNA	double strand DNA
DAPK	death-associated protein kinase
eIF-2	eukarytoic initiation factor-2
ERK	Extracellular-related kinase
FADD	Fas associated death domain
Fas	Fibroblast associated
FBS	fetal bovine serum
FLIP	Flice-like inhibitory protein
GAS	gamma-interferon activated sequence
GM-CSF	granulocyte macrophage colony stimulating factor
Gp120	HIV surface glycoprotein
HAART	highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
HLA	human leukocyte antigen
hrs	hours
IFN	interferon
IFN-γ-R	interferon-gamma receptor
IL	interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IRF	interferon regulatory factor
IP-10	IFN-γ-inducible protein 10
JAK	Janus associated kinase
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LC3	light chain-3
LPS	lipopolysaccharide
MAPK	mitogen-associated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MDA5	melanoma differentiation-associated gene 5
MDM	monocyte-derived macrophages
MEOH	methanol
MHC	major histocompatibility complex
MKK	mitogen-activated protein kinase kinase

M/M	monocytes / macrophages
MIG	monokine induced by IFN-γ
Min	minutes
mTOR	mammalian target of rapamycin
Nef	negative regulatory factor
NFκB	nuclear factor κB
NK	Natural killer cells
nm	nanometer
P53	tumor suppressor protein 53
PARP	poly ADP-ribose polymerase
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCD	programmed cell death
PE	phosphatidylethanolamine
PFA	paraformaldehyde
PI3K	Phosphatidyl inositol-3 kinase
PKR	protein kinase R
P-STAT	tyr-phosphorylated STAT
RIG-I	retinoic acid-inducible gene I
ROS	reactive oxygen species
RT	reverse transcription
SD	standard deviation
SEK	stress activated protein/ERK kinase
Sc-siRNA	scrambled siRNA
siRNA	small interfering RNA
ssRNA	single strand RNA
STAT	Signal Transducer and Activator of Transcription
Tat	Trans-activator of transcription
T.E.M.	Transmission electron microscopy
Th	T helper
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-alpha
TRADD	TNF receptor-associated death domain
TRAIL	TNF-related apoptosis inducing ligand
UNSTIM	unstimulated cells
UVRAG	UV irradiation resistance associated tumor suppressor gene
Vpr	Viral protein R
z-VAD	Z-Val-Ala-Asp-CH_2F
α-Fas	anti-Fas antibody
α-TRAIL	anti-TRAIL antibody
r	Pearson's r

Chapter I

General Introduction

1.1000 Human immunodeficiency virus (HIV)

1.1100 Identification and characteristics:

Acquired Immunodeficiency syndrome (AIDS) was discovered in 1981, when a similar phenomenon was observed in a group of patients that shared symptoms of rare diseases and opportunistic infections such as Kaposi sarcoma, Pneumocystis pneumonia, and persistent lymphadenopathy (1,2). This phenomenon was characterized by a significant decrease in CD4+ T cells and defects in cell-mediated immunity (1-3). Three years later, the causative agent of AIDS was identified as Human Immunodeficiency Virus (HIV) (4,5). HIV was classified under the *lentivirus* genus and the family of *Retroviridae* (6). The genome and main structural components of HIV are illustrated in Fig. 1-1 (7). It is an enveloped virus with a size of about 100 nm in diameter. Its genome consists of two identical copies of positive-sense single stranded RNA (ssRNA) that are reverse transcribed into cDNA in infected cells (6-8). Each ssRNA is about 9,500 nucleotides in length, and encodes three structural genes called *gag*, *pol*, *env*, and a complex of several other nonstructural regulatory genes known as *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (Fig 1-1) (6-8). The *gag* gene encodes the viral structural proteins including p24 (capsid), p17 (matrix), p7 (nucleocapsid). The *pol* gene, on the other hand, encodes the various viral enzymes including p32 (integrase), p66 and p51 (reverse transcriptase), p10 (protease). The *env* gene encodes the coat glycoproteins gp120 (surface) and gp41 (transmembrane) (Fig. 1-1). The nonstructural genes include *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*, which encode transactivator of transcription (Tat), regulator of virion protein expression (Rev), negative regulatory factor (Nef), viral infectivity factor (Vif), viral protein R (Vpr) proteins, and viral protein U (Vpu) proteins, respectively (6-8).

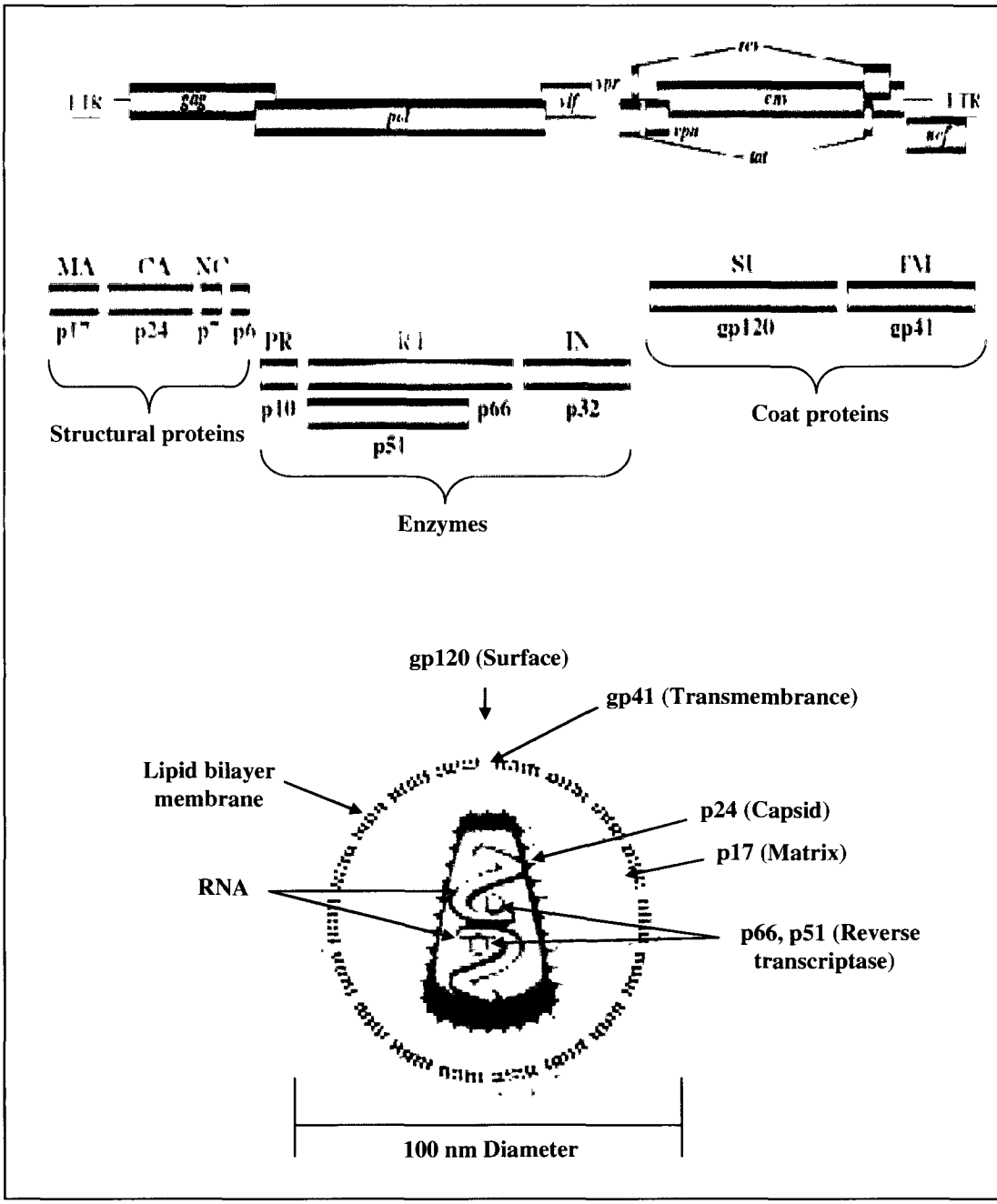

LTR
gag
pol
vif
vpr
env
LTR
vpu
tat
rev
nef
MA
CA
NC
p17
p24
p7
p6
PR
RT
IN
p10
p66
p32
p51
Structural proteins
Enzymes
SU
TM
gp120
gp41
Coat proteins
gp120 (Surface)
gp41 (Transmembrance)
Lipid bilayer membrane
p24 (Capsid)
p17 (Matrix)
RNA
p66, p51 (Reverse transcriptase)
100 nm Diameter

Figure 1-1: HIV genome and structural components.

HIV is an enveloped virus of about 100 nm in diameter. Its genome is composed of two identical copies of positive-sense single stranded RNA (ssRNA) that are reverse transcribed into cDNA in infected cells. Each ssRNA is about 9,500 nucleotides in length, and is composed of three structural genes called *gag*, *pol*, *env*, and a complex of several other nonstructural regulatory genes known as *tat*, *rev*, *nef*, *vif*, *vpr*, *vpu*. The *gag* gene encodes the viral structural proteins including p24 (capsid), p17 (matrix), p7 (nucleocapsid), whereas the *pol* gene encodes the various viral enzymes such as p32 (integrase), p66, and p51 (reverse transcriptase), p10 (protease), and the *env* gene encodes the coat glycoproteins including gp120 (surface), and gp41 (transmembrane). The nonstructural genes *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* encode transactivator of transcription (Tat), regulator of virion protein expression (Rev), negative regulatory factor (Nef), viral infectivity factor (Vif), viral protein R (Vpr), and viral protein U (Vpu) proteins, respectively.

Modified from reference # (7) with permission: HIV genome,
http://www.stanford.edu/group/virus/retro/2005gongishmail/HIV.html

Both structural and nonstructural proteins play a major role in viral entry, replication, and budding out of the infected cells (6,8). In addition, they exhibit an immunomodulatory role thought to be key in HIV pathogenesis and progression to AIDS (6,8,9). To date, two strains of HIV are known to infect humans, HIV-1 and HIV-2. HIV-1 has been found to be the cause of the majority of HIV infections around the world, whereas HIV-2 is more limited to specific regions such as West Africa (8,10).

1.1200 Infection and life cycle:

It has been established that HIV has the ability to infect CD4+ T cells, and cells of the monocytic lineage including dendritic cells and macrophages through its binding via gp120 and gp41 to the CD4 cell surface receptor and chemokine coreceptors (CXCR4 or CCR5), respectively (6,11-14). Though this is not widely accepted, certain reports have also suggested that other cell types, including CD8+ T cells, can be infected with HIV under certain conditions (15). HIV can be sub-categorized based on tropism and its binding to the chemokine receptors CXCR4 or CCR5 (8,10). M-tropic strains bind CCR5, and exhibit a tropism for monocytic lineage cell types expressing CCR5. This is the predominant strain at the early stages of the disease. T-tropic strains, however, bind CXCR4, target CD4+ T cells, and are more predominant in the late stages of the disease. Dual-tropic or transitional strains are able to bind both CXCR4 and CCR5, infect both cell types, and are associated with intermediate stages of the disease (8,10). The virus life cycle is illustrated in Fig. 1-2 (16).

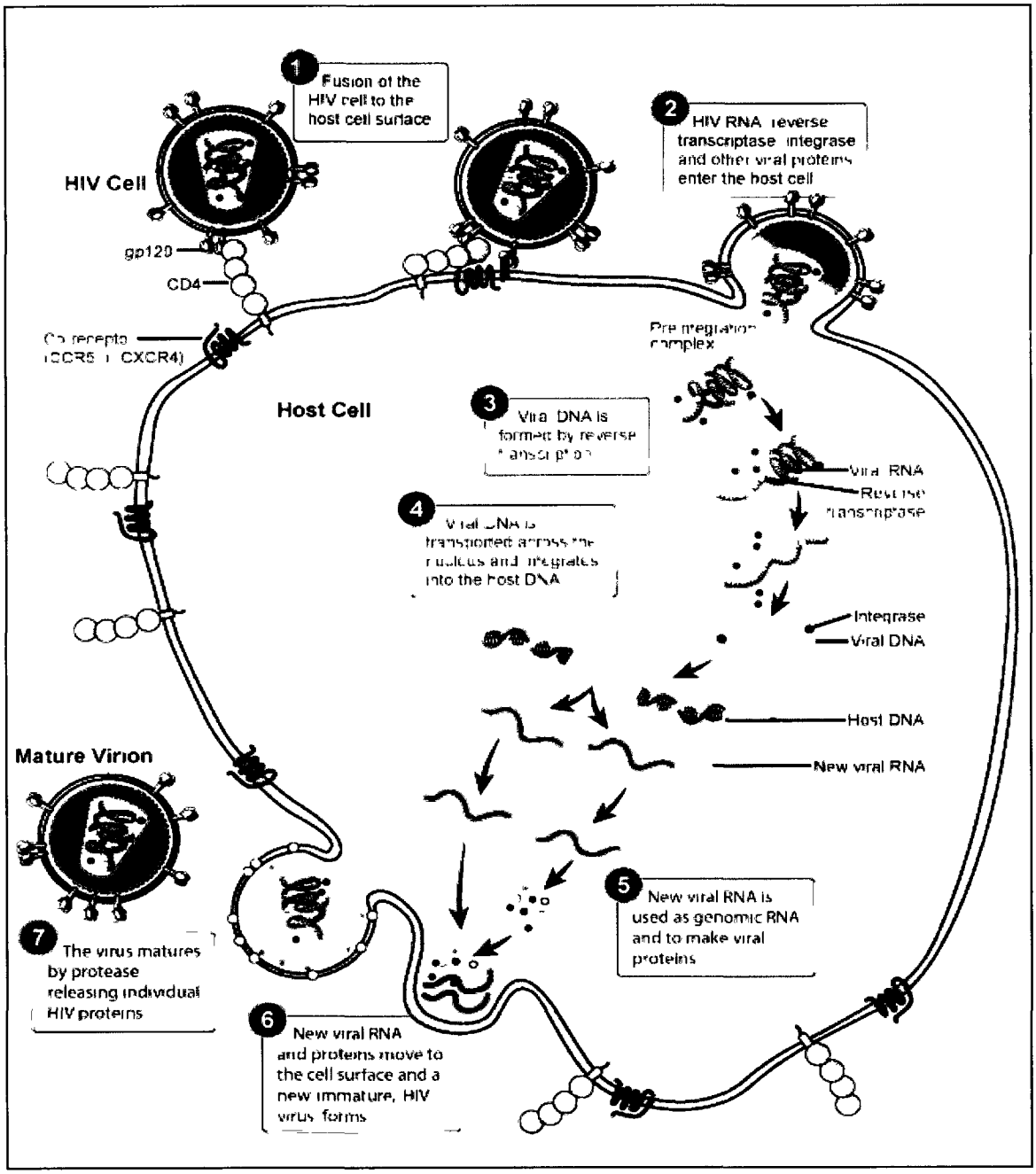

1 Fusion of the HIV cell to the host cell surface
HIV Cell
gp120
CD4
Co receptor (CCR5 or CXCR4)
2 HIV RNA reverse transcriptase integrase and other viral proteins enter the host cell
Pre integration complex
Host Cell
3 Viral DNA is formed by reverse transcriptase
Viral RNA
Reverse transcriptase
4 Viral DNA is transported across the nucleus and integrates into the host DNA
Integrase
Viral DNA
Host DNA
New viral RNA
Mature Virion
5 New viral RNA is used as genomic RNA and to make viral proteins
7 The virus matures by protease releasing individual HIV proteins
6 New viral RNA and proteins move to the cell surface and a new immature, HIV virus forms

Figure 1-2: HIV life cycle.

The HIV replication cycle is initiated when the virus, via its envelope proteins (gp120 and gp41), attaches to the host target cell through the cell surface receptor (CD4) and coreceptor (CXCR4 or CCR5). This is followed by fusion of the virus with the host cell membrane, and the viral genetic material RNA and other proteins are released into the cell. The viral reverse transcriptase converts its genome from ssRNA into dsDNA. Then, the HIV dsDNA enters the nucleus and integrates into the host DNA with the help of HIV integrase. The integrated HIV DNA called a provirus may stay latent (inactive) at this stage for years with no or very little production of new virions. However, when the infected cell receives an activation signal the provirus is activated to start utilizing the host enzymes including RNA polymerase to drive viral transcription. The HIV protease cuts HIV precursor proteins into mature proteins and new virions are assembled. Finally, the newly assembled virions exit from the infected cell by budding, taking a portion of the cell membrane as part of their envelope and are then ready to infect a new cell.

Adapted from reference # (16) with permission:
http://www3.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/hivReplicationCycle.htm

HIV, via its envelope proteins (gp120 and gp41), attaches to the host target cell through the cell surface receptor (CD4) and coreceptor (CXCR4 or CCR5) (6,16). This is followed by fusion of the virus with the host cell membrane, and the viral genetic material (RNA) and other proteins are released into the cell (6,16). The viral reverse transcriptase converts its genome from ssRNA into dsDNA. Then, the HIV dsDNA enters the nucleus and integrates into the host DNA with the help of HIV integrase. The integrated HIV DNA is called a provirus and may stay latent (inactive) at this stage for 2-15 years with no or very little production of new virions. However, when the infected cell receives an activation signal the provirus is activated to begin utilizing the host enzymes such as RNA polymerase to drive viral transcription (6,16). The HIV protease cuts the HIV precursor proteins into small mature proteins and new virions are assembled. Finally, the newly assembled virions exit from the infected cell by budding, taking a portion of the cell membrane as part of their envelope and are then ready to infect a new cell (6,16).

1.1300 Clinical features and treatment:

The course of HIV infection is typically divided into three main stages, based on the clinical features, as briefly described in Fig. 1-3 (17). The first stage is the acute infection period in which the patient feels flu- or mononucleosis-like symptoms such as fever, rash, swollen lymph nodes for 4-8 weeks. At this stage, the plasma viral load level is very high ($>10^6$ copies/mL), and is associated with a quick drop in CD4+ T cell count (<500 cell/mm^3). With the induction of a CD8 T cell response killing virus infected cells, plasma viral load drops and CD4 T cell counts recover (18). The second stage is a prolonged (2-15 years) clinical latency or chronic infection period. The patient is generally asymptomatic but is still contagious.

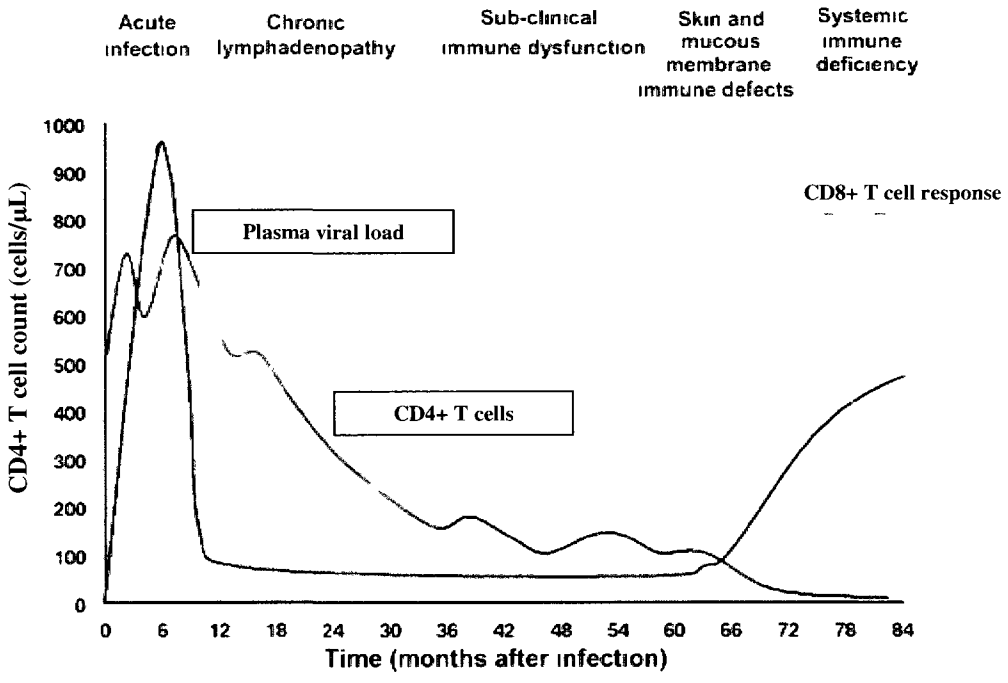

Acute infection
Chronic lymphadenopathy
Sub-clinical immune dysfunction
Skin and mucous membrane immune defects
Systemic immune deficiency
CD4+ T cell count (cells/μL)
1000
900
800
700
600
500
400
300
200
100
0
Plasma viral load
CD8+ T cell response
CD4+ T cells
0
6
12
18
24
30
36
42
48
54
60
66
72
78
84
Time (months after infection)

Figure 1-3: Clinical characteristics of HIV infection.

The course of HIV infection in the absence of antiretroviral therapy is typically divided into three stages based on clinical manifestations. The first stage is the acute infection period (4-8 weeks), and the patient feels flu- or mononucleosis-like symptoms. At this stage, the plasma viral load is very high (>10^6 copies/mL), and is associated with a quick drop in CD4+ T cell counts (<500 cell/mm^3). This is followed by rapid recovery and an increase in CD8+ T cell responses that suppresses HIV replication and controls infection. The second stage is a prolonged (2-15 years) clinical latency or chronic infection period. The patient is generally asymptomatic, and the viral load is relatively low (< 10^4 copies/mL) by, at least in part, the effect of the CD8+ T cell response, while CD4+ T cell counts slowly decrease. The last stage is AIDS (2-4 years), and the patient during this period becomes very susceptible to recurrent infections, opportunistic infections, malignancies, and eventually death. At this stage, the CD8+ T cell response is decreasing, and the viral load rises (> 10^4 copies/mL), while CD4+ T cell counts reach very low levels (< 200 cells/mm^3).

Taken from reference # (17) with permission:
http://pathmicro.med.sc.edu/lecture/hiv_time_course2.jpg

During this period, the viral load is relatively low (< 10^4 copies/mL) by, at least in part, the action of the CD8+ T cell responses, while CD4+ T cell count slowly decreases with disease progression. The last stage is AIDS, which may last for a few years (2-4 years). The patient in this stage becomes very susceptible to recurrent infections, opportunistic infections, malignancies, and eventually death. At this stage, the CD8+ T cell response declines and viral load increases (> 10^4 copies/mL), while CD4+ T cell count drops to very low levels (< 200 cells/mm^3) (Fig 1-3).

Since the identification of HIV, the global population has suffered tremendously from the spread of HIV infection and AIDS. In 2008, the United Nations programme on HIV/AIDS (UNAIDS) reported that over 25 million individuals worldwide have been killed by HIV (19). In addition, there are over 33 million people estimated to be living with HIV, and this number is predicted to rise to over 36 million by the end of 2009 (19). Although, there has been intensive research for over two decades, many aspects of HIV pathogenesis remain unclear. To date, there is no therapy able to cure HIV infection nor a vaccine available to prevent its spread (10). There do exist very effective therapeutics referred to as highly active antiretroviral therapy (HAART). These include nucleoside and non-nucleoside reverse transcriptase inhibitors as well as protease inhibitors, which are given in combination to control HIV replication. HAART has drastically prolonged the lifespan of infected individuals. However, such therapies have substantial limitations because of their side effects and the development of HIV resistance (10,20). Thus, there is constant demand for alternative or more effective therapies. Further, understanding how HIV interacts with immune cells and how it compromises their function and the immune system are critical elements to study further in order to find alternative and more effective treatments for this deadly virus.

1.2000 The immune system

The immune system is a very complex and dynamic network, which can be broadly divided into the innate and adaptive immune systems (21-23). The cellular components of innate immunity include dendritic cells, natural killer (NK) cells, macrophages, and granulocytes, whereas, adaptive immunity is mediated by B and T lymphocytes (21-24). The components of both immune systems act in conjunction and are regulated by soluble mediators known as cytokines and chemokines in order to fight, clear, and protect the host from various pathogens (21-24).

The innate immune system is the first line of defense against invading pathogens. Viral infections including HIV induce the interferon (IFN) response that is characterized by the production and secretion of proinflammatory cytokines including type-I IFN (IFN-α/β). These cytokines have antimicrobial and antiproliferative properties and serve to propagate the adaptive immune responses (25). In humans, cellular RNA molecules are short stem secondary structures. In contrast, RNA viruses produce long dsRNA molecules in the infected cells as a part of their life cycle. Thus, the long dsRNA can be recognized as a foreign molecule and triggers both cellular and humoral innate immune responses (26). There are two well characterized ways in which a cell can recognize pathogens. Extracellular pathogens are recognized by different Toll like receptors (TLR) expressed on the cell surface or in the endosome such as TLR2, TLR3, TLR4, TLR7, TLR8, and TLR9 (27). Intracellular replicating pathogens, however, are recognized by RNA helicases which are encoded by the retinoic acid-inducible gene I (RIG-I) and/or melanoma differentiation-associated gene 5 (MDA5) (28). Following viral recognition, the activation and translocation of the transcription factor nuclear factor κB (NFκB) and interferon-regulatory factor (IRF)-3 to the nucleus occurs and promotes the transcription of IFN type I (29).

Production of type-I IFN stimulates the surrounding cells to produce a wide range of antiviral proteins including protein kinase R (PKR), myxovirus resistance factor, 2'-5' oligoadenylate synthase/RNaseL and dsRNA adenosine deaminase1, which subsequently leads to the activation of eukaryotic initiation factor (eIF)-2, and translation inhibition of both host and viral mRNA (30).

HIV is commonly transmitted by sexual contact, and thus it initially interacts and activates the innate immune system and antigen presenting cells including macrophages and dendritic cells at the mucosal surfaces (8,31,32). These cells then migrate to the lymphoid tissues and secrete inflammatory cytokines such as IFN type-I (IFN-α/β), interleukin (IL)-1, IL-6, IL-12, and chemokines such as IL-8. This stimulates the adaptive immune system and leads to the activation and differentiation of various T cell populations. Activated CD4+ T cells differentiate into T helper (Th)-1 and Th-2 effectors, and memory CD4+ T cells. The Th-1 and Th-2 subsets of CD4+ T cells were originally defined by their polarized cytokine production patterns (described further in section 1.2200) (33,34). Th-1 cells produce IFN-γ, IL-2, IL-12 and lymphotoxin-α causing enhancement of antigen presentation, phagocytosis, and cell-mediated cytotoxicity. On the other hand, Th-2 cells secrete IL-4, IL-5, IL-9, IL-10, and IL-13 promoting antibody production by plasma cells (34-36). Activated CD8+ T cells differentiate into effectors and memory cytotoxic T cells. Cytotoxic T cells secrete IFN-γ, perforin, and granzymes to kill virus-infected cells. In the lymphoid tissues, HIV interacts and infects other cells such as CD4+ T cells and microglia, and disseminates to other areas such as the brain and gut (8,37). Subsequently, inflammatory cells and cytokines are accumulated during chronic infection and immune activation causing severe reactions and tissue pathology.

This includes destruction of immune cells, mainly CD4+ T cells, and overall impairment of immune functions, which are the hallmarks of chronic HIV infection (8,38-40).

1.2100 Monocytes/Macrophages (M/M):

Monocytes, which are the precursors of macrophages, as a part of the innate immune system, play a major role in controlling and clearing pathogens. M/M exhibit antimicrobial, antifungal, and antiparasitic properties (21-24). They possess phagocytic and endocytic activity. In addition, upon activation they produce cytokines such as IL-1 (α, and β), IL-6, IL-10 and IL-18, which also serve to propagate the adaptive immune response and eventually lead to proliferation and differentiation of T cells. Moreover, they act as antigen presenting cells by uptaking, processing, and presenting antigen in the context of major histocompatibility complex (MHC) class II to CD4+ T cells (21-24). These important M/M functions are largely driven and regulated by the responsiveness of these cells to numerous cytokines such as IFN-γ, IL-10, and Tumor Necrosis Factor (TNF)-α, and signals delivered to them via the TLR family, and different microbial antigens such as bacterial lipopolysaccharide (LPS) and viral proteins including those of HIV (21-24). Studies have shown that M/M functions are impaired over the course of HIV infection, thus contributing to the overall immune dysfunction and appearance of the opportunistic infections observed in HIV-infected patients. Several *ex vivo* and *in vitro* studies have reported that many M/M defects arise during chronic HIV infection including poor phagocytic activity (41-43), altered cytokine and chemokine secretion (44-47), impaired antigen uptake (48), and MHC class II expression (48,49). However, the molecular mechanism by which HIV impairs M/M function remains unclear.

One possible mechanism by which chronic HIV infection may affect M/M function is the modulation of programmed cell death (PCD) and signaling molecules as observed in other cell types including CD4+ and CD8+ T cells and neuronal cells (50). This may occur directly by the action of HIV and its different immunomodulatory proteins such as Gp120, Nef, Tat, and Vpr, or indirectly via its effects on the cytokine secretion profile induced during the course of the disease (51-53).

1.2110 Cell death:

Cell death was initially categorized into two types: necrosis, which is defined as an uncontrolled process of cell death resulting from acute cell injury, and programmed cell death (PCD) which occurs via a cellular cascade of signal transduction events (54). PCD is a normal and an essential physiological process which plays an important role in the regulation of many biological functions including embryo development and tissue differentiation, immune regulation and homeostasis, and normal cell termination (55,56). Thus, dysregulation of PCD contributes to the pathology of various diseases including cancer, neurodegeneration, and immune dysfunction (55,57). PCD has been stratified into at least two distinct forms based on the cells morphological characteristics and the cellular machinery/signaling pathway that are invoked. These include apoptosis (self-killing), and autophagy (self-eating) also known as macroautophagy (58,59). The major differences between PCD (apoptosis and autophagy) and necrosis are summarized in table 1-1 (60-62).

Table 1-1: Comparison between apoptosis, autophagy, and necrosis:

Features	Apoptosis	Autophagy	Necrosis
Main morphological characteristics	Chromatin condensation Nuclear fragmentation	Cytoplasmic vacuolization Loss of organelles	Rapid loss of membrane integrity Organelle swelling
Relative rate	Slow	Slow	Rapid
Caspase activation	Often dependent	Independent	Independent
Lysosomal activity	Not induced	Induced	Not induced
Autophagy-related gene activation	Independent	Dependent	Independent
Ability to be reversed or inhibited in presence of the inducer	Reversible	Reversible	Irreversible

1.2111 Apoptosis or PCD type I:

Apoptosis is a normal physiological process which involves a series of biochemical events leading to DNA fragmentation and cell death (60,63). It is characterized by a variety of morphological changes, including changes to the cell membrane such as phosphatidylserine translocation to the outer surface, loss of membrane asymmetry and attachment, chromatin condensation, cell shrinkage, nuclear fragmentation, and DNA degradation as well as membrane blebbing (63). Apoptosis plays a major role in controlling cellular growth, and thus insufficient or defective apoptosis results in uncontrolled cell proliferation and can lead to cancer, whereas excessive apoptosis may lead to immunodeficiency such as that observed in HIV infection, or the hypotrophy observed in ischemic damage.

Apoptosis can occur via different mechanisms, but the two most common and well characterized pathways include the death receptors signaling cascade (extrinsic pathway), and the mitochondrial stress signaling pathway (intrinsic pathway) (63). There is considerable overlap between these two pathways as shown in Fig. 1-4. Briefly, the extrinsic pathway is initiated when a ligand of the TNF family (TNF-α, TRAIL, Fas) binds to its death receptor on the cell surface. This leads to the activation of molecules downstream of death receptors called TNF receptor associated death domain (TRADD) and Fas associated death domain (FADD). Subsequently, specific enzymes known as initiator cysteine aspartate protease (caspases), such as caspases 8 and 10, bind to TRADD and FADD and thus become activated (63). Activation of the initiator caspases causes cleavage and activation of downstream effector caspases including caspase 3, 6, 7. This leads to nuclear poly (ADP-ribose) polymerase (PARP) cleavage, an enzyme involved in DNA repair, and DNA fragmentation and cell death eventually (63).

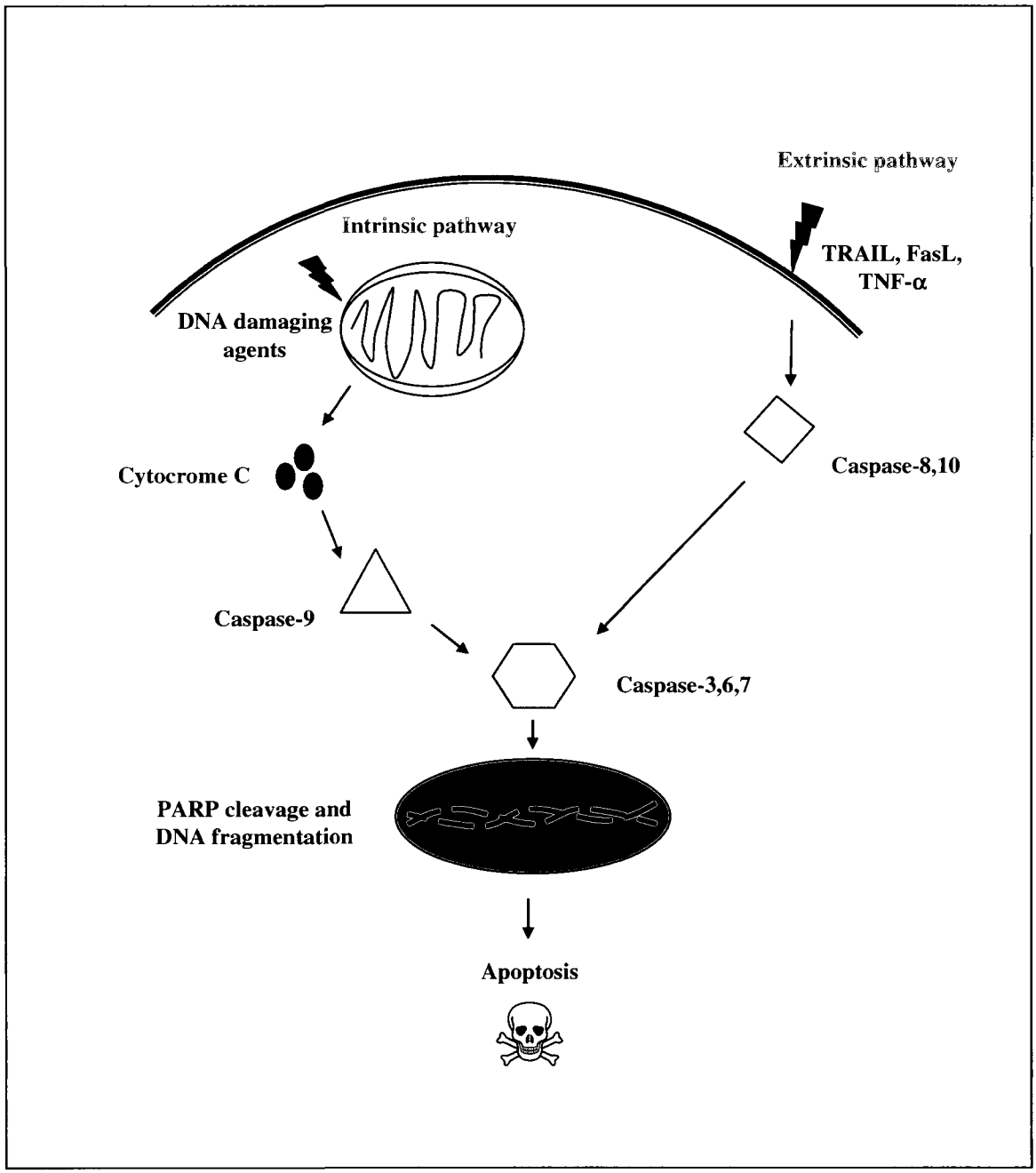

Extrinsic pathway
Intrinsic pathway
TRAIL, FasL,
TNF-α
DNA damaging
agents
Cytocrome C
Caspase-8,10
Caspase-9
Caspase-3,6,7
PARP cleavage and
DNA fragmentation
Apoptosis

Figure 1-4: General overview of apoptosis (PCD type I) pathways.

Classical apoptosis occurs by induction of extrinsic or intrinsic pathways. The extrinsic pathway is triggered when a ligand of the TNF family (TNF-α, TRAIL, Fas) binds to its death receptor on the cell surface. This leads to the activation of initiator caspases such as caspase 8, and 10. Activation of the initiator caspases causes cleavage and activation of the downstream effector caspases such as caspase 3, 6, 7 which then leads to PARP cleavage, DNA fragmentation, and cell death. On the other hand, the intrinsic pathway is activated by disruption of the mitochondrial membrane potential, which is regulated by a balance between the antiapoptotic and proapoptotic proteins of Bcl2 family including Bcl2, Bcl-XL and Bax, Bad, respectively, leading to cytocrome c release and activation of caspase 9. Activation of caspase 9 results in cleavage and activation of the effector caspases 3, 6, 7 which leads to PARP cleavage, DNA degradation, and cell death.

On the other hand, activation of the intrinsic pathway is usually initiated by the disruption of the mitochondrial membrane potential, which is regulated by a balance between antiapoptotic and proapoptotic proteins of the Bcl2 family including Bcl2, Bcl-XL and Bax, Bad, respectively. This leads to cytocrome c release and activation of the initiator caspase 9. Activation of caspase 9 results in cleavage and activation of the effector caspases 3, 6, 7. This causes PARP cleavage, and subsequently DNA degradation, and cell death (63).

1.2112 Autophagy or PCD type II:

Autophagy is another normal physiological and evolutionarily conserved catabolic cellular process that is associated with the activation of a cascade leading to the destruction of bulk cytoplasmic contents including proteins and organelles (60,64,65). This process was initially characterized as a cell survival mechanism during cellular starvation or limited nutrition in which degradation of cytoplasmic proteins by autophagy results in production of nutrients and energy that prolong cell survival (54). Studies have revealed that autophagic degradation of cellular proteins can occur by several mechanisms including macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy which is referred to as autophagy throughout the thesis involves a sequestration of the cytoplasmic material by a double membrane vesicle called an autophagosome, which is then delivered to the lysosome for further degradation (66). Microautophagy, on the other hand, involves a direct engulfment of cytoplasmic contents by the lysosome (67). Chaperone-mediated autophagy is also a receptor-mediated process that involves the transportation of a specific proteins to the lysosome (68). This mechanism of autophagy has been described to be essential for immunity, antigen presentation, and killing of

intracellular pathogens by macrophages (69,69,70,70-74). It has been shown that activation of chaperone-mediated autophagy results in the delivery of cytoplasmic antigens to MHC class II loading compartments and improvement of epitope presentation at the cell surface to CD4+ T cells (75-84). Unlike microautophagy and chaperone-mediated autophagy, only macroautophagy has been associated with cell death and will be the focus in my thesis (54).

Autophagy has been extensively studied in yeast, and most of the genes involved in its regulation are thus named autophagy-related genes (atg) (85-88). However, many of these genes have mammalian counterparts. For example, *atg6* and *atg8* are named *beclin-1* and *light chain (LC)-3*, respectively (89,90). It has been revealed that autophagy plays an important role in the regulation of many cellular processes including cell death and intracellular homoeostasis (56,91-93). A study in the mouse model has elucidated that double knockdown of beclin-1 was lethal during embryogenesis, whereas, single knockdown resulted in appearance of spontaneous tumors (94).

Studies have revealed that activation of the autophagy pathway could be triggered by different cellular stresses such as metabolic stress, inflammatory cytokines, and chemotherapeutic and pharmaceutical agents (82,93,95). It is a very complex process that remains incompletely understood. However, the best defined mechanism involves regulation of different Atg proteins at four stages of the process. A simplified schematic representation of the autophagy pathway is shown in Fig. 1-5. The initiation stage is triggered by derepression of the mammalian target of rapamycin (mTOR) (95).

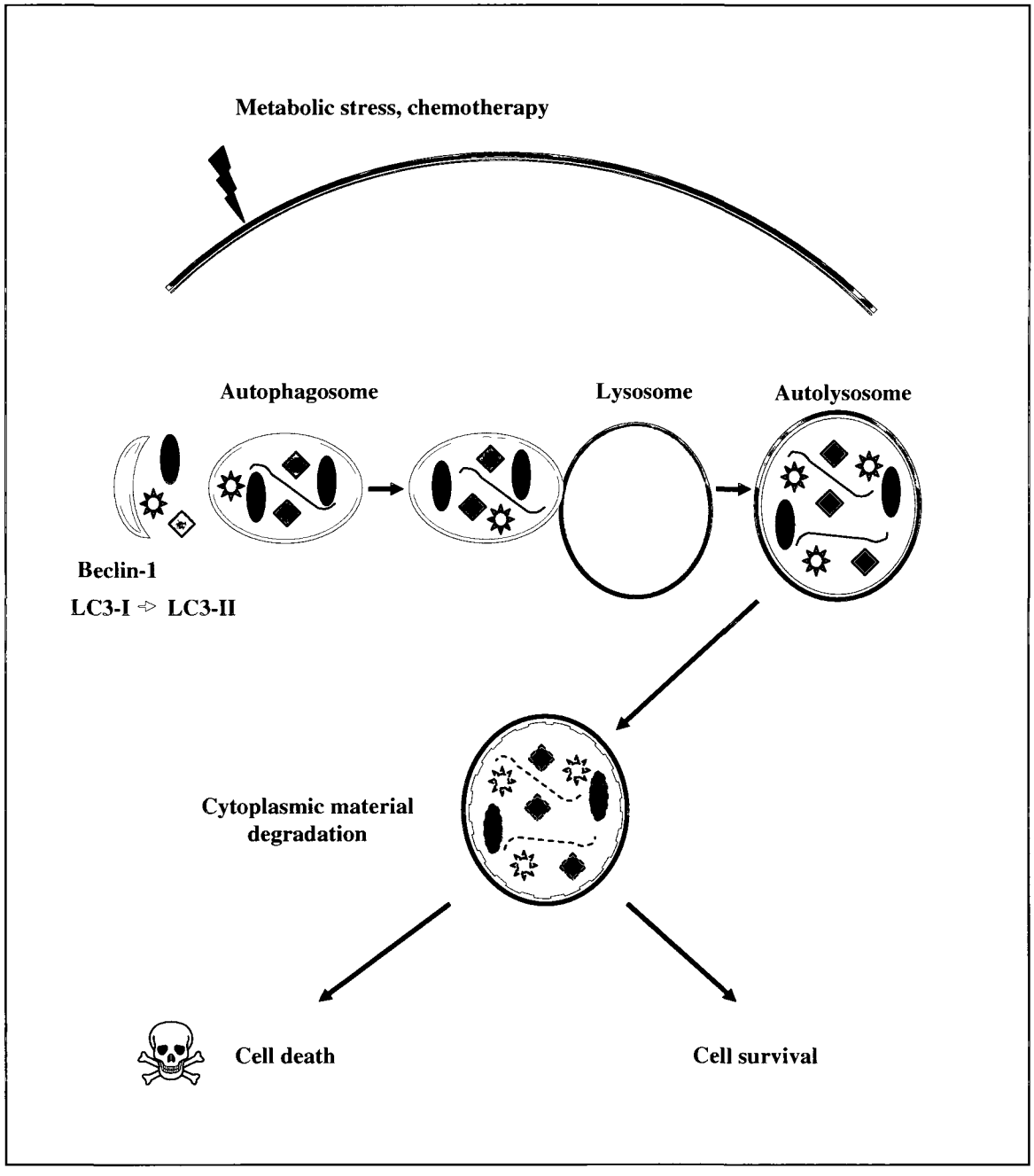

Metabolic stress, chemotherapy
Autophagosome
Lysosome
Autolysosome
Beclin-1
LC3-I ⇨ LC3-II
Cytoplasmic material degradation
Cell death
Cell survival

Figure 1-5: Schematic representation of autophagy (PCD type II) pathway.

Autophagy is initiated by metabolic stress such as nutrients/serum starvation or by chemotherapeutics or pharmaceutical agents (e.g. etoposide, rapamycin, respectively). It starts with a sequestration of a portion of cytoplasmic materials including proteins and organelles into a double membrane vesicle called an autophagosome. Subsequently, the autophagosomes fuse with endosomes and lysosomes forming autolysosomes. Finally, degradation of the cytoplasmic material in the autolysosome compartment may eventually result in cell survival or cell death.

Modified from reference # (93).

MTOR is a serine/threonine kinase that inhibits the autophagy cascade at the initiation stage by phosphorylating Atg13, leading to its disassociation from a protein complex composed of Atg17 and the Atg1 kinase. When mTOR action is inhibited, dephosphorylation of Atg13 occurs and then becomes accessible to binding to Atg17 and Atg1 to form a protein complex leading to the induction of the autophagy pathway. Subsequently, the vesicle nucleation stage is initiated by the activation of a class-III PI3K protein called mammalian Vps34. Activation of Vps34 requires the formation of a protein complex composed of Atg6/beclin-1, UV irradiation resistance associated tumor suppressor gene (UVRAG), and kinase Vps15/p150 (95). The vesicle elongation stage is then activated, and this can occur by two steps. The first involves binding of Atg5 to Atg12 by the action of two proteins Atg7 and Atg10 (95). The second step involves binding of phosphatidylethanolamine (PE) to Atg8/LC3 by the action of Atg4, Atg7, and Atg3. This lipid binding results in conversion of the cytosolic form of LC3-I to the autophagosome associated form LC3-II. As a result, a portion of the cytoplasm that contains intracellular organelles and proteins, is sequestered by a double membrane vesicle called an autophagosome (95). Subsequently, the autophagosome maturation stage occurs by fusion of autophagosomes with endosomes and lysosomes leading to the formation of autolysosomes (93,95). The cytoplasmic materials and the inner membrane of autolysosomes are degraded within this compartment (93).

Autophagy is characterized by increased cytoplasmic vacuolization (formation of double membraned vesicles) and an increased expression of autophagy related proteins including Atg6/beclin-1 and Atg8/LC3 (62). Although it is called PCD type II, studies have revealed that through incompletely understood mechanisms, activation of the autophagy pathway promotes cell survival under certain

circumstances while others promote cell death (54,91). For instance, a recent report has shown that treatment of fibroblast L929 cells with the pan caspase inhibitor (z-VAD) resulted in cell death which depended on the induction of autophagy. However, nutrients/serum starvation of these cells induced autophagy that served to enhance their survival when stimulated with z-VAD (96). Another study has shown that knocking down Atg5 inhibits IFN-γ-induced autophagy and cell death in Hela cells. In the same study, ectopic expression of Atg5 in the same cells caused cell death (97).

To date, it is not clear what drives autophagy to cause cell death *vs* survival. Further, there appear to exist a complex and poorly understood interplay between autophagy and apoptosis (95,98). A number of reports have suggested that blocking apoptosis pathways enhances cell death via the autophagy pathway and vice versa (99-101). Further studies have demonstrated interactions between these two pathways at the molecular level. For example, it has been shown that bcl2 interacted with beclin-1 and Atg5 during autophagic cell death in Bax-/-, Bak-/- fibroblast cells (102). Another study has shown that FADD interacted with Atg5 during IFN-γ-induced autophagy and cell death in Hela cells (97). Moreover, it has been demonstrated that downregulation of caspase 8 resulted in upregulation of beclin-1, Atg7, and autophagy-induced cell death in the fibroblast cell line L929 cells (103).

1.2120 Monocyte/Macrophage cell death:

Although evidence has shown that both CD4+ T cells and monocytic lineage cells are susceptible to HIV infection and replication, only CD4+ T cells are progressively depleted from the host over the course of infection (104,105). It has been suggested that CD4+ T cell depletion is directly linked to HIV pathogenesis and progression to AIDS. This is supported by studies demonstrating that treatment of

HIV+ patients with HAART resulted in a significant restoration of CD4+ T cell counts (105,106), and improved immunity. Thus, the mechanism underlying CD4+ T cell depletion has been an interest for many researchers, and numerous *in vitro* and *ex vivo* reports have been published in this regard (107,108). Studies have shown that destruction of resting or HIV infected CD4+ T cells can occur by several mechanisms including syncytium formation, induction of classical extrinsic and intrinsic apoptosis pathways (9,107,108), as well as recently, the autophagy pathway (109,110). This CD4+ T cell depletion can result from the effects of HIV and its proteins including Gp120, Tat, Nef, and Vpr on CD4+ T cells. Also, it can occur by the action of immune cells due to chronic immune activation (111-113). It has been shown that binding of HIV envelope to CD4 and chemokine coreceptors induced apoptosis in CD4+ T cells (114-118). Other studies have shown that HIV Tat, and Nef were able to induce apoptosis via FasL expression and caspase 8 activation (51,119,120), whereas Vpr induced apoptosis via the mitochondrial pathway (121). Further, it has been revealed that treatment of monocytes with HIV Tat led to TRAIL production and apoptosis of bystanding CD4+ T cells (53,122). Furthermore, recent reports have shown that HIV and its Gp120 protein affected the autophagy pathway in CD4+ T cells. It was shown that exogenous Gp120 induced cell death in uninfected CD4+ T cells via activation of the autophagy pathway (123). Another *in vitro* study has revealed that HIV infection inhibited autophagy in infected CD4+ T cells (124).

Unlike CD4+ T cells, M/M are not progressively depleted from the host. This may be due to their relative resistant to PCD during HIV infection. These cells have the ability to harbor a great amount of the virus without being destroyed (124). Thus, it is believed that M/M are one of the major sources of HIV reservoirs (125). The molecular mechanisms responsible for the resistance of these cells to cell death are

still not clear. One *ex vivo* study has revealed a role for IL-13 in the protection of monocytes spontaneous cell death from HIV+ patients (126). It is possible that the increased resistance of M/M to cell death observed during HIV infection may be due to the alteration of cytokine responsiveness. Thus, understanding the molecular mechanism underlying the resistance of M/M during HIV infection may be important for elimination of these viral reservoirs.

1.2200 Cytokines:

As mentioned above, cytokines are small secreted proteins that function as mediators to regulate both the innate and adaptive immune responses (21). They mainly transmit the biochemical message from the extracellular environment to the nucleus of the targeted cell via cytokine-cytokine receptor interaction and triggering of complex intracellular signal transduction (127,128). They can affect cell function in a paracrine as well as an autocrine manner. There are many cytokines produced by the immune system. Certain cytokines are associated with the initial response to an infection or inflammation and are referred to as inflammatory cytokines. Other cytokines are induced according to the nature of the infectious antigens and the type of immune responses produced against these antigens which differ in their action and function. For instance, infection with *Influenza virus*, *Vaccinia virus*, or *Listeria monocytogenes* is known to induce a Th-1 immune response (129). This type of immune response is associated with the production of cytokines such as IL-2, IFN-γ, and IL-12, which regulate cell-mediated immunity including delayed hypersensitivity reactions, activation of macrophages and leukocyte cytolytic processes, and result in the protection and elimination of intracellular pathogens (34,127,130). On the other hand, infection with *Nippostrongylus barsiliensis* or *Leishmania major* is known to

induce a Th-2 response (129). This immune response is characterized by secretion of cytokines such as IL-4, IL-5, IL-9, IL-10, and IL-13 that predominantly regulate antibody-mediated immunity and generally lead to the protection and clearance of extracellular antigens/pathogens (34,127,130). During chronic HIV infection, both types of immune response and their associated cytokines are dysregulated, and thus may alter M/M effector functions and homeostasis (131,132).

In my research, the focus was on cytokines that play an important role in regulating M/M effector function and cell survival such as IFN-γ, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-4, as summarized in table 1-2. IFN-γ is a potent pleiotropic cytokine produced from Th-1, NK cells, and CD8+ T cells. It has a critical role in the regulation of both innate and adaptive immunity (133,134). It inhibits Th-2 and promotes Th-1 cell differentiation. Also, it inhibits viral replication and regulates cell death (133,134). Moreover, it activates monocytes and macrophages, increases MHC class II expression, promotes antigen processing and presentation, and enhances their phagocytic, antimicrobial, and tumoricidal activity (135-140). For instance, it has been shown that treatment of M/M with IFN-γ resulted in enhanced phagocytic activity against many pathogens including *Aspergillus fumigatus, Cryptococcus neoformans, Listeria monocytogenes, Mycobacterium avium, Toxoplama cruzi and gondii* (42,137,141). Other studies have revealed that the lack of IFN-γ responses, such as in IFN-γ, IFN-γ receptor (IFN-γ-R), or STAT1 deficient mice, or in patients with mutation in IFN-γ-R, lead to impaired immunity and susceptibility to infection by several opportunistic microbes and other pathogens (142-146).

Table 1-2: Cytokines and their effects on monocyte/macrophage function:

Cytokine	Producer cells	Effects on monocytes/macrophages
IFN-γ	Th-1 lymphocytes, activated NK cells, and CD8 T cells	Upregulates the expression of MHC class I and II, and activates pathogen killing.
IL-10	T cells, Macrophages	Potent suppressor of monocytes/macrophage function (e.g downregulates MHC class II expression, antigen presentation, phagocytosis).
GM-CSF	T cells, Macrophages	Stimulates growth and differentiation of myelomonocytic lineage cells. Enhances phagocytosis
IL-4	Th-2 lymphocytes	Induces expression of MHC class II, induces endocytosis, and mannose receptor expression.

GM-CSF is a 22 kDa protein secreted by macrophages and T cells. It facilitates growth and differentiation of monocyte and granulocyte lineages. It also enhances M/M effector functions including phagocytic, antimicrobial and antiparasitic activity (147,148). IL-10 is a potent immunosuppressive and anti-inflammatory cytokine produced by macrophages and T cells. It downregulates MHC class II molecule expression and presenting antigens to CD4+ T cells (149,150). It also inhibits the expression of costimulatory molecules, B7.1/B7.2, on monocytes and macrophages as well as the production of various cytokines such as TNF-α, IL-1, IL-2, IFN-γ, IL-3, and GM-CSF (149,151,152). In addition, it suppresses macrophage nitric oxide production, and anti-fungal activity (153). Moreover, it stimulates proliferation and differentiation of B cells, and polarizes T cells towards a Th-2 type response (35,154).

IL-4 is a 20 kDa cytokine secreted by Th-2 lymphocytes. It has dual immunoregulatory functions (36). It enhances macrophage cytotoxicity, MHC class II and mannose receptor expression (155-160). On the other hand, it inhibits cytokine secretion such as TNF-α, IL-1, IL-6, IL-18, GM-CSF and granulocyte colony-stimulating factor (G-CSF) (161-170). It also suppresses cytokine-induced macrophage activation, oxidative burst, and intracellular killing (138,171). Moreover, it downregulates monocyte adhesion, and CD14 expression (172,173), monocyte-mediated cytotoxicity, nitric oxide production, and anti-fungal activity (153,174). It has been reported that during the course of HIV disease progression many inflammatory and anti-inflammatory cytokines such as TNF-α, IFN-β, IFN-γ, IL-18, IL-2, IL-10, and IL-4 are dysregulated and may play a role in alteration of M/M functions and PCD (131,175-183).

1.2300 Cytokine signaling pathways:

Cytokine signaling pathways can be defined as biochemical signaling cascades that are triggered within minutes to relay the information required for various cellular functions (184-188). Most cytokines share a common mechanism of signal transduction in which cytokine-cytokine receptor binding causes the assembly of the specific receptor subunits. Subsequently, a number of tyrosine kinases from the Src and Syk families are activated leading to the signal transduction through mainly three major signaling pathways: the Janus Associated Kinase (JAK)/Signal Transduction and Activator of Transcription (STAT), the Phosphoinositide 3-kinase (PI3K), Mitogen-activated protein kinase (MAPK) (189-191). These signaling pathways form a very complex and evolutionarily conserved network.

A general overview of these cascades is illustrated in Fig. 1-6. Briefly, when ligand-receptor interaction occurs subsequent events are activated based on the type of receptor. For example, a receptor with kinase activity (growth factor receptor) is usually autophosphorylated directly leading to the creation of a docking site for an adapter protein complex called Grb2/SOS (son of sevenless) (192). As a result, SOS is recruited to the plasma membrane where it encounters and activates a small G protein named Ras (192-194). Activated Ras induces activation of several downstream signaling molecules including a serine/threonine kinase called Raf which in turn activates the MAPK, and PI3K signaling pathways (192,194,195). PI3K signaling molecules can also be activated directly via the p110α catalytic subunit of the PI3K (194).

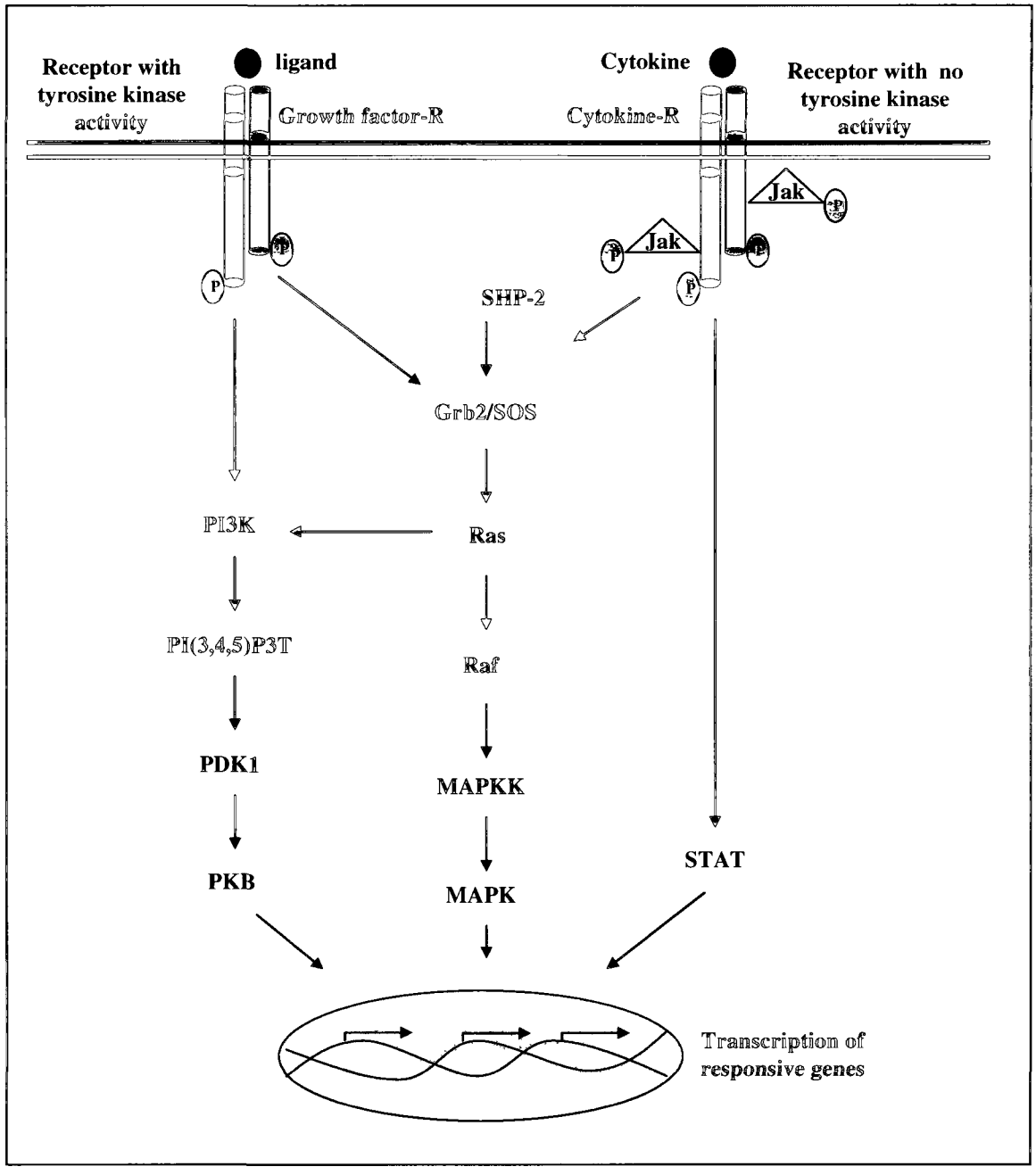

Receptor with tyrosine kinase activity
ligand
Growth factor-R
Cytokine
Cytokine-R
Receptor with no tyrosine kinase activity
Jak
Jak
P
SHP-2
Grb2/SOS
PI3K
Ras
PI(3,4,5)P3T
Raf
PDK1
MAPKK
PKB
MAPK
STAT
Transcription of responsive genes

Figure 1-6: Schematic overview for the cytokine / growth factor intracellular signaling pathways.

Upon ligand-receptor binding signal transduction occurs based on the type of the receptor. Receptors that have tyrosine kinase activity are usually autophosphorylated at tyrosine residues directly and create docking sites for different signaling molecules that have SH2 and PTB domains. Grb2/SOS complexes bind to docking sites and lead to recruitment of SOS to the plasma membrane where they interact with Ras. Subsequently, activated Ras activates several downstream molecules including Raf, MAPKK, and MAPK. PI3K signaling pathway can be activated directly via the p110α catalytic subunit of the PI3K. Receptors with no kinase activity, on the other hand, generally require activation of cytoplasmic domain associated receptor JAKs. Subsequently, activation of JAKs will lead to phosphorylation of the receptor and creation of docking sites for several signaling molecules including the STAT signaling pathway.

Modified from reference # (192).

A receptor with no kinase activity (e.g. cytokine receptor) generally requires activation of membrane associated kinases such as JAKs for its phosphorylation. Subsequently, activated JAKs can activate STAT signaling pathway directly or interact and activate Grb2/SOS which in turn activate PI3K and MAPK (188,192,196,197).

Evidence has also demonstrated a complex cross talk between these pathways. For instance, it has been shown that JAK2 is responsible for the activation of STAT, Erk MAPK, and Akt signaling pathways in response to growth hormone in hepatoma and preadipocyte cells (198). Another report has demonstrated a role for Akt in serine phosphorylation of the STAT1 transcription factor and upregulation of gene expression in response to IFN-γ (199).

1.2310 JAK/STAT signaling pathway:

The Janus Associated Kinase (JAK)/Signal Transduction and Activator of Transcription (STAT) pathway is one of the major signaling pathways involved in cytokine responses. The general sequence of events in the JAK/STAT signal transduction pathway is illustrated in Fig. 1-7. Initially, cytokine-receptor interaction triggers tyrosine transphosphorylation of the JAKs. This is followed by phosphorylation of receptor tails by JAKs and recruitment of the latent STAT proteins via their Src homology 2 (SH2) domains to the activated (tyrosine phosphorylated) receptor. This is followed by STAT tyrosine phosphorylation. Activated STATs form dimers that are translocated into the nucleus where they bind to STAT responsive elements (184,185,187), and subsequently promote transcription of STAT responsive genes such as cytokine-inducible SH2-containing protein (CIS), members of IRF family, and many other genes (200-202).

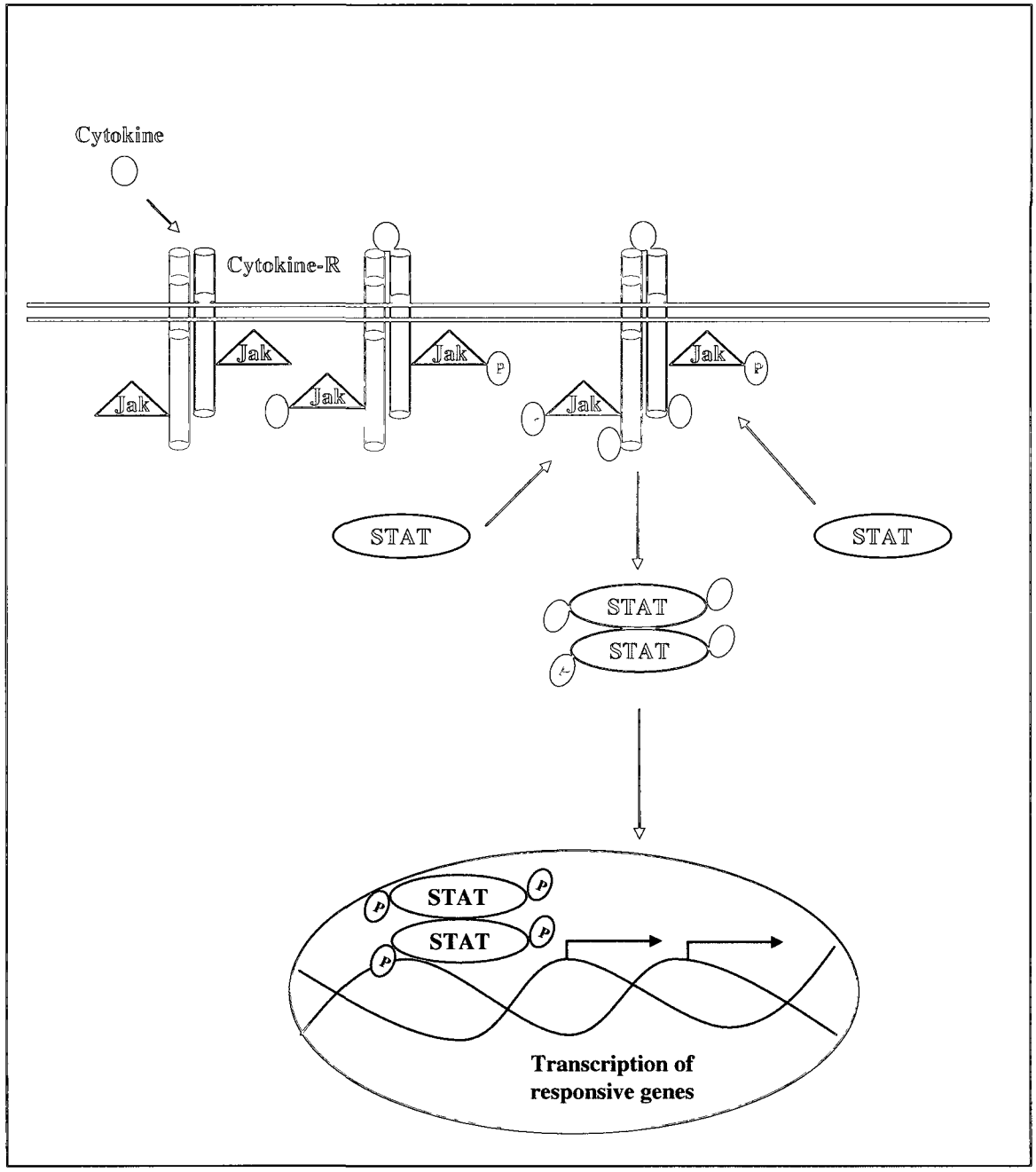

Cytokine
Cytokine-R
Jak
Jak
Jak
Jak
Jak
Jak
P
P
STAT
STAT
STAT
STAT
P
P
P
P
STAT
STAT
Transcription of
responsive genes

Figure 1-7: General model for the JAK-STAT signaling pathway.

Cytokine-receptor interaction triggers tyrosine transphosphorylation (tyr-p) of the JAKs. This is followed by tyr-p of receptor tails by JAKs and recruitment of the latent STAT proteins via their SH2 domain to the activated receptor. This is followed by tyr-p of STAT. Activated STATs form dimers and translocate into the nucleus where they bind to sequence specific responsive elements in the promoter of STAT responsive genes, and activate their transcription.

Modified from reference # (186).

In mammalian cells, four JAKs (Jak1, Jak2, Jak3 and Tyk2) and seven STAT proteins (STAT1, 2, 3, 4, 5a, 5b, and 6) with their different isoforms have been identified. STAT proteins have been shown to play an important role in regulating and maintaining both innate and adaptive immune responses (summarized in table 1-2) (184-187). For instance, studies have demonstrated a correlation between impairment of JAK/STAT signaling and increased susceptibility to many infections including HIV (141,143,146,203). It has been shown that impairment of IFN-γ-induced JAK/STAT signaling caused a defect in the upregulation of MHC class I, and IRF-1 expression, and increased sensitivity to HIV infection in a subclone of U937 cells (203).

A number of reports have suggested that defective cytokine responsiveness could exist in different cell types during chronic HIV infection. It has been shown that IL-2-induced STAT5 activation was impaired in CD8+ T cells from a subset of HIV-infected patients naive to therapy, but was restored, at least in part, after HAART treatment (50). In other *in vitro* studies, activation of STAT5 in response to IL-2 was inhibited by HIV-1 infection, or by Gp120 and anti-CD4 antibody pretreatment of CD4+ T cells (204,205). Furthermore, GM-CSF-induced STAT5 activation in monocyte-derived macrophages (MDM) is inhibited by HIV-1 infection *in vitro* (206). It has also been shown that HIV and its Gp120 and Nef proteins are capable of activating STAT1 and STAT3 in monocytic cell lines and MDM (207-209). However, the effect of *in vivo* chronic HIV infection on cytokine responsiveness in monocytes is unknown.

Table 1-2: STATs proteins and their role in the immune system:

STAT proteins	Activating cytokines	Examples of STAT responsive genes	Phenotype of knockout mice
STAT1	IFNs, IL-6, IL-10	IRF-1, ISG54, MIG, GBP, CIITA	Impaired IFN and innate immune responses, increased susceptibility to tumors, opportunistic and viral infections
STAT2	IFNs	IRF-1, ISG54	Impaired Type-I IFN responses.
STAT3	IL-2, IL-6, IL-10	JunB, SAA3, JAB, C-reactive protein, BclxL.	Embryonic lethal
STAT4	IL-12	IFN-γ, IRF-1, MHC class II, CD23, Fc-γRI	Defect in IL-4 and IL-12 responses, and impaired Th-1 differentiation.
STAT5 a, b	Numerous e.g. IL-2, IL-7, IL-15, GM-CSF	CIS, IL-2R-α, β-casein, osm, pim1, p21	Impaired growth and proliferation, defect in IL-2 responses.
STAT6	IL-4, IL-13	IL-4R-α, C-γ-1, C-γ-4	Defect in IL-4 responses, and impaired Th-2 differentiation.

1.2320 PI3K signaling pathway:

Phosphoinositide 3-kinases or phosphatidylinositol-3-kinases (PI3Ks) are a family of enzymes that has serine/threonine kinase activity. These enzymes can be activated by various stimuli such as growth factor, antigens, cytokines (210,211), and are capable of phosphorylating the third position hydroxyl group of the inositol ring of phosphatidylinositol (PtdIns) (210,212). This family is composed of four classes, which differ in their structure and functions (known as Ia, Ib, II, and III). However, all of them contain at least one catalytic domain and one regulatory domain (210,212). Many of PI3K cellular functions rely on the ability of PI3Ks to activate protein kinase B (PKB, also known as Akt) (Fig. 1-5). In humans, three Akt genes have been identified named *akt1*, *akt2*, and *akt3*.

PI3-kinases have been shown to play a major role in diverse cellular functions, including cell growth, proliferation, differentiation, survival, and migration (213-215). Thus, dysregulation of this pathway may influence different cellular responses that are associated with tumors (210). It has also been reported that there is a basal activation of the PI3K/Akt pathways in macrophages that is required for their survival (216). Reports have demonstrated a critical role for PI3K signaling in chronic immune activation by promoting cell survival. For instance, a study by Pauline Chugh *et al.* has revealed that *in vitro* HIV infection and its protein Tat was sufficient to activate PI3K/Akt pathway in macrophages. Interestingly, PI3K/Akt inhibitors including Miltefosine, an antiprotozoal drug known to inhibit PI3K/Akt pathway, significantly reduced HIV-1 production from infected macrophages and increased susceptibility to cell death in response to extracellular stress as compared to uninfected cells (217). Another study has shown that inhibition of Akt phosphorylation is required for TRAIL-induced cell death in HIV infected macrophages (218).

1.2330 MAPK signaling pathway:

Mitogen-activated protein kinases (MAPKs) are also a family of enzymes that have serine/threonine kinase activity. This family of kinases is generally activated in response to various extracellular stimuli such as growth factors and inflammatory signals, as well as cellular stress. They regulate different cellular processes including mitosis, proliferation, differentiation, and cell death (219). The MAPK family is composed of three major subfamilies of kinases known as the extracellular receptor kinases (ERKs), the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) and the p38 MAP kinases (220). Activation of a specific MAP kinase requires activation of a small GTP binding protein (e.g. Ras) which involves a phosphorylation of a series of downstream kinases (Fig. 1-5) (193). Activation of MAPK kinase kinase (MAPKKK) (e.g. Raf) leads to the activation of downstream MAPK kinase (MAPKK), and finally the specific MAPK (221,222). The ERK MAPK family is found in two isoforms called Erk1 and Erk2. Both isoforms are phosphorylated by members of the MEK family, which are often activated by extracellular stimuli such as growth factors, LPS and chemotherapeutic agents (195,223,224). The JNK family is found in three isoforms named JNK1, JNK2, and JNK3 (225), while the P38 family is found in five different isoforms called P38 (SAPK2), P38β, P38β2, P38γ (SAPK3), and P38δ (226,227). Both JNK and P38 MAPKs are phosphorylated by SAPK/Erk kinases (SEKs) and mitogen-activated protein kinase kinases (MKKs), which are usually induced by inflammatory cytokines as well as different environmental stresses such as endotoxins, oxidative stress, protein synthesis inhibitors, and ultraviolet (UV) irradiation (225,228-230). MAPKs have been shown to activate various downstream transcription factors such as

activator transcription factor (ATF)-2, SP-1 (a member of Sp/KLF family) and activator protein (AP)-1, and even STAT3 (230-233).

Several studies have demonstrated a role for MAPK in regulating monocyte function and cell death. For example, it has been shown that the HIV Tat protein stimulates IL-10 production via activation of calcium/MAPK signaling pathways in human monocytes (234-236). Another report has suggested that HIV Vpr is capable of inducing the activation of all MAPK members ERK, P38 and JNK, and caspase dependent cell death in primary monocytes and the monocytic cell line THP-1 cells (237). However, only blocking of the JNK MAPK pathway appeared to be required for preventing Vpr-induced monocyte PCD (237).

1.3000 Rationale

M/M are critical in clearing many pathogens via their phagocytic and antimicrobial properties. Evidence has shown that many of M/M functions become defective during the course of chronic HIV infection. However, the mechanism of this impairment remains unclear. One plausible mechanism by which HIV alters M/M function is through the modulation of cytokine responsiveness and PCD. Cytokine responsiveness via the JAK/STAT signaling pathway plays a major role in the regulation of different cellular functions including proliferation, differentiation, and cell survival. Kryworuchko *et al.* have shown that IL-2-induced STAT5 activation is impaired in CD8+ T cells from a subset of HIV-infected patients naive to therapy, but was restored under HAART (50). However, the effect of *in vivo* chronic HIV infection on the JAK/STAT signaling pathway and cell death in monocytes remained to be investigated. Thus, the focus in my thesis was to evaluate cytokine responsiveness via the JAK/STAT signalling pathway in monocytes from HIV+

patients and determine the biological significance and the molecular mechanism responsible for the alterations observed.

1.4000 Hypothesis

Abnormal JAK/STAT signaling and cytokine responsiveness in monocytes from HIV+ patients may result in impaired monocyte function and cell death, and thus contribute to the immune deficiency observed in the course of HIV infection.

1.5000 Objectives

The main aim of this study was to investigate whether defects in cytokine responsiveness existed in monocytes from HIV+ patients, and to elucidate the biological significance and the molecular mechanism responsible for the alterations observed. The specific objectives were as follows:

1. Evaluate and compare cytokine dependent JAK/STAT signal transduction in monocytes from HIV- controls and chronically-infected HIV+ patients
2. Investigate the molecular mechanisms responsible for the HIV-induced hyperactivation of STAT1 observed in response to IFN-γ
3. Determine the biological impact of the IFN-γ-induced STAT1 hyperactivation in monocytes from HIV+ patients, particularly focusing on its effect on PCD

In general and irrespective of chronic HIV infection, the mechanisms responsible for monocyte PCD and its regulation by IFN-γ and IL-10 have not been well characterized. Therefore, subsequent objectives were designed to address this knowledge gap as follows:

4. Investigate what form of PCD occurs in primary human monocytes

5. Determine the molecular mechanisms involved in the regulation of monocyte PCD by IFN-γ and IL-10

Chapter II

Materials and Methods

2.010 Patient characteristics:

Samples from HIV- volunteers (n=12) were collected at the Infectious Diseases and Vaccine Research Center in the Children's Hospital of Eastern Ontario. Chronically-infected HIV+ patient samples were obtained from the Immunodeficiency Clinic at Ottawa General Hospital in collaboration with Dr. Jonathan Angel. The samples included those from patients on antiretroviral therapy (ART) for >1 year (n=28) which are characterized by low viral loads [<50 copies/mL] and relatively high CD4 counts [603 (291-1002) cells/μL] [mean (range)], and those from patients off therapy (off ART) for >6 months (n=20) which manifested by high viral loads [94 (6-300) x 10^3 copies/mL] and relatively low CD4 counts [312 (9-733) cells/μL].

2.020 Cytokines, antibodies, and other reagents:

The recombinant human cytokines used for cell stimulation were: IFN-γ (10 ng/mL) (Pierce, Rockford, IL, USA), IFN-α2a (100 U/mL) (PBL Biomedical Laboratories, New Brunswick, NJ, USA), GM-CSF (10 ng/mL), IL-10 (10 ng/mL), IL-4 (4 ng/mL), and TRAIL (100 ng/mL) (R&D Systems, Minneapolis, MN, USA). The recombinant HIV proteins Gp120 (1μg/mL), and Tat (100 ng/mL) were obtained from the National Institute of Health AIDS Reference and Reagent Program. HIV Vpr peptides (1-45) and (52-96) (0.1-1.5 μM) were synthesized by Genemed Synthesis Inc (San Francisco, CA). The anti-human monoclonal antibodies (mAbs) used for flow cytometry were: PerCp-labeled CD14 (monocyte specific marker), Alexa fluor 488-labeled phospho-STAT1, phospho-STAT3, phospho-STAT5, phospho-Erk1/2, and IgG2a Isotype control, PE-labeled phospho-STAT6, phospho-p38, STAT1, and IgG2a Isotype control, FITC-conjugated HLA-DR, and IgG1 and IgG2b Isotype controls

(BD Biosciences), PE-IFN-γ-Receptor1 (IFN-γ-R1) (Biolegend, San Diego, CA, USA), purified-IFN-γ-R2 (Cell Science, Canton, MA, USA), FITC-conjugated goat F $(ab)_2$ anti-mouse IgG (H+L) secondary antibody (Cedarlane Laboratories). Primary antibodies (Ab) used for western blotting were: Phospho-ser 737 STAT1 (Millipore), STAT1, STAT3 (Santa Cruz), β-actin (Sigma), GAPDH, phospho-tyr STAT1, phospho-ser Akt, Akt, Phospho-P38, Phospho-Erk1/2, Caspase 3, Beclin-1, and LC3-B (New England Biolabs Ltd, Pickering, ON, CA). The secondary antibodies were horseradish peroxide-conjugated goat anti-rabbit (H+L), and goat anti-mouse (H+L) antibodies (Bio-Rad), neutralizing antibodies: anti-TRAIL (BD), Anti-FasL (Biolegend). The pharmaceutical inhibitors used were: chloroquine (6-50 μM), and 3-methyladenine (5-10 mM) autophagy inhibitors (Sigma), Jak inhibitor I (50-300 nM), a pan Jak inhibitor, LY294002 (2.5-10 μM), a PI3K inhibitor, PD98059 (25-50 μM), an Erk MAPK specific inhibitor, z-VAD-FMK (0.625-5 μM), a general caspase inhibitor, rapamycin (2 μM), a positive control for activation of the autophagy pathway, (CalBiochem), and staurosporine (2 μM), a positive control for cell death, (Sigma). The small interfering RNAs used were: FITC-non silencing siRNA, Akt1/2 siRNA, Akt3 siRNA, and LC3B siRNA, were purchased and prepared as instructed by the manufacturer (Santa Cruz), non-silencing siRNA, STAT1 siRNA (Qiagen), and STAT3 siRNA were also purchased and ready to use according to the manufacturer's instructions (New England Biolabs Ltd).

2.030 Cell lines and tissue culture:

The human monocytic cell lines U937, THP-1, the myeloblastic cell line HL-60, and the human T cell line Jurkat were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). These cell lines were regularly maintained by

culturing in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma Chemical Company, St. Louis, MO) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) (GIBCO Laboratories, Grand Island, NY) and 100 U/mL penicillin, 100 µg/mL gentamicin. Cells were maintained at 37°C, and 5% CO_2 in a humidified incubator. Cells were washed, resuspended in fresh growth medium and transfered into a new T-75 cm^2 flask (BD Biosciences, MA, USA) after reaching confluency at approximately 10^6 cells/mL. In order to maintain them under consistent culture conditions, cells were frozen in FBS containing 10% dimethyl sulfoxide (DMSO) in cryotubes vials (Nunc), kept stored at 150°C freezer, and thawed when needed.

2.040 Isolation and cryopreservation of peripheral blood mononuclear cells (PBMCs):

Whole blood was collected in sodium heparin-containing 10 mL BD Vacutainer tubes. Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood by density gradient centrifugation on Ficoll-Paque™ Plus (GE Healthcare). The blood was layered onto 4 mL of Ficoll-Paque™ Plus in 15 mL centrifuge tubes and centrifuged for 30 minutes at 400 g with the brake off. The PBMCs in the interphase layer were collected and washed two times with sterile PBS and a third wash with culture medium containing 10% heat-inactivated fetal bovine serum (FBS-IMDM), 100 U/mL penicillin, 100 µg/mL gentamicin antibiotics. Cells were resuspended in 10 mL of 10% FBS-IMDM and an aliquot was diluted 10 fold and then stained with Trypan Blue solution (0.4%). Live cell number was determined by counting cells which excluded the dye on a hemacytometer (238,239). Frozen samples were cryoperserved in FBS containing 10% DMSO at concentration of $5X10^6$ cells/mL and stored at -80°C freezer for future use.

2.050 Monocyte isolation:

CD14+ monocytes were positively selected from PBMCs using anti-CD14 antibody-conjugated microbeads (Miltenyi Biotec). The procedure was carried out according to the manufacturer's protocol. PBMCs collected from whole blood were washed and resuspended in cold PBS containing 0.5% bovine serum albumin (BSA) and 2mM EDTA at a concentration of 10^7 cells/80 µL. 20 µL of CD14 microbeads/10^7 cells were added and incubated at 4°C for 15 minutes. Cells were then washed in cold PBS (0.5% BSA and 2 mM EDTA) and centrifuged at 300 g for 10 minutes. Cells in the positive fraction were collected using the AUTOMACS system (Miltenyi Biotec). CD14+ monocyte purity was determined by flow cytometry as shown in Fig. 2-1.

2.060 Flow cytometry:

2.061 Intracellular staining:

Frozen PBMCs were thawed, washed three times, and resuspended in 10% FBS-IMDM medium at a concentration of $4X10^5$ cells/mL. Cells were stimulated with IFN-γ (10 ng/mL), GM-CSF (10 ng/mL), and IL-10 (10 ng/mL), IL-4 (4 ng/mL) and incubated at 37°C and 5% CO_2 for 15 minutes. Cells were fixed and permeabilized in 2% paraformaldehyde (PFA) and 100% methanol, respectively, for 10 minutes at 4°C. Cells were then washed with staining buffer (PBS containing 0.1% BSA), and resuspended in 100 µL of the same buffer. Specific monoclonal anti-P-STAT antibodies (90 ng/mL) in parallel with lineage specific anti-CD14 antibody (0.25 µg/100 µL) were added to the cells for 15 minutes at 25°C. The cells were washed with staining buffer and fixed in 2% PFA for further flow cytometric analysis.

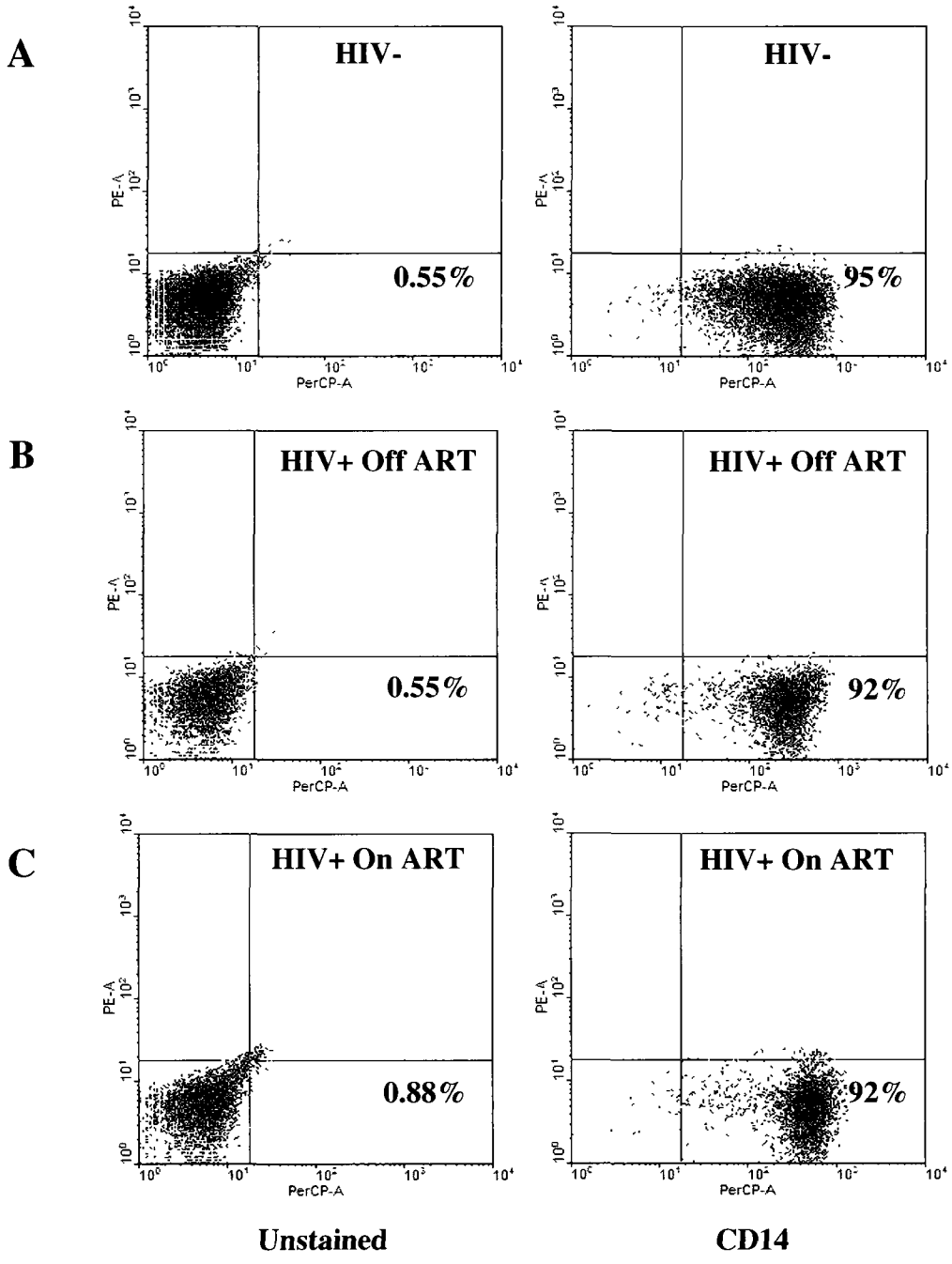

A
HIV-
0.55%
HIV-
95%
B
HIV+ Off ART
0.55%
HIV+ Off ART
92%
C
HIV+ On ART
0.88%
HIV+ On ART
92%
PE-A
PerCP-A
Unstained
CD14

Figure 2-1: The purity of CD14+ monocytes from HIV- and HIV+ individuals.

CD14+ monocytes were purified from PBMCs of HIV- and HIV+ samples by positive selection following the manufacturer's protocol. Cells were then stained with monoclonal anti-CD14 antibody for 15 min, to determine the % of CD14+ in each sample. Within the whole cell gate, 10000 events were acquired. A dot plot analysis for one representative experiment out of at least three performed was plotted. A comparison between unstained and stained cells was carried out. **A)** Dot plot from an HIV- sample shows % of CD14+ monocytes unstained (left) and stained (right). **B)** Dot plot from HIV+ off ART sample shows % of CD14+ monocytes unstained (left) and stained (right). **C)** Dot plot from HIV+ on ART sample shows % of CD14+ monocytes unstained (left) and stained (right).

Data was analyzed using a BD FACS Canto analyzer and WinMDI12.8 software. Fold change was calculated as follows (induced expression levels / basal expression levels).

2.062 Cell surface staining:

PBMCs were washed with PBS (containing 0.1% BSA and 0.1% sodium azide) and resuspended in 100 μL of the same buffer. Fluorescence-labeled monoclonal anti-human antibodies were added to the cells in parallel with monocyte specific anti-CD14-PerCp antibody (0.25 μg/100 μL) for 15 minutes at 25°C. Finally, cells were washed with PBS buffer (0.1% BSA, 0.1% sodium azide), fixed with 2% PFA, and then analyzed using a BD FACS Canto flow cytometer and WinMDI12.8 software.

2.063 Cytometric bead array (CBA) for human chemokine detection:

Purified monocytes ($4X10^5$ cells) from the patient study groups were left unstimulated or stimulated with IFN-γ (10 ng/mL) for 24 hrs. Supernatants from these cells were then collected and assayed by the CBA human chemokine kit I (BD) following the manufacturer's instructions. This assay was designed to detect several human chemokines in a single sample by flow cytometry using microbeads conjugated with specific antibodies for different chemokines and PE conjugated detection antibodies. Briefly, the human chemokine I standard (20X bulk) was prepared by adding 200 μL of assay diluent to lyophilized chemokine standard 1 vial and incubated for 15 minutes. This was followed by making 2 fold serial dilutions in 8 tubes. Then, mixed human chemokine I capture beads were prepared by mixing 10 μL of each chemokine capture beads with each sample. The test samples were prepared

by diluting 25 μL of each supernatant with equal volume of sample diluents to make total volume of 50 μL. Then, 50 μL of the mixed capture beads were added to the standard and test tubes. This was followed by adding 50 μL of human chemokine I PE-detection reagent and incubated for 3 hrs at room temperature. The FACS tubes were washed and resuspended in 300 μL of wash buffer, and analyzed using the FACS Canto flow cytometer. Data obtained from flow cytometry was further analyzed by FCAP software (BD) for determination of the chemokine concentration in pg/mL.

2.064 Cell viability assay:

Cell viability was assessed by two methods depending on cellular condition, Propidium Iodide (PI) (PI/RNase staining buffer, BD) staining and cell cycle analysis was performed on fixed and permeabilized cells, but Annexin-v-FITC/PI (Molecular probes) staining was carried out on fresh cells. The principle of cell cycle analysis is that after fixation and complete permeabilization of the cells, PI will access and bind to DNA. Based on the DNA content during the cell cycle, fluorescence intensity varies accordingly such that cells in the G0/G1 phase have less DNA than those in the G2/M phase. Dead or dying cells in principle have lower amounts of DNA and are defined as a sub-G0 or sub-diploid phase (240,241).

The principle of annexin-v-FITC staining is that annexin-v-FITC (also known as human vascular anticoagulant) is a calcium (Ca^{+2})-dependent phospholipid-binding protein that has high affinity to phosphatidyalserine (PS). In normal viable cells, PS is located on the inner surface of the cell membrane. In early stage of programmed cell death however, the PS is translocated to the outer surface of the cell membrane. Therefore, it will be accessible to binding by FITC-labeled annexin-v and detectable

by flow cytometry. PI is a cell impermeable DNA stain, and thus it stains only the late stage of programmed cell death (240,242).

CD14+ monocytes were purified from the PBMCs using CD14 microbeads (Miltenyi Biotec) (described above). Purified CD14+ monocytes ($4X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL), IL-10 (10 ng/mL), or pretreated with IL-10 for 2 hrs followed by IFN-γ for 24 hrs. For PI staining and cell cycle analysis, cells were fixed in 2% PFA, permeabilized in 100% methanol, then stained intracellular (IC) with PI (250 μL/$5X10^5$ cells) for 15 min and analyzed by flow cytometry. For annexin-v-FITC/PI staining, cells were washed with annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and resuspended in 100 μL of the same buffer. Annexin-v-FITC (5 μL/100 μL of cell suspension) and PI (10 μg/ 100 μL) were added to the cells and incubated at 37°C for 15 minutes. Finally, 400 μL of annexin-binding buffer was added and the samples were run on a flow cytometer using a BD FACS Canto analyzer. Data was analyzed using FACSDiva software (BD).

2.065 Detection of caspase 8 activation:

Activation of caspase 8 was measured using the caspase 8 detection kit (Calbiochem). The assay was designed to detect the active form of caspase 8 by conjugating a cell permeable inhibitor specific for activated caspase 8 with a fluorochrome that can be measured using a fluorescence based instrument. Purified monocytes ($4X10^5$ cells) were left untreated or treated with IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or pretreated with IL-10 for 2 hrs followed by IFN-γ for 24 hrs. Cells were then assayed using the caspase 8 detection kit. Cells were then stained with 1.3 μL of FITC-IETD-FMK and incubated at 37°C for 30 minutes. Cells were washed

twice with 500 μL of wash buffer and centrifuged at 3000 rpm for 5 minutes. Cells were resuspended in 200 μL of wash buffer and analyzed using a BD FACS Canto flow cytometer analyzer and FACSDiva software (BD).

2.070 Stimulation with HIV proteins:

HIV Tat protein and Vpr peptides were prepared as follows: recombinant HIV Tat protein obtained from the National Institute of Health AIDS Reference and Reagent Program, and treated with polymyxin B coated beads (Sigma-Aldrich) to remove endotoxin from the preparation. The endotoxin levels were measured by the LAL assay and found to be < 0.06 EU/mL (235). HIV Vpr peptides were synthesized by Genemed Synthesis Inc (San Francisco, CA) by automated solid-phase synthesis using 9-fluorenylmethoxycarbonyl, and were purified by reverse-phase high pressure liquid chromatography (>95%). The amino acid sequence of these peptides is as follows:

Vpr(1-45),MEQAPEDQGPQREPYNEWTLELLEELKSEAVRHFPRIWLHNLGQH;

Vpr(52-96),DTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTRQRRARNGASRS

(237).

Primary human monocytes, the monocytic cell lines (U937, THP-1), the myeloblastic HL-60 cells, or the Jurkat T cell line ($4X10^5$ cells) were either treated with HIV recombinant Gp120 (1 μg/mL), or Tat (100 ng/mL) directly in culture medium (10% FBS IMDM). Vpr peptides (0.1-1.5 μM) were added to cells in an isotonic buffer (13 mM HEPES, 2.4% glucose, 68 mM NaCl, 1.3 mM KCl, 4 mM Na_2HPO_4, 0.7 KH_2PO_4, pH 7.2) for 30 minutes as Vpr peptides have a high propensity to bind proteins. The isotonic buffer was replaced with culture medium. Subsequently, cells were incubated over a time course (2-48 hrs) and followed by

stimulation with IFN-γ (10 ng/mL) for another 15 minutes. Cells were then stained with P-STAT and analyzed by flow cytometry as described above.

2.080 Enzyme linked immunosorbant assay (ELISA):

Patient plasma or supernatants from IL-10 (10 ng/mL), IFN-γ (10 ng/mL), IL-10 + IFN-γ, treated and untreated cultured monocytes were assayed for TNF-related apoptosis-inducing ligand (TRAIL) by ELISA (Diaclone, Stamford, CT, USA) following the manufacturer's instructions. Briefly, 100 μL of samples or a serially diluted standard provided were loaded into a 96-well biotinylated anti-TRAIL antibody precoated plate. The plate was then incubated for 3 hrs at room temperature. The plate was washed 5 times, and streptavidin-horseradish peroxidase conjugate mixed with biotinylated anti-human monoclonal antibody was added and incubated for 30 minutes. After washing, substrate solution (TMB) was added for detection. Finally, the reaction was stopped using H_2SO_4 and the colorimetric signal was measured by absorbance at 450 nm using a spectrophotometer, the iMARK microplate reader (Bio-Rad).

2.090 Real-time polymerase chain reaction (RT-PCR):

Total cellular RNA from purified monocytes was extracted using the RNeasy Mini kit (Qiagen, Mississauga, ON, CA). Briefly, $5X10^5$ monocytes were homogenized in a denaturing buffer containing guanidine-isothiocyanate, which prevents RNA degradation. Cellular lysate was then passed through genomic DNA eliminator spin column to purify RNA from genomic DNA. Equal volume of 70% ethanol was added to total RNA to enhance its binding to the membrane of the RNeasy spin column when the sample passed through. This was followed by two

washes with a buffer containing ethanol and then total RNA was eluted with 30 μL of RNase-free water.

Total RNA was reverse transcribed using the high capacity cDNA Archive kit (Applied Biosystems (AB), Streetsville, ON, CA) according to the following protocol for each reaction: 1 μg of RNA, 5 μL of 10X RT, 2 μL of 25 mM dNTP, 5 μL of 10X random primer, 2.5 μL RT were mixed together, and Rnase-free H_2O was added to a total volume 50 μL. cDNA was then generated in a thermocycler at 25°C for 10 minutes then 37°C for 2 hrs. cDNA was amplified in a 7500 real-time PCR System (AB) using TaqMan Gene Expression Assays. These were predesigned and validated primers for IRF-1 and GAPDH (product ID Hs00233698-m1 and Hs99999905-m1, respectively) purchased from AB. The protocol used for each reaction was the following: 2.5 μL of cDNA, 12.5 μL of TaqMan universal PCR master mix (AB), 1.25 μL of primers, 8.75 μL of RNase-free H_2O. Relative mRNA expression was calculated by the comparative Ct method (243). Fold change was calculated as follows (induced expression levels / basal expression levels). Additionally, to confirm specificity of the PCR reaction, PCR products on 2% agrose gels and the molecular weight of amplicons was determined (data not shown).

2.100 Protein extraction, quantification and western blotting:

Whole cell extracts from purified primary monocytes or monocytic cell lines ($2X10^6$ cells) were prepared using detergent-containing protein lysis buffer. Briefly, cell pellets were mixed with 20-50 μL of lysis buffer, which contains 1% Triton X-100, 10 mM Tris (pH 7.5), 50 mM NaCl, 50 mM NaF, 2 mM EDTA, 1 mM EGTA, and supplemented with 1 mM Sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL Aprotinin, 1 μg/mL Leupeptin (Sigma), and incubated on

ice for 30 minutes. This was followed by centrifugation for 20 minutes, 4°C, at 15000 g. The supernatants were collected and their total protein concentration was determined by a colorimetric based method using the Bradford protein assay kit (Bio-Rad) and bovine serum albumin (BSA) (Sigma-Aldrich) as standard.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 10-25 μg of total lysate was mixed with an equal volume of sample buffer (Bio-Rad) containing 5% 2-β-mercaptoethanol and incubated in a boiling water bath for 5 minutes to denature the proteins. Using a slab gel electrophoresis apparatus (Bio-Rad), the mixture along with prestained molecular weight markers (Bio-Rad) were loaded onto 10-15% SDS-PAGE and run at 100 volts for 1 h and 45 minutes. This was followed by protein transfer onto polyvinylidene difluoride (PFD) membrane using a wet transfer blotting cell apparatus (Bio-Rad). The membrane was then blocked in a washing buffer containing 0.15 mM of Tris-HCl pH 7.6, 1.4 mM of NaCl, 1% Tween 20, and 5% non-fat dry skim milk for 1 h at room temperature then probed with primary Ab (1:1000 dilution) overnight at 4°C. The membrane was washed and probed with horseradish peroxidase-conjugated secondary Ab (1:20000 dilution) for 1 h at room temperature. Protein expression signal was revealed by chemiluminescence using the ECL advance kit (Amersham Biosciences) and a ChemiGenius2 imaging system (SYNGENE). Quantification of the signal was performed using Gene tools software (SYNGENE) by calculating the mean pixel value of the signal after subtracting background.

2.110 Transmission electron microscopy (T.E.M.):

Purified monocytes ($5X10^5$ cells) were either fixed directly or cultured with or without IFN-γ (10 ng/mL) or IL-10 (10 ng/mL) for 24 hrs. Cells were then fixed in

2.5% glutaraldehyde until the day of processing. Cells were fixed with 2% osmium tetroxide, washed, and dehydrated through graded ethanol. Samples were embedded in Spurr's resin and polymerized at 65°C overnight. Thin sections of the samples were made using a Leica Ultracut R ultramicrotome, stained with Uranyl Acetate and Lead citrate, and then screened on a JEOL 1212 transmission EM at 60Kv. This was carried out at the Childern's Hospital of Eastern Ontario E.M. facility with the assistance of Mr. Jeff McClintock.

2.120 Transient transfection with small interfering RNA (siRNA):

Transfection of U937 cells or primary monocytes was performed using a lipid formulation based method and following the Qiagen TransMessenger transfection kit procedure. For transfection of U937, cells were washed, counted and seeded in a 24 well-plate at a concentration of $2X10^5$ cells/500 µL of medium for 24 hrs. For treansfection of primary human monocytes, CD14+ cells were purified from PBMCs (described in section 2.050) and $5X10^5$ cells /transfection were used directly. On the day of transfection, the transfection reagent for each reaction was prepared by mixing enhancer R reagent, a specific RNA condensing reagent, (0.8 µL), and siRNA (0.4 µg), with TransMessenger reagent (2 µL) to form the TransMessenger-siRNA complexes. Cells were washed and resuspended in 300 µL of serum free medium, and then the transfection reagent was added drop wise to cells and incubated at 37°C for 3 hrs. Transfection reagent was removed by centrifugation and replaced with 10% FBS growth medium for a further 24 hrs of culture at 37°C, 5% CO_2. Transfection efficiency was measured by flow cytometry.

2.130 Statistical Analysis:

Pearson's r (two-tailed, $p \leq 0.05$) was used to calculate correlation between continuous variables. Non-paired Student's t-test was used for determination of the statistical significance between patient study groups. Paired Student's t-test was used for determination of the statistical significance between different cell treatments under the same technical conditions.

Chapter III

Amplification of IFN-γ Signaling and Cell Death in Monocytes from Viremic HIV+ Patients

3.000 Results:

3.100 Objective #1: Evaluate and compare cytokine dependent JAK/STAT signal transduction in monocytes from HIV- controls and chronically-infected HIV+ patients

Activation of signaling pathways is generally evaluated by standard biochemical assays such as western blotting, immunoprecipitation, and electrophoresis mobility shift assay (EMSA) (208,244,245). However, these techniques require a large number of cells which is difficult to obtain from HIV+ patients. Thus, a flow cytometry approach was chosen for its numerous advantages over standard biochemical signal transduction assays. It is amenable to perform on HIV+ patient samples, because it is a sensitive and specific technique that does not require large numbers of cells. It also allows analysis and quantification of multiple populations and parameters simultaneously without prior cell purification steps. In addition, it has been shown that tyr-phosphorylartion (Phospho-STAT) staining detected by flow cytometric analysis correlates with standard western blotting (246). Therefore, the first aim of this project was to optimize a flow cytometry based technique that uses monoclonal antibodies specific for the intracellular tyrosine phosphorylated STAT (P-STAT) protein (described in section 2.061).

3.110 Optimize a specific and sensitive assay to detect cytokine-induced JAK/STAT signaling, at the single cell level, in primary human monocytes and monocytic cell lines

It has been well established in the literature which STAT molecules are activated by which cytokines and in which cell types. For example in monocyte and monocytic cell lines, IFN-γ induces activation of STAT1 and 5 (202,247), whereas

IFN-α induces STAT1 and 2 activation (202), IL-4 induces STAT6 activation (246,248), and GM-CSF induces STAT5 activation (249,250), while IL-10 induces activation of STAT1 and 3 (245,251). It has also been shown that STAT1, 5, and 6 were activated in response to IFN-γ, GM-CSF, and IL-4, respectively, in U937 promonocytic cells using the intracellular flow cytometry approach. This data was validated with standard western blotting technique (246). Therefore, U937 cells were used as a control for optimization experiments. U937 cells ($4X10^5$) were unstimulated or stimulated with IFN-γ (10 ng/mL), GM-CSF (10 ng/mL) and IL-4 (4 ng/mL) for 15 minutes (min) at 37°C. The cells were then fixed and permeabilized in paraformaldehyde (PFA) and methanol, respectively, for 10 min at 4°C. The specific monoclonal anti-P-STAT antibodies were added to the cells and STAT activation was analyzed by flow cytometric analysis. Fig. 3-1 shows typical P-STAT staining observed in U937 cells. STAT6 tyr-phosphorylation was induced in response to IL-4 but not to IFN-γ (Fig. 3-1, B). STAT1 tyr-phosphorylation was observed in response to IFN-γ (Fig. 3-1, C). However, STAT5 tyr-phosphorylation was induced in response to GM-CSF (Fig. 3-1, D).

Similar experiments were performed on primary human monocytes from HIV- individuals (Fig. 3-2). STAT1 activation was induced in response to IFN-γ but not to IL-4 (Fig. 3-2, A). In contrast, STAT3 activation was observed in response to IL-10 but not to GM-CSF (Fig. 3-2, B). STAT5 activation was observed in response to GM-CSF and marginally in response to IFN-γ (Fig. 3-2, C). On the other hand, STAT6 activation was specifically induced in response to IL-4 (Fig. 3-2, D). A summary of the results for the cytokine-induced STAT activation detected in primary human monocytes is provided in table 3-1.

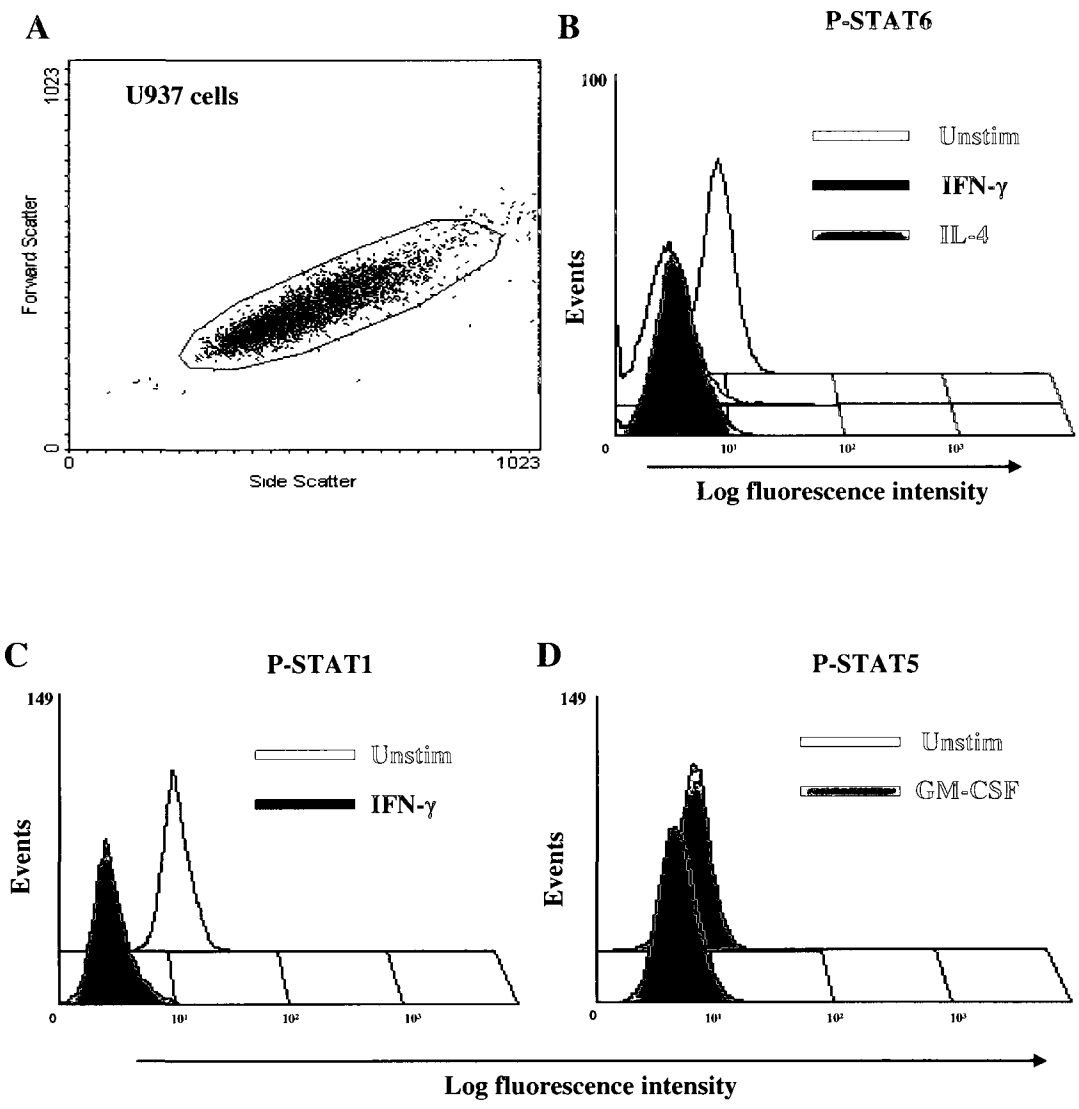

A
U937 cells
1023
0
Forward Scatter
0
1023
Side Scatter
B
P-STAT6
100
Events
Unstim
IFN-γ
IL-4
0
10¹
10²
10³
Log fluorescence intensity
C
P-STAT1
149
Events
Unstim
IFN-γ
0
10¹
10²
10³
D
P-STAT5
149
Events
Unstim
GM-CSF
0
10¹
10²
10³
Log fluorescence intensity

Figure 3-1: Activation of STATs in the U937 monocytic cell line.

U937 cells ($4X10^5$ cells) were stimulated for 15 min with IFN-γ (10 ng/mL), IL-4 (4 ng/mL), and GM-CSF (10 ng/mL), in parallel with unstimulated controls. Cells were then stained with each monoclonal anti-P-STAT antibody to determine which STATs were induced in response to which cytokines. Within the U937 cell gate, 10000 events were acquired. A dot plot and histograms analysis for one representative experiment out of at least three independent experiments performed were plotted (# of events on the y-axis, log fluorescence intensity on the x-axis). A comparison between STAT induction in stimulated and unstimulated cells was performed. **A)** Dot plot shows U937 gated cells for analysis. **B)** STAT6 activation in unstimulated compared to IFN-γ and IL-4 stimulated cells. **C)** STAT1 activation in unstimulated compared to IFN-γ stimulated cells. **D)** STAT5 activation in unstimulated compared to GM-CSF stimulated cells.

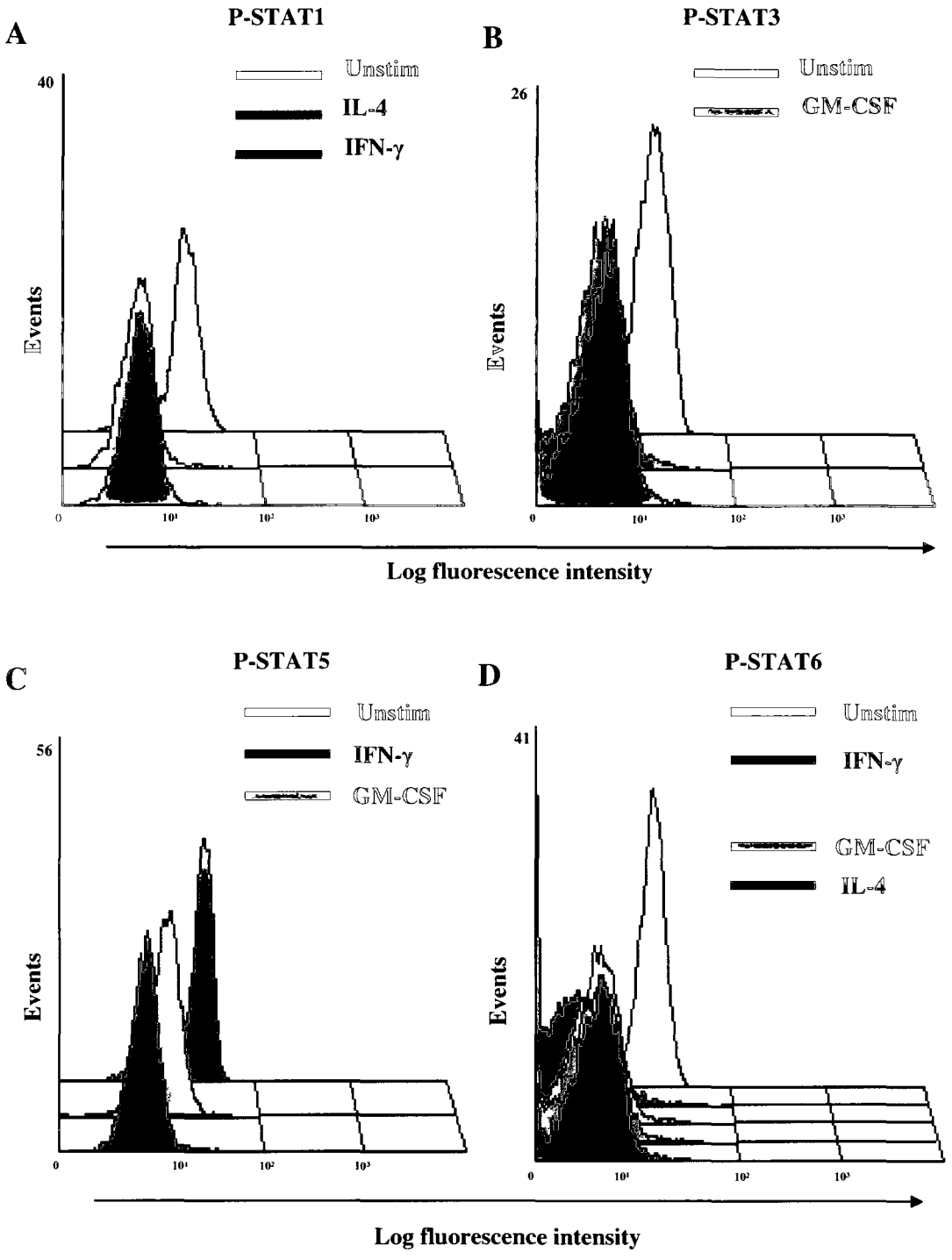

A
P-STAT1
Unstim
IL-4
IFN-γ
40
Events
0
10¹
10²
10³
B
P-STAT3
Unstim
GM-CSF
26
Events
0
10¹
10²
10³
Log fluorescence intensity
C
P-STAT5
Unstim
IFN-γ
GM-CSF
56
Events
0
10¹
10²
10³
D
P-STAT6
Unstim
IFN-γ
GM-CSF
IL-4
41
Events
0
10¹
10²
10³
Log fluorescence intensity

Figure 3-2: Activation of STATs in primary human CD14+ monocytes.

PBMCs ($4X10^5$ cells) were stimulated for 15 min with IFN-γ (10 ng/mL), GM-CSF (10 ng/mL), IL-4 (4 ng/mL), and IL-10 (10 ng/mL), in parallel with unstimulated controls. Cells were stained with each monoclonal anti-P-STAT antibody to determine which STATs were induced in response to which cytokines. Within the CD14+ monocyte gate, 5000 events were acquired. Histogram analysis for one representative experiment out of at least three was plotted (# of events on the y-axis, log fluorescence intensity on the x-axis). A comparison between STAT induction in stimulated and unstimulated CD14+ monocytes was performed. **A)** STAT1 activation in unstimulated compared to IL-4 and IFN-γ stimulated CD14+ monocytes. **B)** STAT3 activation in unstimulated compared to GM-CSF and IL-10 stimulated CD14+ monocytes. **C)** STAT5 activation in unstimulated compared to IFN-γ and GM-CSF stimulated CD14+ monocytes. **D)** STAT6 activation in unstimulated compared to IFN-γ, IL-10, GM-CSF and IL-4 stimulated CD14+ monocytes.

Table 3-1: Summary of the selective STAT activation detected in response to cytokines in primary human monocytes:

Cytokine	STAT1	STAT3	STAT5	STAT6
IFN-γ	++	-	+	-
IFN-α	++	-	-	-
IL-10	+	++	-	-
GM-CSF	-	-	++	-
IL-4	-	-	-	+

++ : High induction.
+ : Low induction.
- : Undetected.

These results showed that flow cytometric analysis of cytokine-induced STAT activation was specific and sensitive as it could be performed on a relatively low number of cells ($2X10^5$/tube). In addition, time course experiments were performed on primary human monocytes for these cytokines. One representative experiment is shown in Fig. 3-3. IFN-γ-induced STAT1 activation was detected as early as 5 min after treatment, and it peaked by 15 min, and remained at this plateau after 60 min (Fig. 3-3).

In order to determine if cryopreservation of cells (described in section 2.040) had any effect on the detection of the JAK-STAT pathway in response to cytokine stimulation in primary monocytes, a comparison between fresh and thawed PBMCs was performed. Fig. 3-4 shows that a clear activation of P-STAT1 in response to IFN-γ was observed in fresh as well as in thawed monocytes. However, no apparent differences were observed between fresh and thawed cells. To confirm this result further, a comparison experiment between the ability of fresh and thawed PBMCs to upregulate HLA-DR expression in response to IFN-γ stimulation, as a biological effect downstream of STAT1 activation, was carried out. IFN-γ stimulation was capable of upregulating HLA-DR expression over 24 and 48 hrs time points on fresh (upper panel) as well as on thawed (lower panel) monocytes. As expected, no significant differences were observed between the two conditions (Fig. 3-5). These results suggested that cryopreservation of PBMCs had negligible effects on monocyte signaling in response to the cytokines and proteins tested.

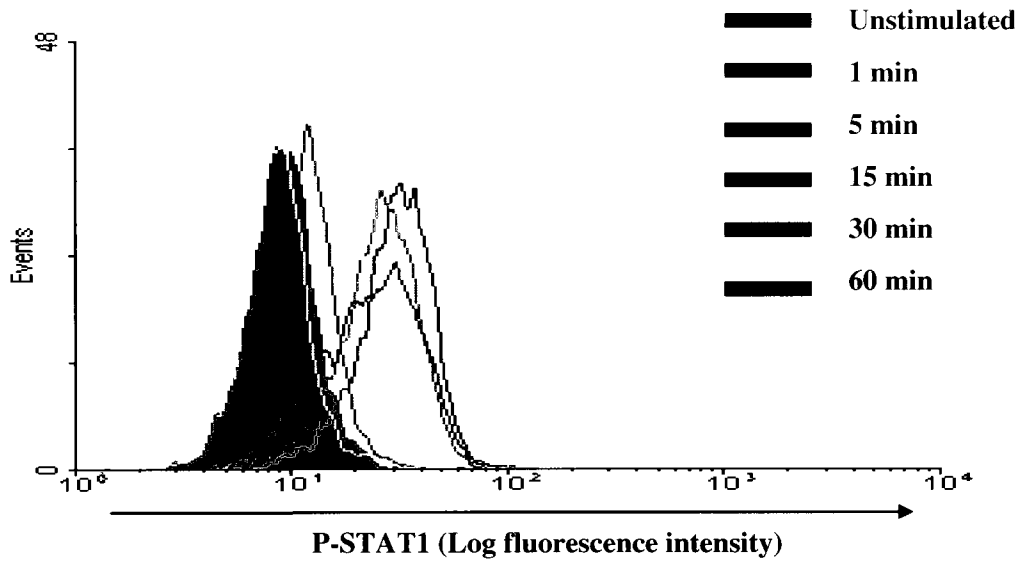

Unstimulated
1 min
5 min
15 min
30 min
60 min
48
Events
0
10⁰
10¹
10²
10³
10⁴
P-STAT1 (Log fluorescence intensity)

Figure 3-3: Time course for P-STAT1 induction in response to IFN-γ in primary human monocytes.

PBMCs ($4X10^5$ cells) were stimulated with IFN-γ (10 ng/mL), in parallel with unstimulated controls for the indicated times. Cells were subsequently stained with monoclonal anti-P-STAT1 antibody and monocyte specific anti-CD14 antibody. Within the CD14+ monocyte gate, 5000 events were acquired. Histograms for one representative experiment out of three were plotted (# of events on y-axis, log fluorescence intensity on x-axis). A comparison between STAT induction in stimulated and unstimulated CD14+ monocytes was performed.

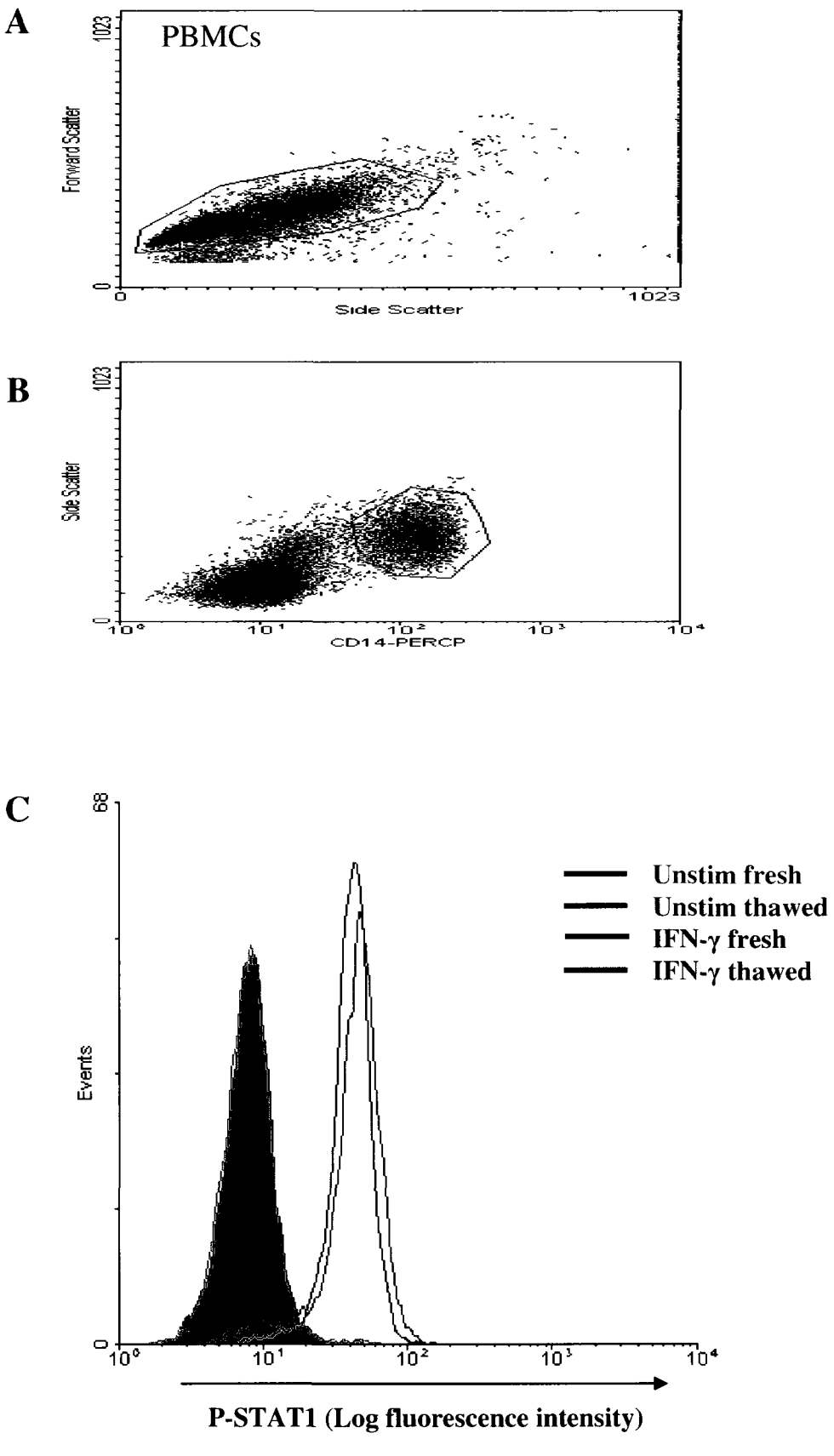

A
PBMCs
Forward Scatter
1023
0
0
1023
Side Scatter
B
Side Scatter
1023
0
10^0
10^1
10^2
10^3
10^4
CD14-PERCP
C
68
Events
0
10^0
10^1
10^2
10^3
10^4
P-STAT1 (Log fluorescence intensity)
Unstim fresh
Unstim thawed
IFN-γ fresh
IFN-γ thawed

Figure 3-4: Activation of STAT1 in fresh and thawed primary human CD14+ monocytes.

Fresh and thawed PBMCs ($4X10^5$ cells) were stimulated for 15 min with IFN-γ (10 ng/mL), in parallel with unstimulated controls. Cells were then stained with monoclonal anti-P-STAT1 antibody and monocyte-specific anti-CD14 antibody. Within the CD14+ monocyte gate, 5000 events were acquired. Dot plots and an overlay of histograms for one representative experiment out of three were generated (# of events on the y-axis, log fluorescence intensity on the x-axis). A comparison between fresh and thawed cells at the level of IFN-γ induced STAT1 activation was performed. **A)** Dot plot shows the PBMC gate. **B)** Dot plot shows the CD14+ monocyte gate. **C)** An overlay of histograms plot of STAT1 activation in unstimulated (fresh and thawed) compared to IFN-γ stimulated (fresh and thawed) CD14+ monocytes.

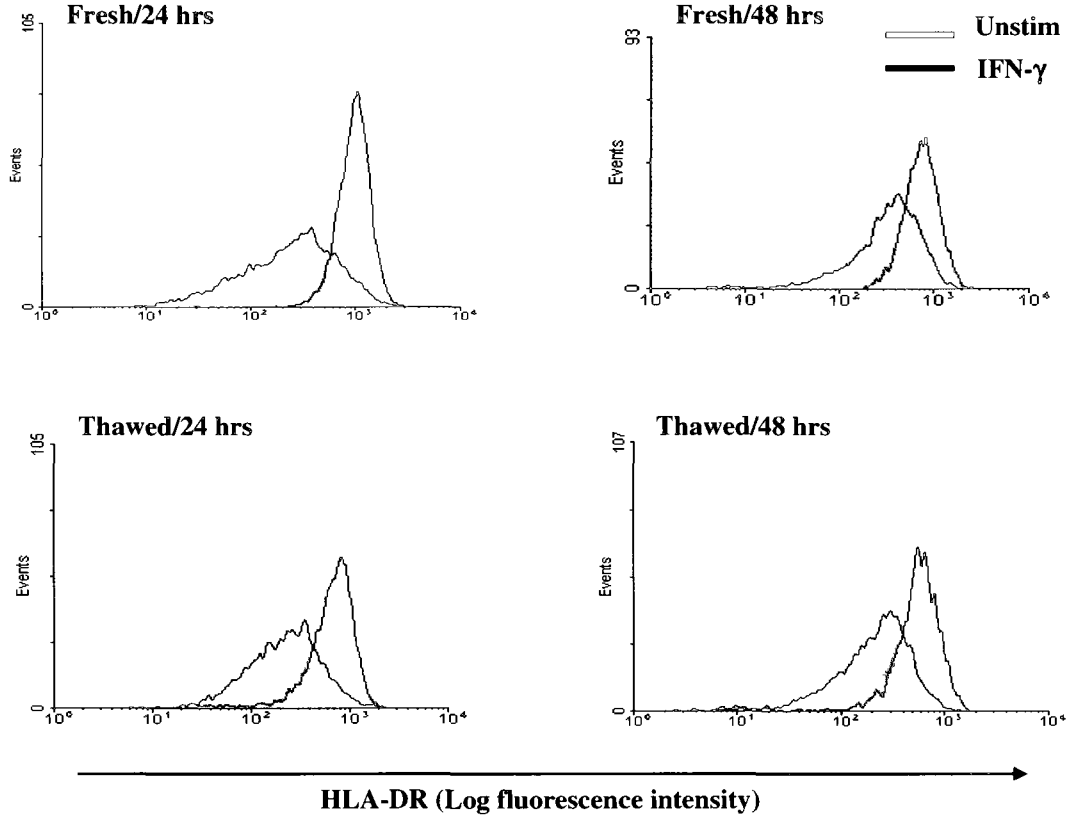

Fresh/24 hrs
Fresh/48 hrs
Unstim
IFN-γ
Thawed/24 hrs
Thawed/48 hrs
Events
HLA-DR (Log fluorescence intensity)

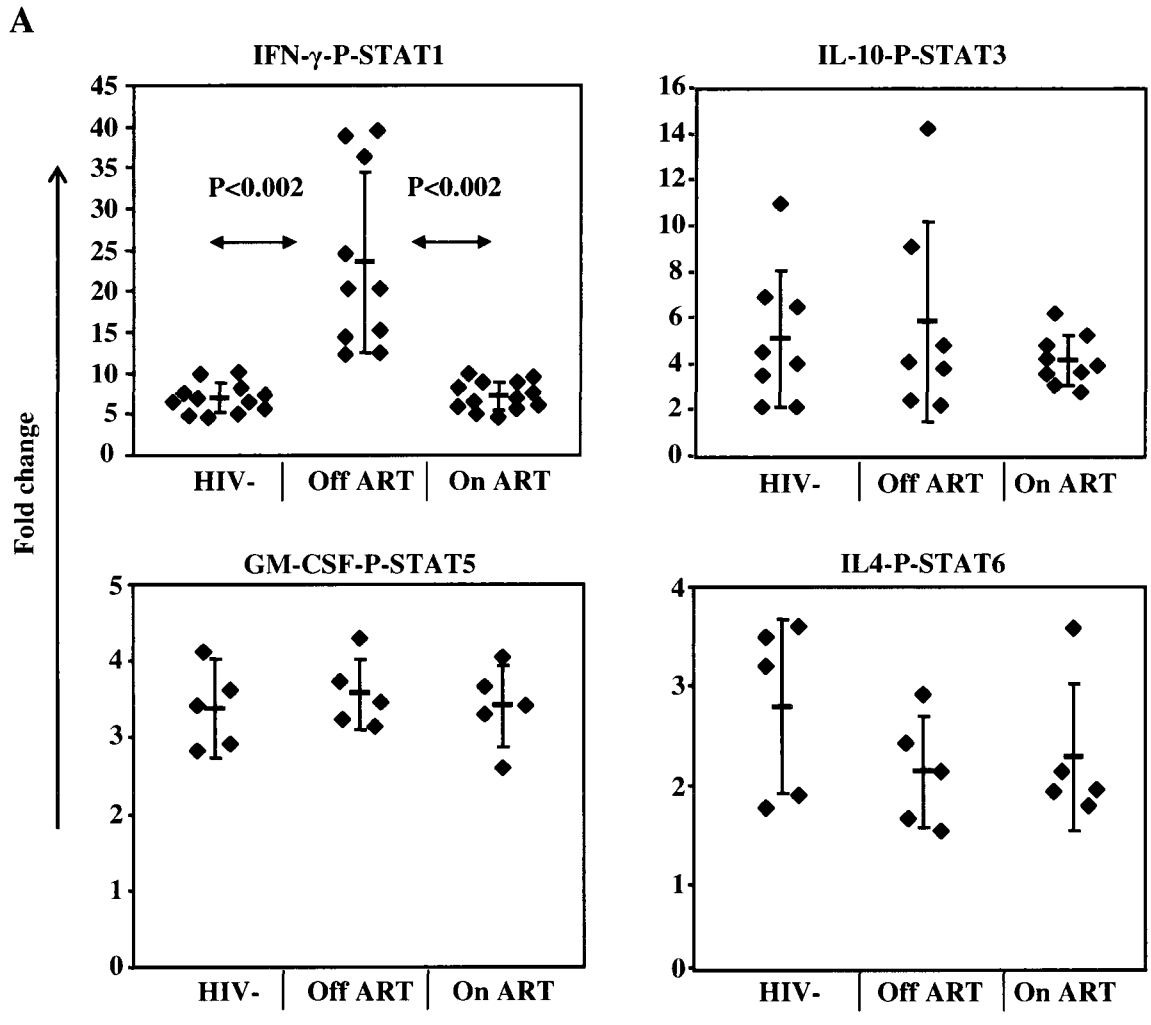
Figure 3-5: IFN-γ induced upregulation of HLA-DR expression in fresh and thawed primary human CD14+ monocytes.

Fresh and thawed PBMCs ($4X10^5$ cells) were stimulated with IFN-γ (10 ng/mL) for 24 and 48 hrs, in parallel with unstimulated controls. Cells were then stained with monoclonal anti-HLA-DR and monocyte-specific anti-CD14 antibodies. Within the CD14+ monocyte gate, 5000 events were acquired. Dot plots and an overlay of histograms for one representative experiment out of three were generated (# of events on the y-axis, log fluorescence intensity on the x-axis). A comparison between fresh (upper panel) and thawed (lower panel) cells at the level of IFN-γ-induced HLA-DR expression at 24 and 48 hrs was performed. Histogram plots of HLA-DR expression in unstimulated compared to IFN-γ stimulated CD14+ monocytes are shown.

3.120 Cytokine dependent STAT activation in CD14+ monocytes from patient study groups

Previous work done by Kryworuchko *et al.* showed IL-2 dependent STAT5 activation defects in CD8 T cells from a subset of HIV+ patients naïve to therapy and their restoration, at least in part, after HAART treatment (50). Therefore, a similar set of patient study groups were selected for this study. Group 1 included 12 HIV- controls. Group 2 included 20 HIV+ patients that were off therapy (off ART) for > 6 months. They had relatively high viral load (94[6-300] $X10^3$ copies/mL [mean(range)]), and relatively low CD4+ T cell counts (312[9-733] cells/μL). Group 3 included 28 ART-treated (>1 year) HIV+ patients. At the time of sampling, they all had undetectable viral loads (<50 copies/mL), and relatively higher CD4+ T cell counts (603[291-1002] cells/μL).

PBMCs ($4X10^5$ cells) from HIV+ patients and HIV- controls were either left unstimulated or stimulated with IFN-γ (10 ng/mL), IFN-α (100 U/mL), IL-10 (10 ng/mL), GM-CSF (10 ng/mL), and IL-4 (4 ng/mL) for 15 min at 37°C. This was followed by staining CD14+ monocytes with relevant anti-P-STAT antibodies and flow cytometric analysis using the FACScan flow cytometry (described in section 2.061). Analysis of the patient samples showed that the specific STAT proteins in response to the appropriate cytokine stimulation were induced in primary monocytes. In CD14+ monocytes from all subjects, P-STAT1 was upregulated in response to IFN-γ, whereas P-STAT3 was induced in response to IL-10 (Fig. 3-6, A). P-STAT5 was activated in response to GM-CSF, while P-STAT6 was activated in response to IL-4 (Fig. 3-6, A). Interestingly, IFN-γ-induced STAT1 activation was significantly upregulated in HIV+ patients off therapy compared to HIV- controls ($p<0.002$) and HIV+ patients on ART ($p<0.002$) (Fig. 3-6, A).


A
IFN-γ-P-STAT1
IL-10-P-STAT3
GM-CSF-P-STAT5
IL4-P-STAT6
Fold change
P<0.002
P<0.002
HIV-
Off ART
On ART

B

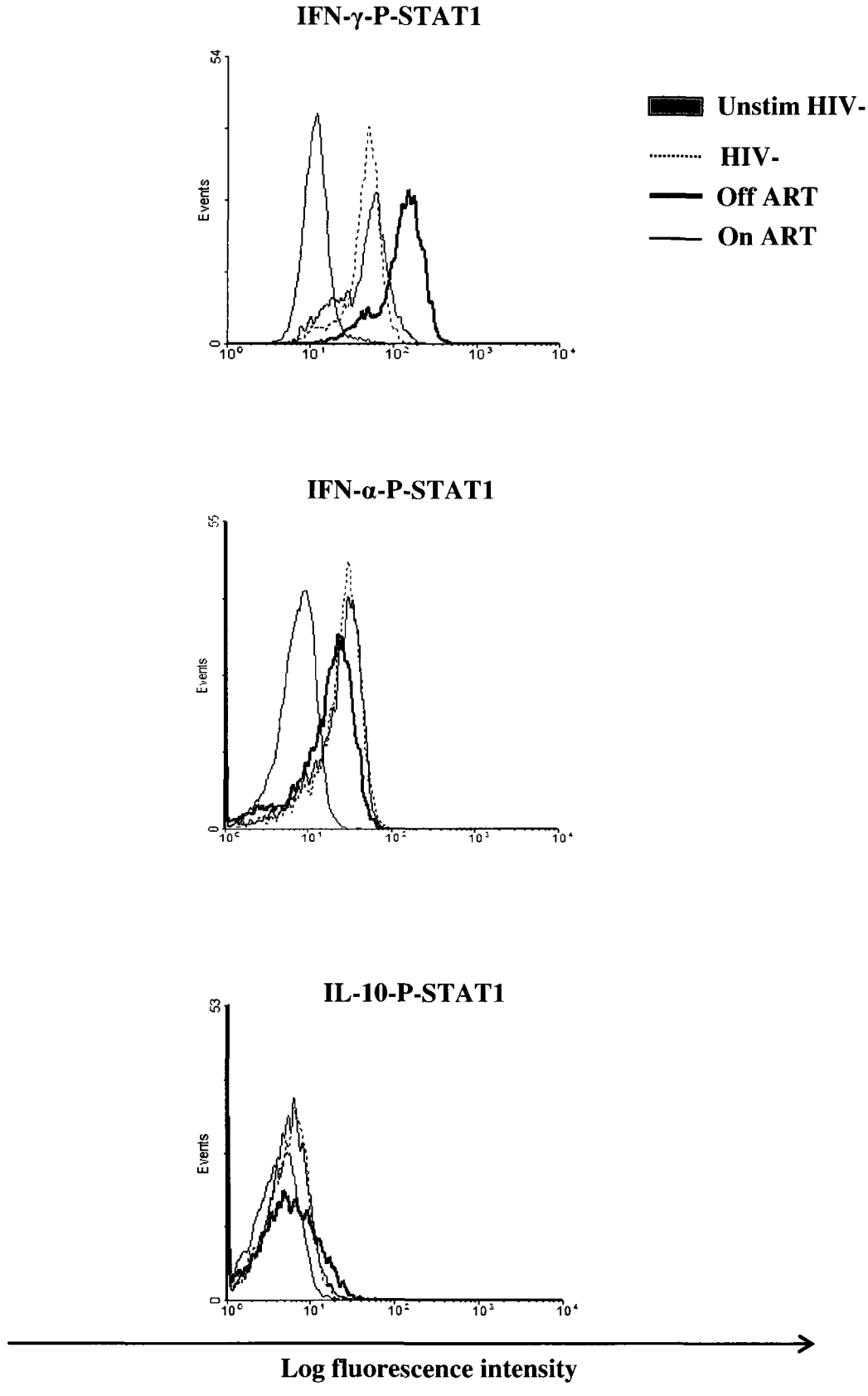

IFN-γ-P-STAT1
Unstim HIV-
HIV-
Off ART
On ART
Events
54
0
10⁰
10¹
10²
10³
10⁴
IFN-α-P-STAT1
55
IL-10-P-STAT1
53
Log fluorescence intensity

Figure 3-6: Activation of specific P-STATs in patient CD14+ monocytes.

Patient PBMCs ($4X10^5$ cells) were stimulated for 15 minutes with IFN-γ (10 ng/mL) or IFN-α (100 U/mL), IL-10 (10 ng/mL), GM-CSF (10 ng/mL), IL-4 (4 ng/mL), in parallel with unstimulated controls. Cells were stained with monoclonal anti-P-STAT combined with monocyte-specific anti-CD14 antibodies. Within the CD14+ monocyte gate, 5000 events were acquired. Comparisons between HIV+ patients off therapy, HIV- controls and ART-treated HIV+ patients were carried out. **A)** The fold change in mean fluorescence intensity of each P-STAT in patient CD14+ monocytes was plotted. Each symbol represents data obtained from one patient. All data was analyzed by Student's *t*-test and p values are indicated where significant. The mean for each study group is represented by a horizontal dash and plotted along with the S.D. (vertical error bars). **B)** An overlay of histograms shows anti-P-STAT1 staining in unstimulated CD14+ monocytes (one representative patient from each group), and in response to IFN-γ (upper graph), IFN-α (middle graph), IL-10 (lower graph) stimulation.

However, there were no significant differences in respective P-STAT induction between the patient groups in response to IL-10, GM-CSF, and IL-4 stimulation (Fig. 3-6, A). To investigate if the hyperactivation of P-STAT1 was unique to IFN-γ, IFN-α- and IL-10-induced P-STAT1 were also examined. Fig. 3-6, B shows P-STAT1 expression in response to IFN-γ (upper plot), IFN-α (middle plot), or IL-10 (lower plot) as compared to unstimulated CD14+ monocytes from one representative patient from each group. Interestingly unlike for IFN-γ, no hyperactivation of IFN-α- or IL-10-induced P-STAT1 was observed in monocytes from off therapy patients (Fig. 3-6, B). Also, basal levels of P-STAT did not vary significantly between patient groups (data not shown). These results suggested that there was a selective alteration in IFN-γ-induced STAT1 activation in monocytes from patients off therapy.

Since hyperactivation of the STAT1 transcription factor in response to IFN-γ was the most significant and clear result compared to the other STATs studied, this observation was pursued further. Thus, I investigated the molecular mechanism and the impact of STAT1 hyperactivation in monocytes from HIV+ patients.

3.200 Objective #2: Investigate the molecular mechanisms responsible for the HIV-induced hyperactivation of STAT1 observed in response to IFN-γ

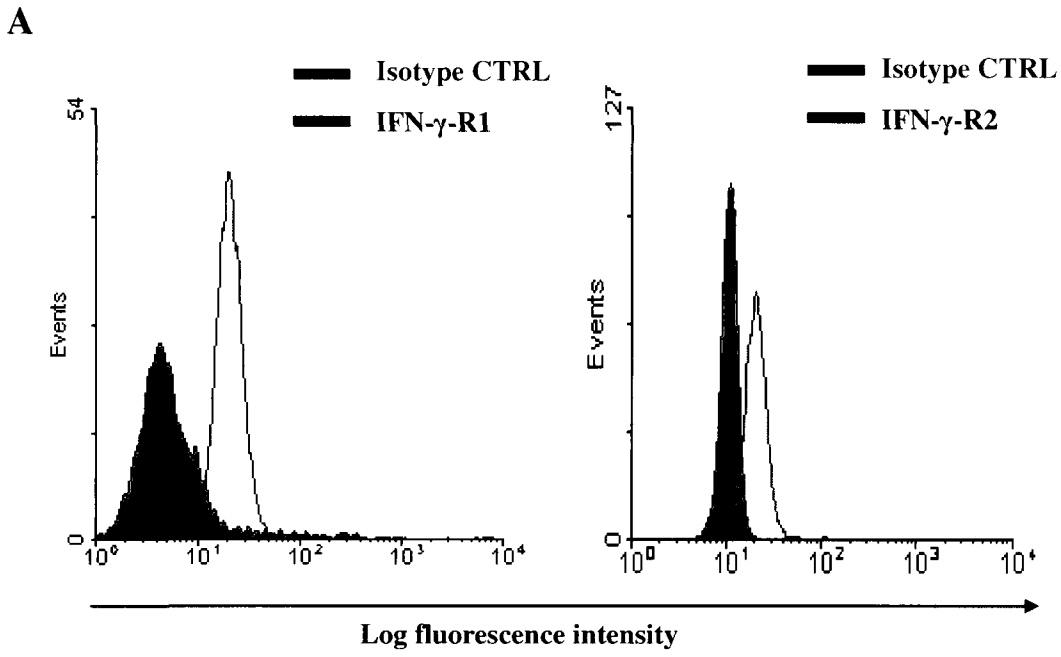
Studies have shown that alteration of IFN-γ receptor (IFN-γ-R) resulted in reduced IFN-γ-induced STAT1 activation and impaired immunity to several pathogens (144,145). Other studies have demonstrated that *in vitro* priming of monocytes with IFNs lead to increased total STAT1 expression and P-STAT1 in response to subsequent stimulation with IFNs (252,253). These reports suggested that the alteration of IFN-γ signaling observed in HIV+ off therapy patients could be

related to either the alteration in the surface IFN-γ receptor expression levels or downstream of the surface receptor such as total STAT1 expression. Thus, to test these possibilities, IFN-γ receptor and total STAT1 expression were evaluated in patient monocytes.

3.210 Assessment of IFN-γ receptor expression in patient CD14+ monocytes

It was hypothesized that the hyperactivation of STAT1 in monocytes from HIV+ patients off therapy may be due to increased IFN-γ receptor expression levels. IFN-γ receptor is composed of two subunits IFN-γ-R1 (α chain) and IFN-γ-R2 (β chain). IFN-γ-R1 is a 90-kDa polypeptide chain, and plays a major role in mediating ligand binding, and signal transduction (200,254). IFN-γ-R2, on the other hand, is a 62-kDa polypeptide chain that plays a minor role in ligand binding, but is required for signal transduction (200,255,256). IFN-γ receptor is expressed on most cell types (254). However, its expression levels vary between different cell types (257). A study from AIDS patients has shown that IFN-γ receptor expression levels were not altered in PBMCs (258).

IFN-γ receptor (R1 and R2 chains) expression on CD14+ monocytes was assessed using specific monoclonal anti-IFN-γ-R1-PE (BioLegend) and anti-IFN-γ-R2-FITC (Cell Sciences) antibodies, in parallel with anti-CD14-PerCp antibody and flow cytometric analysis (described in section 2.062). Optimization experiments were done on monocytes from HIV- individuals. Fig. 3-7, A shows that IFN-γ-R1 (left graph) and IFN-γ-R2 (right graph) are clearly expressed in primary monocytes. Subsequent analysis of IFN-γ-R expression in patient CD14+ monocytes was carried out.


A
Isotype CTRL
IFN-γ-R1
Isotype CTRL
IFN-γ-R2
54
127
Events
Events
0
0
10^0 10^1 10^2 10^3 10^4
10^0 10^1 10^2 10^3 10^4
Log fluorescence intensity

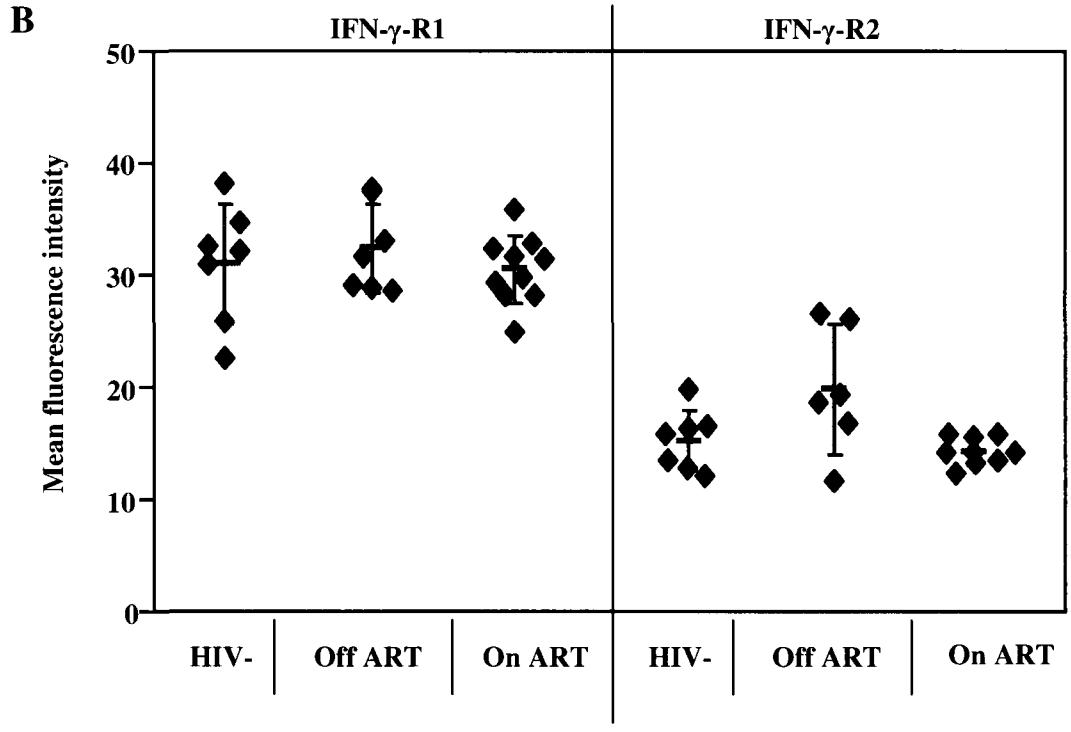

B
IFN-γ-R1
IFN-γ-R2
Mean fluorescence intensity
50
40
30
20
10
0
HIV-
Off ART
On ART
HIV-
Off ART
On ART

Figure 3-7: IFN-γ receptor expression in patient CD14+ monocytes.

Patients PBMCs ($4X10^5$ cells) were stained with monoclonal anti-IFN-γ-R1, anti-IFN-γ-R2 antibodies and monocyte specific anti-CD14 antibody. Within the CD14+ monocyte gate, 5000 events were acquired. **A)** A comparison between isotype control and IFN-γ-R1 (left) and IFN-γ-R2 (right) expression was performed. **B)** A comparison between HIV+ patients off therapy, HIV- donors and ART-treated HIV+ patients was carried out. The mean fluorescence intensity for IFN-γ-R1 (left) and IFN-γ-R2 (right) expression on patient CD14+ monocytes was plotted. Each symbol represents data from one patient. Horizontal dashes represent the mean and are plotted along with S.D. (vertical error bars).

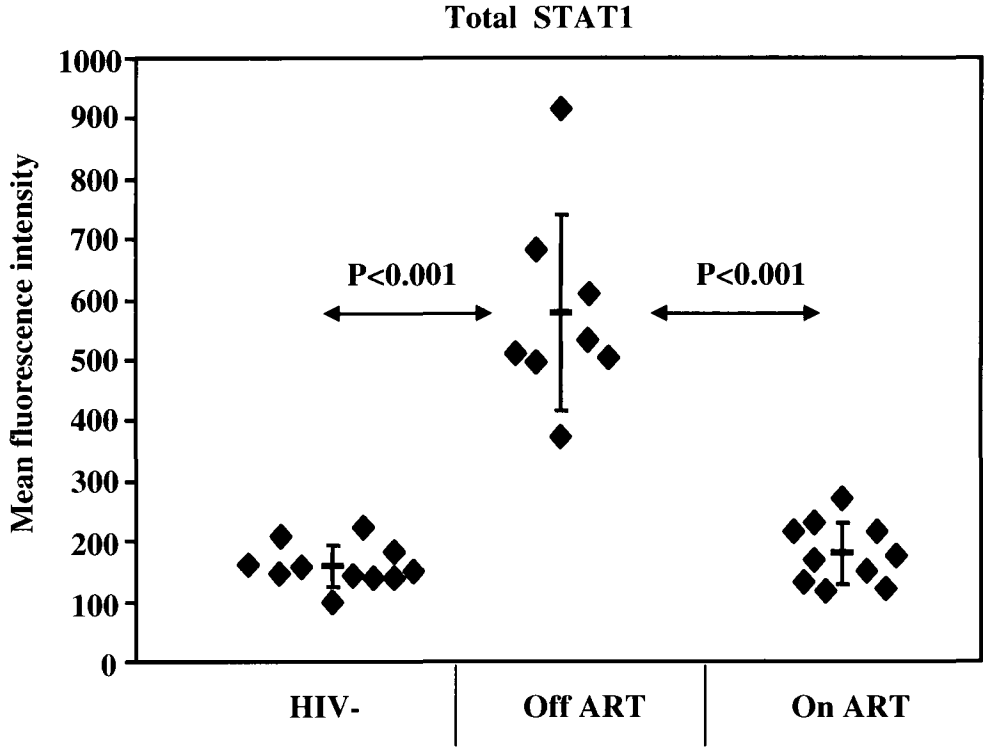
Both IFN-γ receptor subunits R1 and R2 were expressed on monocytes from all subjects, but no statistically significant differences in expression levels between patient study groups were observed (Fig. 3-7, B). This result suggested that STAT1 hyperactivation may be due to a mechanism downstream of cell surface IFN-γ receptor expression.

3.220 Measurement of total STAT1 expression levels in patient monocytes

As mentioned, priming of monocytes with low concentrations of interferons (type I or II) resulted in a selected increase in P-STAT1 in response to restimulation with IFN-γ. Moreover, the priming effect was the result of upregulated total STAT1 expression levels (252,253). Thus, I hypothesized that the hyperactivation of STAT1 may be the result of increases in the level of total STAT1 expression in the cytoplasm available for phosphorylation in response to IFN-γ.

Monocytes from patients were analyzed for total STAT1 expression by flow cytometry. In contrast to IFN-γ receptor, total STAT1 expression was significantly elevated in monocytes from off therapy patients compared to those on ART and HIV- controls (Fig. 3-8, A and B). Interestingly, this finding was correlated with P-STAT1 expression ($r=0.926$, $p<0.001$). Fig. 3-8, B shows an overlay of histograms of total STAT1 expression in unstimulated CD14+ monocytes from one representative patient in each group. A clear increase in total STAT1 level was observed in CD14+ monocytes from the HIV+ patient off therapy compared to that of an ART-treated patient or an HIV- control. This result suggested that the enhanced IFN-γ-induced STAT1 activation observed in off therapy patients was downstream of IFN-γ-R expression and likely due to increased total STAT1 expression levels.

A


Total STAT1
Mean fluorescence intensity
1000
900
800
700
600
500
400
300
200
100
0
P<0.001
P<0.001
HIV-
Off ART
On ART

B

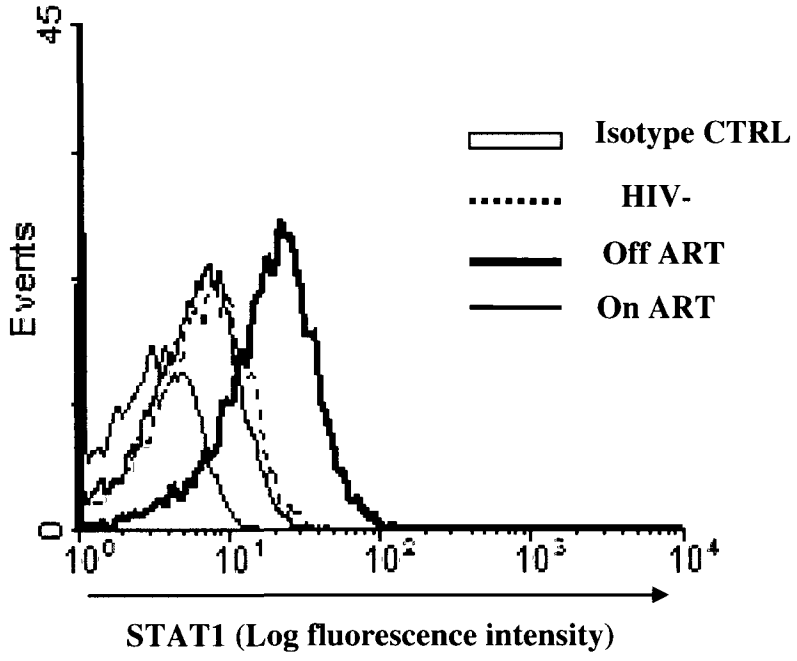

45
Events
0
Isotype CTRL
HIV-
Off ART
On ART
10^0
10^1
10^2
10^3
10^4
STAT1 (Log fluorescence intensity)

Figure 3-8: Total STAT1 expression in patient CD14+ monocytes.

Patient PBMCs ($4X10^5$ cells) were stained with monoclonal anti-total STAT1 antibody along with anti-CD14 antibody. Within the CD14+ monocyte gate, 5000 events were acquired. Comparisons between HIV+ patients off therapy, HIV- controls and ART-treated HIV+ patients were performed. **A)** Mean fluorescence intensity of total STAT1 expression was plotted. Each symbol represents data from one patient. The mean for each study group is represented by a horizontal dash and is plotted along with standard deviation (S.D.) error bars. Statistical analysis was performed using the Student's *t*-test and p values are indicated where significant. **B)** An overlay of histograms shows total STAT1 staining in unstimulated CD14+ monocytes (one representative patient from each group), and isotype control Ab staining.

3.230 Study the effect of immunomodulatory HIV proteins including Gp120, Tat and Vpr on STAT activation in monocytic cells

HIV and immunomodulatory HIV proteins including Gp120, Nef, Vpr, and Tat have been shown to affect many signaling pathways including the JAK/STAT, MAP Kinase, and PI3 Kinase pathways. For example, HIV and HIV proteins Gp120 and Nef have been shown to induce STAT1 and STAT3 activation in MDM and the monocytic cell line U937 (207-209). In other studies from our laboratory, it has been shown that HIV Tat protein stimulated IL-10 production via activation of calcium/MAPK signaling pathways in human monocytes (235,236). In another study HIV Vpr was shown to downregulate Bcl2 expression and induce monocyte apoptosis that was dependent on the activation of JNK MAPK pathway (237). Therefore, I hypothesized that hyperactivation of the STAT1 transcription factor may be due to the action of one or more of the HIV proteins such as Gp120, Vpr, and Tat. Primary human monocytes or the tumor cell lines U937, THP-1, HL-60, Jurkat ($4X10^5$ cells) were cultured in absence or presence of the HIV proteins Gp120 (1 μg/mL), Tat (100 ng/mL), Vpr peptides (1-45) and (52-96) (0.1-1 μM) over (2-48 hrs) time course. Cells were then restimulated with IFN-γ (10 ng/mL) for 15 min. Subsequently, cells were fixed, permeabilized, and stained with monoclonal anti-P-STAT1-Alexa fluor 488 antibody and analyzed by flow cytometry.

In U937 monocytic cells, Gp120 induced STAT1 activation after 24 hrs and the activation increased over time compared to untreated cells that were cultured in medium alone (Fig. 3-9). Gp120 treatment also enhanced STAT1 activation in response to restimulation with IFN-γ for 15 min compared to cells cultured without Gp120 and restimulated with IFN-γ (Fig. 3-9).

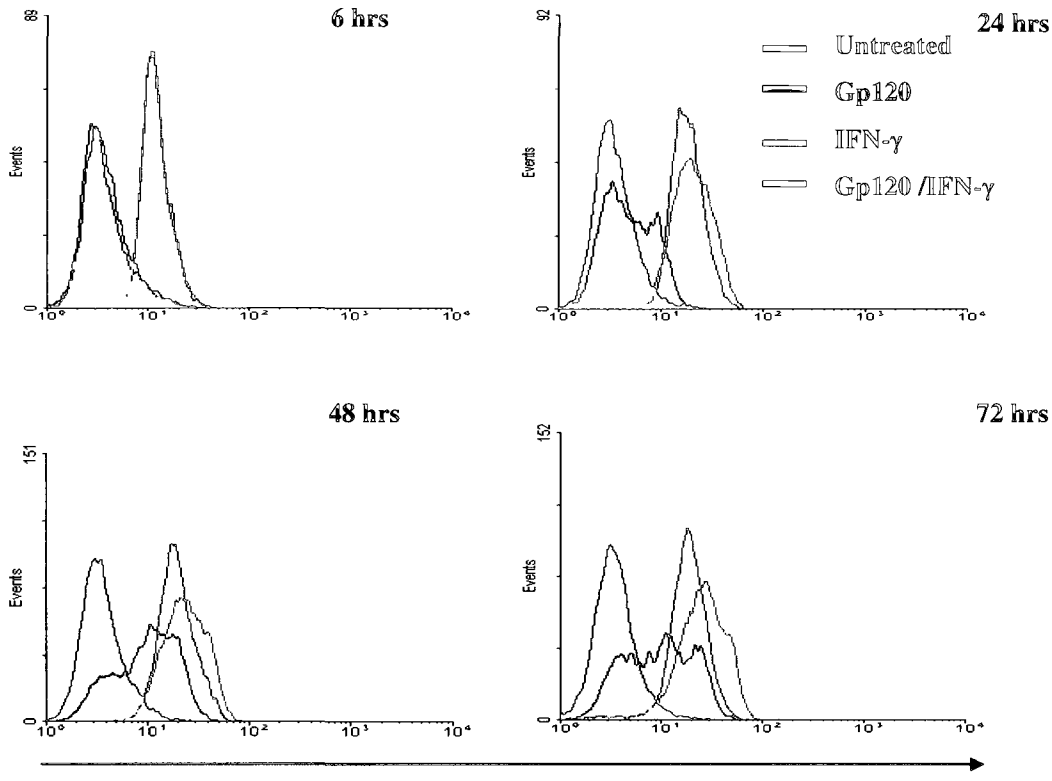

6 hrs
24 hrs
48 hrs
72 hrs
Untreated
Gp120
IFN-γ
Gp120 /IFN-γ
Events
P-STAT1 (Log fluorescence intensity)

Figure 3-9: Gp120 induced STAT1 activation in the U937 monocytic cell line.

U937 cells ($4X10^5$cells) were cultured with or without Gp120 (1 μg/mL) for 6, 24, 48, and 72 hrs. Cells were then restimulated with IFN-γ (10 ng/mL) for 15 min. Cells were immediately stained with anti-P-STAT1 antibody. Within the U937 cell gate, 10000 events were acquired. Overlays of histogram analysis were plotted (# of events on the y-axis, log fluorescence intensity on the x-axis). A comparison between STAT1 activation in Gp120 treated and untreated cells with or without subsequent 15 min restimulation with IFN-γ was performed.

Similar results were observed in the THP-1 monocytic cells and the myeloblastic cell line HL-60 (data not shown). Gp120 appeared not to have the same effect on the Jurkat T cell line (Fig. 3-10).

In the myeloblastic cell line HL-60, the Vpr (52-96) peptide alone was also capable of inducing STAT1 activation within 30 min, which remained detectable after 3 hrs as compared to the Vpr (1-45) peptide (Fig. 3-11). Interestingly, Vpr (52-96) activated STAT1 but not STAT3, STAT5, or STAT6 in HL-60 cells (Fig. 3-12). It has been shown in several reports that Vpr treatment causes cell death in different cell types (237,259,260). Thus, to ensure that HIV Vpr was biologically active, the induction of cell death in response to Vpr stimulation was measured in HL-60 cells. These cells ($4X10^5$) were either left untreated or treated with 1.5 μM of Vpr peptides (1-45) or (52-96) for 6 hrs. Cell death was then evaluated by using PI staining and flow cytometry. Indeed, HIV Vpr (52-96) peptide induced cell death as compared to unstimulated or Vpr (1-45) peptide treated HL-60 cells (Fig. 3-13). Vpr (52-96) peptide also induced STAT1 activation in other monocytic cell lines including THP-1, and U937 cells (data not shown). Similar experiments were performed on primary monocytes. Vpr (52-96) peptide activated the STAT1 transcription factor in a dose dependent manner as compared to Vpr (1-45) in primary monocytes (Fig. 3-14). However, there was no significant effect upon restimulation with IFN-γ. The effect of Vpr peptides on monocyte cell death was also tested. Primary monocytes were purified from PBMCs by positive selection (described in 2.050). CD14+ monocytes ($4X10^5$ cells) were left unstimulated or stimulated with 1 μM of Vpr peptide (1-45) or (52-96) for 6 hrs. Cell death was then measured by intracellular PI staining and flow cytometric analysis.

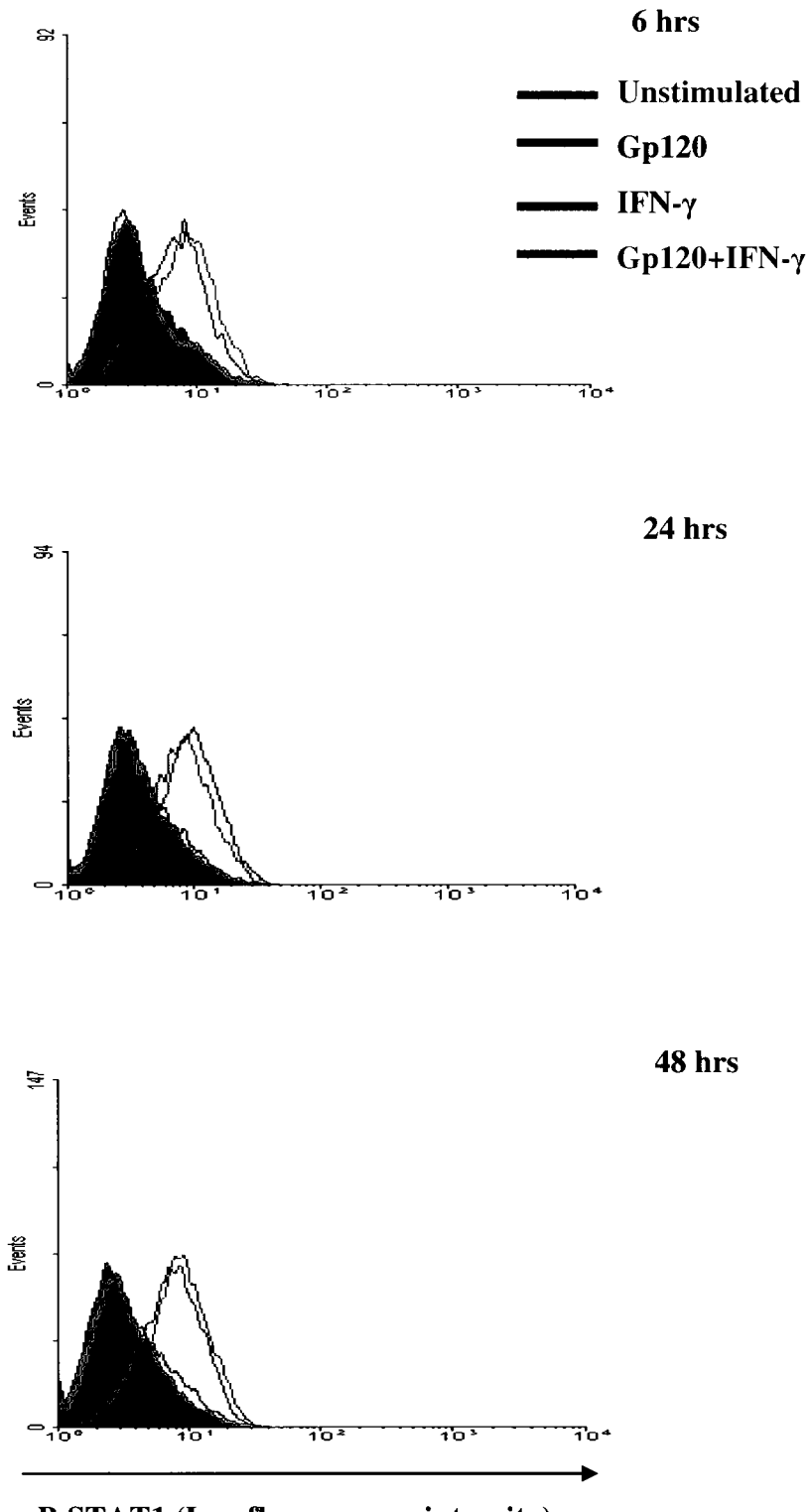

6 hrs
Unstimulated
Gp120
IFN-γ
Gp120+IFN-γ
92
Events
0
10⁰
10¹
10²
10³
10⁴
24 hrs
94
Events
0
10⁰
10¹
10²
10³
10⁴
48 hrs
147
Events
0
10⁰
10¹
10²
10³
10⁴
P-STAT1 (Log fluorescence intensity)

Figure 3-10: Gp120 had no effect on STAT1 activation in the Jurkat T cells.

Jurkat cells ($4X10^5$ cells) were cultured with or without Gp120 (1 μg/mL) for 6, 24, and 48 hrs. Cells were then restimulated with IFN-γ (10 ng/mL) for 15 min. Cells were immediately stained with anti-P-STAT1 antibodies. Within the Jurkat cell gate, 10000 events were acquired. Overlays of histogram analysis were plotted. (# of events on the y-axis, log fluorescence intensity the on x-axis). A comparison between STAT1 activation in Gp120 treated and untreated cells with or without subsequent 15 min restimulation with IFN-γ was performed.

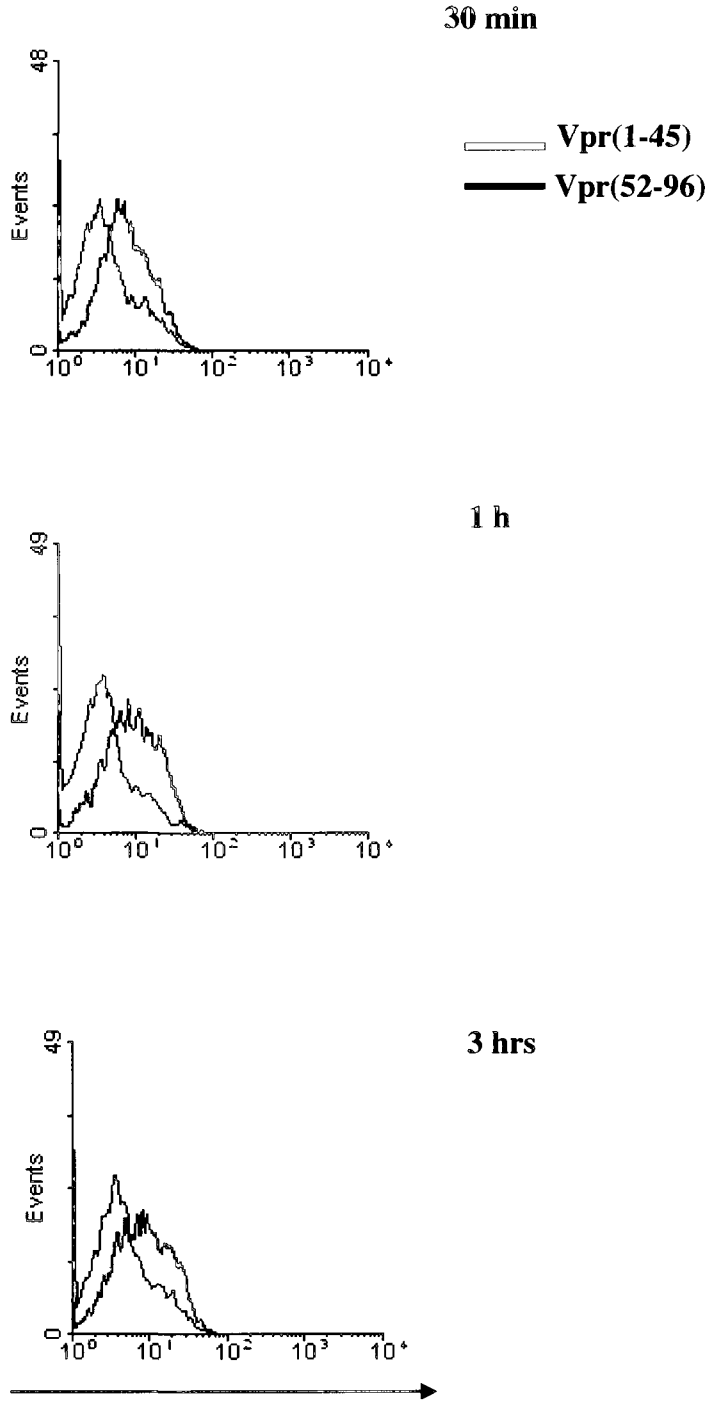

30 min
Vpr(1-45)
Vpr(52-96)
48
Events
0
10^0 10^1 10^2 10^3 10^4
1 h
49
Events
0
10^0 10^1 10^2 10^3 10^4
3 hrs
49
Events
0
10^0 10^1 10^2 10^3 10^4
P-STAT1 (Log fluorescence intensity)

Figure 3-11: Vpr induced STAT1 activation in the HL-60 myeloblastic cells.

HL-60 cells ($4X10^5$ cells) were treated with 1.5 μM of Vpr peptides (1-45) or (52-96) for 30 min, 1 h, 3 hrs time points. Cells were immediately stained with anti-P-STAT1 antibody. Within the HL-60 cell gate, 10000 events were acquired. Overlays of histogram anlysis were plotted. (# of events on the y-axis, log fluorescence intensity on the x-axis). A comparison between STAT1 activation in Vpr (1-45) and Vpr (52-96) treated HL-60 cells was performed.

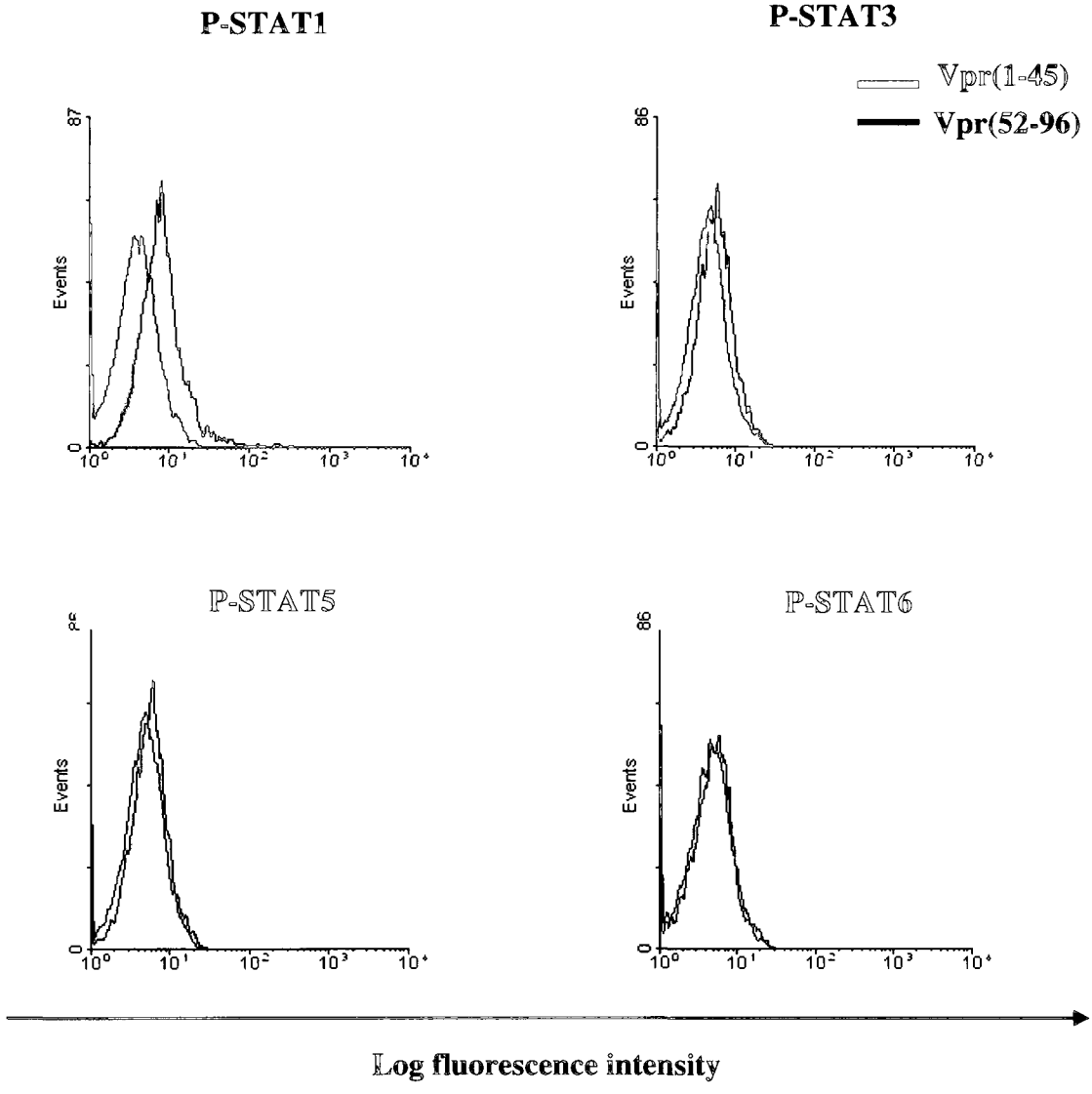

P-STAT1
P-STAT3
Vpr(1-45)
Vpr(52-96)
87
86
Events
0
10^0
10^1
10^2
10^3
10^4
P-STAT5
P-STAT6
Log fluorescence intensity

Figure 3-12: Effect of Vpr on STAT activation in HL-60 cells.

HL-60 cells ($4X10^5$ cells) were treated with 1.5 μM of Vpr peptides (1-45) or (52-96) for 30 min. Cells were immediately stained with anti-P-STAT1, P-STAT3, P-STAT5, P-STAT6 antibodies. Within the HL-60 cell gate, 10000 events were acquired. Overlays of histograms were plotted. (# of events on the y-axis, log fluorescence intensity on the x-axis). Comparisons between STAT1 activation in Vpr (1-45) or Vpr (52-96) treated HL-60 and untreated cells were performed.

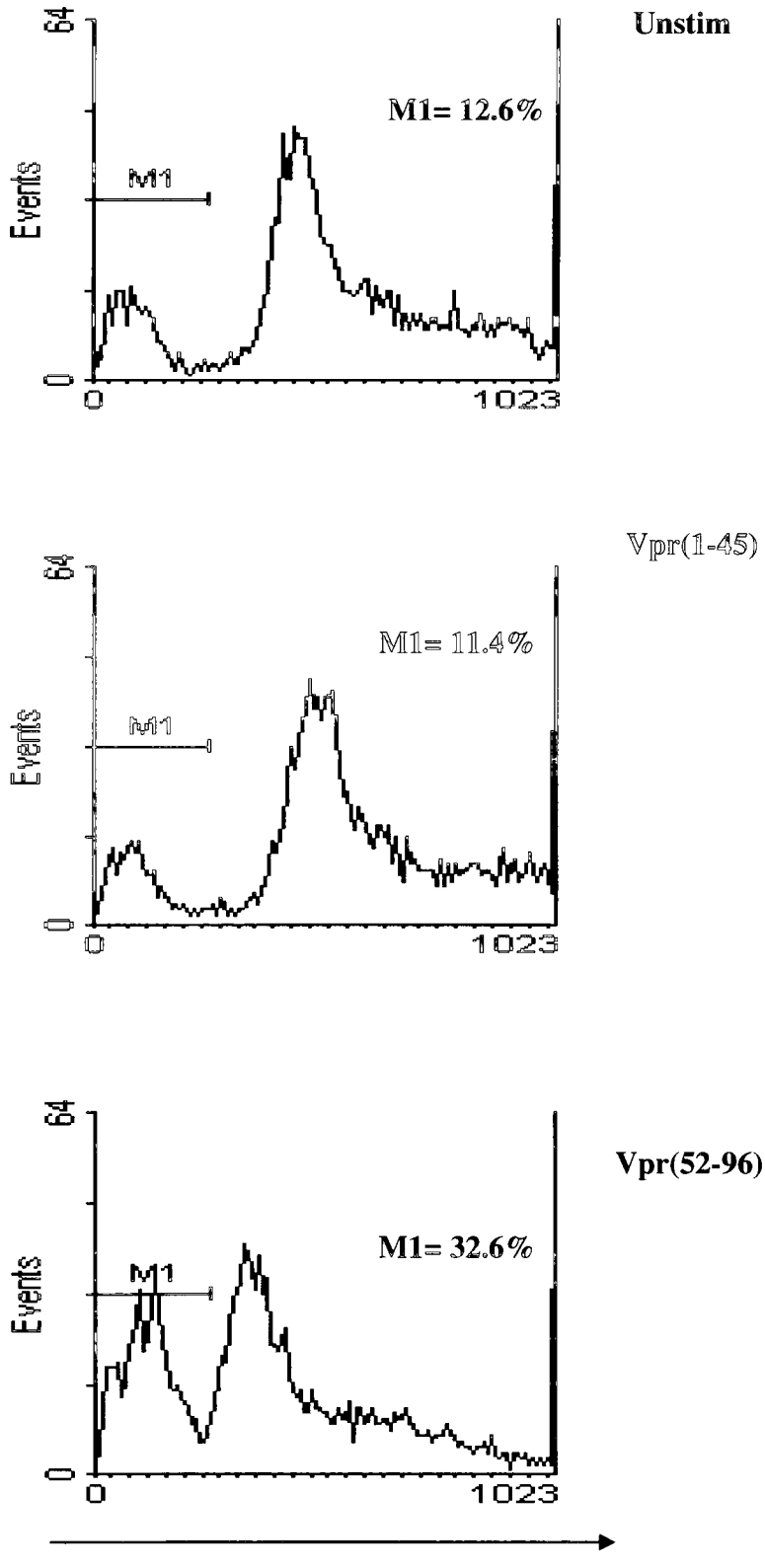
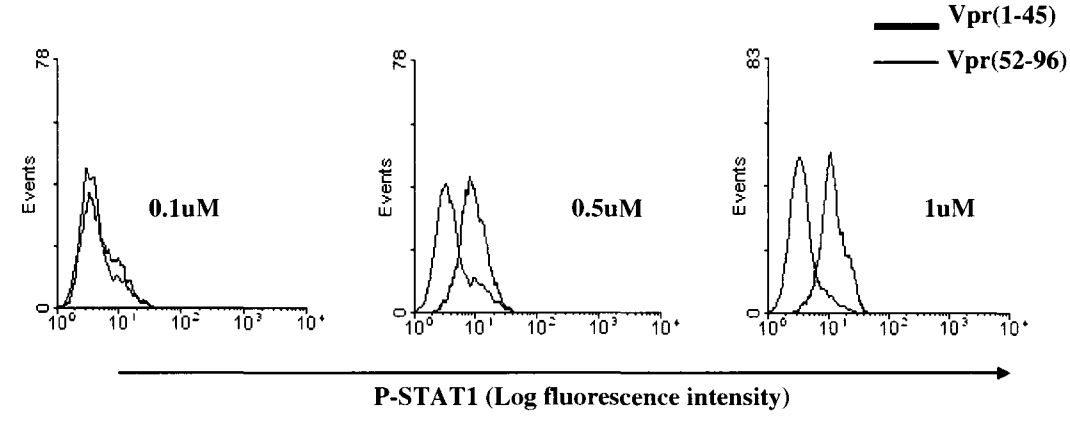

Unstim
M1= 12.6%
M1
Events
64
0
0
1023
Vpr(1-45)
M1= 11.4%
M1
Events
64
0
0
1023
Vpr(52-96)
M1= 32.6%
M1
Events
64
0
0
1023
PI staining (Fluorescence intensity)

Figure 3-13: Vpr induced PCD in HL-60 cells.

HL-60 cells ($4X10^5$ cells) were left untreated or treated with 1.5 μM of Vpr peptides (1-45) or (52-96) for 6 hrs. Cells were then permeabilized, stained with PI, and analyzed by flow cytometry. Within the HL-60 cell gate, 10000 events were acquired. Histograms of PI staining were plotted. (# of events on the y-axis, fluorescence intensity on the x-axis). Comparisons between cell death induced in Vpr (1-45) and Vpr (52-96) treated or untreated HL-60 cells were performed.


Vpr(1-45)
Vpr(52-96)
Events
78
0
0.1uM
0.5uM
83
1uM
P-STAT1 (Log fluorescence intensity)

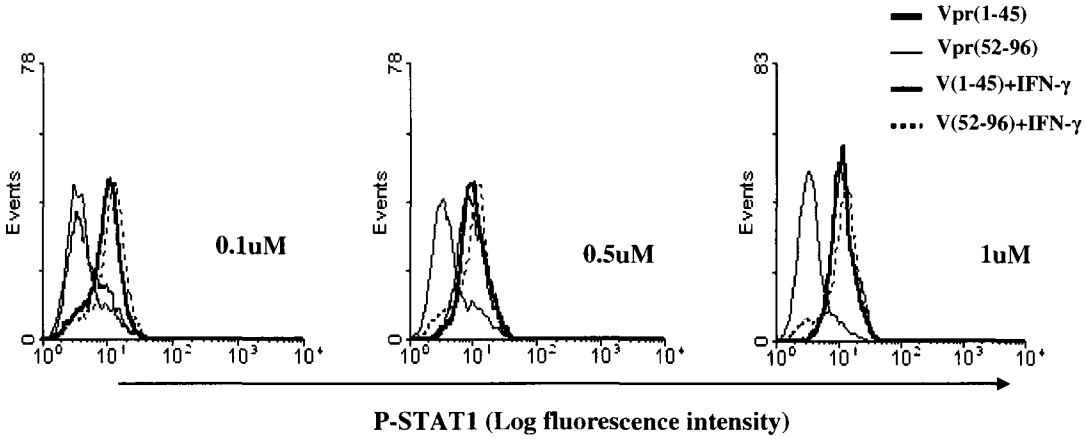

Vpr(1-45)
Vpr(52-96)
V(1-45)+IFN-γ
V(52-96)+IFN-γ
Events
78
0
0.1uM
0.5uM
83
1uM
P-STAT1 (Log fluorescence intensity)

Figure 3-14: Vpr induced STAT1 activation in primary human monocytes.

Purified monocytes ($4X10^5$ cells) were left untreated or treated with different concentrations (0.1-1 μM) of Vpr peptides (1-45) or (52-96) for 30 min. The cells were then restimulated with IFN-γ (10 ng/mL) for 15 min. Cells were immediately stained with anti-P-STAT1 and anti-CD14 antibodies. Within the CD14+ cell gate, 5000 events were acquired. Overlays of histogram were plotted (# of events on the y-axis, log fluorescence intensity on the x-axis). A comparison between STAT1 activation in monocytes treated with Vpr (1-45) and Vpr (52-96) (upper panel) or Vpr peptides with subsequent 15 min restimulation with IFN-γ (lower panel) was performed.

Vpr (52-96) peptide induced cell death in primary monocytes as compared to unstimulated or Vpr (1-45) peptide treated monocytes confirming its biological activity (Fig. 3-15).

Tat did not affect STAT1 phosphorylation in primary monocytes or any of the cell lines tested (Fig. 3-16 and data not shown). These results suggested that the hyperactivation of STAT1 in response to IFN-γ stimulation in monocytes from viremic patients could be due to HIV immunomodulatory proteins such as Gp120, Vpr which are present in the circulation of HIV-infected individuals.

3.300 Objective #3: Determine the biological impact of IFN-γ-induced STAT1 hyperactivation in monocytes from HIV+ patients, particularly focusing on its effect on PCD

STAT1 plays a major role in regulating the immune and proinflammatory responses of IFN-γ. It has been shown that deletion of the STAT1 gene results in defective IFN-dependent biological responses and innate immune responses to viral diseases (141-146). Other studies have revealed that activation of STAT1 could lead to PCD in different cell types (261-263).Therefore, IFN-γ-induced STAT1 responsive genes and PCD were evaluated in patient monocytes.

3.310 Determination of the expression levels of STAT1 responsive genes in patient monocytes

Studies have demonstrated that activation of the STAT1 transcription factor by IFN-γ results in the upregulation of specific downstream genes such as MHC class II (HLA-DR), interferon regulatory factor-1 (IRF-1), IFN-γ-inducible protein 10 (IP-10) or CXCL10, and monokine induced by IFN-γ (MIG) or CXCL9 (196,252,264).

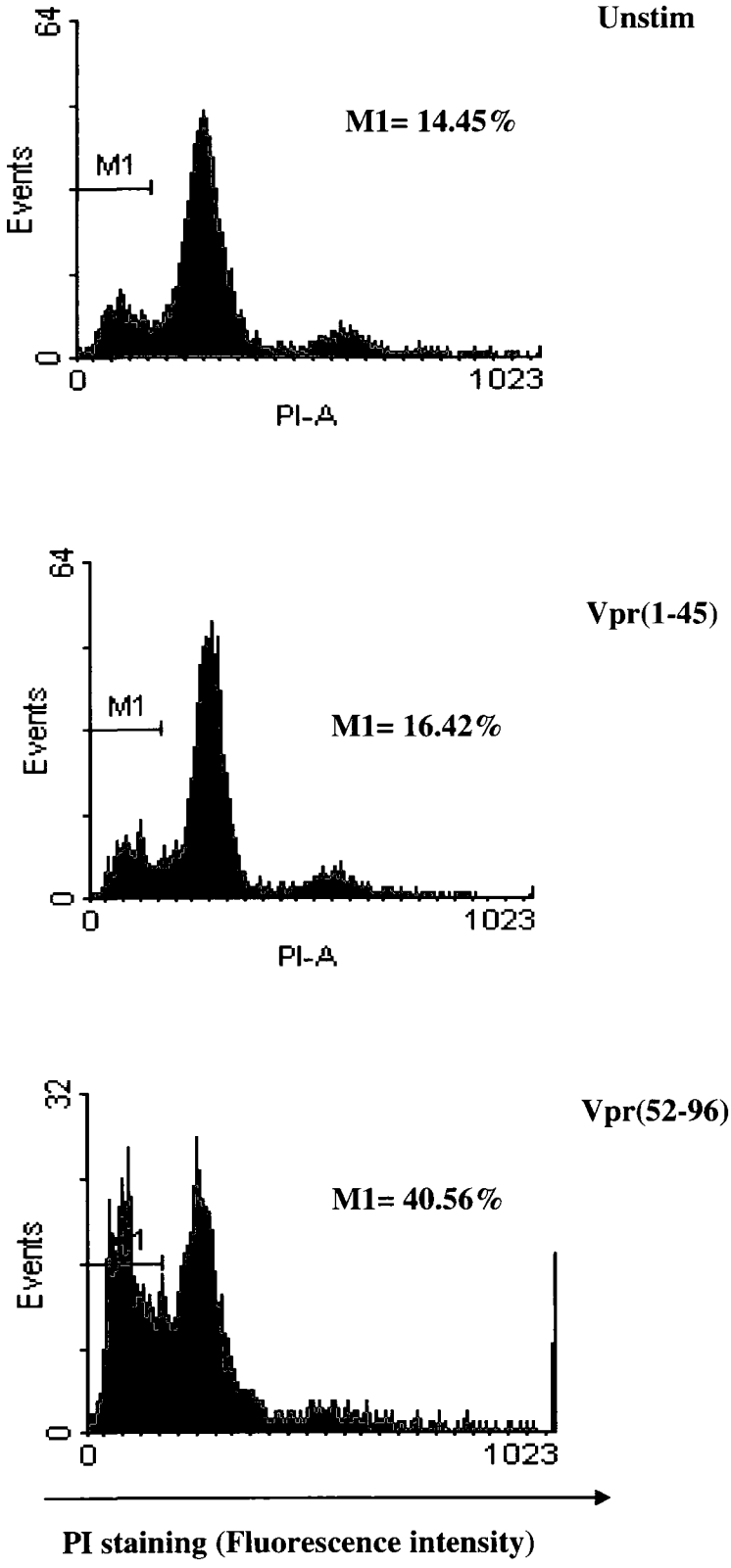

Unstim
M1= 14.45%
M1
Events
64
0
0
1023
PI-A
Vpr(1-45)
M1= 16.42%
M1
Events
64
0
0
1023
PI-A
Vpr(52-96)
M1= 40.56%
M1
Events
32
0
0
1023
PI staining (Fluorescence intensity)

Figure 3-15: Vpr induced PCD in primary human monocytes.

Purified monocytes ($4X10^5$ cells) were treated with 1 μM of Vpr peptides (1-45) or (52-96) for 6 hrs. Cells were immediately stained intracellularly with PI and analyzed by flow cytometer. Within the cell gate, 5000 events were acquired. Histograms analyzing PI staining were plotted (# of events on the y-axis, log fluorescence intensity on the x-axis). Comparisons between cell death induced in Vpr (1-45) and Vpr (52-96) treated or untreated monocytes were performed.

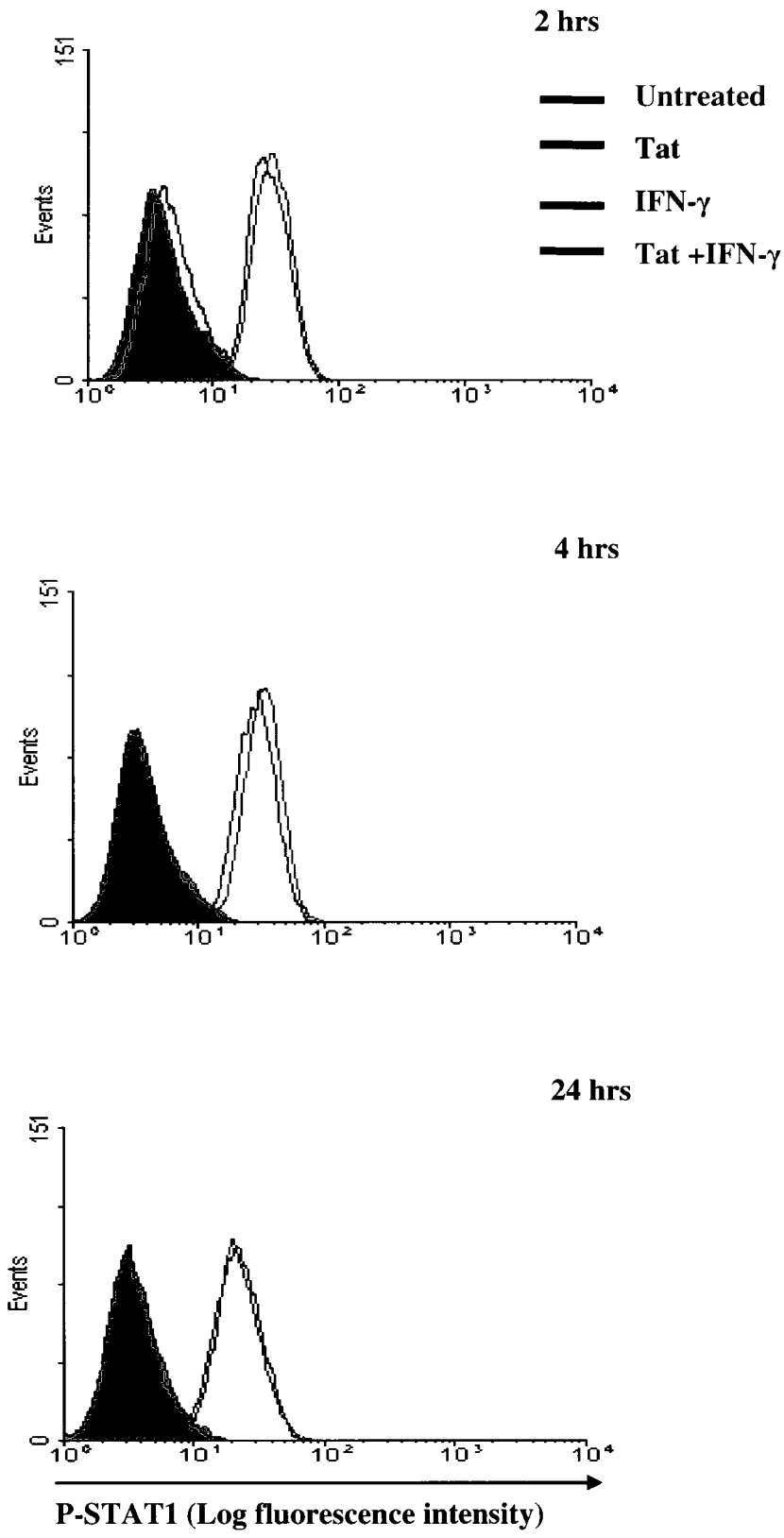

2 hrs
Untreated
Tat
IFN-γ
Tat +IFN-γ
Events
151
0
10^0
10^1
10^2
10^3
10^4
4 hrs
Events
151
0
10^0
10^1
10^2
10^3
10^4
24 hrs
Events
151
0
10^0
10^1
10^2
10^3
10^4
P-STAT1 (Log fluorescence intensity)

Figure 3-16: Tat had no effect on STAT1 activation in U937 cells.

U937 cells ($4X10^5$ cells) were cultured with or without Tat (100 ng/mL) for 2 hrs, 4 hrs, and 24 hrs. The cells were then restimulated with IFN-γ (10 ng/mL) for 15 min. Cells were immediately stained with anti-P-STAT1 antibodies. Within the U937 cell gate, 10000 events were acquired. Histograms were overlaid and plotted (# of events on the y-axis, log fluorescence intensity on the x-axis). A comparison between STAT1 activation in Tat treated and untreated cells with or without subsequent 15 min restimulation with IFN-γ was performed.

Thus, I hypothesized that the impact of IFN-γ-induced STAT1 hyperactivation would be reflected in the effect of this cytokine on STAT1 responsive genes including HLA-DR, IRF-1, CXCL9, and CXCL10.

3.311 Evaluation of HLA-DR expression levels

IFN-γ activates and affects monocyte surface receptors including the human leukocyte antigen (HLA)-DR (156). HLA-DR is a major histocompatibility complex (MHC) class II receptor, which has an important role in antigen presentation (265,266). A study from HIV+ patients has suggested that the expression of HLA-DR is downregulated on monocytes (48). Patient PBMCs ($4X10^5$ cells) were cultured in absence or presence of IFN-γ (10 ng/mL) for 24 hrs. Cells were then washed and stained with specific monoclonal anti-HLA-DR-FITC antibody (BD Biosciences) in parallel with anti-CD14-PerCp antibody for 15 min, and analyzed by flow cytometry as described in section 2.062. Analysis of the patient samples showed that IFN-γ stimulation upregulated HLA-DR expression on monocytes from all subjects, but no significant differences between patient study groups were observed (Fig. 3-17).

3.312 Measurement of IRF-1 mRNA expression levels

IRF-1 belongs to a family of transcription factors called the IRF family. It is induced in response to several stimuli including IFNs, TNF-α, IL-1, IL-6, Leukemia inhibitory factor (LIF), and LPS. It has been shown that IRF-1 plays an important role in the cell cycle and apoptosis, immune regulation, and antiviral defense (202,267,268). Furthermore, studies have demonstrated that *in vitro* infection of PBMC and macrophages with HIV resulted in upregulation of the IRF-1 expression (269,270).

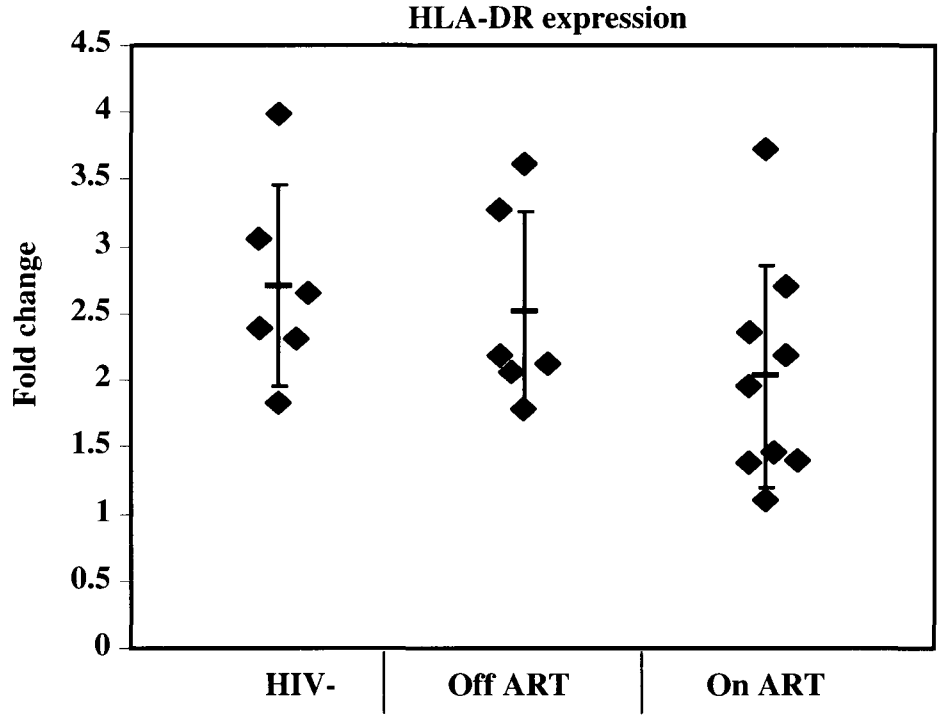

HLA-DR expression
Fold change
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
HIV-
Off ART
On ART

Figure 3-17: IFN-γ upregulated HLA-DR expression in patient monocytes.

Patient PBMCs ($4X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL) for 24 hrs. Cells were stained with anti-HLA-DR-FITC and anti-CD14-PerCp then analyzed by flow cytometry. The fold change in mean fluorescence intensity of each patient sample was plotted. Each symbol represents data from one patient. The mean for each study group is represented by a horizontal dash and plotted along with the S.D. (vertical error bars).

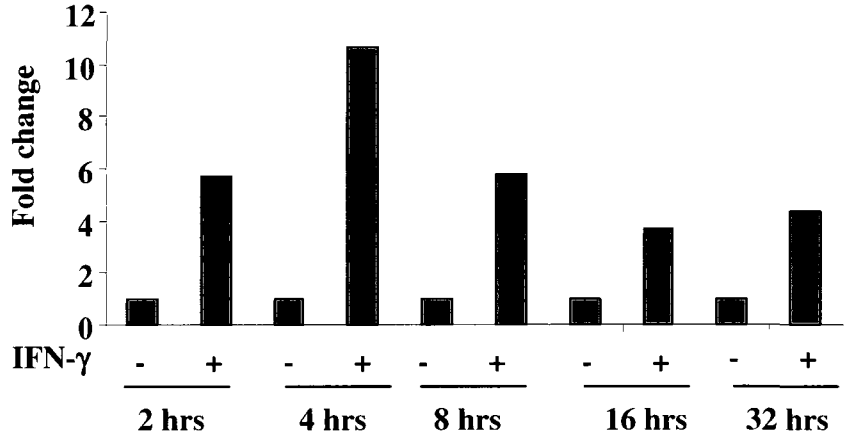
Therefore, IRF-1 expression levels in response to IFN-γ were evaluated using quantitative PCR, as described in section 2.090. Optimization experiments were performed on monocytes from HIV- donors. CD14+ monocytes were purified from the PBMCs of patients using CD14 microbeads (described in section 2.050). Purified CD14+ monocytes ($5X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL) over a (2-32 hrs) time course. Total RNA was extracted, reverse transcribed into cDNA, and subjected to real time-PCR (described in 2.090). Indeed, IFN-γ-induced upregulation of IRF-1 mRNA expression in primary monocytes as early as 2 hrs after stimulation and peaked by 4 hrs, then declined but remained detectable at 32 hrs post stimulation (Fig. 3-18, A). Similar results were also observed in U937 cells (data not shown).

From these experiments, the 4 hrs time point was selected for evaluation in patient monocytes, as samples were limited. Purified monocytes ($5X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL) for 4 hrs. Total RNA for each sample was analyzed for IRF-1 expression by real-time PCR (described in 2.090). IFN-γ stimulation upregulated IRF-1 mRNA expression in monocytes from all subjects (Fig. 3-18, B, and data not shown). Although it appeared that IFN-γ-induced IRF-1 expression was increased in monocytes from ART-treated HIV patients, this was not statistically significant (Fig. 3-18, B).

3.313 Determination of MIG (CXCL9), and IP-10 (CXCL10) expression levels

Chemokines and monokines are small proteins usually produced from activated macrophages and other cell types during inflammatory reactions and viral infection (271-273). IFN-γ regulates macrophage effector functions and induces CXC α-chemokine secretion including IP-10 and MIG.

A


Fold change
12
10
8
6
4
2
0
IFN-γ
- + - + - + - + - +
2 hrs
4 hrs
8 hrs
16 hrs
32 hrs

B

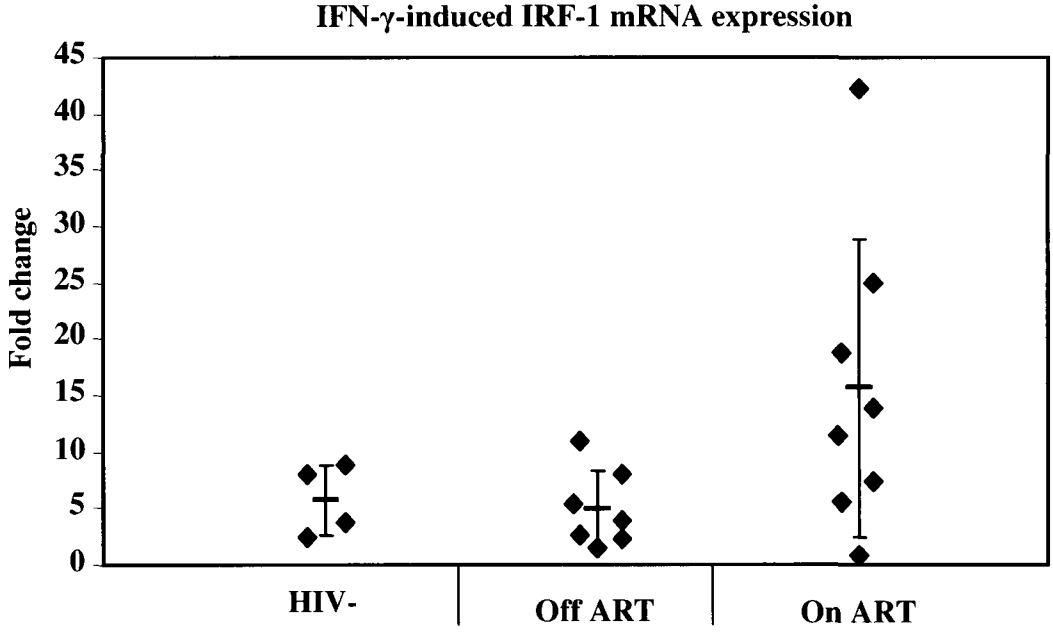

IFN-γ-induced IRF-1 mRNA expression
Fold change
45
40
35
30
25
20
15
10
5
0
HIV-
Off ART
On ART

Figure 3-18: IFN-γ upregulated IRF-1 mRNA expression in patient monocytes.

A) Purified monocytes ($5X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL) for different time points (2, 4, 8, 16, 32 hrs). Cells were collected, and total RNA was extracted and converted to cDNA then analyzed by real-time PCR. The fold change (IFN-γ-stimulated *vs.* unstimulated cells) for each time point was plotted. **B)** IFN-γ-induced IRF-1 mRNA expression in monocytes from patients study groups. Purified monocytes ($5X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL) for 4 hrs. Cells were collected, and total RNA was then extracted and analyzed for IRF-1 expression by real-time PCR. The fold change of IRF-1 expression in each patient sample was plotted. Each symbol represents data from one patient. All data was analyzed by Student's *t*- test and p values are indicated where significant. The mean for each study group is represented by a horizontal dash and plotted along with the S.D. (vertical error bars).

IP-10 and MIG also known as CXCL10 and CXCL9, respectively, are members of the CXC chemokine family and share the CXCR3 receptor. CXCR3 is expressed on activated T and B lymphocytes and on endothelial cells (271-273). IP-10 is involved in delayed hypersensitivity, and attracts monocytes and activated T cells to inflammatory foci (271,273-275). It also promotes selective enhancement of Th-1 responses and increases IFN-γ gene expression (276). MIG is mainly secreted by activated macrophages. It is involved in chronic inflammation as well as in viral and protozoan infections (272,277,278). It also plays an important role in chemotaxis, and T cell activation. It promotes the migration of activated T cells and induces the adhesion of IL-2-stimulated T cells through its receptor (CXCR3) (271,272,274,278). *In vitro* models of HIV infection and *ex vivo* studies in patients have demonstrated the upregulation of several of these genes, including IP-10, and MIG in PBMCs, monocytes and macrophages (274,275,279-281). Therefore, IP-10, MIG, expression levels in response to IFN-γ was measured using a human chemokine kit I (BD), a flow cytometry based expression assay.

Purified monocytes ($4X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL) for 24 hrs. Supernatants of monocyte cultures were then collected and assayed by CBA and flow cytometry, as described in section 2.063. Analysis of supernatants from patient monocytes showed that CXCL9/MIG, and CXCL10/IP-10 expression was upregulated by IFN-γ compared to unstimulated monocytes in all patients studied (Fig. 3-19, and data not shown). IFN-γ-induced CXCL9 was elevated in a few patients (on and off therapy) but the substantial variation within each HIV+ patient group yielded a significant upregulation only in ART patients. In contrast, IFN-γ-induced CXCL10 expression showed no significant differences between patient groups (Fig. 3-19).

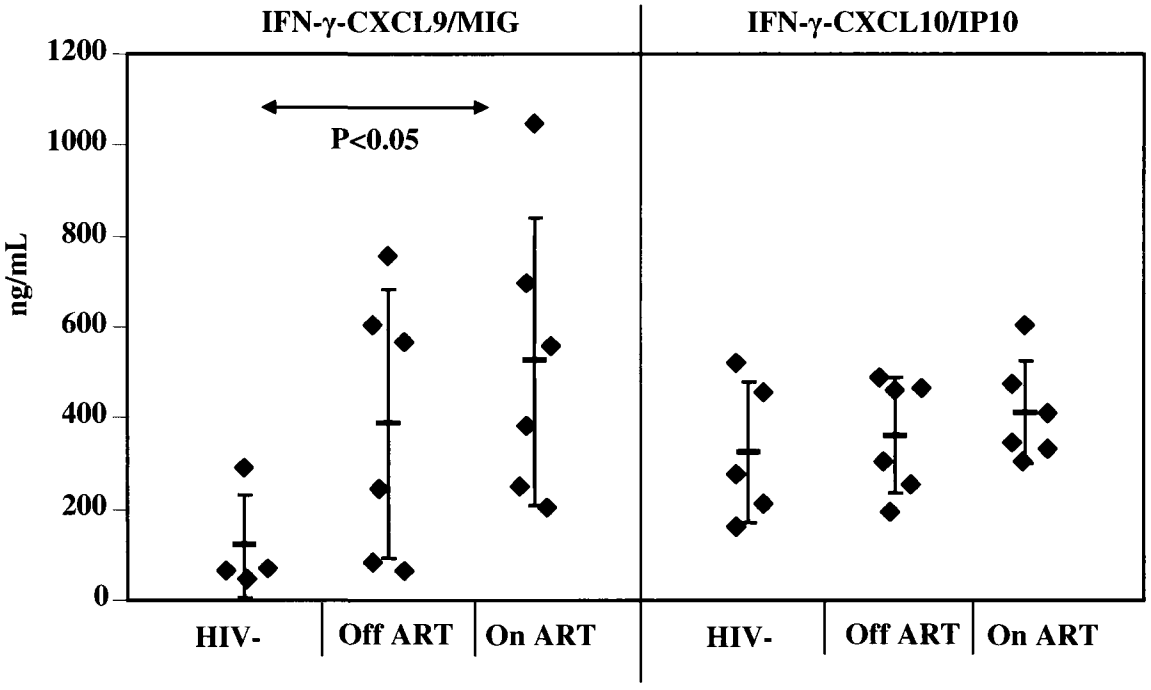

IFN-γ-CXCL9/MIG
IFN-γ-CXCL10/IP10
1200
1000
800
600
400
200
0
ng/mL
P<0.05
HIV-
Off ART
On ART
HIV-
Off ART
On ART

Figure 3-19: IFN-γ upregulated CXCL9, and CXCL10 expression in patient monocytes.

Patient monocytes ($4X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL) for 24 hrs. Supernatants were collected and analyzed by using the CBA chemokine kit and flow cytometry. IFN-γ-induced CXCL9 (left) and CXCL10 (right) expression in monocytes supernatant from patients study groups was plotted. Each symbol represents data from one patient. All data was analyzed by Student's *t*-test and p values are indicated where significant. The mean for each study group is represented by a horizontal dash and plotted along with the S.D. (vertical error bars).

3.320 Evaluation of PCD in monocytes from patient study groups

IFN-γ plays an important role in the activation of M/M effector functions including phagocytosis, antigen presentation, and cytokine secretion. However, there have been reports showing that IFN-γ also induces cell death in different cell types including M/M. For instances, studies have shown that IFN-γ is capable of inducing apoptosis in human monocytes and monocyte-drived macrophages (MDM) (282,283). Further, it has been shown that IFN-γ can induce apoptosis through the activation of the STAT1 and IRF-1 transcription factors in many tumor cell lines such as U937, Jurkat, Raji, and ME180 as well as in Th-1 and Th-2 clones (257,284-286). Studies have also shown that activation of STAT1 by IFN-γ or DNA-damaging agents leads to apoptosis via activation of caspases 1, 2, 3, 7 and 8 or interaction with p53 in the monocytic cells THP-1 and human fibrosarcoma cell lines 2fTGH and U3A (262,263,287,288). Another report has revealed that IFN-γ induced apoptosis by downregulating Bcl2 and upregulating Bak expression in HL-60 cells (289).

IL-10, on the other hand, has inhibitory effects on M/M functions. In addition, it has been shown to inhibit IFN-γ-induced STAT1 signaling and the downstream gene expression including IP-10, and the adhesion molecule ICAM in monocytes (290,291). Further, IL-10 has been shown to inhibit spontaneous cell death in normal monocytes (292,293). Furthermore, studies have shown that IFN-γ and IL-10 are elevated in chronic HIV+ patients (178-181,183,294,295).

Therefore, I hypothesized that the defective M/M function during chronic HIV infection may result from abnormal regulation of programmed cell death. Moreover, I suspected that the upregulated STAT1 activation in response to IFN-γ found in off therapy patient monocytes may translate into an enhanced susceptibility to PCD. To detect cell death in CD14+ monocytes in response to IFN-γ and IL-10 stimulation,

two flow cytometry based techniques were used to confirm the results. Intracellular staining (IC) with Propidium iodide (PI) dye (BD Biosciences) and cell cycle analysis, as well as annexin-v-FITC and PI staining (Apoptosis Detection Kit; Molecular Probes) as described in section 2.064.

3.321 Optimization of the detection of monocyte PCD in response to IFN-γ and IL-10 stimulation

Detection of PCD was optimized in normal primary monocytes using IC PI staining and cell cycle analysis as well as annexin-v-FITC/PI staining approaches. Purified monocytes ($4X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL), IL-10 (10 ng/mL), or pretreated with IL-10 for 2hrs followed by IFN-γ for 24 hrs, or treated with staurosporine (2 μM), a known inducer of apoptosis as a positive control, for 2 hrs. Cell viability was then measured by annexin-v-FITC/PI staining (described in section 2.064). There was a detectable level of spontaneous cell death as determined by % of annexin-v-FITC positive staining or % of annexin-v-FITC/PI positive staining (Fig. 3-20, A). IFN-γ stimulation enhanced monocyte cell death (Fig. 3-20, A). Interestingly, IL-10 stimulation inhibited spontaneous as well as IFN-γ-induced cell death. As expected, staurosporine treatment induced cell death in monocytes (Fig. 3-20, A). Since the trend observed with annexin-v-FITC positive staining was similar to that observed with annexin-v-FITC/PI positive staining, I defined the % of cell death as the % of annexin-v-FITC single positive cells plus the % of annexin-v-FITC/PI double positive cells, as indicated by the red box in Fig 3-20, A. Fig. 3-20, B shows that there was a basal level of spontaneous monocyte cell death over 24 hrs of culture that was slightly increased further after 48 hrs.

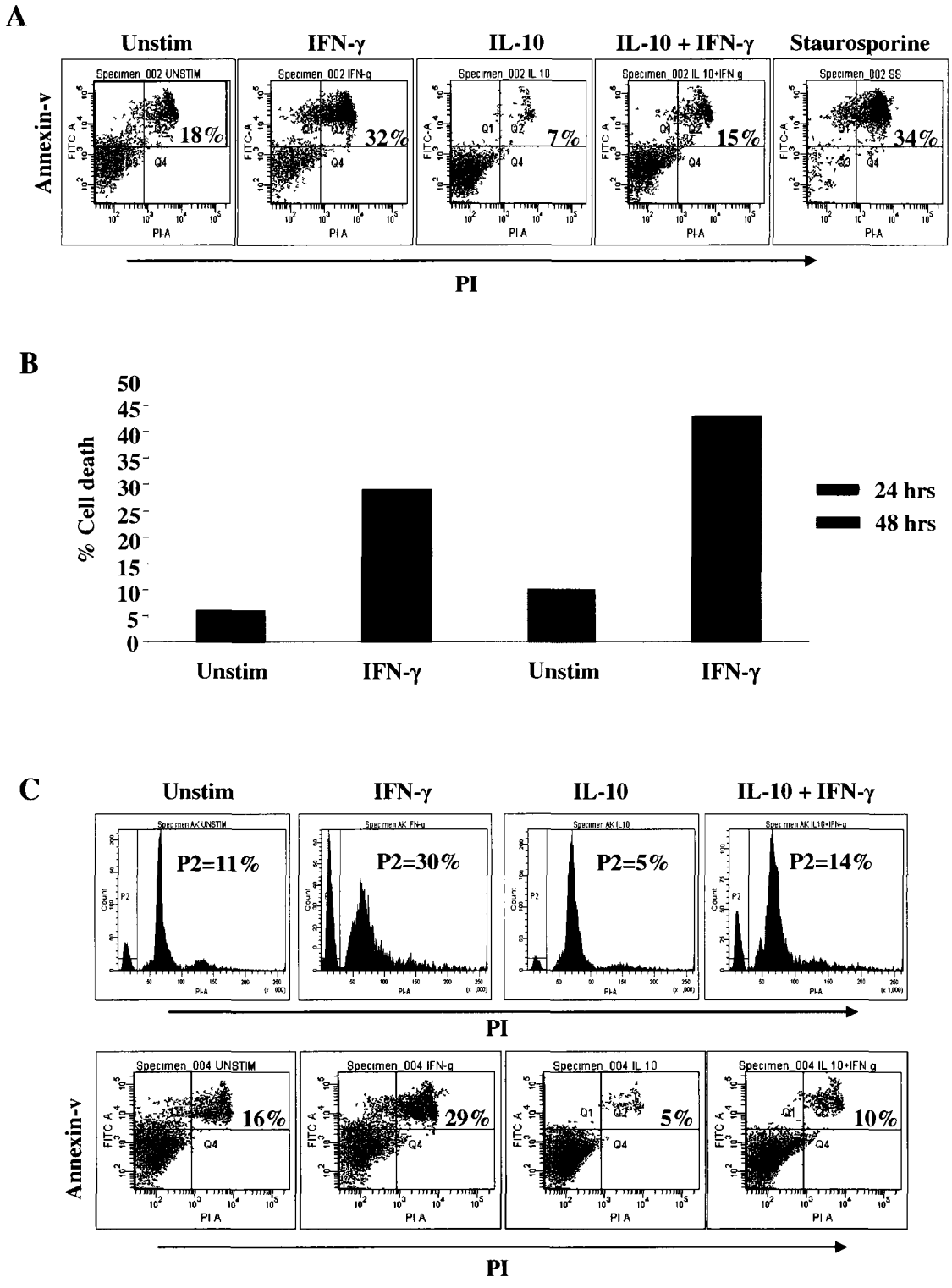

A
Unstim
IFN-γ
IL-10
IL-10 + IFN-γ
Staurosporine
Annexin-v
PI
18%
32%
7%
15%
34%
B
% Cell death
24 hrs
48 hrs
Unstim
IFN-γ
Unstim
IFN-γ
C
Unstim
IFN-γ
IL-10
IL-10 + IFN-γ
P2=11%
P2=30%
P2=5%
P2=14%
Annexin-v
PI
16%
29%
5%
10%

Figure 3-20: IFN-γ induced PCD in primary human CD14+ monocytes.

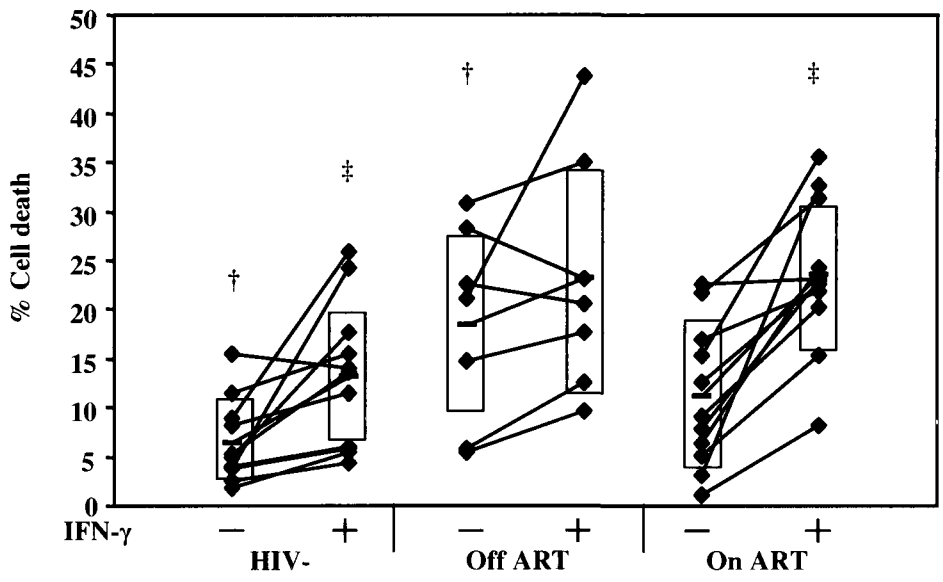
A) Purified CD14+ monocytes ($4X10^5$ cells) were cultured with or without IFN-γ (10 ng/mL), IL-10 (10 ng/mL), or IL-10 and IFN-γ together for 24 hrs or treated with staurosporine (2 μM) for 2 hrs. Cells were then stained with annexin-v-FITC and PI and analyzed by flow cytometry. % Cell death was calculated by summing % annexin-v-FITC positive (quadrant Q1) and % PI positive cells (quadrant Q2). **B)** A bar graph plot shows the % of cell death observed in unstimulated and IFN-γ stimulated monocytes over a 24 and 48 hrs time frame. **C)** Evaluation of monocytes cell death by two different methods. Purified monocytes ($4X10^5$ cells) were cultured with or without IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or IL-10 for 2 hrs followed by IFN-γ for 24 hrs. Cells were either permeabilized, and then stained intracellularly with PI or stained directly with annexin-v-FITC/PI and analyzed by flow cytometry. Two representative experiments were performed under the following conditions: no stimulation, IFN-γ, IL-10, IL-10 + IFN-γ simulation and analyzed by the two different assays. Upper panel shows histograms of PI staining and DNA content. Cell death was determined by their subdiploid DNA content and the % measured by applying the P2 gate as shown in each plot. Lower panel shows dot plots and % of annexin-v-FITC/PI staining of the same set of monocyte stimulations.

IFN-γ stimulation drastically enhanced monocyte cell death further over a (24 and 48 hrs) time course in a time dependent manner. To confirm these results, intracellular PI staining (cell cycle analysis) and annexin-v-FITC/PI staining were compared. Purified monocytes ($4X10^5$ cells) were left untreated or treated with IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or with IL-10 and IFN-γ together for 24 hrs. Cell viability was then assayed by PI staining and cell cycle analysis or by annexin-v-FITC/PI staining (described in section 2.064). Both techniques showed that there were detectable levels of spontaneous monocyte cell death that were enhanced further with IFN-γ stimulation (Fig. 3-20, C). Interestingly, IL-10 inhibited both spontaneous as well as IFN-γ-induced monocyte cell death.

3.322 Evaluation of IFN-γ-induced cell death in patient CD14+ monocytes

Subsequent experiments on patient monocytes were performed. Purified monocytes ($4X10^5$ cells) from each subject were left unstimulated or stimulated with IFN-γ (10 ng/mL) for 24 hrs. Cells were IC stained with PI for cell cycle analysis and flow cytometry (described in section 2.064). IFN-γ upregulated monocyte cell death compared to unstimulated cells in HIV- controls and ART patients (Fig. 3-21, A). Despite a similar trend being noted in most off therapy patients, this was not statistically significant. Interestingly, spontaneous cell death was significantly elevated in monocytes from off therapy patients compared to HIV- controls and correlated with the increased total STAT1 expression levels observed (Fig. 3-8; r=0.924, p=0.025). Also striking was that monocyte cell death in the presence of IFN-γ remained higher in ART patients compared to HIV- controls. Fig. 3-21, B shows representative data obtained from an HIV- control and an off therapy HIV+ patient.

A


% Cell death
50
45
40
35
30
25
20
15
10
5
0
†
‡
IFN-γ
–
+
HIV-
Off ART
On ART

B

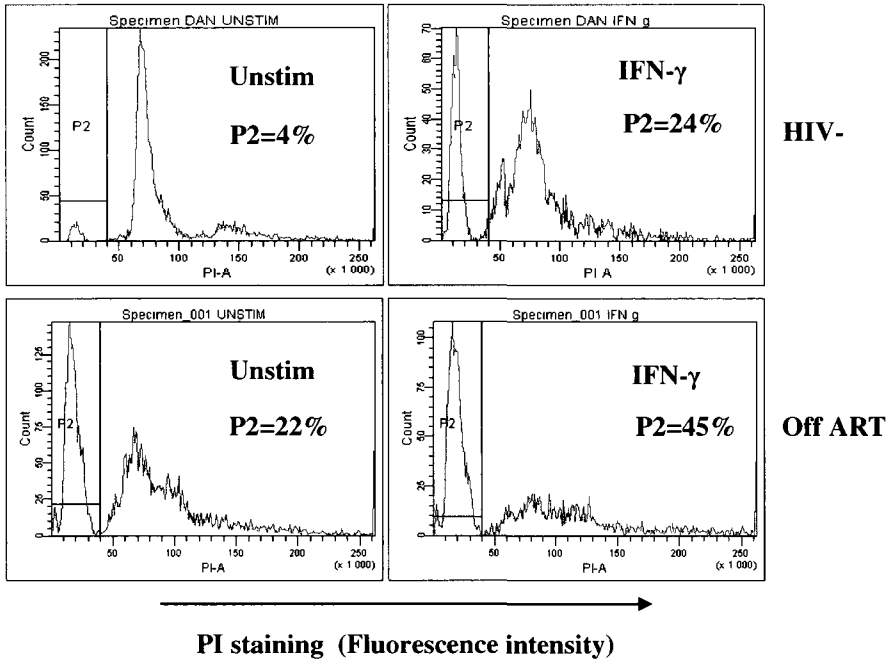

Specimen DAN UNSTIM
Unstim
P2=4%
Specimen DAN IFN g
IFN-γ
P2=24%
HIV-
Specimen_001 UNSTIM
Unstim
P2=22%
Specimen_001 IFN g
IFN-γ
P2=45%
Off ART
Count
P2
PI-A
PI A
(x 1 000)
PI staining (Fluorescence intensity)

Figure 3-21: Detection of spontaneous and IFN-γ-induced cell death in patient monocytes.

Purified monocytes ($4X10^5$ cells) were cultured with IFN-γ (10 ng/mL) for 24 hrs. Subsequently, cells were permeabilized, stained with PI, and analyzed for DNA content by flow cytometry. **A)** The % of cell death for each patient was plotted. Each joined pair of symbols represents data for a given patient's monocytes after a 24 hrs culture in the presence or absence of IFN-γ. †: $p<0.02$, comparing spontaneous cell death in monocytes from HIV+ patients off therapy vs. HIV- controls; ‡: $p<0.005$ comparing cell death after IFN-γ stimulation in HIV+ patients on ART vs. HIV- controls. All data was analyzed by Student's *t*-test and p values are indicated where significant. The mean for each study group is represented by a horizontal dash and plotted along with the S.D. (box). **B)** Histograms plotting the fluorescence intensity of intracellular PI staining in unstimulated (left plots) and IFN-γ stimulated (right plots) monocytes cultured for 24 hrs from a representative HIV- control (upper panels) and an HIV+ patient off ART (lower panels) are shown. Cell death was defined by their subdiploid DNA content and the % calculated by applying the P2 gate as shown.

3.323 Study the effect of IL-10 stimulation on spontaneous and IFN-γ-induced PCD in monocytes from HIV+ patients

As mentioned earlier, IL-10 was initially used as a negative control since it has been shown to inhibit monocyte cell death in some studies (292,296,297). Thus, the effect of IL-10 stimulation was tested in some of the patient samples by flow cytometry. Purified monocytes were left unstimulated or stimulated with IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or IL-10 and IFN-γ together for 24 hrs. Cell death was then evaluated by IC PI staining and flow cytometry. In these experiments, IL-10 was capable of inhibiting spontaneous (Fig. 3-22, A) and IFN-γ-induced monocyte PCD (Fig. 3-22, B) in HIV- controls as well as in HIV+ patients. Also, similar results were observed by using annexin-v-FITC/PI staining. Fig. 3-22, C shows that annexin-v-FITC/PI staining of monocytes from an HIV- control (upper panel) and HIV+ on ART (lower panel). This preliminary result supports my first finding that unlike IFN-γ, IL-l0 induced STAT signaling in monocytes from HIV+ patients was not altered. Since spontaneous and IFN-γ-induced monocyte cell death were significantly elevated in HIV+ patients, further studies were designed to investigate the mechanisms underlining the increased susceptibility of HIV+ patient monocytes to cell death.

3.324 Expression levels of secreted TRAIL in plasma and supernatants of cultured patient monocytes

It has been shown that IFN-γ stimulation sensitized different cell types to TRAIL-induced cell death (261,298). Other studies have demonstrated that IFN-γ induced TRAIL expression and secretion in monocytes and macrophages (139,299,300). Other reports have revealed that both types of IFNs were increased in HIV+ patients (179,295,301).

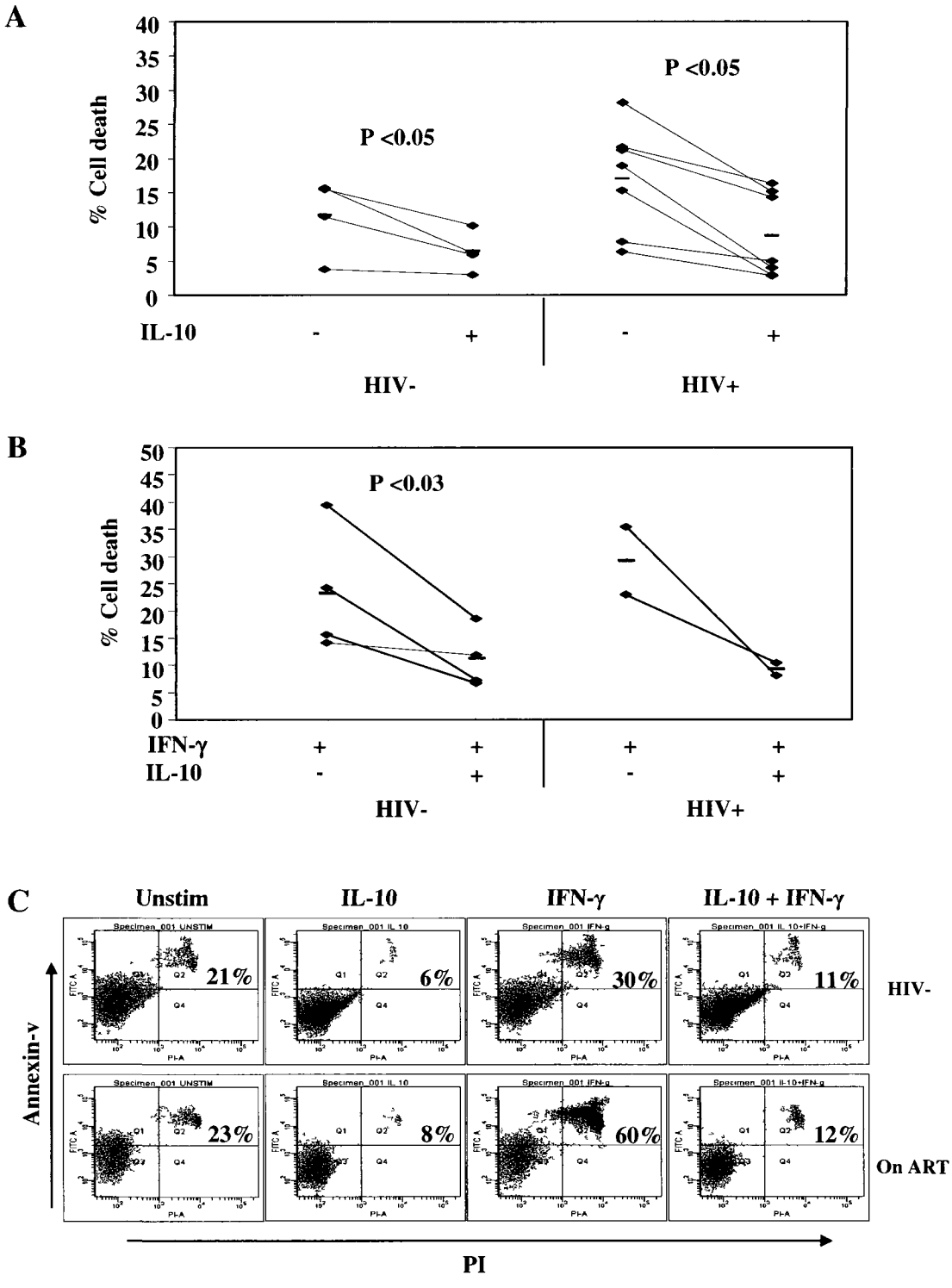

A
% Cell death
P <0.05
P <0.05
IL-10
HIV-
HIV+
B
% Cell death
P <0.03
IFN-γ
IL-10
HIV-
HIV+
C
Unstim
IL-10
IFN-γ
IL-10 + IFN-γ
21%
6%
30%
11%
23%
8%
60%
12%
HIV-
On ART
Annexin-v
PI

Figure 3-22: IL-10 inhibited spontaneous and IFN-γ-induced cell death in patient monocytes.

Purified monocytes ($4X10^5$ cells) were left untreated or treated with IL-10 (10 ng/mL), IFN-γ (10 ng/mL) or IL-10 for 2 hrs followed by stimulation with IFN-γ for 24 hrs. Subsequently, cells were either permeabilized and stained with PI, or stained directly with annexin-v-FITC/PI and analyzed for DNA content by flow cytometry. The % of monocyte cell death for each patient was plotted. Each joined pair of symbols represents data for a given patient after a 24 hrs monocyte culture in the presence or absence of IL-10. **A)** IL-10 inhibited spontaneous cell death in monocytes from HIV- controls and HIV+ patients. **B)** IL-10 inhibited IFN-γ-induced monocyte cell death in HIV- controls and HIV+ patients. Cell death was defined by their subdiploid DNA content. **C)** Dot plots show annexin-v-FITC/PI staining of monocytes from an HIV- control and HIV+ patient on ART under the same culture conditions: no stimulation, IL-10, IFN-γ, IL-10 + IFN-γ. The % of cell death is indicated on each graph. Statistical analysis was performed using the paired Student's *t*-test and p values are indicated where significant.

In support of a role for TRAIL is that *in vitro* HIV-infected macrophages exhibited increased cell death in response to exogenous addition of this molecule (218,302). Thus, I hypothesized that IFN-γ-induced monocyte cell death may be related to the increased expression of TRAIL in the monocyte environment.

Patient plasma and supernatants from 24 hrs IFN-γ (10 ng/mL) treated and untreated monocytes ($4X10^5$ cells) were collected and assayed for TRAIL expression levels by ELISA (Diaclone) (described in section 2.080). Plasma TRAIL levels were significantly increased in HIV+ patients off therapy compared to HIV- controls and patients on ART (Fig. 3-23, A). Spontaneous cell death and plasma TRAIL appeared to exhibit a similar trend, but this was not statistically significant (r=0.542, p=0.165). TRAIL secretion in supernatants of cultured monocytes showed that there were low basal levels of TRAIL detected in some subjects. However, IFN-γ induced TRAIL production in monocytes from most patients studied (Fig. 3-23, B).

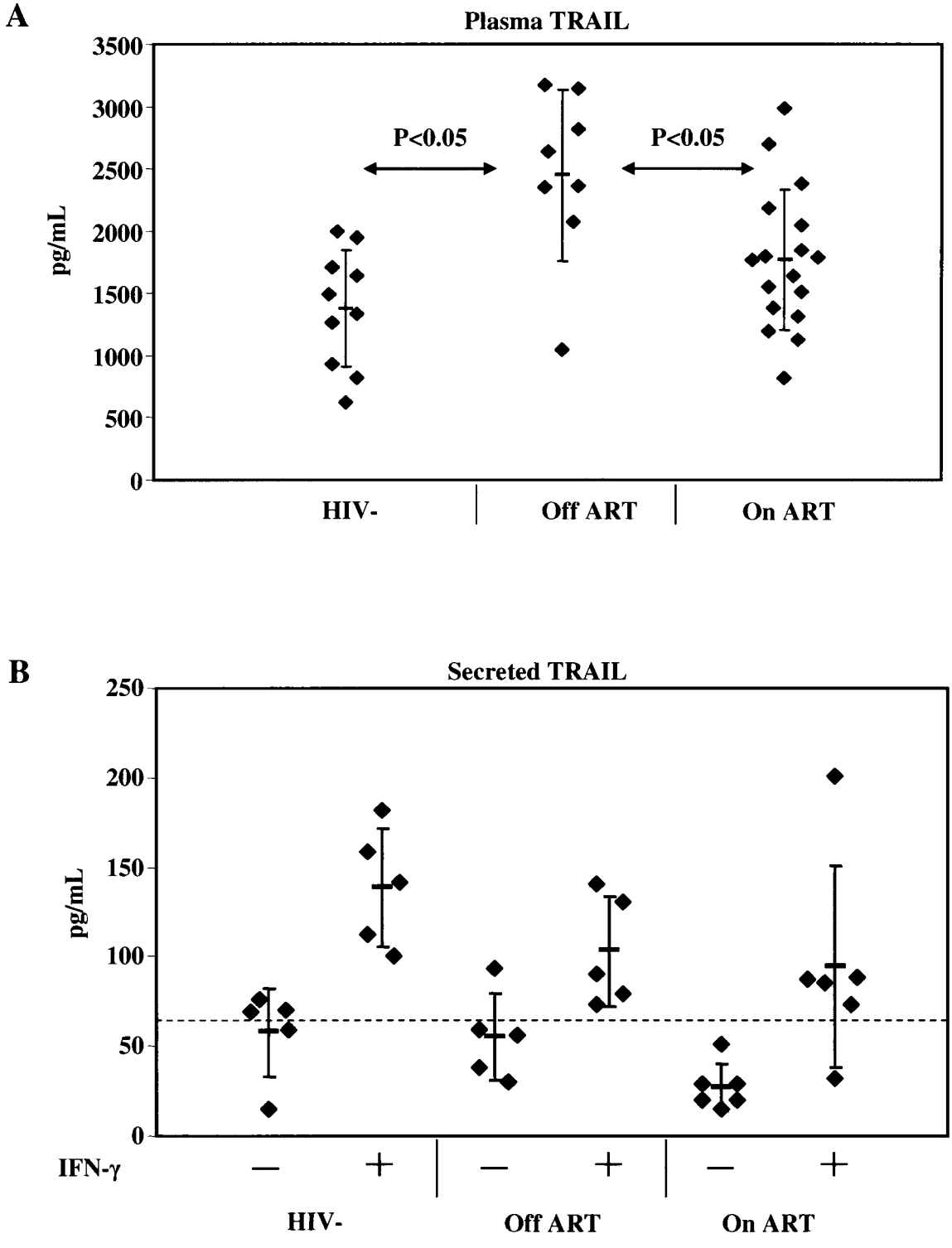

A
Plasma TRAIL
3500
3000
2500
2000
1500
1000
500
0
pg/mL
P<0.05
P<0.05
HIV-
Off ART
On ART
B
Secreted TRAIL
250
200
150
100
50
0
pg/mL
IFN-γ
—
+
—
+
—
+
HIV-
Off ART
On ART

Figure 3-23: Increased levels of TRAIL in plasma from HIV+ off therapy patients but not in supernatant of cultured monocytes.

A) Patients plasma was directly assayed by ELISA. TRAIL levels for all subjects were plotted and expressed as pg/mL. **B)** Supernatant from purified monocytes ($4X10^5$ cells) cultured in presence or absence of IFN-γ (10 ng/mL) for 24 hrs were collected, and subsequently subjected to ELISA. IFN-γ-induced TRAIL expression in medium was plotted and expressed as pg/mL. Each symbol represents data from one patient. The mean for each study group is represented by a horizontal dash and plotted along with the S.D. (vertical error bars). All data was analyzed by Student's *t*-test and p values are indicated where significant. The dotted line indicates the limit of detection.

Chapter IV

Differential Regulation of Monocyte PCD by IFN-γ

and IL-10 via the Autophagy Pathway

The regulation of human monocyte PCD by IFN-γ and IL-10 stimulation was particularly an interesting and important finding, and has not been well characterized. Therefore, subsequent studies were designed to investigate what form of PCD occurs in primary human monocytes, and to determine the molecular mechanisms involved in the regulation of monocyte PCD.

4.000 Results:

4.100 Objective #4: Investigate what form of PCD occurs in primary human monocytes

4.110 Evaluation of monocyte PCD in response to IFN-γ and IL-10 stimulation

It was shown above by IC PI staining and cell cycle analysis that IFN-γ enhanced monocyte PCD in HIV- and HIV+ patients but IL-10, interestingly, could rescue spontaneous and IFN-γ-induced monocyte PCD. This result was further confirmed in normal monocytes from healthy controls using annexin-v-FITC/PI staining (described in section 2.064). Purified primary monocytes were cultured with or without IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or IL-10 and IFN-γ together for 24 hrs. Monocytes were then stained with annexin-v-FITC/PI and analyzed by flow cytometry. Fig. 4-1 shows that indeed there were detectable levels of spontaneous monocyte cell death and IFN-γ enhanced this further. Interestingly, pretreatment with IL-10 diminished spontaneous and IFN-γ-induced monocyte PCD significantly (Fig. 4-1), confirming my previous findings. In order to understand the mechanism by which monocyte PCD is regulated, investigation of what form of PCD occurs under these conditions was examined.

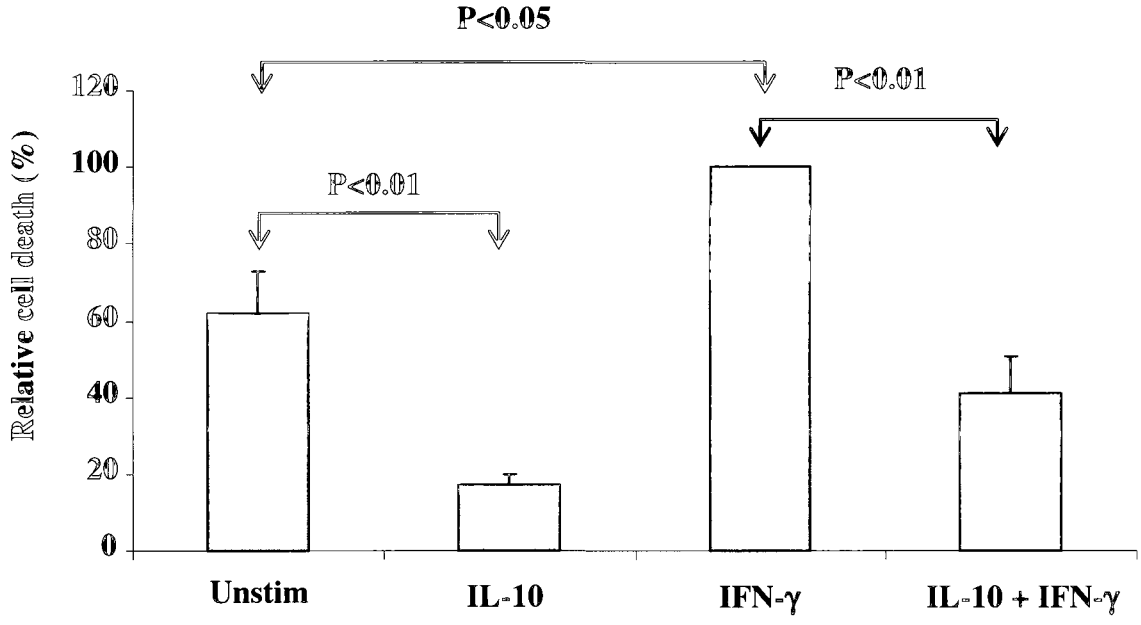

P<0.05
P<0.01
P<0.01
Relative cell death (%)
120
100
80
60
40
20
0
Unstim
IL-10
IFN-γ
IL-10 + IFN-γ

Figure 4-1: IL-10 inhibited spontaneous and IFN-γ-induced PCD in normal primary monocytes.

Purified monocytes ($4X10^5$ cells) were cultured with or without IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or IL-10 for 2 hrs followed by IFN-γ for 24 hrs. Cells were stained with annexin-v-FITC/PI and analyzed by flow cytometry. The average % cell death observed in seven independent experiments with the same conditions analyzed by annexin-v-FITC/PI staining was plotted along with standard deviation (S.D.) error bars. Cell death for all conditions was normalized to that of IFN-γ and presented as relative cell death. Statistical analysis was performed using the paired Student's *t*-test and p values are indicated where significant.

4.120 Determination of the involvement of classical apoptosis and the caspase cascade in monocyte PCD

Several studies have demonstrated that IFN-γ was able to induce apoptosis by activating caspases including caspase 1, 3, 7 in different model cell lines (257,262,287,288,303). Another report has shown that IL-10 and IL-4 diminished monocyte apoptosis by inhibiting caspase 8 activation and increasing Flice-like inhibitory protein (FLIP) expression, a natural antagonist of caspase 8 (292). Thus, investigation of the role of classical apoptosis pathways in the induction of monocyte cell death was carried out. The induction of TRAIL secretion in the media and activation of molecules downstream including caspase 8, and 3 in monocytes were evaluated after stimulation with IFN-γ and IL-10.

4.121 Measurement of TRAIL secretion in response to IFN-γ and IL-10 stimulation

Although TRAIL levels in monocyte supernatants from the patient study groups were measured and no significant differences were observed, this did not exclude the effects of secreted TRAIL on monocyte cell death and was investigated in parallel. Purified monocytes were left untreated or treated with IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or IL-10 and IFN-γ together for 24 and 48 hrs. Culture supernatants were assayed for TRAIL secretion by ELISA (described in section 2.080). With a trend similar to monocyte PCD, there were detectable levels of spontaneous TRAIL secreted in the media over 24 and 48 hrs time points and IFN-γ enhanced this further (Fig. 4-2). In contrast, IL-10 suppressed spontaneous and IFN-γ induced TRAIL secretion.

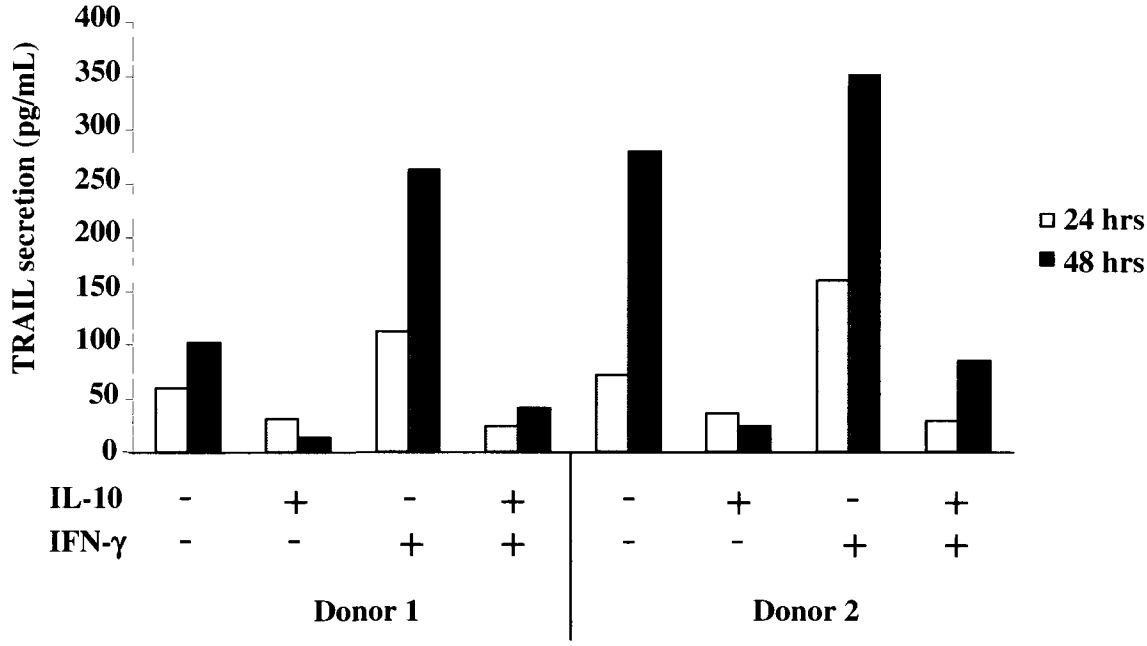

TRAIL secretion (pg/mL)
400
350
300
250
200
150
100
50
0
24 hrs
48 hrs
IL-10 - + - + - + - +
IFN-γ - - + + - - + +
Donor 1
Donor 2

Figure 4-2: IL-10 inhibited spontaneous and IFN-γ-induced TRAIL secretion.

Purified monocytes ($4X10^5$ cells) were cultured with or without IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or IL-10 for 2 hrs followed by IFN-γ for 24 hrs and 48 hrs as indicated on the graph. Supernatant from these cultures was collected and subjected to ELISA. Results from two representative donors were shown. TRAIL was secreted spontaneously overtime and enhanced further in response to IFN-γ but was inhibited with IL-10 stimulation.

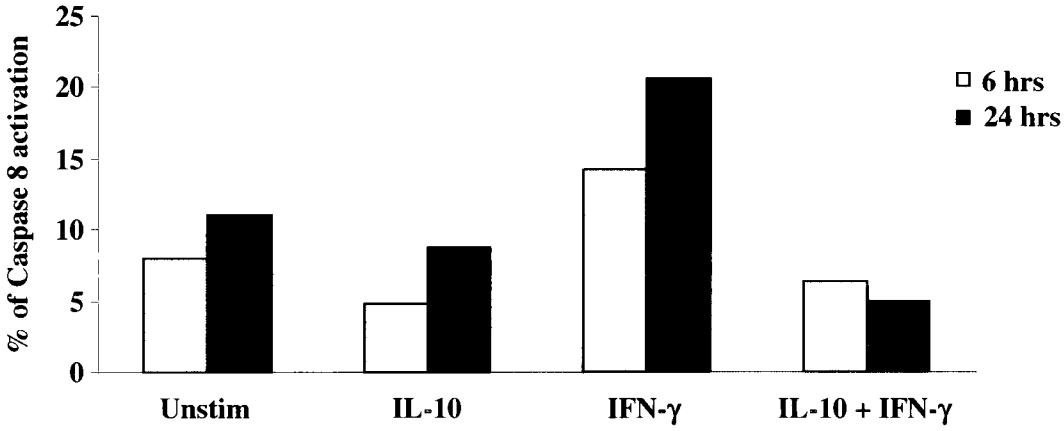
4.122 Evaluation of caspase 8 activation in monocytes after stimulation with IFN-γ and IL-10

Since TRAIL was secreted spontaneously and enhanced by IFN-γ in primary monocytes, investigation of the activation of molecules downstream of death receptor signaling was carried out. The activation of the initiator caspase 8 in monocytes was examined using the caspase 8 detection kit (Calbiochem). Purified monocytes ($4X10^5$ cells) were left unstimulated or stimulated with IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or with both IL-10 and IFN-γ and for 6 and 24 hrs. Cells were then stained with FITC-IETD-FMK, which binds caspase 8 specifically, and analyzed by flow cytometry (described in section 2.065). There were detectable levels of caspase 8 activation in 6 hrs monocyte cultures that were increased further over a 24 hrs period (Fig. 4-3, A). IFN-γ stimulation enhanced caspase 8 activation further as compared to unstimulated monocytes in a time dependent manner (Fig. 4-3, A). IL-10 stimulation, however, diminished both spontaneous and IFN-γ-induced caspase 8 activation in these cells over the same time frame (Fig. 4-3, A). Fig. 4-3, B shows the mean % of caspase 8 activation observed over 24 hrs in at least 3 independent experiments. Indeed and consistent with PCD and TRAIL secretion, caspase 8 activation was upregulated spontaneously in cultured monocytes and enhanced further with IFN-γ stimulation. Interestingly, IL-10 stimulation inhibited spontaneous and IFN-γ-enhanced caspase 8 activation significantly.

4.123 Evaluation of caspase 3 activation in monocytes after stimulation with IFN-γ and IL-10

Activation of molecules downstream of caspase 8, the effector caspase 3 and its cleavage was evaluated under the same conditions by western blotting.

A


% of Caspase 8 activation
25
20
15
10
5
0
6 hrs
24 hrs
Unstim
IL-10
IFN-γ
IL-10 + IFN-γ

B

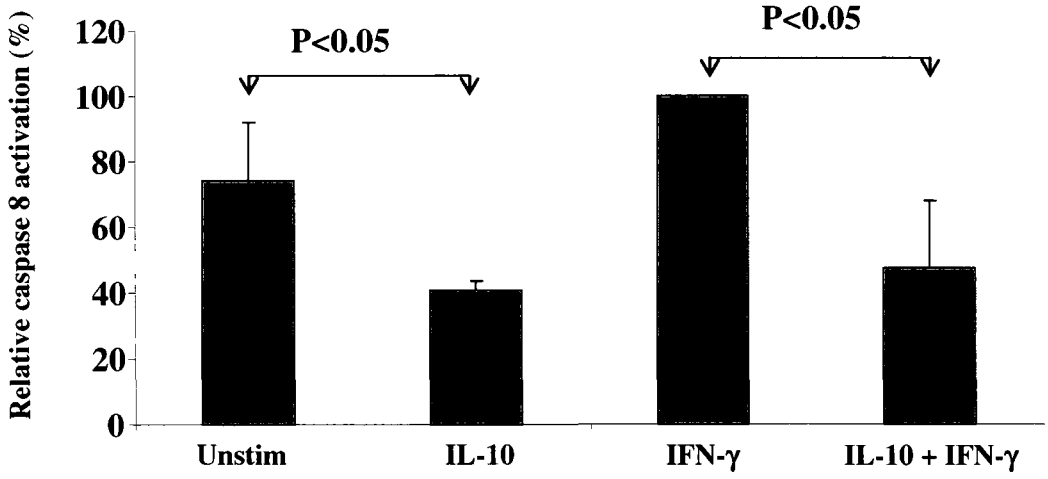

Relative caspase 8 activation (%)
120
100
80
60
40
20
0
P<0.05
P<0.05
Unstim
IL-10
IFN-γ
IL-10 + IFN-γ

Figure 4-3: IL-10 diminished spontaneous and IFN-γ-enhanced caspase 8 activation.

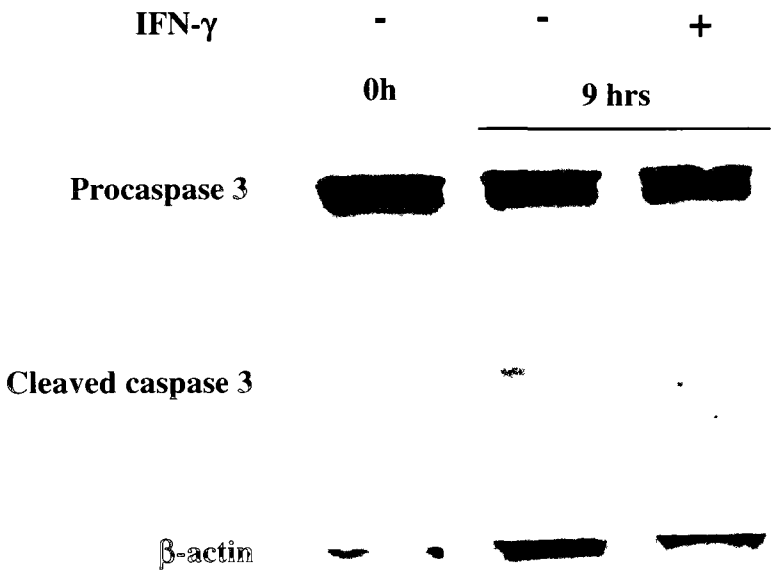
Purified monocytes ($4X10^5$ cells) were cultured with or without IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or IL-10 for 2 hrs followed by IFN-γ for 6 and 24 hrs. Cells were stained with FITC-labeled IETD-FMK and analyzed by flow cytometry. **A)** A representative time course experiment was plotted, and shows the % of activated caspase 8+ cells in cultured monocytes and in presence of IFN-γ, IL-10, or IL-10 and IFN-γ together. **B)** The average % of caspase 8+ cells obtained from three independent experiments was plotted along with S.D error bars after normalization to 24 hrs of IFN-γ stimulation. Statistical analysis was performed using the paired Student's *t*-test and p values are indicated where significant.

Purified monocytes ($2X10^6$ cells) were either collected directly or cultured with or without IFN-γ (10 ng/mL), IL-10 (10 ng/mL), or IL-10 and IFN-γ together for 8-9 hrs. Total cellular lysates were then collected and assayed by western blotting for caspase 3 expression as described in section 2.100. There were detectable levels of caspase 3 cleavage following 8-9 hrs of monocyte culture as compared to uncultured cells (Fig. 4-4 A, B lanes 1 and 2). However, there was no additional effect on caspase 3 cleavage in response to IFN-γ stimulation (Fig. 4-4 A, B lanes 3 and 4, respectively). Interestingly, unlike the trends observed with TRAIL production and caspase 8 activation, IL-10 had no preventative effects on caspase 3 cleavage in untreated or IFN-γ treated monocytes (Fig. 4-4, B lanes 3 and 5). These results suggested that the induction of cell death by IFN-γ and the cytoprotective effects of IL-10 may not be associated with simple caspase activation effects.

4.124 Evaluation of monocyte PCD in the presence of death receptor neutralizing antibodies or a general caspase inhibitor

It is established that the extrinsic and the intrinsic pathways of apoptosis occur via the activation of the caspase cascade. In order to study more specifically whether these pathways were involved in spontaneous and IFN-γ-induced monocyte PCD, the effect of blocking death receptor triggering or downstream caspase activation on monocyte PCD was examined. Purified monocytes ($4X10^5$ cells) were either untreated or treated with IFN-γ (10 ng/mL) in the presence or absence of increasing concentrations of neutralizing monoclonal antibodies for TRAIL (1-9 μg/mL), FasL (0.04-5 μg/mL) or the general caspase inhibitor, z-VAD-FMK (0.625-5 μM), for 24 hrs.

A


IFN-γ
-
-
+
0h
9 hrs
Procaspase 3
Cleaved caspase 3
β-actin

B

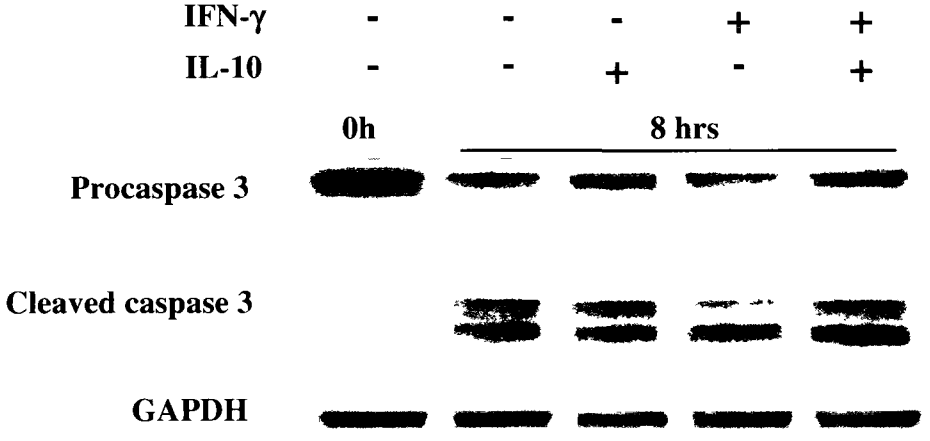

IFN-γ
-
-
-
+
+
IL-10
-
-
+
-
+
0h
8 hrs
Procaspase 3
Cleaved caspase 3
GAPDH

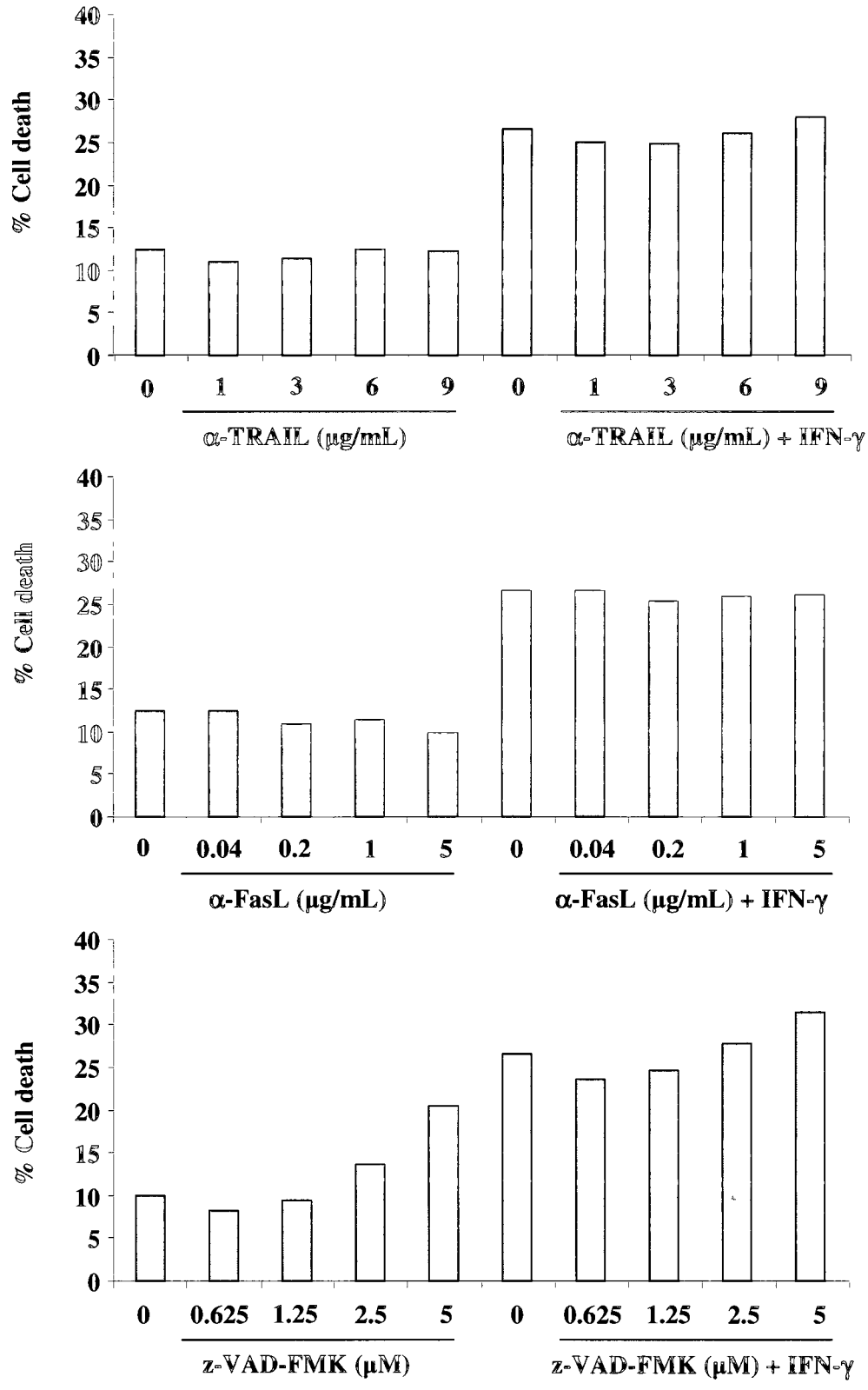
Figure 4-4: IL-10 and IFN-γ had no effects on caspase 3 cleavage in primary monocytes.

Purified monocytes ($2X10^6$ cells) were cultured with or without IL-10 (10 ng/mL), IFN-γ (10 ng/mL), or IL-10 for 2 hrs followed by IFN-γ for 8 or 9 hrs. Total cellular lysates were extracted and assayed by western blotting for caspase 3 cleavage. Detection of β-actin or GAPDH served as a loading control. **A)** Caspase 3 cleavage in unstimulated and after IFN-γ stimulation. **B)** Caspase 3 cleavage in unstimulated as well as with IFN-γ or IL-10 stimulations. A representative experiment out of at least three independent experiments performed is shown.

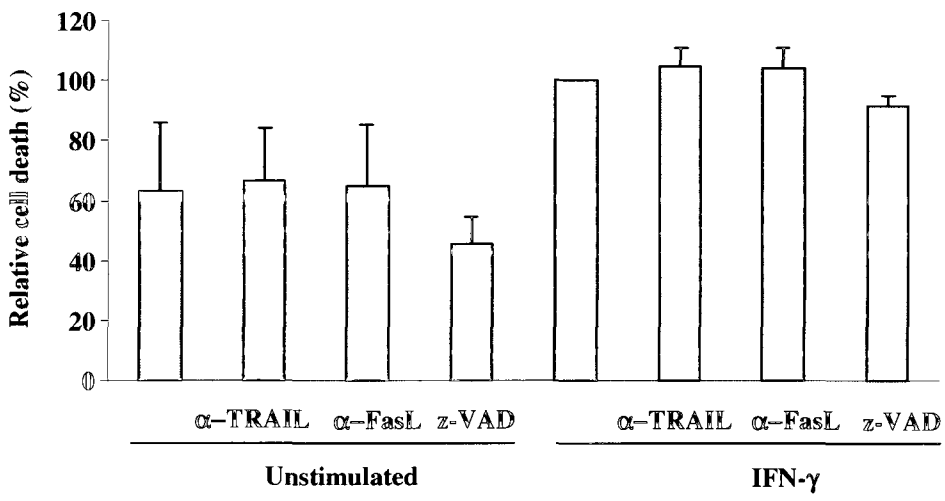
Monocyte PCD was then evaluated by annexin-v-FITC/PI staining and flow cytometric analysis. Under all conditions, there were detectable levels of spontaneous monocyte PCD and IFN-γ increased this further (Fig. 4-5, A, and B). Interestingly, neither the neutralizing antibodies for TRAIL, FasL, nor the general caspase inhibitor could inhibit spontaneous or IFN-γ-induced monocyte PCD. In fact, higher concentrations of z-VAD-FMK appeared even to enhance spontaneous and IFN-γ-induced monocyte PCD further (Fig. 4-5, A).

To ensure that the reagents used were biologically active, the same neutralizing antibodies and caspase inhibitor were tested using different approaches. In the first approach, U937 and Jurkat cells ($4X10^5$) were cultured with or without human recombinant TRAIL (100 ng/mL) and in presence or absence of anti-TRAIL antibodies (0.5-1 μg/mL) for 24 hrs. Cell death was then determined by flow cytometry. In both cell lines, the human recombinant TRAIL was capable of inducing PCD, and anti-TRAIL antibody as expected prevented TRAIL-induced cell death in these cells (Fig. 4-5, C). In the second approach, primary monocytes were collected at 0 h or left untreated or treated with z-VAD-FMK for 8 hrs. Subsequently, cleavage of caspase 3 was assayed by western blotting. There was a detectable level of spontaneous caspase 3 cleavage (Fig. 4-5, D, lane 2 compared to lane 1), and z-VAD-FMK was capable of inhibiting spontaneous caspase 3 cleavage in a dose dependent manner (Fig. 4-5, D lanes 3-5). These results indicated that indeed spontaneous and IFN-γ-induced monocyte PCD appeared to be independent of caspase activation and other mechanisms may be involved. It is possible that caspases may serve some other role in monocytes such as promotion of differentiation, as previously reported (304-307).

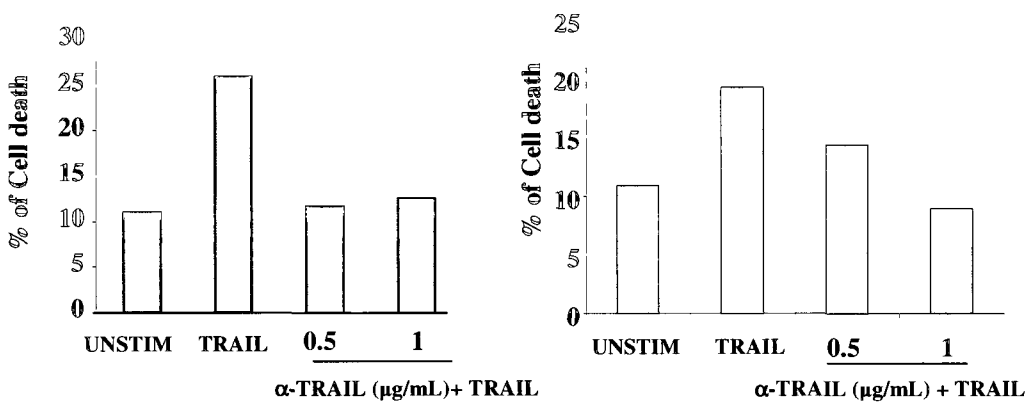
A


% Cell death
40
35
30
25
20
15
10
5
0
0 1 3 6 9 0 1 3 6 9
α-TRAIL (μg/mL)
α-TRAIL (μg/mL) + IFN-γ
% Cell death
0 0.04 0.2 1 5 0 0.04 0.2 1 5
α-FasL (μg/mL)
α-FasL (μg/mL) + IFN-γ
% Cell death
0 0.625 1.25 2.5 5 0 0.625 1.25 2.5 5
z-VAD-FMK (μM)
z-VAD-FMK (μM) + IFN-γ

B


Relative cell death (%)
120
100
80
60
40
20
0
α–TRAIL
α–FasL
z-VAD
α–TRAIL
α–FasL
z-VAD
Unstimulated
IFN-γ

C


% of Cell death
30
25
20
15
10
5
0
UNSTIM
TRAIL
0.5
1
α-TRAIL (μg/mL)+ TRAIL
% of Cell death
25
20
15
10
5
0
UNSTIM
TRAIL
0.5
1
α-TRAIL (μg/mL) + TRAIL

D

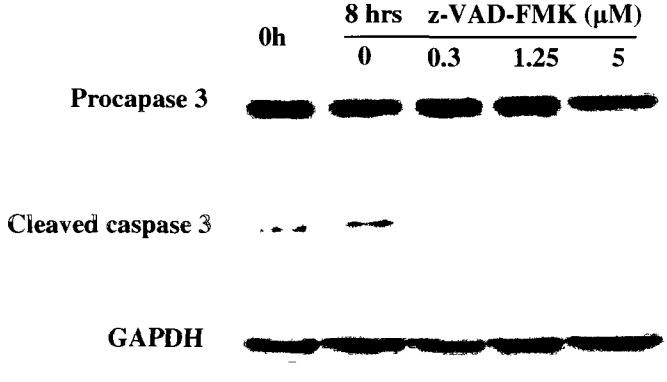

0h
8 hrs
z-VAD-FMK (μM)
0
0.3
1.25
5
Procapase 3
Cleaved caspase 3
GAPDH

Figure 4-5: Spontaneous and IFN-γ-induced monocyte PCD could not be blocked with neutralizing antibodies for TRAIL, FasL, or with the general caspase inhibitor z-VAD-FMK.

Purified monocytes ($4X10^5$ cells) were cultured with or without IFN-γ (10 ng/mL) and in the presence or absence of α-TRAIL (1-9 μg/mL), α-Fas (0.04-5 μg/mL), z-VAD-FMK (0.625-5 μM) for 24 hrs. Cells were stained with annexin-v-FITC/PI and analyzed by flow cytometry. **A)** A representative experiment shows the effect of TRAIL neutralizing antibodies (upper graph), FasL neutralizing antibodies (middle graph), and a general caspase inhibitor z-VAD-FMK (lower graph) on monocyte PCD. **B)** The average of % cell death obtained from three independent experiments performed was normalized to IFN-γ and plotted along with standard deviation (S.D.) error bars. **C)** Anti-TRAIL antibodies were capable of blocking TRAIL-induced cell death in U937 cells (left graph) and Jurkat cells (right graph). U937 and Jurkat cells ($4X10^5$ cells) were cultured with or without recombinant TRAIL (100 ng/mL) and in the presence or absence of α-TRAIL antibody (0.5-1 μg/mL). The % of cell death from one representative experiment out of two performed was plotted. **D)** z-VAD-FMK inhibited spontaneous caspase 3 cleavage in primary monocytes. Purified monocytes ($2X10^6$ cells) were either collected directly or cultured in presence or absence of z-VAD-FMK (0.3-5 μM) for 8 hrs. Total cellular lysates were then extracted and subjected to western blotting for caspase 3 cleavage. A representative experiment out of at least three independent experiments performed is shown.

4.130 Investigation of the role of the autophagy in monocyte PCD

The findings above suggested that classical apoptosis was not involved in the monocyte PCD under the conditions tested. Moreover, the characteristics of PCD observed including the staining pattern with IC PI and cell cycle analysis as well as increased levels of surface annexin-v staining over 48 hrs were not consistent with necrosis (61). Therefore, subsequent approaches were designed to examine the induction of autophagy as an alternative pathway leading to monocyte PCD.

4.131 Measurement of spontaneous and IFN-γ-induced monocyte PCD in the presence of autophagy inhibitors

I hypothesized that monocyte PCD may involve the autophagy pathway. To test this hypothesis, I applied two common pharmaceutical inhibitors that block the autophagy pathway at two different stages of the process. The inhibitors were 3-methyladnine (3-MA) and chloroquine (Cq) (early and late stages, respectively) (62,95). CD14+ monocytes ($4X10^5$ cells) were left untreated or pretreated with increasing concentration of 3-MA (5-10 mM) or Cq (6.25-25 μM) for 2 hrs. Next, cells were either unstimulated or stimulated with IFN-γ (10 ng/mL) for 24 hrs. Monocyte PCD was evaluated by annexin-v-FITC/PI staining and flow cytometric analysis. As expected, there was a detectable level of spontaneous monocyte PCD and IFN-γ stimulation enhanced this further (Fig. 4-6, A and B). Interestingly, both 3-MA and Cq significantly inhibited spontaneous and IFN-γ induced monocyte PCD, though Cq had a weaker effect (Fig. 4-6, B).

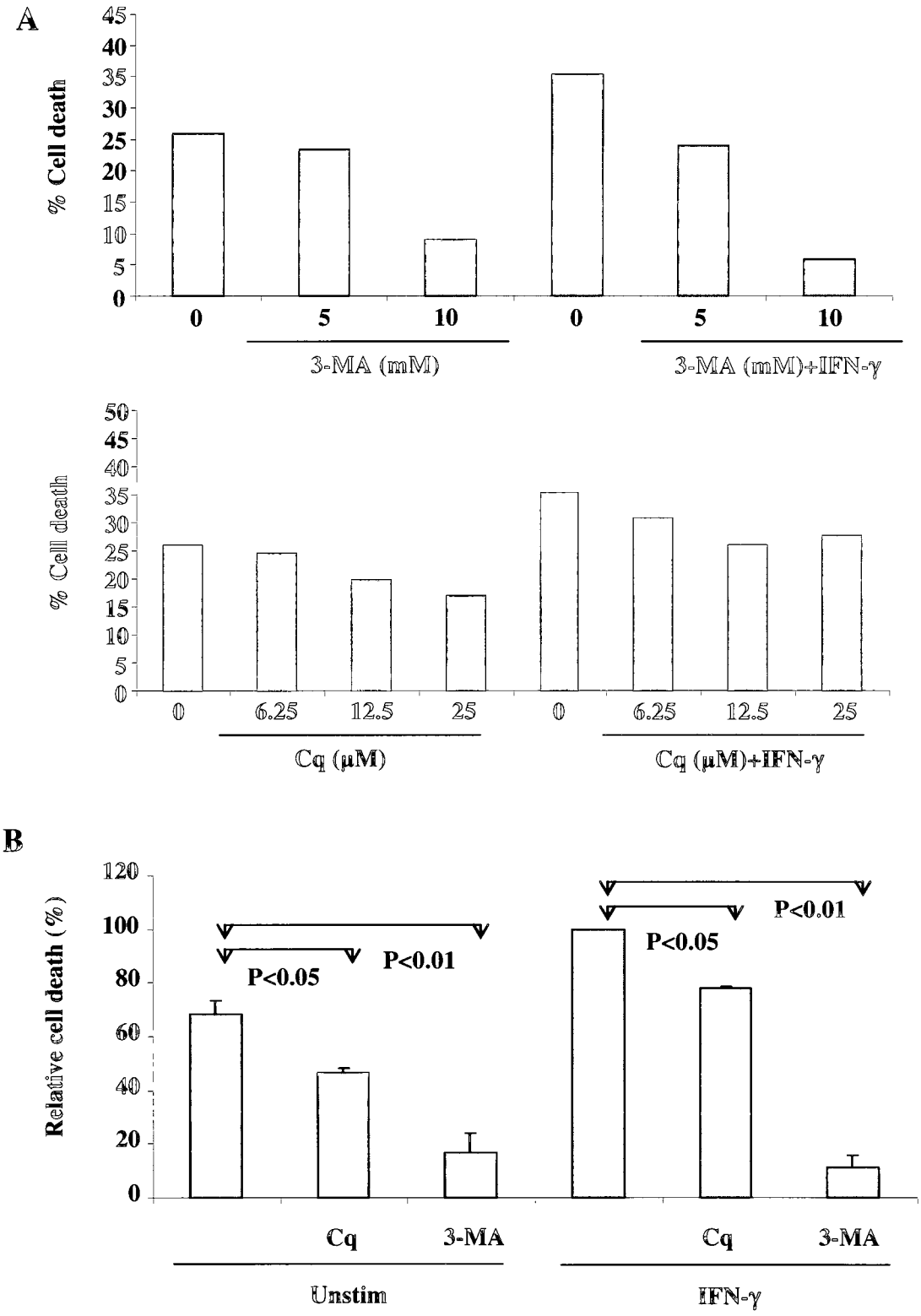

A
% Cell death
45
40
35
30
25
20
15
10
5
0
0
5
10
0
5
10
3-MA (mM)
3-MA (mM)+IFN-γ
% Cell death
50
45
40
35
30
25
20
15
10
5
0
0
6.25
12.5
25
0
6.25
12.5
25
Cq (μM)
Cq (μM)+IFN-γ
B
Relative cell death (%)
120
100
80
60
40
20
0
P<0.05
P<0.01
P<0.05
P<0.01
Cq
3-MA
Cq
3-MA
Unstim
IFN-γ

Figure 4-6: Spontaneous and IFN-γ-induced monocyte PCD was diminished by the autophagy inhibitors Cq and 3-MA.

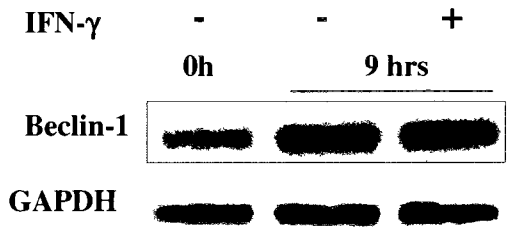
Purified monocytes ($4X10^5$ cells) were left untreated or treated with Cq (6.25-25 μM), 3-MA (5-10 mM) for 2 hrs, and then cultured with or without IFN-γ (10 ng/mL) for 24 hrs. Cells were stained with annexin-v-FITC/PI and analyzed by flow cytometry. **A)** A representative experiment out of at least three independent experiments performed was plotted. **B)** The average % cell death was normalized to IFN-γ and plotted along with standard deviation (S.D.) error bars. Statistical analysis was performed using the paired Student's *t*-test and p values are indicated where significant.

4.132 Expression levels of the autophagy related genes, beclin-1 and LC3, in monocytes in response to IFN-γ and IL-10 stimulation

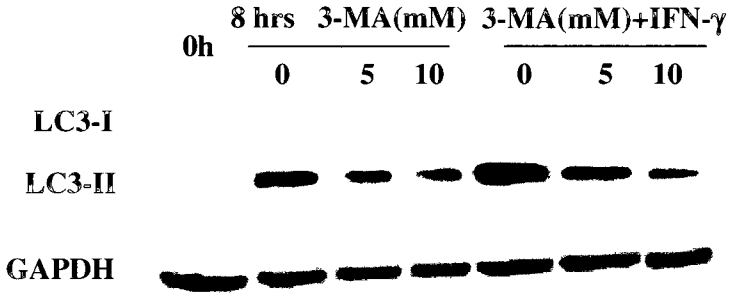
Studies have shown that activation of autophagy pathways involves upregulation of several atg genes that are essential for the process to occur. Two of the most critical genes involved in autophagy activation in mammalian cells are *beclin-1* and *LC3* (308,309). Beclin-1 is a 60 kDa protein localized in the cytoplasm and mitochondria (89). It is usually ubiquitously expressed in most cell types (310). LC3 is a cytoplasmic protein found within a cell in two distinct forms LC3-I and LC3-II (16 and 14 kDa, respectively) (90). LC3-I is the cytosolic form that results from the cleavage of LC3 at the carboxy terminal end after protein synthesis. On the other hand, LC3-II is the converted form of the LC3-I that is produced via a lipidation process during autophagy induction to allow autophagosome formation (311-315). The detection of LC3 in autophagosomes and the conversion of LC3-I to LC3-II are commonly used as markers for autophagy activation (308).

To further investigate the involvement of autophagy in monocyte PCD, the expression of autophagy related proteins, beclin-1 and LC3-II, in monocytes were measured by western blotting. Purified monocytes ($2X10^6$ cells) were left untreated or pretreated with 3-MA (5-10 mM) or Cq (25 μM) for 2 hrs. Next, cells were either left unstimulated or stimulated with IFN-γ (10 ng/mL), IL-10 (10 ng/mL), or with IL-10 and IFN-γ together for another 8-9 hrs. Total cellular lysates were extracted and subjected to western blotting for beclin-1 and LC3-II expression. Basal levels of beclin-1 expression were detected at 0 h (Fig. 4-7, A lane 1), and increased after 9 hrs of culture (Fig. 4-7, A lane 2). However, this was not enhanced further by IFN-γ or IL-10 stimulation (Fig. 4-7, A lane 3, and data not shown).

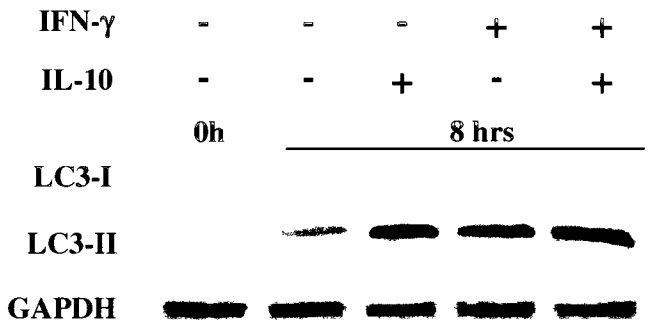
A


IFN-γ - - +
0h 9 hrs
Beclin-1
GAPDH

B


0h 8 hrs 3-MA(mM) 3-MA(mM)+IFN-γ
0 5 10 0 5 10
LC3-I
LC3-II
GAPDH

C


IFN-γ - - - + +
IL-10 - - + - +
0h 8 hrs
LC3-I
LC3-II
GAPDH

D

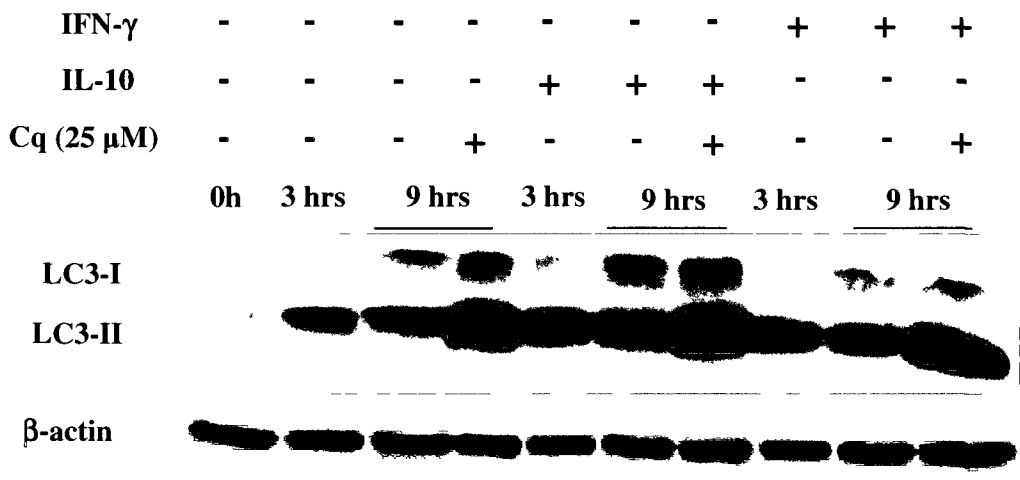

IFN-γ - - - - - - - + + +
IL-10 - - - - + + + - - -
Cq (25 μM) - - - + - - + - - +
0h 3 hrs 9 hrs 3 hrs 9 hrs 3 hrs 9 hrs
LC3-I
LC3-II
β-actin

Figure 4-7: Increased levels of LC3-II expression in cultured monocytes and following IFN-γ or IL-10 stimulation.

Purified monocytes ($2X10^6$ cells) were pretreated with Cq (25 μM), 3-MA (10 mM) for 2 hrs followed by stimulation with IFN-γ (10 ng/mL), IL-10 (10 ng/mL) for 8-9 hrs. Total cellular lysates extracted and subjected to western blotting for beclin-1, LC3-II expression, and GAPDH, β-actin as controls. **A)** Expression of beclin-1 in unstimulated and IFN-γ stimulated monocytes. **B)** Expression of LC3-II in unstimulated and IFN-γ stimulated monocytes, and the effect of 3-MA inhibitor. **C)** Expression of LC3-II in unstimulated as well as in IL-10 and/or IFN-γ stimulated monocytes. **D)** Expression of LC3-II in unstimulated and in IL-10 or IFN-γ stimulated monocytes, and the effect of Cq treatment. A representative experiment out of at least three performed is shown.

Similar to beclin-1, low basal levels of LC3-II expression were detectable at 0 h, which were increased further upon 8 hrs of culture (Fig. 4-7, B lane 1, 2). Interestingly, IFN-γ stimulation enhanced LC3-II expression levels further (Fig. 4-7, B lane 5). As expected, 3-MA treatment attenuated spontaneous and IFN-γ-induced LC3-II expression in these cells (Fig. 4-7, B lanes 3, 4, and 6, 7, respectively). Surprisingly, IL-10 stimulation was also capable of upregulating LC3-II expression in monocytes (Fig. 4-7, C lane 3) compared to unstimulated and uncultured monocytes (Fig. 4-7, C lanes 1 and 2). In fact, IL-10 stimulation enhanced IFN-γ-induced LC3-II expression further (Fig. 4-7, C lane 5). To confirm the induction of autophagy in response to IL-10 and IFN-γ stimulation, cells were treated with Cq that blocks LC3-II turnover by inhibiting autolysosomes formation and LC3-II degradation. Consistently, there was spontaneous upregulation of LC3-II expression over time (Fig. 4-7, D lanes 2, 3) compared with uncultured monocytes (Fig. 4-7, D lane 1). IL-10 and IFN-γ stimulation enhanced LC3-II expression further (Fig. 4-7, D lanes 5, 6, and 8, 9, respectively). As expected, Cq treatment resulted in the accumulation of LC3-II under all conditions (Fig. 4-7, D lanes 4, 7, 10). These results suggested that spontaneous and IFN-γ-induced monocytes PCD as well as the cytoprotective effects of IL-10 were associated with the induction of autophagy.

4.133 Assessment of the morphological characteristics of monocytes after stimulation with IL-10 or IFN-γ

To confirm these results further, the morphological characteristics of monocytes after stimulation with IL-10 or IFN-γ were examined by electron microscopy. Purified monocytes were either fixed directly or cultured with or without IL-10 (10 ng/mL), and IFN-γ (10 ng/mL) for 24 hrs. Subsequently, cells were fixed,

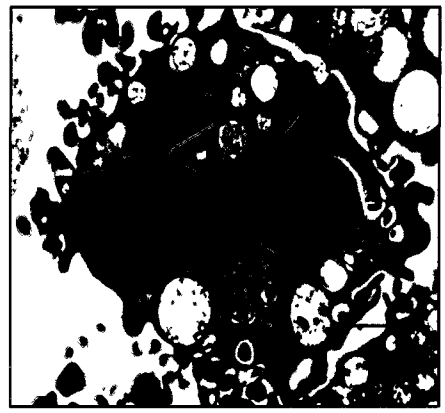
processed and examined by transmission electron microscopy (T.E.M.) (described in section 2.110). There was a clear induction of cytoplasmic vacuolization that was consistent with autophagy after 24 hrs of culture as well as in response to IL-10, or IFN-γ stimulation as compared to uncultured monocytes 0 h (Fig. 4-8). Together, these results suggested that indeed the autophagy pathway plays a role in spontaneous and IFN-γ-induced monocytes PCD as well as in the cytoprotective effects of IL-10.

4.200 Objective #5: Investigation of the molecular mechanisms involved in the regulation of monocyte PCD by IFN-γ and IL-10

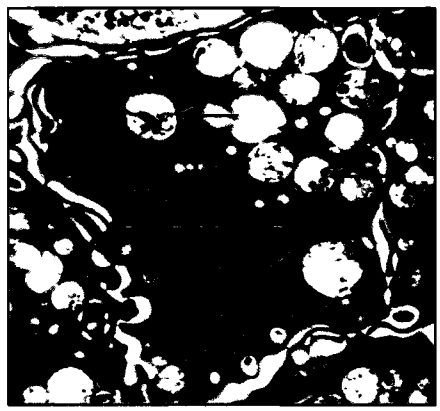
4.210 Measurement of monocyte PCD induced in response to IFN-γ and IL-10 stimulation after blocking the JAK/STAT and PI3K pathways

The above results suggested that monocyte PCD appeared to occur via the autophagy pathway. Cytokines are known to activate three main signaling pathways (the JAK/STAT, PI3K, and MAPK) in different cell types including monocytes (202,245). I showed that IFN-γ and IL-10 were able to activate JAK/STAT and PI3K but not MAPK signaling pathways in normal primary monocytes as described below in section 4.230. Thus, investigation of the role of the JAK/STAT and PI3K pathways in the regulation of monocyte PCD and autophagy was carried out. Pharmaceutical inhibitors specific for JAK/STAT and PI3K signaling were used to block these pathways. Jak inhibitor I (a general Jak inhibitor) and LY294002 (a general PI3K inhibitor) are cell-permeable compounds used commonly for blocking their respective signaling pathways effectively in different cell types (316-320). Purified monocytes ($4X10^5$ cells) were pretreated for 2 hrs with increasing concentrations of Jak inhibitor (Jak inhibitor I) (50-300 nM), PI3K inhibitor (LY294002) (2.5-10 μM), or Erk-MAPK inhibitor (PD98059), added as a control.

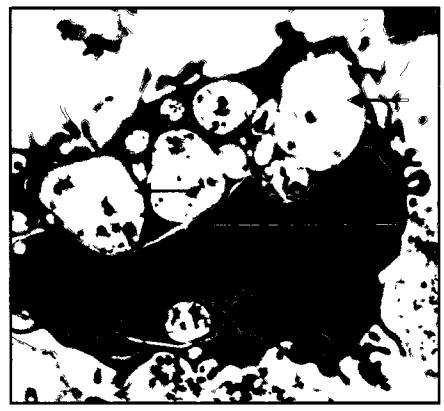
0 h UNSTIM



24 hrs UNSTIM



24 hrs IL-10



24 hrs IFN-γ

Figure 4-8: Cytoplasmic vacuolization in unstimulated as well as in IL-10 and IFN-γ stimulated monocytes.

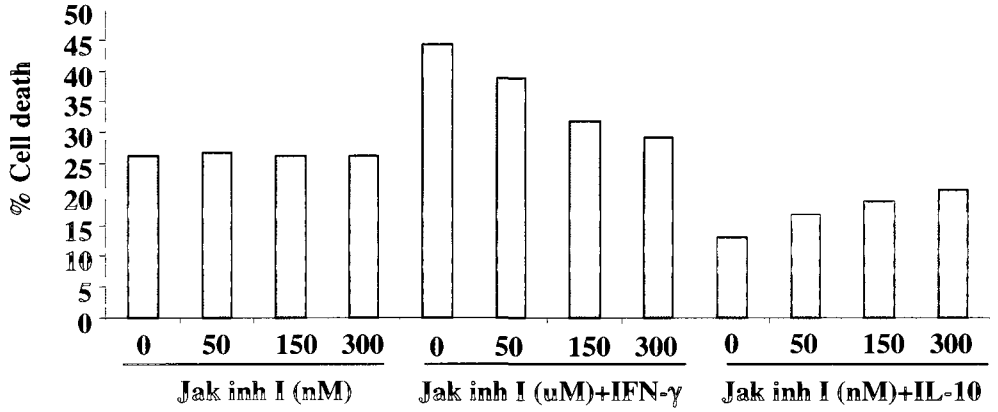
Purified monocytes ($5X10^5$ cells) were cultured with or without IL-10 (10 ng/mL), or IFN-γ (10 ng/mL) for 24 hrs. Cells were fixed, processed, and assayed by transmission electron microscope (T.E.M). T.E.M images show the presence of autophagic vacuoles consistent with autophagy as indicated with the red arrows.

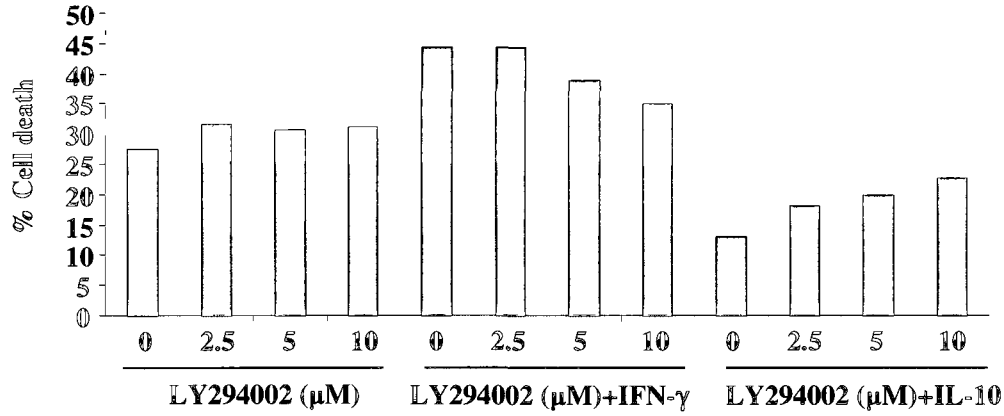
Next, cells were either left unstimulated or stimulated with IFN-γ (10 ng/mL) or IL-10 (10 ng/mL) for 24 hrs. Monocyte PCD was then evaluated by annexin-v-FITC/PI staining and flow cytometry. Blocking the JAK/STAT or PI3K pathways significantly reduced monocyte PCD levels induced by IFN-γ stimulation (Fig. 4-9, A and C). Interestingly, blocking the same pathways also attenuated the cytoprotective effects of IL-10 (Fig. 4-9, A and C). There were no significant effects of these inhibitors on spontaneous monocyte cell death (Fig. 4-9, A). Also, there were no significant effects of blocking the Erk MAPK pathway on IFN-γ-induced cell death or IL-10-induced cell survival of primary monocytes (Fig. 4-9, B.) These results suggested that the regulation of monocyte PCD by IFN-γ and IL-10 was dependent on the activation of the JAK/STAT and PI3K signaling pathways.

4.220 Role of the JAK/STAT and PI3K pathways in regulating LC3-II expression and autophagy in response to IFN-γ and IL-10

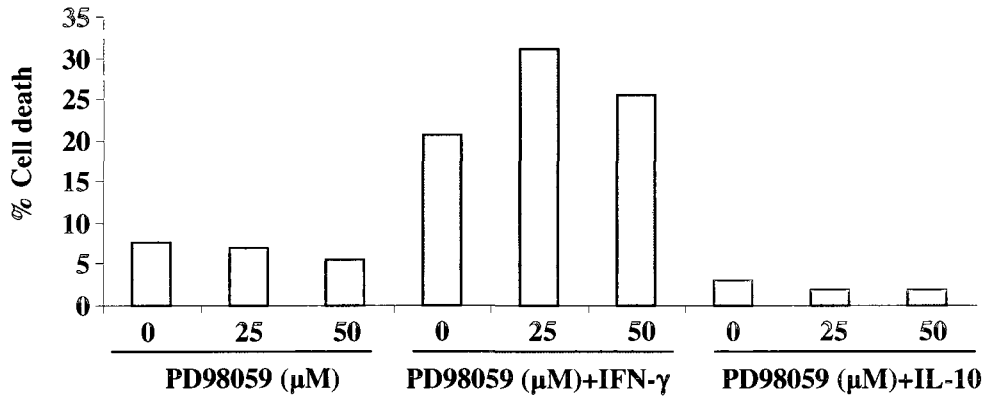
Pharmaceutical inhibitors were similarly used to investigate the role of JAK/STAT and PI3K signaling pathways in the regulation of autophagy and LC3-II expression in monocytes. Purified monocytes ($2X10^6$ cells) were pretreated with Jak inhibitor I (150 nM) or LY294002 (10 μM) for 2 hrs. Next, cells were either left unstimulated or stimulated with IFN-γ (10 ng/mL) or IL-10 (10 ng/mL) for 8 hrs. Cells were then collected and LC3-II expression was evaluated by western blotting. Blocking the JAK/STAT or PI3K pathways significantly reduced IL-10 or IFN-γ-induced LC3-II expression in monocytes (Fig. 4-10, A, B and C). This result suggested that the regulation of autophagy by IFN-γ and IL-10 occurred via JAK/STAT and PI3K signaling pathway activation in primary monocytes.

A

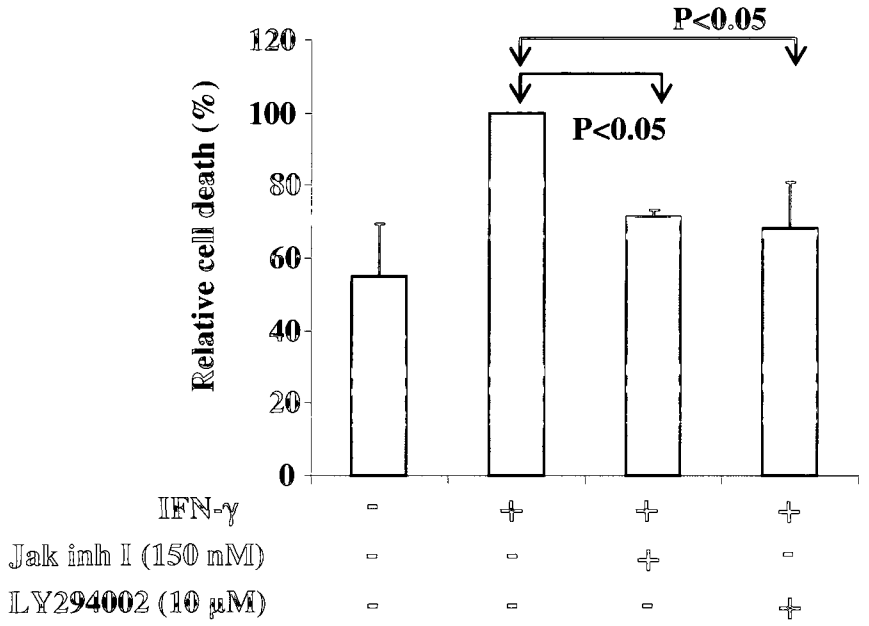

% Cell death
50
45
40
35
30
25
20
15
10
5
0
0 50 150 300 0 50 150 300 0 50 150 300
Jak inh I (nM)
Jak inh I (uM)+IFN-γ
Jak inh I (nM)+IL-10


% Cell death
50
45
40
35
30
25
20
15
10
5
0
0 2.5 5 10 0 2.5 5 10 0 2.5 5 10
LY294002 (μM)
LY294002 (μM)+IFN-γ
LY294002 (μM)+IL-10

B


% Cell death
35
30
25
20
15
10
5
0
0 25 50 0 25 50 0 25 50
PD98059 (μM)
PD98059 (μM)+IFN-γ
PD98059 (μM)+IL-10

C


P<0.05
P<0.05
Relative cell death (%)
120
100
80
60
40
20
0
IFN-γ
Jak inh I (150 nM)
LY294002 (10 μM)

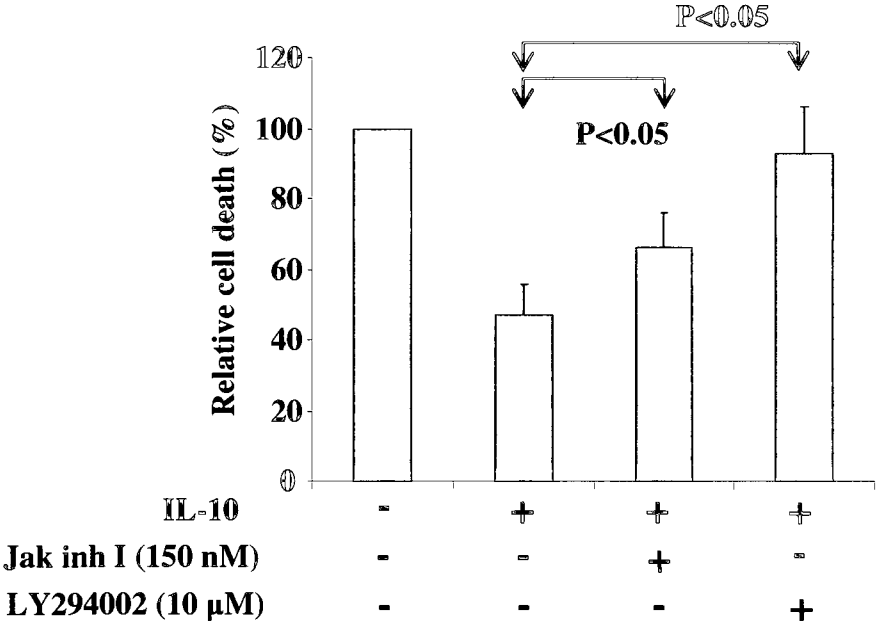
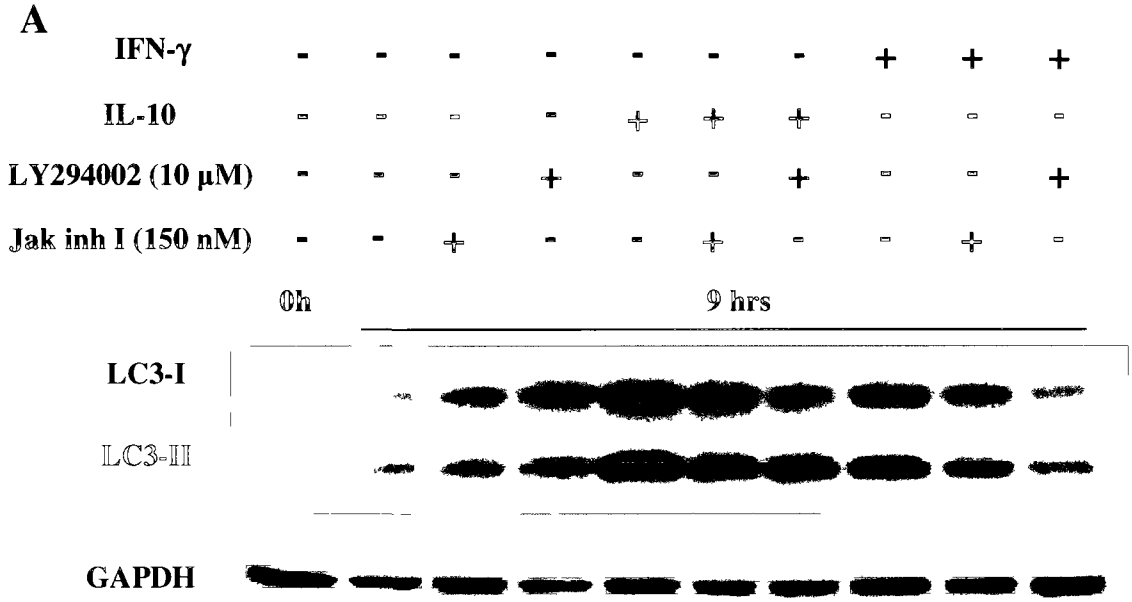
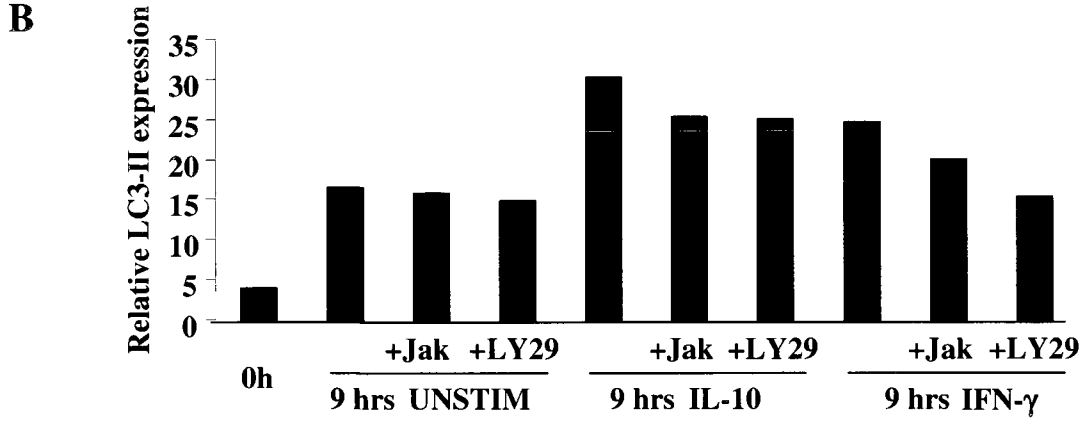
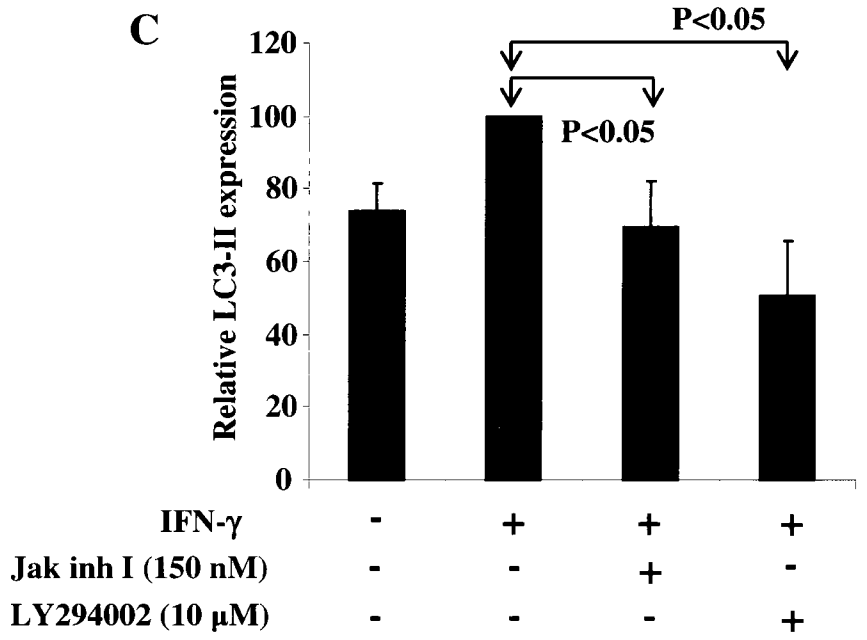

P<0.05
P<0.05
Relative cell death (%)
120
100
80
60
40
20
0
IL-10
Jak inh I (150 nM)
LY294002 (10 μM)

Figure 4-9: Blocking the JAK/STAT or PI3K pathways inhibited IFN-γ-induced monocyte PCD as well as the IL-10-induced cytoprotective effects.

Purified monocytes ($4X10^5$ cells) were pretreated with Jak inhibitor I (50-300 nM), LY294002 (2.5-10 μM), PD98059 (25-50 μM) for 2 hrs followed by cultured with or without IFN-γ (10 ng/mL) IL-10 (10 ng/mL) for 24 hrs. Cell death was determined using annexin-v-FITC/PI staining and flow cytometry. **A)** A representative experiment shows the % cell death in response to IFN-γ or IL-10 stimulation, and effects of Jak inhibitor I, or LY294002 treatments. **B)** A representative experiment shows the % cell death in response to IFN-γ or IL-10 stimulation, and the effects of different concentrations of PD98059. **C)** The average % cell death in response to IFN-γ (upper graph) or IL-10 (lower graph) stimulation and the effects of Jak inhibitor I (150 nM) or LY294002 (10 μM) obtained from three independent experiments were plotted along with standard deviation (S.D.) error bars. The % of cell death for all conditions was normalized to values obtained following IFN-γ simulation (upper graph) or to values of unstimulated cells (lower graph). Statistical analysis was performed using the paired Student's *t*-test and p values are indicated where significant.


A
IFN-γ
IL-10
LY294002 (10 μM)
Jak inh I (150 nM)
0h
9 hrs
LC3-I
LC3-II
GAPDH


B
Relative LC3-II expression
35
30
25
20
15
10
5
0
0h
+Jak +LY29
9 hrs UNSTIM
+Jak +LY29
9 hrs IL-10
+Jak +LY29
9 hrs IFN-γ


C
P<0.05
P<0.05
Relative LC3-II expression
120
100
80
60
40
20
0
IFN-γ - + + +
Jak inh I (150 nM) - - + -
LY294002 (10 μM) - - - +

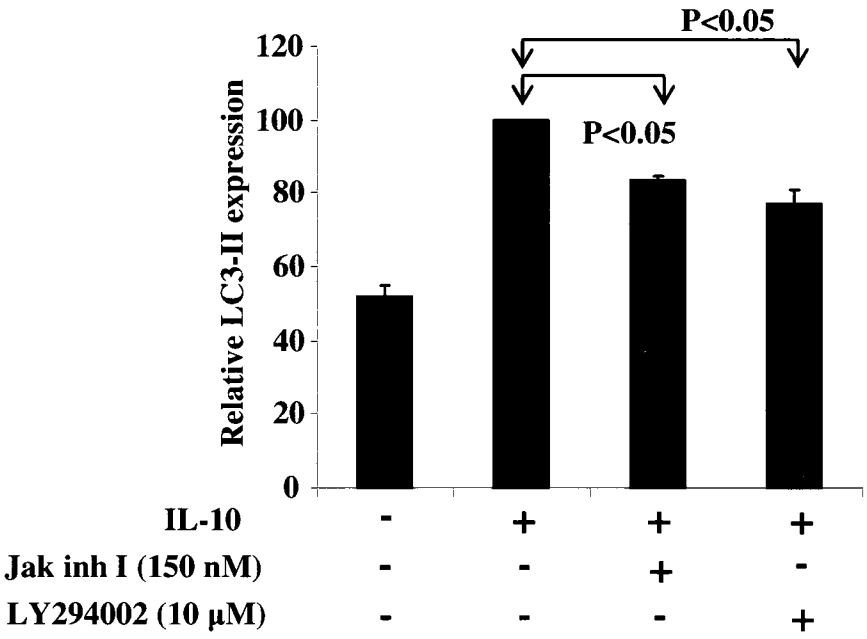

P<0.05
P<0.05
Relative LC3-II expression
120
100
80
60
40
20
0
IL-10 - + + +
Jak inh I (150 nM) - - + -
LY294002 (10 μM) - - - +

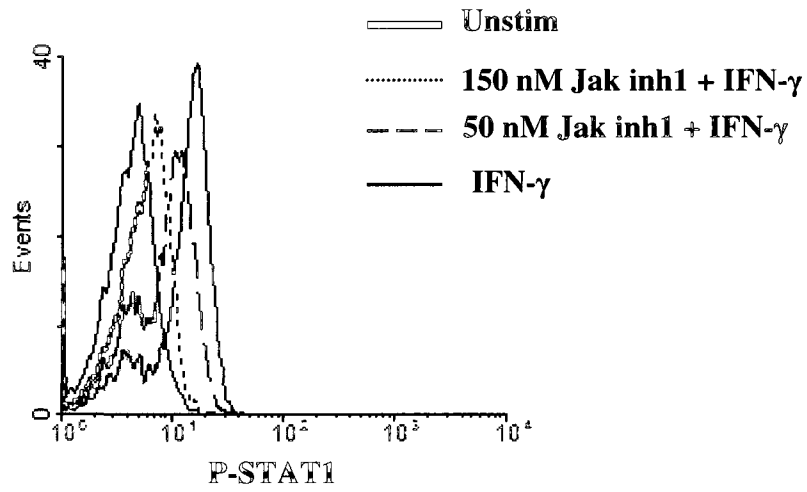
Figure 4-10: Blocking the JAK/STAT or PI3K pathways diminished IFN-γ- and IL-10-induced LC3-II expression in primary monocytes.

Purified monocytes ($2X10^6$ cells) were left untreated or treated with Jak inhibitor I (150 nM) or LY294002 (10 μM) for 2 hrs followed by stimulation with IFN-γ (10 ng/mL) or IL-10 (10 ng/mL) for 8 hrs. Total cellular extracts were collected and subjected to western blotting for LC3-II expression. **A)** A representative western blotting experiment shows LC3-II expression in unstimulated and IL-10 or IFN-γ stimulated monocytes, and the effects of Jak inhibitor I and LY294002 treatments. **B)** The western blot signals obtained in A were quantified, normalized to the GAPDH results, and data presented as relative LC3-II expression in a bar graph. **C)** The average relative LC3-II expression obtained from three independent experiments was plotted along with standard deviation (S.D.) error bars. Upper graph shows LC3-II expression in unstimulated and IFN-γ stimulated monocytes, and the effects of Jak inhibitor I and LY294002 treatments. Lower graph shows LC3-II expression in unstimulated and IL-10 stimulated monocytes, and the effects of treatments with Jak inhibitor I and LY294002. Statistical analysis was performed using the paired Student's *t*-test and p values are indicated where significant.

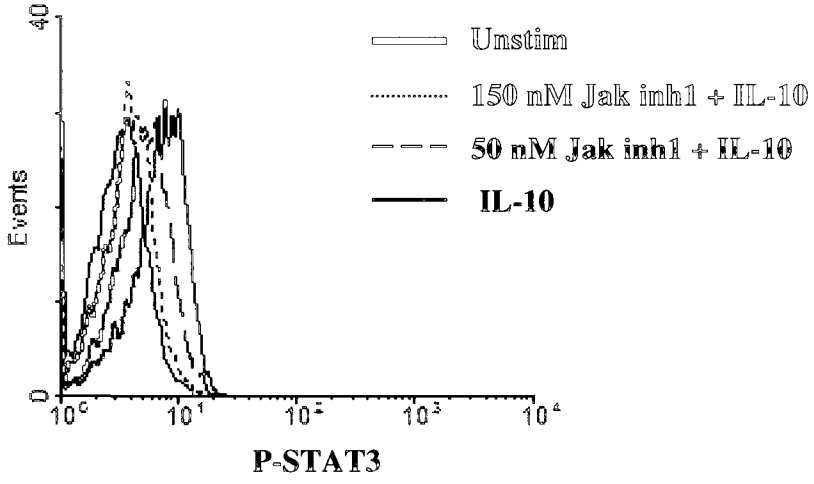
4.230 Evaluation of the activation of the signaling pathways induced in response to IFN-γ and IL-10 stimulation

As mentioned, cytokines such as IFN-γ and IL-10 have been shown to activate different signaling pathways including the JAK/STAT, PI3K, and MAPK pathways in monocytes under certain conditions (202,245). Thus, investigation of which signaling pathways are activated in primary monocytes in response to IFN-γ and IL-10 was carried out. At the same time, I examined the effect of the pharmaceutical inhibitors such as Jak inhibitor I and LY294002 on their respective signaling in primary monocytes. Purified monocytes were left untreated or pretreated with different concentration of Jak inhibitor (Jak inhibitor I) (50-150 nM) or PI3K inhibitor (LY2940002) (5-10 μM) for 2 hrs. This was followed by restimulation with IFN-γ (10 ng/mL) or IL-10 (10 ng/mL) over a (15-60 min) time course. Cells were either stained intracellularly for P-STAT1, P-STAT3, P-Erk1/2, P-P38 expression and analyzed by flow cytometry or collected and analyzed by western blotting. IFN-γ induced STAT1 activation within 15 min, and Jak inhibitor I attenuated STAT1 activation (Fig. 4-11, A). In contrast, IL-10 induced STAT3 activation within 15 min while Jak I inhibitor diminished STAT3 activation (Fig. 4-11, B). Interestingly, pretreatment of monocytes with IL-10 for 2 hrs followed by IFN-γ resulted in attenuation of IFN-γ-induced STAT1 activation in primary monocytes (Fig. 4-11, C). Both IFN-γ and IL-10 induced Akt activation within 15 min, which remained detectable after 1 hr. (Fig. 4-12, A and B). As expected, the PI3K inhibitor attenuated IFN-γ- and IL-10-induced Akt activation. There were relatively high levels of P-P38 and P-Erk1/2 detected in unstimulated monocytes, with no significant effects observed upon IFN-γ or IL-10 stimulation (Fig. 4-13, A, and B).

A


Unstim
150 nM Jak inh1 + IFN-γ
50 nM Jak inh1 + IFN-γ
IFN-γ
Events
40
0
10⁰
10¹
10²
10³
10⁴
P-STAT1

B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50 nM Jak inh1 + IL-10
IL-10
Events
40
0
10⁰
10¹
10²
10³
10⁴
P-STAT3

C

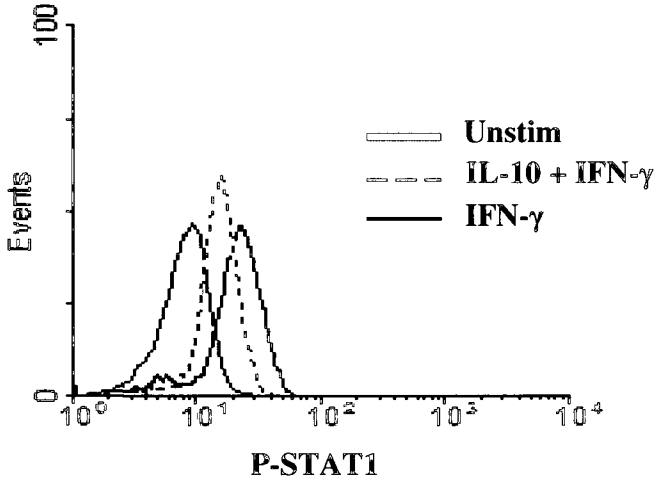
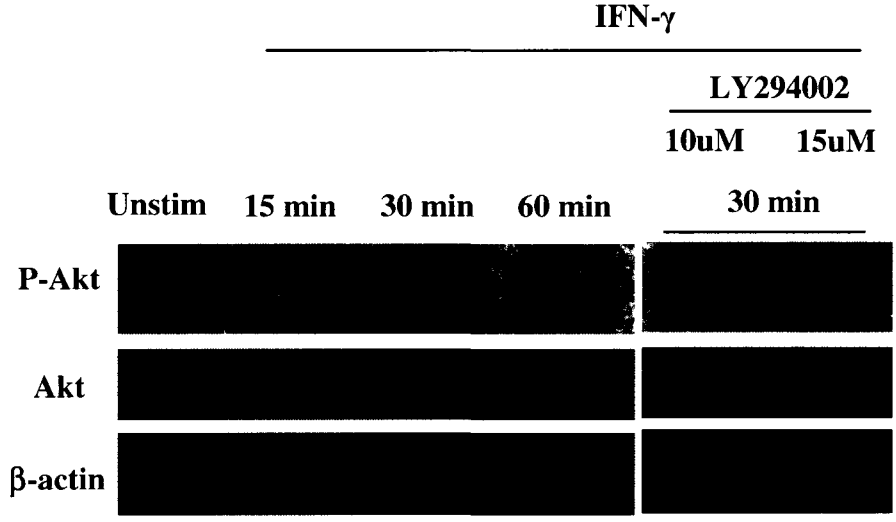

Unstim
IL-10 + IFN-γ
IFN-γ
Events
100
0
10⁰
10¹
10²
10³
10⁴
P-STAT1

Figure 4-11: IFN-γ and IL-10 activated the JAK/STAT signaling pathway, and IL-10 attenuated IFN-γ-induced STAT1 activation in primary monocytes.

Purified monocytes ($4X10^5$ cells) were left untreated or treated with Jak inhibitor I (50-150 nM) for 2 hrs followed by IFN-γ (10 ng/mL) or IL-10 (10 ng/mL) for 15 min. Cells were then fixed, stained with the indicated P-STAT antibodies, and analyzed by flow cytometry. Within the monocyte gate, 5000 events were acquired. **A)** Histogram overlays show P-STAT1 expression in response to IFN-γ stimulation, and the effects of the treatment with different concentrations of Jak inhibitor I. **B)** Histogram overlays show P-STAT3 expression in response to IL-10 stimulation, and the effects of the treatment with various concentrations of Jak inhibitor I. **C)** Purified monocytes ($4X10^5$ cells) were left unstimulated or stimulated with IL-10 (10 ng/mL) for 2 hrs followed by restimulation with IFN-γ (10 ng/mL) for 15 min. Cells were fixed, stained with P-STAT1 antibody and analyzed by flow cytometry. Histogram overlays show P-STAT1 expression in response to stimulation with IFN-γ alone or IL-10 and IFN-γ together. One representative experiment out of at least three independent experiments performed is shown.

A


IFN-γ
LY294002
10uM
15uM
Unstim
15 min
30 min
60 min
30 min
P-Akt
Akt
β-actin

B

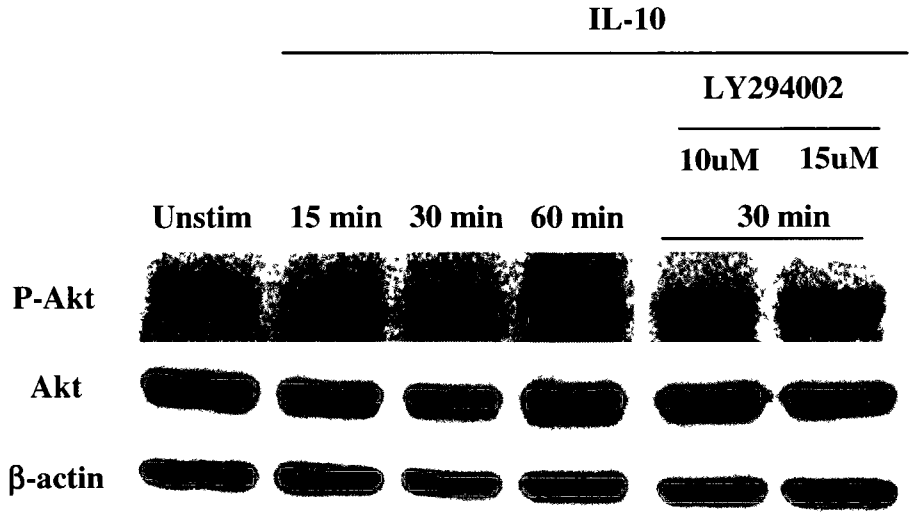
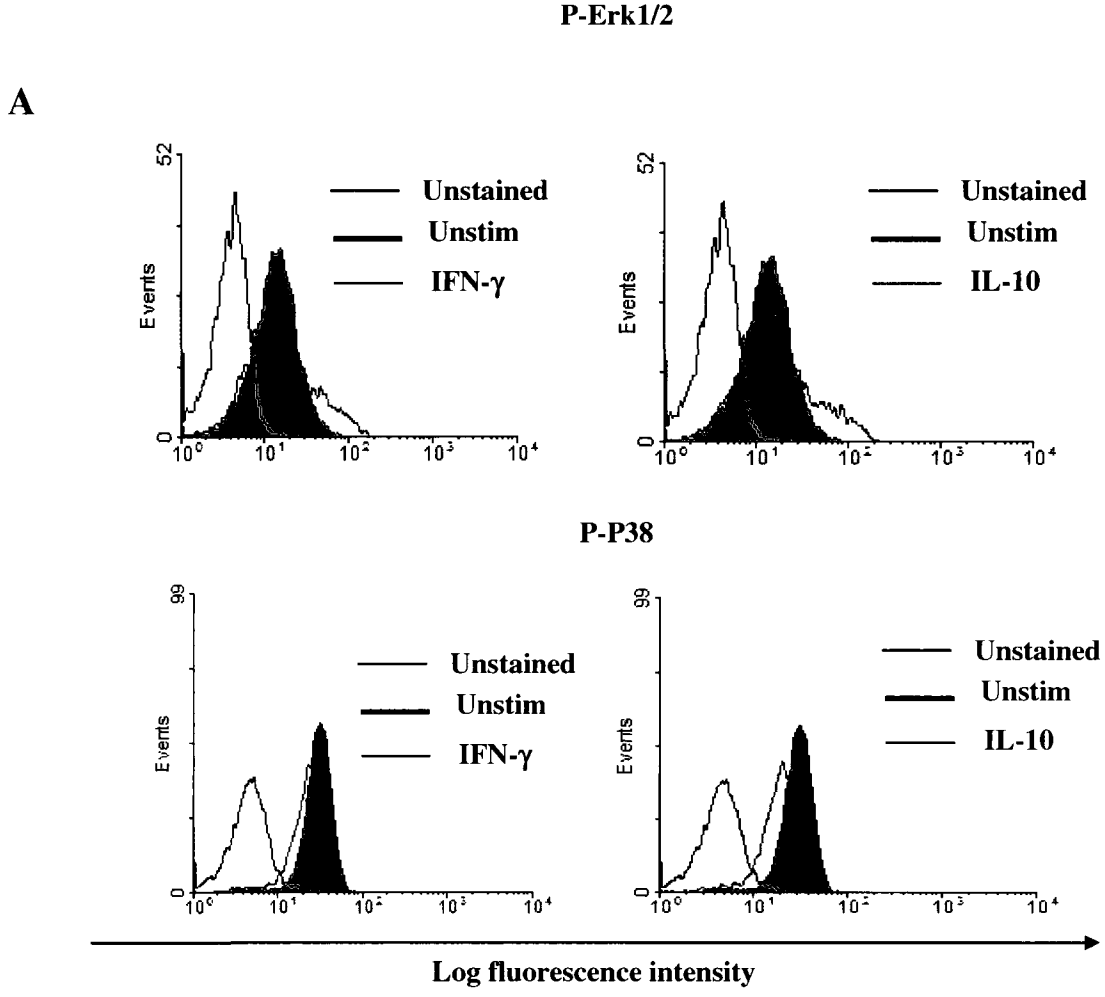

IL-10
LY294002
10uM
15uM
Unstim
15 min
30 min
60 min
30 min
P-Akt
Akt
β-actin

Figure 4-12: IFN-γ and IL-10 induced the Akt signaling pathway in primary monocytes.

Purified monocytes ($2X10^6$ cells) were either left unstimulated or stimulated directly with IFN-γ (10 ng/mL), IL-10 (10 ng/mL) for 15-60 min, or pretreated with LY294002 (5-10 μM) for 2 hrs followed by IFN-γ and IL-10 for 30 min. Total cellular lysates were extracted and assayed by western blotting for Akt activation. **A)** A representative blot shows P-Akt expression in response to IFN-γ stimulation in monocytes, and the effect of LY294002 treatment. **B)** A representative blot shows P-Akt expression in response to IL-10 stimulation in monocytes, and the effect of LY294002 treatment. One representative experiment out of at least three independent experiments performed is shown.


P-Erk1/2
A
Unstained
Unstim
IFN-γ
IL-10
Events
52
0
P-P38
99
Log fluorescence intensity

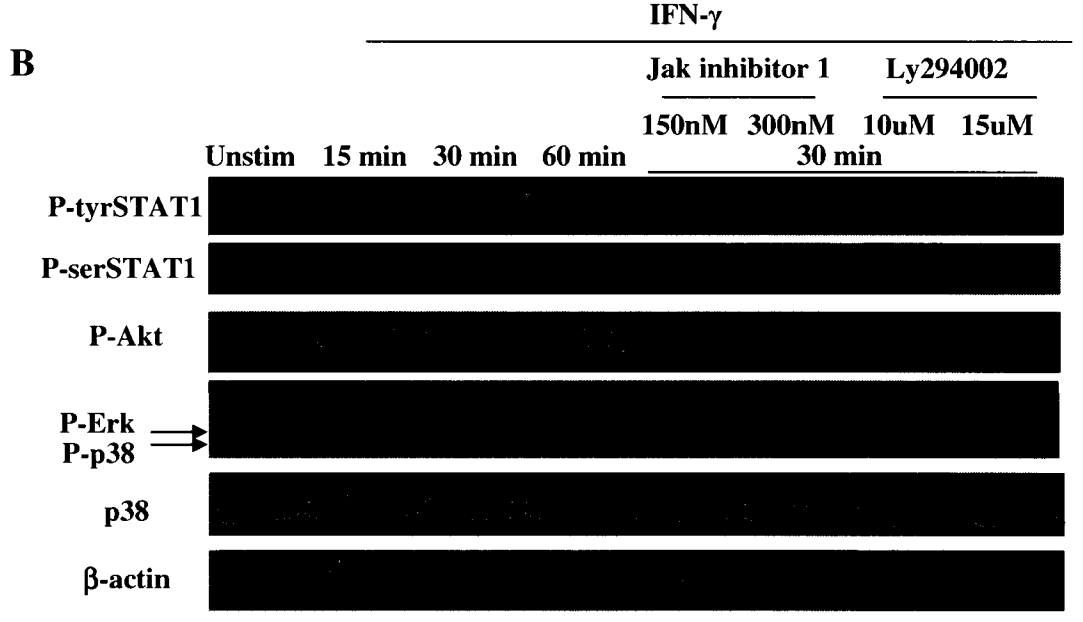

B
IFN-γ
Jak inhibitor 1
Ly294002
150nM
300nM
10uM
15uM
Unstim
15 min
30 min
60 min
30 min
P-tyrSTAT1
P-serSTAT1
P-Akt
P-Erk
P-p38
p38
β-actin

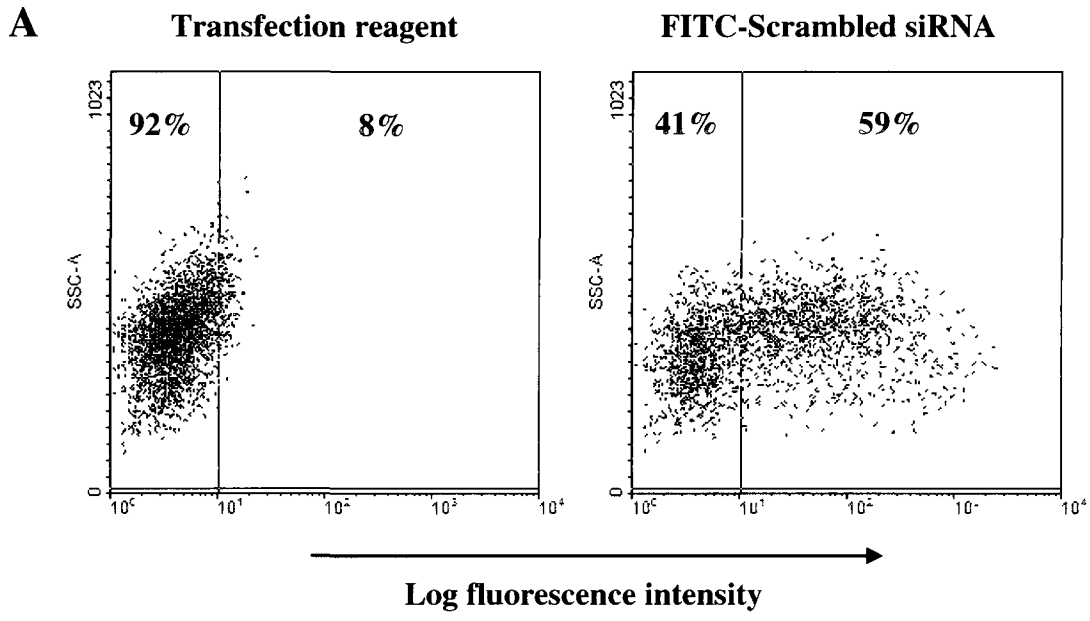
Figure 4-13: IFN-γ or IL-10 had no significant effects on the MAPK signaling pathways in primary monocytes.

A) Purified monocytes ($4X10^5$ cells) were left unstimulated or stimulated with IFN-γ (10 ng/mL) for 15 min. Cells were then stained with P-Erk1/2, P-P38, and analyzed by flow cytometry. Within the monocyte gate, 5000 events were acquired. An overlay of histograms shows expression levels of P-Erk1/2 (upper graphs), and P-P38 (lower graphs) in unstimulated and IFN-γ or IL-10 stimulated monocytes. **B)** Purified monocytes ($2X10^6$ cells) were left untreated or treated with LY294002 (5-10 μM) for 2 hrs followed by IFN-γ (10 ng/mL) or IL-10 (10 ng/mL) for 30 min. Total cellular lysates were extracted and assayed by western blotting. Representative blots show the expression of P-STAT1, P-Akt, P-Erk and P-P38 in response to IFN-γ stimulation over a 60 min time frame in monocytes, and the effects of Jak inhibitor I and LY294002 treatments. One representative experiment out of at least three independent experiments performed is shown.

It appeared that Jak inhibitor I and LY294002 had inhibitory effects on STAT1 and Akt activation, respectively, in response to IFN-γ stimulation (Fig. 4-13, B), confirming the existence of a crosstalk between these pathways (198,199). Interestingly however, neither Jak inhibitor I nor LY294002 had any significant effect on P-P38 and P-Erk1/2 expression levels (Fig. 4-13, B). P-JNK was not detected under these conditions (data not shown).

4.240 Confirmation of the induction of autophagy and LC3-II expression in response to IFN-γ and IL-10 using specific siRNA

As with the majority of pharmaceutical inhibitors, it has been reported that autophagy inhibitors can exhibit non-specific effects (321-323). Therefore, subsequent experiments were performed to confirm the activation of the autophagy pathway and LC3-II expression in monocytes using a specific siRNA targeting the LC3 gene. Purified monocytes ($5X10^5$ cells) were transfected with scrambled siRNA control, siRNA LC3B for 24 hrs, or FITC-scrambled control siRNA for 8 hrs using TransMessenger kit (Qiagen) as described in section 2.120. Monocyte transfection efficiency was then measured by flow cytometry and the knockdown of LC3-II expression was determined by western blotting. Fig. 4-14, A shows that approximately 50% of primary monocytes were transfected with FITC-scrambled control siRNA compared with monocytes treated with reagent alone. LC3B siRNA diminished LC3-II expression levels in primary human monocytes (Fig. 4-14, B). However, this result was not consistent due to poor transfection efficiency.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Transfection reagent
FITC-Scrambled siRNA
92%
8%
41%
59%
SSC-A
1023
0
Log fluorescence intensity

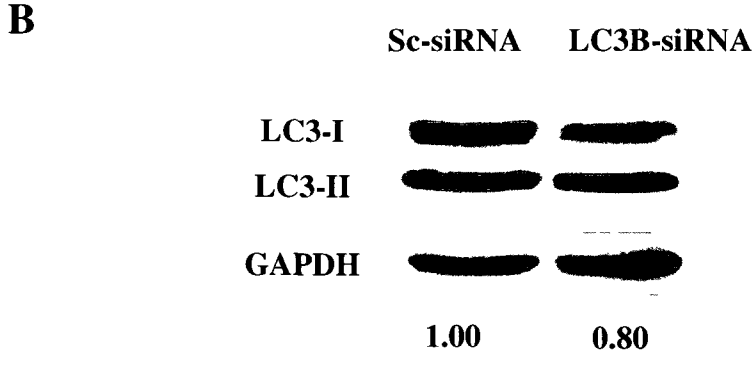

B
Sc-siRNA
LC3B-siRNA
LC3-I
LC3-II
GAPDH
1.00
0.80

Figure 4-14: LC3B siRNA failed to inhibit LC3-II expression significantly in transfected primary monocytes.

A) Purified monocytes ($5X10^5$ cells) were treated with transfection reagent from TransMessenger kit (Qiagen) alone or with FITC-scrambled siRNA following the manufacturer's procedure. Cells were then analyzed by flow cytometry. A dot plot from one representative experiment out of at least three performed was plotted. A comparison between cells treated with transfection reagent alone and cells transfected with FITC-conjugated scrambled siRNA was carried out. Dot plots show the % of monocytes treated with reagent alone (left) or with FITC-scrambled siRNA (right). **B)** CD14+ monocytes ($5X10^5$ cells) were transfected with LC3B siRNA and scrambled siRNA for 24 hrs. Total cellular lysates were extracted and subjected to western blotting for LC3-II expression or GAPDH as control. A representative experiment shows LC3-II expression in scrambled and LC3B siRNAs transfected cells. Quantification of the bands was carried out by normalizing to GAPDH and values were expressed relative to scrambled siRNA.

Since experiments in transfected primary monocytes were not successful, a tumor cell line model was used. It has been shown that IFN-γ was able induce autophagy in the monocytic model cell line U937 (140). Thus, the same cell line was used to study autophagy and LC3-II expression in response to IFN-γ and IL-10. U937 cells ($2X10^6$) were either untreated or treated with IL-10 (10 ng/mL), IFN-γ (10 ng/mL), IL-10 + IFN-γ, and rapamycin (2 μM) (as a positive control) for 8 hrs. Total cell lysate was then assayed by western blotting for LC3-II expression. Similar to primary monocytes, there was a detectable basal expression of LC3-II after 8 hrs of culture (Fig. 4-15, lane 1) and enhanced further with IL-10, IFN-γ, IL-10 + IFN-γ, and rapamycin (Fig. 4-15, lanes 2-5).

To confirm this result further, U937 cells were transfected with siRNA LC3B, and the upregulation of LC3-II expression in response to IL-10 and IFN-γ stimulation was evaluated. U937 cells ($5X10^5$) were transfected with FITC-scrambled siRNA control for 8 hrs or with scrambled siRNA control, and siRNA LC3B for 24 hrs using TransMessenger kit (described in section 2.120). Cell were then stimulated with IFN-γ (10 ng/mL) and IL-10 (10 ng/mL) for another 8 hrs. Transfection efficiency was then evaluated by flow cytometry and the LC3-II expression was determined by western blotting. Unlike in primary monocytes, 92% of U937 cells were transfected with FITC-scrambled siRNA compared with cells treated with reagent alone (Fig. 4-16, A). In this case, LC3-B siRNA was capable of significantly blocking the upregulation of LC3-II expression in response to IL-10 and IFN-γ stimulation in U937 cells (Fig. 4-16, B).

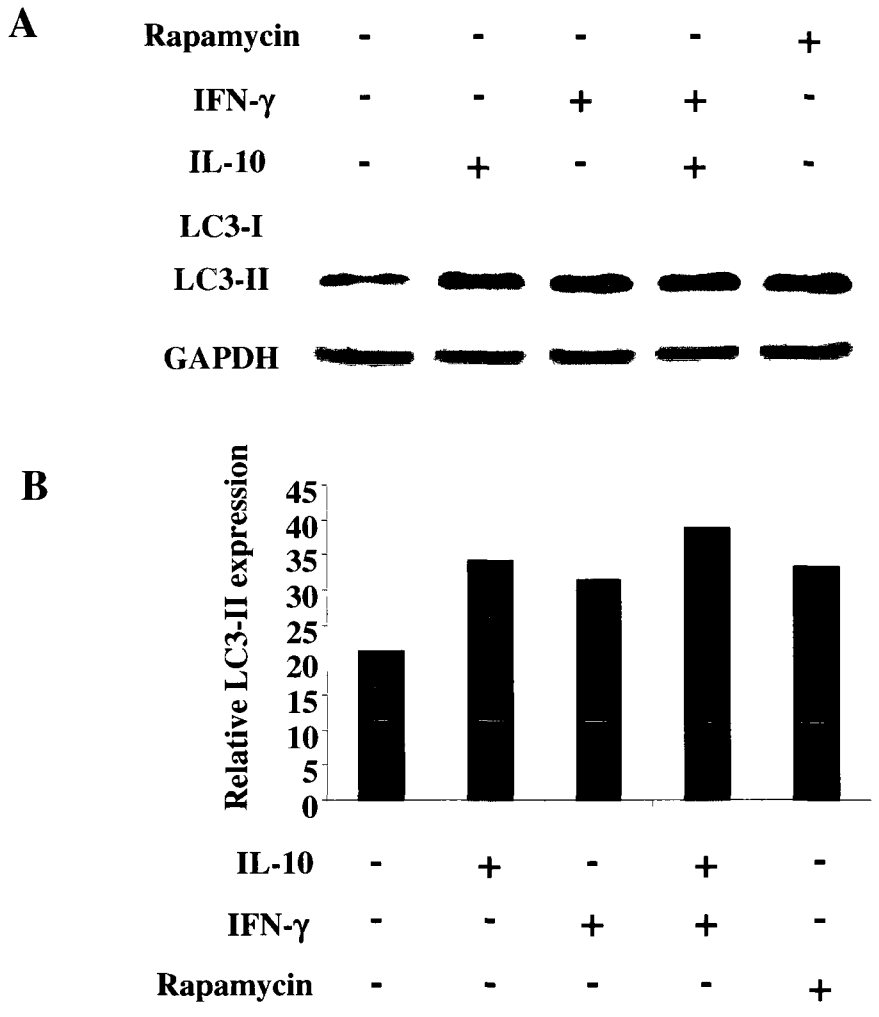
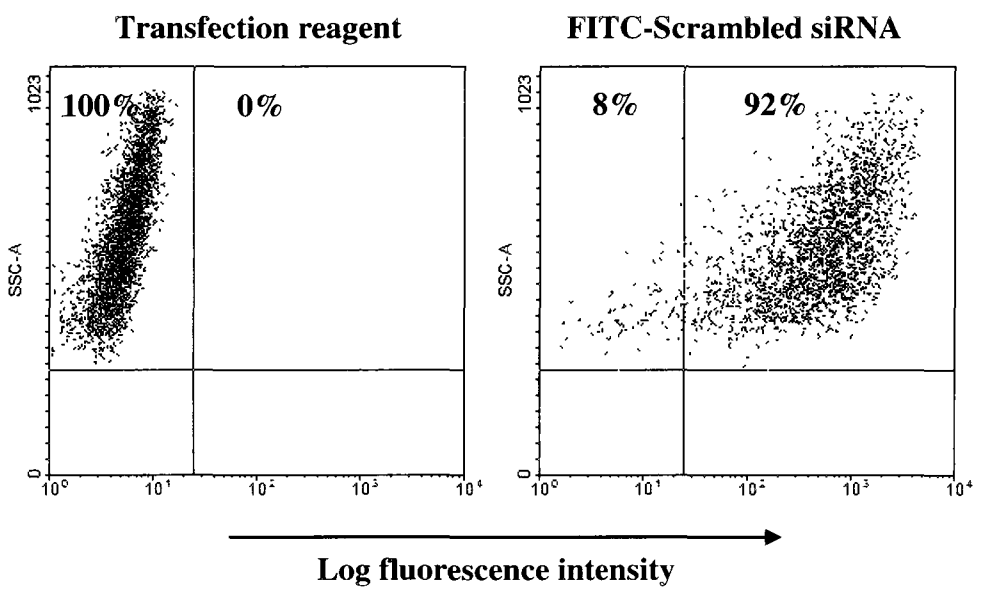

A
Rapamycin - - - - +
IFN-γ - - + + -
IL-10 - + - + -
LC3-I
LC3-II
GAPDH
B
Relative LC3-II expression
45
40
35
30
25
20
15
10
5
0
IL-10 - + - + -
IFN-γ - - + + -
Rapamycin - - - - +

Figure 4-15: Increased expression levels of LC3-II in response to IL-10 and IFN-γ stimulation in U937 cells.

U937 cells ($2X10^6$ cells) were left unstimulated or stimulated with IL-10 (10 ng/mL), IFN-γ (10 ng/mL), IL-10 + IFN-γ, or rapamycin (2 μM) for 8 hrs. Total cellular proteins were extracted and subjected to western blotting for LC3-II expression. **A)** A representative experiment shows the expression levels of LC3-II in response to IL-10, IFN-γ, IL-10 + IFN-γ, and rapamycin stimulation. **B)** Western blot sginals in A were quantified, normalized to GAPDH results and data expressed as relative LC3-II expression. One representative experiment out of at least four experiments performed is shown.

A


Transfection reagent
FITC-Scrambled siRNA
100%
0%
8%
92%
SSC-A
1023
0
Log fluorescence intensity

B

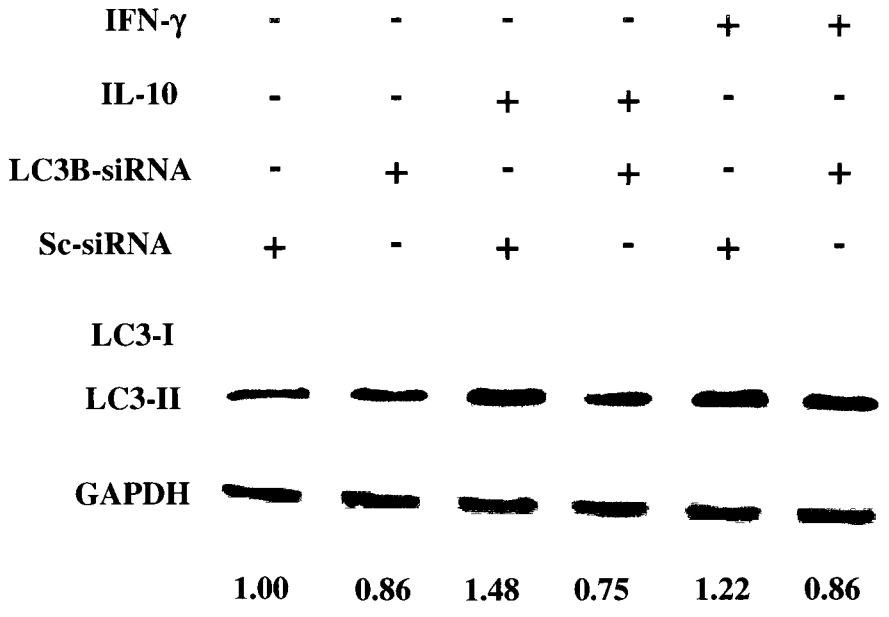
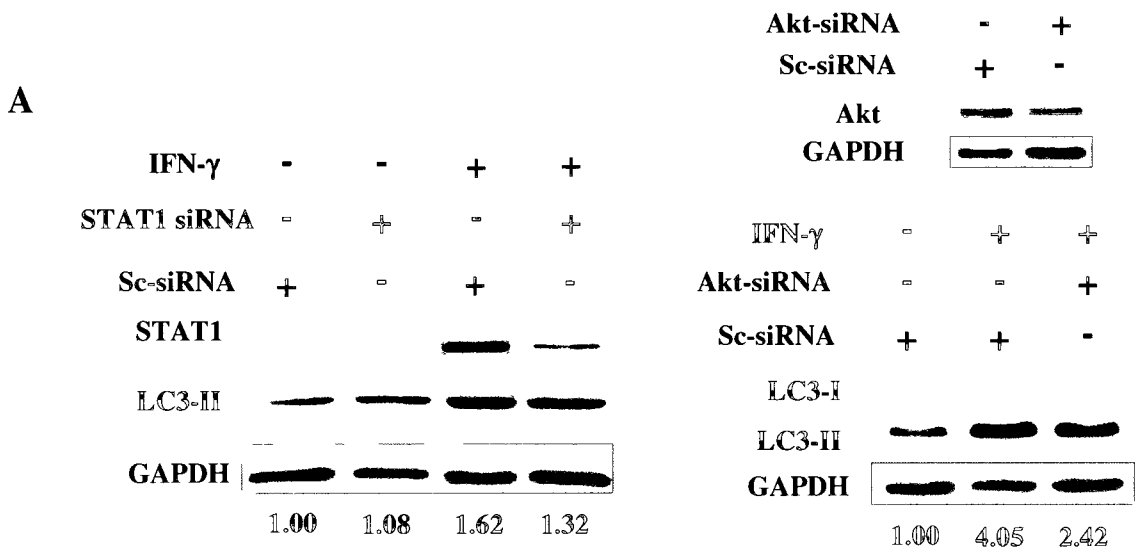

IFN-γ - - - - + +
IL-10 - - + + - -
LC3B-siRNA - + - + - +
Sc-siRNA + - + - + -
LC3-I
LC3-II
GAPDH
1.00 0.86 1.48 0.75 1.22 0.86

Figure 4-16: Attenuation of IL-10 and IFN-γ-induced upregulation of LC3-II expression in U937 cells transfected with LC3B siRNA.

A) U937 cells ($5X10^5$ cells) were treated with transfection reagent only from TransMessenger kit (Qiagen) or transfection reagent containing FITC-scrambled siRNA following the manufacturer procedure. Cells were then analyzed by flow cytometry. Within the whole cell gate, 10000 events were acquired. A dot plot from one representative experiment out of at least three performed was plotted. A comparison between cells treated with transfection reagent alone and cells transfected with FITC-conjugated scrambled siRNA was carried out. Dot plots show the % of transfected U937 cells with transfection reagent (left), FITC-scrambled siRNA (right). **B)** U937 cells ($5X10^5$ cells) were transfected with LC3B and scrambled siRNA for 24 hrs, followed by stimulation with IL-10 (10 ng/mL), IFN-γ (10 ng/mL) for 8 hrs. Total cellular proteins were extracted and subjected to western blotting for LC3-II expression and that of GAPDH as control. Quantification of the bands was performed by normalizing to GAPDH and values were expressed relative to scrambled siRNA. A representative experiment out of at least three performed is shown.

4.250 Confirmation of the role of the JAK/STAT and PI3K pathways in the regulation of LC3-II expression in response to IFN-γ and IL-10 using specific siRNA

In an attempt to confirm the involvement of JAK/STAT and PI3K signaling pathways in the regulation of LC3-II expression and autophagy, transfection with siRNAs specific for STAT1, 3 and Akt was performed in the same model cell line. U937 cells were transfected with specific siRNA for STAT1, STAT3, or Akt along with scrambled siRNA control for 24 hrs (described in section 2.120). Cells were then stimulated with IL-10 (10 ng/mL), or IFN-γ (10 ng/mL) for another 8 hrs, and LC3-II expression was measured by western blotting. Targeting STAT1 (Fig. 4-17, A left plot) or Akt (Fig. 4-17, A right plot) with specific siRNAs significantly reduced the induction of LC3-II expression in response to IFN-γ stimulation. Similarly, using specific STAT3 siRNA (Fig. 4-17, B left plot) or Akt siRNA (Fig. 4-17, B right plot) significantly diminished IL-10-induced LC-3II expression in U937 cells. These results strengthen the findings observed with pharmaceutical inhibitors and suggested that activation of JAK/STAT and PI3K may be required for IFN-γ- and IL-10-induced autophagy activation and LC3-II expression in monocytic cells.


A
IFN-γ
STAT1 siRNA
Sc-siRNA
STAT1
LC3-II
GAPDH
1.00 1.08 1.62 1.32
Akt-siRNA
Sc-siRNA
Akt
GAPDH
IFN-γ
Akt-siRNA
Sc-siRNA
LC3-I
LC3-II
GAPDH
1.00 4.05 2.42

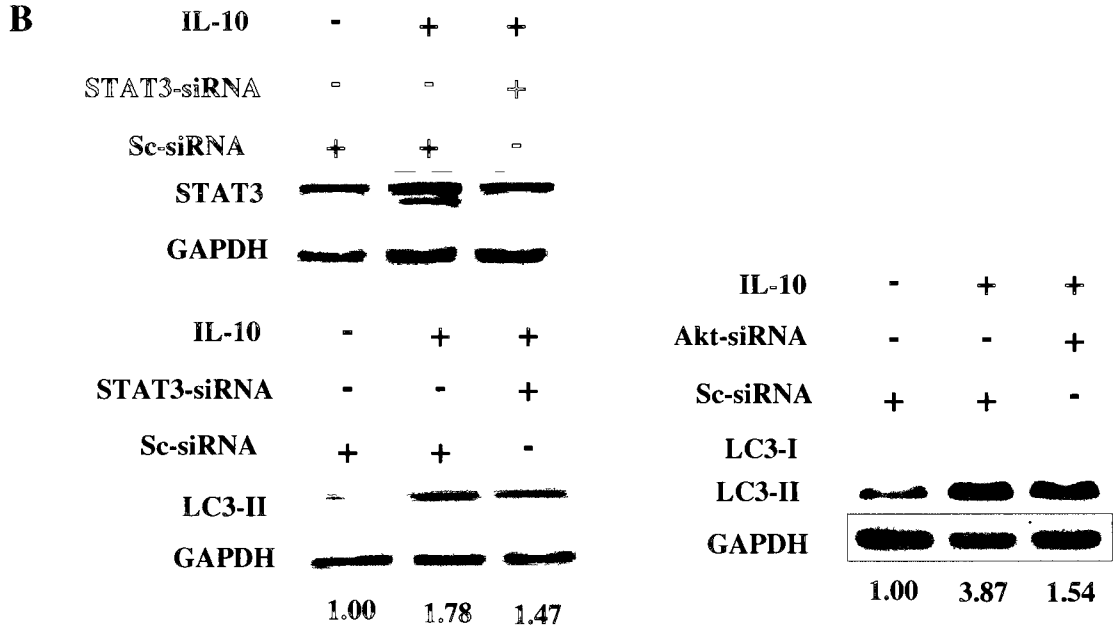

B
IL-10
STAT3-siRNA
Sc-siRNA
STAT3
GAPDH
IL-10
STAT3-siRNA
Sc-siRNA
LC3-II
GAPDH
1.00 1.78 1.47
IL-10
Akt-siRNA
Sc-siRNA
LC3-I
LC3-II
GAPDH
1.00 3.87 1.54

Figure 4-17: Knocking down STAT1, STAT3, Akt with specific siRNAs attenuated the upregulation of LC3-II expression in response to IFN-γ or IL-10 stimulation in U937 cells.

U937 cells ($5X10^5$ cells) were transfected with STAT1, STAT3, Akt siRNA and scrambled siRNA for 24 hrs followed by stimulation with IL-10 (10 ng/mL), IFN-γ (10 ng/mL) for another 8 hrs. Total cellular proteins were extracted and subjected to western blotting for LC3-II expression. **A)** A representative experiment shows the expression levels of LC3-II in response to IFN-γ stimulation in U937 cells transfected with scrambled, STAT1 (left plot), or Akt (right plot) siRNAs. **B)** A representative experiment shows the expression levels of LC3-II in response to IL-10 stimulation in U937 cells transfected with scrambled, STAT3 (left plot), or Akt (right plot) siRNAs. Quantification of the bands was performed by normalizing to GAPDH and values were expressed relative to scrambled siRNA. One representative experiment out of at least three independent experiments performed is shown.

Chapter V

Discussion

5.10 Amplification of IFN-γ signaling and cell death in monocytes from viremic HIV+ patients

Cytokine responsiveness via the JAK/STAT signal transduction plays a major role in regulating different monocyte/macrophage effector functions including differentiation, phagocytosis, cytokine secretion, and cell survival. However, the effect of *in vivo* HIV infection on JAK/STAT signaling pathway and PCD in monocyte has not been investigated. Thus, I hypothesized that chronic HIV infection could alter JAK/STAT signaling in response to cytokine in monocytes and subsequently impair their effector function and promote cell death.

I demonstrated for the first time that among the responses to cytokines tested (IFN-γ, IFN-α, IL-10, IL-4, and GM-CSF) in terms of STAT activation in monocytes, only IFN-γ showed a significant upregulation of P-STAT1 induction in HIV+ patients off therapy compared to HIV- controls and patients on ART. Furthermore, this potentiation of IFN-γ-induced P-STAT1 was due to increased total STAT1 expression levels. The treatment with HIV Gp120 and Vpr proteins was capable of upregulating P-STAT1 expression in normal primary monocytes and monocytic cell lines. Among IFN-γ-induced STAT1 responsive genes (HLA-DR, IRF-1, CXCL9, and CXCL10) studied, only CXCL9 expression was significantly elevated in HIV+ patients on ART. Interestingly, spontaneous monocyte cell death was elevated in HIV+ patients off therapy but not ART patients compared to HIV- controls. IFN-γ-induced monocyte PCD was increased in HIV+ patients compared to HIV- controls irrespective of ART. Interestingly, IL-10, a known IFN-γ antagonist, was capable of inhibiting spontaneous and IFN-γ-induced monocyte PCD in both HIV- controls and HIV+ patients.

Since STAT1 activation in response to IFN-γ was highly upregulated in HIV+ patients off therapy, I investigated the mechanisms responsible for this alteration. As

there were no significant differences in IFN-γ-R expression levels between patient groups, focus fell on a downstream mechanism. Studies have shown that such observations are reminiscent of a priming phenomenon by which type I IFNs may enhance innate antiviral immune responses (324,325). Indeed, priming of macrophages with low doses of IFNs (type I or II) enhanced subsequent IFN-γ signaling in these cells via a selective increase in total STAT1 expression (252,253). Interestingly, total STAT1 expression levels were found to correlate significantly with that of P-STAT1 from the off therapy patients. Treatments of monocytes and monocytic cell lines with HIV Gp120 or Vpr resulted in the upregulation of STAT1 activation. In agreement with these results, other *in vitro* studies have demonstrated that HIV infection or treatment with its proteins Gp120 or Nef were able to upregulate STAT1 expression in MDM and U937 cells (207,208). Thus, the amplification of IFN-γ-induced STAT1 activation observed in these patients may be a manifestation of *in vivo* monocyte priming by cytokines such as IFN-α, or HIV and its immunomodulatory proteins known to be present at high levels in the circulation of such patients (326). Notably, a somewhat similar STAT1 amplification may also occur in other chronic inflammatory disorders including ulcerative colitis, Crohn's disease, rheumatoid arthritis, Systemic Lupus Erythermatosus and chronic hepatitis C virus-infected patient liver cells (252,327-331). To date, other chronic viral infections have not been studied at this level in patient monocytes.

Interestingly, upregulation of P-STAT1 appeared to be unique to IFN-γ since this was not observed in response to either IFN-α or IL-10 (Fig. 3-6, B and C). Contributing to this selective effect may be that IFN-γ induces upstream Jak1 and Jak2 activation whereas IFN-α and IL-10 activate Jak1 and Tyk2 (332). It is also possible that STAT1 may be preferentially recruited to the IFN-γ receptor, and both of

these possibilities would need to be investigated further. A recent study has demonstrated an impairment of IFN-α-induced STAT1 activation in HIV+ patient off therapy (333). This supports my findings that IFN-α failed to hyper-induce P-STAT1. As can been seen in Fig. 3-6, B a slight impairment in IFN-α-induced STAT1 can be appreciated in the off therapy patient shown but this observation was not pursued further in this thesis. Another report has suggested the existence of an impairment in GM-CSF-induced STAT5 activation, and a hyperactivation of ERK MAPK in response to LPS (334). As mentioned, my studies failed to reveal any alterations in GM-CSF induced P-STAT5 in patient monocytes. One explanation for this apparent discrepancy is the that study by Lee *et al.* was conducted on pediatric patients and gating on CD33+ myeloid cells, while my study was conducted on adult volunteers and gating on CD14+ monocytes.

To investigate the biological impact of the hyperactivation of P-STAT1 in response to IFN-γ, I evaluated the expression of STAT1-dependent genes including HLA-DR, IRF-1, CXCL9, and CXCL10 (264). Although the expression of the STAT1 responsive genes studied was upregulated by IFN-γ, their levels did not parallel differences in IFN-γ-induced P-STAT1 induction observed between patient groups. IFN-γ-induced CXCL9 was significantly elevated only in monocytes from ART patients compared to uninfected controls. However, such results need to be interpreted with caution, considering the limited sample number and the large standard deviation obtained. I suggest that these genes are under complex regulatory control involving more than STAT1 alone, particularly in the context of HIV infection and ART.

IFN-γ plays a major role in activating monocyte effector function including phagocytosis, antigen presentation, and cytokine secretion. However, it can also

induce cell death via STAT1 activation in different cell types (283,335). In contrast to T cells, cell death in monocytes from HIV-infected patients was not well established. I demonstrated that spontaneous monocyte cell death was elevated in HIV+ patients off therapy but not ART patients compared to HIV- controls. In agreement with this finding, one study found that spontaneous cell death was elevated in monocytes from patients included based on HIV-seropositivity (336). In another report, such a fate could be averted by *ex vivo* IL-13 stimulation, but this was not compared to uninfected controls (126). My further analyses suggested that spontaneous monocyte cell death may be related to increased constitutive levels of total STAT1.

I demonstrated that IFN-γ-induced monocyte cell death was elevated in HIV+ patients compared to HIV- controls, irrespective of seemingly effective ART (undetectable viral load, stable CD4 counts). This may reflect the adverse effects of ART on cells of this lineage (337). The mechanism by which IFN-γ enhanced cell death in patient monocytes is not known but appears to be unrelated to tyr-P-STAT1 induction (Fig. 3-6, A). IFN-γ did upregulate TRAIL secretion by monocytes but this was low compared to plasma and showed no significant differences between patient groups. Since STAT1-ser-phosphorylation is also important for survival or apoptosis, depending on the cell type (338,339), future studies to investigate this in HIV+ patient monocytes are warranted.

IL-10 exhibits antagonistic effects on IFN-γ-induced monocyte function including inhibition of phagocytosis and downregulation of MHC class II. However, its effects on cell death have not been extensively investigated. I showed that IL-10 is capable of inhibiting spontaneous and IFN-γ-induced PCD in monocytes from HIV+ patients as well as HIV- controls. However, the results showing the effects of IL-10

on monocyte PCD should be strengthened by increasing the sample size. To investigate the molecular mechanism by which PCD could occur in monocytes, I evaluated TRAIL expression in the supernatant of cultured monocytes and in patient plasma. Secreted TRAIL showed no significant differences between patients groups. However, circulating levels of TRAIL were increased, particularly in viremic HIV+ patients, confirming other findings (Fig. 3-24, A; (340). Though plasma TRAIL concentrations appeared to parallel spontaneous cell death results, this did not reach statistical significance.

As mentioned, the status of monocyte cell death, particularly in HIV+ patients, was largely uninvestigated. It appears from *ex vivo* experiments that monocytes from HIV+ patients under certain conditions, such as with IFN-γ treatment, are more sensitive to cell death whereas under other conditions, such as Fas treatment (341), they are more resistant to cell death compared to those from HIV- controls. However, *in vivo* these cells appear not to be progressively depleted from the host thus favoring the maintenance of this cellular reservoir for HIV. Studies have revealed also that cytokines such as TNF-α, IL-10, and IL-13 have a role in enhancing monocyte survival (126,292,296,297,342). Such inflammatory and anti-inflammatory cytokines have been reported to be elevated in serum from patients with HIV infection (130,131,178,180-183,294,301). Thus, it is possible that many factors contribute to the regulation of M/M survival and PCD during chronic HIV infection.

It is interesting to point out that a $CD16^{+}/CD14^{low}$ subset of blood monocytes is expanded in HIV+ patients (44,343,344), and recent observations suggest that this population likely represents an important viral reservoir even under HAART (124). Although I did not analyze this marker specifically, my results could not be attributed

solely to an expansion of the CD16+ subset. However, to rule out this possibility directly, further studies are warranted.

In summary, I demonstrated that monocytes from chronically-infected HIV+ patients exhibit reduced survival *ex vivo* compared to HIV- controls and that there are significant alterations in their expression of STAT1 and signaling capacity in response to IFN-γ. These results may reflect the chronic nature of the immune activation observed, particularly in viremic HIV+ patients, and may also be significant in view of the potential utility of IFN-γ as an adjunct immunotherapy for HIV-related opportunistic infections (137,345,346). However, IL-10 may have a role in increasing monocyte survival during *in vivo* HIV infection.

5.20 Differential regulation of monocyte PCD by IFN-γ and IL-10 via the autophagy pathway

Since monocyte PCD had not been well characterized and its regulation by IFN-γ and IL-10 at the molecular level has not been investigated, I performed studies to address these questions. I demonstrated for the first time that spontaneous and IFN-γ-induced monocyte PCD and the cytoprotective effects of IL-10 are caspase independent but rather autophagy dependent. I also showed that activation of the JAK/STAT and PI3K pathways are required for autophagy activation and modulation of monocyte PCD in response to both IFN-γ and IL-10.

As mentioned, my work establishes that spontaneous and IFN-γ-induced monocyte PCD as well as the cytoprotective effects of IL-10 may be independent of caspases. However, one study has suggested that spontaneous monocyte cell death occurred via Fas-FasL interaction, but could only be partially inhibited by neutralizing antibodies specific to Fas or FasL (347). My results were not consistent with this

report and clearly demonstrate that neutralizing death receptors triggering using antibodies against TRAIL or Fas or blocking caspase activation with a general caspase inhibitor (z-VAD-FMK) had no significant effects on spontaneous or IFN-γ-induced monocyte PCD. These results argue that spontaneous and IFN-γ-induced monocyte cell death were indeed caspase independent and other mechanisms downstream of death receptor are involved in monocyte cell death.

Furthermore, I showed that IL-10 could inhibit spontaneous monocyte cell death which confirms other reports (292,293,296,297). In addition, I demonstrated for the first time that IL-10 could attenuate IFN-γ-induced monocyte cell death. IL-10 blocked the early events of the classical apoptosis cascade including TRAIL secretion and caspase 8 activation. Surprisingly however, neither IFN-γ nor IL-10 had any significant effects on the activation of the effector caspase 3, consistent with the caspase independent component of monocyte PCD. One report has suggested that the Th-2 cytokines IL-10 and IL-4 could block monocyte cell death by inhibiting caspase 8 activation and inducing Flice-like inhibitory protein (FLIP) expression (292). This was consistent with my caspase 8 results however, Eslick *et al.* did not investigate the downstream effector caspases such as 3, 6, or 7 which are ultimately required for PARP cleavage and DNA fragmentation (292). It appears that activation of caspases in monocytes may have an alternative role in monocyte that may be distinct from its classical cell death functions (304-307). This requires further investigation.

Since it appeared that classical apoptosis pathways were not involved in the monocyte PCD observed, I investigated the alternatives, namely, the autophagy cascade. Particularly novel findings were that both spontaneous and IFN-γ-induced monocyte PCD occurred, at least in part, via the activation of the autophagy pathway. Strikingly, the cytoprotective effects of IL-10 were also associated with enhanced

autophagy activation. This was demonstrated in experiments with two commonly used autophagy inhibitors, 3-MA and Cq. Both inhibitors were capable of blocking spontaneous and IFN-γ-induced PCD in monocytes. Also, upregulation of LC3-II expression and the increased in the cytoplasmic vacuolization observed spontaneously and in the presence of IFN-γ or IL-10 stimulation were consistent with activation of the autophagy process in monocytes. However, expression of beclin-1 did not appear to be further enhanced by IFN-γ or IL-10 stimulation. It is likely that increases in beclin-1 basal expression levels in cultured monocytes were sufficient to mobilize the autophagy pathway. Interestingly and in keeping with my results, several reports have shown that inflammatory cytokines such as IFN-γ, and TNF-α are capable of inducing autophagy in different cell types including monocytes and macrophages (140,348-350). For example, it was shown that IFN-γ enhanced antigen presentation and the clearance of various pathogens via the activation of the autophagy pathway in different cell types including antigen presenting cells (72,74,265,351-353). Another study has demonstrated that the Th-2 cytokines IL-4 and IL-13 could attenuate IFN-γ-induced autophagy and killing of intracellular pathogens in the murine macrophage cell line RAW264.7 and the human monocytic cell U937 (140). It is clear that autophagy plays a critical role in killing intracellular pathogens. However, the effect of activating autophagy by such inflammatory cytokines on M/M PCD has not been previously investigated.

The mechanism by which autophagy can lead to cell death under certain conditions (IFN-γ) while under other conditions (IL-10) results in cell survival is quite intriguing but not clear at present. Nevertheless, my results suggest that both IFN-γ and IL-10 upregulated LC3-II expression and autophagy. Therefore, it is likely that this occurs at some point downstream or in parallel with LC3-II formation. This issue

requires further investigation of course. Key players in this process may include reactive oxygen species (ROS), tumor supressor protein (p53), and death-associated protein kinase (DAPK). In support of this are several studies showing that ROS (349,354-359), p53 (348,360) and DAPK (361-363) are involved in the regulation of autophagy and cell death in different cell types including macrophages. Two such studies have demonstrated that stimulation of macrophages with LPS and z-VAD together resulted in ROS production, and this ROS was upstream of autophagy-associated cell death in macrophages (364,365). Also, it has been revealed that inflammatory cytokines including IFN-γ are capable of inducing cell death via ROS production, p53 or DAPK activation in macrophages and other cell types (363,366-369). Finally, a recent report has suggested that IL-10 was capable of inhibiting NADPH oxidase 1 production, a substrate for superoxide, induced by IFN-γ or TNF-α in human and mouse epithelial cells (370).

At the signal transduction level, I elucidated that the JAK/STAT and PI3K signaling pathways play a major role in regulating autophagy and monocyte PCD. I showed that blocking of JAK/STAT or PI3K signaling prior to stimulation with IFN-γ or IL-10 was sufficient to attenuate the upregulation of LC3-II expression in primary monocytes and the U937 monocytic cells. Further, I demonstrated that blocking of these pathways prior to cytokine stimulation inhibited both of IFN-γ-induced cell death as well as IL-10-induced cell survival in primary monocytes. These results suggested that JAK/STAT or PI3K signaling pathways are necessary for IFN-γ- and IL-10-induced autophagy activation and their effects on PCD. This role of the JAK/STAT or PI3K signaling pathways in regulating autophagy and PCD is consistent with several reports (96,140,364,371). Activation of STAT1, STAT3, or

Akt in response to cytokine such as IFN-γ, IL-13, IL-4, or LPS + z-VAD induced autophagy in mouse macrophages and human monocytic cell lines (96,140,364).

In summary, I elucidated that spontaneous and IFN-γ-induced monocyte PCD were caspase-independent but rather autophagy-dependent. Strikingly, the cytoprotective effects of IL-10 were also associated with the activation of the autophagy pathway. Regulation of monocyte cell death by IFN-γ and IL-10 involved the activation of the JAK/STAT and PI3K signaling pathways.

Chapter VI

Concluding Remarks

Monocytes/macrophages play a critical role in the defense against infectious pathogens. During chronic HIV infection, many M/M functions become impaired, and thus may contribute to the immunodeficiency observed over the course of the disease and the appearance of opportunistic infections. However, the mechanism underlying these defects is still not clear. As described in this thesis, one of the possible mechanisms by which HIV impairs M/M function is through the alteration of cytokine signaling and programmed cell death. Cytokine responsiveness via the JAK/STAT signaling pathway plays a major role in the regulation of different M/M functions including phagocytosis, differentiation, and cell survival. Previous work had suggested that defects in cytokine signaling exist in lymphocytes from HIV+ patients. This led to the hypothesis that similar defects may be manifested in monocytes, thus contributing to the impairment of monocyte function and overall immunodeficiency.

My results described a pathophysiological state of monocytes that arises during chronic HIV infection, which is characterized by hyperactivated IFN-γ responsiveness and enhanced susceptibility to PCD *ex vivo*. Specifically, I demonstrated for the first time that monocytes from viremic HIV+ patients exhibited an increased STAT1 activation in response to IFN-γ. This appeared to be due to increased total STAT1 levels that may arise as a result of an *in vivo* priming phenomenon (252,253). Such alterations in JAK/STAT signaling could be the result of chronic immune activation that occurs during HIV infection and may thus contribute to monocyte dysfunction as disease progresses (38,39). Early on during HIV infection, mucosal CD4+ memory cells are massively depleted leading to disruption of the intestinal mucosa and interestingly increased bacterial LPS leaking into the circulation (372-374). As a result, inflammatory cytokines such as IFNs and TNF-α are mobilized, as are other factors such as TRAIL and Fas, and concomitantly

viral proteins (vpr, tat, nef) exert their immunomodulatory functions especially during active viral replication. This chronic immune activation may serve to continuously prime and activate monocytes, bring about further destruction of immune cells including circulating CD4+ T cells, and induce tissue pathology (51,105,113,341,375).

Unlike CD4+ T cells, monocytes appear not to be progressively depleted from the host. However, I found that monocytes from HIV+ patients, irrespective of ART, are more sensitive to PCD under certain conditions such as in response to IFN-γ. Interestingly, IL-10, which can be found at elevated levels in the circulation of HIV+ patients, has a suppressive effect on monocyte functions, and thus could rescue monocytes from PCD *in vivo* and maintain these critical viral reservoirs. Recently, monocytes from HIV+ patients appeared to be more resistant to cell death triggered via Fas stimulation *ex vivo* (341). Thus, during chronic HIV infection *in vivo*, a very complex and shifting balance may exist between the positive and negative regulators of PCD. On the one hand, monocytes are exposed to a variety of soluble factors that are known to cause cell death via apoptosis or autophagy pathways such as Fas, TRAIL, IFN-γ, and HIV Vpr. On the other hand, immune activation via triggering of TLRs and the action of factors such as LPS, TNF-α, as well as the anti-inflammatory cytokines (IL-10, IL-13), exert inhibitory effects on monocyte cell death via these same pathways.

Perhaps most interesting are the findings which suggest that monocyte PCD is regulated via the activation of the autophagy pathway rather than apoptosis or caspases. However, it is unclear at present how autophagy under certain conditions (IFN-γ) leads to cell death while others (IL-10) promote cell survival. I hypothesized that there are other autophagy-dependent molecules implicated in distinguishing these

two cellular fates. These include reactive oxygen species (ROS), the tumor suppressor protein (p53), and the death-associated protein kinase (DAPK). It is possible that IFN-γ induces monocyte PCD by activating the autophagy pathway such that expression of ROS, p53, and/or DAPK are enhanced, and thus bringing about cell death. IL-10, on the other hand, may induce its cytoprotective effects by activating autophagy in a manner that either fails to recruit or downregulates the expression of these molecules, thus promoting cell survival. In fact, our laboratory is currently investigating the role of these molecules in distinguishing the suicidal *vs* cytoprotective tendencies of the autophagy cascade in human monocytes.

A model summarizing the regulation of monocyte PCD by IFN-γ and IL-10 under normal conditions as well as in the context of HIV infection is presented in Fig. 6-1. Briefly, in normal primary monocytes, I observed that TRAIL was secreted spontaneously from cultured monocytes and this led to the activation of the initiator caspase 8 and the downstream effector caspase 3. However, activation of caspases had no significant effect on monocyte cell death. I also found that IFN-γ activated STAT1 and Akt signaling pathways induced autophagy leading to cell death. IL-10, on the other hand, activated STAT3 and Akt signaling cascades by which it also induced autophagy, attenuated IFN-γ-induced STAT1 signaling and brought about cell survival. Whether molecules such as ROS, DAPK, and P53 play a role in autophagy induced survival *vs* PCD, remain to be investigated.

Finally, understanding the mechanisms by which the autophagy pathway regulates monocyte PCD during health as well as in the context of chronic immune activation such as during HIV infection may provide important insight towards the development of new strategies that exploit this pathway for the treatment of HIV and other human diseases in which PCD and autophagy figure prominently (56).

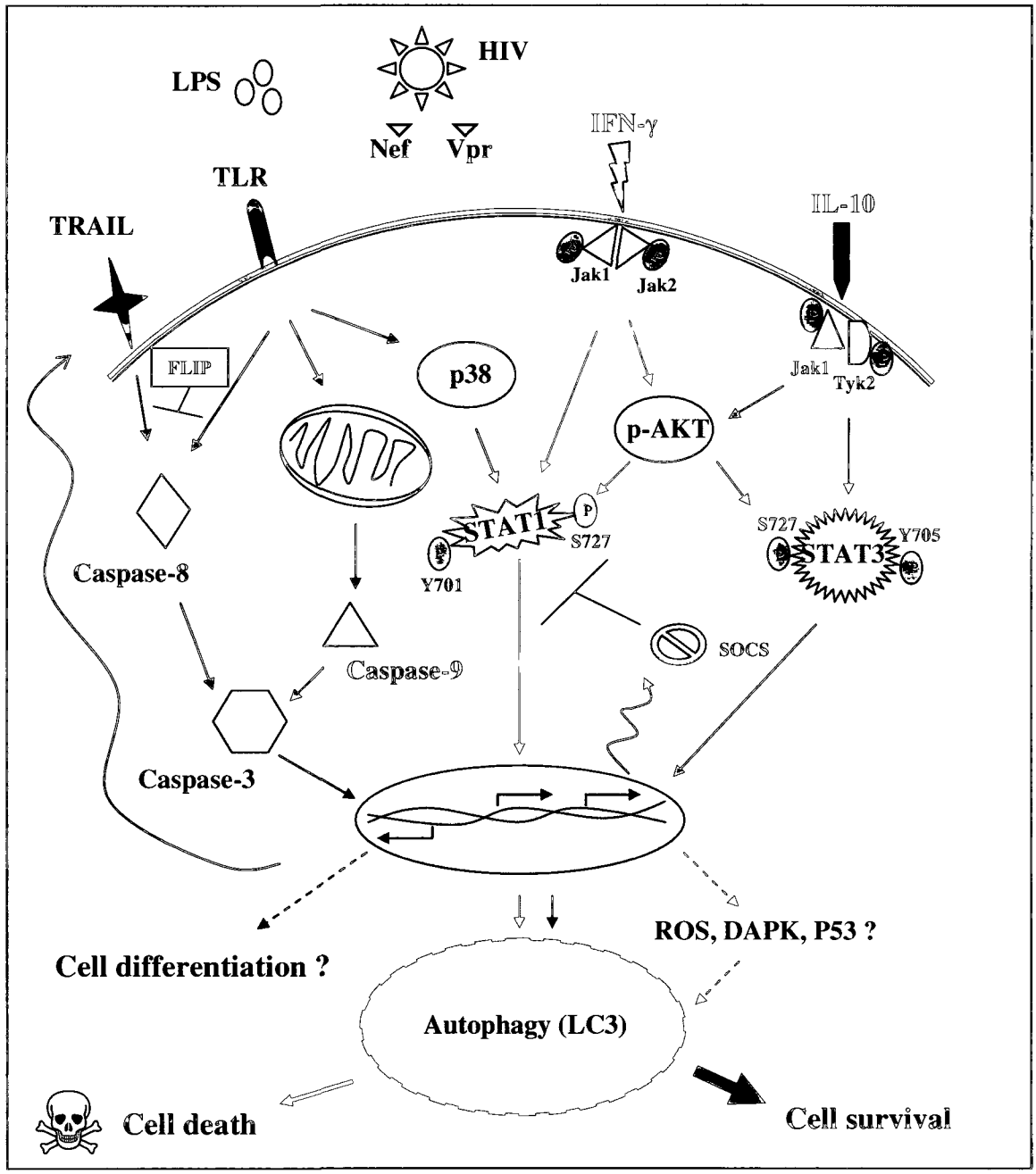

LPS
HIV
Nef
Vpr
IFN-γ
TLR
TRAIL
IL-10
Jak1
Jak2
Jak1
Tyk2
FLIP
p38
p-AKT
STAT1
P
S727
Y701
S727
Y705
STAT3
Caspase-8
Caspase-9
SOCS
Caspase-3
ROS, DAPK, P53 ?
Cell differentiation ?
Autophagy (LC3)
Cell death
Cell survival

Figure 6-1: Proposed model for the regulation of monocyte PCD in health and during chronic HIV infection.

In normal primary monocytes, I observed that TRAIL was secreted spontaneously from cultured monocytes and this led to the activation of the initiator caspase 8 and the downstream effector caspase 3. However, activation of caspases had no significant effect on monocyte cell death. I also found that IFN-γ activated STAT1 and Akt signaling pathways induced autophagy leading to cell death. IL-10, on the other hand, activated STAT3 and Akt signaling cascades by which it also induced autophagy, attenuated IFN-γ-induced STAT1 signaling and brought about cell survival.

During HIV infection, monocytes are constantly exposed to different viral proteins (Gp120, Nef, Vpr) and other factors including LPS, TRAIL, Fas, and IFN-γ, which have dramatic effects on monocyte function and cell death either directly or indirectly. In the direct setting, HIV and Vpr or LPS can affect mitochondrial membrane potential by decreasing Bcl-2 expression leading to cytocrome c release and caspase 9 cleavage. However, in some conditions signals via TLR can lead to caspase 8 activation. This is followed by activation of the downstream effector caspases 3, 6, 7, and DNA fragmentation, and then cell death. In the indirect setting, the HIV Gp120, Nef, and IFN-γ induce STAT1 activation and subsequently upregulation of STAT1 responsive genes such as TRAIL which in turn enhances the activation of caspase 8 and the effector caspases leading to DNA degradation and cell death. Activation of STAT1 could also induce cell death via the activation of the autophagy pathway. However, in the presence LPS, TNF-α, Tat, IL-13, and IL-10 monocyte PCD is prevented and/or reversed by several mechanisms. LPS, TNF-α, and Tat can upregulate the expression of the antiapoptotic genes such as c-IAP-2, Bcl-2 in monocytes. Nef, IL-10, and IL-13 can activate STAT3 which in turn increased the expression levels of FLIP and downregulated caspase 8 activation. Finally, IL-10 can activate autophagy pathway and promote cell survival.

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Curriculum Vitae

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Education

September 2003 to present:

- M.Sc., and Ph.D candidate, Department of Biochemistry Microbiology and Immunology, University of Ottawa.
- Thesis title: HIV-induced dysregulation of IFN-γ signaling and programmed cell death in primary monocytes.
- Supervisor: Dr. Marko Kryworuchko, cosupervisor: Dr. Ashok Kumar.

January 2001:

- Bachelor's degree in Clinical Laboratory Sciences.
- Graduation from college of Applied Medical Sciences, King Saud University in Riyadh.

1996:

- High school diploma with excellent marks, majoring in general sciences.

Work experience

September 2003 to present:

- M.Sc., and Ph.D candidate, Infectious Diseases and Vaccine Research Center, Children's Hospital of Eastern Ontario Research Institute.
- Supervisor: Dr. Marko Kryworuchko and cosupervisor: Dr. Ashok Kumar.
- Techniques: Different applications of flow cytometry including Surface and Intracellular staining, Cell viability assays, and Cytometric beads array,

Western blotting, RNA extraction and Quantitative PCR, ELISA, Microscopy, human PBMCs isolation and purification, transfection and tissue culture.

February 2001 to March 2003:

- Demonstrator in the virology unit, Microbiology department, College of Medicine, King Saud University.
- Training in the virology lab, King Khalid University Hospital.
- Diagnostic virology techniques (Enzyme immunoassays, Immunofluorescent assays, Western blotting, Neutralization assays, and Tissue culture).

2000 to 2001:

- One year Internship, King Faisal Specialist Hospital and Research Center.
- Clinical lab rotation (Microbiology, Hematology & Blood bank, Biochemistry & Toxicology, Histology & Electron Microscopy, Cytology, Immunology, Cytogenetics, I.V.F (*in vitro* fertilization), and Specimen collection).

Publications

Papers:

1- **Alhetheel, A.**, Elsageyer, M., Kumar, A., and Kryworuchko, M. Differential regulation of monocyte programmed cell death by IFN-γ and IL-10 via autophagy. Submitted.

2- Benoit, A., Abdkader, K., Sirskyj, D., **Alhetheel, A.**, Sant, N., Kumar, A., Diaz-Mitoma, F., Kryworuchko, M. Inverse association of repressor growth factor independent-1 with CD8 T cell interleukin (IL)-7 receptor [alpha] expression and limited signal transducers and activators of transcription signaling in response to IL-7 among [gamma]-chain cytokines in HIV patients. AIDS, 2009, 23(11):1341-7.

3- **Alhetheel, A.**, Yakubtsov, Y., Abdkader, K., Sant, N., Diaz-Mitoma, F., Kumar, A., and Kryworuchko, M. Amplification of the STAT1 signaling pathway and its association with apoptosis in monocytes from HIV-infected patients. AIDS, 2008, 22(10):1137-44.

Conference proceedings:

1- **Alhetheel, A.**, Yakubtsov, Y., Abdkader, K., Sant, N., Angel, J., Kumar, A.,

Diaz-Mitoma, F., and Kryworuchko, M. Upregulation of IFN-γ-induced STAT1 Activation and Apoptosis in Monocytes from HIV+ Patients. 13th International Congress of Immunology ICI, 2007, Rio de Janeiro, Brazil.

2- Kryworuchko, M., Abdkader, K., **Alhetheel, A.**, Sant, N., Pasquier, V., Kumar, A., Diaz-Mitoma, F., Keller, H., David, D., Goujard, C., Gilquin, J., Viard, J-P., Joussemet, M., Delfraissy, J-F., and Thèze, J. Analysis of IL-2-induced STAT5 Activation in CD8 and CD4 T Cells from HIV+ Patients. 5th joint meeting of the International Cytokine Society (ICS) and the International Society for Interferon and Cytokine Research (ISICR), 2004, San Juan, Puerto Rico.

Abstracts:

1- **Alhetheel, A.**, Elsagyer, M., Kumar, A., Kryworuchko, M. Regulation of Programmed Cell Death in Monocytes from HIV+ Patients by Interferon-γ and Interleukin-10. OHTN conference, November, 2009.

2- **Alhetheel, A.**, Kumar, A., Kryworuchko, M. Interleukin-10 protects monocytes from spontaneous and interferon-γ-induced programmed cell death in HIV infection. OHTN conference, November, 2008.

3- **Alhetheel, A.**, Kumar, A., Kryworuchko, M. Role of IFN-γ and IL-10 in regulating monocyte programmed cell death in HIV infection. XVII International AIDS Conference, August, 2008.

4- **Alhetheel, A.**, Yakubtsov, Y., Abdkader, K., Sant, N., Angel, J., Kumar, A., Diaz-Mitoma, F., Kryworuchko, M. Upergulation of interferon-γ-induced STAT1 activation and apoptosis in monocytes from HIV+ patients. OHTN conference, November, 2007.

5- **Alhetheel, A.**, Yakubtsov, Y., Abdkader, K., Sant, N., Angel, J., Kumar, A., Diaz-Mitoma, F., Kryworuchko, M. Upregulation of IFN-γ-induced STAT1 activation and apoptosis in monocytes from HIV+ patients. 13th International Congress of Immunology ICI, August, 2007.

6- Benoit, A., Abdkader, K., Sirskyj, D., **Alhetheel, A.**, Sant, N., Angel, J., Kumar, A., Diaz-Mitoma, F., Kryworuchko, M. Reduced IL-7Rα expression in CD8 T cells from HIV+ patients is associated with the transcriptional repressor Gfi-1 and impaired STAT activation in response to IL-7 but not IL-2, IL-15, IL-4, and IL-10. 4th AIS conference, July, 2007.

7- **Alhetheel, A.**, Yakubtsov, Y., Abdkader, K., Sant, N., Angel, J., Kumar, A., Diaz-Mitoma, F., Kryworuchko, M. Upergulation of interferon-γ-induced STAT1 activation and apoptosis in monocytes from HIV+ patients. OHTN conference, November, 2006.

8- **Alhetheel, A.**, Yakubtsov, Y., Abdkader, K., Sant, N., Angel, J., Kumar, A., Diaz-Mitoma, F., and Kryworuchko, M. upergulation of interferon-induced STAT1 activation and apoptosis in monocytes from HIV+ patients. Fourth Northern Lights

Fall Conference Ottawa Congress Centre Canadian Federation of Biological Societies 81 Ottawa Ontario 50th Scientific Conference, October, 2006.

9- **Alhetheel, A.**, Abdkader, K., Sant, N., Angel, J., Kumar, A., Diaz-Mitoma, F., Kryworuchko, M. Amplification of interferon-γ-induced STAT1 transcription factor activation in monocytes from HIV+ patients with chronic infection. XVI International AIDS Conference, August, 2006.

10- **Alhetheel, A.**, Abdkader, K., Sant, N., Angel, J., Kumar, A., Diaz-Mitoma, F., Kryworuchko, M. IFN-gamma-dependent hyperactivation of the STAT1 transcription factoring monocytes from HIV+ patients. OHTN conference, November, 2005.

References

References are available upon request.

APPENDIX

Re: asking a permission for using a figure!

From: **Chad Ishmael**
Sent: March 2, 2010 6:54:13 PM
To: abdulkarim alhetheel

Dear Abdulkarim Alhetheel,

I must apologize for the late reply. You may certainly use the image as a figure in your thesis.

Best regards,

Chad Ishmael

On 21 January 2010 01:00, abdulkarim alhetheel

> Dear Chad Ishmael,
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>
> Thank you very much for your assistance and consideration.
> Best regards,
> Abdulkarim Alhetheel
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> University of Ottawa
> Ottawa, Ontario, Canada

RE: asking a permission for using a figure!

From: **Richard Hunt** (
Sent: January 22, 2010 3:44:15 PM
To: abdulkarim alhetheel

No Problem. Please use it.
RH
Richard Hunt, M.A., Ph.D., FARVO
Director of the Integrated Biomedical Science Graduate Program
Professor of Pathology, Microbiology and Immunology
University of South Carolina School of Medicine

http://pathmicro.med.sc.edu
PLEASE NOTE MY NEW E-MAIL ADDRESS:

From: abdulkarim alhetheel
Sent: Friday, January 22, 2010 12:24 AM
To: Richard Hunt
Subject: asking a permission for using a figure!

Dear Dr. Richard Hunt,

I would like to request your permission to use the image that is pasted in the website

pathmicro.med.sc.edu/ lecture/HIV3.htm, pathmicro.med.sc.edu/ lecture/hiv_time_course2.jpg

and shows the relation between HIV titer, CD4, and CD8 number as a Figure in my Ph.D thesis.

Thank you very much for your assistance and consideration.

Best regards,

Abdulkarim Alhetheel
Ph.D candidate

Faculty of Medicine
Department of Biochemistry, Microbiology, and Immunology
University of Ottawa
Ottawa, Ontario, Canada

Re: asking a permission for using a figure!

From: **info2009**
Sent: February 3, 2010 2:24:51 AM
To:

The image is in the public domain. Feel free to use it and give credit
to U.S. National Institute of Health.

Thank you for visiting our web site.

Web Books Publishing

Quoting

> Dear Sir/Madam,
>
> I would like to request your permission to use the image that is pasted in
> the website:
> www.web-books.com/.../OPS/images/HIVcycle.gif,
> www.web-books.com/eLibrary/ON/B0/B22/05MHIV.html, and entitled
> replication cycle of HIV as figure in my Ph.D thesis.
>
>
> Thank you very much for your assistance and consideration.
>
> Best regards,
>
> Abdulkarim Alhetheel
> Ph.D candidate
>
>
> Faculty of Medicine
> Department of Biochemistry, Microbiology, and Immunology
> University of Ottawa
> Ottawa, Ontario, Canada
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