IMMUNOLOGICAL DETERMINANTS OF TUBERCULOSIS-ASSOCIATED IMMUNE RECONSTITUTION INFLAMMATORY SYNDROME (TB-IRIS) IN HIV-INFECTED PATIENTS ON ANTIRETROVIRAL THERAPY

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FACULTY OF MEDICINE
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TAN HONG YIEN

THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
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ABSTRACT

The advent of combined antiretroviral therapy (cART) has improved the quality of life of human immunodeficiency virus (HIV)-infected individuals by suppressing viral replication leading to reconstitution of the immune system. However, patients initiated on cART at an immunodeficient state i.e. CD4+ T-cell counts <200 cells/μL, may experience immune reconstitution inflammatory syndrome (IRIS) characterized by exaggerated inflammatory responses against an existing opportunistic infection (OI). Tuberculosis (TB) represents the most common OI among individuals with HIV infection, especially in resource-limited settings. TB-associated IRIS (TB-IRIS) often manifests as paradoxical deterioration of treated TB (also known as paradoxical TB-IRIS), or rapid onset of newly diagnosed TB following cART initiation (also known as unmasking TB-IRIS).

Notwithstanding that 10–30% of TB-HIV co-infected patients may develop TB-IRIS, there has not been a report from Malaysia, heretofore. Hence, a retrospective investigation was conducted. The prevalence of TB-IRIS was 16% among TB-HIV co-infected patients at the University Malaya Medical Centre (UMMC). Only disseminated TB was predictive of TB-IRIS (OR=10.7, P=0.032), and that the mortality rates were similar between patients with TB-IRIS (n=1, 5.9%) and TB-HIV without IRIS (n=5, 5.7%). CD4+ T-cell recovery following ART was not different between the two groups.

TB-IRIS reportedly occurs within the first month of ART, and hence it is cumbersome to distinguish it from relapsed or newly acquired TB. Therefore, there is an urgent need to identify appropriate laboratory markers to predict and characterize TB-IRIS. In a prospective cohort at UMMC, a case-control study that comprised the following study groups was established:

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(1) **TB-IRIS (case)**

(2) **TB no IRIS (control)**

(3) **No TB or IRIS (control)**

The levels of twelve cytokines/pro-inflammatory mediators at baseline and at the event of TB-IRIS (or equivalent time-point for control) were compared among the three groups. We found that the plasma IL-18 was higher in TB-IRIS patients at pre-cART and during the event. CXCL10 was higher pre-cART ($P<0.001$), mainly in paradoxical TB-IRIS patients, and during TB-IRIS ($P<0.001$), whereas CXCL8 was only higher during TB-IRIS ($P<0.001$). In contrast, IFN-γ was lower before and during TB-IRIS. Receiver operating curve (ROC) analysis showed that CXCL10 (AUC=0.884, $P<0.0001$) and IL-18 (AUC=0.99, $P<0.0001$) at pre-cART were predictive of TB-IRIS.

Since IL-18 is the signature cytokine for inflammasome activation in monocyte/macrophage, next we investigated the role of inflammasome activation and pyroptosis in the pathogenesis of TB-IRIS. HIV-TB patients exhibited higher proportions of monocytes expressing activated caspase 1 (casp1) pre-cART, compared to HIV patients without TB; patients who developed TB-IRIS exhibited increased casp1 levels following initiation of cART. TB-IRIS patients also had increased *NLRP3* and *AIM2* inflammasome mRNA, compared to controls. Expression of cell death markers (7-AAD and Annexin V by PBMC and plasma mitochondrial DNA (mtDNA) levels) was also higher in TB-IRIS patients. Plasma nitric oxide (NO) levels were lower pre-cART in TB-IRIS patients suggest inadequate inflammasome regulation. Expression of IL-18Rα on CD4+ T cells and NK cells was higher in TB-IRIS patients post cART, providing evidence that receptiveness against IL-18 further increase inflammasome activation and pyroptosis in monocytes.

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ABSTRAK

Kemunculan rawatan kombinasi antiretroviral (cART) telah meningkatkan kualiti hidup bagi individu yang dijangkiti *human immunodeficiency virus* (HIV) dengan menyekat replikasi virus serta pemulihan sistem imun. Walau bagaimanapun, pesakit yang mulakan cART pada tahap imundeficien (jumlah CD4+ sel T <200/μL) mungkin akan mengalami penyakit inflamasi imun pemulihan (Immune Reconstitution Inflammatory Syndrome, IRIS) sering berlaku dengan respons inflamasi yang keterlaluan terhadap jangkitan oportunis yang sediaada (opportunistic infection, OI). Tuberculosis (TB) merupakan OI yang paling biasa di kalangan individu HIV, terutamanya di kawasan sumber penetapan terhad. IRIS berkaitan dengan TB (TB-IRIS) berlaku dengan kemerosotan paradoks pada penyakit TB yang telah dirawat (dikenali sebagai *paradoxical* TB-IRIS), ataupun berlakunya diagnosis TB baru sebaik sahaja cART dimulakan (juga dikenali sebagai *unmasking* TB-IRIS).

Walaubagaimanapun dilaporkan bahawa 10-30% daripada pesakit dijangkiti TB-HIV mungkin boleh mendapat TB-IRIS, akan tetapi tiada laporan dari Malaysia sebelum ini. Oleh itu, satu siasatan retrospektif telah dijalanakan. Prevalens untuk TB-IRIS anatara pesakait TB-HIV ialah 16%. Diseminasi TB dapat meramal TB-IRIS (OR=10.7, P=0.032). Kadar kematian adalah sama antara pesakit-pesakit TB-IRIS (n=1, 5.9%) dan TB-HIV (n=5, 5.7%). Tiada perbezaan dengan pemulihan CD4+ sel T antara dua kumpulan selepas menyusuri cART.

TB-IRIS sering dilapor berlaku antara bulan pertama selepas cART, dan ini adalah sukar untuk membezakan TB yang sediada ataupun TB yang baru dijangkiti. Oleh demikian, adalah perlunya untuk mencari tanda-tanda makmal yang boleh menjangka dan menyifatkan TB-IRIS. Dalam kohot prospektif di UMMC, satu kajian kes-kontrol terdiri daripada kumpulan-kumpulan kajian sedemikian telah diadakan:

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(1) TB-IRIS (kes)

(2) TB tanpa IRIS (kontrol)

(3) Tanpa TB atau IRIS (kontrol)

Tahap-tahap dua belas sitokin-sitokin/ penyusur pro-inflamatori sebelum cART dan semasa TB-IRIS (ataupun titik sewaktu untuk control) telah dibandingkan antara tiga kumpulan yang disebut tadi. IL-18 didapati adalah lebih tinggi pada pesakit-pesakit TB-IRIS sebelum cART dan semasa tindak balas. CXCL10 pula lebih tinggi sebelum cART terutamanya semasa TB-IRIS pardoks ($P<0.001$), CXCL8 pula hanya lebih tinggi semasa TB-IRIS ($P<0.001$). Dengan membuat analisis receiver operating curve (ROC), CXCL10 (AUC=0.884, $P<0.0001$) dan IL-18 (AUC= 0.99, $P<0.0001$) sebelum cART. Kedua-duanya dikenali sebagai tanda jangkaan TB-IRIS.

Memandangkan IL-18 merupakan satu-satunya sitokin penting dalam aktivasi inflamasom dalam monosit dan makrofaj, kami seterusnya menyiasat peranan aktivasi inflamasome dan pirotopsis dalam patogenesis TB-IRIS. Pesakit-pesakit HIV-TB mempaparkan nisbah lebih tinggi untuk monosit yang mengekspres caspase 1 aktif (casp1) sebelum cART, jika dibandingkan dengan pesakit-peakit HIV tanpa TB; casp1 untuk pesakit TB-IRIS meningkat pengekspressan paling tinggi. Pesakit-pesakit TB-IRIS juga ada peningkatan ekspresi mRNA bagiNLRP3 dan AIM2, berbanding kontrol-kontrol. Ekspresi tanda sel mati (7-AAD dan Annexin V bagi PBMC dan DNA mitokondria (mtDNA) dalam plasma) juga tinggi antara pesakit TB-IRIS. Tahap Nitrik oxide (NO) dalam plasma lebih rendah pada pesakit TB-IRIS menyumbangkan bukti kekurangan regulasi terhadap inflamasom. Ekspresi IL-18Rα pada CD4+ T sel dan sel NK adalah tinggi dalam pesakit TB-IRIS selepas cART, memberi bukti bahawa penerimaan terhadap IL-18 semakin meningkat aktivasi inflamasom dan piroptosis dalam monosit.

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Hong Yien

9 November 2015

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ABBREVIATIONS

7-AAD	7-aminoactinomycin D
α	alpha
AIDS	acquired immunodeficiency syndrome
AIM2	absent in melanoma 2
APC	allophycocyanin
ASC	apoptotic speck-like protein containing a CARD
ATT	anti-tuberculosis therapy
ATP	adenosine triphosphate
AUC	area under curve
AZT	zidovudine
β	beta
BFA	brefeldin A solution
BP	binding protein
BSA	bovine serum albumin
cART	combined antiretroviral therapy
CARD	caspase recruitment domain
casp	caspase
CD	cluster of differentiation
CO_2	carbon dioxide
CR	complement receptor
CSF	cerebrospinal fluid
Cy	cyanine
D-PBS	Dulbecco's phosphate-buffered saline
DAMPs	damage-associated molecule patterns
DCs	dendritic cells

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DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleotide triphosphate
ds	double-stranded
E. coli	*Escherichia coli*
Ecto-CRT	calreticulin exposure
EDTA	ethylenediamine tetra-acetic acid
EI	early inhibitors
ELISA	enzyme-linked immunosorbent assay
EQT	equivalent time point
ESAT-6	6 kDa early secretary antigenic target
FACS	fluorescence activated cell sorter
Fas	first apoptotic signal
Fas-L	Fas ligand
FcγR	Fc gamma receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FLICA	fluorescence labelled of inhibitor caspase
FMO	fluorescence minus one
γ	gamma
g	gram
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hank's balanced salt solution
HEPES	hydroxyethyl piperazine ethane sulfonic acid
HIS	heat-inactivated serum
HIV	human immunodeficiency virus

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HLA	human leukocyte antigen
HMGB1	high-mobility group protein B1
hr	hours
HRP	horseradish peroxidase
ICS	intracellular staining
IFN	interferon
Ig	immunoglobulin G
IGIF	IFN-γ inducing factor
II	integrase inhibitors
IL	interleukin
iNOS	inducible nitric oxide synthase
INSHI	International Network for the Study of HIV-Associated IRIS
IQR	interquartile range
ITAMs	immunoreceptor tyrosine-based activation motifs
IP-10	interferon-gamma-inducing protein-10
IRIS	immune reconstitution inflammatory syndrome
k	kilo
Kd	kilodalton
LPS	lipopolysaccharide
LRR	leucine-rich repeat
M	Molar
Mab	monoclonal antibody
MAC	*Mycobacterium avium-intercellulare-scrofulaceum* complex
M-CSF	macrophage-colony stimulating factor
MDM	monocyte-derived macrophages
MEC	medical ethics committee

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MEM	minimum essential medium
MESF	molecules of equivalent soluble fluorochrome
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
min	minutes
MIP	macrophage inflammatory protein
MKK	mitogen-activated protein kinase kinase
ml	milliliter
mRNA	messenger RNA
mt	mitochondria
MTB	*Mycobacterium tuberculosis*
MW	molecular weight
NACHT	nucleotide-binding and oligomerization domain
NAK	nef-associated kinase
NFκB	nuclear factor-kappa B
ng	nanogram
NK	natural killer
NLR	NOD-like receptor
NLRC4	NLR family CARD domain containing 4
NLRP3	NLR family pyrin domain containing 3
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NRTIs	nucleoside reverse transcriptase inhibitors
NO	nitric oxide
NOD	nucleotide binding and oligomerization domain
OD	optical density

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OI	opportunistic infection
PAMPs	pathogens-associated molecule patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PHA	phytohaemagglutinin
PI	protease inhibitors
PMA	phorbol-12-myristate-13-acetate
PPD	purified protein derivative
PR	protease
PS	phosphatidylserine
PYD	pyrin
PYHIN	pyrin and HIN containing domain
QFTGIT	Quantiferon-TB Gold in-tube
RD-1	region of differentiation-1
RNA	ribonucleic acid
ROC	receiver operating characteristic
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
s	soluble
ScR	scavenging receptor
SD	standard deviation
SDHA	succinate dehydrogenase complex, subunit A

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sec	seconds
TB	tuberculosis
TBM	tuberculosis meningitis
TBP	TATA box binding protein
Th	T helper
TLR	Toll-like receptor
TMB	3, 3′,5 ,5′-tetramethylbenzidine
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
UV	ultraviolet
µg	microgram
µl	microlitre
µM	micromolar
V	volts
VL	viral load
x g	times gravitational constant

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CHAPTER 1

BACKGROUND AND REVIEW OF LITERATURE

1.1 Epidemiology of HIV and Tuberculosis

1.1 .1 HIV Pandemic – A Major Global Concern

Human immunodeficiency virus (HIV-1), the etiology cause of acquired immunodeficiency syndrome (AIDS) continues to remain a major global health challenge (UNAIDS 2012) ever since it's initial description made more than two decades ago. Estimates suggest that over 34 million people are infected with HIV-1, and approximately 67% (22.5 million in 2009) of this population belongs to sub-Saharan Africa (UNAIDS 2010). The past 10 years has witnessed unprecedented advances in the improvement of global health and sustenance of development. An UNAIDS report shows that the incidence of HIV in adults and children has markedly declined to 2.5 million in 2011, ~20% lower than in 2001 (UNAIDS 2012). Further, the mortality rates due to AIDS-related ailments have also been reported to decline since the mid-2000s, largely due to increased accessibility to combined antiretroviral therapy (cART) (WHO 2011). By 2015, at least 8 million people living with HIV/AIDS are expected to receive cART in low-and middle-income countries, although an additional 7 million people were enrolled for treatment (WHO 2011).

1.1.2 Epidemiology of Tuberculosis

Tuberculosis (TB) is a bacterial disease described in humans since antiquity, and still remains a major health challenge in most of the developing countries in the modern era. The disease results from an infection with *Mycobacterium tuberculosis* (MTB) (and occasionally by *M. bovis* and *M. africanum*), and is spread almost exclusively via the respiratory route (Zumla *et al* 2001). TB occurs when a person acquires the tubercle

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bacilli, which is usually cleared or controlled by the immune system. Nonetheless, the organism is able to persist in a dormant or latent state in lung tissues for years without causing any overt disease in ~90% of the infected individuals (Harries *et al* 2004). More than a third of the world's population is infected with MTB, with ~95% of the cases and ~98% of all TB deaths occurring mostly in the developing world. Worldwide estimates suggest that over 1.7 million people succumb to TB disease annually. In 2009 alone, >9.4 million incident cases of TB were recorded globally (equivalent to 137 cases per 100,000 population). Of note, southern Africa has the highest prevalence of HIV in newly diagnosed TB cases, and thus reportedly had the worst burden of HIV/TB co-infections in 2009 (UNAIDS 2010) .

1.1.3 Dual Epidemic: The Epidemiology of HIV and Tuberculosis Infections in Malaysia

South East Asia has ~1.7 million people living with HIV infection accounting for ~5% of the total pool of HIV-infected individuals reported from all over the world (UNAIDS 2012). Ever since her first case of AIDS documented in 1986, Malaysia has witnessed over 16,340 deaths due to the pandemic in the past 28 years strategies have been successfully implemented to treat HIV infection and have resulted in markedly increased survival rates in infected individuals (MOH 2014) . A recent annual report suggests that the incidence rates of HIV infection in Malaysia have witnessed a sharp decline from 6978 cases in 2002 to 3393 in 2013, with more than half reported in 2002 (**Figure 1.1**) (MOH 2014) . However, with no foreseeable cure HIV/AIDS continues to pose a serious threat, mainly to young adults aged between 30 and 49 years in the country (MOH 2014).

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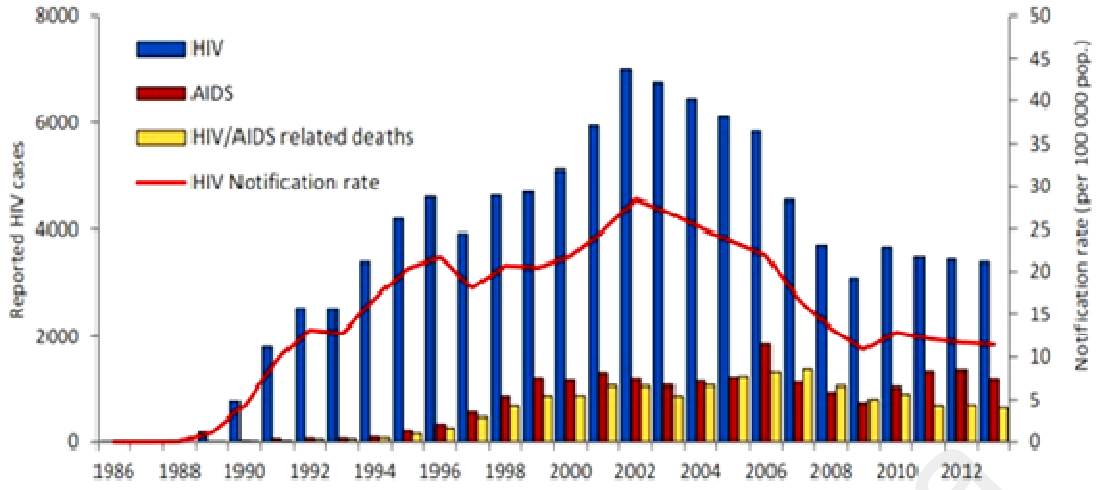

Figure 1.1: Reported HIV and AIDS-Related Deaths in Malaysia (1986 – 2013).

Figure adapted from Global AIDS Response Progress Report 2014, Malaysia, page 13.

Ever since it's introduction in 2002, cART coverage could only reach ~45% (17,369 individuals) of the HIV-infected population in Malaysia until 2013. Although the epidemic was reported to be highly concentrated amongst the injecting drug use (IDU) community, recent reports suggest that trend in the mode of transmission has shifted largely to the sexual route (Fu *et al* 2012). Therefore, HIV infection among IDUs has seen a dramatic decline from 70-80% in the 1990s to 21.5% in 2013 (MOH 2014). At least 80% of the cumulative cases documented in Malaysia are males and the incidence rates appear to rise significantly among females over the years (MOH 2014). Estimates show that since the late 1990s, there has been a continued and rapid spread of HIV among females, with seroprevalence rates increasing from 5% in 1990 to 21% in 2011 (UNAIDS 2013). With the scaling-up of prevention-of-mother-to-child-transmission (PMTC) programmes in antenatal care settings, neonates with HIV infection has occurred at the rate of 1% in 2010 (MOH 2014).

The annual TB case notification rates in Malaysia have decreased from 92 per 100,000 in 2001 to 80 per 100,000 population in 2012 (The World Bank 2014). The actual

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incidence rates of TB has been found to be ~16,000 to 20,000 cases each year (MOH 2014). In 2012, ~6% (1347 cases) of the total 22,124 TB-infected individuals were co-infected with HIV where 32% were receiving cART (WHO 2014) (**Figure 1.2**).

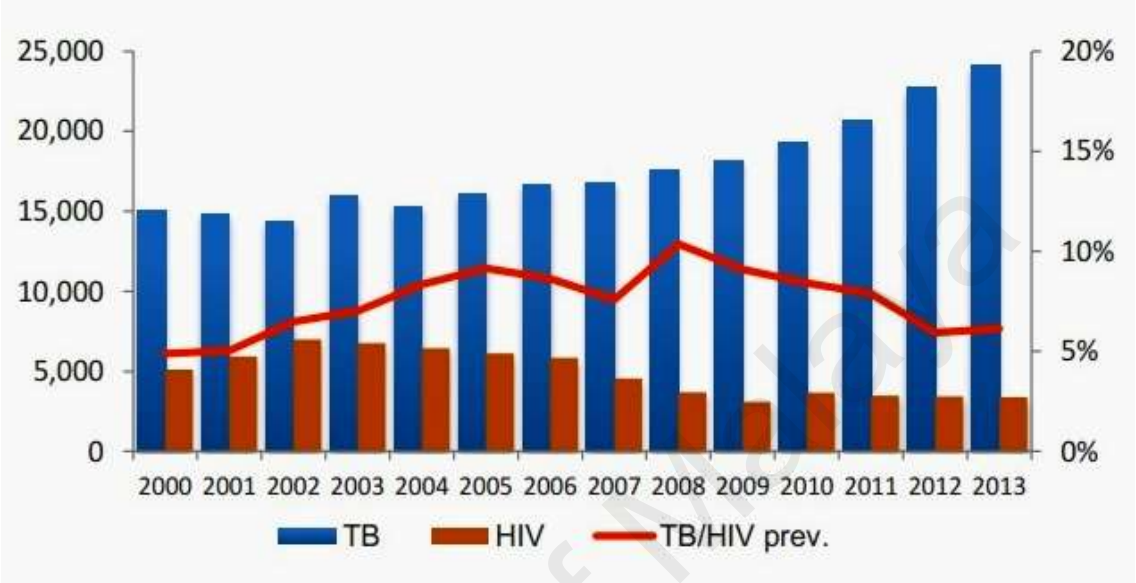

Figure 1.2: New TB, HIV and Prevalence of TB-HIV Co-Infection, Malaysia (1999-2013).

Figure adapted from Global AIDS Response Progress Report 2014, Malaysia, page 22.

1.2 Virology of HIV

1.2.1 HIV Genomic Organization and Structure

HIV-1 is a retrovirus consisting of an outer envelope, a nucleocapsid layer, two identical strands of RNA, viral enzymes and proteins (**Figure 1.3**). The virion is spherical in shape with a diameter of 100-120nm, and is surrounded by a lipoprotein membrane envelope (env) derived from the infected host cell during viral budding. The envelope contain glycoprotein (gp) spikes integrated into the envelope, and is composed of a trimer of the external (gp120) and transmembrane (gp41) subunits (Wyatt *et al* 1998). The matrix protein p17 is anchored to the internal face of the envelope. Beneath the envelope lies a cone-shaped capsid (composed of protein p24) that consists of two

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genomic single-stranded positive sense RNA strands (diploid genome), each consisting of ~9.7 kilobases, and tightly bound to nucleocapsid proteins p6 and p7, and viral enzymes (Gelderblom *et al* 1989; Arnold *et al* 1991). There are 9 viral genes, viz., the structural *env, gag, pol*, the regulatory *rev, nef, tat*, and the accessory *vpr, vpu, vif* genes (Subbramanian *et al* 1994; Frankel *et al* 1998; Seelamgari *et al* 2004).

1.2.2 Life Cycle of HIV

The HIV-1 viral surface protein gp120 binds to the cluster of differentiation 4 (CD4) receptor on the target host cell, inducing a conformational change that enables binding to either a CCR5 or CXCR4 β−chemokine co-receptor (Deng *et al* 1996; Simmons *et al* 1998). The CD4 is expressed on the surface of T lymphocytes, monocytes, macrophages, microglial and dendritic cells (DCs) (Wyatt *et al* 1998). Based on this tropism, HIV-1 can be broadly divided into two categories, those using the CCR5 co-receptor (R5 viruses) and those that use the CXCR4 co-receptor (X4 viruses) [reviewed in (Gorry *et al* 2011)]. During acute infection, the R5 virus utilizes the CCR5 co-receptor that is primarily expressed on activated memory CD4+ T cells and macrophages. During the later stages of HIV disease, ~50% of individuals experience a shift in viral tropism to a predominately X4 type or mixed R5/X4 (dual tropic) virus populations. This switch-over to use CXCR4 (expressed mainly on naïve T-cells), is usually accompanied by a rapid decline in CD4+ T-cell numbers and clinical progression to AIDS (Wyatt *et al* 1998; Berger *et al* 1999; Moore *et al* 2007). After binding to the cell surface, fusion of viral and cell membranes allows viral entry into the cell. By reverse transcription, the RNA genome is subsequently transcribed into a DNA intermediate (un-integrated provirus) that is transported into the nucleus for integration with the host cell genome by viral integrase [reviewed in (Gorry *et al* 2011)]. The process of reverse transcription is highly error-prone, likely resulting from defective

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proof-reading ability of viral RT. As a consequence, the virus is highly mutagenic, allowing it to evade neutralizing antibodies (nAb) and develop resistance to antiretroviral (ARV) agents (Preston *et al* 1988; Roberts *et al* 1988; Coffin 1995). Following integration, production of viral proteins and assembly of new virions takes place at the cell surface [reviewed in (Gorry *et al* 2011)] (**Figure 1.4**).

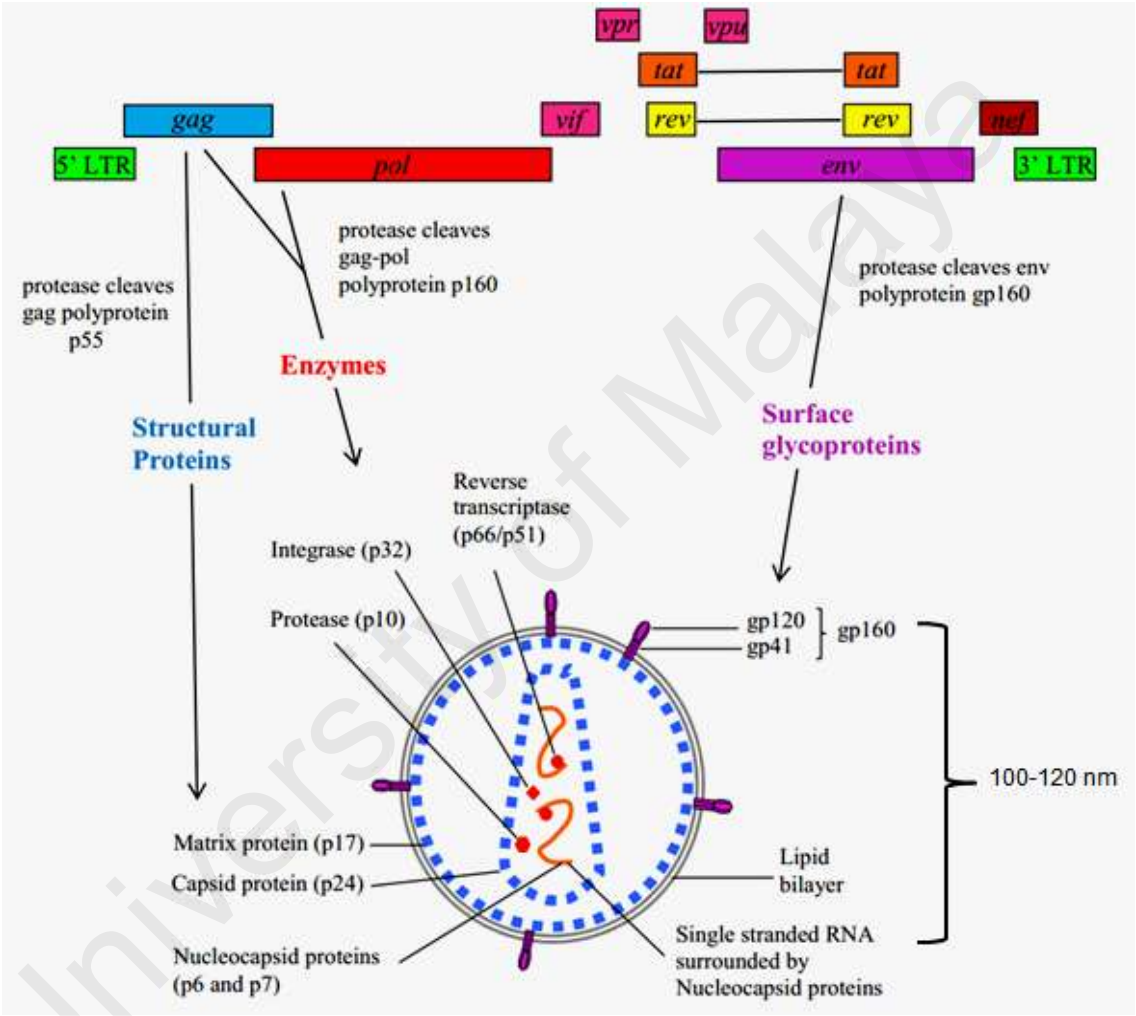

Figure 1.3: Genomic Organization and Viral Structure of HIV-1 (Adapted from (Costin 2007).

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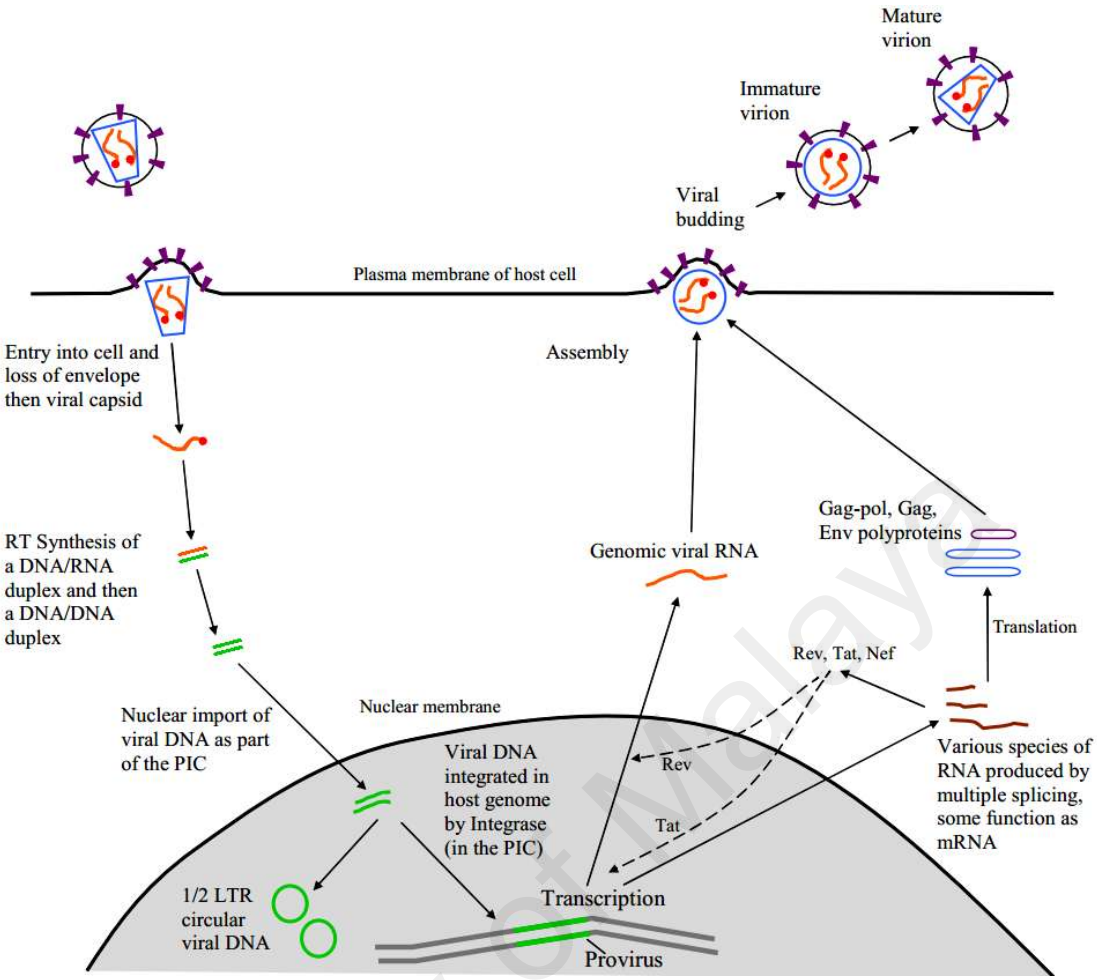

Figure 1.4: Life Cycle of HIV in Target CD4+ T Cells.

HIV-1 enters target cell by fusion. Subsequent steps in the viral life cycle involve reverse transcription of viral RNA, integration of proviral DNA into the host nuclear DNA, and assembly of viral proteins into new virions budding from the cell surface (Figure adapted from (Barre-Sinoussi *et al* 2013).

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1.2.3 Natural History of HIV Infection

Following infection with HIV-1, the virus rapidly multiplies in the infected host, and reaches high levels in plasma within weeks of transmission (**Figure 1.5**) (Daar *et al* 1991). Concurrent to the rapid rise in viremia, the CD4+ T-cell counts decline (Picker 2006). During the primary infection phase, a vast majority of infected subjects develop clinical symptoms, called acute retroviral syndrome (ARS) or seroconversion illness, typically characterized by fever, fatigue, sore throat, myalgia, headache, lymphadenopathy and rash (Tindall *et al* 1991; Schacker *et al* 1996). During this stage, the infected individuals pose an increased risk for disease transmission owing to the high levels of plasma viremia. After an additional period of a few weeks, PVL begins to decrease as HIV-specific immune responses develop (Koup *et al* 1994). During the subsequent phase chronic of infection, plasma viremia stabilizes at a viral set-point, which varies significantly between individuals. The viral set point is predictive of long-term clinical outcomes, where higher levels of viral set-point is associated with a faster depletion of CD4+ T cells and progression to AIDS (Mellors *et al* 1996; Lefrere *et al* 1998). During chronic infection, patients have few clinical symptoms. However, virus replication proceeds rapidly in the blood and lymphoid tissues as CD4+ T cells are continuously destroyed and replenished (Embretson *et al* 1993; Ho *et al* 1995). Over the years, the CD4+ T-cell counts undergo gradual depletion to <200 cells/µl, and the infected individuals becomes susceptible to opportunistic infections (OIs) or cancers leading to terminal AIDS (Selik 2014).

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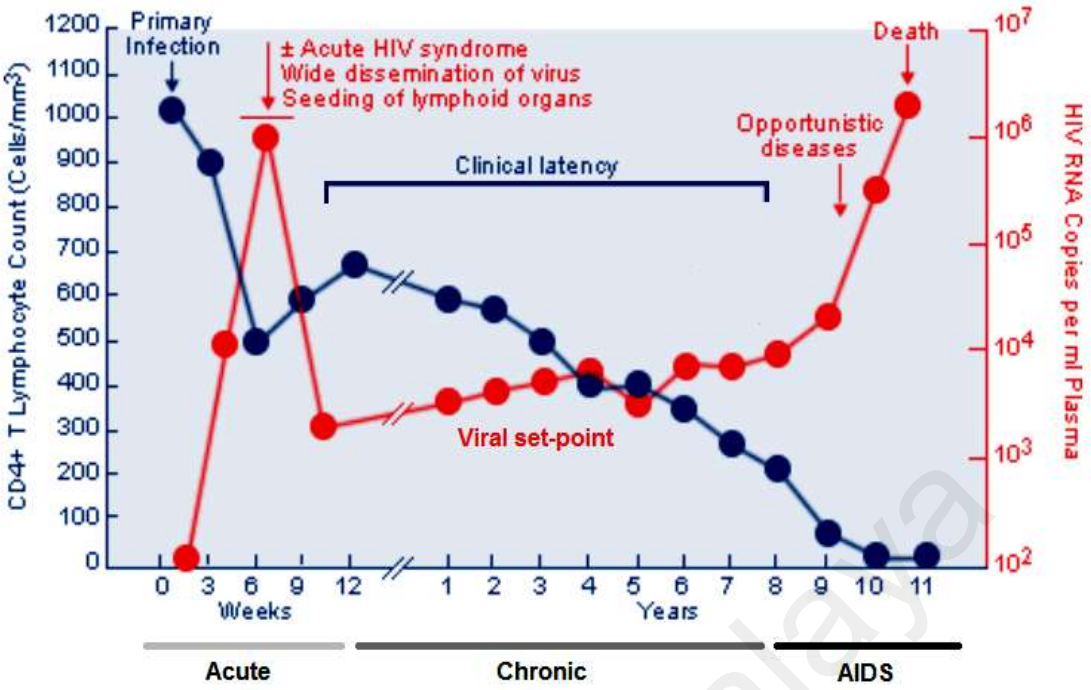

Figure 1.5: Natural History of HIV-1 Infection.

The course of HIV-1infection is typically divided into (i) primary infection, (ii) clinical latency and (iii) advanced disease according to the status of viral replication and CD4+ T-cell counts of the infected individual. (Figure adapted from (Pantaleo *et al* 1993).

1.2.4 Mechanisms of CD4+ T-Cell Depletion

1.2.4.1 HIV-Mediated Direct and Indirect Killing of CD4+ T Cells

Acute HIV infection is characterized by rapid depletion of memory CD4+ T cells in the gut mucosa (Douek *et al* 2003). HIV preferentially infects memory CD4+ T cells owing to their increased activation state and higher expression of CCR5 (Brenchley *et al* 2004). HIV-infected cells are also susceptible to apoptosis via Fas-Fas-ligand (FasL) interactions as HIV appears to enhance the surface expression of these molecules (Estaquier *et al* 1996). However, subsequent studies have shown that during chronic HIV infection more CD4+ T-cell death occurs by direct infection (Haase 1999; Rodriguez *et al* 2006; Mellors *et al* 2007). Furthermore, increased death rate is observed

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also in non-target cells, such as CD8+ T cells and macrophages (Hellerstein *et al* 1999), suggesting the likely involvement of an alternate mechanism. One process whereby HIV triggers apoptosis in uninfected bystander T cells is via the onset of generalized chronic immune activation (CIA) (Gougeon *et al* 1996).

Generalized CIA during chronic HIV infection is characterized by increased expression of immune activation markers e.g. HLA-DR, CD69, CD38 on CD4+ and CD8+ T cells, macrophages, NK cells and DCs (reviewed in (Bosinger *et al* 2011; Miedema *et al* 2013; Paiardini *et al* 2013)). The depletion of CD4+ T cells has been linked with increased levels of these immune activation markers including HLA-DR and CD69 expressions on CD4+ and increased HLA-DR and CD38 expressions on CD8+ T cells (Leng *et al* 2001; Sousa *et al* 2002; Hazenberg *et al* 2003; Deeks *et al* 2004). Generalized CIA could result from exposure to gp120, Vpr, Nef, Tat and HIV protease, which could also cause apoptosis of uninfected bystander CD4+ T cells. The mechanisms of induction of CD4+ T-cell apoptosis by HIV proteins are outlined in **Table 1.1**.

It has also been suggested that generalized CIA results from breakdown of the mucosal barrier due to massive depletion of CD4+ T cells at the gastrointestinal tract. This may lead to translocation of microbial products, such as bacterial lipopolysaccharide (LPS), peptidoglycan (Brenchley 2007), bacterial DNA (16s rDNA) (Jiang *et al* 2009), from the gut lumen into the systemic circulation, causing generalized immune activation via stimulation of Toll-like receptors (TLRs) (Brenchley *et al* 2006).

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Table 1.1: Mechanisms of HIV-Protein-Mediated CD4+ T-Cell Death		
HIV Proteins	**Mechanisms**	**References**
gp120	• Cross-linking with CD4 without TCR ligation and induction of apoptosis • Increase of Fas/FasL expression • Increase of Bax and decrease of Bcl-2	(Marschner *et al* 2002) (Arthos *et al* 2002)
gp41	• Induction of multinucleated syncytia apoptosis • Induction of hemi-fusion-mediated apoptosis	(Ferri *et al* 2000a) (Ferri *et al* 2000b) (Blanco *et al* 2003)
Tat	• Increase expression of Fas/FasL • Increase of pro-apoptotic Bax and decrease of anti-apoptotic Bcl-2	(Sastry *et al* 1996) (Li-Weber *et al* 2000)
Nef	• Increased expression of Fas/FasL • Membrane disorder and lysis of bystander CD4+ T cells	(Xu *et al* 1999) (Zauli *et al* 1999) (Azad 2000)
Vpr	• Arrest of cell cycle • Mitochondria disruption • Membrane disorder and lysis of both CD4+ T-cells and bystander cells	(Watanabe *et al* 2000) (Azad 2000) (Roumier *et al* 2002)
Protease	• Inactivation of anti-apoptotic Bcl-2 • Activation of pro-apoptotic pro-caspase-8	(Strack *et al* 1996) (Nie *et al* 2002)

1.2.4.2 Fibrosis of Lymphoid Tissues

Effective immune responses against infection involve the capture and processing of antigens by mucosal resident DCs followed by their maturation and migration to regional lymph nodes where antigen will be presented to antigen-specific naïve T cells (Banchereau *et al* 1998; Stoll *et al* 2002). However, DCs infected by HIV at mucosal surfaces migrate to lymph nodes are capable of transmitting HIV to interacting CD4+ T cells and follicular DCs residing in lymph nodes (Granelli-Piperno *et al* 1999). As such, a large number of activated CD4+ T cells are inappropriately attracted to and retained in lymph nodes (Biancotto *et al* 2007). Chronic inflammation in lymph nodes can also

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increase deposition of collagen in lymph nodes, leading to fibrosis and disruption of lymphoid architecture (Schacker *et al* 2002). This likely affects the ability of lymphoid tissue to support homeostasis, and therefore, the level of fibrosis correlates inversely with CD4+ T-cell numbers in infected patients (Schacker 2008; Zeng *et al* 2011).

1.2.4.3 Impairment of Haematopoietic Progenitor Cells

Mechanisms of CD4+ T-cell depletion described in the previous section affect mature CD4+ T cells. HIV infection is also associated with impaired production and differentiation of CD34+ haematopoietic progenitor cells (HPCs) from the bone marrow and thymocyte proliferation (Dion *et al* 2004), leading to reduced frequencies of recent thymic CD4+ emigrants and naïve CD4+ T cells in the peripheral circulation (Bonyhadi *et al* 1993; Su *et al* 1995). HIV-mediated depletion of CD34+CD4+ cells could also occur due to a combination of direct HIV infection or indirect mechanisms. Impairment occurs in accessory cells of the bone marrow (e.g. T cells, monocytes, macrophages, micro-vascular endothelial cells and stromal cells) to regulate the development of haematopoietic cells (Moses *et al* 1998; Alexaki *et al* 2008). Furthermore, cells that differentiated from HPCs in the bone marrow can be infected, and could serve as HIV reservoirs throughout the body. The differentiation of naïve T cells in the thymus is critical during early life. Although thymic function decreases with age, production of naïve T cells is maintained in late adulthood. HIV infection causes thymocyte depletion and loss of thymic epithelium leading to thymus involution (Bonyhadi *et al* 1993; McCune 1997). Expression of T-cell receptor (TCR) rearrangement excision circles (a marker of thymic function) was lower in HIV-infected children and adults compared to age-matched uninfected controls. The thymus also contributes to complete immune reconstitution in children and partial recovery in adults initiated with cART (Douek *et al* 1998; Correa *et al* 2002). In HIV-infected individuals with a sustained response to

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cART, CD4+ T-cell counts are determined by thymus function and immune activation (Fernandez *et al* 2006).

1.2.5 AIDS

Patients with AIDS develop OIs, malignancies, cachexia, HIV nephropathy and encephalopathy. The common OIs include MTB, *Pneumocystis jirovecii* pneumonitis (PCP), cerebral toxoplasmosis and disseminated *M. avium-intercellulare-scrofulaceum* (MAI) complex infection. Common malignancies include Kaposi's sarcoma and non-Hodgkins lymphoma. Besides, HIV-infected patients also experience non-AIDS manifestations, namely neurocognitive disorders, peripheral neuropathy and HIV wasting syndrome (Selik 2014). The time from primary infection to development of AIDS is highly variable, but on average is ~8-10 years (Pantaleo *et al* 1993)

1.3 Antiretroviral Therapy

cART suppresses the replication of HIV allowing recovery of the immune system. Nonetheless, HIV is not completely eradicated by cART as it can integrate with the genome of long-lived immune cells (e.g. memory T cells and DCs), which reportedly act as persistent viral reservoirs, and therefore treatment is always life-long (reviewed by (Apostolova *et al* 2011). Ideally, cART should be initiated as early as possible to minimize damage caused to the immune system, and subsequent immune recovery. Currently, the International AIDS Society-USA panel recommends the initiation of cART in symptomatic patients regardless of CD4+ T-cell counts, and for asymptomatic individuals with CD4+ T-cell counts ≤500 cells/µl (Hirnschall *et al* 2013; WHO 2013; Vitoria *et al* 2014). Initiating cART at a CD4+ T-cell count of 500 cells/µl decreases mortality and leads to improved prognosis cART in ≤4 months (following the estimated

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date of HIV acquisition) is associated with enhanced likelihood of recovery of CD4+ T-cell counts to 900 cells/μl (Le *et al* 2013).

1.3.1 Antiretroviral Therapy and Mode of Action

The standard cART regimen consists of a combination of at least three ARV agents to effectively suppress virus replication and decrease the rate of HIV disease progression. The currently available regimens includes nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (IIs), and entry inhibitors (EIs). Each ARV class targets a different step in the viral life cycle (**Figure 1.6**). The most commonly used drugs belong to the class of NRTI/NNRTIs and PIs. NRTI/NNRTIs inhibit HIV RT, which is important for the transition of viral RNA to pro-viral DNA (De Clercq 1998; Squires 2001). PIs disrupt post-translational processing of viral proteins and enzymes (Randolph *et al* 2004). There are newer drug classes such as integrase strand-transfer inhibitors, entry inhibitors and CCR5 antagonists (Greene 2004; Thompson *et al* 2010). The newly developed drugs namely PI darunavir and II raltegravir are better tolerated by HIV-infected individuals compared with older drugs (Grinsztejn *et al* 2007; Ortiz *et al* 2008). Nonetheless, the availability of these drugs is limited in resource-limited settings.

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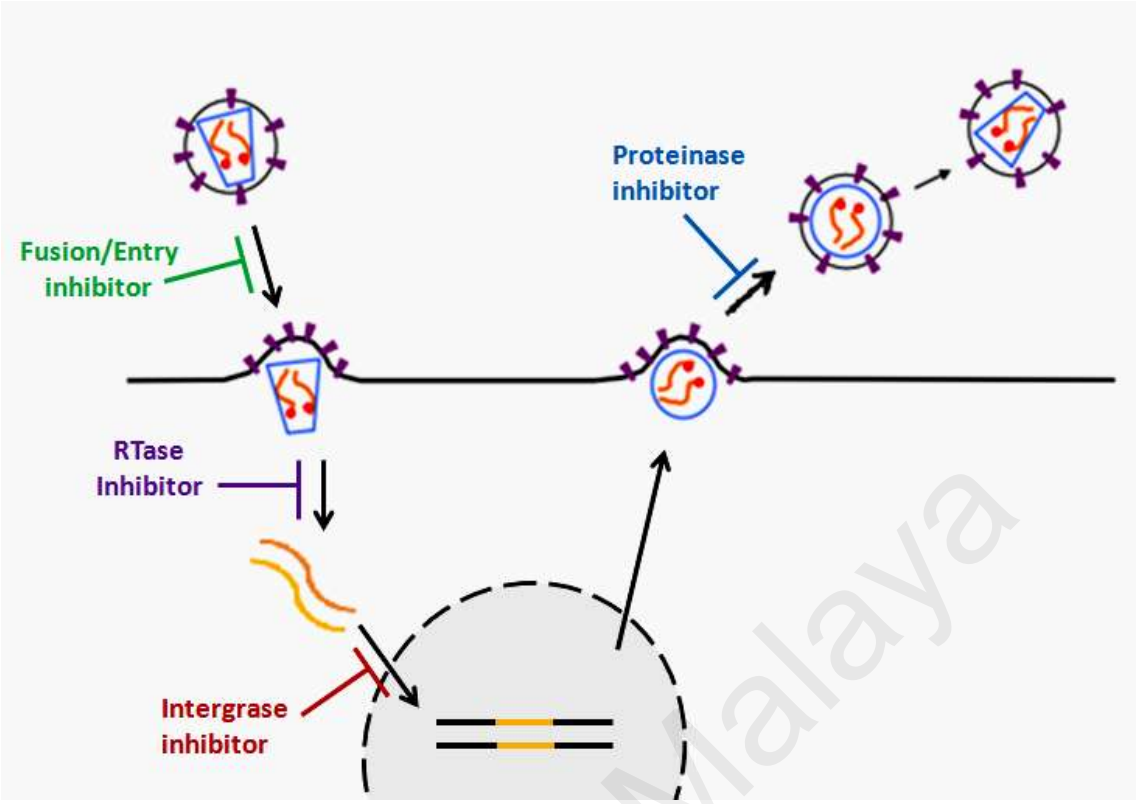

Figure 1.6: Life Cycle of HIV and Mode of Action of Classes of Antiretroviral (ARV) Drugs.

The currently available ARV drugs can be divided into five classes according to the mechanism of interruption of viral life cycle. RT inhibitors inhibit the enzymatic function of viral reverse transcriptase, either compete with natural nucleosides (NRTIs) or by reducing their catalytic activities (NNRTIs) that prevent completion of viral DNA synthesis, thereby preventing the replication of HIV-1. PIs inhibits enzymatic function of HIV-1 protease thereby preventing the generation and maturation of newer virions. IIs prevent the integration of viral DNA into the host genome. FIs prevent fusion between viral envelope and host cell membrane; whilst CCR5 inhibitors block the interaction between HIV and CCR5 co-receptor on the host cell membrane thereby prevent the entering of HIV-1 into cells.

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1.3.2 Differential Rates of CD4+ T-Cell Recovery Following Initiation of cART In HIV-Infected Individuals

The advent of combination ART (cART) in 1996 has resulted in substantial improvement in the prognosis of HIV-1-infected individuals (Egger *et al* 1997; Hogg *et al* 1999). As well as leading to sustained reduction in the incidence of AIDS-related OIs, the introduction of cART substantially decreased the rates of HIV-associated mortality. Furthermore, cART has also reduced the risk of acquisition of TB in 59-80% of the HIV-infected individuals (Egger *et al* 1997; Badri *et al* 2002; Maartens *et al* 2007). Integrated anti-tubercular (ATT) and cART (as opposed to sequential) therapies are associated with >50% reduced rates of mortality (Abdool Karim *et al* 2010). However, not all HIV-infected patients on successful cART (i.e. suppressed plasma HIV RNA levels) achieve optimal CD4+ T cell restoration (e.g. >500 cells/µl after 6 months). Several factors appear to contribute to poor immune recovery including advanced age (Kaufmann *et al* 2005), low baseline (Kaufmann *et al* 2005; Le Moing *et al* 2007; Moore *et al* 2007) or nadir CD4+ T-cell counts (Negredo *et al* 2010) (Schacker *et al* 2010), residual HIV replication (Sigal *et al* 2011), CIA (Fernandez *et al* 2006; Piconi *et al* 2010), high rates of apoptosis (Massanella *et al* 2010), abrogated thymic functions (Franco *et al* 2002; Rubio *et al* 2002; Krathwohl *et al* 2006), gender (Hunt *et al* 2003; Gandhi *et al* 2006), and genetic polymorphisms associated with increased apoptosis (Nasi *et al* 2005; Haas *et al* 2006). While all these factors are known to influence immune reconstitution, there could also be other factors likely contributing to this process.

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1.3.3 Reconstitution of CD4+ T-Cell Counts may not be Associated with Restoration of CD4+ T-Cell Functions

Reconstitution of CD4+ T-cell counts may not be always associated with restoration of T-cell functions. Rather, HIV-infected patients with increased, but stable CD4+ T-cell counts after cART may still display suboptimal CD4+ T-cell responses. This has also been shown in individuals with low nadir CD4+ T-cell counts (Moore *et al* 2007; Piconi *et al* 2010). Responses to tetanus toxoid, CMV, candida and MTB antigens were still lower than the median in healthy controls even after >1 year on cART (Lederman *et al* 2003; Keane *et al* 2004; Burgess *et al* 2006; Sutherland *et al* 2006). Poor antigen-specific CD4+ T-cell responses may reflect limited recovery of TCR repertoire following initiation of cART (Connors *et al* 1997).

1.3.4 Immune Activation is Alleviated by cART but not Normalized

Spontaneous CD4+ T-cell apoptosis and levels of expression of cellular markers of immune activation (CD38 and HLA-DR) were reduced after cART, and remained higher than healthy controls (Roger *et al* 1999; Almeida *et al* 2002). An immediate decrease in Ki67 expression (marker of cell proliferation) on naïve T-cells after initiating cART has also been reported (Hazenberg *et al* 2003). Patients with effective suppression of viral replication but persistent immune activation experience recovery of CD4+ T cells at a slow pace on short-term (median of 21 months) and long-term (median of 103 months) cART (Hunt *et al* 2003). Plasma LPS levels have also been reported to decrease following cART but not normalized (Brenchley *et al* 2006). Decreased levels of LPS (mediator of immune activation) have also been associated with better CD4+ T-cell recovery on cART (Brenchley *et al* 2006).

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1.3.5 Adverse Clinical Events

Emergence of HIV drug resistance resulting from mutations has become a major concern in the long-term management of patients receiving cART (Gilks *et al* 2006). Lists of mutations associated with cART drug resistance are regularly updated by the International AIDS Society-USA Drug Resistance Mutations Group (Johnson *et al* 2013). The direct consequence of drug resistance is treatment failure because the existing drug regimen has poor or no marked effect on the resistant HIV strain. Further, there are also complications resulting from use of cART viz., drug toxicity, drug-drug interactions (e.g. with ATT) and/or increased inflammation. Phenotypic changes include altered distribution of subcutaneous fat, impaired endothelial functions, low levels of high-density lipoprotein (HDL) cholesterol and increased risk of CVD (Dube *et al* 2010). The use of some NRTIs has been associated with lipodystrophy and metabolic syndromes, whilst some NNRTIs can cause severe skin reactions, hepatotoxicity and psychosis. Stavudine (NRTI) and indinavir (PI) potentiate sensory neuropathy in HIV-infected patients (Pettersen *et al* 2006; Smyth *et al* 2007).

Results from the Strategies for Management of Anti-Retroviral Therapy (SMART) study that enrolled patients with plasma HIV RNA levels <400 copies/ml to investigate the role of immune activation in treatment outcome showed that patients had increased levels of high sensitivity C-reactive protein (CRP) and IL-6 (markers of acute inflammation); D-dimer (marker of coagulation and fibrolysis) and cystatin C (marker of impaired renal function) compared to the general population (Neuhaus *et al* 2010) at baseline. Elevation of these markers is associated with mortality and non-AIDS-defining illnesses like cardiovascular and renal diseases (Panichi *et al* 2001; Pepys *et al* 2006). Higher pre-cART sCD14 levels are also reported to independently predict mortality (Sandler *et al* 2012). HIV-infected patients who experienced new AIDS-related OIs or mortality showed higher baseline levels of plasma immune activation

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markers; soluble TNF receptor (sTNFR) family, soluble CD40L and IL-6 compared to controls (Kalayjian *et al* 2010). Furthermore, subjects that are severely immunodeficient (nadir CD4+ T-cell count <100 cells/µl) initiating cART are susceptible to development of immune reconstitution disorders (Manabe *et al* 2007). The most common ones are characterized by inflammatory processes due to restoration of host responses against pathogen-specific antigens. Further, patients may also show atypical presentations resulting from OIs such as mycobacterial, cryptococcal or herpes infections. The constellation of these symptoms is called as immune restoration disease (IRD) or immune reconstitution inflammatory syndrome (IRIS) (French 2009).

1.4 Immune Reconstitution Inflammatory Syndrome (IRIS)

1.4.1 History of IRIS

The first report of restored pathogen-specific immunity resulting in immuno-pathological reactions was reported in 1992, when atypical presentations of *Mycobacterium avium-intercellulare-scrofulaceum* complex (MAC) were seen in patients on AZT monotherapy (French *et al* 1992). MAC as an OI usually presents as disseminated infection, and is associated with anergy towards mycobacterial antigens. However, in the case of IRIS, MAC infection presents as localized infection, and is associated with recovery of delayed type hypersensitivity (DTH) reactions to mycobacterial antigens (French *et al* 1992). IRIS typically occurs within the first few months of initiating cART and can be associated with various bacterial, invasive fungal and chronic viral infections. In general, the pathogenesis of IRIS is thought to be owing to restoration of pathogen-specific immune responses that are pathological rather than protective (reviewed by (French 2009). However, subsequent investigations have suggested that innate immunity could also be involved (Tan *et al* 2011; Barber *et al* 2012; Pean *et al* 2012; Tran *et al* 2014).

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1.4.2 IRIS - Clinical Manifestations

Ever since the first report of MAC-IRIS (French *et al* 1992), the clinical spectrum consisted of ~25 different infections, 2 tumors and 18 other non-infectious (French 2007) manifestations. Of the different pathogens commonly associated with IRIS are mycobacteria such as MAC, MTB and *M. leprae* as well as viruses namely, herpes, hepatitis B, hepatitis C, and JC viruses (Shelburne *et al* 2002; French *et al* 2004; Lawn *et al* 2006). The onset of IRIS following commencement of cART may also be associated with certain autoimmune diseases and sarcoidosis, both of which appear to present an immunopathogenesis that is distinct from the classical IRIS (French 2009). The clinical manifestations of IRIS associated with various infectious pathogens are summarized in Table 1.2.

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Table 1.2 Manifestations of IRIS Associated with Various Pathogens		
Pathogens	**IRIS Manifestations**	**Reference**
Mycobacterium tuberculosis	Lymph node enlargement, pulmonary infiltrates, pleural effusion, cutaneous abscess Tachycardia Meningitis, tuberculoma, radiculomyelitis	(Viskovic *et al* 2013) (Lawn *et al* 2005) (Pepper *et al* 2009)
Mycobacterium avium	Lymph node enlargement, pulmonary infiltrates, pleural effusion, cutaneous abscess. Tuberculoma	(Viskovic *et al* 2013) (Kishida *et al* 2008)
Mycobacterium leprae	Pulmonary infiltrates, hyperaesthesic skin lesions with granulomatous changes	(Batista *et al* 2008)
Cryptococcus neoformans	Lymphadenitis, cavitary pneumonia, meningitis, cryptococcoma	(Lortholary *et al* 2005)
***Candidia* sp.**	Meningitis with vasculitis	
Pneumocystis jirovecii	Exacerbation of pneumonitis after cART	(Crothers *et al* 2003)
Cytomegalovirus	CMV retinitis after cART Immune recovery uveitis Encephalitis, myelitis	(Mutimer *et al* 2002) (Robinson *et al* 2000) (French *et al* 2000)
Human herpesvirus-8 (Kaposi's sarcoma–associated herpes virus)	Kaposi's sarcoma-associated edema after cART	(Bower *et al* 2005)
Varicella-Zoster virus	Dermatomal or multidermatomal zoster Myelitis Brain vasculitis	(Song *et al* 2010) (Clark *et al* 2004) (Newsome *et al* 2009)
HCV	Hepatitis flare and/or liver enzyme level elevation after cART	(John *et al* 1998)
HBV	Hepatitis flare and/or liver enzyme level elevation after cART	(Crane *et al* 2009)
JC	Progressive multifocal leuko-encephalopathy-associated IRIS	(Gray *et al* 2005)
Toxoplasma gondii	Retinitis Toxoplasmic encephalitis	(Sendi *et al* 2006) (Martin-Blondel *et al* 2011)
***Leishmania* sp.**	Uveitis Worsening of tegumentary lesions	(Blanche *et al* 2002) (Posada-Vergara *et al* 2005)

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Of the various clinical manifestations reported, IRIS that involve central nervous system (CNS) is associated with greater clinical severity or morbidity and mortality rates as compared to other IRIS types (French 2009). In countries with high- and middle-income, it has been reported that ~16% of people diagnosed with cryptococcal meningitis experienced CNS-IRIS (C-IRIS) with average mortality rate of 20% (Muller *et al* 2010). In resource-limited countries, C-IRIS is associated with >25% mortality, and represents an important cause of early mortality after cART in these countries (Haddow *et al* 2010). In addition, early initiation of cART in patients with treated cryptococcal meningitis has been associated with higher mortality rates (Makadzange *et al* 2010).

One report suggests that ~68% of adults may likely develop asymptomatic JC polyomavirus (JCV) infection by 59 years of age (Egli *et al* 2009). Due to immunodeficiency, the JCV reactivates causing lytic infection in oligodendrocytes in the white matter of brain resulting in progressive multifocal leukoencephalopathy (PML). There is no established treatment for JCV infection, and therefore cART is the only effective therapy for PML in patients with HIV. However, ~15% of patients experience an exacerbation of PML after commencement of cART (Martin-Blondel *et al* 2011).

1.4.3 Incidence of IRIS

The incidence rates of IRIS in HIV-infected persons from different populations reportedly range from 2-43 % (Breton *et al* 2004). The incidence for IRIS was 11% in South Africa (Eshun-Wilson *et al* 2010), 17% in Thailand (Manosuthi *et al* 2009), 18% in Brazil (Dibyendu *et al* 2011), 19% in India (Agarwal *et al* 2012), and 26% in Cambodia (Laureillard *et al* 2013). A meta-analysis has estimated that the mortality rate of TB-related IRIS is ~3.2% on average (Muller *et al* 2010). Due to lack of

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appropriate facilities to diagnose OIs (particularly TB prior to cART), after commencement of cART represents a significant concern amongst HIV-infected patients in resource-limited countries with incidence ~20 times higher than that from resource-rich nations (Lawn *et al* 2008; Meintjes *et al* 2008). **Table 1.3** summarizes the various studies and the global incidence of TB-IRIS reported from across the world until 2010.

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Table 1.3: Summary of Reported Global Incidence of TB-IRIS

Reference	Country	Study period	Mean age (years)	Median CD4 count (IQR)	Number of patients	Number of patients developing IRIS	Death from IRIS
Tuberculosis-Associated IRIS							
(Narita *et al* 1998)	USA	1996-97	..	..	33	12 (36%)	..
(Wendel *et al* 2001)	USA	1996-2000	..	..	24	3 (13%)	..
(Navas *et al* 2002)	Spain	1995-98	36.3 (..)	35 (18-215)	17	6 (35%)	0
(Breton *et al* 2004)	France	1996-2001	35.0 (..)	100 (..)	37	16 (43%)	..
(Kumarasamy *et al* 2004)	India	2000-03	34.0 (..)	122 (..)	144	11 (8%)	..
(Michailidis *et al* 2005)	UK	2001-03	37.4 (..)	..	55	14 (25%)	..
(Bourgarit *et al* 2006)	France	..	39.1 (10)	32 (15-131)	19	7 (37%)	..
(Manosuthi *et al* 2006)	Thailand	2003-04	34.5 (..)	36 (15-69)	167	21 (13%)	2 (1%)
(Lawn *et al* 2007)	South Africa	2002-05	..	68 (29-133)	160	19 (12%)	2 (1%)
(Park *et al* 2007)	South Korea	1998-2005	38.0 (..)	..	482	9 (2%)	..
(Serra *et al* 2007)	Brazil	2000-03	..	..	84	10 (12%)	0
(Manosuthi *et al* 2009)	Thailand	2006-07	35 (31-42)	43 (23-92)	126	20 (16%)	0
(Eshun-Wilson *et al* 2010)	South Africa	2003-08	36 (..)	..	333	35 (11%)	..
(Agarwal *et al* 2012)	India	2006-08	39 (35-45)	74 (55-110)	103	13 (12.6%)	5 (5%)
(Laureillard *et al* 2013)	Cambodia	2006-09	36 (7.9)	2 (12-62)	597	15 (2%)	6 (1%)

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Cryptococcal Meningitis IRIS							
(Jenny-Avital *et al* 2002)	USA	1998-2001	..	..	10	5 (50%)	..
(Lawn *et al* 2005)	South Africa	2002-05	34.0 (..)	86 (46-146)	434	9 (2%)	6 (1%)
(Shelburne *et al* 2005)	USA	..	..	..	59	18 (31%)	1 (2%)
(Sungkanuparph *et al* 2007)	Thailand	..	34.4 (6.9)	26 (..)	52	10 (19%)	0
(Bicanic *et al* 2009)	South Africa	2005-06	..	..	65	11 (17%)	3 (5%)
Immune Recovery Uveitis							
(Nguyen *et al* 2000)	USA	1995-98	..	..	33	6 (18%)	..
(Karavellas *et al* 2001)	USA	1996-98	..	..	30	19 (63%)	..
(Banker *et al* 2002)	India	1998-2000	37.3	36.5 (22-63)	12	5 (42%)	..
(Arevalo *et al* 2003)	Venezuela	1998-2000	..	..	32	12 (38%)	..
(Ortega-Larrocea *et al* 2005)	Mexico	1996-2003	..	19.7	43	23 (53%)	..
(Lin *et al* 2008)	Taiwan	1995-2006	40.3	16.6 (..)	41	10 (24%)	
Herpes Zoster IRIS							
(Dunic *et al* 2005)	Serbia	2000-01	38.1	..	115	14 (12%)	..
Kaposi's Sarcoma IRIS							
(Bower *et al* 2005)	UK	1996-2004	37.9 (..)	..	150	10 (7%)	..
Progressive Multifocal Leukoencephalopathy (PML)							
(Vidal *et al* 2008)	Brazil	2003-04	37.3 (..)	45 (..)	12	1 (8%)	0

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Other IRIS							
(French *et al* 2000)	Australia	1996-97	..	..	132	33 (25%)	..
(Jevtovic *et al* 2005)	Serbia	1998-2004	41.0	108	389	65 (17%)	1 (<1%)
(Shelburne *et al* 2005)	USA	1997-2003	38.8	..	180	57 (32%)	2 (1%)
(Ratnam *et al* 2006)	UK	2000-02	35.0	174 (82-285)	199	44 (22%)	..
(Murdoch *et al* 2008)	South Africa	2006	34	115 (51-173)	423	43 (10%)	2 (<1%)
(Sharma *et al* 2008)	India	2004-06	..	..	90	20 (22%)	..

Note: Data are mean (SD), median (IQR) or number (%), .. data not reported.

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1.4.4 General Clinical Diagnosis of IRIS

The International Network for the Study of HIV-associated IRIS (INSHI) established in 2006 to harmonize ongoing and future studies on IRIS world-wide employs a similar clinical definition for the diagnosis of IRIS. To date, consensus clinical case-definitions have been developed for TB-IRIS (Meintjes *et al* 2008) and cryptococcal IRIS (Haddow *et al* 2010). Generally, definition of IRIS involves the following criteria:

1. Patients must have received cART and achieved a virological response (>1 log10 decrease in plasma HIV RNA).
2. Clinical deterioration of an infectious or inflammatory condition must be temporally related to the initiation of cART.
3. Symptoms cannot be explained by expected clinical course of associating pathogen, drug toxicity, treatment failure and complete non-adherence.
4. Increase in peripheral blood CD4+ T-cell counts may be used as a marker of immune reconstitution when plasma HIV RNA result is lacking. However, this is not included as a criterion for IRIS as:
   a) Some cases of IRIS occur without an increase in peripheral CD4+ T-cell counts.
   b) CD4+ T-cell counts in the peripheral blood may not reflect function or counts present at the site of infection.
   c) CD4+ T cells are not the only cellular mediators of IRIS. Other immune cells can also contribute to development of IRIS.

The diagnosis criteria specific for TB-IRIS will be discussed later in the Section 1.5.1.

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1.4.5 Risk Factors Associated with Development of IRIS

Owing to lack of surrogate laboratory markers for IRIS, understanding of risk factors is essential for the prompt identification of patients at high risk of developing IRIS after initiation of cART, to be able to provide them with early intervention and clinical monitoring modalities. The major risk factors for development of IRIS are low baseline CD4+ T-cell counts (French *et al* 2000; Jevtovic *et al* 2005; Lawn *et al* 2007; Manabe *et al* 2007; Manabe *et al* 2008). Other risks include a low baseline haemoglobin (Robertson *et al* 2006; Letang *et al* 2010), rapid increase of CD4% after cART (Breton *et al* 2004; Jevtovic *et al* 2005; Valin *et al* 2010) or rapid reduction in PVL (French *et al* 2000; Breton *et al* 2004; Manabe *et al* 2007; Letang *et al* 2010; Valin *et al* 2010).

Antigen burden has also been associated with the onset of IRIS, particularly in patients with low CD4+ T-cell counts (He *et al* 2013). Since IRIS results from dysregulation of inflammation against residual antigens, patients with higher antigen load prior to cART initiation are more susceptible to TB-IRIS. This has also been demonstrated in patients with extrapulmonary and disseminated TB who are at increased risk for TB-IRIS (Manosuthi *et al* 2006; Burman *et al* 2007; Lawn *et al* 2009). This is in line with a finding where paradoxical TB-IRIS patients were 4.6-fold more likely than TB non-IRIS patients to show TB lipoarabinomannan (LAM) antigen in urine specimens (Conesa-Botella *et al* 2011). Furthermore, the level of LAM is inversely associated with CD4+ T-cell counts. These data suggest that patients with higher pathogen loads may display a higher magnitude of inflammation when the immune system undergoes reconstitution.

Short interval between OI treatment and commencement of cART also has been identified as a major risk factor for IRIS (Navas *et al* 2002; Lortholary *et al* 2005; Michailidis *et al* 2005; Shelburne *et al* 2005; Burman *et al* 2007; Narendran *et al* 2013). In patients treated with TB, commencement of cART during the first 4 weeks of TB

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treatment increases the risk of TB-IRIS by ~2.5-fold compared to cART commencement 8 weeks post-TB treatment (Abdool Karim *et al* 2010). Conversely, in a Ugandan study, 22% of patients starting cART early (within 2 months) and 31% of those starting late (after 2 months) developed TB-IRIS (Baalwa *et al* 2008). Therefore, deferring cART 2 months after TB treatment would not necessarily prevent paradoxical TB-IRIS. The ability to clear residual antigens during OI treatment may influence the breadth of inflammation after immune reconstitution by cART.

1.4.6 Therapeutic Strategies for IRIS

While corticosteroids have been shown to reduced death and residual neurological disabilities amongst survival of TB meningitis (HIV-negative) (Prasad *et al* 2008), there have been little evidences to support their use in TB-IRIS. Available evidence suggests that improvement of symptoms occurs in ~82% patients (N=19/23) who developed TB-IRIS; of which 18 received corticosteroids (Pepper *et al* 2009). Another study using double-blinded randomized placebo-controlled trial of patients with paradoxical TB-IRIS found that the use of prednisolone (1.5 mg/kg/day for 2 weeks 0.75 mg/kg/day for 2 weeks) reduced the need for hospitalization and therapeutic procedures, showed rapid recovery from symptoms, as well as improved quality of life of patients with TB-IRIS although prednisolone did not reduce mortality rates (Meintjes *et al* 2010).

Together, these studies suggest that use of corticosteroids could benefit TB-IRIS patients only to a certain extent although inappropriate adjunct corticosteroid therapy for patients with immunosuppression with underlying sub-optimally-treated TB or other untreated OIs could be fatal (Pepper *et al* 2009). Further, usage of corticosteroid has also been associated with progression of herpes zoster, Kaposi's sarcoma and reactivation of latent infections (Elliott *et al* 1992; Volkow *et al* 2008). Therefore,

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potential complications in treating IRIS patients under corticosteroid therapy have stressed the need for predictive and diagnostic markers for prompt recognition of TB-IRIS.

Alternatively, other classes of immunosuppressive agents may also be use. In a case report, Hardwick *et. al.* showed leukotrienes, a pro-inflammatory mediator primarily produced by mast cells could also involved in the pathogenesis of IRIS, as Montelukast (a leukotriene receptor antagonist) appeared to be effective in a syphilis-IRIS and two TB-IRIS cases (Hardwick *et al* 2006). Nonetheless, the use of Montelukast as a therapeutic regimen in clinical IRIS is limited.

1.5 Tuberculosis-Associated IRIS

Despite the diverse association and presentations, TB-IRIS still remains the most commonly occurring form of IRD across the world, and is particularly common in countries with a huge burden of both diseases (Burman *et al* 2007). Manifestations of TB-IRIS range from mild symptoms such as fever, to life-threatening conditions such as respiratory failure. In most cases, the clinical manifestations resemble the original disease, such as fever, malaise, weight loss, cough etc. Commonly presenting features include severe fever, cervical and intra-thoracic lymphadenopathy, and pulmonary infiltrates (Dhasmana *et al* 2008). Tuberculoma, meningitis, cold abscess and ascites are other less common manifestations (Meintjes *et al* 2008).

1.5.1 Definition of Tuberculosis-Associated IRIS

Due to diverse clinical presentations and diagnostic predicaments, there have been difficulties to clearly define IRIS. There is seldom an investigation or biological test available to confirm the diagnosis of paradoxical TB IRIS. Thus, confirmation relies

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largely on case-definitions inclusive of clinical and laboratory data (Dhasmana *et al* 2008). In most cases, diagnosis is mainly reliant on consistent history and exclusion of alternative diagnoses such as non-compliance with treatment for OI, emergence of a new OI, or drug toxicity (Meintjes *et al* 2008). In regions where drug-resistant TB is common, extra efforts have to be made to exclude drug resistance as a cause for deterioration (Dhasmana *et al* 2008). To a certain extent, lack of consensus case-definitions and definitive diagnoses has considerably hindered research on IRIS.

General case-definitions have been previously proposed, and have seldom been validated, and therefore there has been no consensus on their validity and applicability, particularly in resource-constrained settings. To this end, the International Network of HIV-associated IRIS (INSHI) has now modified the previously proposed case-definitions for IRIS to make them applicable in resource-limited settings. The INSHI definitions have since been independently validated by various international research groups (Manosuthi *et al* 2009; Eshun-Wilson *et al* 2010; Haddow *et al* 2010). This definition has 3 main components which includes (i) primary or antecedent requirements, (ii) clinical criteria (viz., major or minor criteria) and (iii) exclusion of possible diagnosis as confirmatory criteria in the definition of paradoxical TB-IRIS (Meintjes *et al* 2008). The primary criteria in the diagnosis of paradoxical TB-IRIS are microbiological confirmation of TB or strong clinical evidence of TB before starting cART in addition to evidence of an initial response to ATT prior to the initiation of cART. Clinical criterion also suggests that the onset of IRIS should be within 3 months of initiating cART and one of the major or two of the minor criteria. The major criteria include new or worsening lymph nodes or cold abscesses and radiological features while minor criteria include worsening of clinical symptoms. The exclusion of alternative explanations for clinical deterioration such as treatment failure, drug resistance, poor adherence or drug toxicity or other OIs form the third component of the

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INSHI case-definition for paradoxical TB-IRIS (Meintjes *et al* 2008). The major revisions incorporated in the INSHI case-definitions that render them applicable in resource-limited settings are the omission of changes in PVL/CD4+ T-cell counts as necessary criteria in confirming IRIS and the inclusion of a time-frame for onset of clinical manifestations for a diagnosis of TB-associated IRIS to be made (Meintjes *et al* 2008).

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Table 1.4 Case-Definitions of Tuberculosis-Associated IRIS by INSHI (adapted from (Meintjes *et al.* 2008)

A. Paradoxical TB-IRIS (both of the two following requirements must be met)

(a) Antecedent Requirements

Diagnosis of TB	1. TB diagnosis was made before starting cART and should fulfil WHO criteria	
Initial Response to TB Treatment	2. patient's condition stabilized/improved on appropriate TB treatment before cART initiation	e.g. cessation of night sweat, fever, cough, and weight loss.

(b) Clinical Criteria

The onset of TB-IRIS manifestations should be within 3 months of cART initiation, re-initiation, or regimen change because of treatment failure.
Of the following, at least **one major** criterion or **two minor** clinical criteria are fufilled:

Major Criteria	1. New or enlarging lymph nodes, cold abscesses, or other focal tissue involvement	e.g. TB arthritis
	2. New or worsening radiological features of TB	examined by chest radiography, abdominal ultrasonography, CT, or MRI
	3. New or worsening CNS TB	eg. meningitis or focal neurological syndrome
	4. New or worsening serositis	eg. pleural effusion, ascites, or pericardial effusion
Minor Criteria	1. New or worsening constitutional symptoms	eg. fever, night sweats, or weight loss
	2. New or worsening respiratory symptoms	eg. cough, dyspnoea, or stridor
	3. New or worsening abdominal pain	Symptom accompanied by peritonitis, hepatomegaly, splenomegaly, or abdominal adenopathy

(c) Exclude alternative explanations for clinical deterioration

1. Failure of TB treatment because of drug resistance
2. Poor adherence to TB treatment
3. Another OI or neoplasm
4. Drug toxicity or reaction

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Table 1.4 Case-Definitions of Tuberculosis-Associated IRIS by INSHI (adapted from (Meintjes *et al.* 2008)		
B. Unmasking TB-IRIS (provisional) (one of the of the following criteria must be met)		
(a) Antecedent Requirements **Patient is not receiving treatment for TB when cART is initiated and then presents with active TB within 3 months of starting cART**		
Diagnosis of TB	1. Patient is not receiving treatment for TB when cART is initiated and 2. Presents with active TB within 3 months of starting cART.	
(b) Clinical Criteria		
	1. Heightened intensity of clinical manifestations, particularly if there is evidence of a marked inflammatory component to the presentation.	e.g. TB lymphadenitis, TB abscesses with prominent acute inflammatory features, pulmonary TB complicated by respiratory failure.
	2. Clinical course that is complicated by a paradoxical reaction after initiation TB treatment	

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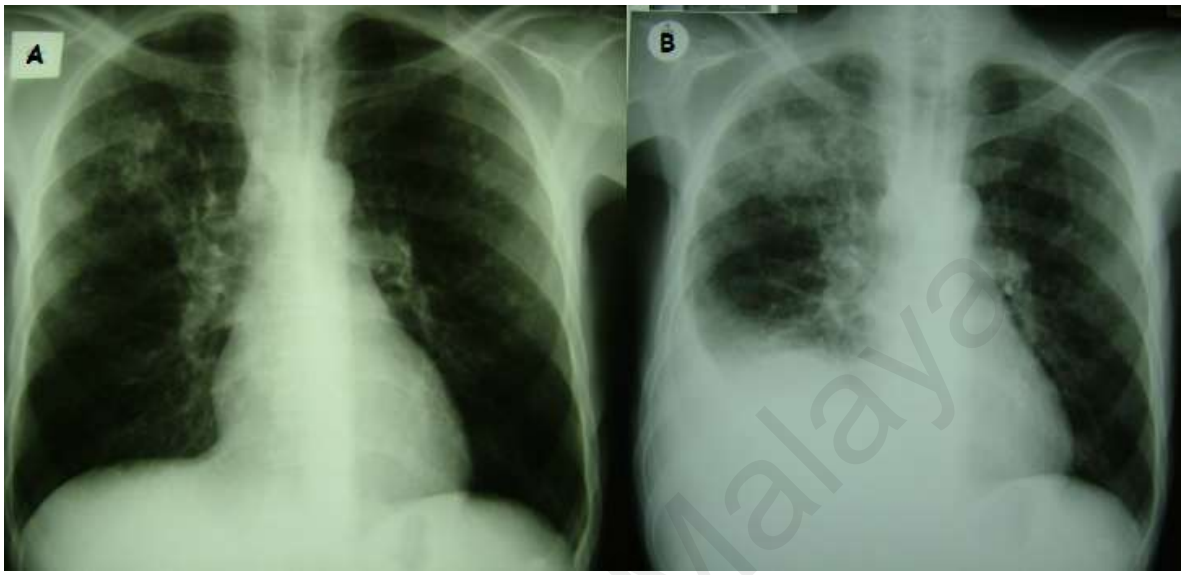
Figure 1.7: Worsening Pulmonary Infiltrates and New Pleural Effusion



Note: A 49-year-old HIV-infected man with CD4 of 29 cells/µl was diagnosed with drug-susceptible pulmonary TB (chest radiograph A). He started cART 2 weeks after TB treatment. Two weeks later the patient developed recurrent TB symptoms, worsening of pulmonary infiltrate and new pleural effusion due to paradoxical TB-IRIS (chest radiograph B). The TB sputum culture and pleural aspirate TB culture were negative at the time of TB-IRIS. (Figure adapted from Meintjes, G. PhD thesis, 2011. Fig. 1 pg. 43)

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1.5.2 Immunopathogenesis of Tuberculosis-IRIS

The immunopathogenesis of IRIS varies according to the causative pathogens. Histopathological examinations have shown that IRIS provoked by viruses, such as JCV (Gray *et al* 2005) and CMV (Mutimer *et al* 2002) is associated with infiltration of inflammatory CD8+ T cells into the affected tissue or organ; whereas IRIS provoked by fungi, such as *Histoplasma* sp. and *Cryptococcus* sp. (Lortholary *et al* 2005; Breton *et al* 2006); protozoans, such as *Leishmania* sp. (Blanche *et al* 2002); and by mycobacteria such as *M. tuberculosis, M. leprae* (Batista *et al* 2008), is associated with granulomatous inflammation. As IRIS usually occurs during the initial weeks to months of effective cART during early phase of immune reconstitution (refer to Section 1.3.2), it is believed that exaggerated inflammatory responses experienced by IRIS patients is driven by rapid immune recovery. However, the precise pathogenic mechanisms are not completely understood. The pathogenesis of TB-IRIS is related to recovery of MTB-specific immunity, and previous studies have hinted the contribution of various components and cells of the immune system to development of IRIS (discussed in following sections).

1.5.2.1 Recovery of Delayed Hypersensitivity Responses

The first report to suggest that mycobacterial IRIS coincided with recovery of mycobacteria-specific immune responses was in patients who developed a localized form of MAC disease, such as lymphadenitis, after starting AZT mono-therapy in the early 1990's (French *et al* 1992). Reversion of tuberculin or MAC purified-protein derivative (PPD) skin tests from being anergic to positive was observed among these patients (French *et al* 1992). Furthermore, the same phenomena was also observed among paradoxical TB-IRIS patients where patients anergic to PPD prior to cART developed strong positive skin tests around the time of IRIS (Narita *et al* 1998). These

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results suggest that recovery of DTH responses may be associated with the onset of IRIS. However, certain patients with MAC or TB-IRIS failed to experience CD4+ T-cell count rise at the time of IRIS (Narita *et al* 1998; Phillips *et al* 2005) suggesting that IRIS may not be relevant to recovery of CD4+ T-cell counts in the periphery.

1.5.2.2 Recovery of MTB-Specific T-Cell Responses

Though paradoxical TB-IRIS is characterized as exuberant inflammation and elevation of pro-inflammatory cytokines such as IL-6 (Stone *et al* 2002; Morlese *et al* 2003), the cause of this inflammation was unknown. It has been suggested that TB-IRIS could be associated with restoration of MTB-specific Th1 responses with either recirculation or proliferation of T cells after successful cART (Autran *et al* 1997; Bourgarit *et al* 2006). In order to confirm this hypothesis, MTB-specific T cells in the peripheral blood has been investigated extensively using enzyme-linked immunospot (ELISpot) assays, whole blood IFN-γ release assays (IGRA) and flow cytometry (Bourgarit *et al* 2006; Meintjes *et al* 2008; Tan *et al* 2008; Bourgarit *et al* 2009; Elliott *et al* 2009; Tieu *et al* 2009; Antonelli *et al* 2010). Bourgarit *et al*. (2009) and others showed that TB-IRIS could be associatepd with massive expansion of PPD-specific IFN-γ-producing T cells. Interestingly, such expansions were not evident in TB-IRIS patients when CMV antigens or ESAT-6 were used as antigenic stimuli (Bourgarit *et al* 2006; Tan *et al* 2008; Elliott *et al* 2009). Furthermore, it was subsequently reported that these PPD-specific T cells were highly activated, multifunctional (IFN-γ+ TNF-α+ IL-2+), and belongs to the CD4+ effector memory T cell phenotype (Antonelli *et al* 2010). However, results from several other studies suggested otherwise. Two independent studies from TB endemic regions (Malaysia and South Africa) have shown that though PPD-specific IFN-γ-producing T cell expansion was seemingly higher among TB-IRIS patients, but such expansion may also occur in some of the TB no IRIS patients

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(controls). Furthermore, some patients who had high numbers of mycobacteria-specific T cells on ELISpot failed to develop TB-IRIS (Meintjes *et al* 2008; Tan *et al* 2008). In addition, a Thai study found no significant difference with the levels Th1 cytokine (IL-2, IL-12, and IFN-γ) production following PPD and RD1 antigen stimulation between patients who developed TB-IRIS and controls prior to cART or at time of IRIS (Tieu *et al* 2009). Collectively, these data suggest that the expansion of IFN-γ-producing T cells may not be relevant to TB-IRIS. Studies that characterized the cellular immune responses in IRIS are summarized in **Table 1.5**.

1.5.2.3 Exuberant γδT-Cell Responses

A part from most abundant αβT cells, others also observed an elevation in the frequency of γδT cells among TB-IRIS patients both at baseline and at TB-IRIS event (Bourgarit *et al* 2009). Since γδT cells can respond to mycobacterial phospho-antigens (Constant *et al* 1994; Poquet *et al* 1996) and produce large amounts of IFN-γ (Rojas *et al* 2005), their elevation in TB-IRIS patients implicate their likely role in the pathogenesis of TB-IRIS. However, these findings have not been replicated by more investigations.

1.5.2.4 Dysregulated Release of Cytokines

a) Diminished Th1/Th2 Homeostasis: Several studies have shown that TB-IRIS is associated with elevation of a wide range of Th1 cytokines including IL-2, IP-10, TNF-α and IL-1β (Bourgarit *et al* 2006)) but not Th2 cytokines, suggesting that TB-IRIS may result from imbalance in Th1/Th2 cytokine secretion. However, later studies showed that IFN-γ and IL-5 concentrations in whole blood mitogen-stimulated cultures demonstrated no differences in concentrations when comparing TB-IRIS cases and controls, suggesting that TB-IRIS is seldom attributed to an imbalance in Th1- and Th2 homeostasis (Oliver *et al* 2010).

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b) Cytokine Storm: It has been suggested that paradoxical TB-IRIS is caused by "cytokine storm" (Ruhwald *et al* 2007). However, data generated thus far are heterogenous. Tadokera *et al*. (2011) showed that there were elevations in a wide range of cytokines and chemokines among TB-IRIS patients when compared to TB-HIV co-infected patients who did not develop IRIS. Of all cytokines, TNF-α, IFN-γ and IL-6 remained significant after adjustment for multiple comparisons (Tadokera *et al* 2011) suggesting that TB-IRIS could indeed be a cytokine storm.

c) Perturbation in Adaptive and Innate Inflammatory Mediators: Data from Haddow *et al*. 2011 did not demonstrate any elevation in pro-inflammatory cytokine levels (including IL-1β, IFN-γ, TNF-α and IL-6) among paradoxical TB-IRIS patients as compared to TB-HIV controls. Conversely, they showed that TB-IRIS patients had lower IL-10 and MCP (monocyte chemotactic protein)-1, as well as higher CRP:IL-10 ratio (inflammatory/anti-inflammatory ratio) relative to controls. These findings reflected deficit in monocyte and regulatory T cell (Treg) activity as well as higher pro-inflammatory roles among TB-IRIS patients (Haddow *et al* 2011). As opposed to this finding, another team found exuberant innate immune responses (Oliver *et al* 2010) speculating that TB-IRIS could be associated with higher levels of CXCL10 and IL-18 and low levels of CCL2 in unstimulated samples. Though the findings for soluble biomarkers are heterogenous reflecting the complexity of the various immune factors; these data collectively suggest that TB-IRIS could be associated with dysregulated cytokine productions. The plasma biomarkers proposed to be predictive of IRIS and some other potential markers are summarized in **Table 1.6**.

1.5.2.5 Role of Innate Immune System

Considering the aforementioned findings, it is clear that recovery of mycobacteria-specific T-cell frequencies and functions alone may not be sufficient to explain the

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occurrence of TB-IRIS and that recovery of innate immune functions may play an important role in disease pathogenesis (Sereti *et al* 2010).

a) Role of Myeloid Cells: The finding of higher levels of CXCL10 and IL-18 in TB-IRIS patients is an evidence for monocyte activation in TB-IRIS (Oliver *et al* 2010). CXCL10 (also known as IFN-γ-inducible protein-10 (IP-10)) is a chemoattractant for effector T cells; whereas IL-18 is a macrophage-derived inducer of IFN-γ that contributes to protective responses to TB. The results were interpreted to indicate that production of IL-18 and CXCL10 augments effector T-cell responses to MTB antigens in TB-IRIS (Sereti *et al* 2010). A similar finding was also observed in a mouse model of MAC-IRIS that demonstrated marked alterations in blood and tissue CD11b+ myeloid cells (Barber *et al* 2010). In line with this, a case-control study on TB-IRIS from Malaysia also suggested a role for TLR-2-induced pro-inflammatory cytokine production by monocytes and DCs in TB-IRIS pathogenesis. At 24 weeks of cART, TB-IRIS patients showed significant higher expression of TLR-2 on monocyte and TNF-α production following lipomannan stimulation without concurrent increase in IL-10 production (Tan *et al* 2011).

Evidence indicates that neutrophils may also contribute to the pathogenesis of TB-IRIS. Compared with non-IRIS TB meningitis (TBM) patients, TBM-IRIS patients were shown to have much higher neutrophil counts in their cerebrospinal fluid (CSF). Furthermore, the combination of high CSF TNF and low IFN-γ at TBM diagnosis predicted development TBM-IRIS (Marais *et al* 2013).

A recent microarray investigation of RNA from monocytes of patients with TB-IRIS demonstrated that 100 genes related to "inflammatory disease", "immunological disease", "cellular movement", "hematological system development and function", and "immune cell trafficking" were perturbed to at least 1.5 folds in patients relative to

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controls (Tran *et al* 2014). These findings suggest that monocytes likely to involved in thw pathogenesis of TB-IRIS. Another ther study have shown that CD14++CD16- classical monocyte subsets, but not the CD16+ subsets is a predictor of TB-IRIS (Andrade *et al* 2014). The study also suggested that immune activation may be caused by mycobacterial antigens, which however requires more confirmed findings.

b) NK Cell Activation and Degranulation: Apart from myeloid cells, the involvement of NK cells in TB-IRIS has also been examined. In one South African cohort, patients with unmasking TB-IRIS showed increased expression of CD69 and HLA-DR on NK cells (Conradie *et al* 2011). In a separate clinical trial in Cambodia, the ability of NK cells to degranulate at baseline was found significantly higher in paradoxical TB-IRIS than in non-IRIS controls (Pean *et al* 2012) demonstrating the potential role of NK cells in the onset of clinical TB-IRIS.

1.5.2.6 Dysfunctions in Immune Regulation

a) Role of Tregs: Given the irrelevance of Th1/Th2 imbalance in the development of TB-IRIS, it has been hypothesized that paradoxical TB-IRIS may reflect a relative delay in the recovery rates of Treg cell numbers and functional deficit (Lim *et al* 2007; Lim *et al* 2008). However, the data generated have been inconclusive thus far. Two studies have demonstrated that deficiency of Tregs seldom play a role in TB-IRIS (Meintjes *et al* 2008; Tan *et al* 2008). However, others have demonstrated an expansion of $CD127^{low}$ Foxp3+ CD25+ Tregs and a higher ratio of Treg to effector/memory subsets in TB-IRIS patients (Seddiki *et al* 2009). Sediki et al also demonstrated (*in vitro* suppression assays) a reduced functional ability and reduced release of IL-10 by suppressor cells in TB-IRIS patients, suggesting that while Treg numbers are increased, therefore their ability to down-regulate aberrant immune responses is impaired.

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b) Role of Killer Inhibitory Receptors (KIR): In spite of the elevated levels of γδT cells, the cells also lacked killer inhibitory receptors viz., CD94/NKG2, CD158a and CD158b on mycobacteria-specific Vδ2 TCR γδ suggesting that this potent IFN-γ-producing cell may be deficiently regulated (Bourgarit *et al* 2009).

1.5.2.7 Immunopathogenesis of Unmasking TB-IRIS

"Unmasking" TB-IRIS is less well characterized than paradoxical TB-IRIS with fewer cases reported. Cases described include patients presenting with rapid onset of severe respiratory manifestations (Goldsack *et al* 2003; John *et al* 2005; Meintjes *et al* 2008), one of whom required mechanical ventilation for adult respiratory distress syndrome (RDS) associated with miliary TB (Goldsack *et al* 2003). A fatal case of unmasking TB-IRIS presenting after 6 weeks on cART was shown at post-mortem to have extensive infiltrates involving the upper lobe of the right lung with histological appearance compatible with bronchiolitis obliterans organizing pneumonia (BOOP) with predominantly macrophages infiltration (Lawn *et al* 2009). Neutrophils are likely involved given that suppurative lymphadenitis and abscess formations are frequently the features of TB-IRIS (Lawn *et al* 2009). Complicated neurological involvement in unmasking TB-IRIS cases has also been described, as pyomyositis has been by others (Chen *et al* 2009).

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Table 1.5 Characteristics of Cellular Responses Causing IRIS Against Various Infectious Pathogens.

Pathogen(s)	Assay	Patients Origin	Grouping/ Study subjects(n)	Median (IQR) pre-cART CD4+ T-cells (cells/µl)	Mechanism(s) of IRIS	Reference(s)
M. tuberculosis	ELISpot	African	P.TB-IRIS=7 TB no IRIS=12	32 60	1. ↑ of PPD-specific IFN-γ-producing T-cells in IRIS group. 2. T-cell response to ESAT-6 was not significant	(Bourgarit *et al* 2006)
M. tuberculosis	ELISpot	South African	P.TB-IRIS=39 (treated for TB and HIV) TB no IRIS=25 (treated for TB and HIV) TB no IRIS=31 (untreated for TB and HIV)	51 (29-106) 45 (23-122) 195 (111-331)	1. ↑ of both ESAT-6 and PPD-specific IFN-γ-producing T-cells in P.TB-IRIS group. 2. ↑ of PPD-specific IFN-γ-producing T cells also found in non-IRIS groups.	(Meintjes *et al* 2008)
	Quanti FERON-TB Gold In-Tube assay	Cambodian	P.TB-IRIS=15 TB non-IRIS=55 cART-assoc. TB=11 No TB no IRIS=216	69 (41.5% were <50 cells/µL)	1. Greater ↑ in IFN-γ and 2. PPD-skin test were positive at week 12 and 24 in participants with TB-IRIS. 2. IFN-γ responses to PPD & ESAT-6 corrected with pre-cART CD4+ T-cell counts and were higher in individuals with cART-associated TB.	(Elliott *et al* 2009)

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M. tuberculosis	FCM	African	TB-IRIS=11 TB no IRIS=13	TB-IRIS=37 TB no IRIS=56	Expansion of Vδ2+ subset of TCRγδ+ T-cells with down-modulated killer inhibitory receptor (KIR) in IRIS group.	(Bourgarit *et al* 2009)
M. tuberculosis	FCM	South African	Unmasking TB-IRIS=18 TB no IRIS=31 HIV only=58	Unmasking TB-IRIS=115 TB no IRIS=61 HIV only=118	↑ CD69 and HLADR surface expression of NK cells in unmasking TB-IRIS group.	(Conradie *et al* 2011)
M. tuberculosis	FCM	Cambodian	TB-IRIS=37 TB no IRIS=91 HIV only=49	TB-IRIS=27 TB no IRIS=27 HIV only=85 ($p<0.001$)	↑ degranulation activity by NK cells in TB-IRIS group.	(Pean *et al* 2012)
M. tuberculosis	Microarray	Ugandan	TB-IRIS=18 TB no IRIS=18	TB-IRIS= 42 TB no IRIS=58	Dysregulation of the complement system of monocytes in TB-IRIS group.	(Tran *et al* 2013)
M. tuberculosis	FCM	Indian North American	P.TB-IRIS=26 TB no IRIS=22 No TB no IRIS=8	200 32	Frequency of CD14++CD16- monocytes ↑ in P.TB-IRIS group compared to TB non-IRIS group.	(Andrade *et al* 2014)
MAC	FCM	Australian	P.Mac-IRIS=8	P.Mac-IRIS=26	1. Expansion of Tregs in Mac-IRIS	(Seddiki *et al*

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					group. 2. Reduced of functional capacity of suppressor cells and diminished IL-10 among MAC-IRIS patients	2009)
M. avium	Mouse model	--	--	--	1. IRIS is associated with impaired, rather than augmented, T-cell expansion and function. 2. CD11b+ myeloid cells expansion in peripheral and lung for MAC-IRIS group.	(Barber *et al* 2010)
M. tuber-culosis & Crypto-coccus IRIS	ELISPOT	Malaysian Chinese	P.IRIS=5 No IRIS=8	< 200	1. TB-IRIS patients displayed elevated IFN-γ responses and/or plasma IgG to PPD, but none responded to ESAT-6. 2. Cryptococcus IRIS elevated IFN-γ responses to crypto antigens.	(Tan *et al* 2008)
Crypto-coccus IRIS	IGRA	South African	P.C-IRIS=27 C no IRIS=63	C-IRIS=16 C no IRIS=36 p=0.015	Lower cryptococcus-specific IFN-γ responses pre-cART in cyptococcus meningitis-IRIS (C-IRIS) compared to CM no IRIS patients.	(Chang *et al* 2013)
Cross-IRIS (Majority MAC-IRIS)	FCM -PMA stimulation	American (consisted of African, Latino,	IRIS=16 No IRIS=29	IRIS=19 No IRIS= 19	1. At pre-cART, IRIS patients displayed higher frequencies of effector memory, PD-1, HLA-DR, and Ki67 CD4+ T-cells than	(Antonelli *et al* 2010)

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		Caucasians, & others)			patients without IRIS. 2. Elevated PD-1 and Ki67 expression in regulatory T cells of IRIS patients. 3. Higher frequencies of PD-1– expressing effector memory CD8+ T-cells were observed in IRIS, compared with non-IRIS.	
***Cross-IRIS* (Majority *MAC*-IRIS)**	FCM	American (consisted of African, Latino, Caucasians, & others)	IRIS= 19 No IRIS=48 (OI-matched)	IRIS=14; no IRIS=22	1. Selective expansion of polyfunctional (IFN-γ+ IL-2+ TNF+ and IFN-γ+ IL-2− TNF+) pathogen-specific CD4+ T cells during the event in IRIS group 2. PD-1 expression was elevated pre-cART in IRIS patients both on CD4+ and CD8+ T cells and majority expressing co-stimulatory ICOS and inhibitory CTLA-4 and LAG-3 molecules.	(Mahnke *et al* 2012)
***Cross-IRIS* (Majority Pneumocystis pneumonia)**	FCM	Multicenter Strategy Trial (consisted of American & South African)	IRIS=19 No IRIS= 39 (OI-matched)	IRIS=18; no IRIS=14; p=0.037	T-cell activation phenotypes were similar (Ki67, CD25+, CD127+, HLADR+, CCR5+, CD57+, PD1+, and LAG3+) in IRIS and non-IRIS group.	(Grant *et al* 2012)

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Note: ELISpot= Enzyme-linked immunosorbent spot assay; IGRA= Interferon-gamma releasing assay; FCM= Flow cytometry; P.TB-IRIS= Paradoxical TB-RIS; *Mac*= *Mycobacterium avium-intercellulare-scrofulaceum* complex; PMA= Phorbol acetate myristate; OI= Opportunistic infection.

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Table 1.6 Biomarkers Predictive and Characteristic of IRIS

Markers	Immunological function(s)	References
Neopterin	A macrophage-specific activation marker triggered by Th1 cytokine IFN-γ. An indicative marker for proinflammatory immune responses. Serum neopterin is particularly high in TB-HIV co-infected patients with CD4+ T-cell counts <200mm^3 (Immanuel *et al* 2005). Neopterin derivatives might modulate immune response by interfering with the cellular redox balance, activating redox-sensitive transcription factors, or inducing in specific cell types (Wirleitner *et al* 2005).	Up to date, no work has been done to correlate the serum levels of neopterin with IRIS.
Ferritin	Ferritin plays an essential role in iron homeostasis by binding and sequestering intracellular iron (Camaschella *et al* 2009). Severe hyperferritinemia was found in patients with TB infection (Visser *et al* 2011).	Till date, no work has been done to correlate ferritin with IRIS.
Soluble/ sCD163	A haptoglobin-hemoglobin (Hp-Hb) scavenger receptor (Kristiansen *et al* 2001; Schaer *et al* 2007), expressed by monocytes and macrophages, is important in resolution of inflammation (Schaer *et al* 2007). A recent study showed that serum levels of sCD163 in HIV-infected individuals were no different from non-HIV-infected individuals, (Tippett *et al* 2011), explained by monocyte tolerance to endotoxin. Further investigations into the effect of chronic bacterial translocation on CD163 expression and shedding in HIV-1 infection, and correlation with other markers of inflammation, may uncover a better understanding of chronic immune activation (CIA) in HIV-1 infection. Andrade *et al*. (2014) found that sCD163 is significantly higher in TB-IRIS patients compared to non-TB-IRIS at pre-cART.	(Andrade *et al* 2014)
Soluble/ sCD14	CD14 act as membrane bound receptor for LPS-LBP complex. The soluble form is shed of by activated macrophages. *In vitro* and mice experiments have shown that sCD14 can exert both pro-inflammatory and anti-inflammatory functions, depending on the circulating levels of LPS and LPS-binding protein (Haziot *et al* 1994; Haziot *et al* 1995). Andrade *et al*. (2014) found that sCD14 was significantly higher in TB-IRIS patients compared to non-TB-IRIS during IRIS event.	(Andrade *et al* 2014)

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CCL2	Also known as monocyte chemoattractant protein-1 (MCP-1). Induced by IL-18, IFN-β, IFN-γ, TNF-α, IL-4, IL-6, IL-15 (Fantuzzi *et al* 2003). Constitutively expressed at high levels on peripheral monocytes. It plays an important role in mast cells recruitment and activation in the lung. When compared to controls, CCL2 was found at low levels in TB-IRIS patients and thought to be associated with monocyte dysfunctions (Oliver *et al* 2010).	Oliver *et al*. 2010
TNF-α	Mainly produced by CD14+CD16+DR++ pro-inflammatory monocytes that accounts only for 10% of all monocytes (Belge *et al* 2002). Unmasking TB-IRIS had higher plasma CRP, TNF-α and CRP: IL-10 ratio during TB-IRIS onset than controls (Haddow *et al* 2011).	(Haddow *et al* 2011)
IL-18	Pro-inflammatory cytokine belonging to the IL-1 family. Produced constitutively by immune and non-immune cells: monocytes/macrophages, APCs and epithelial cells. Massively produced through the inflammasome pathway. IL-18 stimulates IFN-γ production, but can also acts in synergy with IL-12 to sustain the Th-1 immune response, induces chemokines and cell-adhesion molecules, stimulates inflammatory cytokine secretion such as IL-1 and TNF-α (Nakanishi *et al* 2001; Mazodier *et al* 2005). Andre *et al* (2012) found that PBMC-derived monocytes exposed to combined IL-10 and IL-1α exhibit functional impairment (Andre *et al* 2012). Production of IL-18 was reported to be augmented in HIV infection (Sailer *et al* 2007; Iannello *et al* 2009) and shed off from membrane upon antigenic stimulation (Bellora *et al* 2012). Significantly elevated among TB-IRIS patients at pre-cART (Oliver *et al* 2010) and 2 weeks post-cART (Conesa-Botella *et al* 2012).	(Oliver *et al* 2010) (Conesa-Botella *et al* 2012)
CXCL10	CXCL10 is secreted by several cell types in response to IFN-γ. These cells include CD4+ T cells, NK, NKT, monocytes, pDCs. Therefore, CXCL10 is also known as IFN-γ-inducing protein-10 (IP-10). TB-IRIS after commencing cART is associated with increased CXCL10 responses to *M. tuberculosis* antigens (Oliver *et al* 2010)	(Oliver *et al* 2010) (Conesa-Botella *et al* 2012) (Oliver *et al* 2012)
CXCL8	Also known as IL-8. Secreted by macrophages after phagocytosis. A potent chemoattractant, which attracts monocyte/macrophages, mast cells and regulate neutrophil activation and degranulation. Macrophages carry out their	(Conesa-Botella *et al* 2012)

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	functions via phagocytosis and killing, or via the release of reactive oxygen species (ROS) and cytokines such as TNF-α and interleukin (IL)-12 ,IL-6, and IL-8 (Klimp *et al* 2002; Fujihara *et al* 2003).	
	The serum concentrations of pro-inflammatory IL-1β, IL-6, and IL-8 were greater at both baseline and at the time of TB-IRIS (Conesa-Botella *et al* 2012).	(Grant *et al* 2012)
	A cross-IRIS study (predominantly *Pneumocystis jirovecii* pneumonia) found elevated CXCL8 associated with IRIS at pre-cART.	
IL-10	HIV-1 infection can result from impaired signals delivered by the co-stimulatory CD28-B7 pathway and the altered production of immunoregulatory cytokines, in particular IL-10. HIV+ individuals have shown defects in IL-10 production by CD4+ and CD8+ T cells, whereas monocytes constitute the major IL-10-producing cell type (Daftarian *et al* 1995). The anti-inflammatory cytokine IL-10 suppresses antigen-specific Th1 responses (Torheim *et al* 2009).	
	Low levels of IL-10 during paradoxical TB-IRIS event have also been reported (Haddow *et al* 2011).	(Haddow *et al* 2011)
IL-6	IL-6 acts both as a pro-inflammatory and an anti-inflammatory myokine. It could be produced by monocytes upon stimulation by IL-32 (Kim *et al* 2005) and also produced by pDCs, which will facilitate differentiation of plasma cells.	(Stone *et al* 2002)
	Levels of IL-6 and soluble IL-6 receptor are increased in patients who experience IRIS after cART (Stone *et al* 2002). Higher concentrations of TNF, IL-6, and IFN-γ in serum were observed among TB-IRIS patients. Plasma IL-6 levels in HIV patients may differ significantly when assayed by ELISA or multiplex bead array assays (MBAA) (Cozzi-Lepri *et al* 2011).	(Tadokera *et al* 2011) (Cozzi-Lepri *et al* 2011)
IFN-γ	A cytokine secreted by activated T cells and NK cells. IFN-γ-deficient mice were killed by a sub-lethal dose of the intracellular pathogen *M. bovis* (Dalton *et al* 1993).	(Antonelli *et al* 2010)
	IFN-γ production (IGRA assay) is regarded as the key contributor of pathogen-specific Th1 responses in TB-IRIS (Bourgarit *et al* 2009). Direct measurement of IFN-γ from serum was significantly higher in TB-IRIS patients than controls (Antonelli *et al* 2010; Tadokera *et al* 2011). High baseline IFN-γ and TNF were strong predictors of IRIS (Grant *et al* 2012). Low IFN-γ (IGRA assay) was found in cryptococcosis-IRIS patients (Chang *et al* 2013)	(Tadokera *et al* 2011) (Grant *et al* 2012) (Chang *et al* 2013)

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IL-18 binding protein (IL-18BP)	Secreted by endothelial cells and macrophages upon stimulation by IFN-γ. It binds to neutralize IL-18 and thus regulates downstream inflammatory responses by other cells with IL-18 receptor. Isoforms 'a' (most abundant) and 'c' are functional while isoforms 'b' and 'd' are not (Dinarello *et al* 2013). Oliver *et al* found that IL-18BP had no association with TB-IRIS.	(Oliver *et al* 2012)

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1.6 Aims and Objectives:

Since TB-IRIS is the most common amongst all IRIS types, the current studies focus on TB-IRIS in order to accumulate ample sample size for comparison with non-TB-IRIS groups. The aims and specific objectives of this study are:

AIM 1) To examine the prevalence, clinical spectrum, risk factors, and impact of TB-IRIS on CD4+ T-cell recovery at our setting.

AIM 2) To investigate plasma markers that characterize and predict TB-IRIS, as well as marker(s) that differentiate unmasking and paradoxical TB-IRIS.

AIM 3) To investigate immune pathogenesis of TB-IRIS.

Specific objective 1: To investigate inflammatory pathway associated with TB-IRIS (guided by results obtained in AIM 2)

Specific objective 2: To investigate the phenotypes and functions of lymphocytes associated with TB-IRIS.

Specific objective 3: To investigate the regulatory mediators controlling inflammation in non-TB-IRIS patients

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CHAPTER 2: MATERIALS AND METHODS

2.1 Study Population

For the retrospective investigation (Chapter 3), patients who received ATT at the Direct Observed Therapy Strategy (DOTS) Clinic of UMMC (Kuala Lumpur, Malaysia), between 1 January 2006 and 31 January 2010 were considered. Demographic information, medical history before cART initiation, baseline laboratory information, results of imaging studies, and information on clinical events after cART initiation were retrieved from patients' medical files. For the case-control study (Chapter 4 and 5), the study participants were recruited from the Immune Reconstitution Cohort, a prospective observatiponal study on immune reconstitution and it's consequences in immunodeficient HIV-infected patients initiating cART. Patients were enrolled at the Infectious Disease out-patient Clinic of UMMC. Blood samples were collected at weeks 0, 6, 12, 24 and 48 plus an additional sample during an IRIS event from patients (suspect). Samples were collected only once from healthy controls. Patients with no evidence of IRIS and healthy controls were selected to match with IRIS patients by sex, ethnicity and age. Institutional ethics approval (Ref. No. 661.8) was obtained for the study and informed consent was given by all participants. The inclusion and exclusion criteria for the observational cohort were as follows:

Table 2.1 Inclusion & Exclusion Criteria

Inclusion criteria	Exclusion criteria
1) Age: >18 years old	1) Defaulted cART (non-adherence)
2) Starting cART with <200 CD4+ T cells/µl	2) Autoimmune disease (e.g. Type-1 Diabetes, SLE etc)
3) Naïve for cART at enrolment	3) Immune cell-related malignancies such as lymphoma
	4) Pregnancy

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2.2 Specimens

Blood samples were collected in ethylene diamine tetra acetic acid (EDTA)-treated tubes and centrifuged. Plasma was collected following centrifugation of whole blood at 1000g for 10 minutes and stored at -80ºC until use. The remaining blood was diluted (1:2) with phosphate-buffered solution (PBS; 1.44% w/v Na_2HPO_4, 0.24%w/v KH_2PO_4, 0.2% w/v KCl, 8% w/v NaCl), and peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll gradient technique (Ficoll®Paque Plus, GE Healthcare, Uppsala, Sweden) following centrifugation at 700g for 20 minutes. The isolated cells were washed twice in cold PBS, enumerated using an inverted microscope (by trypan blue exclusion of non-viable cells) and stored in liquid nitrogen in suspensions of 10 x 10^6 cells/ml freezing media (10% dimethyl sulphoxide, DMSO; Sigma-Aldrich, St Louis, MO) in heat-inactivated fetal calf serum, FCS (SAFC Biosciences, Lenexa, KS) until further use.

2.3 Measurement of Plasma HIV RNA and CD4+ T-Cell Counts

Plasma HIV RNA levels and CD4+ T-cell counts were available from the clinical diagnostic laboratory at pre-cART and at least twice during the first year of cART (i.e. approximately week 12-24 and/or week 24-48). Plasma HIV RNA was measured using the COBAS Amplicor HIV-1 Monitor Test, v.1.5 (Roche Diagnostics, Indianapolis, IN, USA). Viral load was <50 HIV-1 RNA copies/ml is regarded as undetectable. CD4+ T-cell counts were quantified by flow cytometry.

2.4 ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of cytokines in plasma samples or culture supernatants. The optical density (OD) generated is proportional to the concentration of the cytokines of interest and can be

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quantified in comparison to a standard of known concentration. ELISA methods employed in Chapter 4 and Chapter 5 are described in detail in the respective sections.

The list of Quantikine® ELISA kits used is given below: sCD163, sCD14, IFN-γ (DC1630, DC140, and DIF50; R&D Systems, Minneapolis, MN, USA); IL-6, IL-10, TNF-α, CCL2, CXCL8, CXCL10 (550799, 550613, 550610, 559017, 550999, and 550926; BD Biosciences, San Jose, CA, USA). Absorbance was read at 450 nm using Biotek™ Eon™ microplate spectrophotometers (Winooski, Vermont, USA).

Table 2.2: List of ELISA Kits and Lowest Detection Limit to Each Respective Kits.

Cytokines	ELISA kit	Catalog number	Lowest detection limit
Ferritin	Service provided by Clinical Investigation Lab, UMMC	-	-
Neopterin	IBL International	RE59321	1.35 nmol/l
sCD163	Quantikine ELISA, R&D Systems	DC1630	1.56 ng/ml
sCD14	Quantikine ELISA, R&D Systems	DC140	250 pg/ml
IL-18	recombinant human IL-18, primary and secondary antibodies from R&D Systems	B001-5, D044-3, and D045-6	15.6 pg/ml
IL-18BPa	Quantikine ELISA, R&D Systems	DBP180	26.6 pg/ml
IFN-γ	Quantikine ELISA, R&D Systems	DIF50	15.6 pg/ml
CXCL10	BD Biosciences	550926	7.8 pg/ml
TNF-α	BD Biosciences	550610	4.7 pg/ml
CCL-2	BD Biosciences	559017	15.6 pg/ml
IL-6	BD Biosciences	550799	9.4 pg/ml
CXCL8	BD Biosciences	550999	9.4 pg/ml
IL-10	BD Biosciences	550613	7.8 pg/ml
IL-1β	Quantikine ELISA, R&D Systems	DLB50	3.9 pg/ml

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2.5 Flow Cytometry

Cryopreserved PBMC samples were used in our flow cytometric investigations. Briefly, the PBMCs were thawed in RPMI and cell numbers were calculated. Subsequently, the PBMCs were washed in FACS buffer (PBS, 0.5-1% BSA or 5-10% FBS) and stained with conjugated monoclonal antibodies (mAbs) specific for cellular surface markers for 15 minutes at room temperature. If required, staining of intracellular markers were performed using kits or reagents, according to manufacturer's protocols. All staining was performed in the dark. After staining, the cells were resuspended in 200µl FACS buffer, fixed with a drop of 1% paraformaldehyde and kept at 4ºC before data acquisition. Data were acquired using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed using the FlowJo program v.5.7.2 (Tree Star, Ashland, OR, USA).

2.6 RNA Isolation and Quantitative RT-PCR

In Chapter 5, 2.5 x 10^6 PBMC were suspended in 1ml of RPMI/10% FCS in 24-well plate and incubated for 2hr at 37ºC with 5% CO_2 to facilitate monocyte adherence. After 2hr, non-adherent cells were removed and the adherent monocytes were washed twice with PBS. To the adherent monocytes, ice cold PBS was added and incubated in ice for 30 min to render detachment of the monocytes. The cells were spun and cell pellet was resuspended in 350µl of RLT-lysing buffer (Qiagen, Valencia, CA). Total RNA was extracted from the lysates using the RNeasy Mini Kit (Qiagen) as per manufacturer's instructions and stored at -80ºC until further use.

Predesigned TaqMan® Gene Expression Assays were purchased from Applied Biosystems as follow:

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Table 2.3: List of Quantitative PCR Assay.

Gene	Amplicon (bp)	Assay number	Cat. #
NLRP1	82	Hs00248187_m1	4331182
NLRP3	84	Hs00918082_m1	4331182
NLRC4	98	Hs00892666_m1	4331182
AIM2	106	Hs00915710	4331182
Casp-1	76	Hs00354836_m1	4331182
ASC	61	Hs00203118	4331182
TBP	91	Hs00427620_m1	4331182
SDHA	124	Hs00417200_m1	4331182

Additionally, mRNA for iNOS was quantified using the following primers:

iNOS-F 5'-TAGAGGAACATCTGGCCAGG

iNOS-R 5'-TGGCAGGGTCCCCTCTGATG

The RNA concentration was determined by a spectrophotometer and samples were diluted to give a RNA working concentration of ~10ng/µl. First strand cDNA synthesis was performed with High Capacity cDNA Reverse Transcription kit (ABI, cat. 4374966) using 2µg of total RNA according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qPCR) was performed using TaqMan Gene Expression Master Mix and Gene Expression Assay Kits. Briefly, 1µl of Taqman Gene Expression assay, 10µl of 2X buffer, 0.5µl of RT-enzyme and 8.5µl of diluted mRNA were used for each reaction.

HPRT-1, TBP and SDHA were used as an endogenous control throughout. qPCR was performed on an ABI ViiA 7 real-time PCR system under the following thermal cycling conditions: initiation/enzyme activation at 95°C for 15 seconds followed by 40 cycles annealing-extension step at 60°C for 1 minute. Normalization was performed by

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averaging the Ct values of the two housekeeping genes. The expression levels of the target mRNA in each sample were calculated by the $2^{-\Delta Ct}$ method.

2.7 Statistical Analysis

Data are presented as medians, interquartile range (IQR) and means ± standard deviation (SD) as indicated. Categorical variables were compared using chi-square or Fisher's exact test; whilst continuous variables with the Kruskal-Wallis test or Mann-Whitney U-test where appropriate. Paired non-parametric variables were compared using Wilcoxon signed-rank test. P values for multiple comparisons were adjusted by Bonferroni correction (n-1) where n = number of comparisons. Statistical analysis and figures were performed using GraphPad Prism 4.0 software (GraphPad, La Jolla, CA, USA) and regression analyses were performed using SPSS v20.

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CHAPTER 3: TUBERCULOSIS-ASSOCIATED IMMUNE RECONSTITUTION INFLAMMATORY SYNDROME IN TB-HIV CO-INFECTED PATIENTS IN MALAYSIA: PREV, RISK FACTORS, AND TREATMENT OUTCOMES

3.1 Introduction

TB represents one of the major health concerns among HIV-infected patients, especially across resource-limited countries. South-East Asia harbors >40% of the global burden of TB cases (2012). In 2011, the incidence of TB in Malaysia was estimated at 81 cases per 100,000 populations. Of a total of 21,000 TB cases, 1630 (9%) were co-infected with HIV (WHO 2012). Concurrent treatment of TB and HIV infection remains a challenge due to high pill burden, drug toxicity, drug-drug interactions and, TB-associated immune reconstitution inflammatory syndrome (TB-IRIS), which affects up to 30% of patients in resource-limited settings. There has been no data reported from Malaysia.

TB-IRIS results from exaggerated inflammatory responses attributed to dysregulated immune recovery after commencing cART (French 2009). This syndrome can either present as a clinical deterioration of existing disease (paradoxical TB-IRIS) or appearance of new disease associated with previously subclinical infection (unmasking TB-IRIS) after initiation of cART (French 2009).

Notwithstanding that the symptoms and signs can be as mild as solitary lymph node inflammation, TB-IRIS could also manifest as potentially life-threatening complications, including meningitis and acute respiratory distress syndrome (ARS). Hence, it is important that TB-IRIS be differentiated from the other clinical complications of cART. Here, we retrospectively followed-up patients who were initiated on ATT and cART at the UMMC. We aimed to investigate the prevalence of

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TB-IRIS, risk factors, and treatment outcomes including mortality rates and long-term CD4+ T-cell recovery in the recruited patients.

3.2 Methods

3.2.1 Study Population and Setting

The source population for this retrospective cohort was patients who received ATT at the Direct Observed Therapy Strategy (DOTS) Clinic at UMMC (Kuala Lumpur, Malaysia), from 1 January 2006 to 31 January 2010. New patients with pulmonary TB were given a standard 6-month ATT rpegimen that consisted of 2 months isoniazid, rifampicin, pyrazinamide, ethambutol (H,R,Z,E) followed by 4 months of HR. Of the 1579 TB patients identified, 153 were recruited in the investigation who were co-infected with HIV-1 and were on ATT. Patients with insufficient data, with existing NTM infection, or who were transferred to another centre or died before receiving ATT were excluded. Further, we also excluded 47 patients who did not commence cART due to death, transfer to another centre or default from follow-up (**Figure 3.1**). All participants with Malaysian nationality were BCG vaccinated and were screened and treated according to the WHO Malaysia CPG guidelines for Management of Tuberculosis (Malaysia Health Technology Assessment Section (MaHTAS) 2012). The study was approved by the ethics committee of UMMC (reference number: 661.8).

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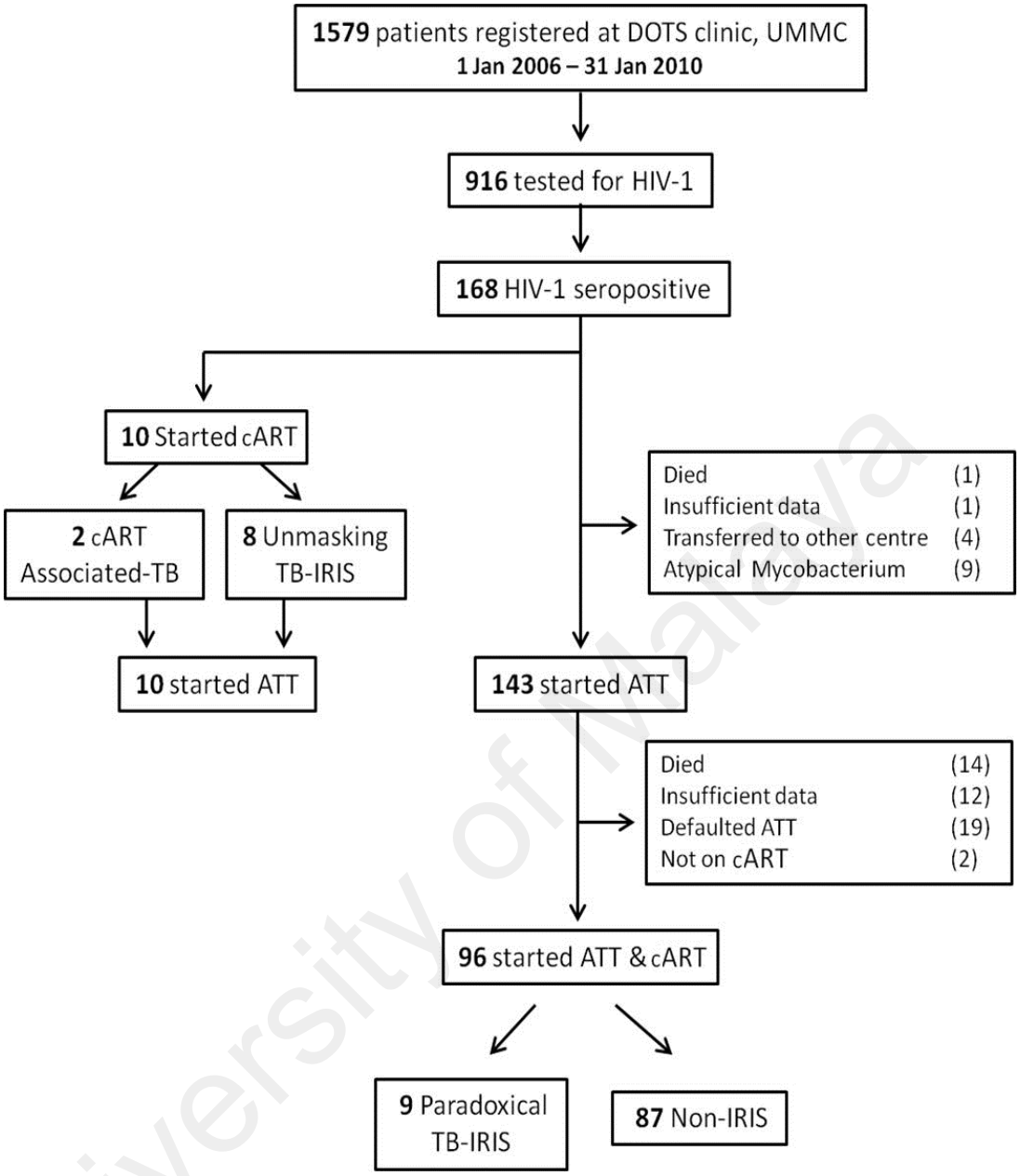

Figure 3.1: Flow Diagram for Retrospective Study.

168 adult (≥18 years old) HIV patients who registered at DOTS clinic from 31 Jan 2006 to 1 Jan 2010. DOTS: Direct Observed Therapy Strategy, ATT: Anti-tuberculosis treatment, cART: combined Anti-retroviral therapy, UMMC: University Malaya Medical Centre.

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3.2.2 Data Collection

Demographic information, medical history prior to cART initiation, baseline laboratory information, results of imaging studies, and information on clinical events after cART initiation were retrieved from medical files of the patients. We also examined the medical files for 18-months post-cART to assess treatment outcomes.

3.2.3 Definitions

Based on the World Health Organization (WHO) criteria (WHO 2010), a case of TB was defined as a patient who received a full course of ATT, while a definite case of TB required MTB complex to be identified from clinical specimens, either by culture or by a newer diagnostic method, such as molecular line probe assay. Extra-pulmonary TB was defined as TB or definite TB (as defined above) that involves organs other than the lungs, e.g. pleura, lymph nodes, skin, gastro-intestinal tract and meninges. Disseminated TB was defined as TB or definite TB (as defined above) that involved more than one organ. Previously published case-definitions were used to identify cases of TB-IRIS (Meintjes *et al* 2008).

All possible TB-IRIS cases were evaluated by a committee that comprised of physicians specializing in infectious diseases (n=2) or clinical immunology (n=1) to verify the diagnosis. The cases were subsequently assigned to 1 of 4 groups:

(1) **Non-IRIS:** TB-HIV co-infected patients who did not develop inflammatory disease after commencement of cART.

(2) c**ART-associated TB:** HIV patients who presented with active TB after initiation of cART but did not exhibit an exaggerated inflammatory reaction.

(3) **Paradoxical TB-IRIS:** TB-HIV co-infected patients with TB disease at baseline, who were receiving ATT and who subsequently developed clinical deterioration within the first 3 months of cART.

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(4) **Unmasking TB-IRIS:** HIV patients with subclinical TB on commencement of cART and was 'unmasked' within the first 3 months of cART associated with an exaggerated inflammatory reaction.

Due to limitations in diagnostic capacity, alternative diagnoses (ie. drug toxicity, failure/poor adherence to ATT) could not be completely ruled out for all events. Hence, an event was regarded as "probable" when IRIS was the most likely cause of clinical deterioration and as "possible" when IRIS was plausible where alternative explanations were equally plausible (Lawn *et al* 2008; Haddow *et al* 2009).

3.2.4 Statistical Analysis

Our primary analysis compared between patients with non-TB-IRIS, paradoxical TB-IRIS, and unmasking TB-IRIS. Comparison of categorical variables was tested using chi-square test or Fisher's exact test while continuous variables (e.g., viral load, CD4) were compared using the non-parametric Kruskal-Wallis test, as these variables did not follow normal distribution.

Potential risk factors for TB-IRIS such as, pre-cART demographic variables, HIV parameters, TB diagnosis, and time interval between ATT and cART were evaluated by a simple logistic regression followed by adjusted logistic regression. The odds ratio and 95% confidence interval (95% CI) were estimated. Cox regression analysis was used to assess longitudinal CD4+ T-cell recovery between TB-IRIS and non-TB-IRIS patients. CD4+ T-cell recovery was defined as the time taken to achieve CD4+ T-cell counts of >500 cells/µl. All analyses were conducted using SPSS, v.20 and p values <0.05 was considered as statistically significant.

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3.3 Results

3.3.1 Demographic and Baseline Characteristics

One hundred and fifty three patients who commenced ATT were included in the baseline demographic analysis (**Table 3.1**); 125 (81.7%) were male; 62 were Chinese (40.5%), median age was 39 years (inter-quartile range [IQR], 34-49). Patients co-infected with HIV (n=153) had a median baseline CD4+ T-cell count of 52 cells/μl (IQR= 12-130). Co-infections with HBV and HCV were 8.5% and 28.1% respectively. Sputum AFB smear was positive in 34% (n=52) of patients and 48.4% (n=74) were culture positive. Almost half the patients had disseminated TB (n=75, 49%). Tuberculin skin test was only performed on 49 (32%) TB-HIV co-infected patients, of whom 8 (16%) showed induration diameter of >10mm (data not shown).

3.3.2 Incidence of TB-IRIS Events

Of the 153 TB-HIV cases enrolled, 106 commenced both cART and ATT. Twenty two potential TB-IRIS cases were evaluated by the end-point review committee. Nine of the 96 (9.4%) cases were confirmed to have developed paradoxical TB-IRIS within 12 weeks of initiating cART: one patient developed both probable paradoxical TB-IRIS of the lymph nodes and possible paradoxical TB-IRIS of the brain. In addition, 8 patients developed unmasking TB-IRIS prior to recruitment into the study. There were inadequate data to determine the prevalence of unmasking TB-IRIS. Further, two patients developed cART-associated TB and were not considered for data analysis. The remaining 87 patients experienced no adverse effects from starting cART (**Table 3.3**).

Five cases were excluded due to rifampicin resistance (n=1), not fulfilling criteria for TB-IRIS (n=2), decline in plasma HIV RNA of $<1\log_{10}$ (n=1), and no viral load data at disease onset (n=1). Clinical manifestations in patients with TB-IRIS are presented in **Table 3.2**.

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Table 3.1: Demography of Study Participants.	
Characteristics (n=153)	**Started ATT (%)**
Gender, male	125 (81.7)
Age (year), median (IQR)	39 (34, 48.5)
Nationality/ethnicity	
Immigrants†	16 (10.5)
Malay	46 (30.1)
Chinese	62 (40.5)
Indian	26 (17)
Others	4 (2.6)
Alcohol Use	25 (12.3)
Smoking	72 (47.1)
Route of Transmission	
Heterosexual	81 (53)
Drug addiction	42 (27.5)
MSM	2 (1.3)
Unknown	28 (18.3)
HIV Monitoring, median (IQR)	
Baseline CD4+ T-cell count (cells/µl)	52 (12.8, 130.3)
Baseline viral load (copies/ml)	111335 (100000, 370262)
Other Medical Conditions & Co-Infections	
Diabetic mellitus	20 (13.1)
Positive HBV surface Antigen	13 (8.5)
Positive anti-HCV Antibody	43 (28.1)
TB Diagnosis	
Sputum AFB positive	52 (34)
TB culture positive	74 (48.4)
Forms of TB	
Pulmonary TB	55 (36)
Extra-pulmonary TB	23 (15)
Disseminated TB	75 (49)
Drug-Resistant TB	
MDR	2 (1.3)

Note: †Immigrants were Bangladeshi (n=1), Burmese (n=3), Philippino (n=2), Indian (from mainland India) (n=3), Indonesian (n=2), Liberian (n=2), Nigerian (n=1), South African (n=1) and Vietnamese (n=1).

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Table 3.2: Manifestations in Patients with TB-IRIS (n=17).	
Manifestation	**No. of Patients (%)**
Clinical	
New-onset fever	8 (47.1)
New-onset cough	3 (17.6)
New-onset cervical adenopathy	2 (11.8)
Worsening cervical adenopathy	4 (23.5)
New-onset subcutaneous cold-abscess	3 (17.6)
Worsening cold abscess	1 (5.9)
Radiological	
New-onset intra-abdominal lymphadenopathy	10 (58.8)
New-onset pulmonary infiltrates	1 (5.9)
New-onset pleural effusion	3 (17.6)
Worsening meningitis	1 (5.9)

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Table 3.3: Baseline Characteristics of Study Participants Stratified by TB-IRIS Status.				
Characteristics	**Patients with TB at Baseline Before cART**		**Unmasking TB-IRIS**	
(n=104)#	**Non TB-IRIS (n=87)**	**Paradoxical TB-IRIS (n=9)**	**(n=8)**	***P Value***
Male, n (%)	71 (81.6)	9 (100)	6 (75)	0.322
Age	39 (34, 47)	41 (30.5, 51)	40 (33.3, 53)	0.969
Pre-cART				
CD4+ T-cell count	65.5 (14, 164.3)	12 (5, 69)	23 (9.3, 79.3)	0.04*
Viral load (log)	5.14 (5, 5.6)	5 (4.9, 5.9)	5.2 (5, 5.8)	0.859
Baseline Haematology				
Haemoglobin	109 (98, 127)	103 (96.9, 116)	94.5 (83.5, 106.3)	0.064
Total WBC	6.4 (4.8, 7.9)	5.2 (15.5, 28.5)	5.9 (3, 7.6)	0.722
Baseline Liver Function				
Albumin	26 (22, 32)	23 (15.5, 28.5)	28.5 (18.3, 29.8)	0.377
ALT	44 (32, 70)	52 (38.5, 74.5)	39 (31.8, 61.5)	0.589
AST	39 (26, 60)	38 (33, 49.5)	47 (41, 69)	0.49
GGT	84 (50, 158.3)	133 (84.5, 285)	93 (26.3, 396)	0.288
Pre-cART CRP	4.8 (1.6, 9)	4.6 (3.7, 8.2)	12.6 (5.3, 15.2)	0.241
Other Medical Conditions & Co-Infections, n (%)				
Diabetes mellitus	10 (11.5)	0 (0)	3 (37.5)	0.053
HBV	8 (9.2)	0 (0)	0 (0)	0.432
HCV	20 (23)	1 (11.1)	1 (12.5)	0.586
MTB Disease^, n (%)				
AFB smear positive	26 (31.3)	4 (44.4)	1 (12.5)	0.523
MTB culture positive	40 (46)	6 (66.7)	2 (25)	0.147
Disseminated TB	31 (35.6)	9 (100)	5 (62.5)	0.001*
Interval (Day)				
ATT to cART	61.5 (30.3, 87)	36 (32, 65.5)	-	0.159
cART to paradoxical TB-IRIS	-	27 (12, 64.5)	-	-
cART to unmasking TB-IRIS	-	-	19 (14.5, 65.3)	-

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Note: All data are expressed as median (IQR) unless specified. P values are calculated by chi-square test for categorical variable and Kruskal-Wallis test for continuous variables. IQR, interquartile range. #Two cART-associated TB cases were excluded from TB-IRIS analysis, *Fisher exact test, ^Data on MTB disease were collected before cART in paradoxical TB-IRIS cases and at presentation in unmasking TB-IRIS cases.

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3.3.3 Analysis of Risk Factors for TB-IRIS

The pre-cART characteristics for non-TB-IRIS, paradoxical TB-IRIS, and unmasking TB-IRIS patients are shown in **Table 3.3**. The three groups were similar with respect to age, gender, pre-cART HIV viral load, total white blood cell count, liver function tests and plasma CRP levels. Patients who developed TB-IRIS had significantly lower CD4+ T-cell counts at baseline than non-TB-IRIS patients (p=0.04). Furthermore, cases of disseminated TB were more prevalent among TB-IRIS patients as compared to non-IRIS patients (p=0.001). In addition, TB-IRIS patients had a trend of lower haemoglobin levels at baseline (p=0.064) and lower rates of diabetes mellitus before cART (p=0.053). cART was initiated at a median (IQR) duration of 61.5 (30.3-87) and 36 (32-65.5) days after ATT, for non-TB-IRIS and paradoxical TB-IRIS patients, respectively (p=0.159). The median time to unmasking TB-IRIS development was 19 days (IQR=14.5-65.3).

Using a logistic regression model, we investigated the association of pre-cART characteristics with subsequent development of TB-IRIS (**Figure 3.2**). The variables that showed a significant (or trending towards significant) relationship with development of TB-IRIS, (low haemoglobin, low CD4+ T-cell count, presence of disseminated TB and lower rate of diabetes mellitus) were included in this predictive model. Variables associated with increased risk of TB-IRIS in other studies (log_{10} viral load >5, AFB smear positivity and interval between ATT to cART) were also included in the model. An increased risk of developing paradoxical TB-IRIS within the first 3 months of cART was associated with disseminated TB (OR=10.74 [95% CI=1.22, 94.3]; p=0.032). The association between TB-IRIS and lower haemoglobin level, baseline CD4+ T-cell count <100 cells/μl and diabetes mellitus were not statistically significant after adjustment for potential confounding factors (**Figure 3.2**).

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Figure 3.2

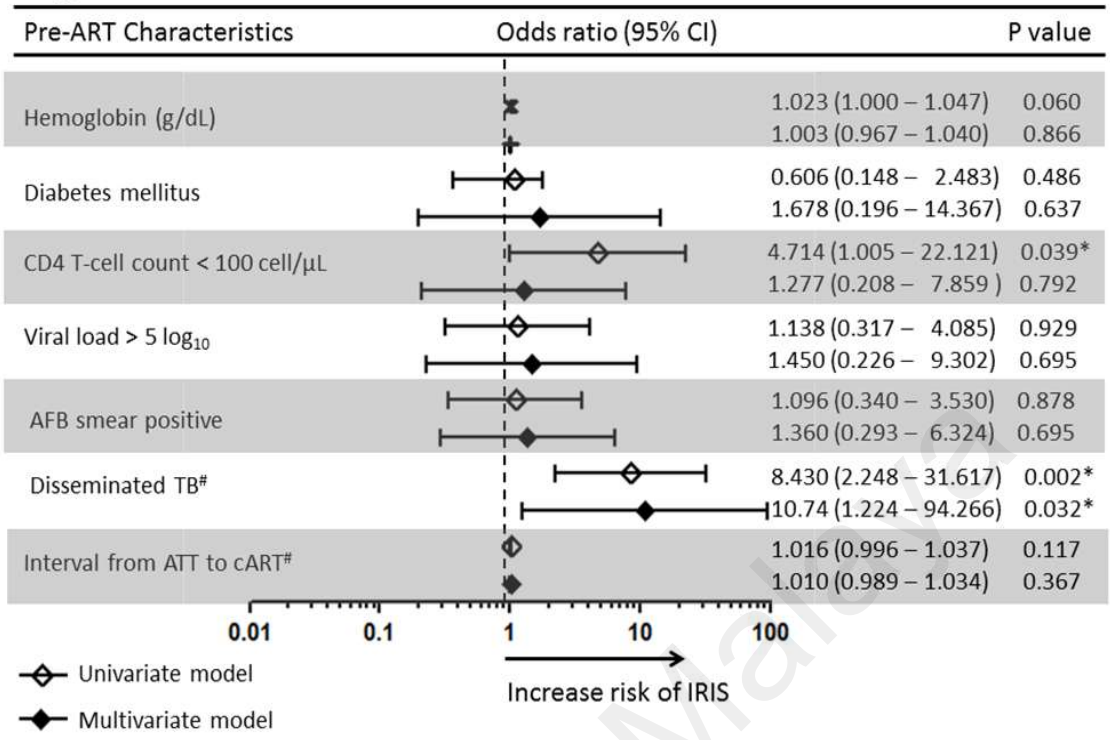

Figure 3.2. **Associations of Baseline Clinical and Laboratory Characteristics with TB-IRIS Events Following the Commencement of cART**.

The variables that showed a significant relationship with development of TB-IRIS (from **Table 3.1**) *i.e.* haemoglobin, CD4+ T-cell counts and disseminated TB were included in this predictive model. Additional variables that associated with increased risk of TB-IRIS *i.e.* viral load and AFB smear positivity and interval from ATT to cART were also included in the model. Association of all variables with risk for TB-IRIS was assessed in simple logistic and adjusted logistic regression models. Odds ratios for values below or above threshold levels were displayed in forest plot, median and 95% CI were calculated. The Hosmer-Lemeshow value for this model was p=0.825. CI, Confidence Interval; #, calculation did not include unmasking TB-IRIS cases; *, represent statistical significant and $p<0.05$.

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3.3.4 Treatment Outcomes

The completion rate of ATT for patients with and without TB-IRIS was 70.6% vs 79.3%, respectively. The mortality rate was similar in TB-IRIS (n=1, 5.3%) and non-TB-IRIS (n=5, 5.8%) patients. The TB-IRIS patient who died had unmasking TB-IRIS that presented with an inflammatory syndrome and signs suggestive of TB in the second month of cART. This patient commenced ATT only after AFB was detected at week 27 of cART. Treatment failure (smear positive on sputum) after the completion of ATT was higher in TB-IRIS (n=2, 10.5%) compared to non TB-IRIS (n=3, 3.5%) patients. Default rate on cART was similar in TB-IRIS and non TB-IRIS patients (n=1, 5.3%; n=5, 5.8%), respectively.

3.3.5 CD4+ T-Cell Recovery on cART in TB-IRIS and Non-TB-IRIS Patients

To assess if TB-IRIS affects long term CD4+ T-cell recovery in TB-HIV co-infected patients, survival analyses were performed to compare the time taken to achieve a CD4+ T-cell count of >500 cells/µl in TB-IRIS and non-TB-IRIS patients (**Figure 3.3**). The Cox regression survival plot showed that the rate of CD4+ T-cell recovery in TB-IRIS patients was not significantly different from that of TB-HIV co-infected patients who did not develop TB-IRIS (p=0.363).

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Figure 3.3

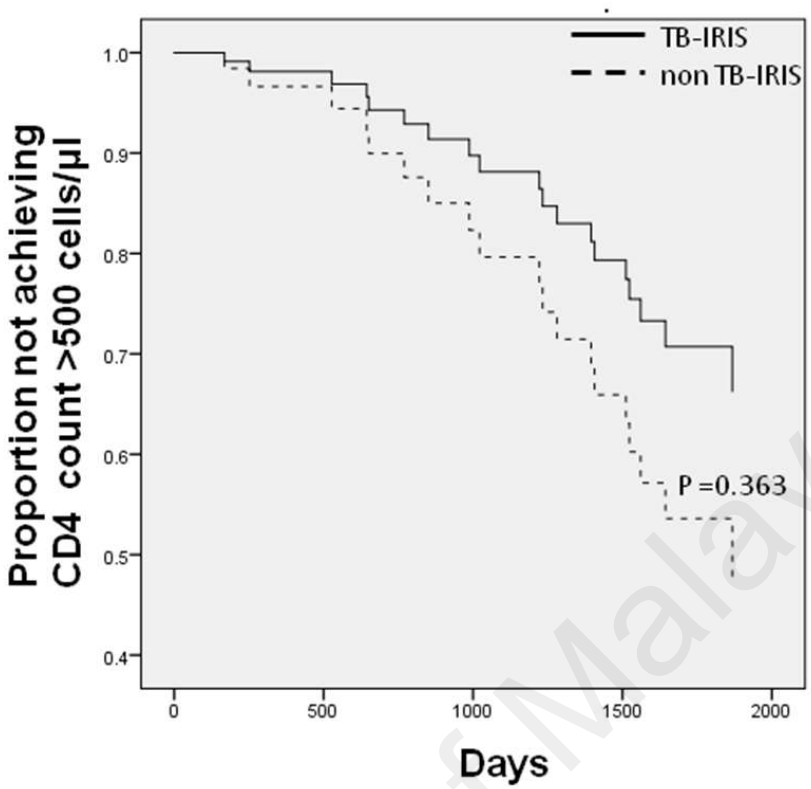

Figure 3.3: Cox Regression Analysis of Time to Achieve CD4+ T-cell Counts of >500/µl.

Cox regression survival plots of time taken to achieve a CD4+ T-cell count of >500 cells/µl were compared in TB-IRIS non-TB-IRIS patients, controlling for age, baseline CD4+ T-cell count and HIV viral load. The analysis showed that the rates of CD4+ T-cell recovery were not significantly different between TB-IRIS and non-TB-IRIS patients.

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3.4 Discussion

MTB is among the most frequently reported pathogens associated with IRIS. The prevalence of TB-IRIS varies throughout the world and is highly dependent on the population studied, specifically the degree of immunodeficiency and OI burden. Earlier studies from developed countries reported a higher incidence of TB-IRIS (29-43%) (Narita *et al* 1998; Breen *et al* 2004; Breton *et al* 2004; Michailidis *et al* 2005). For resource-limited settings, the reported prevalence of TB-IRIS ranges between 12% in South Africa (Lawn *et al* 2007) , 12-18% in Brazil (Serra *et al* 2007; Dibyendu *et al* 2011), 13% in India (Agarwal *et al* 2012), 5-13% in Thailand (Manosuthi *et al* 2006; Aramaki *et al* 2010; Umphonsathien *et al* 2011), and 29% in Uganda (Baalwa *et al* 2008). In this retrospective study, we report a prevalence of paradoxical TB-IRIS in Malaysia of 9.4% (9 out of 96 patients), which is in line with data from other developing countries.

Paradoxical and unmasking TB-IRIS are associated with the restoration of immune responses against mycobacterial antigens, which culminates in an inflammatory response that cause clinical disease. Currently available data suggest that both adaptive and innate immune responses contribute to this process (Chang *et al* 2013). Perturbation in innate immune responses has been clearly established in paradoxical TB-IRIS (Oliver *et al* 2010). However, while Th1 responses reportedly increase, it still remain unclear as to what extent this drives inflammation (Vignesh *et al* 2013). By contrast, markers of heightened Th1 responses against MTB antigens are more prominent than markers of innate immune responses in unmasking TB-IRIS (Elliott *et al* 2009; Oliver *et al* 2012).

Diagnosis of HIV infection in resource-limited settings appears to be challenging and is often delayed, with many patients seeking HIV care only after developing advanced immunodeficiency, and presenting with one or more OIs. This has been clearly reflected by the low CD4+ T-cell counts and high co-infection rates in our study population,

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whereby median baseline CD4+ T-cell count was 52 cells/µl, and 44% of TB-HIV co-infected patients presenting with disseminated TB and 47% with multiple OIs (data not shown). In the current study, a logistic regression model identified more advanced immunodeficiency (low CD4+ T-cell counts), presence of disseminated TB, low haemoglobin levels and diabetes mellitus as predictors of TB-IRIS. Whilst these findings were in agreement with other studies (except with diabetes) (Martin-Blondel *et al* 2012), unlike others, we found no association between high baseline viral load, or interval between ATT to cART, and increased risk of TB-IRIS. For instance, it has been demonstrated an independent association of a short interval between ATT to cART with a relative risk of 20.2 (IQR=2.0-201.5) for developing TB-IRIS (Narendran *et al* 2013). The discrepancy between that study and our investigation may have resulted from differences in the interval between ATT and cART in the two studies [median (IQR)=28 (14-47) days compared with 57 days (31-85) in our study].

Our adjusted logistic regression model has demonstrated that disseminated TB was the only independent factor associated with TB-IRIS. Interestingly, we found no associations between lower baseline CD4+ T-cell counts, higher baseline viral load and shorter interval between ATT to cART with increased risk of TB-IRIS. These findings suggest an interaction between these variables and underline the importance of pre-cART mycobacterial antigen load in the pathogenesis of TB-IRIS. In a rabbit model of aerosol TB infection, the development and severity of TB-IRIS were dependent on the antigen load at the time of immune reconstitution (Manabe *et al* 2008). Furthermore, others have demonstrated that high urinary mycobacterial lipoarabinomannan (LAM) levels before cART initiation in TB-HIV co-infected patients increased the risk of TB-IRIS (Conesa-Botella *et al* 2011). Measures that reduce pathogen load in HIV-TB patients prior to commencing cART might therefore decrease the risk of TB-IRIS. However, delaying cART to reduce the mycobacterial antigen load with ATT in order to

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reduce the risk of TB-IRIS, is contra-indicated in patients with CD4+ T-cell counts <50/µl because delaying of cART increases the overall mortality (Abdool Karim *et al* 2010). Further research on possible measures to increase pathogen clearance before cART initiation is urgently required.

The mortality rate for TB-IRIS patients (5.9%) in our study was similar to that for non-TB-IRIS patients (5.7%) and lower than that documented by others, which have reported mortality ranging between 10% and 38% (Manosuthi *et al* 2006; Lawn *et al* 2007; Dibyendu *et al* 2011; Agarwal *et al* 2012; Gupta *et al* 2012; Manosuthi W 2012). Notably, the only fatal case of TB-IRIS in our study was observed in a patient with unmasking TB-IRIS. Others have demonstrated that HIV-infected patients who developed TB during the first 3 months of cART have a 3.5-fold increase in mortality rates relative to patients presenting with TB at other times (Koenig *et al* 2009). Hence, it is important to detect, and commence treatment for subclinical TB in patients with HIV before commencement of cART (French 2012).

Using a Cox regression model, we showed that long term CD4+ T-cell recovery was not significantly different between TB-IRIS and non-IRIS patients (p=0.363). This finding is similar to that of others (Kumarasamy *et al* 2013), who have found that TB-IRIS patients had higher CD4+ T-cell counts than non-TB-IRIS patients at the time of initiating cART and at 6, 18 and 24 months following initiation of cART ($p<0.05$). These findings suggest that TB-IRIS seldom have a negative impact on the recovery of CD4+ T-cell levels.

Our investigations do have certain limitations, mainly the small sample size and secondly, the retrospective nature, which potentially precluded us from having documented the missed clinical events that were not recorded in the medical records.

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Similarly, clinical features that may have been potential risk factors for TB-IRIS have not been recorded.

In conclusion, we have shown that TB-HIV co-infected patients presenting with disseminated TB exhibit the highest risk for developing TB-IRIS. Treatment strategies for enhancing effective pathogen clearance prior to commencing cART in HIV-TB co-infected patients should be prioritized to prevent the development of TB-IRIS.

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CHAPTER 4: PLASMA IL-18 ARE A BIOMARKER OF INNATE RESPONSES THAT PREDICT AND CHARACTERIZE TUBERCULOSIS-ASSOCIATED IMMUNE RECONSTITUTION INFLAMMATORY SYNDROME

4.1 Introduction

Tuberculosis (TB) is the most common OI among individuals with HIV infection, especially in resource-limited countries. The use of cART has reduced morbidity and mortality in HIV-infected patients but may be complicated by an IRIS in patients who commence cART with a low CD4+ T-cell counts (Chang *et al* 2014). Following initiation of cART, 10-30% of TB-HIV co-infected patients experience a paradoxical deterioration of treated TB, or a rapid onset of newly diagnosed TB associated with an exaggerated inflammatory response, conditions that are referred to as paradoxical TB-IRIS and unmasking TB-IRIS, respectively (Meintjes *et al* 2008). Since TB-IRIS usually occurs within the first month of cART, it could lead to diagnostic predicaments to distinguish between relapsed and newly acquired TB. Corticosteroid therapy is effective in patients who experience severe TB-IRIS (Meintjes *et al* 2010). Nonetheless, the potential for adverse effects limit its use. Therefore, there is an urgent need for laboratory markers to predict and identify TB-IRIS (Murdoch *et al* 2007; Meintjes *et al* 2010).

Risk factors for the development of an IRIS in HIV-infected patients are reported to be a low CD4+ T-cell count at cART initiation (Grant *et al* 2010), robust immunological and virological response to cART (Shelburne *et al* 2005), high antigenic burden of an underlying OI at cART initiation (Breton *et al* 2004) and early initiation of cART after OI treatment (Shelburne *et al* 2005; Lawn *et al* 2007). However, the immunological mechanisms underlying the development of an IRIS are not clearly understood. Several studies have demonstrated an expansion of MTB-specific IFN-γ-producing T cells

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(Bourgarit *et al* 2006; Antonelli *et al* 2010) or elevation of polarized T-helper 1 (Th1) responses (Mahnke *et al* 2012) among TB-IRIS patients. However, these responses may also occur in non-IRIS patients (Meintjes *et al* 2008; Tan *et al* 2008), questioning their relevance.

Accumulating evidence suggests that innate immune responses play a role in the pathogenesis of TB-IRIS (Oliver *et al* 2010; Tan *et al* 2011; Barber *et al* 2012). A recent microarray investigation of RNA from monocytes of patients with TB-IRIS demonstrated that 100 genes related to "inflammatory disease", "immunological disease", "cellular movement", "hematological system development and function", and "immune cell trafficking" were perturbed at least 1.5 fold in patients relative to controls (Tran *et al* 2014). These findings suggest that monocytes contribute to the pathogenesis of TB-IRIS. Given the strong interplay between innate and adaptive immune responses, the onset of TB-IRIS may stem from protective immune responses that are excessive in magnitude resulting from dysregulation of the interaction between innate and adaptive immune responses (Barber *et al* 2012).

Here, we sought to identify and compare plasma biomarkers of innate immune responses that predict and characterize TB-IRIS, including interleukin (IL)-18, which is increased in TB-IRIS patients (Oliver *et al* 2010) and is a signature cytokine of the nucleotide-binding domain and leucine-rich repeat pyrin containing protein-3 (NLRP3) inflammasome (Novick *et al* 2013). We established that elevated plasma levels of IL-18 and CXCL10 predicted TB-IRIS and that levels of IL-18BPa, the most abundant splice variant of IL-18 binding protein (IL-18BP) that regulates the biological activity of IL-18 (Novick *et al* 1999), may not be sufficient to regulate the biological functions of IL-18. These findings suggest that TB-IRIS is associated with an exaggerated inflammatory response that includes increased IL-18 production, which may be inadequately regulated.

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4.2 Methods

4.2.1 Study Groups and Design

The study was initially conducted on samples from patients selected from a prospective observational cohort study of 200 HIV-infected cART-naive adults (>18 years) initiating cART between 2005 and 2012, with a CD4+ T-cell count of <200 cells/μL at the UMMC (Kuala Lumpur cohort) (Tan *et al* 2014). Symptomatic patients were screened for TB by chest radiograph and/or sputum examination and those with TB completed a median of 5 weeks (range 1-20 weeks) of ATT prior to cART initiation. Participants were reviewed 4, 12, 24 and 48 weeks after cART initiation. Blood samples were collected at each follow-up visit and additional samples were collected from those who developed symptoms suggestive of paradoxical or unmasking TB-IRIS. Informed consent was obtained from all participants and the study was approved by the Medical Ethics Committee of the UMMC (MEC Ref. No: 673.33).

We excluded patients who were non-adherent to ATT or cART, non-responders due to ATT or cART drug resistance, and those who developed malignancy or multiple diagnoses of an IRIS. Paradoxical and unmasking TB-IRIS were later classified by an expert panel, which was blinded from test results, according to diagnostic criteria of the International Network for the Study of HIV-associated IRIS (Haddow *et al* 2009). Demographic and clinical information on patients are listed in **Supplementary Table 4.1**. All cases where plasma samples were available for both baseline (within 2 weeks before cART initiation) and TB-IRIS (within 2 weeks of symptom onset), or EQT in controls, were included. Controls were matched 2:1 to TB-IRIS cases based on baseline TB status, age and CD4+ T-cell counts (within 50 cells/μl).

Participants were classified into three groups according to the following definitions (Haddow *et al* 2011):

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1) **TB-IRIS (case):** HIV-infected patients who had TB prior to initiation of cART and were treated with ATT, who subsequently experienced disease classified as TB-IRIS (paradoxical TB-IRIS); or HIV-infected patients who had no evidence of TB prior to initiation of cART and subsequently presented with TB that exhibited exaggerated inflammation after commencing cART (unmasking TB-IRIS).
2) **TB no IRIS (control):** HIV-infected individuals who had TB prior to initiation of cART and were treated with ATT, with no clinical deterioration after commencing ART.
3) **No TB or IRIS (control):** HIV-infected individuals who did not have TB prior to initiation of cART and did not develop TB-IRIS after commencing cART.

To confirm the findings for IL-18, samples were analyzed from patients selected from a randomized controlled clinical trial (NCT 933790) conducted at the National Institute for Research in Tuberculosis (NIRT), Chennai, India (Chennai cohort) (Narendran ; Andrade *et al* 2014). Fifty seven patients with newly diagnosed sputum culture-confirmed pulmonary TB were enrolled. Patients (n=48) were selected based on the criteria described elsewhere (Narendran *et al* 2013). Baseline investigations were repeated at event in the paradoxical TB-IRIS group (n=26) and at week 6 of cART in patients who had uneventful cART initiation (n=22).

4.2.2 Laboratory Investigations

In the Kuala Lumpur cohort, serum/plasma levels of neopterin (RE59321; IBL International GmBH); sCD163, sCD14, IFN-γ (DC1630, DC140, and DIF50; R&D Systems); IL-6, IL-10, TNF-α, CCL2, CXCL8, CXCL10 (550799, 550613, 550610, 559017, 550999, and 550926; BD Biosciences) were measured by ELISA. The IL-18 ELISA kits consisted of recombinant human IL-18, primary and secondary antibodies

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(B001-5, D044-3, and D045-6; R&D Systems). The assay was performed on Nunc Maxisorp™ 96-well plates (62407-162; Thermo Scientific). Ferritin levels were measured in the clinical diagnostic laboratory of UMMC. IL-18BPa was assayed by ELISA (DBP180, R&D Systems). All analytes were assayed according to manufacturers' protocols. The bio-availability of free circulating IL-18 (fraction of cytokine not bound to its inhibitor, IL-18BP) was estimated using the law of mass action (Novick *et al* 2001). Briefly, bioavailability of IL-18 was calculated using the molecular weights of IL-18 (18.4 kDa) and IL-18BP (17.6 kDa) and their interaction at a ratio of 1:1 with dissociation constant (Kd) of 0.4 nM (Kim *et al* 2000):

$$K_d = \frac{[Ligand]\cdot[Receptor]}{[Ligand \cdot Receptor]}$$

and was applied as follows:

$$x = \frac{-b + \sqrt{b^2 - 4c}}{2}$$

where χ is $[\text{IL-18}]_{free}$, b is [IL-18BP] –[IL-18] + Kd, c is –Kd•[IL-18] and [ ] the molar concentration of the analyte (Migliorini *et al* 2010).

In the Chennai cohort, IL-18 was assayed in cryopreserved EDTA-treated plasma using an ELISA kit from R&D systems (7620), following the manufacturer's instructions.

4.2.3 Statistical Analysis

The primary analysis was to compare biomarkers among the 3 groups of patients before cART and at the time of TB-IRIS or equivalent time-point (EQT) in controls. Comparisons of categorical variables were tested using chi-square test or Fisher's exact test while continuous variables were tested using the non-parametric Kruskal-Wallis test for multiple group comparisons. If significant differences were seen, each marker was tested separately using Mann-Whitney U tests applying Bonferroni correction. The

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predictive power of pre-cART levels of biomarkers was examined using receiver operating characteristic (ROC) analysis. A cut-off value was obtained from ROC analysis and the odds-ratio for predicting TB-IRIS was calculated using a binary regression model. Correlations were estimated using Spearman's test. Statistical analyses were performed using SPSS, ver.20 and Prism, version 5.02 (GraphPad) softwares. Statistical significance was defined as $p<0.05$ except in analyses that included a Bonferroni correction, where a p value of 0.0042 was considered to be significant.

4.3 Results

4.3.1 Baseline Demography

The pre-cART characteristics for the Kuala Lumpur cohort patients categorized as TB-IRIS (n=15) (comprising paradoxical TB-IRIS (n=9) and unmasking TB-IRIS (n=6), TB no IRIS (n=14), and no TB or IRIS (n=15) are shown in **Supplementary Table 4.1**. We observed that the three patient groups had similar pre-cART CD4+ T-cell counts (p=0.189), CD4+ T-cell percentages (p=0.253), age (p=0.384) and HIV viral load (p=0.739). Furthermore, no significant difference was observed in the interval from ATT to cART initiation between patients with paradoxical TB-IRIS and the TB no IRIS controls (p=0.493). However, there was a significant difference in the form of TB disease at baseline between TB-IRIS and TB no IRIS patients ($p<0.001$), whereby disseminated TB was more prevalent among TB-IRIS patients (66.7%) as compared to TB no IRIS patients (33.3%).

4.3.2 Increased Plasma Levels of IL-18 and Decreased Levels of IFN-γ Prior to Initiation of cART were Consistently Associated with TB-IRIS

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Comparison of all three study groups in the Kuala Lumpur cohort revealed that patients experiencing TB-IRIS exhibited higher plasma IL-18 levels pre-cART compared to the TB no IRIS or the no TB or IRIS controls (both p<0.0001) (**Figure 4.1E**). During the TB-IRIS event (one sample was obtained per patient, ranging from 29-75 days post cART initiation), levels of IL-18 remained higher in comparison with control groups (p=0.0001 and <0.0001 respectively) (**Figure 4.2E**).

As compared to the TB no IRIS or the no TB or IRIS controls, TB-IRIS patients also displayed lower plasma levels of IFN-γ both pre-cART (p=0.003 and <0.0001, respectively) and during TB-IRIS (p=0.0008 and <0.0001, respectively) (**Figure 4.1F, 4.2F**). There was no difference in IFN-γ levels between TB no IRIS controls compared with the no TB or IRIS controls at baseline and the event/EQT (**Figure 4.1F, 4.2F**). Together, we showed that increased plasma levels of IL-18 and decreased plasma levels of IFN-γ prior to cART were associated with the subsequent development of TB-IRIS.

4.3.3 The Development of TB-IRIS was Associated With Increased Plasma Levels of CXCL10 and CXCL8

In view of the previous observation that plasma levels of the chemokines CXCL10 and CCL2 were different in HIV patients with TB-IRIS compared to TB/HIV patients who did not develop an IRIS (Oliver *et al* 2010). Next, we investigated if plasma levels of CXCL10, CCL2 or CXCL8 could be used as potential biomarkers for the prediction or diagnosis of TB-IRIS. Pre-cART plasma levels of CXCL10 were increased in TB-IRIS (p=0.0002) (**Figure 4.1G**). Further analysis of the data revealed that the higher CXCL10 levels were mostly in patients with paradoxical TB-IRIS (**Supplementary Figure 4.1**). During TB-IRIS, CXCL10 and CXCL8, were higher in TB-IRIS patients (p<0.0001 and p<0.0001, respectively) compared to controls (**Figure 4.2G,** and **4.2K**, respectively).

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4.3.4 Higher Plasma Levels of sCD14 were Associated with HIV-TB Co-Infection

To determine if other biomarkers of monocyte and macrophage activation had the potential to predict the onset of TB-IRIS, we also compared plasma levels of ferritin, neopterin, TNF-α, IL-6, IL-10, soluble(s) CD163 and sCD14 in the different study groups. None of these biomarkers were predictive to TB-IRIS (**Figures 4.1A-D, H-L**; **4.2A-D, H-L**). Although sCD163 and sCD14 levels were higher in TB-IRIS patients than no TB or IRIS controls, there were no significant differences between TB-IRIS and TB no IRIS controls. Of note, we observed that, compared with the no TB or IRIS controls, TB no IRIS controls also had higher levels of sCD14 at both pre-cART and EQT ($p<0.0001$ and $p<0.0001$, respectively) (**Figures 4.1D, 4.2D**) These results suggest that increased plasma levels of sCD14 are tightly associated with HIV-TB co-infection.

4.3.5 Plasma Levels of IL-18 and CXCL10 Prior to cART Initiation Predicted the Development of TB-IRIS

Using ROC analyses, the plasma levels of both IL-18 and CXCL10 prior to cART initiation were observed to be strong candidate biomarkers for predicting paradoxical TB-IRIS (AUC=0.99, $p<0.0001$ and AUC=0.884, $p<0.0001$, respectively) (**Figures 4.3A and 4.3B**).

The lower AUC observed for CXCL10 compared with IL-18 may reflect the observation that IL-18 levels were higher pre-cART in both paradoxical and unmasking TB-IRIS whereas CXCL10 levels were higher in only paradoxical TB-IRIS (**Supplementary Figure 4.1**). In contrast, plasma levels of IFN-γ were not predictive to TB-IRIS (**Figure 4.3C**). Based on the ROC analysis, we determined that a cut-off value >8200 pg/ml for IL-18 was strongly predictive of TB-IRIS (OR 175; 95% CI=14-216, $p<0.0001$).

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4.3.6 Plasma Levels of IL-18 Binding Protein may be Low in TB-IRIS Patients

Having demonstrated that plasma levels of IL-18 were elevated two-fold in patients with both paradoxical and unmasking TB-IRIS both pre-cART and during TB-IRIS, and given the role of IL-18 in the NLRP3 inflammasome (Dinarello *et al* 2013), we hypothesized that IL-18, in particular, is linked to the immunopathogenesis of TB-IRIS. IL-18 activity is regulated by IL-18BP, a soluble molecule that binds mature IL-18 with high affinity and prevents its interaction with cell surface receptors. Hence, it restricts the bio-availability of IL-18 (Novick *et al* 1999). Therefore, we next assessed the circulating levels of this regulatory molecule, and demonstrated that although TB-IRIS patients had higher plasma levels of IL-18BPa prior to cART initiation and during TB-IRIS, the levels of free circulating IL-18 were higher in TB-IRIS patients compared to controls (**Figure 4.4A**). ROC analysis showed that, like plasma IL-18 levels, free IL-18 levels were predictive of TB-IRIS (AUC=0.847, p=0.00026 (**Figure 4.4B**). These observations suggest that the balance between IL-18 and IL-18BP in plasma may be critical in setting the stage for TB-IRIS by determining the bio-availability of free IL-18.

4.3.7 Higher Plasma IL-18 Levels in Paradoxical TB-IRIS were In Agreement with the Chennai Cohort

To confirm the potential use of plasma IL-18 levels as a biomarker for predicting TB-IRIS, we assayed plasma levels of this cytokine in an independent cohort of patients from Chennai, South India. Consistent with the results observed in the Kuala Lumpur patients, paradoxical TB-IRIS patients from Chennai exhibited higher plasma IL-18 levels pre-cART (p=0.0043) and during the TB-IRIS event (p=0.0004) compared to HIV/TB patients who did not develop TB-IRIS (**Figure 4.5A**). In addition, the ROC analysis also validated the observations in the Kuala Lumpur patients by demonstrating

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that plasma IL-18 levels predicted paradoxical TB-IRIS (AUC=0.742, p=0.004) (**Figure 4.5B**). A cut-off value of 520 pg/ml for IL-18 predicted TB-IRIS with an OR of 3.3 (95% CI=1-10, p=0.048) (**Figure 4.5C**). Therefore, the predictive value was lower than that observed in the Kuala Lumpur cohort (**Figure 4.3D**).

4.4 Discussion

There are currently no biomarkers that predict TB-IRIS in HIV/TB patients. Inflammation is a prominent feature of an IRIS, and monocytes/macrophages play a critical role in both inflammatory responses and control of MTB infection. Thus, we investigated plasma biomarkers associated with inflammation, with a particular focus on monocytes/macrophages, in a case-control study of HIV/TB patients with either paradoxical or unmasking TB-IRIS. We demonstrated higher plasma IL-18 levels before cART and during TB-IRIS, when compared with controls, and confirmed this in an independent cohort of HIV-infected patients with TB-IRIS. Furthermore, ROC analyses demonstrated that IL-18 levels before cART were the strongest candidate biomarker for predicting both paradoxical and unmasking TB-IRIS. However, differences in the plasma IL-18 level that predicted TB-IRIS, and the certainty with which this prediction could be made, were observed in the two cohorts of patients studied. This finding might reflect the use of different assay methods or differences in patient characteristics and requires further investigation.

Inflammation plays a pivotal role in defending the host from infectious agents, but can become dysregulated and cause deleterious consequences to the host (Serhan *et al* 2007). In the absence of effective T-cell responses, the immune system 'defaults' to innate immune responses and 'upstream' inflammatory markers produced by innate immune cells are particularly important as they govern the downstream inflammatory pathways (Mogensen 2009; Schenten *et al* 2011). In the current study, we observed that

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HIV-TB co-infection was associated with potent monocyte/macrophage activation, as evidenced by elevation of sCD14 prior to cART initiation and during the TB-IRIS event. However, this biomarker did not appear to be as informative as IL-18 for predicting TB-IRIS.

Our findings confirm that plasma IL-18 levels are increased during TB-IRIS (Oliver *et al* 2010) and, furthermore, provide evidence that they predict the development of both paradoxical and unmasking TB-IRIS better than the other biomarkers examined in this study. IL-18 is a potent pro-inflammatory cytokine predominantly produced by activated monocytes/macrophages. It was initially discovered as IFN-γ-inducing factor (IGIF), and has a critical role in enhancing Th1 immune responses. IL-18 is present in monocytes/macrophages as a biologically inactive precursor, pro-IL-18, and requires further cleavage by intracellular cysteine protease caspase-1 via activation of the NLRP3 inflammasome (Novick *et al* 2013). Biologically active IL-18 secreted by monocytes/macrophages induces activation of NF-κB and FasL expression, as well as induction of both CC and CXC chemokines from a wide range of cells (Dinarello *et al* 1998). Furthermore, IL-18 also enhances NK cell cytotoxicity, neutrophil activity and IFN-γ production by T cells and NK cells (Dinarello *et al* 2013).

We would therefore expect the elevation of IL-18 to be accompanied by elevation of IFN-γ among TB-IRIS patients but observed that TB-IRIS was associated with lower plasma IFN-γ levels. Various findings for serum/plasma IFN-γ levels have been reported in different cohorts of TB-IRIS patients (Haddow *et al* 2011; Tadokera *et al* 2011; Conesa-Botella *et al* 2012), including two cohorts of patients also investigated previously by a few other authors of our work (Andrade *et al* 2014). A study of tuberculous meningitis (TBM)-IRIS patients reported similar findings to ours, whereby low CSF IFN-γ levels at TBM diagnosis predicted the development of an IRIS (Marais *et al* 2013). Data from our own studies of TB-IRIS (this study and (Andrade *et al* 2014))

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suggest that plasma IFN-γ levels are related to CD4+ T-cell counts pre-cART. Thus, patients in the Chennai cohort (Andrade *et al* 2014) had the highest IFN-γ levels and a median baseline CD4+ T-cell count of ~200 cells/μl, patients in a South African cohort (Andrade *et al* 2014) had intermediate IFN-γ levels and a median baseline CD4+ T-cell count of ~100 cells/μl and patients in the Kuala Lumpur cohort reported here had the lowest IFN-γ levels and a median CD4+ T-cell count of ~50 cells/μl. Therefore, we proposed that any effect of IL-18 activity on plasma IFN-γ levels is over-ridden by the effect of CD4+ T-cell deficiency.

IL-18 activity is regulated by IL-18BP, a soluble molecule that binds mature IL-18 with high affinity and prevents its interaction with cell surface receptors, hence limiting its biological activity (Novick *et al* 1999). The expression of IL-18BP is regulated by IFN-γ. The promoter region of the IL-18BP gene contains an IFN regulatory factor 1 response element that increases IL-18BP gene expression upon IFN-γ stimulation (Muhl *et al* 2000; Hurgin *et al* 2002). Therefore, IL-18 indirectly increases the production of its own inhibitor in a negative feed-back loop (Boraschi *et al* 2006). We postulated that the inflammatory response driven by increased IL-18 production among TB-IRIS patients might be fuelled by a relative deficiency of IL-18BP and tested this by assaying IL-18BPa levels and calculating free circulating IL-18 levels in plasma. We demonstrated that free IL-18 levels were 40% higher in TB-IRIS patients compared to controls. Published data indicate that IL-18BPa is usually present with a 20-fold molar excess over IL-18 in the plasma of healthy individuals (Novick *et al* 2001). Another *in vitro* study showed that at least a 2 molar excess of IL-18BP was required to neutralize 95% of IL-18 biological activity (Kim *et al* 2000). Therefore, our results suggest that the biological activity of IL-18 may be inadequately regulated in patients with TB-IRIS.

We also observed increased production of CXCL10 and CXCL8 during TB-IRIS, confirming the findings of others for CXCL10 (Oliver *et al* 2010). These chemokines

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reportedly play an important role in the recruitment and activation of T cells, NK cells and neutrophils at the sites of infection (Hoffmann *et al* 2002; Christensen *et al* 2006; Deshmane *et al* 2009). This finding is in line with histopathology findings in biopsy material, which demonstrate that TB-IRIS is associated with granulomata characterized by mixed inflammatory cell infiltrates (Lawn *et al* 2009; Martin-Blondel *et al* 2012). Furthermore, TBM-IRIS patients also exhibit a marked increase in CSF neutrophils (Marais *et al* 2013).

There are several limitations in this study. While the matching strategy led to cases and controls having similar clinical characteristics, our control groups may have been heterogeneous for non-IRIS events. Also due to the difficulties in defining latent TB infection, we do not know how many of the no TB or IRIS controls were ever exposed to MTB or had resolved previous infection. Finally, patient numbers were small and, although our findings on IL-18 in Malaysian patients were confirmed by similar results obtained in an independent cohort of HIV patients with paradoxical TB-IRIS in India, caution must be exercised in interpreting the findings using ROC analyses.

In summary, our data underpin the role of innate immune responses in the immunopathogenesis of TB-IRIS, particularly the role of IL-18, CXCL10 and CXCL8. The elevation of IL-18 among TB-IRIS patients raises the possibility that the pathogenesis of TB-IRIS includes an NLRP3 inflammasome disorder and, furthermore, that the biological activity of IL-18 may be inadequately regulated by IL-18BP. Future study of inflammasome activation and regulation in TB-IRIS is warranted. In addition, plasma levels of IL-18 should be validated as a biomarker of TB-IRIS, and possibly other types of IRIS in large prospective clinical studies.

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SUPPLEMENTARY TABLE 4.1: Clinical Characteristics of Paradoxical and Unmasking TB-IRIS after Commencement of Combination Antiretroviral Therapy.

Study Subject	Patient Group	Age (years)	Gender	Baseline CD4+ T-Cell Count (cells/ul)	Clinical/Radiological Presentation at Diagnosis	TB Culture	Day b/w ATT and cART	Clinical/Radiology Manifestation of TB-IRIS	Day b/w cART and IRIS
# 1	Paradoxical TB-IRIS	45	M	49	Pulmonary TB with pleural effusion and extensive mediastinal lymphadenopathy	Positive	39	High grade fever, increase in size of cervical lymph node	21
# 2	Paradoxical TB-IRIS	44	M	3	Pleural TB , pericardial effusion, cervical and mediastinal lymphadenopathy	Not done	34	Fever (prolonged), increase in size of supraclavicuar lymph node	74
# 3	Paradoxical TB-IRIS	41	M	0	Pulmonary TB with supraclavicular, bilateral cervical and axillary lymphadenopathy,	Positive	29	Cold abscesses, hepatomegaly, new cervical lymph node, increase in size of bilateral supraclavicular lymph node	95
# 4	Paradoxical TB-IRIS	39	M	106	Cervical lymphadenopathy	Not done	49	Recurrence of fever, increase in size of existing lymph node, swelling of new supraclavicular lymph node	31
# 5	Paradoxical TB-IRIS	32	M	116	Pulmonary TB with lymphadenopathy	Positive	16	New lung lesions, increase in size of subcarinal lymph node, new hypodense spleen lesions, fever, cough with haemotypsis	49
# 6	Paradoxical TB-IRIS	44	M	57	Pleural TB and para-aortic lymph node	Positive	82	Fever, cough, new cervical lymph nodes	32
# 7	Paradoxical TB-IRIS	43	M	35	Pulmonary TB with abdominal and mediastinal lymphadenopathy	Positive	31	Increased pulmonary air space opacity	35
# 8	Paradoxical TB-IRIS	41	M	59	Pulmonary TB with lymphadenopathy, bowel wall thickning at the proximal ileum	Positive	36	Fever, worsening of pulmonary lesion, bowel wall thickening in proximal iliem and caecum, new mediastinal, mesenteric and paracaval	35

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								lymphadenopathy	
# 9	Paradoxical TB-IRIS	37	M	116	Pulmonary TB with mediastinal, hilar, inguinal, paraaortic, axillary lymphadenopathy	Negative	14	Low grade fever, increase in size of mediastinal, hilar, axillary, paracaval, paraaortic, iliac and inguinal lymph nodes	28
# 10	Unmasking TB-IRIS	34	M	24	Right apical ground glass changes and cavity. Multiple cavitating lesions bilaterally.	Negative	-	High grade fever, cough, short of breath, tachypnoeic, worsen consolidation cavity of right lung	11
# 11	Unmasking TB-IRIS	31	F	0	Bilateral lower lobe haziness	Negative	-	Bronchiectasis, cough, weight loss, chest pain accompanied by breast lump (with biopsy AFB positive)	77
# 12	Unmasking TB-IRIS	54	M	14	Mild scar over left lower zone	Negative	-	Worsening lung lesions with fibrocystis, mediastinal lymphadenopathy, cough, prolonged fever	25
# 13	Unmasking TB-IRIS	44	M	16	Ground glass appearance	Negative	-	High grade fever (prolonged), short of breath, new mediastinal and bilateral hilar lymph node, hepatomegaly	8
# 14	Unmasking TB-IRIS	44	M	22	Right lung lesions and haziness, submandibular lymph nodes	Negative	-	Worsening lung lesions, new splenic lesions, supraclavicular, mediastinal, para tracheal, pre-cardial, and hilar lymph nodes	42
# 15	Unmasking TB-IRIS	43	M	15	Ground glass appearance	Negative	-	Skin biopsy demonstrated necrotizing granulomatous inflammation, cough, short of breath	75

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Figure 4.1

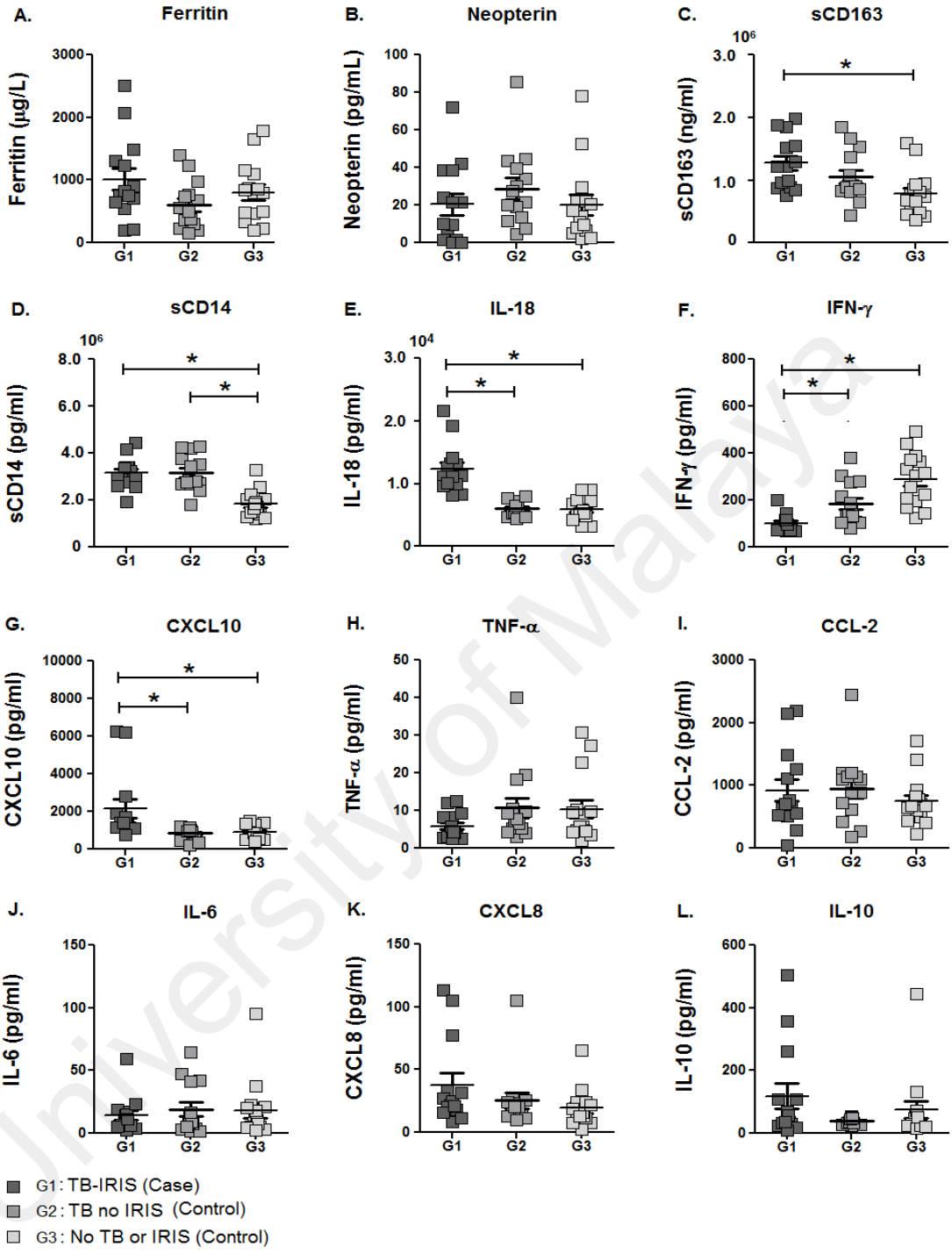

Figure 4.1: Comparison of Plasma Levels of Biomarkers in TB-IRIS Patients and Controls at Baseline.

(A) Ferritin, (B) Neopterin, (C) sCD163, (D) sCD14, (E) IL-18, (F) IFN-γ, (G) CXCL10, (H) TNF-α, (I) CCL2, (J) IL-6, (K) CXCL8, (L) IL-10. Levels of biomarkers were compared across the 3 patient groups by Kruskal-Wallis test. Post-hoc Mann-Whitney U tests were then performed for those biomarkers with a Kruskal-Wallis test p value of <0.05.* p<0.0042 (adjusted for multiple comparisons).

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Figure 4.2

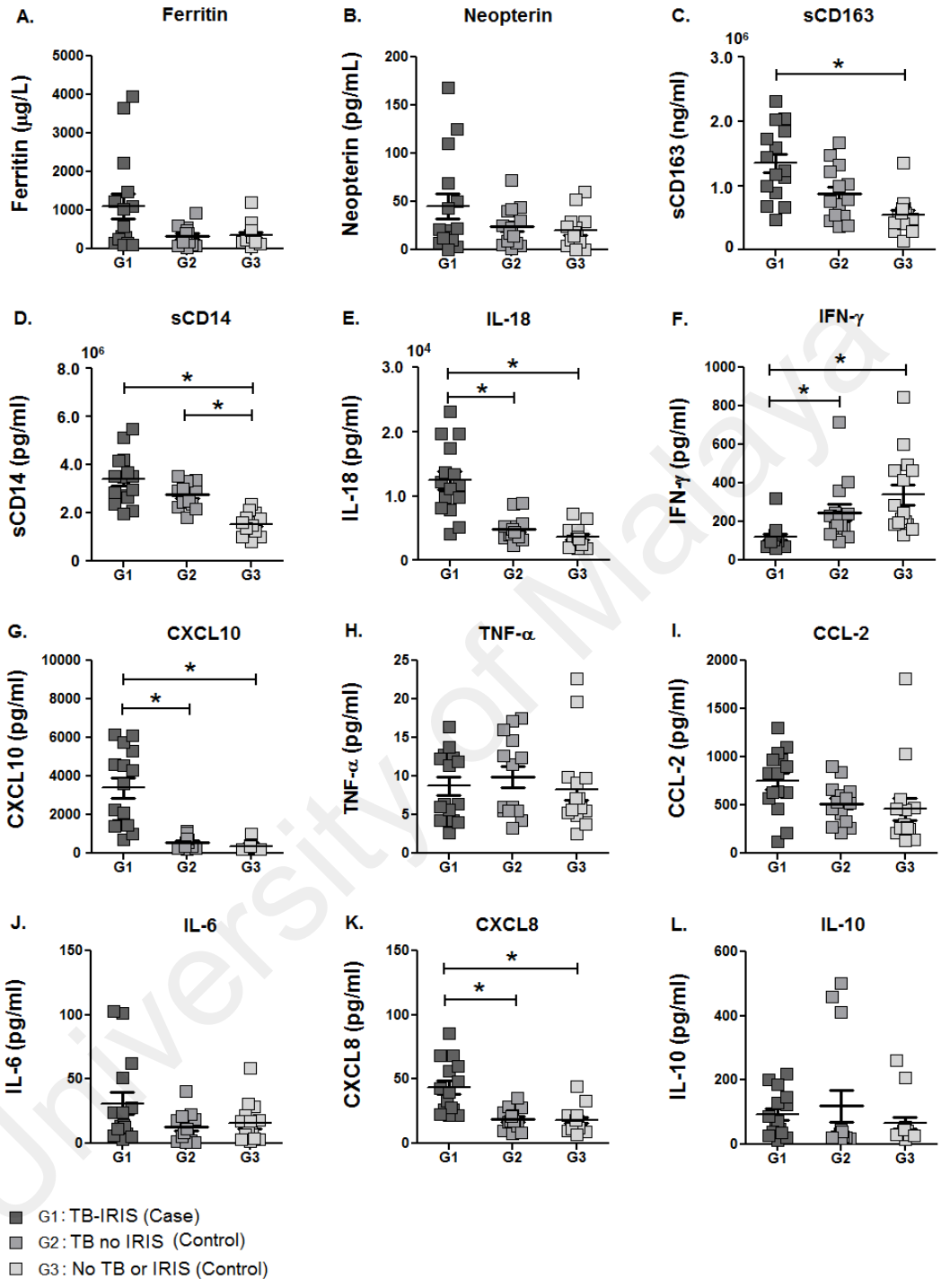

Figure 4.2: Comparison of Plasma Levels of Biomarkers in TB-IRIS Patients and Controls at Clinical Events.

(A) Ferritin, (B) Neopterin, (C) sCD163, (D) sCD14, (E) IL-18, (F) IFN-γ, (G) CXCL10, (H) TNF-α, (I) CCL2, (J) IL-6, (K) CXCL8, (L) IL-10. Levels of biomarkers were compared across the 3 patient groups by Kruskal-Wallis test. Post-hoc Mann-Whitney U tests were then performed for those biomarkers with a Kruskal-Wallis test p value of <0.05. * p<0.0042 (adjusted for multiple comparisons).

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Figure 4.3

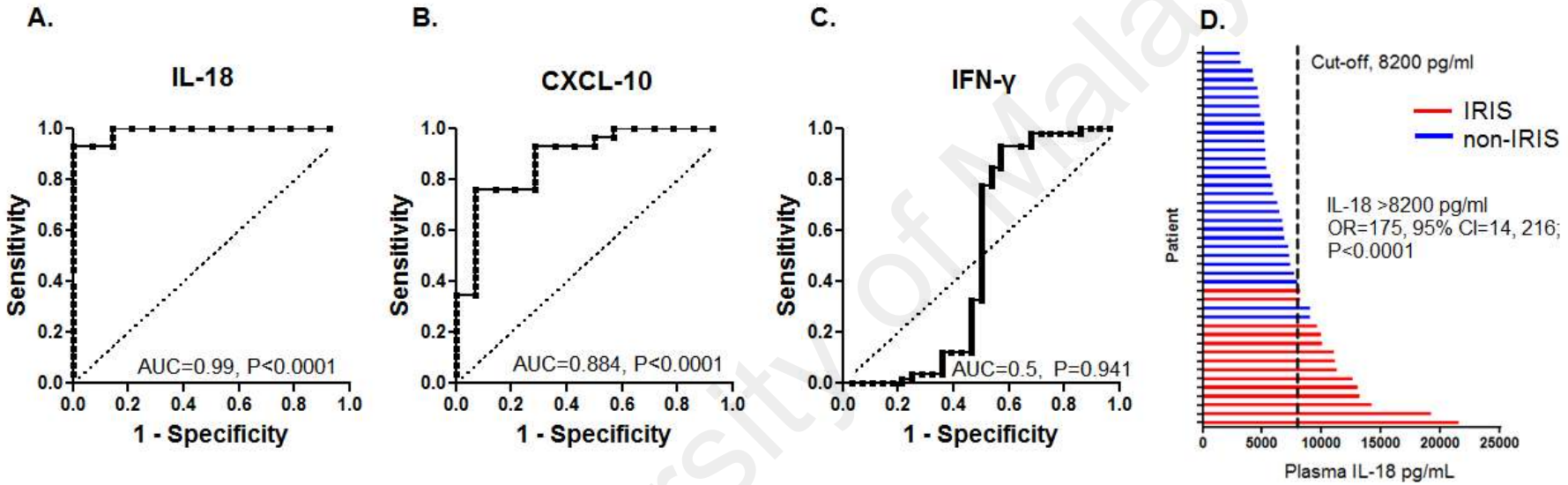

Figure 4.3: Receiver Operating Characteristic Curves for Prediction of TB-IRIS.

(A) IL-18, (B) CXCL-10, (C) IFN-γ and (D) Cut-off of IL-18 value that predicts TB-IRIS. AUC, area under curve.

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Figure 4.4

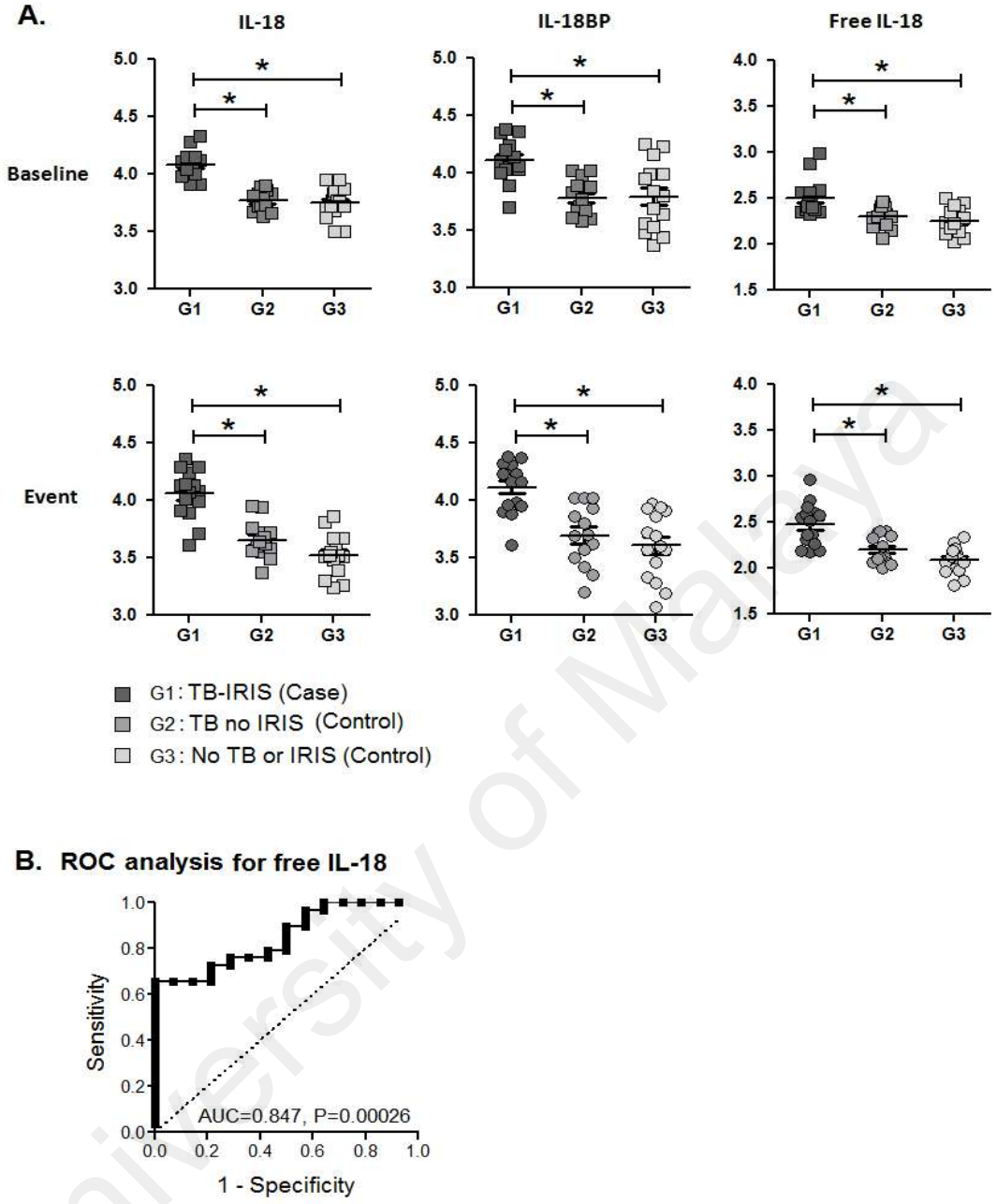

Figure 4.4: Plasma Levels of IL-18, IL-18BPa and Free Circulating IL-18 in TB-IRIS Patients and Controls.

(A) Plasma levels at baseline and time of clinical event. Samples at time of TB-IRIS episode were only collected for case patients; whilst samples in the control groups were selected at an equivalent time-point (EQT) post-cART. Levels of biomarkers were compared across the 3 patient groups and post-hoc Mann-Whitney U tests were then performed for those biomarkers with a Kruskal-Wallis test p value of <0.05. * p<0.0042 (adjusted for multiple comparisons). (B) ROC analysis for the prediction of TB-IRIS using pre-cART levels of free circulating IL-18. AUC, area under curve.

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Figure 4.5

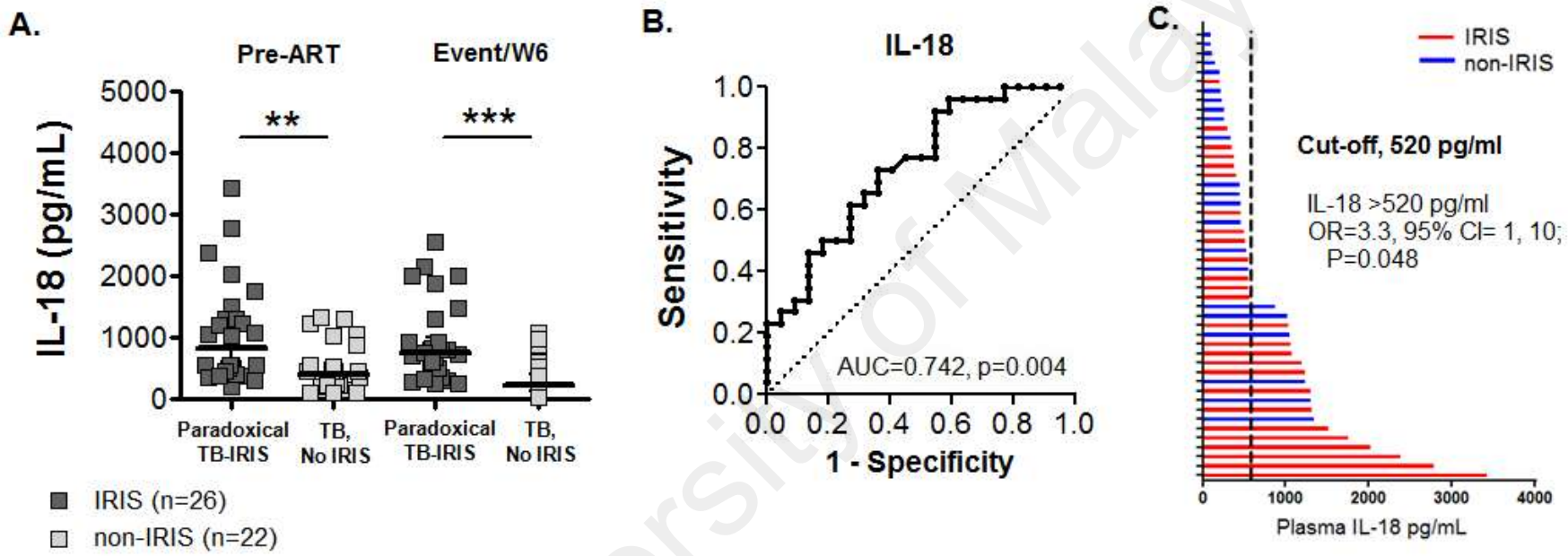

Figure 4.5: Plasma Levels of IL-18 and ROC Analysis for Chennai Cohort.

(A) Comparison of plasma levels of IL-18 between paradoxical TB-IRIS and TB no IRIS. Samples at time of TB-IRIS episode were only collected for case patients; whilst samples in the control groups were selected at 6 weeks post-cART. (B) ROC analysis for the prediction of paradoxical TB-IRIS. (C) Cut-off value of IL-18 that predicts TB-IRIS. W6, 6 weeks, AUC, area under curve. Levels of IL-18 was compared using Mann-Whitney U test, ** $p<0.01$, *** $p<0.001$.

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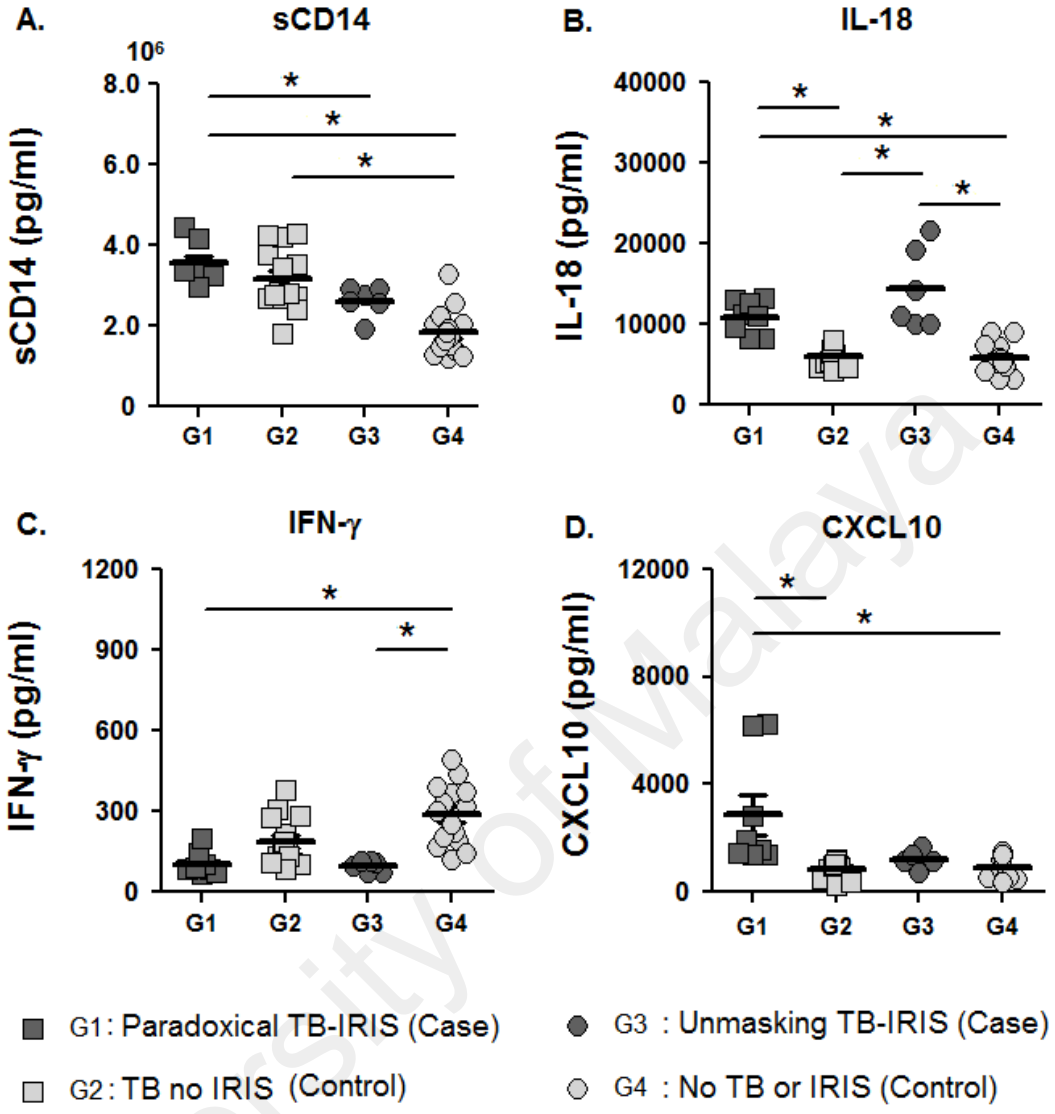
Supplementary Figure 4.1



Supplementary Figure 4.1: Comparison of Plasma Levels of Biomarkers in TB-IRIS Patients and Controls at Clinical Events.

(A) sCD14, (B) IL-18, (C) sCD163 and (D) CXCL10. Levels of biomarkers were compared across the 3 patient groups by Kruskal-Wallis test. Post-hoc Mann-Whitney U tests were subsequently performed for those biomarkers with a Kruskal-Wallis p value of <0.05. * p<0.0042 (adjusted for multiple comparisons).

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CHAPTER 5: ABERRANT INFLAMMASOME ACTIVATION AND PYROPTOSIS CHARACTERISE TUBERCULOSIS-ASSOCIATED IMMUNE RECONSTITUTION INFLAMMATORY SYNDROME

5.1 Introduction

Prior studies have found multiple immunological abnormalities in association with TB-IRIS, including hyperactivation of MTB antigen-specific T cells (Bourgarit *et al* 2006; Tan *et al* 2008) leading to expansion of highly activated (Antonelli *et al* 2010) polyfunctional CD4+ T cells (Mahnke *et al* 2012), excessive production of pro-inflammatory cytokines (Tadokera *et al* 2011; Meintjes *et al* 2012; Narendran *et al* 2013; Ravimohan *et al* 2015), dysfunction of NK cells (Conradie *et al* 2011; Pean *et al* 2012), increased frequency of neutrophils (Marais *et al* 2013), and perturbation in NKT cells (Bourgarit *et al* 2009; Wilkinson *et al* 2015). Nonetheless, the immunopathological mechanisms underlying the development of TB-IRIS are not completely understood, and the cross-talk between different arms of the immune system in TB-IRIS still remain ambiguous.

Recent investigations suggest that TB-IRIS could be associated with expansion of CD14++CD16- monocytes (Andrade *et al* 2014) and activation of monocytes (Tan *et al* 2011; Tran *et al* 2014), which underline the importance of monocytes in TB-IRIS. Furthermore, three geographically-independent HIV infection cohorts from Cambodia, Malaysia, and India have reported that plasma levels of interleukin (IL)-18 could potentially serve as a predictor and/or biomarker of TB-IRIS (Oliver *et al* 2010; Tan *et al* 2015). IL-18 is a pro-inflammatory cytokine belonging to the IL-1 family, mainly produced by monocytes/macrophages and other cells (Gracie *et al* 2003). IL-18 is initially produced as pro-IL-18, a biologically inactive precursor, and is subsequently processed by caspase 1 (casp1) to biologically active IL-18 (Novick *et al* 2013). The

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casp1 itself is generated as a zymogen (inactive enzyme precursor), which is activated via inflammasomes (Netea *et al* 2010; Dinarello *et al* 2013).

5.1.1 Inflammasome: Composition, Structure and Function

The inflammasome is an intracellular multi-protein complex, which following activation assemble together to mediate the secretion of inflammatory casp1, and eventual release of IL-18 and IL-1β (Martinon *et al* 2002; Latz *et al* 2013). The name *inflammasome* is derived from the word *inflammation*, which reflects the function of the complex, and "*some,* is from the Greek word *soma* for *body*. The name also reflects similarities with the apoptosome, which triggers apoptosis (Zou *et al* 1999). Typically an inflammasome consist of three proteins; a scaffold, an adaptor and an effector.

Scaffold protein: Most scaffold protein of inflammasomes contains a member of the nucleotide binding and oligomerization domain (NOD)-like receptor (NLR) family. The domain organization of NLR usually includes **(i)** an amino-terminal protein–protein interaction domain such as a caspase recruitment domain (CARD) or pyrin domain (PYD); **(ii)** a NACHT domain that is required for nucleotide binding and self-oligomerization; and **(iii)** a variable number of carboxy terminal leucine-rich repeat (LRR) motifs involved in sensing pathogen molecules. Similar to the role of mammalian TLRs at the cell surface, these NLRs are sensors for pathogens-associated molecule patterns (PAMPs), and detect conserved microbial components within the intracellular compartments (Kawai *et al* 2006). In addition to these NLRs, the HIN-200 protein absent in melanoma 2 (AIM2), was recently shown to trigger casp1 activation in response to cytoplasmic double-stranded DNA (dsDNA) (Burckstummer *et al* 2009; Hornung *et al* 2009) (**Figure 5.1**)

Adaptor protein: The apoptotic speck-like protein containing a CARD (ASC) is an adaptor protein that plays a central role in the interaction between NLRs and

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casp1 in each of these inflammasome complexes. The ASC contains an N-terminal pyrin domain and a C-terminal CARD domain. This allows one end of ASC to bind the N-terminal pyrin domain of inflammasome-forming NLRs while the other end bind the N-terminal CARD domain allowing recruitment of pro-casp1 (Lamkanfi *et al* 2009; Netea *et al* 2010; Schroder *et al* 2010) (**Figure 5.1**)

Effector protein: Formation of the inflammasome results in autocleavage of pro-casp1 to its active form. Subsequently, casp1 will recruit inactive forms of the cytokines pro-IL-1β and pro-IL-18 and processes them to their active forms (Netea *et al* 2010). Later, these cytokines are rapidly secreted into the extracellular environment leading to inflammation and recruitment of inflammatory cells to the site of injury (Lamkanfi *et al* 2009; Schroder *et al* 2010). To date, four different receptors capable of initiating inflammasome assembly have been characterized. Three belongs to the NLR family (NLRP1b, NLRP3 and NLRC4) and one is an AIM2 protein, which is also a part of PYHIN (pyrin and HIN containing domain) family of proteins (Lupfer *et al* 2012) (**Figure 5.1**).

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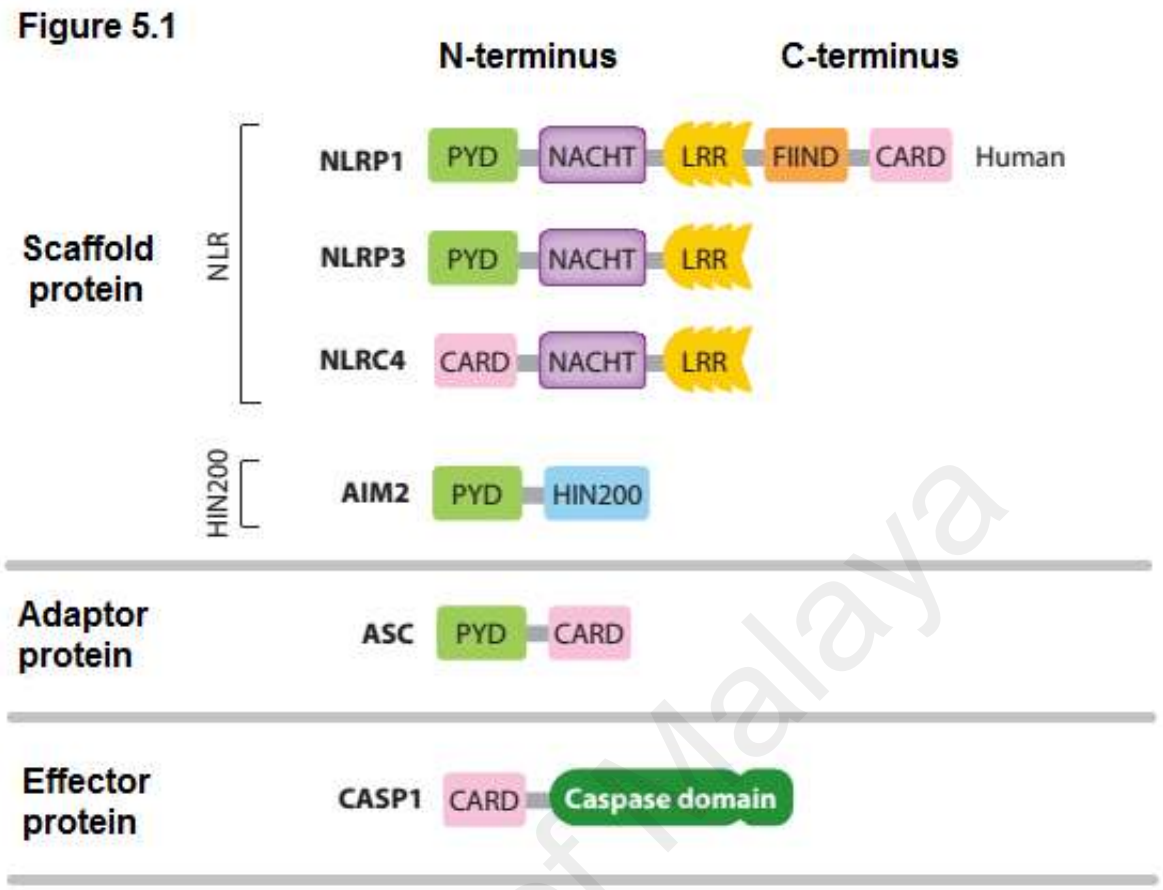

Figure 5.1: Structure of Inflammasome Components.

A subset of NLR protiens (eg. NLRP1, NLRP3 and NLRC4) as well as the AIM2 protein can assembles to form scaffold of inflammasome complexes. NLRs contained a PYD or CARD domain followed by NACHT and a variable number of LRRs. AIM2 characterized presence of N-terminal PYD domain followed by a HIN200, a DNA-binding domain. The adaptor protein, ASC contains a PYD followed by CARD domain; whilst the effector protein ie. pro-casp1 contains a CARD domain followed by casp1. Figure adapted from (Lamkanfi *et al* 2012)

Abbreviations: *NLR*, Nod-like receptor; *PYD*, pyrin; *CARD,* caspase recruitment domain; *NACHT,* nucleotide-binding and oligomerization domain; *LRR,* leucine-rich repeat; *AIM2,* absent in melanoma 2; *ASC,* apoptosis-associated speck-like protein containing a CARD; *CASP,* caspase.

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5.1.2 Overview of Stimuli for Inflammasome Activation.

Depending of the type of stimuli, the NLR proteins (ie. NLRP1, NLRP3 and NLRC4) well as the AIM2 assemble to form inflammasomes in a stimulus-specific manner.

NLRP1: Cells exposed to *Bacillus anthracis* lethal toxin (Boyden *et al* 2006), leading to activation of mitogen-activated protein kinase kinase (MKK) processing (Duesbery *et al* 1998), and K+ (Ali *et al* 2011) and Ca+ (Fink *et al* 2008; Muehlbauer *et al* 2010) effluxes may also activate NLRP1 inflammasome.

NLRP3: Cells exposed to various microbial PAMPs (Bauernfeind *et al* 2011; Bulua *et al* 2011; Kayagaki *et al* 2011), endogenous DAMPs such as ATP, uric acid crystals (Lamkanfi *et al* 2009; Kanneganti 2010) as well as other crystalline particles/nanomaterials such as silica, asbestos (Tschopp *et al* 2010), or even UVB radiation (Keller *et al* 2008) may activate the NLRP3 inflammasome.

NLRC4: Unlike the NLRP3 inflammasome, NLRC4 is less studied. The most notable stimuli for NLRC4 are Gram-negative bacteria components ie. flagellin and their type III secretion systems (Amer *et al* 2006; Miao *et al* 2006; Lamkanfi *et al* 2007)

AIM2: AIM2 can be stimulated by cytosolic double-stranded DNA in the cells mostly infected by bacteria such as *Francisella tularensis* (Fernandes-Alnemri *et al* 2010; Jones *et al* 2010), and *Listeria monocytogenes* (Sauer *et al* 2010), as well as certain DNA viruses, namely cytomegalovirus and vaccinia virus (Rathinam *et al* 2010) or even cytosolic mitochondrial DNA (Nakahira *et al* 2011).

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Figure 5.2

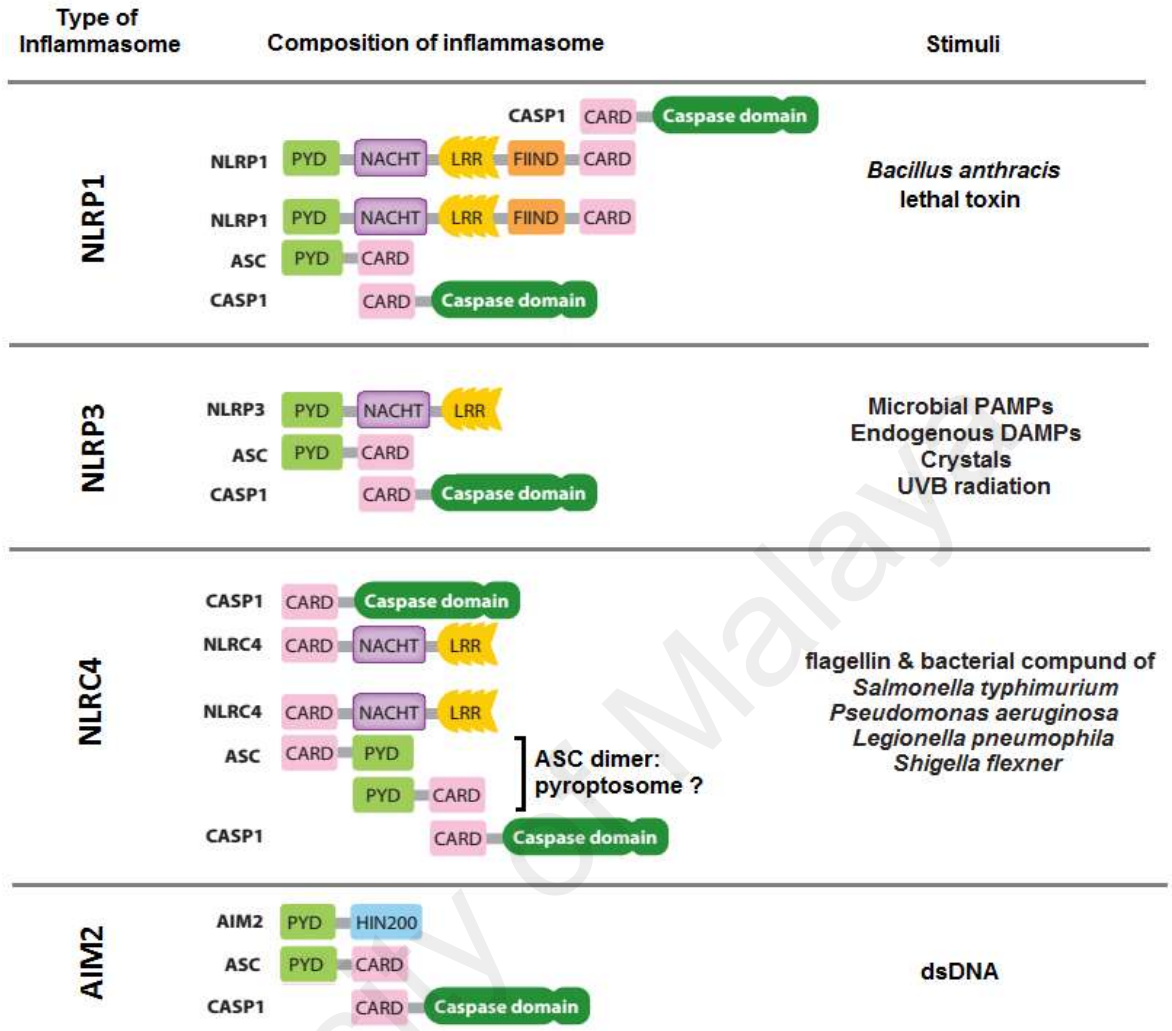

Figure 5.2 Composition and Stimuli of Distinct Inflammasomes.

The PYD domains of AIM2, NLRP1 and NLRP3 recruit the adaptor protein ASC. Alternatively, NLRP1 and NLRC4 may directly interact with the CARD of casp1. NLRC4 may also recruit casp1 indirectly through formation of ASC dimer. **a)** The NALP1 inflammasome recognizes the cytosolic lethal toxin of anthrax. **b)** The NALP3 inflammasome recognizes multiple PAMPs, DAMPs, as well as other crystalline particle such uric acid, silica, and asbestos particles. **c)** The NLRC4 inflammasome senses the cytosolic flagellins and type III or IV secretion system of Gram-negative bacteria. **d)** AIM2 directly binds dsDNA in the cytosol to induce casp1 activation. Once activated, the casp1 will processes the pro-IL-1β and pro-IL-18 precursors to mature cytokines and release into the systemic circulation. Figure adapted from (Lamkanfi *et al* 2012).

Abbreviations: *NLR*, Nod-like receptor; *PYD*, pyrin; *CARD,* caspase recruitment domain; *NACHT,* nucleotide-binding and oligomerization domain; *LRR,* leucine-rich repeat; *AIM2,* absent in melanoma 2; *ASC,* apoptosis-associated speck-like protein containing a CARD; *CASP,* caspase.

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5.1.3 Inflammasome Activation and Pyroptosis

Activation of the inflammasome is also associated with the onset of a form of cell death termed pyroptosis (Fernandes-Alnemri *et al* 2007). Pyroptosis, by definition is a proinflammatory cell death that requires casp1 activity and is characterized mainly in myeloid cells infected with pathogenic bacteria such as *S. typhimurium and F. tularensis* (Jones *et al* 2010; Lamkanfi *et al* 2010; Miao *et al* 2011). However, such cell death may also occur in cells of the central nervous and cardiovascular systems that are often associated with disease conditions (Bergsbaken *et al* 2009).

Pyroptosis has also been implicated as a mechanism of clearance of intracellular pathogens (Terra *et al* 2010). Bacterial pathogens that replicate within macrophages are sensed by inflammasome leading to casp1 activation, which eventually results in pyroptosis. The bacteria are then released from macrophages, which markedly shorten their ability to replicate in the intracellular compartment. The released bacteria are later subjected to additional clearance mechanisms, including phagocytosis by neutrophils (Miao *et al* 2011).

Pyroptosis is a programmed cell-death mode that morphologically distinctly from apoptosis. Pyroptosis involve early plasma membrane rupture, loss of osmoregulation, leading to cytoplasmic swelling andcell burst (Lamkanfi *et al* 2010), thereby releasing the pro-inflammatory cytoplasmic contents into the extracellular space. As opposed to apoptosis, the cell undergoes shrinkage, condensation and fragmentation of nuclear DNA and formation of apoptotic body of which generally non-inflammatory (Taylor *et al* 2008; Lamkanfi *et al* 2011). Nonetheless, apoptosis and pyroptosis also share certain similarities in several biochemical features such as requirement of caspases (although the caspases involved differ), nuclear condensation, and fragmentation of genomic DNA (Lamkanfi *et al* 2010). The **comparison** between apoptosis and pyroptosis are listed in **Table 5.1**.

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Table 5.1: Comparison Between Apoptosis and Pyroptosis		
	Apoptosis	**Pyroptosis**
Mode of cell death	Programmed	Programmed
Initiators	TNF-α, Fas-L, TRAIL, infectious pathogens	DAMPs, microbial infections
Signalling pathway	Mitochondrial pathway, caspase-3, -6, -7 - dependent	NLR-caspase-1 dependent inflammasome
Terminal cellular events	Non-lytic cell shrinkage, DNA fragmentation & apoptotic bodies	Lytic, rapid loss of plasma membrane integrity, pore formation and swelling of cells
Inflammation & immunogenicity	Non-inflammatory +	Pro-inflammatory ++
DAMPs & pro-inflammatory mediators release	Ecto-CRT, HMGB1 and ATP released	HGMG1 and ATP released IL-1α, IL-1β, IL-18, IL-6

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5.1.4 Aim

The goal of the study was to investigate the role inflammasome activation and pyroptosis in monocytes derived from HIV-infected patients presenting with TB-IRIS.

5.2 Materials and Methods

5.2.1 Patient Enrolment and Selection of TB-IRIS Cases and Controls

Patients were selected from a prospective observational cohort study of 200 HIV-infected cART-naïve adults (>18 years) initiating cART between 2005 and 2012 at the UMMC, and biological specimens (plasma and PBMCs) were collected as previously described (Tan *et al* 2015). Participants were classified into three groups as TB-IRIS (including both paradoxical and unmasking TB-IRIS cases), TB no IRIS (controls) and no TB or IRIS (controls) according to the definitions previously described (Tan *et al* 2015).

5.2.2 Collection of Peripheral Blood Mononuclear Cells

Ten milliliters of blood was collected from all subjects by venepuncture in lithium heparin Vacutainer® tubes (BD Biosciences, Franklin Lakes, USA). PBMCs were extracted from the whole blood samples within 3 hours post-collection by using density-gradient centrifugation (Ficoll®Paque Plus, GE Healthcare, Uppsala, Sweden). The cell viability was determined by trypan blue staining (0.4%). The cells were then were resuspended in 10% DMSO in FBS and cryopreserved until use.

5.2.3 Antibodies and Immunophenotyping of Monocytes, T Cells and NK Cells

Immunostaining of monocytes and T cells was performed according to the manufacturer's protocol (BD Biosciences, USA). Fluorochrome-labelled monoclonal antibodies (mAbs) against human CD14-Alexa Fluor 488 (cat. 557718), CD14-APC

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(cat. 555399), CD16-PE-Cy7 (cat. 557744), CD16-PE (cat. 55407), CD64-APC-H7 (cat. 561190), CD11B-PE (cat. 557701), CD69-APC-H7 (cat. 562884), CD38-PE (cat. 555460), CD56-PE-Cy7 (cat. 557747), CD36-PerCP-Cy5,5 (cat. 561536) were from BD Pharmingen. The antibodies against human CD3-PerCP (cat. 347344), CD4-APC-H7 (cat. 340584) and CD8-APC (cat. 340584) were procured from BD Biosciences. The antibody against IL-18Rα, CD218-FITC (cat. 313810) was from BioLegend.

5.2.4 Assay of Activated Caspase 1

PBMCs ($5x10^5$) were suspended in 100µL FACS buffer (5% FCS in PBS) and were stained with FLICA® 660 Caspase 1 (ImmunoChemistry Technologies, Cat. #9122), a fluorescent-labelled inhibitor of caspases probe, that specifically detects activated casp1. The staining was done according to the manufacturer's protocols. Briefly, cells were incubated with FLICA-casp1 reagent for 30 min at 37°C and washed 3 times in washing buffer (Doitsh *et al* 2014). Subsequently, cells were stained with anti-CD14 and anti-CD16 as described in the following sections, and events were acquired on a BD FACSCanto™ II system (BD Biosciences, USA).

5.2.5 Assay of Cell Markers of Pyroptosis

Cellular expression of markers of apoptosis and pyroptosis was determined by flow cytometry after staining of PBMC with FITC-annexin-V and 7-AAD (cat. 640922, Biolegend, USA). Annexin V stains phosphatidylserine (PS), the inner leaflet of cell membranes and expression is common to both apoptosis (Koopman *et al* 1994) and pyroptosis (Miao *et al* 2011). Conversely, 7-AAD is an impermeable dye that does not stain apoptotic cells and only stains pyroptotic cells by entering the cell through pores on the cell membrane (Fink *et al* 2006; Miao *et al* 2011)

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5.2.6 Assay of Plasma Nitric Oxide and Cytokines

Plasma levels of NO were measured using Griess reagent procured from Promega (cat. TB229). Data on plasma IFN-γ and IL-18 have been previously published (Tan *et al* 2015).

5.2.7 RNA Isolation, cDNA Synthesis and Quantification of mRNA

Monocytes were enriched by a plastic adherence method (de Almeida *et al* 2000). Briefly, $2.5x10^6$ PBMCs were transferred into a polystyrene 24-well plate (BD, cat. 353047), and incubated at 37°C for 30 minutes in a 5% CO_2 incubator. Non-adherent cells were washed three times with PBS. Adhered monocytes were gently harvested and re-suspended in 380µL of RLT lysis buffer and RNA was extracted using RNAeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA concentrations were measured at 260/280nm in a Take3 micro-volume plates (Bio-Tek). First strand cDNA synthesis was performed with High Capacity cDNA Reverse Transcription kit (ABI, cat. 4374966) using 2µg of total RNA according to manufacturer's instructions.

The quantitative PCR assay of inflammasome related mRNA was performed in an ABI ViiA 7 real-time PCR system using TaqMan Gene Expression Master Mix and Gene Expression Assay Kits (*NLRP1*: Hs00248187-m1, *NLRP3*: Hs00918082-m1, *NLRC4*: Hs00892666_m1, *AIM2*: Hs00915710-m1, *ASC*: Hs00203118, *Caspase-1*: Hs00354836-m1). *iNOS* mRNA was measured using iNOS-F 5'-TAGAGG AACATCTGGCCAGG and iNOS-R 5'-TGGCAGGGTCCCCTCTGATG. *TBP* (Hs00427620_m1) and *SDHA* (Hs00417200_m1) were used as endogenous RNA controls. The PCR were performed using the following thermal cycling profile: 10 min of initiation at 95ºC, followed by 40 cycles of denaturing at 95ºC for 15 s and annealing-extension at 60ºC for 1 min. The expression levels of the target mRNA in

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each sample were calculated by the $2^{-\Delta Ct}$ method. Normalization was performed by averaging the Ct values of the two housekeeping genes (Belibasakis *et al* 2012).

5.2.8 Quantitation of Plasma mtDNA

Cell-free mtDNA was extracted using a previously published plasma fractionation method (Lauring *et al* 2012). Pre-cART plasma levels of mtDNA obtained were measured by quantitative real-time PCR as per standard defined protocols using a pair of primer CoxF (5' ATG ACC CAC CAA TCA CAT GC 3'), CoxR (5' ATC ACA TGG CTA GGC CGG AG 3') (Lauring *et al* 2012). Further, we also designed a TaqMan® probe targeting this region (5' FAM-CCA TGA CCC CTA ACA GGG GC-MGB 3')

5.2.9 Statistical Analysis

The primary analysis was to compare biomarkers among the three groups of patients pre-cART and at the time of TB-IRIS, or between 4-12 weeks post-cART in controls. Continuous variables were tested using the non-parametric 1-way ANOVA (Kruskal–Wallis) for multiple group comparisons followed by pair-wise comparison by Mann-Whitney U tests for biomarkers with a Kruskal-Wallis test $p<0.05$ (**Figure 1B** and **1D**, **Figure 2**, **Figure 3B** and **3D** middle panel, **Figure 4A** and **4B**, **Figure 5**, **Figure 6B** lower panel and **6C**). Three-way subset analyses for piechart were performed using non-parametric 1-way, followed by pair-wise comparision using Mann-Whitney u test (**Figure 6B** upper panel). Spearman rank tests were used to assess the correlation between two continuous variables. Wilcoxon matched-pairs test for paired analyses for pre- and post-cART (**Figure 1C** and **Figure 6D-F**). Spearman rank test was used to compare the correlation between two continuous variables (**Figure 1E**, **Figure 3C** and **3D** right panel, **Figure 4C**, **Figure 5B** as in heatmap). Statistical analyses were performed using Prism, version 5.02 (GraphPad Software Inc., San Diego, California,

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USA). Binary regression was performed using SPSS, version 20 (Armonk, New York, USA) and heatmap (**Figure 5B**) was generated using Plotly (https://plot.ly/). Data are expressed as mean ± standard deviation and statistical significance was defined as *p<0.05, **p<0.01, ***p<0.001, **** p<0.0001.

5.2.10 Ethics Approval

The study was approved by the Medical Ethics Committee of UMMC (MEC Ref. No: 673.33). Written informed consent was obtained from all the study participants.

5.3 Results

5.3.1 Development of TB-IRIS was Associated with Elevation of Activated Casp1 In Monocytes Pre-cART

To determine if increased IL-18 production in TB-IRIS (Oliver *et al* 2010; Tan *et al* 2015) might reflect inflammasome activation, we first measured the expression of activated casp1 using a fluorescent-labelled inhibitor of caspases (FLICA)-casp1 probe, which only produces flurescence upon binding to activated casp1, in the PBMCs of HIV patients with TB-IRIS, and controls with TB no IRIS, and no TB or IRIS (**Figure 5.3A**). We found that before cART was commenced (pre-cART), the proportion of monocytes expressing activated casp1 was higher in patients with TB who subsequently developed TB-IRIS (n=16) (median: 77.5; IQR: 71.5-88.4) or did not develop TB-IRIS (n=16) (median: 81.1; IQR: 72.9-89.1) compared to those patients who did not have TB (n=19) (median: 69.9; IQR: 54.9-75.3)(p=0.004). There were similar findings for lymphocytes. These differences were not apparent during TB-IRIS or post-cART (between 4 and 12 weeks post-cART initiation) in controls (**Supplementary Figure 5.1A**).

Having shown that the proportion of monocytes and lymphocytes expressing activated casp1 was higher pre-cART in HIV patients with TB compared to those without TB, we

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next compared the expression level of activated casp1, assessed by mean fluorescence intensity (MFI), in the TB-IRIS and TB no IRIS groups. There was no difference pre-cART (**Figure 5.3B**) but expression of activated casp1 was higher in TB-IRIS patients (median=1050; IQR=1000-1134) during the TB-IRIS event compared to TB no IRIS (median=897; IQR=784-985; p=0.005) and no TB or IRIS (median=792; IQR=734-839; p<0.0001) patients post-cART (**Figure 5.3B**). We also examined the increase in expression of activated casp1 in monocytes of TB-IRIS patients, compared with pre-cART values, and demonstrated an average increment of 1.15 fold (p=0.017, Wilcoxon paired test) (**Figure 5.3C**). A similar pattern for increase in both proportions of casp1+ lymphocytes, and level of expression (MFI) of activated casp1 in lymphocytes, was also observed post-cART, although there was no difference between values pre-cART and during TB-IRIS (Wilcoxon paired-test) (**Supplementary Figure 5.1B, 5.1C**). The elevation of activated casp1 expression among TB-IRIS patients at event was further confirmed by an analysis of the level of *casp1* in monocytes (**Figure 5.3D**). These findings provide evidence that HIV-infected patients with TB have increased proportions of monocytes expressing activated casp1 before cART is commenced and that the level of expression further increases and to a greatest degree in individuals who develop TB-IRIS after commencement of cART.

To further examine the relationship between increased expression of activated casp1 in monocytes and development of TB-IRIS, we assessed the relationship of TB-IRIS with activated casp1 expression (MFI) in monocytes pre-cART in combination with other risk factors previously identified (Tan *et al* 2015) (baseline CD4+ T-cell count, TB clinical presentation (pulmonary versus extra pulmonary versus disseminated TB), and the interval between anti-TB therapy and initiation of cART) using a binary regression model (graph not shown) adjusted for gender and age. We found that the level of activated casp1 was associated with development of TB-IRIS to a greater degree than

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the other risk factors, whereby every increase of 150 units in MFI of casp1 expression was associated with increased risk of TB-IRIS by 2.49 [95% CI=1.03 - 6.02; (p=0.043)]. Furthermore, activated casp1 expression in monocytes was strongly correlated with plasma IL-18 levels post-cART (r=0.64; p<0.0001) (**Figure 5.3E**). Together, our findings indicate that HIV/TB co-infected patients exhibit increased proportions of casp1+ monocytes pre-cART and that the level of activated casp1 expression increases to the greater degree in individuals who develop TB-IRIS after commencement of cART.

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Figure 5.3

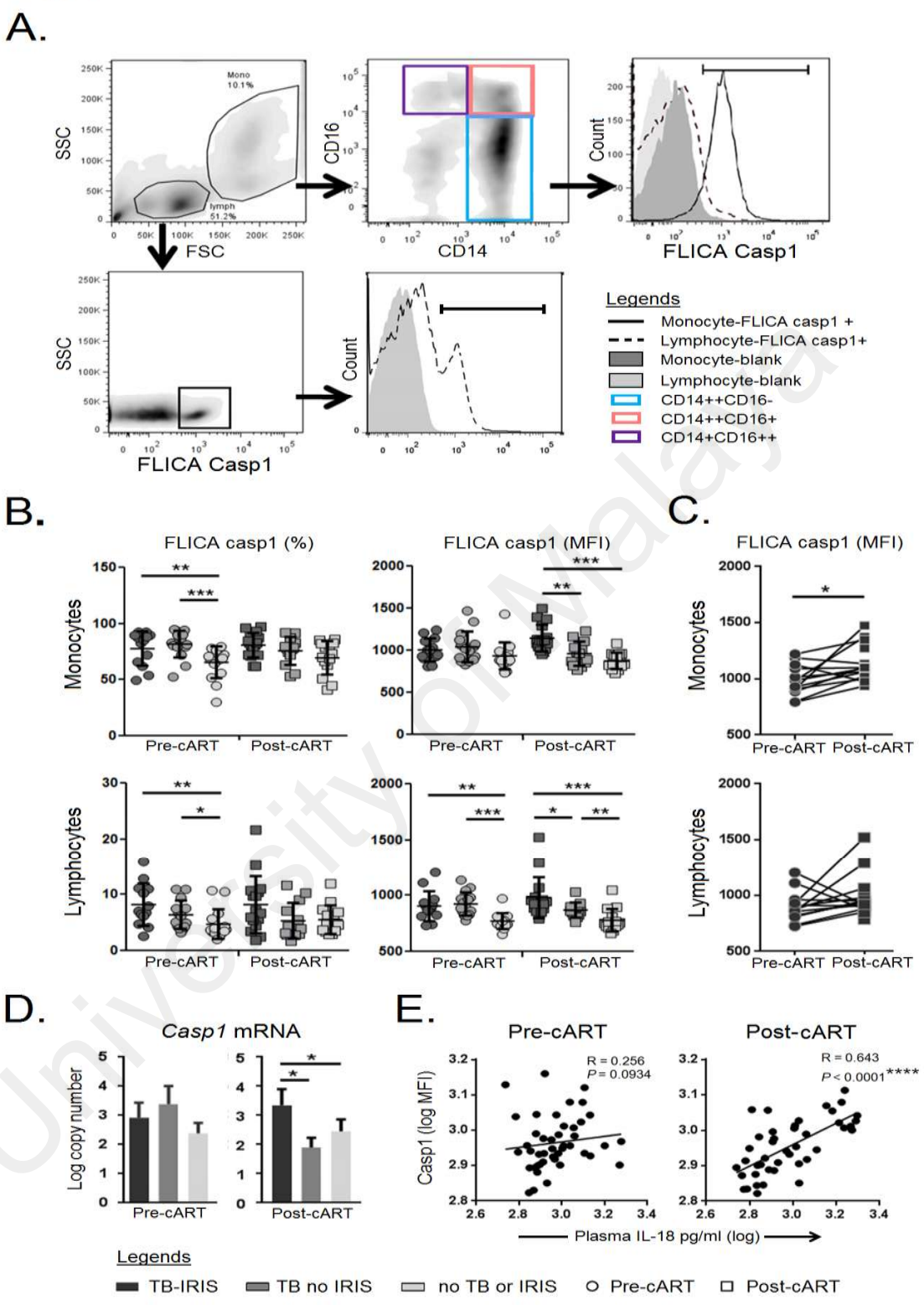

Figure 5.3. Expression of Activated Caspase-1 in Monocytes and Lymphocytes of HIV-Infected Patients with TB-IRIS, TB no IRIS and no TB or IRIS.

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(A) Representative FACS plots showing the distribution of monocyte subsets (top bars), defined by CD14 and CD16 markers, and lymphocytes (bottom bars) expressing activated casp1. **(B)** Percent of total monocytes and lymphocytes expressing activated casp1 and their respective mean fluorescence intensity (MFI) at baseline (pre-cART) and at the time of IRIS or weeks 4-12 following cART in patients with TB-IRIS (post-cART) (n=16), TB no IRIS (n=16) or no TB or IRIS (n=19). **(C)** Changes in activated casp1 expression at baseline (pre-cART) and post-cART. **(D)** Quantification of *Casp1* normalized against stable housekeeping genes *TBP* and *SDHA* in monocytes. **(E)** Correlation between plasma IL-18 levels and activated casp1 expression in monocytes (MFI) pre- and post-cART. Data were analyzed using Kruskal-Wallis test across three patient groups. Post-hoc Mann-Whitney U tests were then performed for those biomarkers with a Kruskal-Wallis test p value of <0.05. Wilcoxon matched-pairs test for paired analyses within each study group. Spearman rank test to compare the correlation between two continuous variables. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Abbreviations: Casp1, caspase 1; *TBP*, TATA box binding protein; *SDHA,* succinate dehydrogenase complex, subunit A

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5.3.2 The Onset of TB-IRIS was Associated with Upregulation of Inflammasome Gene Expression

Given that inflammasomes are the activators of casp1, we next examined the relationship between development of TB-IRIS and markers of inflammasome activation. Firstly, we used qRT-PCR to analyse the differential expression of *NLRP1, NLRP3, NLRC4* and *AIM2* mRNA in monocytes pre-cART and at the time of TB-IRIS or post-cART in patients with TB no IRIS and no TB or IRIS. Pre-cART, *NLRP1* and *NLRC4* levels were higher in TB no IRIS patients compared to TB-IRIS and no TB or IRIS patients. *AIM2* levels were higher in both TB-IRIS and TB no IRIS as compared to no TB or IRIS, whilst *NLRP3* was not different among the three groups of patients (upper panel **Figure 5.4A**). During TB-IRIS, *NLRP1, NLRP3,* and *AIM2* levels were higher in TB-IRIS patients compared to the other two groups post-cART (lower panel **Figure 5.4A**). In addition, when compared with the expression level of mRNA of the internal reference genes (*TBP* and *SDHA*), expressions of *NLRP1, NLRP3, NLRC4* and *AIM2* were 2.9-fold, 167-fold, 3.2-fold and 7.9-fold higher, respectively, during TB-IRIS. When mRNA expression levels during TB-IRIS were compared to levels pre-cART, we found that the fold-changes for *NLRP1, NLRP3, NLRC4, and AIM2* were 1.1 fold, 1.6 fold, 1.3 fold and 2.2 fold, respectively (**Figure 5.4B**). These findings provide evidence that TB-IRIS is associated with up-regulation of genes encoding components of the NLRP3 and AIM2 inflammasomes, and possibly other inflammasomes.

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Figure 5.4

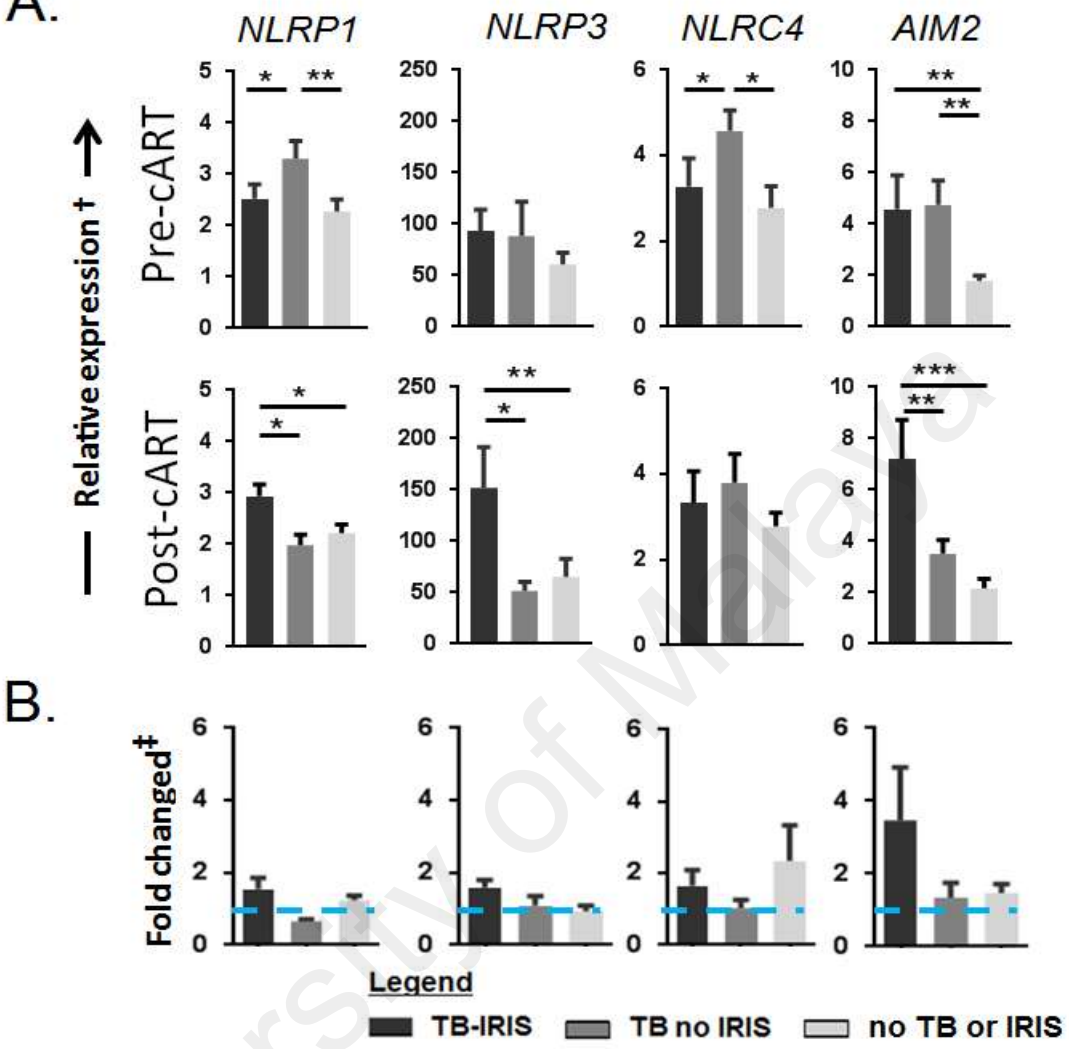

Figure 5.4. Expression Profile of mRNA of Inflammasomes in Patients with TB-IRIS, TB no IRIS and no TB or IRIS.

(A) Comparison of mRNA expression profile of 4 major inflammasomes *NLRP1, NLRP3, NLRC4* and *AIM2* across the three patient groups. Data were analyzed using the Kruskal-Wallis test followed by a post-hoc Mann-Whitney U tests for those genes with a Kruskal-Wallis test p value of <0.05. *p<0.05, **p<0.01, ***p<0.001. **(B)** Fold change of inflammasome-related mRNA. The blue line indicates fold change = 1. †, normalized against housekeeping genes *TBP* and *SDHA*; ‡ levels of mRNA at event normalized to baseline mRNA levels.

Abbreviations: NLRP, nucleotide-binding domain and leucine-rich repeat protein; NLRC4, Nucleotide-binding oligomerization domain, NOD, (NOD)-like receptor C4; AIM2, absent in melanoma 2.

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5.3.3 TB-IRIS was Associated with Markers of Cell Death with Changes of Pyroptosis Most Conspicuous Pre-cART

Apart from it's association with increased production of IL-18, casp1 also facilitates pro-inflammatory programmed cell death, termed pyroptosis (Bergsbaken *et al* 2009). Therefore, we determined the proportion of expression of Annexin V and 7-aminoactinomycin D (7-AAD) in PBMCs, monocytes and lymphocytes of TB-IRIS patients and control groups. Pyroptosis is characterized by expression of 7-AAD whereas apoptotic cell death is characterized by expression of Annexin V without 7-AAD (**Figure 5.5A**) (Miao *et al* 2011). Pre-cART, proportions of 7-AAD+ PBMCs were higher in TB-IRIS patients (median=6.3%, IQR=2.6-11.2%) compared to TB no IRIS (median=4.4%, IQR=1.6-5.4%, p=0.046) and no TB or IRIS (median=1.6%, IQR=1.1-2.9%, p=0.0001) patients (**Figure 5B**). At TB-IRIS event, proportions of 7-AAD+ PBMC (median=4.4%, IQR=3.2-9) and Annexin V+/7-AAD- PBMC (median=24.3%, IQR= IQR=22-28.4%) were higher among TB-IRIS patients as compared to the TB no IRIS (median=2.2%, IQR=1.2-6%, p=0.036; median=13.3%, IQR=9.2-18.4%, p<0.0001, respectively) and no TB or IRIS (median=1.4%, IQR=0.8-3%, p=0.0005; median=11.1%, IQR=7.5-15.5%, p<0.0001 respectively) (**Figure 5.5B**). There was also a similar trend in monocytes (**Figure 5.5B**) but not lymphocytes (data not shown).

Next, we correlated the proportion of PBMCs expressing markers of cell death with expression level (MFI) of activated casp1 in monocytes. Activated casp1 expression did not correlate with markers of cell death pre-cART but correlated with molecules associated with pyroptosis (r=0.502, p=0.0006) and apoptosis (r=0.468, p=0.0016) during TB-IRIS (**Figure 5.5C**).

Pyroptosis appears to have an association with release of intracellular contents into the systemic circulation (LaRock *et al* 2013), and hence we determined if an increase in

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inflammatory cellular components was evident in the plasma of TB-IRIS patients compared with control groups. To address this, we used a well-evaluated method to measure levels of cell-free mitochondrial DNA (mtDNA) (Lauring *et al* 2012), which indicates disruption of the cellular membrane. Plasma cell-free mtDNA pre-cART was higher in TB-IRIS patients (log copy number, median=3.8, IQR=3.4-4.1) compared to TB no IRIS (log copy number, median=3.3, IQR=2.8-3.4; p=0.0008) and no TB or IRIS (log copy number, median=3.4, IQR=3.1-3.6; p=0.005) patients. At event, the TB-IRIS patients showed higher cell-free mtDNA in plasma compared to the other groups, although this was not statistically significant (Kruskal-Wallis test, p=0.065) (**Figure 5.5D, middle panel**). Using the Spearman test, we also investigated the correlation of plasma mtDNA with IL-18, activated casp1 expression and markers of cell death. We found that mtDNA levels correlated with IL-18 levels alone ($r=0.4225$, $p<0.0001$ for combined pre- and post-cART values) (**Figure 5.5D**, right panel). Taken together, our data suggest that TB-IRIS is associated with a higher degree of cell death amongst PBMC, with changes of pyroptosis dominating pre-cART and evidence of both pyroptosis and apoptosis post-cART.

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Figure 5.5

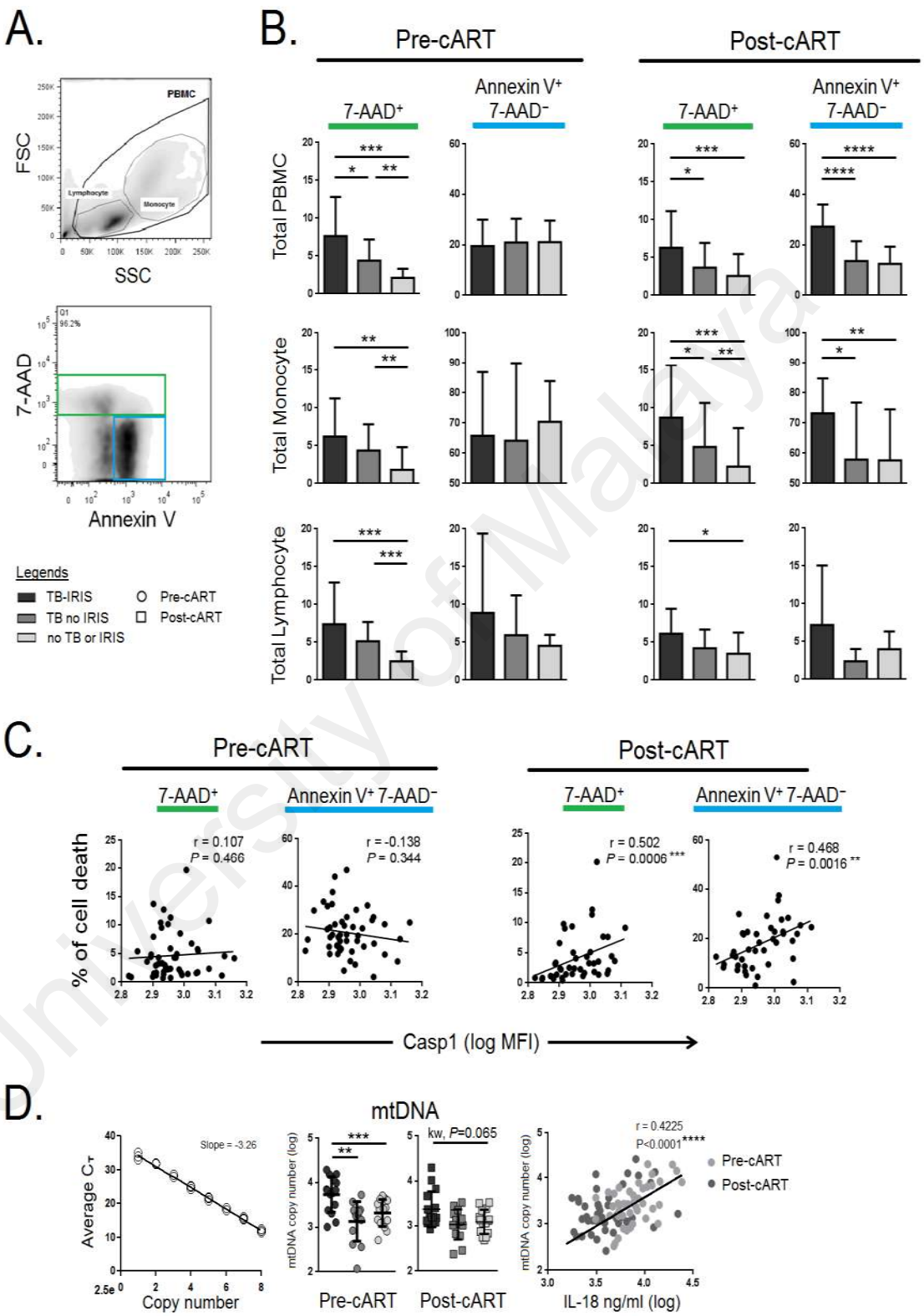

Figure 5.5. Cellular Expression of Markers of Pyroptotic Cell Death Among Patients with TB-IRIS, TB no IRIS and no TB or IRIS.

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(A) Representative FACS plots showing the distribution of pyrototic (7-AAD+) as well as apoptotic (Annexin V+/7-AAD-) cell death. **(B)** Comparisons of percent of pyroptotic cell death between patient groups TB-IRIS, TB no IRIS and no TB or IRIS. **(C)** Correlation analysis between expression of activated casp1 (log MFI) and markers of pyroptosis and apoptosis in monocytes of all three patient groups. **(D)** Plasma levels of mtDNA distinguished individuals with TB-IRIS and correlated with IL-18 levels. *Left panel:* shows dynamic range for qPCR assay for mtDNA; *middle panel:* shows comparison of plasma mtDNA level between the three clinical groups; *right panel*: shows correlation between mtDNA and IL-18 at baseline. Data were analyzed using the Kruskal-Wallis test across the three patient groups. Post-hoc Mann-Whitney U tests were then performed only for those biomarkers with a Kruskal-Wallis test p value of <0.05. Spearman rank test were used to assess the correlation between two continuous variables. KW, Kruskal-Wallis test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Abbreviations: 7-AAD, 7-Aminoactinomycin D; mtDNA, Mitochondrial DNA

5.3.4 TB-IRIS was Associated with Correlates of Decreased Regulation of Inflammasome Activity

Activation of inflammasomes is a tightly regulated process, and two factors, interferon (IFN)-γ (Mishra *et al* 2013) and nitric oxide (NO) (Mao *et al* 2013; Mishra *et al* 2013; Rayamajhi *et al* 2013) have been shown to down-regulate the activity of the NLRP3. We previously reported that plasma INF-γ levels were decreased among TB-IRIS patients both pre-cART and at the time of TB-IRIS (Tan *et al* 2015). Here, we investigated if plasma levels of NO were also lower amongst patients with TB-IRIS. Using the Griess method (Mishra *et al* 2013), we measured plasma NO levels pre-cART and found that they were lowest in TB-IRIS patients (median=30μM, IQR=16-46) followed by TB no IRIS (median=47μM, IQR=23.7-66.7; p=0.0015) and no TB or IRIS

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(median=79 μM, IQR=66-107; p<0.0001). Nevertheless, plasma NO levels were not different between the study groups post-cART (**Figure 5.6A**).

Next, we investigated if TB-IRIS patients had evidence of impaired NO production by monocytes. To address this, we measured the mRNA levels of inducible nitric oxide synthase (*iNOS*) using qRT-PCR. We found that the mRNA levels of *iNOS* were comparable between the three groups pre- and post-cART (**Figure 5.6B**).

Finally, using Spearman correlation test, we investigated if plasma NO levels correlated with plasma levels of IFN-γ and IL-18 and expression of activated casp1 in monocytes. Pre-cART, NO levels correlated positively with IFN-γ (r=0.51, p=0.0004) but inversely with IL-18 (r=-0.44, p=0.003) and activated casp1 expression (r=-0.53, p=0.0001) (**Figure 5.6C**).

Together, these findings provide evidence that NO production is lower pre-cART in HIV patients who subsequently developed TB-IRIS, in association with lower production of IFN-γ. The negative correlation of NO and IFN-γ levels with IL-18 levels and casp1 expression in monocytes suggests that inflammasome activity is inadequately regulated in patients who develop TB-IRIS after commencing cART.

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Figure 5.6

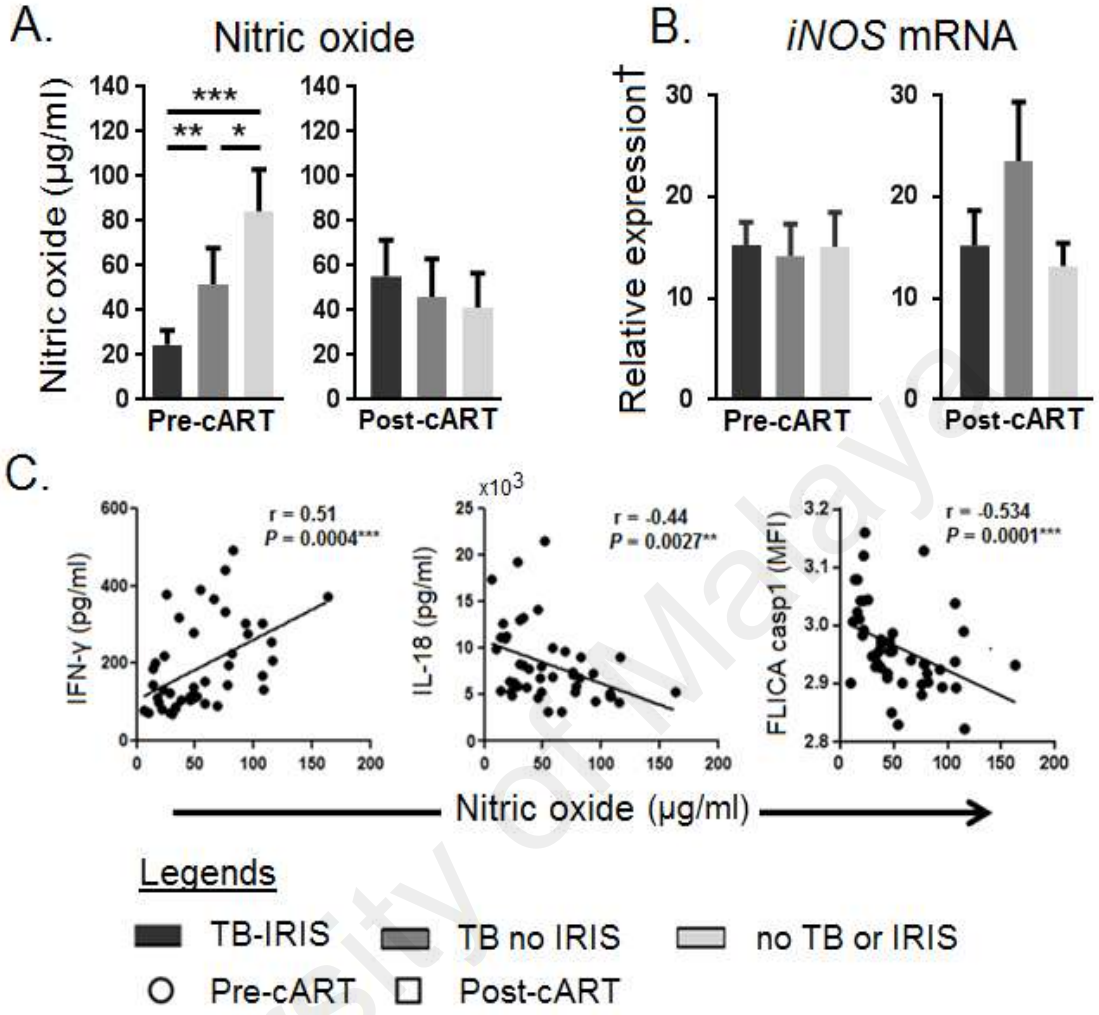

Figure 5.6. Plasma Levels of NO and Cellular Expression of iNOS Among Patient Groups, and Correlation with IFN-γ, IL-18 and Caspase-1.

(A) Plasma levels of NO among TB-IRIS, TB no IRIS and no TB or IRIS pre-cART at time of TB-IRIS or 4-12 weeks post-cART. **(B)** *iNOS* expression in monocyte among the 3 groups of patients pre- and post-cART. **(C)** Correlation between plasma levels of NO and plasma levels of IFN-γ or IL-18 and monocyte expression of activated Casp1. Data were analyzed using the Kruskal-Wallis test across the three patient groups. Post-hoc Mann-Whitney U tests were then performed only for those biomarkers with a Kruskal-Wallis test p value of <0.05. Correlation was analysed using Spearman's rank test. *p<0.05, **p<0.01, ***p<0.001.

Abbreviations: Casp1, caspase 1; *iNOS,* inducible nitric oxide synthase

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5.3.5 Expression of CD64 on Monocytes was Associated with Monocyte Expression of Casp1 in TB-IRIS

Prior studies have shown that the development of TB-IRIS is independently associated with expansion of CD14++CD16- monocytes (Andrade *et al* 2014), which prompted us to investigate if monocyte activation in TB-IRIS is associated with markers of inflammasome activation and pyroptosis in monocytes. Hence, we studied the surface expression levels (MFI) of CD64 (Fcγ receptor 1), CD11b, CD69 and CD38 on CD14++CD16-, CD14++CD16+, and CD14+CD16++ monocyte subsets and compared the data across the three clinical groups. We found that all these activation markers were increased to a variable degree in all three monocyte subpopulations across the three groups of patients, with CD64 expression showing consistent elevation in all three monocyte subsets. CD64 expression was higher in TB-IRIS patients when compared to no TB or IRIS pre-cART, and was also higher compared to TB no IRIS and no TB or IRIS patients post-cART (**Figure 5.7A**).

Next, we investigated if these monocyte activation markers correlated with activated casp1 expression levels (MFI) in CD14++CD16-, CD14++CD16+ and CD14+CD16++ monocyte subpopulations. Using Spearman correlation test, we found that the levels of CD64 correlated with activated casp1 expression in all the three monocyte subpopulations (R=0.754 in CD14++CD16-, R=0.587 in CD14++CD16+ and R=0.558 in CD14+CD16++; $p<0.0001$ for all correlations) (**Figure 5.7B**). In contrast, there were no correlations with the other monocyte activation markers. These findings suggest that CD64 expression on monocytes is a correlate of activated casp1 expression, and possibly inflammasome activation, in TB-IRIS patients.

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Figure 5.7

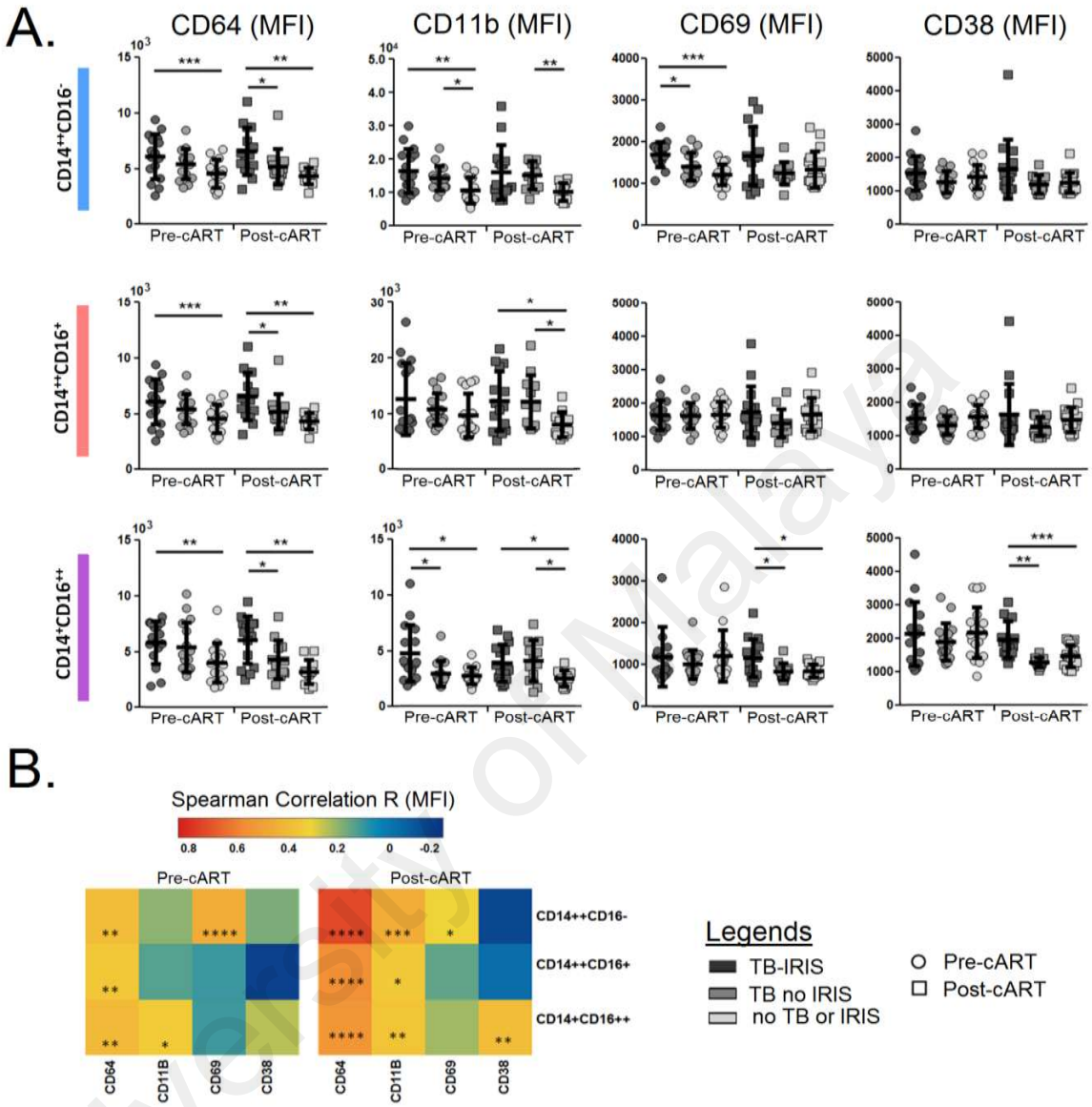

Figure 5.7. The Association Between Monocyte Activation and TB-IRIS and Correlation with Caspase-1.

(A) Expression (mean fluorescence intensity, MFI) of monocyte activation markers CDp64, CD11b, CD69 and CD38 on circulating CD14++CD16-, CD14++CD16+ and CD14+CD16++ monocytes pre- and post-cART. **(B)** Heatmap depicting the overall pattern of correlation between expression of monocyte activation markers and activated casp1 expression in respective monocyte subsets at pre- and post-cART. Data were analyzed using the Kruskal-Wallis test across the three patient groups. Post-hoc Mann-Whitney U tests were then performed only for those biomarkers with a Kruskal-Wallis test p value of <0.05. Spearman rank test to compare the correlation between two continuous variables. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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5.3.6 Development of TB-IRIS was Associated with Increased Expression of IL-18Rα on NK Cells and CD4+ T Cells Post-cART

To assess the interplay between monocytes, T cells and NK cells in TB-IRIS, we also assessed the frequency and activation status of NK cells and expression of IL-18Rα (CD218) on CD4+ and CD8+ T cells, and NK cells by flow cytometry (**Figure 5.8A**). First, we found that the frequency of circulating CD56+ NK cells was lower in TB-IRIS patients compared with TB no IRIS and no TB or IRIS patients pre-cART. In addition, surface expression of CD56 on NK cells post-cART was highest in the TB-IRIS group compared to the other two groups (**Figure 5.8B**), suggesting that there was an increase of terminally differentiated NK cells (Loza *et al* 2004) among TB-IRIS patients. We also found that the proportion of IL-18Rα-expressing CD4+ T cells and NK cells were higher among TB-IRIS and TB no IRIS patients compared with no TB or IRIS patients pre-cART (**Figure 5.8C**). At TB-IRIS event, the proportion of both IL-18Rα+ CD4+ T cells and CD56+ NK cells, and the level of IL-18Rα expressions on these cells were also higher in TB-IRIS patients compared with TB no IRIS and no TB or IRIS patients (**Figure 5.8C**). Using a Wilcoxon paired-test, we demonstrated that IL-18Rα was increased post-cART compared to pre-cART in TB-IRIS patients. Changes in IL-18Rα in the other two groups were not significant. (left panel of **Figure 5.8D, E** and **F**). Taken together, our data indicate that the onset of TB-IRIS is associated with increased proportions of terminally differentiated NK cells and higher expression of IL-18Rα on CD4+ T cells and NK cells.

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Figure 5.8

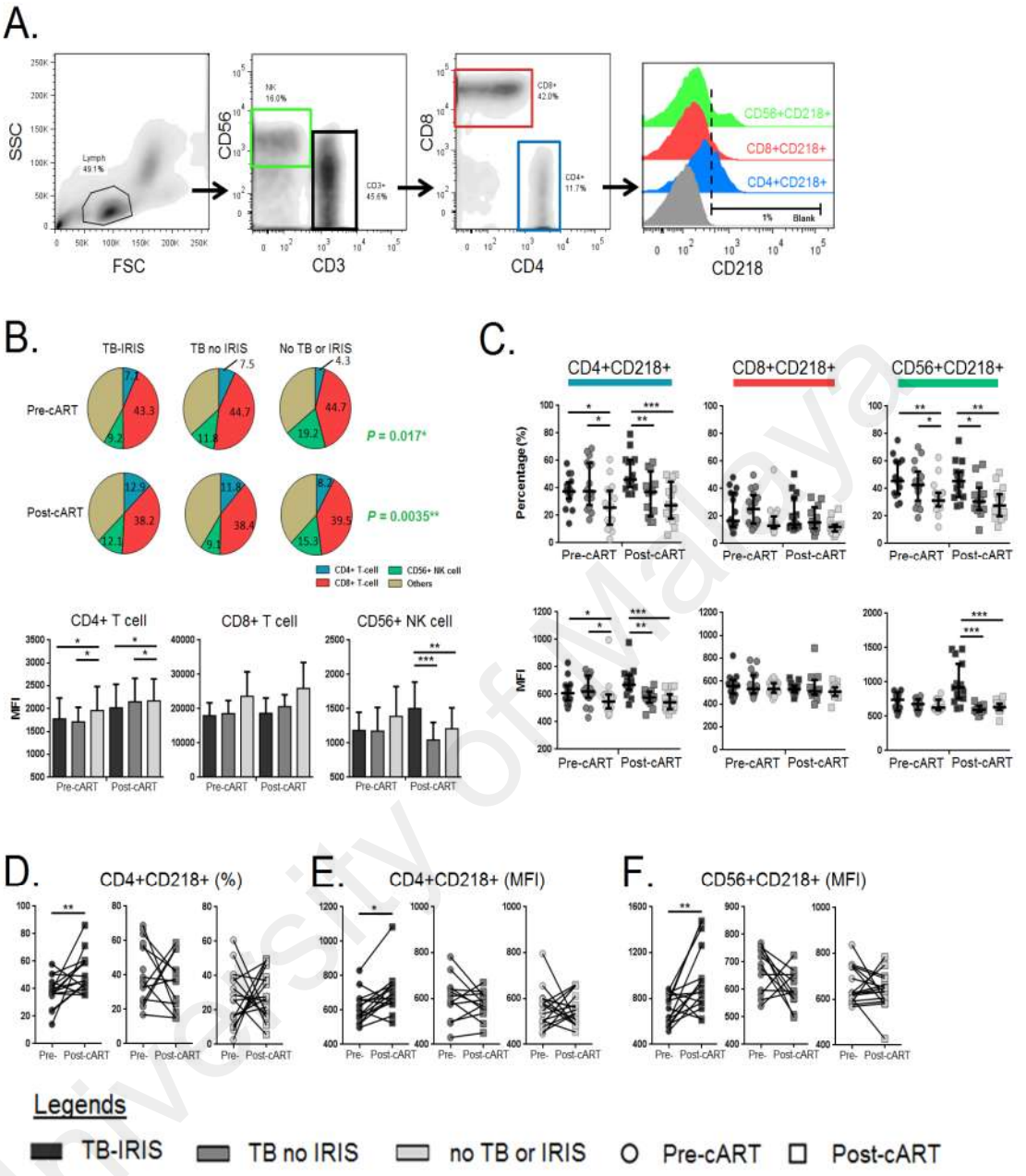

Figure 5.8. Expression of CD218 (IL-18Rα) on T cells and NK cells of patients with TB-IRIS, TB, no IRIS and no TB or IRIS.

(A) Representative FACS plots showing the distribution of CD4+ T cells, CD8+ T-cells and NK cells expressing CD218. **(B)** Percent of circulating CD4+ T-cells, CD8+ T-cells and CD3-CD56+ NK cells were compared pre-cART and at the time of TB-IRIS or week 4-12 or following iniation of cART in patients with TB-IRIS (n=16), TB no IRIS (n=16) or no TB or IRIS(n=19). **(C)** Percentage and MFI of total lymphocytes expressing CD218. **(D-F)** Changes in CD218 expression following iniation of cART. Data were analyzed using the Kruskal-Wallis test across the three patient groups. Post-hoc Mann-Whitney U tests were then performed only for those biomarkers with a Kruskal-Wallis test p value of <0.05. Wilcoxon matched-pairs test for paired analyses within each study group. *p<0.05, **p<0.01, ***p<0.001.

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5.4 Discussion

The immunologic mechanisms underlying the development of TB-IRIS are not clearly understood, and there are currently no effective therapeutic strategies to prevent the onset of TB-IRIS. We have previously shown a robust association between increased plasma IL-18 levels and TB-IRIS (Oliver *et al* 2010; Tan *et al* 2015), suggesting that inflammasome activation may have a role in the immunopathogenesis of TB-IRIS. Here, we have shown that HIV-infected patients with untreated or recently treated TB exhibited an increased proportion of monocytes expressing activated casp1 before cART is commenced, and that the level of activated casp1 expression increases further, and to the greatest degree, in those patients who develop TB-IRIS after the initiation of cART. Upregulation of *NLR3* and *AIM2* inflammasomes during TB-IRIS provides further evidence to inflammasome activation. These changes were associated with evidence of pyroptotic cell death, including increased plasma mtDNA levels, before cART was commenced providing evidence for both pyroptosis and apoptosis during TB-IRIS. Lower pre-cART plasma levels of NO demonstrated here, and IFN-γ demonstrated previously (Tan *et al* 2015), in patients who developed TB-IRIS provide evidence that inflammasome activity may be inadequately regulated. We also demonstrated a correlation between activated casp1 expression and CD64 expression in monocytes suggesting that CD64+ monocytes might be a marker of inflammasome activation in monocytes. Finally, plasma IL-18 levels correlated negatively with NO levels but positively with mtDNA levels pre-cART and positively with casp1 expression in monocytes during TB-IRIS, providing evidence that IL-18 may be a marker of inflammasome activation and pyroptosis. Futhermore, upregulation of IL-18Rα was greatest on the CD4+ T cells and NK cells of patients with TB-IRIS providing further evidence that IL-18 is directly involved in the inflammatory responses observed in TB-IRIS.

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Cellular immune responses against MTB primarily involves the coordinated activity of innate immune responses mediated by monocytes/macrophages and DCs, and adaptive immune responses mediated by CD4+ and CD8+ T cells and non-classical T cells (O'Garra *et al* 2013). In resting macrophages, MTB is capable of blocking phagosome-lysosome fusion as well as inhibiting phagosome acidification (Vergne *et al* 2004; Meena *et al* 2010) to ensure its intracellular survival and replication. These immune evasion strategies may be overcome by IFN-γ activation of macrophages along with NO production (MacMicking *et al* 1997; Yang *et al* 2009; Gallegos *et al* 2011). However in HIV-infected patients, CD4+ T-cell deficiency results in decreased IFN-γ activation of macrophages and intracellular killing of MTB (Diedrich *et al* 2011; Tomlinson *et al* 2014) leading to accumulation of MTB intracellularly and eventual activation of inflammasomes (Dorhoi *et al* 2012). Here, we provide evidence that failure to control MTB replication in monocytes/macrophages because T cell responses are impaired by HIV-induced CD4+ T cell depletion, leads to 'default' immune control mechanisms that include inflammasome activation in monocytes, and that this is greatest in those HIV/TB patients who subsequently develop TB-IRIS after cART is commenced.

We observed different inflammasome activation profiles between TB-IRIS and TB no IRIS patients, whereby increased AIM2 and NLRP3 inflammasome activity was observed in TB-IRIS patients whilst increased NLRP1 inflammasome activity was observed in TB no IRIS patients. NLRP3 inflammasomes respond to LPS (Lamkanfi *et al* 2009) and MTB-specific antigens, such as 6-kDa early secretary antigenic target (ESAT-6) (Mishra *et al* 2010; Lee *et al* 2013); whereas AIM2 responds to dsDNA of viral, bacterial and even host origin (Lamkanfi *et al* 2009). One possible explanation for activation of the AIM2 inflammasome observed in our study is that NLRP3 inflammasome and/or Casp1 activation results in pyroptotic cell death leading to the release of intracellular contents, including mtDNA, into the systemic circulation

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(Bergsbaken *et al* 2009). Consequently, the mtDNA activates the AIM2 inflammasome of other monocytes, amplifying the inflammation cascade (Nakahira *et al* 2011). This proposal is supported by our observation that increased pyroptotic cell death and cell-free mtDNA in plasma were clearly evident among TB-IRIS patients.

We previously reported that plasma levels of IL-18 were higher in TB-IRIS patients both pre- and post-cART compared to levels in control groups, and that the biological activity of IL-18 was not adequately regulated by IL-18-binding protein (IL-18BP) (Tan *et al* 2015). It is currently unclear why TB-IRIS patients do not show exaggerated inflammatory responses before commencement of cART. Here, we have demonstrated increased expression of IL-18Rα on T cells and NK cells among TB-IRIS patients during TB-IRIS. Neutrophils also express IL-18Rα (Hirata *et al* 2008), and it is likely that T cells, NK cells and neutrophils of TB-IRIS patients become more responsive to IL-18 after commencement of cART. Our findings suggest a significant interaction between monocytes/macrophages, T cells, NK cells and possibly neutrophils via the IL-18 signalling pathway.

Although inflammasome activation and pyroptosis contribute to pathogen clearance and induction of adaptive immune responses (Eisenbarth *et al* 2008; Conforti-Andreoni *et al* 2011), dysregulated NLRP3 inflammasome activity is regarded as deleterious in patients with sepsis (Cinel *et al* 2009). We propose that this could also be the case with TB-IRIS patients. As the pro-inflammatory effects of inflammasome activation can be severe, their activation must be tightly regulated (Lamkanfi *et al* 2012). Both IFN-γ and NO are known to regulate inflammasome activity at different stages of activation. It has been reported that NO is able to down-regulate the expression inflammasome-related genes, including *NLRP3*, *ASC*, *casp1* (Mishra *et al* 2013), as well as inhibiting NLRC4 and AIM-2 inflammasome-mediated caspase-1 activation (Mao *et al* 2013). IFN-γ, on the other hand, has no appreciable effect in altering the expression of inflammasome-

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related genes but it does elicit a regulatory effect by inhibiting the docking of ASC and casp1 to the assembled inflammasome (Mishra *et al* 2013). In the absence of effective CD4+ T-cell responses, it is likely that the IFN-γ and NO are not produced in sufficient amounts to regulate casp1 activity induced by NLRP3 and AIM2, thereby leading to the excessive casp1 activity seen in TB-IRIS patients. Contrary to the activation of NLRP3 and AIM2, the activation of NLRP1 observed in TB no IRIS patients appears to be less pathogenic. One possible reason could be that NLRP1 is regulated by IL-18 (Masters *et al* 2012) produced as a consequence of inflammasome activation, leading to less severe inflammation. Together with our previous observation that plasma IFN-γ levels were low in patients with TB-IRIS (Tan *et al* 2015), our current data suggests that NLRP3 and AIM2 activity in TB-IRIS patients is inadequately regulated by IFN-γ and NO.

Detection of apoptosis and pyroptosis using annexin V and 7-AAD may have certain limitations. Although it has previously been demonstrated that 7-AAD stains pyroptotic cells by entering the cell through pores on the cell membrane (Fink *et al* 2006; Miao *et al* 2011), apoptotic cells are destined to undergo secondary necrosis and could lose their membrane intergrity to become 7-AAD+ (Zimmermann *et al* 2011; Zembruski *et al* 2012). In this context, it is possible that annexin V and 7AAD staining may not clearly distinguish pyroptotic cells from cells that have undergone secondary necrosis. Unlike *in vitro* studies where inhibitors of casp3 (e.g. DEVD-CHO) and inhibitors of casp1 (e.g. pralnacasan) can be employed to confirm the type of cell death (Callus *et al* 2007), such an approach is seldom used to confirm *in vivo* cell death. An alternate approach would be to use microarray strategies to analyse multiple genes involved in the different pathways of cell death. Despite this uncertainty on the use of annexin V and 7AAD as markers of the type of cell death, we showed that TB-IRIS is associated with a higher level of mtDNA in plasma, in association with activated inflammasomes and that the

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amount of cell death correlated with the levels of casp1; which concertedly reflect the pro-inflammatory nature of pyroptosis.

In conclusion, we have provided evidence for increased inflammasome activation and pyroptosis in monocytes of HIV-TB patients, and that patients who develop TB-IRIS show increased levels of inflammasome activation and cell death (pyroptosis and apoptosis) after the commencement of cART. Furthermore, we have provided evidence for the regulation of inflammasome activity by NO and IFN-γ, which could be ineffective in those HIV-TB patients who develop TB-IRIS. We interpret these findings as evidence for increased inflammasome activation and pyroptosis in monocytes of HIV-infected patients with TB, in an attempt to control mycobacterial replication when T cell-dependent monocyte/ macrophage activation becomes less effective. This likely primes the monocytes and macrophages to exaggerate the inflammatory responses when HIV replication is suppressed by cART, which is the greatest in patients who develop TB-IRIS.

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CHAPTER 6: CONCLUSIONS AND FUTURE STUDIES

There are three major themes studied in this thesis: 1) In Chapter 3, the prevalence and risk factors associated with TB-IRIS were investigated. 2) In Chapter 4, the immunological correlates of TB-IRIS were investigated and certain putative predictive markers of TB-IRIS were identified and 3) in Chapter 5, the underlying immunological mechanisms of development of TB-IRIS were studied, with special reference to the roles of inflammasome and activation of monocytes. Our findings could likely provide insights into the underlying mechanisms of IRIS pathogenesis, and help identify specific predictive markers to promptly diagnose and treat TB-IRIS. This could also set stage for the development of newer improved therapeutics in HIV-infected patients commencing on cART.

6.1 Prevalence of TB-IRIS in UMMC, Malaysia

The prevalence of TB-IRIS varies throughout the world and is highly dependent on the population studied, specifically the degree of immunodeficiency and OI burden. For resource-limited settings, the prevalence of TB-IRIS ranges from 12% in South Africa (Lawn *et al* 2007), 12-18% in Brazil (Serra *et al* 2007; Dibyendu *et al* 2011), 13-18% in India (Agarwal *et al* 2012), 5-13% in Thailand (Manosuthi *et al* 2006; Aramaki *et al* 2010; Umphonsathien *et al* 2011), and 29% in Uganda (Baalwa *et al* 2008); However, there has been paucity of data available from Malaysia. Here, we conducted a retrospective investigation, and reported that the prevalence of TB-IRIS in Malaysia was 16% (17 out of 106 patients), which is similar to other developing countries and the mortality rate was <5.6%. Although the prevalence was seemingly low, the incidence of IRIS is increasing, and represents a significant clinical problem due to the increased access to cART especially in resource-limited countries.

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6.2 Risk Factors and Clinical Outcome of TB-IRIS.

As for the risk factors for development of TB-IRIS, our adjusted logistic regression model has shown that disseminated TB was the only independent factor associated with TB-IRIS. Unlike previously reported (reviewed in (Martin-Blondel *et al* 2012), we found no associations between lower baseline CD4+ T-cell counts, higher baseline viral load and shorter interval between ATT to cART with increased risk of TB-IRIS. Nonetheless, the mortality rate for TB-IRIS was 5.9% and was similar as compared to 5.7% in HIV/TB co-infected patient without IRIS. The CD4+ T-cell recovery following cART was not different between those who had TB-IRIS and those who did not, and is consistent with previously reported by others (Bonnet *et al* 2013).

6.3 Plasma Markers that Predict and Characterize TB-IRIS

Because TB-IRIS usually occurs within the first month of initiation of cART, it could be difficult to distinguish from relapsed or newly acquired TB. Therefore, there is an urgent need for laboratory markers to predict and identify TB-IRIS (Murdoch *et al* 2007; Meintjes *et al* 2010). In order to identify markers predictive of TB-IRIS, we conducted a case-control study by comparing the levels of 12 cytokines/pro-inflammatory mediators in plasma that included ferritin, neopterin, sCD163, sCD14, IL-18, IFN-γ, CXCL-10, TNF-α, CCL2, IL-6, CXCL8 and IL-10. The levels of these mediators were compared at pre-cART and post-cART across the three groups of patients ie. **i)** TB-IRIS (case), **ii)** TB no IRIS (control) and **iii)** no TB no IRIS (control). Using ROC analysis, the study indentified that IL-18 to be the best predictive marker for development of TB-IRIS.

Prior studies have proposed several biomarkers for predicting TB-IRIS, including plasma IFN-γ (Haddow *et al* 2011), IFN-γ after exposing T cells to ESAT-6 (IFN-γ releasing assay, IGRA, e.g. QuantiFERON®) (Bourgarit *et al* 2006), IL-6, TNF-α

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(Tadokera *et al* 2011), CXCL10, CCL2, IL-18 (Oliver *et al* 2010; Conesa-Botella *et al* 2012), sCD163 and sCD14 (Andrade *et al* 2014). However, only CXCL10, CCL2, IL-18 were analysed against their sensitivities and specificities by receiver operating characteristic (ROC) analyses. Here, we investigated the potential of IL-18 using 3 specimens from three different geographical locations (i.e. Cambodia, Malaysia and India), and demonstrated the robustness of IL-18 as predictive marker for TB-IRIS.

6.4 Role of High IFN-γ Levels in TB-IRIS is Less Clear

IL-18 is a potent pro-inflammatory cytokine produced predominantly by activated monocytes/ macrophages. IL-18 was initially discovered as an IFN-γ-inducing factor, and plays a critical role in augmenting Th1 responses. Hence, we speculated a far higher role of IFN-γ in the development of TB-IRIS. However, we found that the contribution of IFN-γ to development of TB-IRIS was relatively negligible. Our findings differ from those of others for serum/plasma IFN-γ levels (Haddow *et al* 2011; Tadokera *et al* 2011; Conesa-Botella *et al* 2012) and production of IFN-γ by IGRA (Bourgarit *et al* 2006; Tan *et al* 2008; Bourgarit *et al* 2009; Elliott *et al* 2009; Vignesh *et al* 2013). Nonetheless, the discrepancies could likely be attributed to differences in experimental design (*ex vivo* stimulation, culture supernatant vs. plasma etc) as well as use of analytical techniques (sandwich ELISA, cytokines beads array, ELISpot or intracellular cytokine staining and flow cytometric analysis) to confirm the findings. Our finding is supported by a recent study on tuberculous meningitis (TBM)-IRIS patients, which has reported that low IFN-γ levels in the CSF of TBM cases could predict IRIS (Marais *et al* 2013).

Interestingly, the plasma IFN-γ levels in two of our cohorts (the KL cohort and the Chennai cohort studied in Chapter 4) and a South Africa cohort (studied by one of our collaborators) also appear to be associated with pre-cART CD4+ T-cell counts. Whilst

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the Chennai cohort [24] showed the highest levels of IFN-γ and a median baseline CD4+ T-cell count of ~200 cells/ml, the South African cohort [24] found intermediate levels of IFN-γ and a median baseline CD4+ T-cell count of ~100 cells/ml. Patients in our Kuala Lumpur cohort (reported here) showed the lowest IFN-γ levels and a median CD4+ T-cell count of ~50 cells/ml. Therefore, the differences in plasma IFN-γ levels in the different cohorts could likely be owing to the residual CD4+ T-cell functions at pre-cART.

6.5 High Intracellular Pathogen Load: Potential Trigger of TB-IRIS

The early secretary antigenic target (ESAT-6) represents a potent immunogenic antigen derived from MTB. It is a virulence factor capable of modulating host immune responses (Andersen *et al* 1995; Sorensen *et al* 1995). ESAT-6 reportedly inhibits *IL-12p40* expression by macrophages, and therefore may indirectly inhibit the ability of T cells to produce IFN-γ via reduced secretion of IL-12 by APCs (Pathak *et al* 2007). Previous findings have also shown that ESAT-6 can directly inhibit IFN-γ production by T cells in a dose-dependent manner. Low concentrations of ESAT-6 could induce IFN-γ, but higher concentrations appear to inhibit IFN-γ production via down-regulation of ATF-2 and c-Jun, two promoters essential for production of IFN-γ (Wang *et al* 2009). Hence, low plasma IFN-γ levels observed among TB-IRIS patients as evident from our study, could in part be attributed to the inhibitory effects of ESAT-6. Furthermore, the RD-1 locus of MTB genome has been associated with activation of casp1 via inflammasome pathways and release of IL-18 (Kurenuma *et al* 2009; Mishra *et al* 2010; Welin *et al* 2011). Taken together, the increase of IL-18 and decrease of IFN-γ levels observed in our TB-IRIS patients could likely be due to the immunomodulating effects of ESAT-6.

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6.6 Heightened Inflammasome Activity is Associated with TB-IRIS

We have previously shown a robust association between increased plasma IL-18 levels and TB-IRIS (Oliver *et al* 2010; Tan *et al* 2015), which underpins the likely role of inflammasome activation with development of TB-IRIS. In Chapter 5, we demonstrated that TB-IRIS is associated with increased expression of activated casp1 both before cART and during TB-IRIS event that involves activation of NLRP3 and AIM2 inflammasomes.

Furthermore, it has also become clear that activated casp1 could also results in pyroptosis, a casp1-dependent pro-inflammatory cell death characterized by rupture of plasma membrane, swelling of cells and release of pro-inflammatory intracellular contents into the systemic circulation (Fink *et al* 2006; Bergsbaken *et al* 2009). The release of pro-inflammatory intracellular contents (measured as cell-free plasma mtDNA) will further activate bystander cells via TLRs (or inflammasomes) to promote inflammation as observed in clinical TB-IRIS (Broderick *et al* 2014).

6.7 Regulation of Inflammasome Activation by IFN-γ

Given that activation of inflammasome leads to potent immune responses, dysregulated inflammasome activity reportedly results in deleterious effects in patients with sepsis (Cinel *et al* 2009), and therefore are tightly regulated (Lamkanfi *et al* 2012). IFN-γ and NO are known to regulate inflammasome activity at different stages of activation. It has been reported that NO is able to down-regulate the expression inflammasome-related genes, including *NLRP3*, *ASC*, *casp1* (Mishra *et al* 2013), as well as to inhibit *NLRC4* and *AIM-2*-mediated casp1 activation (Mao *et al* 2013). Notwithstanding that IFN-γ has no appreciable effect in altering the expression of inflammasome-related genes, it does elicit a regulatory effect by inhibiting the docking of ASC and casp1 to the assembled inflammasome (Mishra *et al* 2013).

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In the absence of effective CD4+ T-cell responses, it is likely that IFN-γ and NO are not produced in sufficient amounts to regulate casp1 activity induced by NLRP3 and AIM2. Hence, this could result in excessive casp1 activity as seen in TB-IRIS patients. The role of IFN-γ and NO in regulating the activation of inflammasome is theorized and illustrated in **Figure 6.1**.

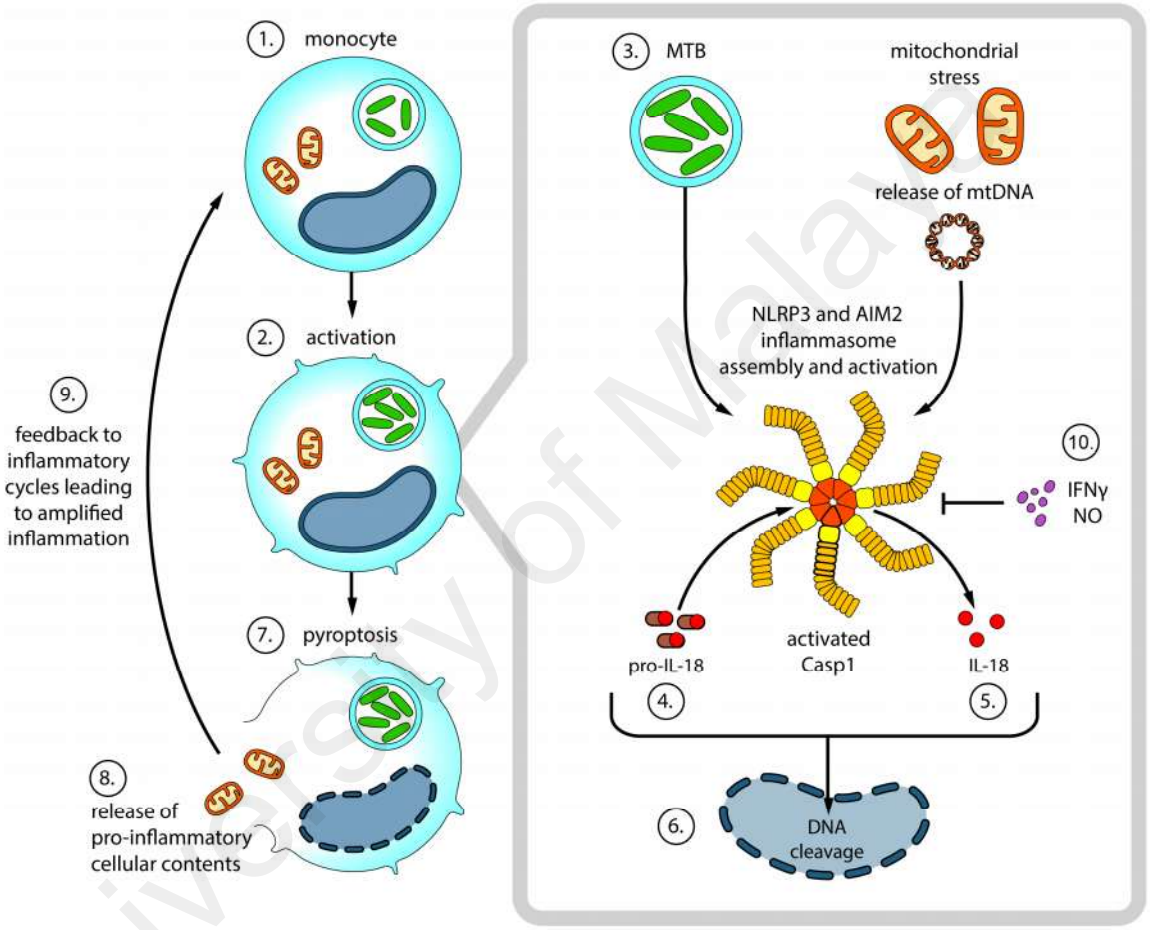

Figure 6.1 Proposed Model of Inflammasome-Mediated Pathogenesis of TB-IRIS.

(1-2) In a microenvironment with low levels of IFN-γ, MTB escape monocyte/macrophage bactericidal effectors by interfering with phagosome-lysosome fusion (Herbst *et al* 2011) survive and continue to replicate intracellularly (de Chastellier 2009) leading to mitochondrial stress (Jamwal *et al* 2013). (**3**) Accumulation of intracellular MTB and release of mtDNA leads to the assembly of NLRP3 and AIM2 followed by activation of casp1. (**4-5**) IL-18 is a potent pro-inflammatory cytokine that is initially synthesized in an inactive form (pro-IL18) requiring processing by casp1 to assume activity. (**6-9**) As well as processing IL-18, casp1 also facilitates an

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inflammatory form of programmed cell death termed pyroptosis, leading to egress of mtDNA into the systemic circulation, feeding back to amplify inflammatory responses. (**10**) IFN-γ and NO negatively regulate NLRP3 and AIM2.

Abbreviations: Casp1, caspase 1; MTB, *Mycobacterium tuberculosis*, mtDNA, ; NLRP3, nucleotide-binding domain and leucine-rich repeat protein 3; AIM2, absent in melanoma 2; NO, nitric oxide.

6.8 Regulation of Biological Activity of IL-18 by IFN-γ

The highly potent pro-inflammatory cytokine IL-18 is produced at the downstream aspect of inflammasome activation. A plethora of cells are known to express IL-18 receptor (CD218). In Chapter 5, we have shown that CD218 is elevated among TB-IRIS patients following the initiation of cART. Nevertheless, the biological activity of IL-18 appears to be regulated by IL-18BP, a soluble molecule that binds mature IL-18 with high affinity and prevents it's interaction with cell surface receptors (competitive inhibitor) to limit its biological activities (Novick *et al* 1999). Interestingly, the expression of IL-18BP is induced by IFN-γ. It has been shown that the promoter region of IL-18BP contains an IFN regulatory factor 1 response element that increases IL-18BP gene expression upon IFN-γ stimulation (Muhl *et al* 2000; Hurgin *et al* 2002). Therefore, IL-18 indirectly increases the production of it's own inhibitor in a negative feed-back loop (Boraschi *et al* 2006).

In Chapter 4, we theorized that the inflammatory response driven by augmented IL-18 among TB-IRIS patients might be fuelled by a deficiency of IL-18BP, and we examined this possibility by assaying the plasma levels of IL-18BPa and the circulating IL-18 levels. We found that the circulating IL-18 levels were 40% higher in the plasma of TB-IRIS patients relative to controls. Previously published data is in agreement with our finding in that IL-18BPa could occur 20-fold molar excess than IL-18 in healthy individuals (Novick *et al* 2001). Another *in vitro* study showed that at least a 2 molar

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excess of IL-18BP would be required to neutralize 95% of the biological activity of IL-18 (Kim *et al* 2000). Hence, our results strongly suggest that the biological activity of IL-18 may have been inadequately regulated.

Interestingly, we also observed over-production of three chemokines, CXCL10, CCL2 and CXCL8 during TB-IRIS. These chemokines have been shown to play an important role in the recruitment and activation of T cells, monocytes, macrophage and neutrophils (Hoffmann *et al* 2002; Christensen *et al* 2006; Deshmane *et al* 2009) at the site of inflammation. This finding is also in line with other histopathological findings in stating that TB-IRIS could be associated with granulomata characterized by mixed inflammatory cell infiltrates (Lawn *et al* 2009; Martin-Blondel *et al* 2012). The association of these biomarkers with TB-IRIS immunopathology is summarized in a model of disease pathogenesis (see **Figure 6.2**).

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Figure 6.2 Potential Mechanism of TB Antigen-Induced Immune Dysregulation and Development of TB-IRIS

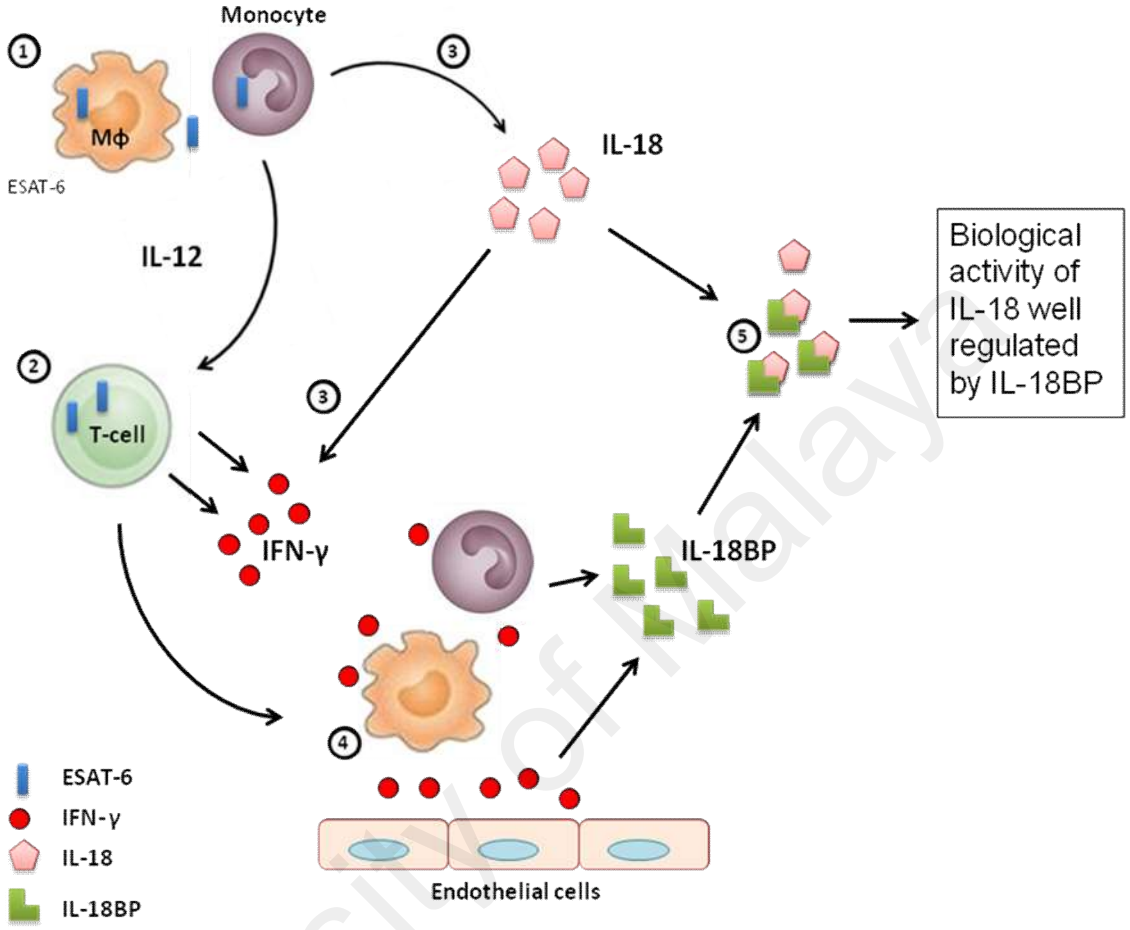

Figure 6.2a. In the absence of a high mycobacterial load, there is a low level of ESAT-6 and therefore the inhibitory effects of ESAT-6 is negligible, and hence the production of IL-12 by macrophages, and IFN-γ synthesis by T cells is relatively efficient **(1-2)**. ESAT-6 induces the production of IL-18 by monocyte/macrophages, and IL-18 will further enhance the production of IFN-γ by CD4+ T cells **(3)**. Subsequently, IFN-γ will induce monocytes/macrophages and endothelial cells to produce IL-18-binding protein (IL-18BP), a natural occurring regulatory protein that binds to IL-18 and neutralizes its biological activity **(4-5)**.

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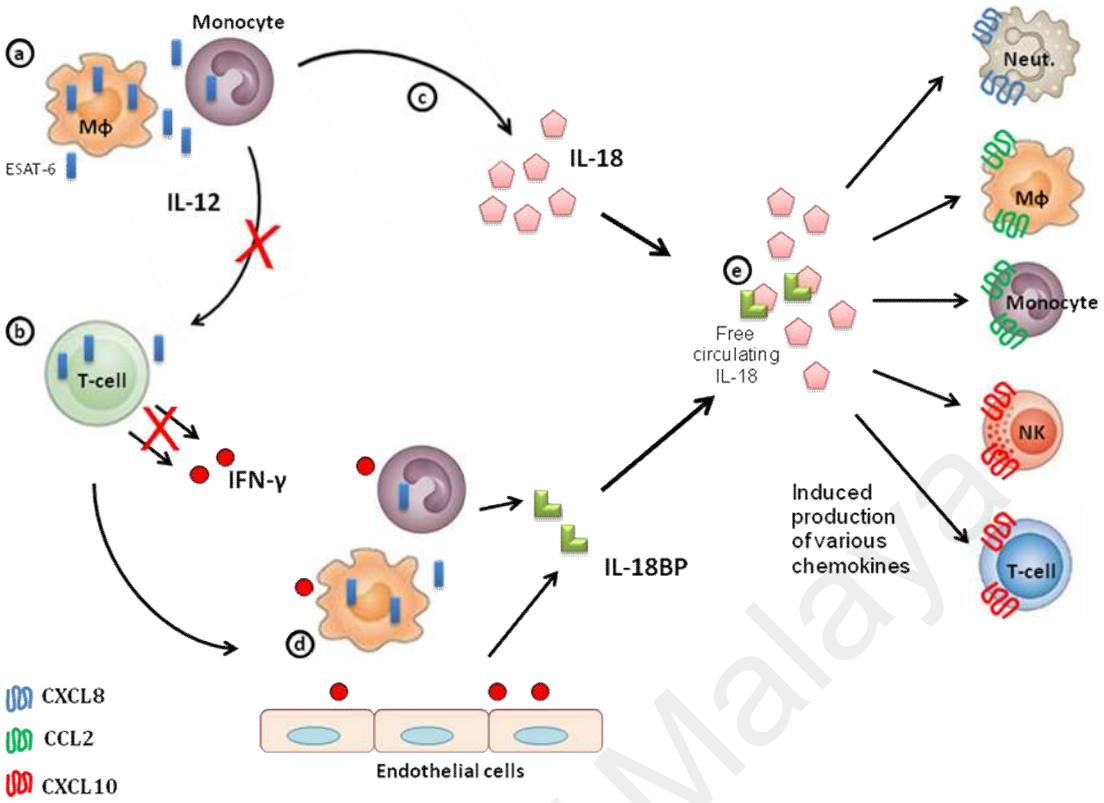

Figure 6.2b. In the presence of high mycobacterial load and abundance of *M. tuberculosis* antigens, ESAT-6 inhibits the production of IL-12 by macrophages, inhibiting the capacity of T cells to produce IFN-γ indirectly **(a)**. ESAT-6 also inhibits T cell production of IFN-γ directly via down-regulation of ATF-2 and c-Jun (promoters for *IFN-γ*), leading to the reduced IFN-γ levels in TB-IRIS patients **(b)**. Whilst ESAT-6 induces the secretion of IL-18 by monocyte/macrophages **(c)**, the IFN-γ produced by T cells is insufficient to induce production of IL-18BP **(d)**. The biological activity of IL-18 remains unregulated **(e)**, leading to an exaggerated inflammatory response that includes recruitment of neutrophils, T cells and monocytes resulting from increased production of chemokines.

Abbreviations: ESAT-6, 6 kDa early secretory antigenic target; IFN-γ, interferon-gamma; IL-18BP, interleukin-18 binding protein, Neut., neutrophils; Mφ, macrophages; NK, natural killer cells; ATF-2, activating transcription factor 2; c-Jun, protein encoded by *JUN* gene, subtype c.

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6.9 Conclusion and Future Studies

Collectively, our data suggests that in the absence of effective CD4+ T-cell responses (reflected also by deficient IFN-γ production), the innate immune "defaulted" to innate immunity as evidenced by the exuberant activation of inflammasome and casp1 activity followed by excessive production of IL-18. The ensuing innate immune responses become unregulated due to insufficient production of IFN-γ, which in turn regulates the activation of inflammasome as well as induction of IL-18BP to modulate the biological activities of IL-18.

Several animal models have been developed in the past to investigate into the roles of IFN-γ in TB-IRIS (Manabe *et al* 2008; Barber *et al* 2010). However, animal models to conduct investigations on inflammasome and IL-18 have not been developed till date. Furthermore, MCC950 has been recognized as a specific blocker that inhibits the activation of NLRP3 but not the AIM2, NLRC4 or NLRP1 inflammasomes. The role of NLRP3 in the development of TB-IRIS should also be investigated in animal models using MCC950.

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LIST OF PUBLICATIONS

- **Tan HY**, Yong YK, Lim SH, Ponnampalavanar S, Omar SFS, Kamarulzaman A, Crowe SM, French MA (2014). **Paradoxical Tuberculosis-Associated IRIS Among HIV Co-Infected Patients in Malaysia: Incidence, Risk Factors, and Treatment Outcomes.** *Sexual Health* (11):532–539.

- **Tan HY**, Yong YK, Andrade BB, Shankar EM, Ponnampalavanar S, Omar SFS, Narendran G, Kamarulzaman A, Swaminathan S, Sereti I, Crowe SM, French MA (2015). **Plasma IL-18 Levels are a Biomarker of Innate Immune Responses that Predict and Characterize Tuberculosis-Associated Immune Reconstitution Inflammatory Syndrome.** *AIDS* (29): 421-431.

- **Tan HY**, Yong YK, Shankar EM, Paukovics G, Ellegård R, Larsson M, Kamarulzaman A, French MA, Crowe SM (2016). **Aberrant Inflammasome Activation and Pyroptosis Characterise Tuberculosis-Associated Immune Reconstitution Inflammatory Syndrome.** *The Journal of Immunology, 196(10*): 104052-4063.

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APPENDICES

List of Presentations

- **Tan HY**, Yong YK, Ong LY, Lee YM, Omar SFS, Shasheela P, Pang YK, Kamarulzaman A. **Impact of Active Tuberculosis on the Immune Recovery of HIV-Infected Individuals Receiving HAART.** *Oral-Poster Presentation at XVIII International AIDS Conference (Vienna, Austria 18-23 July 2010). Abstract No. WEPDA20.*

- **Tan HY**, Yong YK, Shankar EM, Kamarulzaman A, Crowe SM, French MA. **Plasma Biomarkers of Innate Immune Responses Characterize and Predict Tuberculosis-Associated Immune Reconstitution Inflammatory Syndrome (TB-IRIS).** *Poster Exhibition at The 20th International AIDS Conference (Melbourne, Australia, 20-25 July 2014). Abstract No. A64100640447.*

Travel Scholarships

- Oral-poster presentation at XVIII International AIDS Conference (Vienna, Austria 18-23 July 2010). Abstract no. WEPDA20.

  Title: Impact of Active Tuberculosis on the Immune Recovery of HIV-Infected Individuals Receiving HAART.

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