Phenotypical and functional characterization of polarized human macrophages

By

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Abstract

Macrophages can be polarized into M1 and M2 macrophages based on the composition of the milieu. Human macrophages have been poorly characterized. In this study, various macrophage subsets were generated by treating monocyte-derived macrophages (MDMs) with IFNγ (M1), IL-4 (M2a), LPS and IL-1β (M2b) or IL-10 (M2c) which were characterized with respect to their cell surface marker profile and functional profile in the context of cytokine production, susceptibility to HIV infection and apoptosis. Each polarization state demonstrated a unique cell surface marker profile and cytokine profile. In addition M1 macrophages were shown to produce IFNγ post TLR stimulation. Moreover, M1 macrophages were highly sensitive to apoptosis following Smac mimetic treatment. Furthermore, M2a and M2c macrophages were resistant to apoptosis, induced by PI3K blockage and IAPs degradation respectively, and at the same time supported productive HIV infection unlike the other macrophage subsets. These findings might lead to better understanding of HIV reservoir formation and be used to develop therapies to eradicate it.

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List of abbreviations

AIDS	Acquired immunodeficiency syndrome
Akt	Protein kinase B
anti-IFNγR	Anti-interferon gamma receptor
APCs	Antigen presenting cells
APOBEC3	Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G
BAD	Bcl-2-antagonist of cell death
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BIR	Baculoviral IAP Repeat
BSA	Bovine serum albumin
CARD	Caspase associated recruitment domain
CCL2	Chemokine (C-C motif) ligand 2
CCR2	C-C chemokine receptor type
CD	Cluster of differentiation
cDNA	Complementary DNA
cIAP	Cellular inhibitor of apoptosis protein
CLRs	C-type lectins
Ct	Cycle threshold
CX_3CR1	CX3C chemokine receptor 1
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor type
DAMPs	Danger-associated molecular patterns

DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
Env	Envelope
ERK	Extracellular signal-regulated kinases
FADD	FAS-associated death-domain protein
FLIP	FADD-like IL-1β-converting enzyme-inhibitory protein
G0	Gap 0
G1	Gap 1
Gag	Group-specific antigen
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
HIV	Human immunodeficiency virus
HLA/MHC	Human Leukocyte Antigen/Major Histocompatibility Complex
HLADR	Human leukocyte antigen-D related
IAPs	Inhibitors of apoptosis proteins
IFN	Interferon
IL-	Interleukin-
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid

LTR	Long terminal repeat
MAPK	Mitogen-activated protein kinases
Mcl1	Myeloid-cell leukemia sequence 1
Mcl-1	Myeloid cell leukemia-1
M-CSF	Macrophage colony-stimulating factor
MDMs	Monocyte-derived macrophages
MIP	Macrophage inflammatory protein
miRNA	MicroRNA
mRNA	Messenger RNA
MX2	IFN-induced myxovirus resistance 2
MyD88	Myeloid differentiation primary response gene (88)
Nef	Negative regulatory factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLRs	Nucleotide-binding oligomerization domain receptors
nuc	Nucleosome
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIC	Pre-integration complex

PMBCs	Peripheral blood mononuclear cells
Pol	Polymerase
Poly I:C	Polyinosinic:polycytidylic acid
PRRs	Pattern recognition receptors
PTEN	Phosphatase tensin homolog
RANTES	Regulated on activation, normal T cell expressed and secreted
Rev	Regulator of expression of virion proteins
RING	Really Interesting New Gene
RLRs	RIG-I like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SAMHD1	SAM domain and HD domain-containing protein 1
SIV	Simian immunodeficiency virus
Smac	Second mitochondria-derived activator of caspases
STAT	Signal transducer and activator of transcription
TAMs	Tumor associated macrophages
Tat	HIV trans-activator
TGF-β	Transforming growth factor beta
Th	T helper
TIR	Toll-IL-1R resistance
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alpha

TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter-inducing interferon-β
VCCs	Virus containing compartments
VEGF	Vascular endothelial growth factor
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Virus protein U
XIAP	X-linked inhibitor of apoptosis protein

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Introduction

Plasticity of the immune response

The host's defense against invading pathogens relies on the efficient immune response comprising the innate immune response and the adaptive immune response. The adaptive immune response consists of clonal selection of lymphocytes that are specific for an antigen and that mediate long-lasting immunity[1]. In contrast, innate immunity is not specific in its target and does not generate a memory response[2]. The cells of the innate immune response, such as neutrophils and macrophages, serve to provide a rapid response to the invading pathogen. They recognize repeated molecular motifs that are conserved on pathogens by non-clonal receptors. This leads to the induction of pro-inflammatory cytokines, phagocytosis and the up-regulation of co-stimulatory molecules in antigen presenting cells. These cells serve as a bridge between the two arms of the host defense[3].

In innate or adaptive immunity, differentiated hematopoietic cells must have the capacity to coordinate their function in a manner that will mount an appropriate host defense response and clear the pathogen while limiting damage that might occur to surrounding tissues. A mechanism by which such functions can be undertaken is by micro-changes in the phenotype of the cell in response to the milieu hereby referred to as polarization. The principle that governs polarization is associated with a small number of signals that can lead to a pronounced change in cell phenotype and function promoting a differentiation of the distinct cell population. Polarized cells are able to mount the appropriate response with respect to the context of the environment[4]. The cells of the hematopoietic system have a considerable amount of plasticity as demonstrated by the ability of the hematopoietic

progenitor cells to develop into cells of any hematopoietic lineage[5], but the ability of the differentiated cells to further undergo marked phenotypic changes is limited.

Innate immunity

The innate immune system serves at the first line of defense in the host and is responsible for mediating acute inflammation associated with tissue injury or infection with a pathogen[6]. Acute inflammatory response is designed to rapidly eliminate pathogens and is normally limiting[7]. During the inflammatory response the cells of the innate immune response, such as monocytes, neutrophils, macrophages, dendritic cells and natural killer cells are rapidly activated and mobilized to the site of injury or following pathogen invasion[8]. Tissue injury and infection causes epithelial cells to release chemokines that recruit neutrophils[9]. Neutrophil activation leads to the degranulation of proteolytic enzymes and antimicrobial proteins. This also leads to the production of reactive oxygen species (ROS) and an oxidative burst which further degrades nucleic acids and lipids present in the environment[10]. Innate immune cells express pattern recognition receptors (PRRs) that can recognize conserved repeated motifs on a variety of pathogens called pathogen-associated molecular patterns (PAMPs). Activation of PPRs by virtue of their agonists leads to the production of pro-inflammatory cytokines as well as destruction of the ligand via phagocytosis or release of cytotoxic agents[6]. Macrophages and dendritic cells represent an essential component of an immune response.

Macrophages and dendritic cells are professional antigen presenting cells (APCs) and serve as the bridge between innate and adaptive immunity. Upon receptor engagement, pathogens undergo phagocytosis, are broken down and their associated antigens are presented to T cells by virtue of HLA/MHC (Human Leukocyte Antigen/Major

Histocompatibility Complex) class II molecules expressed on the surface of APCs[11]. Some viruses, such as cytomegalovirus, evade the immune response by down-regulating the expression of MHC molecules. Natural killer cells are important components of the innate immune response as demonstrated by their ability to kill infected cells that do not express MHC [12].

Macrophage origins

Macrophages arise from circulating peripheral blood mononuclear cells (PMBCs) that infiltrate tissues in a steady state or during the course of inflammation[13]. Monocytes develop from common myeloid progenitor cells that develop into monoblasts. These monoblasts give rise to pro-monocytes which develop into monocytes. Monocytes are known to replenish the tissue macrophage population[14]. In the murine model, monocytes that are circulating in the blood are not a homogenous population. There are studies suggesting that monocytes have distinct subpopulations with various maturation states and varied affinities for recruitment to inflammatory sites[15]. The maturation state of monocytes that leave the blood may define their function. Two distinct murine monocyte subsets have been identified on the basis of their maturation state as well as cell surface marker expression[16]. The monocytes that spend the least amount of time circulating in the blood are termed "inflammatory" and express CCR2 and GR1 and low levels of CX_3CR1. Resident monocytes represent the other end of the maturation spectrum and spend the most time in circulation and are $CCR2^{-}GR1^{-}$ CX_3CR1^{high} [16]. In mice, these two monocyte populations are equally represented but this is not the case in humans[17]. Human monocytes can also be divided into two categories based on their cell surface marker expression: classical monocytes are

$CD14^{high}CD16^{-}$ and non-classical monocytes are $CD14^{+}CD16^{+}$[17]. In humans, classical monocytes represent 90% of the monocytes[17].

In mice, according to one theory, inflammatory monocytes are thought to give rise to tissue macrophages and dendritic cells[18]. Furthermore, monocytes that are not immediately recruited to tissues circulate in the blood and associate with the endothelial layers of blood vessels[19]. These monocytes are also responsible for replenishing tissue-resident macrophage populations in a steady state[19]. It has been shown that their human counterparts, CD16+ monocytes, are better suited to migrate across endothelial monolayers than $CD16^{-}$ monocytes[20]. This enhances their migration into non-inflamed tissues and allows them to serve as an immediate source of monocytes during inflammation.

Monocytes released from the bone marrow circulate in the bloodstream for 3 days before infiltrating tissues to maintain tissue resident macrophage populations[13]. Tissue resident macrophages are named according to the tissue in which they are present such as osteoclasts in the bone or microglia in the brain but their functions are that of a macrophage[21]. Tissue macrophages can also proliferate to generate mature macrophage populations. This is evident with respect to microglial cells which can sustain themselves and their function[22]. Furthermore, macrophages have been shown to be present in the yolk sac prior to hematopoiesis[23]. Recent evidence has demonstrated the presence of a separate embryonic lineage of macrophages that have the ability to persist during adulthood[24]. For example, microglia are derived from yolk sac cells and Langerhans cells develop from fetal liver and yolk egg sac cells[25, 26]. This discovery of microglia and Langerhans cells having prenatal origins is in contrast to the model in which monocytes replenish macrophages. This was further confirmed with experiments in a mouse model where the mouse lacked the

development of hematopoietic stem cells due to the absence of the transcription factor Myb. Yolk sac macrophages from these Myb-deficient mice gave rise to F4/80^{hi} macrophages in the skin, liver, lung, spleen, brain and pancreas. In the control mice, these cells exist with F4/80^{lo} macrophages that are derived from a hematopoietic source[27]. Other studies have also confirmed the presence of prenatal F4/80^{hi} macrophages that do not require blood monocytes for replenishment under steady state[28]. However, the gastro-intestinal tract is a region in the body that harbors tissue resident macrophages that are replenished by blood monocytes even in the steady state[29]. Indeed, many tissues harbor macrophages of both prenatal and hematopoietic origins[27, 28]. Thus, the current understanding of macrophage replenishment from blood derived monocytes needs to be expanded to include macrophages from prenatal origins.

Macrophage polarization

Macrophages are present in nearly all the tissues of the body. Tissue resident macrophages can undergo polarization into macrophage subtypes to fine-tune their response based on the signals present in the tissue (Figure 1).

Macrophages are very plastic cells that respond rapidly to stimuli generated by tissue injury or infection. Stimuli produced by the innate immune cells exert a marked but transient effect on macrophage physiology. Signals from antigen-specific immune cells, such as Th 1 cells, are more long-lived in their consistency and lead to maintenance of the polarization state[30]. Additionally, macrophages also respond to the cytokine-mediated signals in an autocrine manner. Changes induced by these stimuli induce enhanced microbicidal activity of macrophages. In other cases, these environmental cues can make the macrophage more permissive to infections and less able to make cytokines that can combat the infection[31].

Figure 1. Development and Differentiation of macrophages.

Circulating blood monocytes infiltrate tissues and differentiate into tissue resident macrophages.

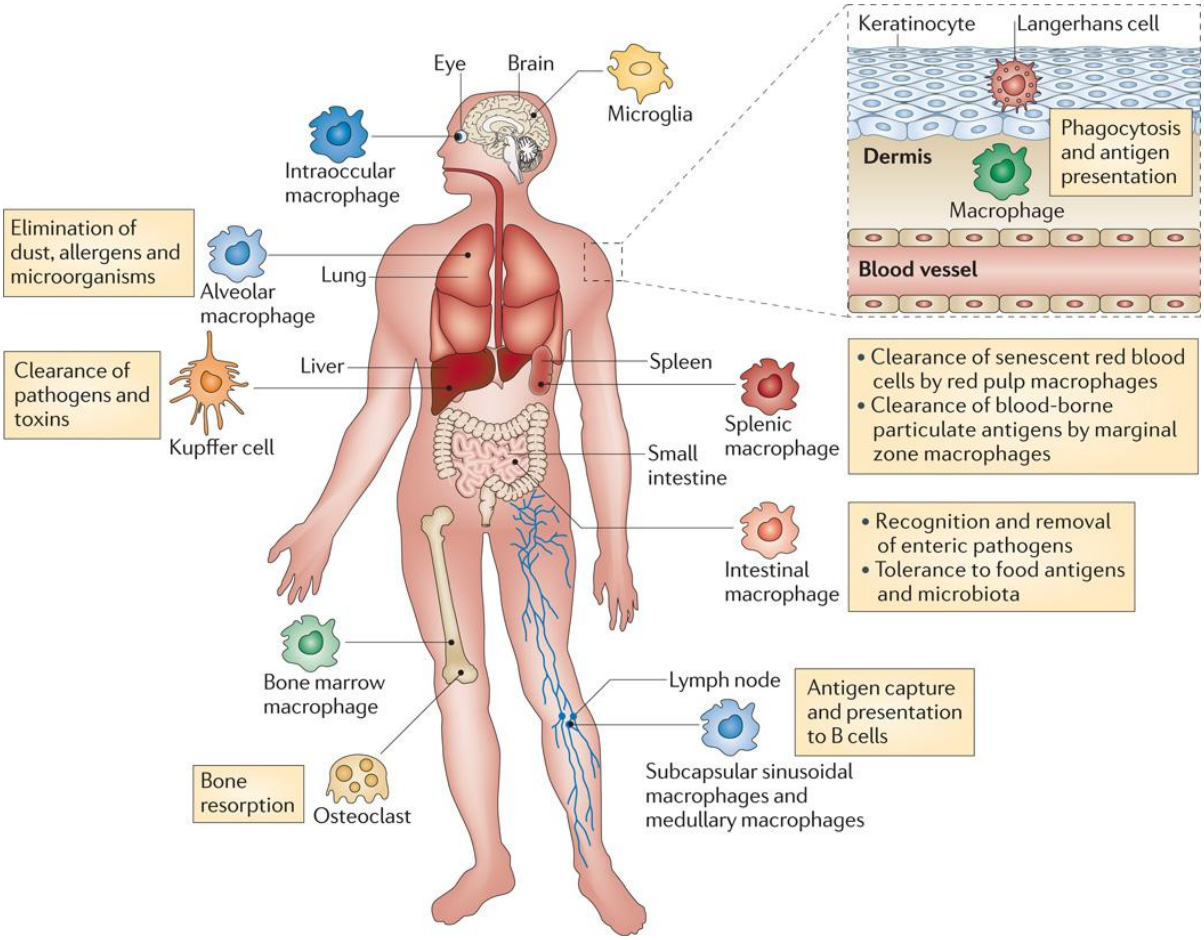


Figure 1 (Murray and Wynn, 2011)

Macrophages have been classified as being either M1 or M2 polarized in a linear representation where the two polarization states are at opposite ends of the scale. M1 polarization is used to describe classically activated macrophages and M2 polarization is used for alternatively activated macrophages[32]. Alternatively activated macrophages denote a more heterogeneous polarization state that includes several subtypes: M2a, M2b and M2c. This classification has persisted despite evidence demonstrating biochemical and physiological differences between M2 polarized subtypes[33].

Macrophage polarization *in vitro*

There is a lack of an experimental standard for macrophage polarization. The *in vitro* model used varies from study to study using the following as guidelines. M1 polarized macrophages can be derived *in vitro* by treating monocytes with either M-CSF or GM-CSF and IFNγ in the presence or absence of LPS[34]. M2a polarized macrophages are derived with treating monocytes with M-CSF and IL-4 or IL-13[34]. M2b and M2c macrophages can be induced by treatment of monocytes with M-CSF and a variety of factors, including IL-10, LPS, IL-1β, immune complexes, apoptotic cells, glucocorticoids and TGF-β[35]. There are studies that classify macrophages generated using GM-CSF as M1 polarized macrophages and those in M-CSF as M2 polarized macrophages[36]. While there are differences between macrophages generated using either GM-CSF or M-CSF as demonstrated in their transcriptome analysis, but these differences are not enough to classify the treatments as M1 and M2 respectively[37,38]. In the system outlined in this study, M-CSF was used to treat monocytes to differentiate them into monocyte-derived macrophages as M-CSF is used predominately for macrophage generation[38]. Furthermore, to demonstrate the phenomenon of polarization, it was apt to start form the same baseline for each of the polarization states.

Thus, MDMs were used for this purpose. There is also no consensus on when to administer the polarizing stimuli. Approaches range from treating the monocytes with M-CSF and the polarizing stimulus in concert for 6 days to generating MDMs and then treating the cells with the stimuli[39, 40]. Here, MDMs were generated and then treated for 48 hours with the appropriate stimuli to induce polarization. This highlights the current problem with macrophage polarization studies; therefore I sought to characterize the polarized macrophages in this system.

Classically Activated Macrophages and their role in disease

Classically activated macrophages or M1 polarized macrophages are the macrophages that are found during cell-mediated immune responses. In early characterization of M1 polarized macrophages, the combination of both interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) was thought to be required to polarize macrophages into a population that possessed high microbicidal and tumouricidal properties and secreted high levels of pro-inflammatory cytokines[41]. Innate and adaptive immune cells, such as T cells and NK cells, both have the ability to produce IFNγ. Natural killer (NK) cells serve as the early source of IFNγ for M1 polarization. NK cells produce IFNγ in response to stress or infections which primes and activates macrophages to secrete pro-inflammatory cytokines and increases their production of oxygen and nitrogen radicals as well as superoxide anions which promotes their killing ability[42]. Thus, NK cells help macrophages in combatting infections. However, NK cells do not produce IFNγ on a long-term basis and therefore cannot support M1 polarization indefinitely. The adaptive immune response is required to sustain M1 macrophage polarization to maintain clearance of infection and

defense against intracellular pathogens. Antigen specific T helper 1(T_H1) cells produce IFNγ which maintains M1 polarization and sustain host defense [31].

An important component of the host defense mediated by M1 macrophages is the production of pro-inflammatory cytokines. However, excessive production of pro-inflammatory cytokines can also lead to damage of host tissue. It is known that IL-23, IL-6, and IL-1 are required to promote differentiation and expansion of T cells into highly pro-inflammatory T_H17 cells secreting IL-17 cytokine which has been linked with inflammatory autoimmune pathologies[43-45]. M1 polarized macrophages have also been implicated in the immunopathology associated with several autoimmune disorders, such as rheumatoid arthritis and inflammatory bowel disease[46, 47].

M1 polarized macrophages play an important role in clearing intracellular pathogens[48]. Humans that have genetic mutations in IFNγ pathways or mice that lack IFNγ expression have been shown to be more susceptible to viral, protozoal and bacterial infections[49]. For example, treating *Leishmania donovani* infected macrophages with IFNγ did not result in clearance of the pathogen due to the impairment of IFNγ signaling and consequent M1 polarization by *L.donovani*[50]. *Mycobacterium tuberculosis* evades the macrophage immune response by a similar mechanism. *M. tuberculosis* expresses a lipoprotein that disrupts IFNγ signaling and interferes with the pathway involved in antigen presentation[51]. The evolution of such mechanisms by pathogens highlights the importance of M1 polarization in host defense.

Alternatively activated macrophages and their role in disease

Alternatively activated macrophages or M2a polarized macrophages or wound healing macrophages arise in response to IL-4 or IL-13 which are produced during tissue

injury[52]. Mast cells and basophils are the major producers of IL-4; however other granulocytes may also produce IL-4[53]. Eosinophils and basophils also produce IL-4 in response to chitin which is found in some fungi and parasites[54]. IL-4 stimulation of macrophages leads to higher arginase activity. This enzyme allows M2a polarized macrophages to contribute to the extracellular matrix by converting arginine into ornithine, which is a precursor of collagen and polyamines[55]. The polyamines, thus produced, can suppress clonal expansion of surrounding lymphocytes by influencing cytokine production[56]. In contrast to M1 polarized macrophages, murine macrophages treated with IL-4 produced very little pro-inflammatory cytokines and as a consequence are not able to clear intracellular pathogens[33].

T_H2-associated immune responses are important in the lungs and intestines where they are induced by disturbances in the integrity of mucosal surfaces[54]. These responses are not limited to mucosal tissues and can also occur in response to helminth infections[57]. As for M1 polarized macrophages, M2a macrophage polarization also needs to be controlled. M2a polarized macrophages can also have a harmful effect on the host if the matrix promoting ability of these macrophages is not properly regulated. For example, in chronic schistosomiasis, excessive activation of M2a polarized macrophages leads to tissue fibrosis[58]. There was a reduction in tissue fibrosis when macrophages were treated with IL-4 specific antibodies which led to a decrease in M2a polarized macrophage population. Tissue fibrosis was also diminished with macrophages that lacked the receptor for IL-4[58]. Mice with experimental asthma have been shown to harbor M2a polarized macrophages in their lungs and this population is thought to play a part in increased airway resistance and mucus formation[59].

Studies have shown that M2a polarized macrophages allow survival of some intracellular pathogens[60-62]. *Cryptococcus neoformans*, an intracellular fungus, multiplies at a higher rate in mice that overexpress IL-13 as compared to wild type mice. It was also shown that mice deficient in IL-13 expression did not support intracellular fungal growth[63]. IL-13 production was also linked to M2a polarization and enhanced hyper-reactivity in the airways of the mice[63]. Polyamine synthesis in M2a polarized macrophages has been linked to increased survival of *Leishmania major*[61]. Furthermore, using murine *Yersinia enterocolitica* infection model it was demonstrated that the clearance of the pathogen was associated with M1 polarization of macrophages while M2a polarized macrophages allowed for growth of the bacterium[64].

Regulatory macrophages and their role in disease

Regulatory macrophages, generated in the later stages of the adaptive immune response, serve to limit inflammation and dampen the immune response [65]. Although many signals can generate regulatory macrophages, production of IL-10 by this population of macrophages is their key feature[33]. IL-10 production has been shown to be regulated by activation of MAPK extracellular-signal-regulated kinase (ERK) signaling[66]. Another cardinal feature of regulatory macrophages is that they down-regulate IL-12 production, therefore a high IL-10 to IL-12 ratio can also be used to demarcate regulatory macrophages[67].

Regulatory macrophages can be divided into M2b and M2c polarized macrophages. M2c polarization is predominately induced with IL-10, but can also be induced by transforming growth factor-β (TGF-β), or glucocorticoids[68]. M2b polarization can be induced with immune complexes, LPS or IL-1R ligands[68]. Although microbial signals, like LPS,

lead to the induction of M1 polarization they can also, under certain conditions, result in endotoxin tolerance. This means that upon re-challenge there is an attenuated response at the macrophage and even at the organism level[69]. The immunosuppressive phenotype that is found during sepsis is mirrored during *in vitro* generation of LPS tolerance. Thereby, the host prevents excessive inflammation related to tissue damage via tolerance[69]. However, LPS tolerance is not so much a lack of response as it is an adaptive response that results in a shift in macrophage function from microbicidal to immune-regulatory phenotype [70, 71].

Regulatory macrophages, similar to M2a polarized macrophages, allow for susceptibility to infection by pathogens. For example, *Leishmania* spp. bind host IgG and use Fc receptors on macrophages to gain entry into the cells. This triggers downstream signaling events that lead to the development of regulatory macrophages[72]. These macrophages by virtue of their ability to dampen the immune response allow for the pathogen to evade the immune response. Pathogens have evolved to either limit M1 polarization or to promote the generation of regulatory macrophages to avoid eradication. *Bacillus anthraci'*s lethal toxin has the ability to interfere with MAPK signaling and thereby limiting the secretion of pro-inflammatory cytokines[73]. *Coxiella burnetii* infection down-regulates genes associated with M1 polarization like TNF but promotes M2 macrophages polarization. The promotion of M2 polarization may explain the persistence of this intracellular bacterium[74]. Thus, macrophage polarization must be tightly regulated to limit host damage as well as susceptibility to infection.

Tumour-associated macrophages (TAMs) or M2d macrophages

TAMs that have been isolated from solid tumours display a M2 polarized state. In fact, TAMs represent a novel M2 polarization state denoted as M2d polarization[75]. M2d

macrophages arise from circulating monocytes infiltrating the tumour environment in response to chemotactic factors such as MCSF, CCL2 and VEGF[76, 77]. M2d polarization occurs in response to the tumour milieu but the exact mechanisms are not known. Thus far, leukemia inhibitory factor and IL-6 have been identified as factors that could promote M2d polarization in conjunction with MCSF in the tumour microenvironment[75]. Poor outcomes in cancer have been linked to increased numbers of TAMs[78]. IL-4 and IL-13 also play a role in tumour progression as well as feeding M2 polarization[79]. Recently, IFNγ has been shown to reverse the immunosuppressive nature of TAMs. Thus, IFNγ could potentially be used as local therapy to suppress the formation of TAMs and to push macrophages to M1 polarization as well as activate T cells to promote an anti-tumour response[80].

In summary, macrophages have been associated with having either a protective role or a causative role in cancers[81]. M1 polarized macrophages have been shown to have protective role by mediating tumour killing mechanisms and counteracting tumour-associated macrophages (TAMs) and M2 polarized macrophages. TAMs and M2 polarized macrophages have been shown to limit the immune response to tumours and promote tumour growth and metastasis[82]. M1 polarized macrophages also promote the Th1 response associated with the anti-tumour response[83].

Innate immunity and Toll-like receptors (TLRs)

The recognition of the repeated molecular motifs found on pathogens such as LPS, peptidoglycan or lipoteichoic acid forms the core of the innate immune response. These molecular motifs are collectively known as pathogen-associated molecular patterns (PAMPs) [1]. The receptors that are involved in recognizing PAMPs are known as pattern recognition

receptors (PRRs). There are several classes of PRRs such as C-type lectins (CLRs), Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I like receptors (RLRs).

TLRs are type 1 transmembrane proteins and were the first PRRs to be discovered. They are expressed on NK cells, epithelial cells, endothelial cells, neutrophils, dendritic cells and macrophages[84]. TLRs all share the same structure and are composed of three domains: an N-terminal ectodomain that has leucine-rich repeats that are involved in co-receptor interactions and recognition of receptors, a transmembrane domain, and a C-terminal cytoplasmic domain with Toll-IL-1R resistance (TIR) signaling domain[85]. TLRs sense various microbial products including nucleic acids, polysaccharides, proteins and lipids. In addition to recognizing microbial patterns, they can also sense danger-associated molecular patterns (DAMPs). DAMPs, such as host DNA and chromatin, are usually stored in compartments that are not accessible to TLRs. However, during inflammation or injury, the contents of these compartments become exposed to TLRs[86]. There are at least 13 TLRs that have been discovered and each has their own specificity to microbial ligands[87]. TLRs can be divided into two main groups: surface bound TLRs and nucleic acid sensing TLRs[88]. The surface bound group includes TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 and they recognize microbial membrane products such as lipids, proteins, and lipo-proteins. The other group composed of TLR3, TLR7, TLR8, and TLR9 sense microbial nucleic acids and are located in endoplasmic reticulum, endosomes and lysosomes[88].

When stimulated with agonists, TLRs recruit adaptor molecules to initiate downstream signaling[89]. TLR signaling can be classified by virtue of the adaptor molecules that are involved in the pathways: dependent on adaptor MyD88 or TIR domain-containing adaptor inducing IFN-β (TRIF) (MyD88 independent) [89]. All TLRs, except TLR3 and TLR4,

signal exclusively through the MyD88 dependent pathway. TLR4 is unique due to its ability to signal through both MyD88 and MyD88 independent pathways[90]. TLR 2, 3 and 4 ligands reflect all kinds of microbial ligands and thus were used to characterize the cytokine profile of the polarized macrophages.

Macrophages and cytokines

Macrophages are a major source of cytokines produced in the body and play a central role in the immune response, hematopoiesis and inflammation by virtue of their cytokine production[91]. While T cells require days before they secrete cytokines upon activation, macrophages have the ability to respond immediately within hours of activation via *de novo* mRNA synthesis and subsequent protein expression[91]. Macrophages can release pro-inflammatory cytokines, such as TNFα, IL-1β, IL-6, and interferons, which recruit immune cells, such as neutrophils, by causing chemokine release in nearby cells and up regulating adhesion molecule expression to facilitate endothelial transmigration[92]. Macrophages also secrete cytokines, such as IL-12 and IL-23, which play an important role in differentiation and regulation of Th cells[93]. M1 polarized macrophages are characterized as producing copious amounts of pro-inflammatory cytokines which help promote antigen-specific Th1 and Th17 response[94]. Macrophages also secrete cytokines such as IL-10 and IL-22 which aid in inhibiting the immune response and regulating reparative processes. M2 polarization is associated with high levels of IL-10 and low levels of IL-12 production[94].

Interferon γ production in macrophages

IFNγ is a Type II IFN and is produced by NK cells and Th1 cells. IFNγ activates phagocytes by up-regulating their lysosomal enzymatic activity and increasing the level of reactive oxygen species[95]. Recently there has been evidence in murine and human models

suggesting that macrophages can also produce IFNγ. IL-12 stimulation of peritoneal murine macrophages has been show to result in IFNγ secretion[96]. LPS has also been shown to increase the steady-state levels of IFNγ mRNA in murine macrophages[97]. IL-12/IL-18 treatment of murine peritoneal macrophages results in IFNγ production in a STAT4-dependent manner[98]. These results have also been extended to murine bone marrow-derived macrophages treated with IL-12/IL-18[99]. *In vivo* and *in vitro* models of pulmonary mycobacterial infection studies have shown that murine lung macrophages can also produce IFNγ[100]. In humans, macrophages have been shown to produce IFNγ in certain disease states. Macrophages from patients suffering from pulmonary sarcoidosis released IFNγ while macrophages from healthy controls failed to do so[101]. *In vitro* infection of human alveolar macrophages with *Mycobacterium tuberculosis* has been shown to result in IFNγ protein release as well as an increase in mRNA levels[102]. The primary criticism in these studies is the possible contamination of macrophages with other cells such as T cells or NK cells. To this end, a study examined IFNγ production in human macrophages at a single-cell level by immunohistochemistry and by enzyme-linked immunosorbent spot (ELISPOT) analysis. The study demonstrated IFNγ production in macrophages that had been cultured with IL-12 and IL-18 as well *ex vivo* production in macrophages from bronchoalveolar lavage samples from patients who underwent flexible bronchoscopy[103]. One study reports the production of IFNγ in human monocytes upon IFNγ treatment, but there are no reports on IFNγ production in healthy human macrophages or polarized macrophages[104]. Therefore, I investigated whether polarized macrophages produced IFNγ prior to or following stimulation with TLR ligands.

HIV infection in macrophages

Macrophages, unlike T cells, maintain their numbers during HIV infection. They have been shown to produce large amounts of intracellular and extracellular HIV both *in vivo* and *in vitro* without being subjected to death due to productive infection[105]. They serve as a constant source of virus, even when CD4+ T cell numbers are low[105]. Productively-infected macrophages can transfer virus to CD4+ T cells by fusing with them and thereby acting as viral dissemination agents[106]. Furthermore, direct contact between infected macrophages with CD4+ and CD8+ lymphocytes leads to lymphocyte apoptosis[107-109]. Infected macrophages can also cause apoptosis in neurons and astrocytes without direct contact. This bystander effect is mediated by the release of Fas ligand by infected macrophages and its initiation of the extrinsic apoptosis pathway via Fas-Fas ligand binding[110].

HIV and its entry into macrophages

Human immunodeficiency virus (HIV) is a lentivirus and is the causative agent of acquired immunodeficiency syndrome (AIDS). The viral core is composed of p24, the viral capsid, in which lies two strands of viral RNA. The core also contains the enzymes necessary for viral replication: reverse transcriptase, protease, ribonuclease, and integrase. The viral genome also encodes three structural proteins (Gag, Pol and Env) and six accessory proteins (Vpr, Vpu, Vif, Nef, Rev, Tat) [111]. HIV entry into a target cell requires two steps: the first is the binding of the viral glycoprotein gp120 to CD4 on the target cell and the second step involves the interaction with a chemokine receptor (CXCR4 or CCR5)[112]. The virus generates DNA copies of its genome using a reverse transcriptase and using integrase integrates the DNA copies into the host cell genome. Subsequent viral copies are made at the expense of the host own replication machinery[111].

Invading pathogens are faced with harsh conditions inside and outside of a cell. The extracellular environment is protected by innate and adaptive immune responses by factors such as antibodies and complement[113]. The cytoplasmic environment is protected by intracellular innate immune receptors and mechanisms such as autophagy[114,115]. Furthermore, intracellular pathogens must possess strategies to enter and exit vesicles or the cytoplasm[116]. Virus containing compartments (VCCs) limit access to innate and adaptive immune factors and protect viruses from recognition by intracellular pathogen receptors. While these VCCs resemble late endosomes and are rich in markers for multivesicular bodies, there are distinct from either due to their neutral pH and tubular connections to the extracellular milieu[117]. Hence, VCCs provide protection against acidic environments and serve to transport the virus to other target cells.

The presence of budding, mature and immature virions in VCCs has provided evidence that HIV-1 uses VCCs as an assembly site in macrophages[118-120]. These compartments do not necessarily form in response to HIV as similar compartments exist in uninfected macrophages yet their function pre-infection is not known[121]. These compartments become more apparent as the cells mature and become more pronounced during HIV infection[121,122]. Most VCCs, not all, are usually connected to the extracellular environment by narrow channels that permit the passage of small molecules such as dyes and horseradish peroxidase[120,123]. The accumulation of HIV-1 in VCCs make macrophages a reservoir that can remain localized in its tissue or migrate to other tissues[124]. The interaction of an infected macrophage and a CD4 T cell can lead the formation of a viral synapse by virtue of CD4 on the T cells and HIV-1 Gag and Env on the macrophage[125]. The idea of

VCC migration to these viral synapses has been supported by time-course experiments and the presence of the HIV virions at the interphase[126].

HIV persistence in macrophages

The elimination of HIV infection is hampered by HIV latency and the persistence of viral reservoirs[127]. CD4+ T cells and macrophages constitute the major reservoirs of HIV[128]. These latently infected cells are poorly recognized by the immune system and can persist for a long time even during antiretroviral therapy administration[129]. There are marked differences in the infection of these two cell types. The number of CD4+ T cells falls as the infection progresses as the virus has a cytopathic effect on T cells. The antigen induced T cell proliferation stage has been linked to the formation of the viral reservoir. The cells that evade the cytolytic effects of the virus turn to resting stage and this is linked with memory T cell establishment[130]. These resting cells' genomes have integrated HIV provirus that cannot replicate[128]. The activation of these resting cells by activating stimuli can lead to virus replication[131].

Latency is a reversible state in an infected cell during which the cell does not produce infectious particles[132]. There are two kinds of latency: pre-integration latency and post integration latency[133]. When HIV-1 enters the cell, it reverse transcribes its genome into DNA which is assembled into a pre-integration complex (PIC) [134]. The PIC, composed of viral DNA and proteins, is shuttled to the nucleus where it integrates into the host cell's genome. Pre-integration latency is a function of the inability of the PIC to reach the nucleus as well as inefficient reverse transcription[135]. This form of latency is common *in vivo,* especially in CD4 T cells. In resting CD4 T cells the rate of reverse transcription is much slower, as compared to active T cells, making the viral genome vulnerable to decay[136].

However, macrophages can accommodate unintegrated HIV-1 DNA for at least 30 days[137]. Macrophages can also facilitate sustained viral replication from this unintegrated DNA as well induce CXCL9 and CXCL10 in response to it[137]. This suggests that macrophages can facilitate low levels of viral replication and virus mediated activities promoting pathogenesis in infected individuals. However, there are only a few studies that have found unintegrated viral DNA in macrophages from infected patients and they are from brain tissue[138,139]. Restriction factors, such as APOBEC3, SAMHD1 and MX2 and a limited dNTPs pool are also factors that contribute to restricted replication in macrophages and might aid in pre-integration latency. SAMHD1 hydrolyzes dNTPs into nucleosides thus diminishing the dNTPs pool and rendering viral transcription inefficient[140]. MX2 prevents the integration of proviral DNA into the host chromatin thus restricting HIV at a post-entry stage[141].

Post-integration latency is the silencing of HIV gene expression after viral integration into the genome. This kind of latency is responsible for the persistence of the virus and its dissemination upon reactivation[142]. HIV integrates into euchromatin, which is actively transcribed, in macrophages[143]. Two nucleosomes, nuc-0 and nuc-1, interact with the promoter of HIV 1 regardless of the integration site of viral genome. Furthermore, HIV replication is only possible once nuc1 has been displaced suggesting chromatin remodeling is important in establishing HIV-1 latency in macrophages[144]. Viral gene expression is also partly controlled by miRNAs[145]. In PBMCs, HIV inhibits the expression of miRNA cluster miR-17/92 to promote its replication[146]. Furthermore, the lesser susceptibility of monocytes to HIV infection as compared to MDMs might be explained by the expression of anti-HIV miRNAs. Indeed, suppression of theses miRNAs in monocytes led to increased HIV

replication and the addition of their mimics to MDMs protected MDMs from productive HIV infection[147].

Macrophage polarization and HIV infection

Immune system activation plays an important role in the resolution of many infectious diseases[148]. For example, pathogenic simian immunodeficiency virus (SIV) infection is linked to a prolonged and excessive activation of the immune response which constitutes a difference between non-pathogenic SIV infection and pathogenic SIV infection[149]. The activation status of the immune system is also linked to HIV pathogenesis[150]. Prolonged immune activation during chronic HIV infection creates a milieu for viral replication even in the presence of antiretroviral therapy[151]. In advanced HIV disease, immune activation is a stronger determinant in survival than higher viral burden or virus chemokine co-receptor usage[152]. Acute HIV infections leads to gastrointestinal damage and the increased translocation of gut microbial products, such as LPS. This suggests that during chronic HIV infection systemic immune activation is caused by these factors, such as LPS, and determines the rate of AIDS onset[153]. Since macrophages are one of the primary targets of LPS-mediated immune activation and play a key role in the pro- and anti-inflammatory immune response, it is important to study the effect of immune activation and macrophage polarization with respect to HIV infection. Macrophages also react to signals from opportunistic co-infections and therefore can also be activated by various bacterial, viral or fungal entities. Hence, the dominant polarization profile will be a function of all these stimulations.

The location of a macrophage also dictates its ability to support HIV infection. Intestinal macrophages are typically resistant to HIV infection unlike alveolar and vaginal

macrophages[154]. Intestinal macrophages express CD4 but have lower expression levels of the chemokine HIV co-receptors CCR5 and CXCR4 as well as innate immune receptors[155]. Cytokines also modulate the ability of macrophages to support HIV infection. IFNα/β treatment of macrophages has been shown to limit HIV infection[156]. Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) is a cytidine deaminase that creates C to U mutations in newly transcribed viral DNA. IFNα treated macrophages have shown increased levels of APOBEC3G mRNA[157]. Macrophages constitutively transcribe NF-κB and this property makes them ideal for HIV infection due to the presence of a NF-κB responsive site in HIV-LTR[158]. However, IL-6 stimulated macrophages promote HIV infection independent of NF-κB activation[159]. Certain cytokines can have varying effects on HIV infection depending on the differentiation state of macrophages and the exposure timing. IL-4 and IL-13 are known to enhance viral replication in monocytes but limit it in monocyte-derived macrophages[160]. Pre-treating macrophages with TNFα before infection leads to limiting infection, however treating latently infected macrophages with TNFα leads to NF-κB activation and increase in viral transcription[161,162].

Macrophage polarization can also affect the susceptibility of macrophages to HIV. A study showed that macrophage polarization resulted in a poor HIV infection as compared to MDMs[163]. This study looked at M1 and M2a polarized macrophages and suggested that M1 polarization led to inhibition at a pre-integration point and M2a polarization at a post-integration point[163]. Overall, this suggests that macrophage polarization might be a model through which HIV establishes latency. Therefore, it was of interest to explore HIV infection in the model of macrophage polarization of this study which includes M2b and M2c macrophages to further expand the understanding of HIV latency.

Macrophage resistance to apoptosis

Macrophages are suited to their role as HIV reservoirs due to their inherent resistance to apoptosis. Apoptosis is a form of cell death that is important in tissue development and immune regulation. It serves as a mechanism to control macrophage activation. Apoptosis is brought about by the activation of caspases, which are cysteine proteases involved in the cleavage of certain proteins and this process eventually leads to cell death[164]. Apoptosis can be mediated through death receptor ligation or through disruption of the mitochondrial membrane integrity. Before differentiation, monocytes are highly susceptible to death receptor mediated apoptosis[165]. However, macrophages are resistant to apoptosis mediated by death receptor ligation, antineoplastic factors and ionizing irradiation[166,167]. There are three mechanisms in macrophages that help maintain their viability: the presence of anti-apoptotic molecule FLIP (FADD-like IL-1β-converting enzyme-inhibitory protein), the constitutive expression of NF-κB and the PI3K-Akt pathway.

Peripheral blood isolated monocytes express both death receptor FAS and the FAS ligand and are highly sensitive to caspase 8 dependent FAS-mediated apoptosis[168]. Cytokine activation induces the expression of anti-apoptotic molecule FLIP in monocytes. FLIP inhibits caspase 8 activation after FAS ligand interactions by binding to FADD (FAS-associated death-domain protein). Activated macrophages have been implicated in the pathogenesis of rheumatoid arthritis[169]. FLIP has been found to be highly expressed in macrophages that were obtained from the joints of people with rheumatoid arthritis and this might pertain to a mechanism by which macrophages persist in the disease[170].

In vitro differentiated macrophages constitutively express NF-κB. Inhibition of NF-κB expression led to caspase 9 activation and the loss of mitochondrial membrane integrity

via down-regulation of A1[171]. The phosphatidylinositol 3-kinase (PI3K) pathway is also involved in the activation of NF-κB. It protects against apoptosis through phosphorylation of BAD (Bcl-2-antagonist of cell death), caspase-9 inhibition and suppression of FAS ligand. Akt1 activation is mediated by PI3K in macrophages[172]. Akt suppression via PI3K inhibition using pharmacological inhibitor LY294002 or using a dominant negative form of Akt-1 resulted in the loss of mitochondrial potential, caspase 3 and 9 activation, and DNA fragmentation which resulted in apoptosis[172]. This phenomenon was independent of NF-κB or death receptor interactions and involved suppression of the Bcl-2-family member Mcl1 (myeloid-cell leukemia sequence 1)[172].

The PI3K/Akt pathway has been studied for its role in cell survival and has been nominated as a target for anti-cancer therapies due to its key activation during tumorigenesis[173]. Recently, it has also been shown that HIV-1 triggers the PI3K/Akt pathway making primary human macrophages resistant to apoptosis [174]. Normally, PI3K/Akt pathway is negatively regulated by the phosphatase tensin homolog (PTEN), which prevents the activation of Akt [175]. HIV-1 Tat, an accessory viral protein, promotes cell survival by lowering PTEN levels in infected macrophages[174]. Thus, PI3K/Akt pathway inhibitors could be used as a therapy to interfere with HIV reservoir formation.

The role of IAPs in macrophage survival

Inhibitors of apoptosis proteins (IAPs) provide another mechanism by which cells maintain their viability[176]. Expression of the IAP gene was able to prevent actinomycin-D induced apoptosis in baculovirus-infected insect cells which led to the discovery of IAPs[177]. XIAP, cIAP1 and cIAP2 are the most studied IAPs from the eight mammalian ones that are known. IAPs are composed of three main domains. Members of IAPs can contain up to

three Baculoviral IAP Repeat (BIR) motifs in their N-terminus. BIRs are 70 amino acid long domains that facilitate protein-protein interactions[178]. Each of the BIR domains of XIAP has a distinct specificity for caspases: BIR 3 binds caspase 9 whereas BIR1 and BIR 2 are involved in binding caspase 3 and 7[179]. Additionally, IAPs are made up of a RING (Really interesting new gene) domain in the C terminal end involved in ubiquitin ligase activity and of a CARD (caspase associated recruitment domain) which also serves in protein interaction[180]. Studies involving overexpression of IAPs have shown that these proteins have a role in preventing apoptosis. Although cIAP 1/2 and XIAP can bind caspases, it is only XIAP that has been shown to down regulate caspase activity[181]. IAPs can inhibit caspases by binding their active site as is the case for XIAP or they can ubiquitinate caspases making them the target of proteasomal degradation[182,183].

Another mechanism by which IAPs maintain cell viability is by binding to pro-apoptotic molecules from mitochondria such as Smac (second mitochondria-derived activator of caspases). Smac is a protein derived from the mitochondria that has the ability to bind with IAPs and cause cell death via apoptosis[184]. The last four N-terminal residues of Smac bind to XIAP by virtue of its BIR3 domain causing the displacement of caspase 9 and prevent caspase inhibition[185]. Smac can simultaneously bind BIR 2 and BIR3 on XIAP releasing caspase 3 and caspase 7 and reinitiating the caspase cascade[186].

Smac also has a role in receptor-mediated apoptosis. Two signals are required to induce activation of caspase 3 via TRAIL and FAS ligand interactions: the first signal is the activation of caspase 8 via its cleavage and the other is a cytochrome-c and caspase 9 independent signal from the mitochondria. Smac has been discovered to be this second signal that releases caspase 3 from XIAP[187].

High IAPs levels have also been associated with cancers such as leukemia and lymphomas by virtue of their anti-apoptotic properties. They are a factor in poor prognosis and play a role in resistance to chemotherapy[188]. In light of this, there is interest in designing compounds that would mimic the 4 N terminal residues of Smac and would allow for caspase 9 displacement from XIAP and re-initiation of the caspase cascade. Smac mimetics are small molecules that act like the 4 N terminal residues of Smac and have the affinity for the BIR3 domain of XIAP[189]. Smac mimetics can induce cells death in tumour cell lines as well as cause resistant cell lines to become more susceptible to cell death[190,191]. Smac treatment was shown to cause cell death via TNFα as this effect was abrogated during caspase 8 knockdown and TNFα blockade[192,193]. For these reasons, Smac mimetics are being actively developed as potential therapeutics for cancer therapy[194].

Rationale

In infections or in inflammatory episodes the differentiated hematopoietic cells of the innate and adaptive immunity must regulate their functions in such a way as to be able to efficiently clear the injury or invading pathogens without causing damage to the surrounding tissue. One mechanism by which this is carried out is fine-tuning of the immune response with respect to the milieu and this is achieved by promoting a polarized state that can effectively re-establish homeostasis. Macrophages operate at the interface of the innate and adaptive immunity and through their plasticity can display polarized states. Macrophages can be divided into two main polarization states: M1 and M2. These polarization states are acquired due to a change in type, timing or amount of micro-environmental signals[4]. These states are far more flexible than tissue-specific differentiation and macrophages can switch from one state to the other.

The majority of studies on macrophage polarization have occurred in the murine model. In fact, murine macrophages are very well characterized with distinct markers that delineate polarization states in mice such as arginase-1 and Ym1 in M2 polarized states[195]. However human polarized macrophages are poorly characterized. There are no such phenotypic markers that are only present in one polarization state of human macrophages. Furthermore, studies that do explore macrophage polarization are limited to M1 and M2a polarized macrophages. Thus, this study was undertaken to characterize macrophage polarization using a non-exhaustive panel of surface markers with respect to M1, M2a, M2b and M2c macrophages.

However, markers alone are not sufficient in characterizing macrophage polarization states[196]. Thus, another aim of this study was to characterize the macrophage polarization

states with respect to their functionality. Macrophages are potent producers of cytokines in response to pathogen recognition, thus it was of interest to characterize the polarized macrophages with respect to cytokine production. Macrophages were activated using agonists for TLR 2, 3 and 4 to take into account MyD88-dependent and -independent pathways.

Furthermore, macrophages are one of two cell types that are infected with HIV and act as viral reservoirs and disseminators[105]. This is facilitated by their ability to be resistant to apoptosis. Cell survival in macrophages is mediated in part via PI3K pathway and IAPs. Thus, it was important to characterize the polarized macrophages with respect to susceptibility to HIV infection as well as susceptibility to apoptosis.

Hypothesis

Polarized macrophages manifest a characteristic cell surface marker profile and functional profile related to cytokine production, resistance to apoptosis and susceptibility to HIV infection.

Objectives

1. To determine the cell surface marker profile for each polarization state
2. To examine the functional profile by:
    a. Determining the cytokine profile for each polarization state
    b. Determining which polarized macrophage subsets are resistant /susceptible to *in vitro* HIV infection
    c. Determining which polarized macrophages subsets are resistant/ susceptible to apoptosis induced by PI3K/Akt blockage.

Materials and Methods

Macrophage polarization

Blood was obtained from healthy donors as per the protocol approved by the Ottawa Health Sciences Network Research Ethics Board. Informed consent was obtained from the donors verbally and in written form. Peripheral blood mononuclear cells (PBMCs) were isolated by collecting the buffy coat generated after Ficoll Paque (GE Healthcare, Buckinghamshire, UK) density centrifugation at 1600 rpm for 45 minutes. PBMCs were re-suspended (4×10^6 cells/ml) in Iscove's Modified DMEM 1X media (Sigma-Aldrich) and 1 ml was seeded into 12 well polystyrene plates (Thermoscientific, Rochester, New York) to isolate monocytes by adherence method. Monocytes were allowed to adhere at 37 °C, 5% CO_2/air mixture for 3 hours. Non-adherent cells were washed off using Iscove's Modified DMEM 1X media. The adherent monocytes were cultured for 6 days in Iscove's Modified DMEM 1X media supplemented with 10% fetal bovine serum (GE Healthcare), 10 units/mL of penicillin/gentamicin (Sigma-Aldrich) and 10 ng/ml recombinant MCSF (R&D Systems, Minneapolis, Minnesota) to generate monocyte-derived-macrophages (MDMs). The media was changed every second day. MDMs were polarized using appropriate stimuli for 2 days: IFNγ (20 ng/ml) (Thermoscientific, Rochester, New York) for M1 macrophages, IL-4 (20 ng/ml) (R&D Systems, Minneapolis, Minnesota) for M2a macrophages, LPS (1 µg/ml) (Sigma-Aldrich) and IL-1β (10 ng/ml) (R&D Systems, Minneapolis, Minnesota) for M2b macrophages and IL-10 (10 ng/ml) (R&D Systems, Minneapolis, Minnesota) for M2c macrophages.

Cell surface marker analysis by flow cytometry

Polarized macrophages were washed with PBS and stained using phycoerythrin-conjugated antibodies for CD80, CD86, CD14, TLR4, CD163, CD200R and HLADR (BD Biosciences, Franklin Lakes, New Jersey) for 1 hour at 4°C in the dark. The cells were washed with 1X PBS (Sigma-Aldrich) to remove excess antibodies. Fluorescence was measured via flow cytometry using a FACSCanto flow cytometer and FACSDiva software (BD Biosciences, Franklin Lakes, NJ). Histograms were plotted using WinMDI version 2.8 software (J. Trotter, Scripps Institute, San Diego, CA).

Cytokine analysis by flow cytometry

Cytokine secretion was measured using Human Th1/Th2/Th9/Th17/Th22 13plex FlowCytomix Multiplex from eBioscience according to manufacturer's instructions. The bead-based immunoassay is designed to detect multiple analytes in the same sample. In this assay, the beads are coated with antibodies to the respective analyte and can be distinguished by their size and spectral address. 25 µl of the sample supernatant was incubated with the bead mixture and then incubated with a mixture of biotin-labelled antibodies against the analytes to be measured. Streptavidin-Phycoerythrin, which binds to the biotin and emits fluorescence, was then added to the mixture. Fluorescence was measured via flow cytometry using a FACSCanto flow cytometer and FACSDiva software (BD Biosciences, Franklin Lakes, NJ).

Cytokine analysis by ELISA

IL-23

Human IL-23 Duoset (R&D Systems, Minneapolis, MN), a plate based enzyme-linked immunosorbent assay (ELISA), was used to measure IL-23 levels in macrophage

supernatants. The assay was carried out on Costar high binding 96 well ELISA plates (Corning Incorporated, Corning, NY). The plates were coated overnight at 4° C with 100 µl/well of capture antibody (60 µg/ml) in PBS. The following day, plates were washed three times with 300 µl/well of 0.05% Tween 20 (Biorad) in PBS using Elx50 ELISA washer (Biotek, Winooski, VT). The plates were blocked with 300 µl/well of 1% Bovine serum albumin (BSA) in PBS for 2 hours at room temperature. Following blocking, the plates were washed as previously described and 100 µl of samples and standards were added and allowed to incubate overnight. The following day, the plates were washed as previously described and 100 µl/well of detection antibody (400 ng/ml) in 1% BSA in PBS was added. The plates were incubated at room temperature for 2 hours and then washed as previously described. 100 µl/well of Streptavidin-HRP in 1% BSA in PBS was added and the plates were incubated at room temperature for 30 minutes. The plates were washed and 100 µl/well of substrate (BioFX Labs, Owing Mills, MD) was added. 50 µl/well of Stop Solution (BioFX Labs, Owing Mills, MD) was added to stop the reaction. The plates were read at 490nm using iMark Microplate reader (Biorad, Mississauga, Ontario) and data was processed using Micro Plate Manager 6 software.

IFNγ

Human IFNγ Duoset (R&D Systems, Minneapolis, MN) ELISA was used to measure IFNγ levels in macrophage supernatants. The plates were coated overnight at 4°C with 100 µl/well of capture antibody (4 µg/ml) in PBS. The following day, plates were washed three times with 300 µl/well of 0.05% Tween 20 in PBS using Elx50 ELISA washer. The plates were blocked with 300µl/well of 1% BSA in PBS for 2 hours at room temperature. Following blocking, the plates were washed as previously described and 100 µl of samples

and standards were added and allowed to incubate overnight. The following day, the plates were washed as previously described, and 100 µl/well of detection antibody (200 ng/ml) in 1% BSA in PBS and 2% heat inactivated normal goat serum was added. The plates were incubated at room temperature for 2 hours and then washed as previously described. 100 µl/well of Streptavidin-HRP in 1% BSA in PBS was added and the plates were incubated at room temperature for 30 minutes. The plates were washed and 100 µl/well of substrate (BioFX Labs, Owing Mills, MD) was added. 50 µl/well of Stop Solution (BioFX Labs, Owing Mills, MD) was added to stop the reaction. The plates were read at 490 nm using iMark Microplate reader (Biorad, Mississauga, Ontario) and data was processed using Micro Plate Manager 6 software.

RNA isolation and semi-quantitative RT-PCR analysis of IFNγ

RNeasy Plus Mini kit (Qiagen, Hilden, Germany) was used to extract total RNA from cells, according to the manufacturer's instructions. A master mix was prepared as follows using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, California): 7.5 µL 10 X reverse transcriptase buffer, 3 µL dNTPs, 7.5 µL random primers, 3.75 µL reverse transcriptase and 53.25 µL ddH_2O. 25 µl of this master mix was added to the isolated RNA samples and GeneAmp PCR System 2700 amplifier (Applied Biosystems, Carlsbad, CA) was used to carry out RT-PCR for 2 hours to yield cDNA. The PCR program was carried out as follows for 40 to 50 cycles: initial incubation for 2 minutes at 50°C and 10 minutes at 95°C, denaturation cycles at 95°C for 15 seconds that were followed by annealing and elongation for 2 minutes at 60°C. For real time PCR the generated cDNA was mixed with 12.5 µL Taqman DNA polymerase, 1.25 µL primer pairs for either IFNγ (Hs00989291_m1) or β-actin (ACTB Hs99999903_m1) and 8.75 µL ddH_2O. The samples

were run using 7500 Real Time PCR System for 2 hours. Cycle threshold (Ct) values in amplification plots (Applied Biosystems) were used to calculate transcript levels to the control β-actin.

Measurement of IFN γ bioactivity in macrophage supernatants

MDMs and M1 polarized macrophages were treated with LPS for 24 hours. Supernatants from unstimulated and LPS stimulated MDMs and M1 polarized macrophages were collected. MDMs were treated with anti-IFNγR antibodies (40 μg/ml) or isotype control (40 μg/ml) (BioLegend, San Diego, USA) for 2 hours to block IFNγ binding to the receptor. These blocked MDMs were treated with the previously collected supernatants for 24 hours to induce expression of TLR4. The cells were stained with antibodies against TLR4 and fluorescence was measured by flow cytometry using a FACSCanto flow cytometer and FACSDiva software (BD Biosciences, Franklin Lakes, NJ). Histograms were plotted using WinMDI version 2.8 software (J. Trotter, Scripps Institute, San Diego, CA).

Apoptosis analysis by intracellular propidium iodide (PI) staining

Apoptosis was measured in macrophages following treatment with either LY294002 (Calbiochem, La Jolla, California), which inhibits PI3K activity, or LCL-161, a SMAC mimetic, or both LY294002 and LCL-161for 48 hours after polarization as previously mentioned. Cells were harvested and washed with 1X PBS and fixed with methanol at 4°C for 15 min. Cells were washed again with PBS and treated with 25 μl of 10 μg/ml RNase A, followed by staining with 25 μl of 1 mg/ml PI solution (Sigma-Aldrich) at 4°C for 1 hour. The amount of DNA present was analyzed using a FACSCanto flow cytometer (BD Biosciences) and the FACSDiva software. Apoptotic cells were represented as having

subdiploid levels of DNA as marked by the area next to the G0/G1 peak (2N DNA) and were quantified by histogram analyses. Cells which displayed minimal light scatter were not gated on during the analysis.

HIV infection and p24 ELISA

Dual tropic HIV clinical isolate, CS204, was a gift from Dr. F. Diaz-Mitoma (Children's Hospital of Eastern Ontario, Ottawa, Canada) [197]. HIV_{CS204} stocks were grown in CD14 THP-1 cells. The stocks were inactivated for 1 hour at 37°C in 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and the concentration of p24 was measured by ELISA using the HIV-1 $p24^{CA}$ antigen capture assay kit, following the manufacturer's instructions (AIDS & Cancer Virus Program, National Cancer Institute, Fredrick, MD).

Polarized macrophages and MDMs were infected with 700pg of viral stocks of HIV_{CS204} supernatants for 2 hours. The cells were washed with PBS and supernatants were collected on days 0, 2, 5 7, 9 and p24 concentration was measured at those time points using the HIV-1 $p24^{CA}$ antigen capture assay kit, following the manufacturer's instructions.

Statistical analysis

Graphs were generated analysis was carried out using GraphPad Prism 5. Means were compared by the Student's t test or ANOVA followed by Tukey's post-hoc test and results are expressed as a mean ± standard error of the mean of at least three experiments, unless otherwise stated.

Ethics Statement

All healthy participants gave informed written consent based on the study protocol approved by the Ottawa Health Sciences Network Research Ethics Board. .

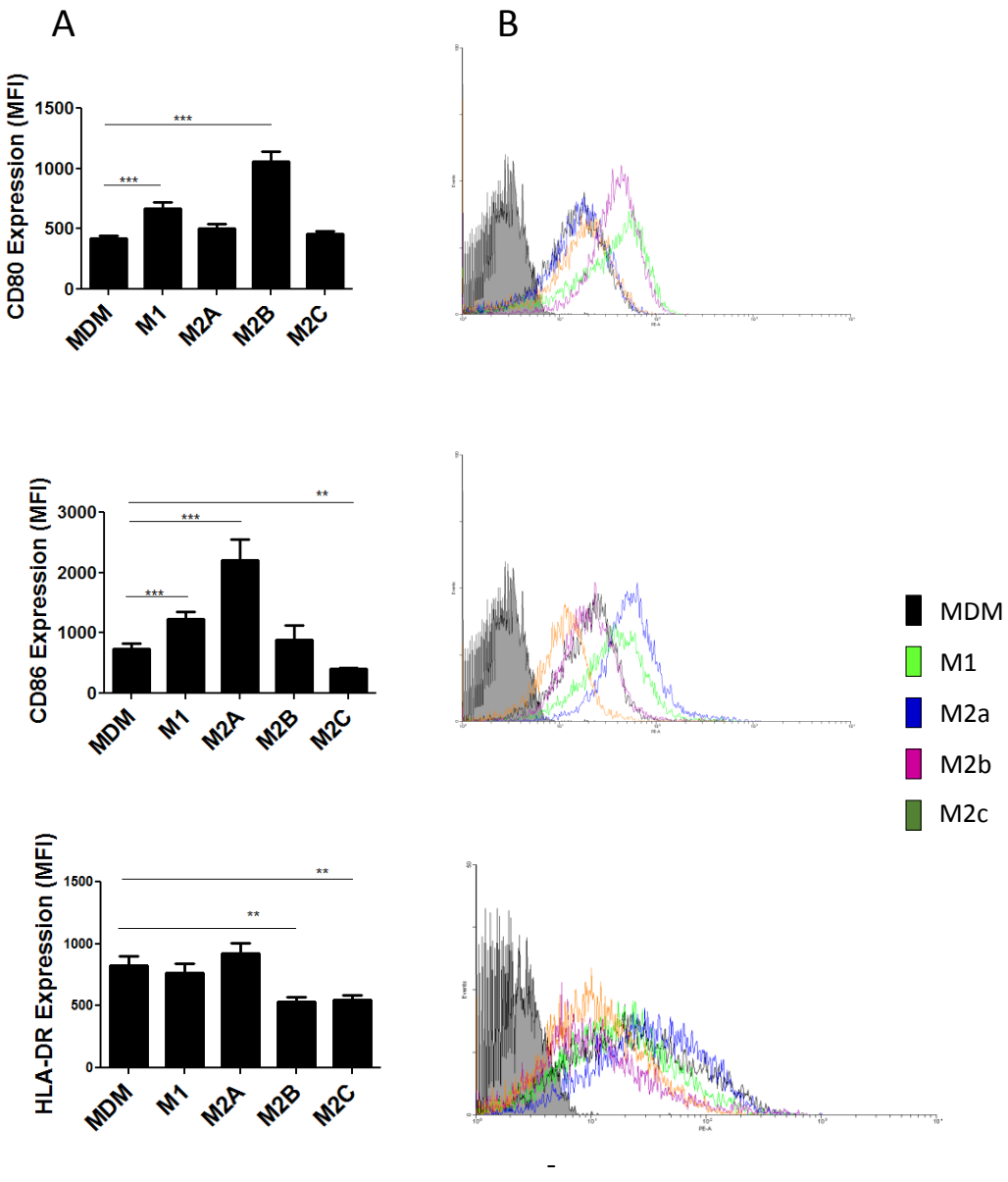
Results

Cell surface marker profile of polarized macrophages

Macrophages can polarize into classically or alternatively activated, M1 or M2, populations depending on environmental cues. M1 and M2 polarized macrophages can be characterized by a differential expression of the cell surface molecules that are generally present on macrophages, including CD80, CD86, HLA-DR, CD14, TLR4, CD163 and CD200R. MDMs were therefore analyzed for the expression of these molecules following different stimulations. MDMs were treated for 48 hours to induce different polarization states with the following stimuli: IFNγ for M1 macrophages, IL-4 for M2a macrophages, LPS and IL-1β for M2b macrophages and IL-10 for M2c macrophages. The cells were stained with antibodies against CD80, CD86, HLA-DR, CD14, TLR4, CD163 and CD200R. This non-exhaustive panel was selected based on literature reports, and the involvement of these molecules in macrophage activation[198-200]. The expression of the cell surface molecules was determined by flow cytometry analyses setting the expression levels in unpolarized MDMs as the base line. M1 macrophages were found to have significantly higher CD80 and CD86 expression compared to MDMs (Figure 2). There was no change in the expression levels of the other molecules in M1 polarized macrophages. M2a macrophages exhibited a significantly higher expression of CD86 and CD200R and significantly lower expression of CD14 and TLR4 as compared to MDMs. M2b polarized macrophages had significantly higher CD80 and CD14 expression and lower HLA-DR expression as compared to MDMs.

Figure 2. Cell surface marker characterization of polarized macrophages.

MDMs were polarized using indicated stimuli for 2 days: IFNγ (20 ng/ml) for M1 macrophages, IL-4 (20 ng/ml) for M2a macrophages, LPS (1 μg/ml) and IL-1β (10 ng/ml) for M2b macrophages and IL-10 (10 ng/ml) for M2c macrophages. Polarized macrophages were stained with antibodies against the mentioned cell surface molecules and fluorescence was measured by flow cytometry. MFI values (A) were obtained using FACSDiva software and histograms (B) from one representative experiment are shown. Bar graphs represent mean ±SEM, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$ with n=10 (CD80, CD86, HLA-DR, CD14, CD163 and CD200R) and n=4 (TLR4).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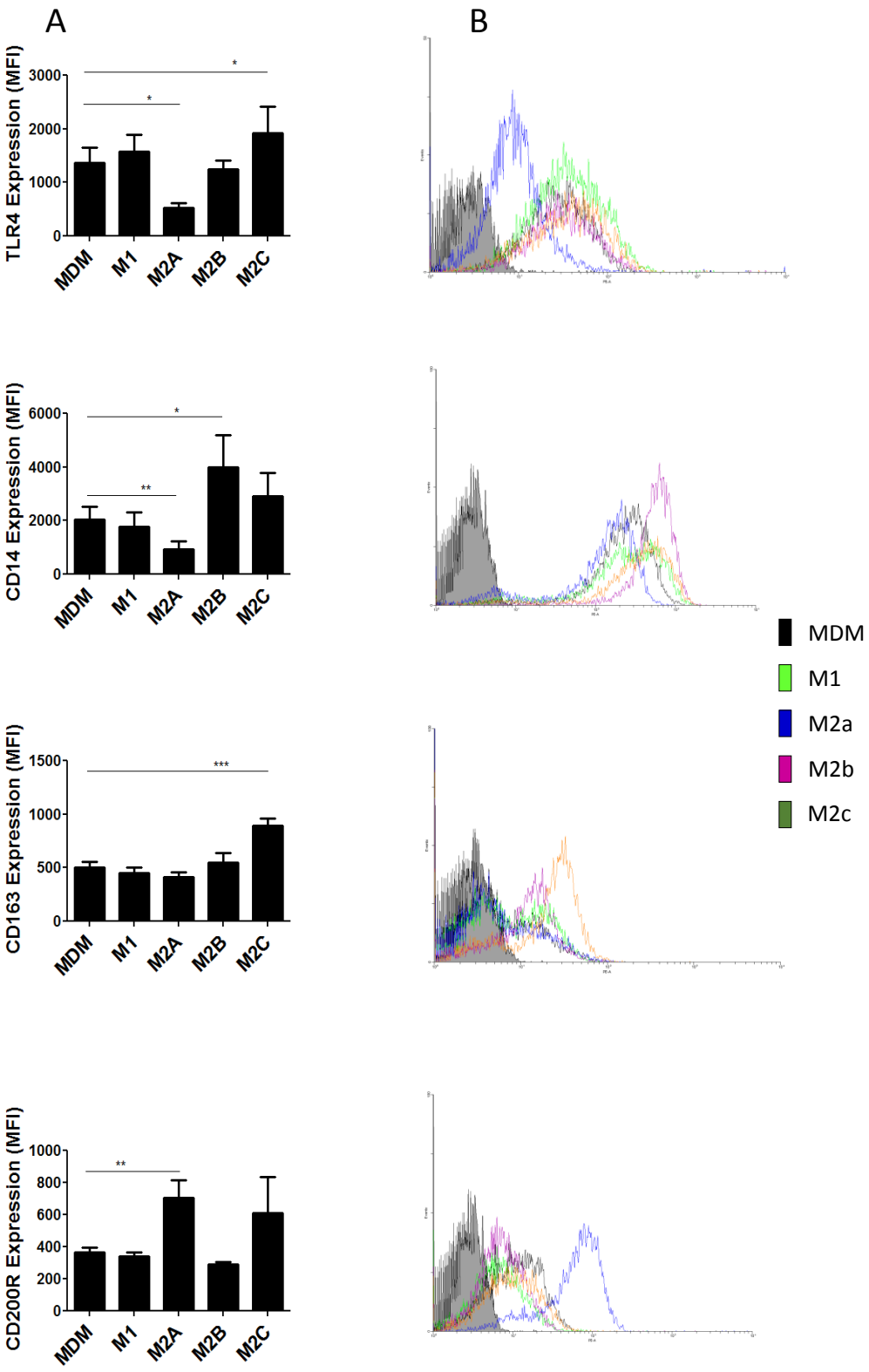
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200
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MDM
M1
M2a
M2b
M2c

The expression levels of other molecules were similar to that of unpolarized macrophages. M2c polarized macrophages were characterized by a significant decrease in CD86 and HLA-DR expression and a significant increase in CD163 expression compared to MDMs. Thus, each polarization state has its own distinct profile with respect to these markers. In summary, the following are the phenotypic characteristics of each polarization state: M1 polarized macrophages are $CD80^{high}$ and $CD86^{high}$, M2a macrophages are $CD86^{high}$, $CD200R^{high}$, $TLR4^{low}$ and $CD14^{low}$, M2b macrophages are HLA-DR^{low}, $CD80^{high}$ and $CD14^{high}$, and M2c macrophages are $CD86^{low}$, HLA-DR^{low}, $TLR4^{high}$, and $CD163^{high}$.

Functional profile of polarized macrophages

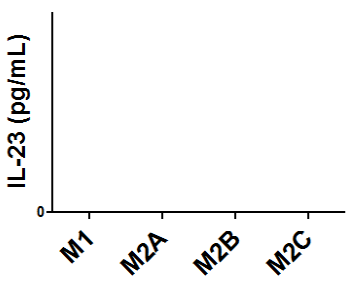
Differential cytokine production by polarized macrophages

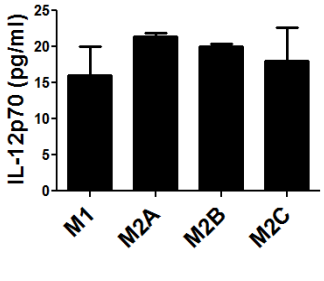
Basal cytokine profile of polarized macrophages

Cytokines are important messengers in mediating and coordinating the immune response. Therefore, to understand the functions of the polarized macrophages it was also important to analyze the profile of cytokines produced by these distinct populations. To analyze the cytokine profile, MDMs were polarized as previously described. The polarizing stimulus was removed after 48 hours, fresh media was added and the cells were incubated for additional 24 hours. The cell culture supernatants were examined for the production of key cytokines involved in the immune responses including: IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p70, IL-13, IL-17A, IL-22, IL-23 and TNFα using the FlowCytomix kit. The results showed that IL-2, IL-4, IL-9, IL-13 or IL-17A was not produced by any of the polarized states (Figure 3). Unstimulated M1 polarized macrophages did not produce significant levels of any of the cytokines.

Figure 3. Basal cytokine production in polarized macrophages.

MDMs were polarized using indicated stimuli for 2 days: IFNγ (20 ng/ml) for M1 macrophages, IL-4 (20 ng/ml) for M2a macrophages, LPS (1 μg/ml) and IL-1β (10 ng/ml) for M2b macrophages and IL-10 (10 ng/ml) for M2c macrophages. Polarizing stimuli were removed after the 2 days, fresh media was added and the cells were incubated for another 24 hours. Cytokine levels in the culture supernatants were measured using flow cytometry as described in Materials and Methods. Bar graphs represent mean ±SEM, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$ with at least n=5.


IL-23 (pg/mL)
0
M1
M2A
M2B
M2C


IL-12p70 (pg/ml)
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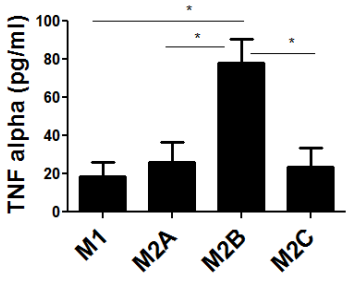
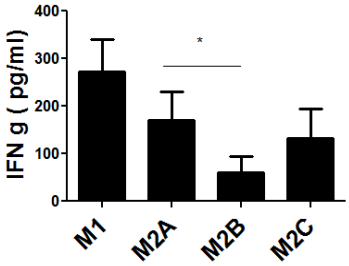
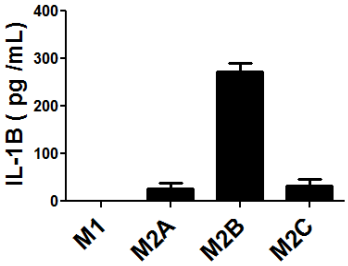
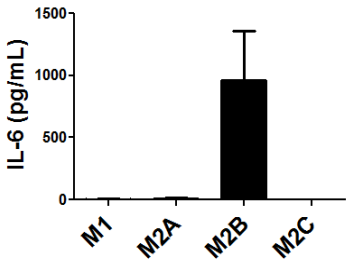
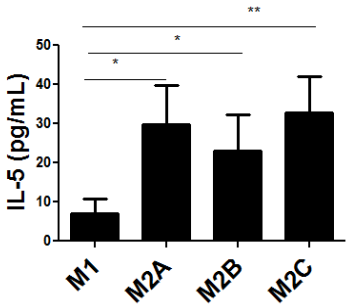
20
15
10
5
0
M1
M2A
M2B
M2C

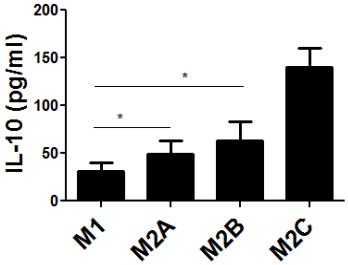

TNF alpha (pg/ml)
100
80
60
40
20
0
*
*
*
M1
M2A
M2B
M2C

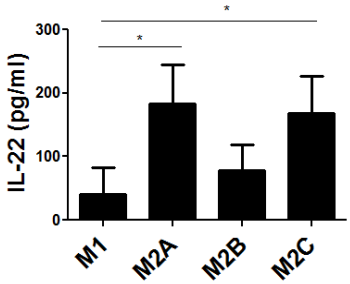

IFN g (pg/ml)
400
300
200
100
0
*
M1
M2A
M2B
M2C


IL-1B (pg /mL)
400
300
200
100
0
M1
M2A
M2B
M2C


IL-6 (pg/mL)
1500
1000
500
0
M1
M2A
M2B
M2C


IL-5 (pg/mL)
50
40
30
20
10
0
**
*
*
M1
M2A
M2B
M2C


IL-10 (pg/ml)
200
150
100
50
0
*
*
M1
M2A
M2B
M2C


IL-22 (pg/ml)
300
200
100
0
*
*
M1
M2A
M2B
M2C

In contrast, the M2b polarized macrophages produced significant levels of TNFα and IL-6 compared to the other macrophage populations analyzed. The M2a and M2c polarized macrophages exhibited a similar cytokine profile manifested by enhanced production of IL-22 and IL-5 and low levels of IL-1β and IL-6. The M2 polarized macrophages produced enhanced levels of IL-10 as compared to M1 macrophages. Significance was not calculated for the M2c polarized macrophages since IL-10 was used to generate this polarization state. It was therefore not obvious whether the measured IL-10 levels were derived from the secreted IL-10 or represented remnant following polarization. Thus, M2c macrophages were excluded when calculating significance for IL-10. In summary, unstimulated M1 macrophages did not produce significant levels of any of the cytokines. M2b macrophages produced TNFα, IL-6, IL-5 and IL-10. M2a and M2c macrophages had a similar cytokine profile and produced IL-5, IL-10 and IL-22.

Cytokine profile of polarized macrophages after TLR 2, 3 and 4 stimulation

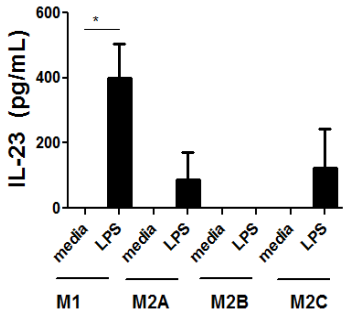
Stimulation of innate immune cell receptors leads to the activation of macrophages and results in cytokine production and eventually clearance of the pathogen[73]. It was of interest to determine the cytokine profile of the polarized macrophages following activation of receptors, such as TLRs, known to be stimulated by bacterial and viral pathogens. Ligands against two cell surface bound TLRs and one against intracellular membrane bound TLR were used: LPS (TLR4), LTA (TLR2) and Poly I:C (TLR3). Polarized macrophages were treated with LPS, LTA or Poly I:C for 24 hours and cytokine induction was measured using the FlowCytomix kit. M1 polarized macrophages led to secretion of predominately and significantly higher levels of pro-inflammatory cytokines IL-23, IL-12p70, TNFα and IFNγ upon stimulation with LPS (Figure 4), LTA (Figure 5), and Poly I:C (Figure 6) as compared

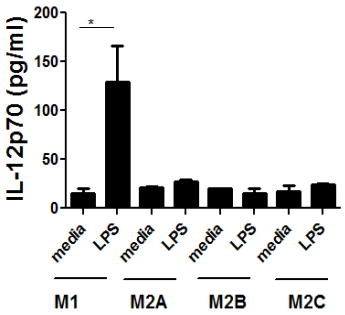
to unstimulated cells. In contrast, none of the M2 polarized macrophages produced significant of levels of IL-23, IL-12p70, TNFα or IFNγ upon stimulation. M2b polarized macrophages were poor inducers of the cytokines tested, regardless of the TLR stimulation used, with the exception of IL-10. IL-10 was significantly induced by M2b polarized macrophages when stimulated with LPS (Figure 4). Although stimulation of M1 polarized macrophages with different TLR ligands exhibited similar profiles of cytokine production, there were also some differences. For example, LPS stimulation of M1 polarized macrophages also resulted in the induction of IL-1β, IL-6, IL-5, IL-10 and IL-22 (Figure 4). LTA stimulation of M1 polarized macrophages resulted in the induction of IL-1β, IL-6, IL-5 and IL-10 (Figure 5) and Poly I:C stimulation led to the induction of IL-6, IL-5 and IL-10 (Figure 6).

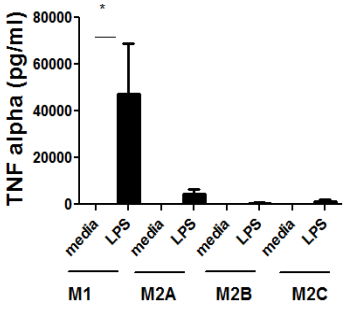
Likewise, stimulation of M2a and M2c polarized macrophages with LPS resulted in the induction of IL-1β, IL-6, IL-5 and IL-10 (Figure 4), while stimulation with LTA led to the induction of IL-1β, IL-6 and IL-10 (Figure 5), and stimulation with Poly I:C resulted in IL-10 production and a decrease in IL-22 secretion (Figure 6). Moreover, there were several differences between the M2a and M2c polarization states with respect to their response to TLR agonists. In particular, M2c macrophages, but not M2a macrophages, induced IL-22 upon LPS stimulation (Figure 4). However, M2a macrophages, but not M2c macrophages, induced IL-22 and IL-5 upon LTA stimulation (Figure 5). M2c macrophages induced IL-6 and high levels of IL-10 when stimulated with Poly I:C (Figure 6).

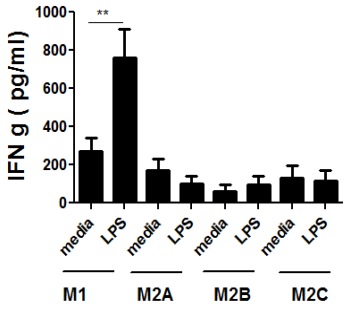
Figure 4. Cytokine production in polarized macrophages following LPS stimulation.

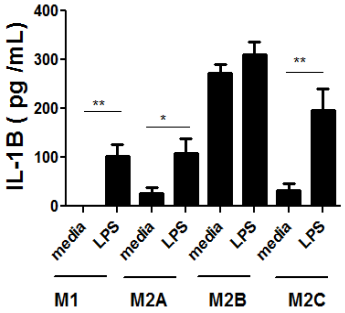
MDMs were polarized using appropriate stimuli for 2 days: IFNγ (20 ng/ml) for M1 macrophages, IL-4 (20 ng/ml) for M2a macrophages, LPS (1 μg/ml) and IL-1β (10 ng/ml) for M2b macrophages and IL-10 (10 ng/ml) for M2c macrophages. Polarizing stimuli were removed after the 2 days and polarized macrophages were treated with LPS (1 μg/ml) for 24 hours. Cytokine levels in the supernatants were measured using flow cytometry as described in Materials and Methods. Bar graphs represent mean ±SEM, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$ with at least n=5.

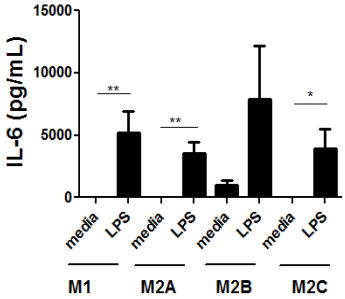

IL-23 (pg/mL)
600
400
200
0
*
media
LPS
M1
M2A
M2B
M2C

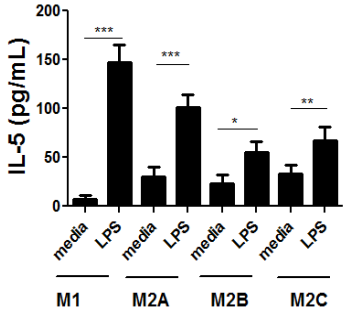

IL-12p70 (pg/ml)
200
150
100
50
0
*
media
LPS
M1
M2A
M2B
M2C

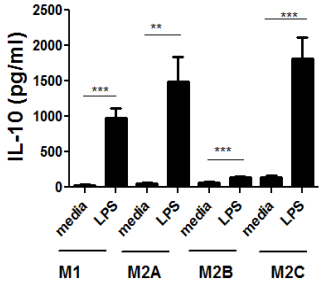

TNF alpha (pg/ml)
80000
60000
40000
20000
0
*
media
LPS
M1
M2A
M2B
M2C


IFN g (pg/ml)
1000
800
600
400
200
0
**
media
LPS
M1
M2A
M2B
M2C


IL-1B (pg /mL)
400
300
200
100
0
**
*
**
media
LPS
M1
M2A
M2B
M2C


IL-6 (pg/mL)
15000
10000
5000
0
**
**
*
media
LPS
M1
M2A
M2B
M2C


IL-5 (pg/mL)
200
150
100
50
0
***
***
*
**
media
LPS
M1
M2A
M2B
M2C


IL-10 (pg/ml)
2500
2000
1500
1000
500
0
***
**
***
***
media
LPS
M1
M2A
M2B
M2C

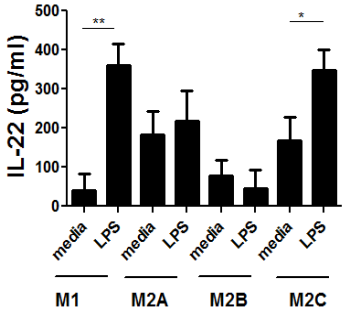
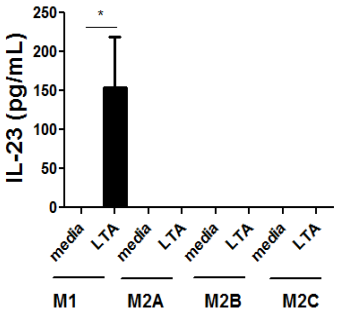
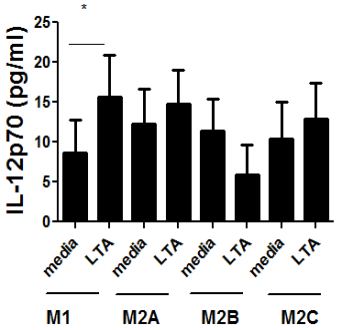
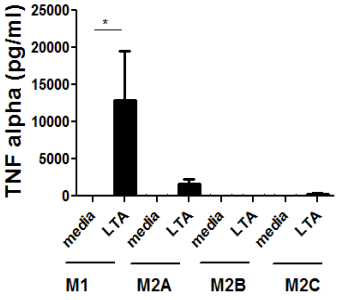

IL-22 (pg/ml)
500
400
300
200
100
0
**
*
media
LPS
M1
M2A
M2B
M2C

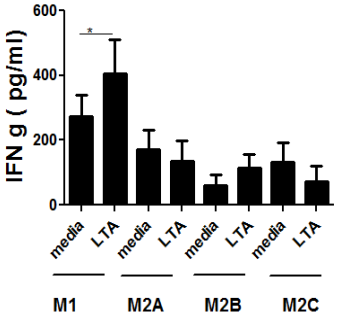
Figure 5. Cytokine production in polarized macrophages following LTA stimulation.

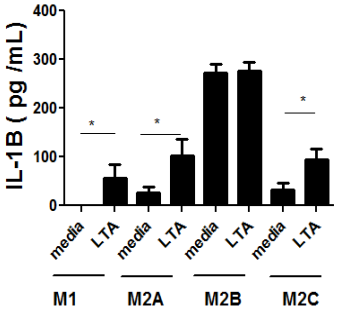
MDMs were polarized as mentioned previously. Polarizing stimuli were removed after the 2 days and polarized macrophages were treated with LTA (5 µg/ml) for 24 hours. Cytokine levels in the supernatants were measured using flow cytometry as described in Materials and Methods. Bar graphs represent mean ±SEM, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$ with at least n=5.

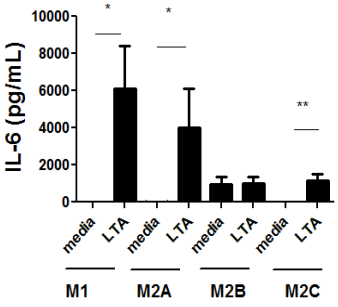

IL-23 (pg/mL)
250
200
150
100
50
0
*
media
LTA
M1
M2A
M2B
M2C

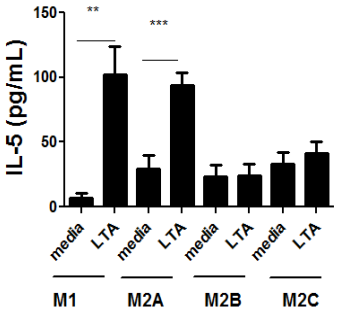

IL-12p70 (pg/ml)
25
20
15
10
5
0
*
media
LTA
M1
M2A
M2B
M2C

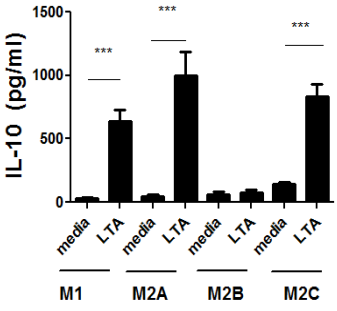

TNF alpha (pg/ml)
25000
20000
15000
10000
5000
0
*
media
LTA
M1
M2A
M2B
M2C


IFN g (pg/ml)
600
400
200
0
*
media
LTA
M1
M2A
M2B
M2C


IL-1B (pg /mL)
400
300
200
100
0
*
media
LTA
M1
M2A
M2B
M2C


IL-6 (pg/mL)
10000
8000
6000
4000
2000
0
*
**
media
LTA
M1
M2A
M2B
M2C


IL-5 (pg/mL)
150
100
50
0
**
***
media
LTA
M1
M2A
M2B
M2C


IL-10 (pg/ml)
1500
1000
500
0
***
media
LTA
M1
M2A
M2B
M2C

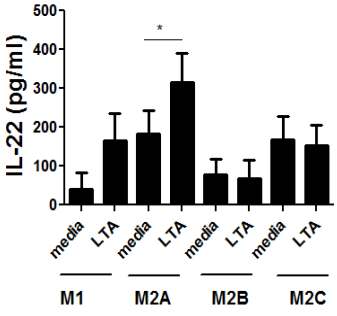
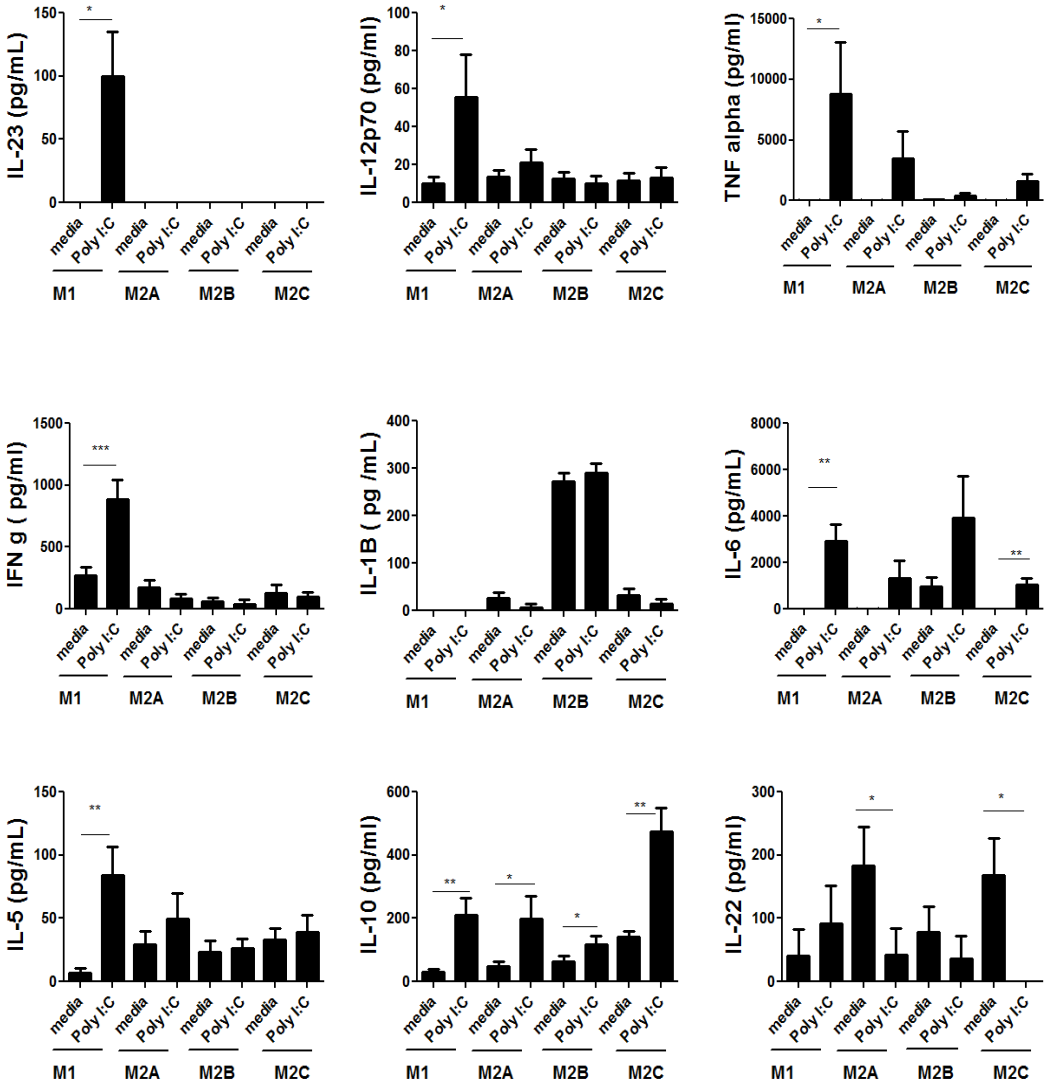

IL-22 (pg/ml)
500
400
300
200
100
0
*
media
LTA
M1
M2A
M2B
M2C

Figure 6. Cytokine production in polarized macrophages following Poly I:C stimulation.

MDMs were polarized as described previously. Polarizing stimuli were removed after the 2 days and polarized macrophages were treated with Poly I:C (50 µg/ml) for 24 hours. Cytokine levels in the supernatants were measured using flow cytometry as described in Materials and Methods. Bar graphs represent mean ±SEM, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$ with at least n=5.


IL-23 (pg/mL)
IL-12p70 (pg/ml)
TNF alpha (pg/ml)
IFN g (pg/ml)
IL-1B (pg /mL)
IL-6 (pg/mL)
IL-5 (pg/mL)
IL-10 (pg/ml)
IL-22 (pg/ml)
media
Poly I:C
M1
M2A
M2B
M2C

Taken together (summarized in Table 1), M1 polarized macrophages display a pro-inflammatory cytokine profile, M2b macrophages generally display a hypo-responsiveness to TLR stimulation with exception to IL-10 induction and M2a and M2c macrophages display a similar cytokine profile characterized by induction of IL-10 family of cytokines including IL-22 and IL-10.

TLR 2, 3 and 4 stimulation leads to IFNγ mRNA expression in M1 polarized macrophages

IFNγ is usually produced by T cells and NK cells[201]. However, my results showed that M1 but not M2 polarized macrophages secreted significant levels of IFNγ in response to LPS, LTA and Poly I:C stimulations (Figures 4-6). There are a few reports of IFNγ production by murine and human macrophages. Murine peritoneal macrophages have been shown to produce low levels of IFNγ when stimulated with IFNγ or IL-12[202,203]. IFNγ production by human alveolar macrophages has been documented in certain disease conditions[204,205]. In view of my results showing production of IFNγ by M1 macrophages, I next validated these observations by comparing the ability of M1 and M2 macrophages to induce IFNγ mRNAs upon stimulation with TLR ligands. For this, M1 and M2a polarized macrophages were treated with LPS, LTA or Poly I:C for 4 hours and IFNγ mRNA transcription was assessed by qRT-PCR (Figure 7). M1 polarized macrophages showed a significant increase in mRNA transcription levels as compared to the control M2a polarized macrophages when treated with LPS (Figure 7A), LTA (Figure 7B) or Poly I:C (Figure 7C). Taken together, the results show that following stimulation with either TLR 2, 3 or 4 agonists, M1 polarized macrophages actively transcribe IFNγ mRNA and synthesize IFNγ protein.

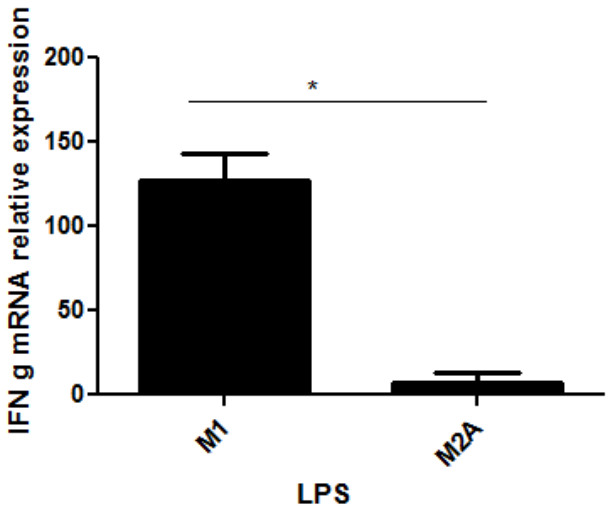
Table 1. Cytokines induced upon TLR stimulation in polarized macrophages

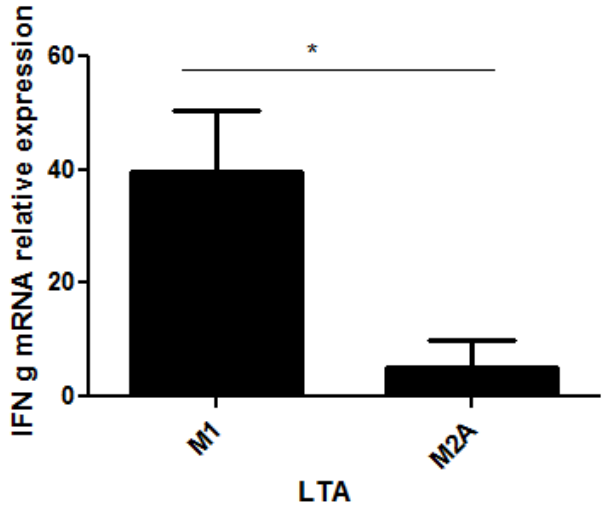
M1	M2A	M2B	M2C
IL-23[a, b, c]	IL-1β[a, b]	IL-10[a, c]	IL-1β[a, b]
IL-12p70[a, b, c]	IL-6[a, b]		IL-6[a, b, c]
TNFα[a, b, c]	IL-5[a, b]		IL-5[a]
IFNγ[a, b, c]	IL-10[a, b, c]		IL-10[a, b, c]
IL-1β[a, b]	IL-22[b]		IL-22[a]
IL-6[a, b, c]			
IL-5[a, b, c]			
IL-10[a, b, c]			
IL-22[a]			

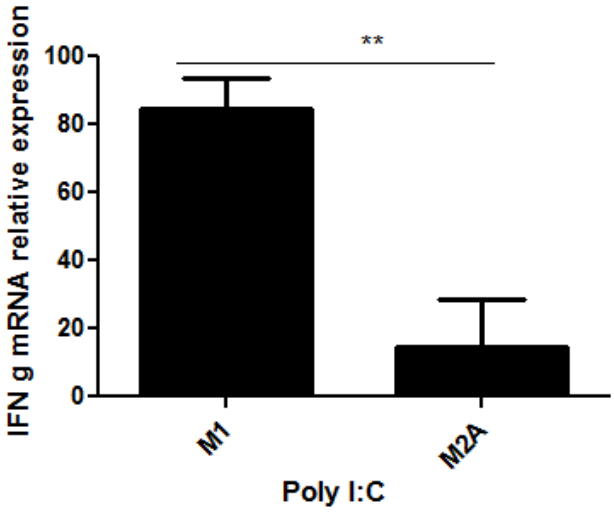
a- LPS
b- LTA
c- Poly I:C

Figure 7. *De novo* IFNγ synthesis in M1 polarized macrophages.

MDMs were polarized using appropriate stimuli for 2 days: IFNγ (20 ng/ml) for M1 macrophages and IL-4 (20 ng/ml) for M2a macrophages. Polarizing stimuli were removed after the 2 days and polarized macrophages were treated with either (A) LPS (1 µg/ml), (B) LTA (5 µg/ml) or (C) Poly I:C (50 µg/ml) for 4 hours. IFNγ expression as measured by qRT-PCR. Bar graphs represent mean ±SEM, * $p \leq 0.05$; ** $p \leq 0.005$; with at least n=3.


IFN g mRNA relative expression
200
150
100
50
0
*
M1
M2A
LPS


IFN g mRNA relative expression
60
40
20
0
*
M1
M2A
LTA

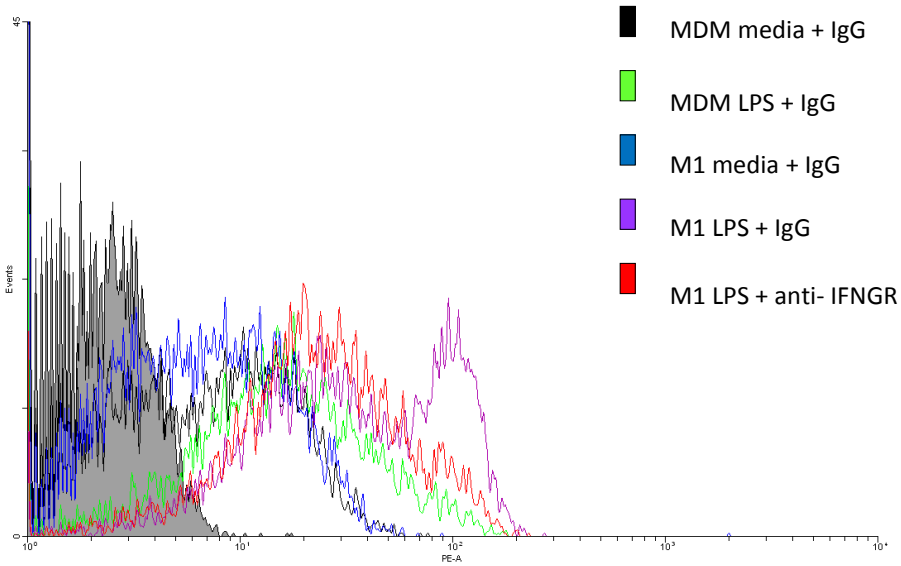

IFN g mRNA relative expression
100
80
60
40
20
0
**
M1
M2A
Poly I:C

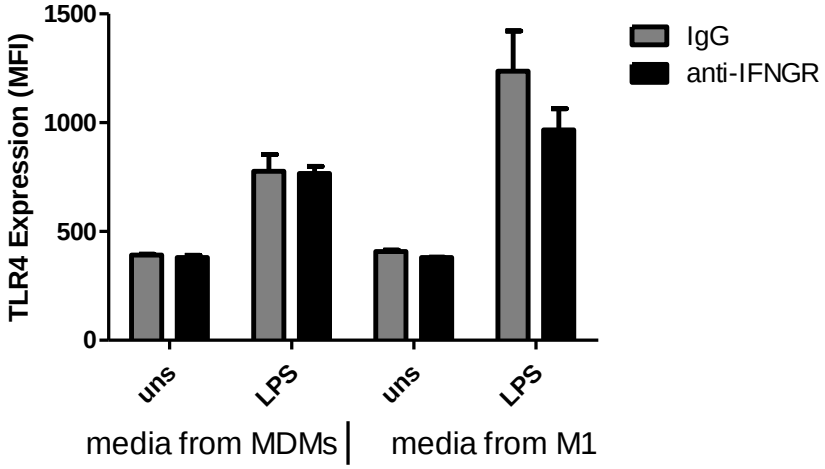
IFNγ produced in macrophages is biologically active

NK cell- and T cell-derived IFNγ acts on macrophages to promote their activation and by engaging the mechanisms that limit bacterial growth[206]. It was therefore of interest to determine whether IFNγ derived from M1 macrophages would have the same functional potential. IFNγ is known to up-regulate the surface expression of TLR4 in macrophages[207]. Therefore, I determined whether IFNγ produced by M1 macrophages after TLR stimulation is capable of inducing TLR4 expression in macrophages. For this, M1 polarized macrophages and unpolarized MDMs controls were stimulated with LPS for 24 hours to induce IFNγ secretion. Supernatants were collected from unstimulated, LPS-stimulated MDMs and M1 polarized macrophages and the effect of secreted IFNγ on TLR4 expression was determined by flow cytometry. To determine if the IFNγ secreted by M1 polarized macrophages was biologically active, anti-IFNγR antibody was employed to block the functional activity of the endogenously produced IFNγ. MDMs were treated for 2 hours with anti-IFNγR antibody (40 µg/ml) or with isotype control IgG (40 µg/ml) followed by treatment with the previously collected culture supernatants for 24 hours. The effect of IFNγ in culture supernatants on TLR4 expression was then measured. Culture supernatants from unstimulated MDMs showed basal levels of TLR4 expression in the macrophages (Figure 8). This expression was slightly increased when the culture supernatants from LPS-stimulated MDMs was used for stimulation. Treatment with media from unstimulated M1 polarized macrophages shows the same TLR4 expression levels as treatment with culture supernatants from unstimulated MDMs. In contrast, treatment with the media from LPS-stimulated M1 polarized macrophages shows a marked increase in TLR 4 expression.

Figure 8. M1 polarized macrophage derived IFNγ is biologically active and up-regulates TLR4 expression.

MDMs and M1 polarized macrophages were stimulated with LPS (1 μg/ml) for 24 hours. Supernatants from LPS stimulated and unstimulated MDMs and M1 polarized macrophages were collected. MDMs were pre-treated with anti-IFNγR antibodies or isotype control antibodies for 2 hours. The cells were then stimulated with the collected supernatants for 24 hours. The cells were stained with PE-conjugated antibody against TLR4 and fluorescence was measured by flow cytometry. Histogram (A) from one representative experiment is shown. MFI values (B) were obtained using FACSDiva software. Bar graphs represent mean ±SEM of three experiments.


MDM media + IgG
MDM LPS + IgG
M1 media + IgG
M1 LPS + IgG
M1 LPS + anti- IFNGR
Events
PE-A


TLR4 Expression (MFI)
1500
1000
500
0
IgG
anti-IFNGR
uns
LPS
uns
LPS
media from MDMs
media from M1

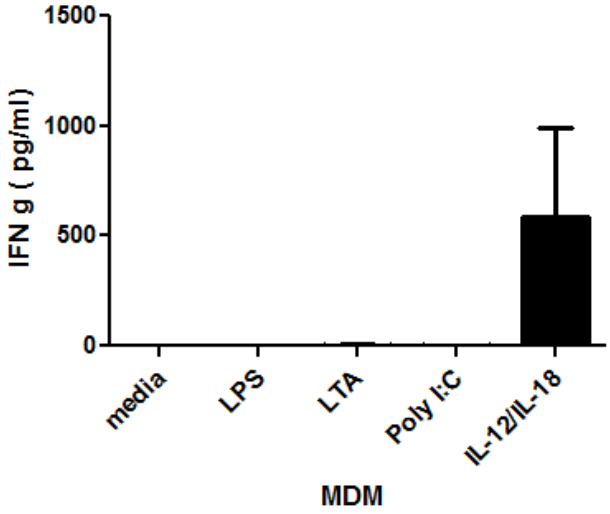
To confirm that this increase in TLR4 expression was mediated by IFNγ present in M1 culture supernatants, MDMs were treated with antibodies to IFNγR to block IFNγ response. Treatment with anti-IFNγR antibody induced TLR4 expression that was comparable to that produced by supernatants from LPS stimulated MDMs. These results therefore demonstrate that IFNγ derived from M1 culture supernatants has the ability to activate macrophages by enhancing expression of TLR4.

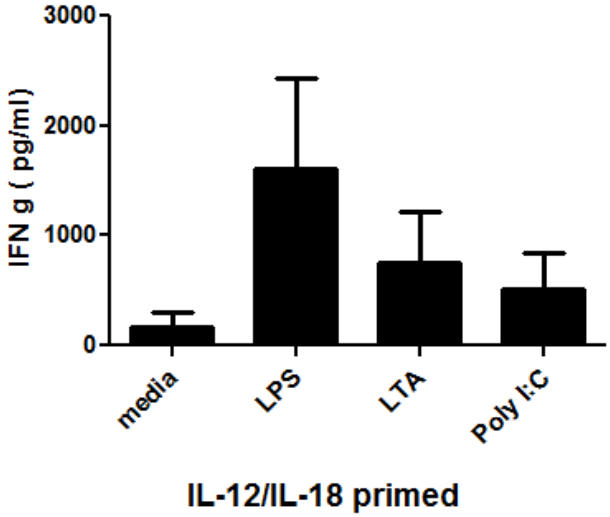
IL-12/IL-18 priming also leads to IFNγ production in macrophages

IL-12 is produced by macrophages early during the innate immune response upon pathogen recognition[208]. It acts on NK cells and T cells to promote IFNγ secretion which in turn activates macrophages[209]. Recently, IL-18 was shown to induce IFNγ in murine and human T cells[210,211]. IL-12 and IL-18 have also been shown to synergize and produce IFNγ in T cells and NK cells in the murine model[210-212]. It has also been shown that IL-12/IL-18 treatment of murine macrophages leads to IFNγ production[99]. Whether IL-12/IL-18 can induce IFNγ in human macrophages is not known. To address this question, MDMs were treated with IL-12 and IL-18 and IFNγ production was measured by ELISA. MDMs did not produce IFNγ, on their own or following TLR stimulation (Figure 9). However, stimulation of MDMs with IL-12 and IL-18 led to the production of IFNγ. It was of interest to determine whether priming MDMs with IL-12/IL-18 and followed by treatment with TLR agonists would enhance IFNγ production. IL-12/IL-18 primed macrophages were treated with TLR agonists for 24 hours and IFNγ was measured. Similarly, as observed following IFNγ polarization, IL-12/IL-18 priming induced IFNγ production in macrophages following TLR 2, 3 and 4 ligand stimulation. These results suggest that MDMs can secrete IFNγ after IL-12/IL-18 treatment on its own and when followed by TLR stimulation.

Figure 9. IL-12/IL-18 primed macrophages produce IFNγ.

(A) MDMs treated with LPS (1 µg/ml), LTA (5 µg/ml) and Poly I:C (50 µg/ml) for 24 hours. MDMs were also treated with IL-12 (10 ng/ml) and IL-18 (10 ng/ml) for 48 hours. IFNγ secretion was measured in the supernatants by ELISA. (B) MDMs were primed with IL-12 (10 ng/ml) and IL-18 (10 ng/ml) for 48 hours. The priming stimulus was removed and the macrophages were treated with LPS (1 µg/ml), LTA (5 µg/ml) and Poly I:C (50 µg/ml) for 24 hours. IFNγ secretion was measured in the supernatants by ELISA. Bar graphs represent mean ±SEM of at least three experiments.


1500
1000
500
0
IFN g (pg/ml)
media
LPS
LTA
Poly I:C
IL-12/IL-18
MDM


3000
2000
1000
0
IFN g (pg/ml)
media
LPS
LTA
Poly I:C
IL-12/IL-18 primed

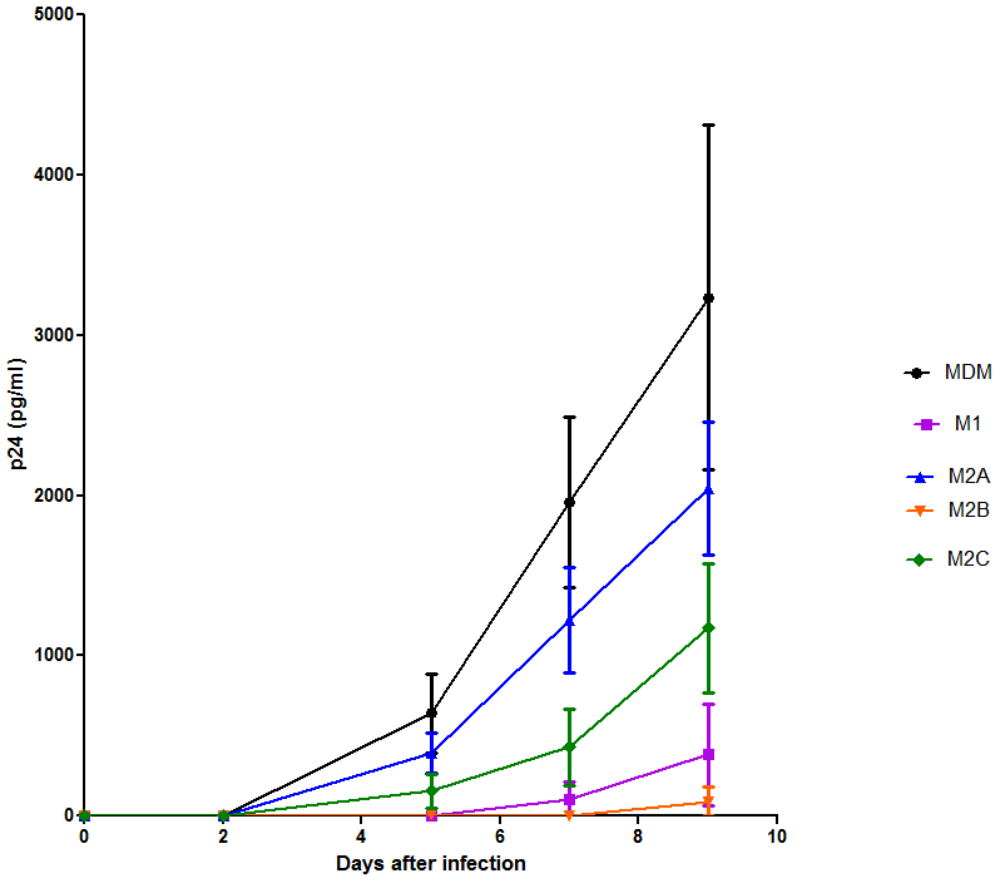
Differential susceptibility of polarized macrophages to *in vitro* HIV infection

M1 and M2b polarization renders macrophages resistant to productive HIV infection

Macrophages constitute one of the major reservoirs of HIV[128]. Cytokines also modulate the ability of macrophages to support HIV infection. For example, stimulation of macrophages with IFNα/β has been shown to limit HIV infection[156]. However, cytokines' effects on HIV infection depend on the differentiation state of macrophages and the exposure timing. IL-4 and IL-13 are known to enhance viral replication in monocytes but limit HIV replication in monocyte-derived macrophages[160]. It was therefore, of interest to determine the effect of differentially polarized macrophages on productive HIV infection. Polarized macrophages and MDMs were infected with 700 pg of viral stocks of HIV_{CS204} supernatants for 2 hours. The cells were washed with PBS and cultured in fresh media. The supernatants were collected on days 0, 2, 5, 7 and 9 and p24 concentration was measured. The results show that MDMs were productively infected with HIV virus. However, the polarized macrophages displayed a diminished capacity to support productive infection as compared to MDMs. Among the polarized states, M2a and M2c polarized macrophages exhibited the most productive HIV infection (Figure 10). In contrast, M1 and M2b polarized macrophages were relatively resistant to HIV infection. M1 polarization did not result in productive infection until day 9 post infection. Similarly, M2b polarized macrophages did not show any infection in 4 out of the 5 donors tested. However, one donor showed low levels of infection 9 days post infection.

Figure 10. M2a and M2c polarized macrophages were productively infected with HIV.

Polarized macrophages and MDMs were infected with 700 pg viral stocks of HIV_{CS204} supernatants for 2 hours. The cells were washed with PBS and supernatants were collected on days 0, 2, 5 7 and 9 and p24 concentration was measured using the HIV-1 $p24^{CA}$ antigen capture assay kit. Data points represent mean ± SEM with n=5.

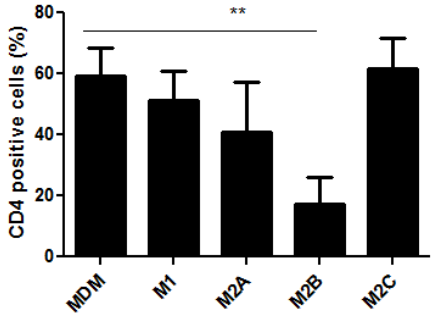

5000
4000
3000
2000
1000
0
p24 (pg/ml)
0
2
4
6
8
10
Days after infection
MDM
M1
M2A
M2B
M2C

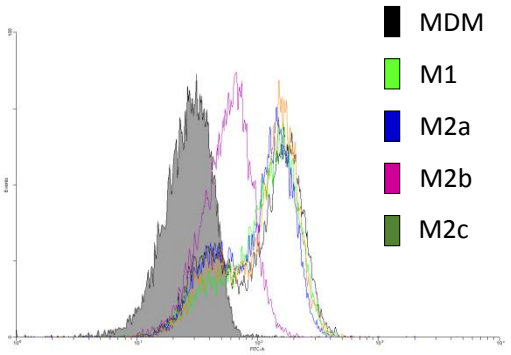
HIV receptor and co-receptor expression does not explain differential susceptibility of polarized macrophages to *in vitro* HIV infection

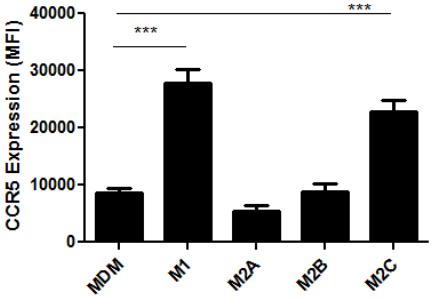
HIV entry into a target cell requires two steps: the first is the binding of the viral glycoprotein gp120 to CD4 on the target cell and the second step involves the interaction with a chemokine receptor (CXCR4 or CCR5)[112]. Resistance to infection induced by M1 and M2b polarized macrophages may be due to the absence or relatively decreased expression of CD4 or HIV co-receptors CXCR4 and CCR5. Therefore, expression levels CD4, CCR5 and CXCR4 were measured in the polarized macrophages. M1 and M2c macrophages expressed similar percentages of CD4 positive cells as MDMs (Figure 11). Furthermore, they expressed higher levels of CCR5 and had a higher percentage of cells that were CXCR4 positive as compared to MDMs. However, M1 macrophages were resistant to productive HIV infection and M2c macrophages allowed for productive HIV infection (Figure 10). M2a and M2b macrophages had similar percentages of CD4 positive cells, CXCR4 positive cells and CCR5 expression as compared to MDMs. However, M2a macrophages were productively infected with HIV and M2b macrophages were resistant to HIV infection. Taken together, differential HIV receptor or co-receptor expression does not explain the differences in HIV infection across polarization states. This suggests there might be other mechanisms by which that macrophage polarization leads to variability in productive HIV infection.

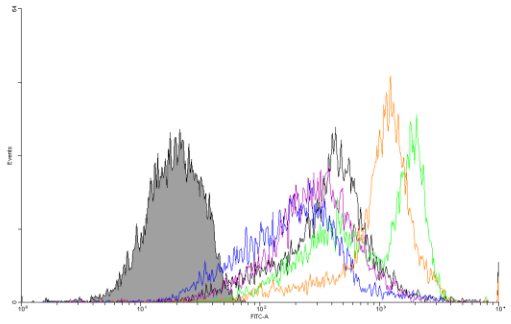
Figure 11. HIV receptor and co-receptor expression in polarized macrophages.

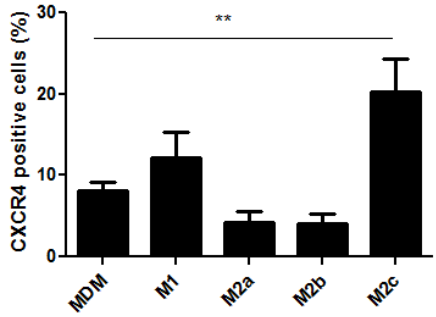
MDMs were polarized using appropriate stimuli for 2 days: IFNγ (20 ng/ml) for M1 macrophages, IL-4 (20 ng/ml) for M2a macrophages, LPS (1 μg/ml) and IL-1β (10 ng/ml) for M2b macrophages and IL-10 (10 ng/ml) for M2c macrophages. Polarized macrophages were stained with antibodies against CD4, CCR5 and CXCR4 and fluorescence was measured by flow cytometry. MFI values and percentages (A) were obtained using FACSDiva software and histograms (B) from one representative experiment are shown. Bar graphs represent mean ±SEM, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$ with at least n=4.

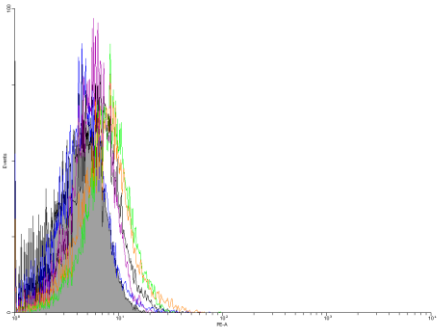

CD4 positive cells (%)
80
60
40
20
0
**
MDM
M1
M2A
M2B
M2C


MDM
M1
M2a
M2b
M2c


CCR5 Expression (MFI)
40000
30000
20000
10000
0
***
***
MDM
M1
M2A
M2B
M2C




CXCR4 positive cells (%)
30
20
10
0
**
MDM
M1
M2a
M2b
M2c



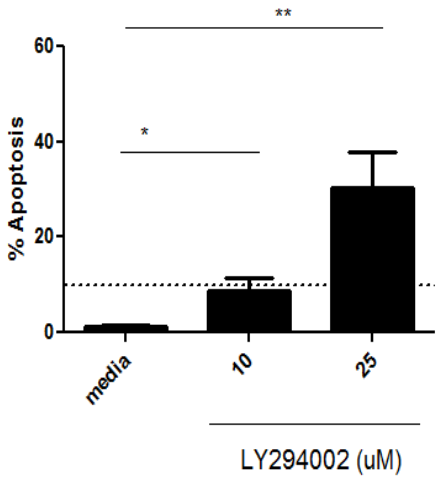
Differential susceptibility of polarized macrophages to apoptosis

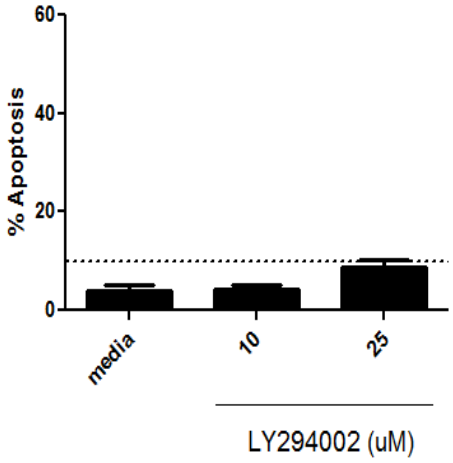
Susceptibility of polarized macrophages to apoptosis induced by blocking PI3K pathway

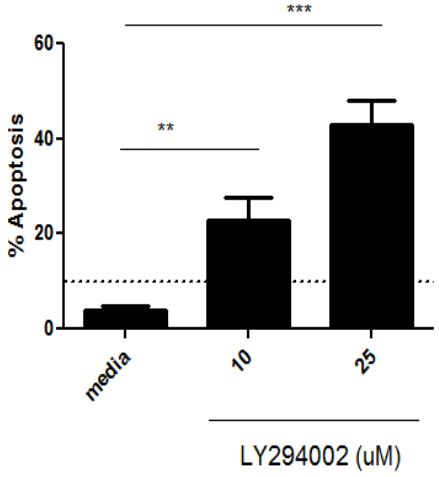
Apoptosis is a form of cell death that is important in tissue development and immune regulation. It serves as a mechanism to control macrophage activation. Macrophage resistance to apoptosis also makes them excellent HIV reservoirs. Previous work in this lab has shown that differentiated macrophages are resistant to Vpr-induced apoptosis due to the overexpression of anti-apoptotic molecules cIAP 1/2[213]. The PI3k/Akt pathway is also important in maintaining macrophage survival. Akt suppression via PI3K inhibition using pharmacological inhibitor LY294002 resulted in the loss of mitochondrial potential in triggering apoptosis in macrophages[172]. It was of interest to determine whether blockage of PI3K activation would lead to apoptosis in polarized macrophages. Polarized macrophages were treated for 48 hours with LY294002 and assessed for apoptosis by intracellular PI staining (Figure 12). Apoptosis in fewer than 10% of the cells was not considered significant. M1, M2b, and M2c polarized macrophages underwent apoptosis upon blocking P13K pathway. However, M2a polarized macrophages were least susceptible to the effects of P13K blockage induced apoptosis. Apoptosis occurred in almost 40% of M1 and M2b macrophages upon treatment with 25μM of LY294002. The same treatment in M2c macrophages resulted in apoptosis of only 20% of the cells. However, less than 10% of cells underwent apoptosis in M2a macrophages at the highest dose of LY294002. This suggests that M2a macrophages are resistant to apoptosis mediated by blocking the PI3K pathway.

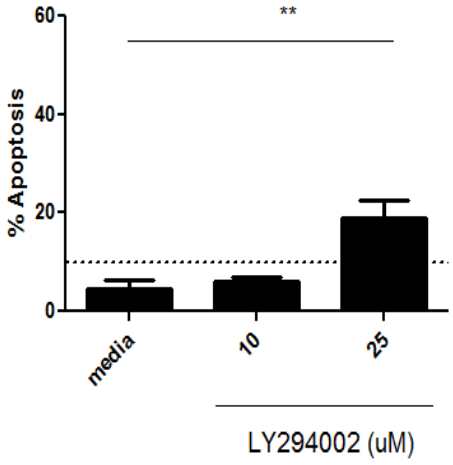
Figure 12. M2a polarized macrophages are least susceptible to apoptosis induced by blocking PI3K/Akt

M1 (A), M2a (B), M2b (C), M2c (D) polarized macrophages were treated with indicated concentrations of LY294002 for 48 hours. Apoptosis was measured by flow cytometry using intracellular PI staining. Bar graphs represent mean ± SEM, * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$ with n=5.


**
*
60
40
20
0
% Apoptosis
media
10
25
LY294002 (uM)


60
40
20
0
% Apoptosis
media
10
25
LY294002 (uM)


***
**
60
40
20
0
% Apoptosis
media
10
25
LY294002 (uM)

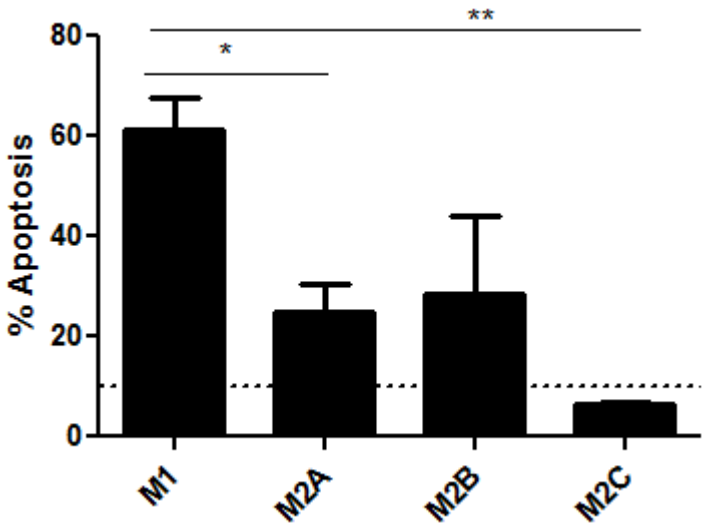

**
60
40
20
0
% Apoptosis
media
10
25
LY294002 (uM)

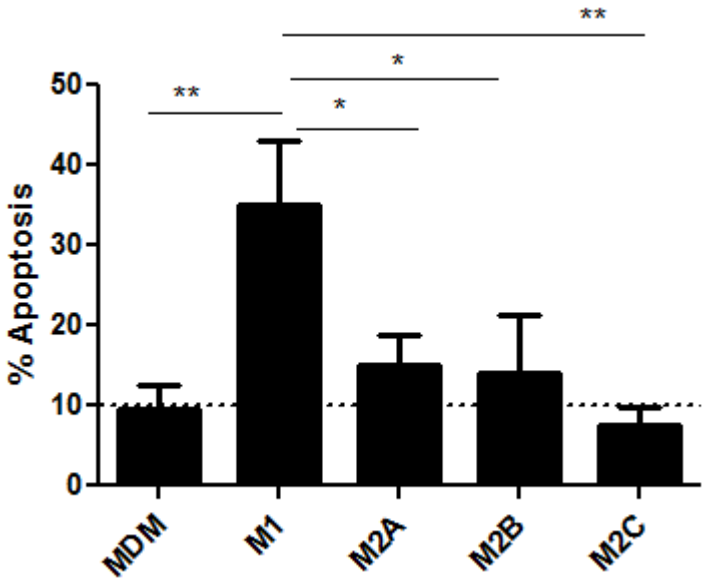
Susceptibility of polarized macrophages to Smac mimetic mediated apoptosis

Inhibitors of apoptosis proteins (IAPs) are another mechanism by which cells maintain their viability[176]. However, it has been shown that Smac mimetics do not cause significant apoptosis in MDMs[213]. Smac mimetics are drugs that degrade IAPs and are being used in cancer therapy to cause apoptosis in cancer cells [194]. It was of interest to determine whether degrading IAPs by LCL-161 (Smac mimetic) would cause apoptosis in the polarized macrophages. Polarized macrophages were treated with 100nm and 10 μM LCL-161 for 48 hours and assessed for apoptosis by intracellular PI staining (Figure 13). M1 polarized macrophages were most susceptible to Smac mimetic mediated apoptosis as compared to the M2 polarized macrophages. M1 macrophages displayed apoptosis in 40% of cells at the lower 100nm dose. Similarly, apoptosis was observed in 60% of M1 macrophages with 10μM LCL-161 treatment. In contrast, at the lower dose, M2a and M2b macrophages showed apoptosis in 15% of the cells and less than 10% of M2c macrophages underwent apoptosis. At the higher dose, M2a and M2b macrophages displayed apoptosis in 20% of the cells at 10μM. However, there were still less than 10% of M2c macrophages that underwent apoptosis even at a higher dose. Taken together, M1 polarization makes MDMs sensitive to apoptosis by LCL-161 treatment and M2c polarization protects MDMs from this effect.

Figure 13. M1 polarized macrophages undergo apoptosis in response to LCL-161 treatment.

Polarized macrophages were treated with (A) 10 μM and (B) 100 nm LCL-161, a SMAC mimetic, for 48 hours. Apoptosis was measured by flow cytometry using intracellular PI staining. Bar graphs represent mean ± SEM, * $p \leq 0.05$; ** $p \leq 0.005$; with at least n=3.


**
*
80
60
40
20
0
% Apoptosis
M1
M2A
M2B
M2C

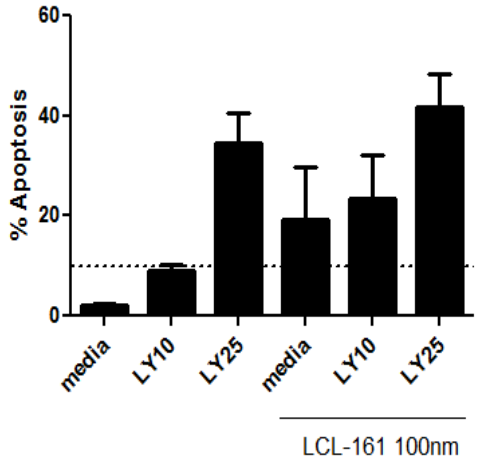

**
*
*
**
50
40
30
20
10
0
% Apoptosis
MDM
M1
M2A
M2B
M2C

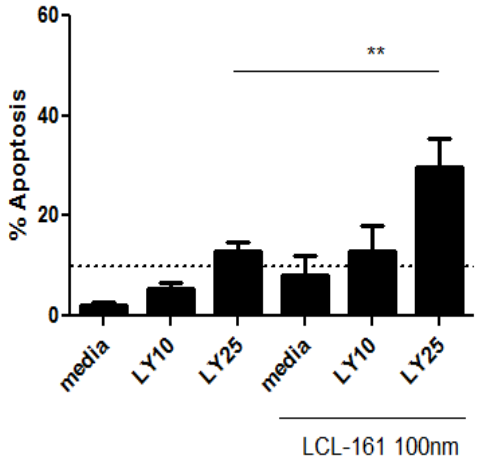
LCl-161 treatment sensitizes M2a polarized macrophages to apoptosis mediated by PI3K blockage

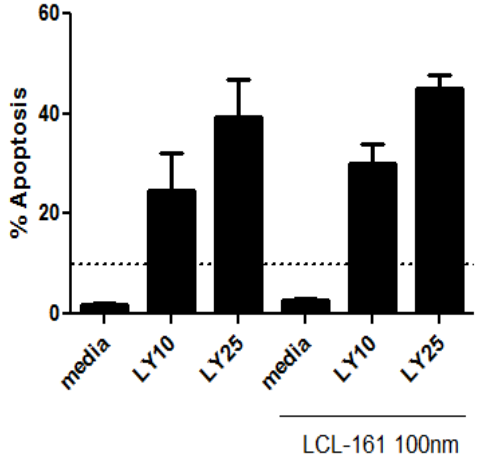
M1 polarized macrophages were shown to be susceptible to apoptosis mediated by degrading IAPs using LCL-161 and M2a macrophages were resistant to apoptosis induced by blocking the PI3K pathway using LY294002. Therefore, it was of interest to determine if one could cause apoptosis in all the polarized macrophages by treating them simultaneously with both LY294002 and LCL-161. To this end, polarized macrophages were treated with LY294002 and LCL-161, alone or together for 48 hours and assessed for apoptosis via intracellular PI staining (Figure 14). M1 macrophages showed apoptosis in 35% of their cells when treated with 25µM LY294002. Furthermore, only 40% of M1 macrophages underwent apoptosis upon simultaneous treatment with 25 µM and LCL-161. Similarly, 40% of M2b macrophages underwent apoptosis when treated with 25µM LY294002 alone and only 45% of the cells underwent apoptosis upon combined treatment with LCL-161. The number of cells undergoing apoptosis was lower in M2c macrophages as seen previously. There was apoptosis in 20% of M2c macrophages with 25µM LY294002 alone. Furthermore, combined treatment led to apoptosis in 25% of M2c macrophages. In contrast, M2a macrophages displayed apoptosis in 10% of their cells when treated with 25µM LY294002. However, simultaneously treating M2a macrophages with LY294002 and LCL-161 led to apoptosis in 30% of the cells. Taken together, there was no significant difference in LY294002 treatment and LY294002/LCL-161 treatment in M1, M2b, and M2c polarized macrophages. However, LCL-161 sensitizes M2a polarized macrophages to apoptosis mediated by PI3K blockage.

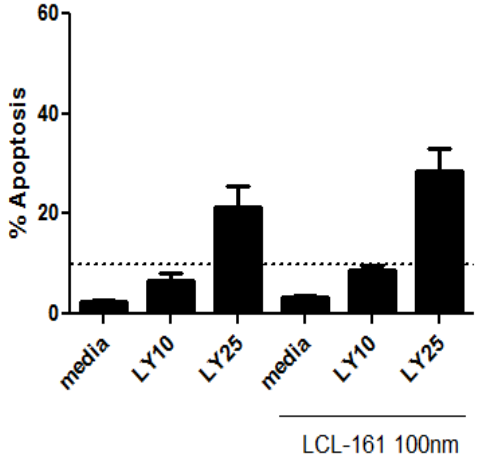
Figure 14. LCL-161 enhanced apoptosis induced by blocking the PI3K pathway in M2a macrophages.

M1 (A), M2a (B), M2b (C), M2c (D) polarized macrophages were treated with indicated concentrations of LY294002 and/or LCL-161 for 48 hours. Apoptosis was measured by flow cytometry using intracellular PI staining. Bar graphs represent mean ± SEM, ** $p \leq 0.005$; with n=5.


60
40
20
0
% Apoptosis
media
LY10
LY25
media
LY10
LY25
LCL-161 100nm


**
60
40
20
0
% Apoptosis
media
LY10
LY25
media
LY10
LY25
LCL-161 100nm


60
40
20
0
% Apoptosis
media
LY10
LY25
media
LY10
LY25
LCL-161 100nm


60
40
20
0
% Apoptosis
media
LY10
LY25
media
LY10
LY25
LCL-161 100nm

Discussion

In innate or adaptive immunity, differentiated hematopoietic cells must regulate their functional response in such a way as to limit damage but as well as to clear infection or inflammation in the surrounding tissue. One mechanism by which this is carried out is fine tuning of the immune response influenced by the milieu to achieve a polarized state of macrophage cells that can effectively contribute to the re-establishment of homeostasis. Macrophages can be polarized into two main populations: M1 and M2. These polarization states are acquired ensuing a change in type, timing or amount of micro-environmental signals. These states which are far more flexible than tissue specific differentiation are developed to quickly respond to environmental changes and allow macrophages to switch from one state to the next.

The aim of this research project was to characterize the polarization states of macrophages using in vitro generated human MDMs as an experimental model. Polarization states of MDMs were characterized with respect to expression of surface markers and their functional profile. The specific functions of polarized macrophages were analyzed in the context of cytokine production, HIV infection and apoptosis. My results reveal distinct differences associated with each polarization state characterized by expression of specific cell surface markers, distinct cytokine production profiles and productive HIV infection and susceptibility to apoptosis.

Cell surface marker profile of polarized macrophages

The first step that was undertaken to characterize the polarization states of MDMs was to determine the expression of cell surface molecules. MDMs were polarized with IFNγ

(M1), IL-4 (M2a), LPS and IL-1β (M2b) or IL-10 (M2c) analyzed for CD80, CD86, HLA-DR, CD14, TLR4, CD163 and CD200R expression. This non-exhaustive panel was selected based on previous reports, as well as the involvement of these molecules in macrophage activation[198-200]. My results, which are in line with literature reports, demonstrate that following IFNγ/LPS polarization M1 macrophages express higher levels CD80 and CD86 as compared to MDMs[198]. There are no reports on the TLR4 expression in polarized macrophages. My results demonstrate that M1 polarized macrophages have higher TLR4 expression as compared to MDMs. These results, which are consistent with literature reports, suggest that the M1 polarized macrophages are capable of responding efficiently to microbial/endotoxin challenge and are capable of engaging the adaptive immune response through enhanced surface expression of co-stimulatory molecules[214].

M2a polarized macrophages were found to have a distinctly higher CD200R expression as compared to MDMs. Binding of CD200 to CD200R present on endothelial cells delivers a down-regulatory signal and exerts an inhibitory effect on macrophage activation. Expression of viral homologues of CD200, such as herpesvirus 8 K14 protein[215], cytomegalovirus e127[216] protein and myxoma virus M141R[217] in the Herpesviridae and Poxviridae families[218], results in asymptomatic infection and inhibition of macrophage function. This suggests that enhanced expression of CD200R by M2a polarized macrophages may contribute to viral pathology. Moreover, expression of CD200R highlights the differences between human and murine macrophages which do not express CD200R following IL-4 stimulation[219].

The results of my study are in contrast to a report suggesting low levels of CD86 expression by M2a macrophages[220]. In this study, M2a macrophages were found to have a

higher CD86 expression as compared to the other polarization states. It was demonstrated that immunizing mice with a mixture of ascaris extract and LPS led to a significant ascaris specific IgE response[221]. This response was not seen when mice were given anti-CD86 antibody simultaneously with the immunization dose suggesting that CD86 is important in mediating an antigen-specific IgE antibody response under those conditions[221]. Human infection with *Ascaris lumbricoides* has been shown to drive a Th2 response[222]. It has also been reported that CD86 promotes T cell differentiation into Th2 cells[223] and CD86 co-stimulation leads to IL-4 production by Th2 cells[224]. Thus, IL-4 promotes M2a polarization which feeds back and may drive the Th2 response by virtue of CD86 engagement. Indeed, it has been demonstrated that alternatively activated macrophages, or M2a macrophages, are essential for survival during helminth infection[225].

M2b and M2c polarized macrophages were found to express low levels of HLA-DR as compared to MDMs. Lower expression levels of HLA-DR have been linked with changes in immune response mediated by stress, such as trauma, burns, surgery and sepsis[226]. Low HLA-DR expression is in agreement with the role of M2b and M2c macrophages to dampen the immune response. Furthermore, M2c macrophages were found to express high levels of CD163 as compared to the other polarization states. High CD163 expression in macrophages has been linked to inflammatory disease, including liver cirrhosis, type 2 diabetes, macrophage activation syndrome, Gaucher's disease, sepsis, HIV infection, rheumatoid arthritis and Hodgkin Lymphoma[227]. CD163 has been shown to prevent tissue inflammation by clearing hemoglobin/haptoglobin complexes and inducing antioxidative enzyme heme oxygenase-1[228]. The soluble form of CD163 plays a role in preventing inflammation by preventing T cell activation[228,229]. Thus, high expression level of CD163 by M2c

macrophages highlights their role in preventing tissue inflammation. Phenotypic characterization of polarized macrophages is essential as it can be used markers to characterize macrophage polarization states during inflammation *in vivo*.

Cytokine production by polarized macrophages

Cytokines are important messengers in mediating and coordinating the immune response. Macrophages release cytokines to orchestrate an anti-bacterial and anti-viral response as well as to recruit other immune cells to the site of infection or injury[230]. Therefore, I also characterized cytokine profiles exhibited by polarized macrophages by examining production of IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p70, IL-13, IL-17A, IL-22, IL-23 and TNFα before and after TLR-2, -3 and -4 agonist stimulation. The majority of studies that have thus far characterized cytokines in polarized macrophages have been performed by mRNA analysis[198]. Recent developments in mRNA/protein analysis techniques have demonstrated that due to factors such as differential regulation of mRNA stability and protein stability that the correlation between mRNA and protein expression is rather poor[231,232]. Fournier et al demonstrated that 26 out of 56 proteins whose expression increased did not demonstrate a corresponding increase in mRNA expression. Furthermore, some of these proteins actually demonstrated a decrease in mRNA expression despite the protein expression increase[231]. Thus, I characterized cytokine profiles of these polarized macrophages by measuring secreted cytokines. My results demonstrated lack of IL-2, IL-4, IL-9, IL-13 or IL-17A production by all polarized states. None of the polarized states produced IL-23 and IL-12p70 at the basal level prior to stimulation. At the basal level, M2b polarized macrophages predominately produced higher levels of IL-6 and TNFα. This is in conjunction with the murine model where macrophages, classified as type II by the author

and polarized with TLR agonists were reported to secrete TNFα and IL-6[233]. TNFα and IL-6 are considered as biomarkers for inflammation and even sepsis[234]. This is consistent with M2b polarized macrophages being implicated in sepsis and inflammatory response[69]. It is these macrophages which will become tolerant to further inflammatory stimuli[69]. Consistent with literature reports, the M2 polarized macrophages were found to produce more IL-10 than M1 polarized macrophages[35]. M2a and M2c polarized macrophages had a similar cytokine profile. This similar cytokine profile might hint at shared transcriptional responses by the polarization stimuli.

Macrophages provide a rapid immune response by recognizing, through Toll-like receptors (TLRs), repeated motifs called pathogen-associate molecular patterns (PAMPs) on invading pathogens[1]. Stimulation of these innate immune cell receptors leads to the activation of macrophage and results in cytokine production and eventually clearance of the pathogen[73]. Polarized macrophages were stimulated with agonists against two cell surface bound TLRs (TLR2 and TLR4) and one against intra cellular membrane bound TLR (TLR3) and cytokine production was measured. In general, TLR-2, -3 and -4 ligands reflect the broad range of microbial ligands and thus were used to characterize the cytokine profile of the polarized macrophages. Activation of the IRF5 transcription factor has been linked to induction of IL-12p40, a subunit of IL-12 and IL-23 as well as to M1 polarization[235]. Consistent with this, M1 polarized macrophages predominately produced IL-23, IL-12p70 and TNFα when stimulated with LPS, LTA or Poly I:C compared to M2 polarized macrophages. This is in contrast to a previous report demonstrating lack of IL-23 and IL-12 production in M1 polarized macrophages[236]. This could be a result of the difference in macrophage generation. Indeed, they cultured macrophages for 6 days in the presence of M-

CSF and followed by co-stimulation with LPS and IFNγ for 24 hours and measured cytokine secretion[236].

My results demonstrate that M2b macrophages did not induce any significant levels of pro-inflammatory cytokines, including IL-23, IL-12p70, TNFα, in response to either TLR 2, 3 or 4 stimulation. My results are in agreement with M2b macrophages being tolerant to inflammatory stimuli such as TLR agonists[69]. In contrast, M2b produced high levels of IL-10, which is the major anti-inflammatory cytokine important in inhibiting the immune response and limiting inflammation-mediated tissue injury[237]. M2a and M2c polarized macrophages had a similar cytokine profile hinting again at the idea of shared transcriptional profiles. M2a and M2c macrophages were polarized using IL-4 and IL-10, respectively. Yet, despite using different cytokines to induce polarization, the polarized macrophages secreted the same cytokines prior and after TLR stimulation. This phenomenon is analogous to what has been observed with M2a macrophages polarized by either IL-4 or IL-13. Though IL-13 and IL-4 are different cytokines they both polarize macrophages to the M2a state and both induce STAT6 phosphorylation. Thus, IL-4 and IL-13 stimulation seems to share a similar transcriptional response by engagement of signal transduction pathways culminating in STAT6 phosphorylation[238]. Similarly, IL-10 and IL-4 could share a transcriptional profile which allows for the similar cytokine profile of M2a and M2c macrophages.

IFNγ production by M1 macrophages

An interesting aspect of this study was the finding of IFNγ production solely by M1 macrophages after TLR 2, 3 and 4 agonist stimulation. IFNγ is known to be produced by NK cells and Th1 cells. IFNγ activates macrophages by up-regulating their lysosomal enzymatic activity and increasing the level of reactive oxygen species[95]. My results show for the first

time that M1 macrophages can secrete IFNγ following TLR 2, 3 and 4 stimulation. I have conclusively demonstrated that M1 macrophages upon TLR stimulation *de novo* synthesized IFNγ mRNA leading to IFNγ production. This was done to discount the possibility that the IFNγ detected in the ELISA was the same that was used to polarize MDMs into M1 macrophages. The results of this study demonstrate that priming MDMs with IFNγ leads to more IFNγ production following TLR 2, 3 and 4 stimulation.

Furthermore, I also demonstrated that IFNγ produced by M1 macrophages was biologically active. Supernatants from LPS stimulated M1 polarized macrophages were able to induce high TLR4 expression and this effect was lost when the MDMs had been pre-treated with IFNGR blocking antibodies, suggesting that the IFNγ present in the supernatant was responsible for the increased TLR4 expression. This finding suggests that IFNγ derived from M1 polarized macrophages has the ability to activate macrophages in an autocrine fashion to further promote the polarization and activated state.

MDMs that are stimulated with LPS are known to produce IL-12 and IL-18[239,240]. IL-12 and IL-18 have also been shown to synergize to promote production of IFNγ in T cells and NK cells[210-212]. MDMs that were co-stimulated with IL-12 and IL-18 were also able to produce IFNγ. Moreover, production of IFNγ by M1 macrophages was also confirmed by polarizing MDMs using IL-12 and IL-18 followed by stimulation with TLR agonists. This suggests that IL-12 and IL-18 produced during the innate immune response can lead to induction of IFNγ production by M1 macrophages contributing to their activation.

Susceptibly of polarized macrophages to *in vitro* HIV infection

Macrophages, in addition to monocytes, represent a reservoir for HIV[128]. Macrophages, unlike T cells, maintain their numbers during HIV infection[105]. They have been shown to produce large amounts of intracellular and extracellular HIV both *in vivo* and *in vitro* without being subjected to death due to productive infection[105]. The location of a macrophage can dictate its ability to support HIV infection. Intestinal macrophages are typically resistant to HIV infection unlike alveolar and vaginal macrophages[154]. The capacity of HIV to have a productive infection in macrophages is modulated by cytokines and macrophage location[157]. Therefore, it was of interest to determine what effect macrophage polarization could have on the productive HIV infection *in vitro*. Virus replication increased progressively in the control MDMs over the course of 10 days. However, M2a polarized macrophages showed the most productive infection as compared to the other polarized macrophages, followed by M2c polarized macrophages. In contrast, M1 polarized macrophages were relatively poor supporters of productive HIV infection whereas M2b polarized macrophages did not support productive HIV infection. These results confirmed findings that M1 polarized macrophages restrict HIV infection strongly as compared to M2a macrophages[241]. My results demonstrate for the first time that M2b polarization restricts productive *in vitro* HIV infection of macrophages and that M2c polarization supports it.

This study explored one avenue to explain the differences in productive infection seen across polarization states by measuring the expression levels of CD4, CXCR4 and CCR5, cell surface receptors required for HIV entry[112]. The differences in productive HIV infection in M1 and M2b versus M2a and M2c macrophages might be explained by differences in the HIV receptor and co-receptor expression levels. My results show that M1

(resistant) and M2c (productive) macrophages expressed similar percentages of CD4 positive cells as MDMs. M1 (resistant) and M2c (productive) macrophages expressed higher levels of CCR5 and had a higher percentage of cells that were CXCR4 positive as compared to MDMs. M2a (productive) and M2b (resistant) macrophages had similar percentages of CD4 positive cells, CXCR4 positive cells and CCR5 expression as compared to MDMs. This suggests that differences in HIV receptor and co-receptor expression may not explain the differences in productive HIV infection in polarized macrophages.

This was in contrast to a report by Cassol et al that examined expression levels of CD4, CXCR4 and CCR5 solely on M1 and M2a macrophages. The polarization stimuli they utilized for M1 polarization was IFNγ and TNFα[241]. In their report, M1 and M2a polarization of human MDMs inhibited productive HIV infection. They found that M1 macrophages had a significantly lower CD4 expression and CCR5 expression remained unchanged. They also found CCR5 expression increased and CXCR4 and CD4 expression decreased in M2a macrophages as compared to unpolarized MDMs[241]. Hence, in their report the differences in productive infection with respect to HIV were attributed to the expression levels of CD4.

Taken together, differential HIV receptor or co-receptor expression does not explain the differences in HIV infection across polarization states. These results suggest that there might be other mechanisms by which macrophage polarization leads to susceptibility or restriction to productive HIV infection. Nevertheless, these results demonstrate that the delicate changes within the alternatively polarized M2a-M2c macrophages impact their function in such a way as to induce a spectrum of permissiveness to HIV infection.

HIV infection is regulated by many factors including viral genotype, cell genotype and environmental factors such as cytokines and their location. Macrophage polarization is also regulated by small changes in macrophage environment. For example, the same cytokine can have opposing effects depending on when it is expressed during HIV infection. The treatment of infected macrophages with TNFα results in an increase in HIV mRNA that peaks after 3-4 days of infection[242]. TNFα acts on cells by binding to two receptors: TNF-R1 and TNF-R2. TNFα increases HIV infection in macrophages following binding of TNFα to TNF-R1 resulting in NF-κB activation[243]. However, pre-treatment of macrophages with TNFα led to signaling via TNF-R2 and inhibition of HIV replication[243]. My results show that M2b polarized macrophages produced high basal levels of TNFα prior to any stimulation with TLR agonist. It is possible that lack of productive HIV infection by M2b macrophages is due to TNFα induction following TLR4 stimulation during their polarization. LPS treatment of MDMs has also been shown to alter the availability of CCR5 on the cell surface and hence restrict HIV entry[244]. However, my results show that M2b polarization did not result in any changes in CCR5 expression as compared to control MDMs. LPS has also been shown to induce chemokines such as MIP-1α, MIP-1β and RANTES. These chemokines act as competitive inhibitors for CCR5 with respect to the virus and thus limit entry [245-247]. LPS treatment has been shown to protect MDMs from HIV infection by induction of heme oxygenase-1, a protective stress-induced protein[248]. Therefore, it is possible that LPS stimulation and subsequent cytokine production may impair productive HIV infection in M2b polarized macrophages.

Restriction factors, such as APOBEC3, SAMHD1 and MX2 and a limited dNTPs pool are also factors that contribute to restricted replication in macrophages[140,141,157].

Cytokine treatment has been shown to modulate the expression of these restriction factors in macrophages. For example, IFNα-treated macrophages have shown increased levels of APOBEC3G mRNA[157]. It is plausible that macrophage polarization results in changes in restriction factor expression thus mediating the differential susceptibility of polarized macrophages to *in vitro* HIV infection observed herein.

HIV gene expression can be silenced following its integration into host genome[142]. It is possible that the HIV genome has integrated in M2b polarized macrophages but is not being actively transcribed. Thus, further studies are needed to investigate HIV integration in polarized macrophages.

Viral gene expression is also partly controlled by miRNAs[145]. In PBMCs, HIV inhibits the expression of miRNA cluster miR-17/92 to promote its replication[146]. Recently, it has been shown that miRNAs could play a role in driving macrophage polarization[249]. Further studies are needed to investigate whether changes in miRNA expression that arise during macrophage polarization could explain the observations in this study.

Susceptibly of polarized macrophages to apoptosis

Macrophages are resistant to cell death induced by many apoptotic stimuli including infection[250]. Monocytes undergo apoptosis when cultured *in vitro*[251]. However, monocyte-derived macrophages become resistant to apoptosis after the differentiation process[252]. This resistance is mediated at least in part by the constant activation of the PI3K/Akt pathway[172]. The PI3K/Akt pathway is important in maintaining macrophage viability. Akt suppression, via PI3K inhibition using pharmacological inhibitor LY294002, resulted in the loss of mitochondrial potential and cumulated in apoptosis in macrophages[172]. Monocyte

differentiation is also accompanied by an increase in the expression of anti-apoptotic molecules, such as IAPs[176]. Macrophages, unlike CD4+ T cells, are resistant to cell death upon HIV infection. However, the susceptibly of polarized macrophages to apoptotic stimuli remains unknown and were the focus of my investigation.

Polarized macrophages were characterized with respect to their ability to undergo apoptosis. M1, M2b and M2c polarized macrophages underwent significant apoptosis in response to PI3K inhibition. Whereas, IL-4 treatment or M2a polarization prevented PI3K blockage mediated apoptosis in macrophages. It is possible that IL-4 is mediating a protective effect in M2a macrophages. It has been shown that Bcl-2 overexpression prevents the release of Cytochrome c from mitochondria upon apoptotic stimuli[253]. It has been shown in T cells that IL-4 can induce Bcl-2 via a PI3K independent pathway[254]. It is possible a similar mechanism occurs during M2a polarization that renders the cells resistant to apoptosis by increasing the expression of anti-apoptotic molecules.

Inhibitors of apoptosis proteins (IAPs) are involved in maintaining cell viability[176]. Smac mimetics are targeted agents that degrade IAPs and are being used in cancer therapy to cause apoptosis in cancer cells [194]. However, it has been shown that Smac mimetics do not cause significant apoptosis in MDMs[213]. It was important to determine whether polarized macrophages would undergo apoptosis with Smac mimetic treatment. For the first time, my results show that M1 polarization led to apoptosis in macrophages when they were treated with a physiological concentration of Smac mimetic. In contrast, M2a, M2b and M2c polarized macrophages did not undergo significant apoptosis. M2c polarized macrophages were resistant to apoptosis even when treated with a much higher dose of Smac mimetic. The differences in susceptibility to Smac mimetic treatment-mediated apoptosis might be

explained by the differential basal TNFα secretion levels in polarized macrophages. A recent study has looked at increasing the efficacy Smac mimetic treatment in cancer therapy by combining it with an innate immune stimulus, such as Poly I:C, to induce TNFα[255]. The study shows that the combination of TNFα and Smac mimetic leads to tumour cells death by caspase-8 dependent apoptosis[255]. My results show that M1 polarized macrophages did not produce significant levels of TNFα prior to TLR 2, 3 or 4 stimulation. In contrast, M2b macrophages were characterized by TNFα production prior to any stimulation with TLR ligands. However, M1 macrophages underwent significant apoptosis in response to Smac mimetic treatment, whereas M2b macrophages did not. Thus, the differences in Smac mimetic mediated apoptosis in polarized macrophages cannot be explained by differences in basal TNFα levels.

M2c polarized macrophages showed resistance to cell death mediated by Smac mimetic treatment. M2c polarized macrophages were generated using IL-10. It is possible that the resistance to apoptosis seen in M2c macrophages might be attributed using IL-10 as a polarizing stimulus. A recent study tested the effect of LCL-161 to induce apoptosis in various melanoma cells lines[256]. They found that cIAP 2 did not undergo degradation in the resistant cell lines and pSTAT 3 was upregulated[256]. IL-10 has been shown to induce STAT 3 in human macrophages[257]. It is possible that the resistance to cell death in M2c macrophages is also mediated by STAT 3. Further studies investigating IAPs' expression and the Jak/STAT pathways are needed to elucidate the mechanism by which M2c macrophages are resistant to apoptosis by Smac mimetic treatment.

Smac mimetic treatment, in concert with LY294002, did not produce significant differences in apoptosis of M1, M2b and M2c polarized macrophages. M2a macrophages

were resistant to apoptosis mediated by the blocking PI3K/Akt pathway. However, M2a polarized macrophages were sensitive to PI3K blockage induced-apoptosis when treated in concert with Smac mimetic. This suggests that the mechanism by which M2a macrophages resisted PI3K-blockage induced apoptosis was through IAPs. However, blocking IAPs alone in M2a polarized macrophages did not lead to significant apoptosis suggesting roles for other anti-apoptotic molecules. Previous work by our lab has shown that knocking down Akt in differentiated macrophages does not result in a decrease in the anti-apoptotic molecule Bcl-xL[258]. It was also shown that Bcl-xL expression becomes independent of PI3k/Akt signaling during monocyte to macrophage differentiation[258]. Similarly, it is possible that during the polarization process the expression of other anti-apoptotic molecules, such as Mcl-1, might also become independent of the PI3K/Akt pathway. The exact mechanism by which polarized macrophages become resistant to apoptosis still needs to be investigated.

In summary, this study was performed to characterize human macrophage polarization with respect to phenotype and function. Each polarization state was found to have its own distinct surface marker and cytokine profiles. My results demonstrate for the first time that M1 and M2b polarization make macrophages less susceptible to *in vitro* HIV infection. Furthermore, M2a and M2c polarization make macrophages less susceptible to apoptosis induced by LY294002 and LCL-161 respectively. Further studies are needed to elucidate the exact mechanisms by which polarization causes these differential effects in macrophages. These studies could be used to develop potential therapies for HIV-infected individuals.

Concluding remarks, significance and future directions

Macrophages form a bridge between innate and adaptive immunity. They can be polarized into M1 and M2 macrophages on the basis of their environment. The main criticism of previous investigations of macrophage polarization is their delimitation in looking at macrophage polarization solely in the context of M1 and M2a macrophages. This study for the first time characterizes all four polarization states (M1, M2a, M2b and M2c) with respect to their cell surface marker expression and functional profile.

The first aim of the study was to look at the expression of a non-exhaustive panel of surface markers usually expressed on macrophages was used to characterize each polarization state. Each polarization state was determined to have its own unique pattern of cell surface marker expression. These phenotypic markers can be used to determine which factors affect macrophage polarization *in vivo*. Furthermore, phenotypic markers can be used to characterize macrophage populations in tissue inflammatory disease conditions *in vivo* to further understand disease pathogenesis. However, macrophage polarization is very plastic and thus presents a challenge in translating these *in vitro* findings to *in vivo* conditions.

The second aim of this study was to characterize the polarized macrophages with respect to cytokine production, *in vitro* HIV infection and susceptibility to apoptosis. This study for the first time provides a comprehensive report on cytokine production in all four polarization states prior to and post TLR stimulation. M1 macrophages were found to predominately produce pro-inflammatory cytokines including IL-23, IFNγ, IL-12p70 and TNFα, M2b macrophages did not respond to TLR stimulation except with the secretion of IL-10. Interestingly, M2a and M2c macrophages shared a similar cytokine profile with respect to IL-6, IL-5, IL-10, IL-1β and IL-22. A novel finding was the production of IFNγ

solely by M1 macrophages post-TLR stimulation. This suggests there is an autocrine macrophage activation mechanism. Thus, this might change the current understanding of macrophage activation. IFNγ priming of macrophages leads to IFNγ production following TLR stimulation. Thus, the molecular signaling pathways activated by IFNγ that lead to IFNγ production following TLR stimulation need to be investigated. In inflammatory autoimmune conditions, macrophages are present which may provide a highly pro-inflammatory environment. It may be important to investigate the presence of such macrophages in these disease conditions.

This study also investigated the effect macrophage polarization might have on *in vitro* HIV infection and it was demonstrated that M1 and M2b macrophages were resistant to productive infection. These results suggest that macrophage polarization is an important factor in the susceptibility of macrophages to HIV infection. The expression levels of the HIV receptor, CD4, and the HIV chemokine co-receptors CXCR4 and CCR5 were analyzed as a means to explain the differential susceptibility to productive HIV infection. However, the changes in receptor and co-receptor expression could not explain the differences in HIV infectivity in the polarized macrophages. Further studies are needed to determine the mechanism responsible for the resistance/susceptibility to infection. The levels of p24 mRNA and HIV genome integration can be analyzed in the macrophages that were not infected. HIV, labelled with green fluorescent protein, could be used to determine the percentage of cells being infected in each polarization state. Furthermore, studies could investigate whether macrophage polarization results in a change in restriction factor expression and miRNA expression in macrophages and thus limits productive HIV infection.

Understanding the role macrophage polarization plays in HIV infection is essential for the development of new techniques to eradicate HIV infected macrophage reservoirs.

The last aim of this study was to investigate the susceptibility of polarized macrophages to apoptosis. Apoptosis was induced by blocking a key survival pathway, the PI3K pathway, and by degrading IAPs alone and in concert by using pharmacological inhibitors. This study demonstrates for the first time that M2a polarized macrophages were resistant to apoptosis mediated by blocking the PI3K pathway. The mechanism responsible for mediating this effect could involve the up-regulation of anti-apoptotic genes such as Bcl-xL and requires further investigation. It also demonstrated for the first time that M1 polarization caused macrophages to undergo apoptosis when treated with Smac mimetics. This effect was not seen in M2 polarized macrophages. M2c macrophages were especially unaffected by Smac mimetics even with higher doses. The mechanism responsible for this might include up-regulation of IAPs in M2c polarized macrophages and this needs to be further investigated by looking at IAPs' expression levels. It is possible Smac mediated apoptosis was enhanced due to the presence of TNFα. My results show that M1 polarized macrophages do not produce significant levels of TNFα prior to TLR stimulation. However, further studies are needed to fully discount the involvement of TNFα. The focus of further investigations could be to determine whether cell death was solely by apoptosis and involved caspases and PARPs (poly ADP ribose polymerases) or whether other forms of cell death such as necroptosis were also involved.

Polarized macrophages were treated with both LCL-161 and LY294002 to determine whether this would induce apoptosis in all the cells. This treatment at a higher concentration did cause M2a polarized macrophages to undergo apoptosis. In the future, these findings can

be confirmed by using specific siRNAs to block the pathway and IAPs. Interestingly, it was the M2a and M2c macrophages that were resistant to apoptosis and were also the most productively infected with HIV. Hence, it would be of interest to determine if blocking PI3K and IAPs would also cause differential apoptosis in HIV-infected polarized macrophages. Furthermore, studies could investigate the expression of anti-apoptotic molecules after macrophage polarization to understand the resistance displayed by these macrophages. Understanding the mechanism by which this occurs may facilitate developing ways to eradicate HIV infected macrophage reservoirs.

References

1. Janeway CA,Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today*. 1992;13(1):11-16.

2. Medzhitov R, Janeway C,Jr. Innate immunity. *N Engl J Med*. 2000;343(5):338-344.

3. Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. *Science*. 1996;272(5258):50-53.

4. Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: Macrophages, mast cells and neutrophils. *Nat Immunol*. 2011;12(11):1035-1044.

5. Shizuru JA, Negrin RS, Weissman IL. Hematopoietic stem and progenitor cells: Clinical and preclinical regeneration of the hematolymphoid system. *Annu Rev Med*. 2005;56:509-538.

6. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell*. 2006;124(4):783-801.

7. Barton GM. A calculated response: Control of inflammation by the innate immune system. *J Clin Invest*. 2008;118(2):413-420.

8. Liaskou E, Wilson DV, Oo YH. Innate immune cells in liver inflammation. *Mediators Inflamm*. 2012;2012:949157.

9. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol*. 2007;25:821-852.

10. Borregaard N. Neutrophils, from marrow to microbes. *Immunity*. 2010;33(5):657-670.

11. van Kasteren SI, Overkleeft H, Ovaa H, Neefjes J. Chemical biology of antigen presentation by MHC molecules. *Curr Opin Immunol*. 2014;26(0):21-31.

12. Jones TR, Hanson LK, Sun L, Slater JS, Stenberg RM, Campbell AE. Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. *J Virol*. 1995;69(8):4830-4841.

13. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*. 2005;5(12):953-964.

14. van Furth R, Cohn ZA, Hirsch JG, Humphrey JH, Spector WG, Langevoort HL. The mononuclear phagocyte system: A new classification of macrophages, monocytes, and their precursor cells. *Bull World Health Organ*. 1972;46(6):845-852.

15. Sunderkotter C, Nikolic T, Dillon MJ, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol*. 2004;172(7):4410-4417.

16. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity*. 2003;19(1):71-82.

17. Passlick B, Flieger D, Ziegler-Heitbrock HW. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood*. 1989;74(7):2527-2534.

18. Randolph GJ, Inaba K, Robbiani DF, Steinman RM, Muller WA. Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo. *Immunity*. 1999;11(6):753-761.

19. Auffray C, Fogg D, Garfa M, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science*. 2007;317(5838):666-670.

20. Randolph GJ, Sanchez-Schmitz G, Liebman RM, Schakel K. The CD16(+) (FcgammaRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. *J Exp Med*. 2002;196(4):517-527.

21. Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. *J Leukoc Biol*. 2011;89(4):557-563.

22. Ajami B, Bennett JL, Krieger C, Tetzlaff W, Rossi FMV. Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. *Nat Neurosci*. 2007;10(12):1538-1543.

23. Naito M, Umeda S, Yamamoto T, et al. Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. *J Leukoc Biol*. 1996;59(2):133-138.

24. Lichanska AM, Hume DA. Origins and functions of phagocytes in the embryo. *Exp Hematol*. 2000;28(6):601-611.

25. Ginhoux F, Greter M, Leboeuf M, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science*. 2010;330(6005):841-845.

26. Hoeffel G, Wang Y, Greter M, et al. Adult langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med*. 2012;209(6):1167-1181.

27. Schulz C, Gomez Perdiguero E, Chorro L, et al. A lineage of myeloid cells independent of myb and hematopoietic stem cells. *Science*. 2012;336(6077):86-90.

28. Yona S, Kim KW, Wolf Y, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*. 2013;38(1):79-91.

29. Bain CC, Scott CL, Uronen-Hansson H, et al. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol*. 2013;6(3):498-510.

30. Gordon S. The macrophage: Past, present and future. *Eur J Immunol*. 2007;37 Suppl 1:S9-17.

31. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-969.

32. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003;3(1):23-35.

33. Edwards JP, Zhang X, Frauwirth KA, Mosser DM. Biochemical and functional characterization of three activated macrophage populations. *J Leukoc Biol*. 2006;80(6):1298-1307.

34. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: Cancer as a paradigm. *Nat Immunol*. 2010;11(10):889-896.

35. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Biosci*. 2008;13:453-461.

36. Joshi S, Singh AR, Zulcic M, et al. Rac2 controls tumor growth, metastasis and M1-M2 macrophage differentiation in vivo. *PLoS One*. 2014;9(4):e95893.

37. Fleetwood AJ, Dinh H, Cook AD, Hertzog PJ, Hamilton JA. GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling. *J Leukoc Biol*. 2009;86(2):411-421.

38. Murray P, Allen J, Biswas S, et al. Macrophage activation and polarization: Nomenclature and experimental guidelines. *Immunity*. 2014;41(1):14-20.

39. Zizzo G, Hilliard BA, Monestier M, Cohen PL. Efficient clearance of early apoptotic cells by human macrophages requires M2c polarization and MerTK induction. *J Immunol*. 2012;189(7):3508-3520.

40. Jaguin M, Houlbert N, Fardel O, Lecureur V. Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. *Cell Immunol*. 2013;281(1):51-61.

41. O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. *Immunity*. 2008;28(4):477-487.

42. Dale DC, Boxer L, Liles WC. The phagocytes: Neutrophils and monocytes. *Blood*. 2008;112(4):935-945.

43. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity*. 2004;21(4):467-476.

44. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006;441(7090):235-238.

45. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med*. 2005;201(2):233-240.

46. Zhang X, Mosser DM. Macrophage activation by endogenous danger signals. *J Pathol*. 2008;214(2):161-178.

47. Szekanecz Z, Koch AE. Macrophages and their products in rheumatoid arthritis. *Curr Opin Rheumatol*. 2007;19(3):289-295.

48. Nathan,Carl. Metchnikoff's legacy in 2008. .

49. Filipe-Santos O, Bustamante J, Chapgier A, et al. Inborn errors of IL-12/23- and IFN-gamma-mediated immunity: Molecular, cellular, and clinical features. *Semin Immunol*. 2006;18(6):347-361.

50. Nandan D, Reiner NE. Attenuation of gamma interferon-induced tyrosine phosphorylation in mononuclear phagocytes infected with leishmania donovani: Selective inhibition of signaling through janus kinases and Stat1. *Infect Immun*. 1995;63(11):4495-4500.

51. Pai RK, Convery M, Hamilton TA, Boom WH, Harding CV. Inhibition of IFN-gamma-induced class II transactivator expression by a 19-kDa lipoprotein from mycobacterium tuberculosis: A potential mechanism for immune evasion. *J Immunol*. 2003;171(1):175-184.

52. Loke P, Gallagher I, Nair MG, et al. Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. *The Journal of Immunology*. 2007;179(6):3926-3936.

53. Brandt E, Woerly G, Younes AB, Loiseau S, Capron M. IL-4 production by human polymorphonuclear neutrophils. *J Leukoc Biol*. 2000;68(1):125-130.

54. Reese TA, Liang HE, Tager AM, et al. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature*. 2007;447(7140):92-96.

55. Kreider T, Anthony RM, Urban Jr. JF, Gause WC. Alternatively activated macrophages in helminth infections. *Curr Opin Immunol*. 2007;19(4):448-453.

56. Cordeiro-da-Silva A, Tavares J, Araújo N, et al. Immunological alterations induced by polyamine derivatives on murine splenocytes and human mononuclear cells. *Int Immunopharmacol*. 2004;4(4):547-556.

57. Wilson MS, Mentink-Kane MM, Pesce JT, Ramalingam TR, Thompson R, Wynn TA. Immunopathology of schistosomiasis. *Immunol Cell Biol*. 2007;85(2):148-154.

58. Hesse M, Modolell M, La Flamme AC, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: Granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol*. 2001;167(11):6533-6544.

59. Munitz A, Brandt EB, Mingler M, Finkelman FD, Rothenberg ME. Distinct roles for IL-13 and IL-4 via IL-13 receptor alpha1 and the type II IL-4 receptor in asthma pathogenesis. *Proc Natl Acad Sci U S A*. 2008;105(20):7240-7245.

60. Shirey KA, Cole LE, Keegan AD, Vogel SN. Francisella tularensis live vaccine strain induces macrophage alternative activation as a survival mechanism. *J Immunol*. 2008;181(6):4159-4167.

61. Kropf P, Fuentes JM, Fahnrich E, et al. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. *FASEB J*. 2005;19(8):1000-1002.

62. Harris J, De Haro SA, Master SS, et al. T helper 2 cytokines inhibit autophagic control of intracellular mycobacterium tuberculosis. *Immunity*. 2007;27(3):505-517.

63. Muller U, Stenzel W, Kohler G, et al. IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with cryptococcus neoformans. *J Immunol*. 2007;179(8):5367-5377.

64. Tumitan AR, Monnazzi LG, Ghiraldi FR, Cilli EM, Machado de Medeiros BM. Pattern of macrophage activation in yersinia-resistant and yersinia-susceptible strains of mice. *Microbiol Immunol*. 2007;51(10):1021-1028.

65. Mosser DM. The many faces of macrophage activation. *J Leukoc Biol*. 2003;73(2):209-212.

66. Lucas M, Zhang X, Prasanna V, Mosser DM. ERK activation following macrophage FcgammaR ligation leads to chromatin modifications at the IL-10 locus. *J Immunol*. 2005;175(1):469-477.

67. Gerber JS, Mosser DM. Reversing lipopolysaccharide toxicity by ligating the macrophage fc gamma receptors. *J Immunol*. 2001;166(11):6861-6868.

68. Hao NB, Lu MH, Fan YH, Cao YL, Zhang ZR, Yang SM. Macrophages in tumor microenvironments and the progression of tumors. *Clin Dev Immunol*. 2012;2012:948098.

69. Medzhitov R, Schneider DS, Soares MP. Disease tolerance as a defense strategy. *Science*. 2012;335(6071):936-941.

70. Biswas SK, Lopez-Collazo E. Endotoxin tolerance: New mechanisms, molecules and clinical significance. *Trends Immunol*. 2009;30(10):475-487.

71. Porta C, Rimoldi M, Raes G, et al. Tolerance and M2 (alternative) macrophage polarization are related processes orchestrated by p50 nuclear factor κB. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(35):14978-14983.

72. Miles SA, Conrad SM, Alves RG, Jeronimo SM, Mosser DM. A role for IgG immune complexes during infection with the intracellular pathogen leishmania. *J Exp Med*. 2005;201(5):747-754.

73. Agrawal A, Pulendran B. Anthrax lethal toxin: A weapon of multisystem destruction. *Cell Mol Life Sci*. 2004;61(22):2859-2865.

74. Benoit M, Barbarat B, Bernard A, Olive D, Mege JL. Coxiella burnetii, the agent of Q fever, stimulates an atypical M2 activation program in human macrophages. *Eur J Immunol*. 2008;38(4):1065-1070.

75. Duluc D, Delneste Y, Tan F, et al. Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. *Blood*. 2007;110(13):4319-4330.

76. Mantovani A, Allavena P, Sica A. Tumour-associated macrophages as a prototypic type II polarised phagocyte population: Role in tumour progression. *Eur J Cancer*. 2004;40(11):1660-1667.

77. Condeelis J, Pollard JW. Macrophages: Obligate partners for tumor cell migration, invasion, and metastasis. *Cell*. 2006;124(2):263-266.

78. Steidl C, Lee T, Shah SP, et al. Tumor-associated macrophages and survival in classic hodgkin's lymphoma. *N Engl J Med*. 2010;362(10):875-885.

79. Gocheva V, Wang HW, Gadea BB, et al. IL-4 induces cathepsin protease activity in tumor-associated macrophages to promote cancer growth and invasion. *Genes Dev*. 2010;24(3):241-255.

80. Duluc D, Corvaisier M, Blanchard S, et al. Interferon-gamma reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. *Int J Cancer*. 2009;125(2):367-373.

81. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest*. 2007;117(5):1155-1166.

82. Nardin A, Abastado JP. Macrophages and cancer. *Front Biosci*. 2008;13:3494-3505.

83. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: Cancer as a paradigm. *Nat Immunol*. 2010;11(10):889-896.

84. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*. 2011;34(5):637-650.

85. Beutler B. Microbe sensing, positive feedback loops, and the pathogenesis of inflammatory diseases. *Immunol Rev*. 2009;227(1):248-263.

86. Kono H, Rock KL. How dying cells alert the immune system to danger. *Nat Rev Immunol*. 2008;8(4):279-289.

87. O'Neill LA. The interleukin-1 receptor/toll-like receptor superfamily: 10 years of progress. *Immunol Rev*. 2008;226:10-18.

88. Cole JE, Mitra AT, Monaco C. Treating atherosclerosis: The potential of toll-like receptors as therapeutic targets. *Expert Rev Cardiovasc Ther*. 2010;8(11):1619-1635.

89. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol*. 2004;4(7):499-511.

90. Hoebe K, Du X, Georgel P, et al. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature*. 2003;424(6950):743-748.

91. Cavaillon JM. Cytokines and macrophages. *Biomed Pharmacother*. 1994;48(10):445-453.

92. Striz I, Brabcova E, Kolesar L, Sekerkova A. Cytokine networking of innate immunity cells: A potential target of therapy. *Clin Sci (Lond)*. 2014;126(9):593-612.

93. Lyakh L, Trinchieri G, Provezza L, Carra G, Gerosa F. Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans. *Immunol Rev*. 2008;226:112-131.

94. Sica A, Mantovani A. Macrophage plasticity and polarization: In vivo veritas. *J Clin Invest*. 2012;122(3):787-795.

95. Foster N, Hulme SD, Barrow PA. Induction of antimicrobial pathways during early-phase immune response to salmonella spp. in murine macrophages: Gamma interferon (IFN-gamma) and upregulation of IFN-gamma receptor alpha expression are required for NADPH phagocytic oxidase gp91-stimulated oxidative burst and control of virulent salmonella spp. *Infect Immun*. 2003;71(8):4733-4741.

96. Puddu P, Fantuzzi L, Borghi P, et al. IL-12 induces IFN-gamma expression and secretion in mouse peritoneal macrophages. *J Immunol*. 1997;159(7):3490-3497.

97. Fultz MJ, Barber SA, Dieffenbach CW, Vogel SN. Induction of IFN-gamma in macrophages by lipopolysaccharide. *Int Immunol*. 1993;5(11):1383-1392.

98. Schindler H, Lutz MB, Rollinghoff M, Bogdan C. The production of IFN-gamma by IL-12/IL-18-activated macrophages requires STAT4 signaling and is inhibited by IL-4. *J Immunol*. 2001;166(5):3075-3082.

99. Munder M, Mallo M, Eichmann K, Modolell M. Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. *J Exp Med*. 1998;187(12):2103-2108.

100. Wang J, Wakeham J, Harkness R, Xing Z. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. *J Clin Invest*. 1999;103(7):1023-1029.

101. Robinson BW, McLemore TL, Crystal RG. Gamma interferon is spontaneously released by alveolar macrophages and lung T lymphocytes in patients with pulmonary sarcoidosis. *J Clin Invest*. 1985;75(5):1488-1495.

102. Fenton MJ, Vermeulen MW, Kim S, Burdick M, Strieter RM, Kornfeld H. Induction of gamma interferon production in human alveolar macrophages by mycobacterium tuberculosis. *Infect Immun*. 1997;65(12):5149-5156.

103. Darwich L, Coma G, Pena R, et al. Secretion of interferon-gamma by human macrophages demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18. *Immunology*. 2009;126(3):386-393.

104. Kraaij MD, Vereyken EJ, Leenen PJ, et al. Human monocytes produce interferon-gamma upon stimulation with LPS. *Cytokine*. 2014;67(1):7-12.

105. Orenstein JM, Fox C, Wahl SM. Macrophages as a source of HIV during opportunistic infections. *Science*. 1997;276(5320):1857-1861.

106. Crowe SM, Mills J, Kirihara J, Boothman J, Marshall JA, McGrath MS. Full-length recombinant CD4 and recombinant gp120 inhibit fusion between HIV infected macrophages and uninfected CD4-expressing T-lymphoblastoid cells. *AIDS Res Hum Retroviruses*. 1990;6(8):1031-1037.

107. Mastino A, Grelli S, Piacentini M, et al. Correlation between induction of lymphocyte apoptosis and prostaglandin E2 production by macrophages infected with HIV. *Cell Immunol*. 1993;152(1):120-130.

108. Badley AD, Dockrell D, Simpson M, et al. Macrophage-dependent apoptosis of CD4+ T lymphocytes from HIV-infected individuals is mediated by FasL and tumor necrosis factor. *J Exp Med*. 1997;185(1):55-64.

109. Herbein G, Mahlknecht U, Batliwalla F, et al. Apoptosis of CD8+ T cells is mediated by macrophages through interaction of HIV gp120 with chemokine receptor CXCR4. *Nature*. 1998;395(6698):189-194.

110. Aquaro S, Panti S, Caroleo MC, et al. Primary macrophages infected by human immunodeficiency virus trigger CD95-mediated apoptosis of uninfected astrocytes. *J Leukoc Biol*. 2000;68(3):429-435.

111. Engelman A, Cherepanov P. The structural biology of HIV-1: Mechanistic and therapeutic insights. *Nat Rev Micro*. 2012;10(4):279-290.

112. Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature*. 1998;393(6686):648-659.

113. Dumenil G. Revisiting the extracellular lifestyle. *Cell Microbiol*. 2011;13(8):1114-1121.

114. Ray K, Marteyn B, Sansonetti PJ, Tang CM. Life on the inside: The intracellular lifestyle of cytosolic bacteria. *Nature Reviews Microbiology*. 2009;7(5):333-340.

115. Randow F, Münz C. Autophagy in the regulation of pathogen replication and adaptive immunity. *Trends Immunol*. 2012;33(10):475-487.

116. Friedrich N, Hagedorn M, Soldati-Favre D, Soldati T. Prison break: Pathogens' strategies to egress from host cells. *Microbiology and Molecular Biology Reviews*. 2012;76(4):707-720.

117. Deneka M, Pelchen-Matthews A, Byland R, Ruiz-Mateos E, Marsh M. In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. *J Cell Biol*. 2007;177(2):329-341.

118. Pelchen-Matthews A, Kramer B, Marsh M. Infectious HIV-1 assembles in late endosomes in primary macrophages. *J Cell Biol*. 2003;162(3):443-455.

119. Deneka M, Pelchen-Matthews A, Byland R, Ruiz-Mateos E, Marsh M. In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. *J Cell Biol*. 2007;177(2):329-341.

120. Welsch S, Keppler OT, Habermann A, Allespach I, Krijnse-Locker J, Krausslich HG. HIV-1 buds predominantly at the plasma membrane of primary human macrophages. *PLoS Pathog*. 2007;3(3):e36.

121. Welsch S, Groot F, Kräusslich H-, Keppler OT, Sattentau QJ. Architecture and regulation of the HIV-1 assembly and holding compartment in macrophages. *J Virol*. 2011;85(15):7922-7927.

122. Pelchen-Matthews A, Giese S, Mlcochová P, Turner J, Marsh M. ß2 integrin adhesion complexes maintain the integrity of HIV-1 assembly compartments in primary macrophages. *Traffic*. 2012;13(2):273-291.

123. Koppensteiner H, Banning C, Schneider C, Hohenberg H, Schindler M. Macrophage internal HIV-1 is protected from neutralizing antibodies. *J Virol*. 2012;86(5):2826-2836.

124. Sharova N, Swingler C, Sharkey M, Stevenson M. Macrophages archive HIV-1 virions for dissemination in trans. *EMBO J*. 2005;24(13):2481-2489.

125. Groot F, Welsch S, Sattentau QJ. Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses. *Blood*. 2008;111(9):4660-4663.

126. Gousset K, Ablan SD, Coren LV, et al. Real-time visualization of HIV-1 GAG trafficking in infected macrophages. *PLoS Pathog*. 2008;4(3):e1000015.

127. Geeraert L, Kraus G, Pomerantz RJ. Hide-and-seek: The challenge of viral persistence in HIV-1 infection. *Annu Rev Med*. 2008;59:487-501.

128. Aquaro S, Caliò R, Balzarini J, Bellocchi MC, Garaci E, Perno CF. Macrophages and HIV infection: Therapeutical approaches toward this strategic virus reservoir. *Antiviral Res*. 2002;55(2):209-225.

129. Shen L, Siliciano RF. Viral reservoirs, residual viremia, and the potential of highly active antiretroviral therapy to eradicate HIV infection. *J Allergy Clin Immunol*. 2008;122(1):22-28.

130. Dooms H, Abbas AK. Control of CD4+ T-cell memory by cytokines and costimulators. *Immunol Rev*. 2006;211:23-38.

131. Chun T, Fauci AS. Latent reservoirs of HIV: Obstacles to the eradication of virus. *Proceedings of the National Academy of Sciences*. 1999;96(20):10958-10961.

132. Eisele E, Siliciano R. Redefining the viral reservoirs that prevent HIV-1 eradication. *Immunity*. 2012;37(3):377-388.

133. Van Lint C, Bouchat S, Marcello A. HIV-1 transcription and latency: An update. *Retrovirology*. 2013;10:67-4690-10-67.

134. Greene WC, Peterlin BM. Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. *Nat Med*. 2002;8(7):673-680.

135. Bukrinsky MI, Sharova N, Dempsey MP, et al. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proceedings of the National Academy of Sciences*. 1992;89(14):6580-6584.

136. Pierson TC, Zhou Y, Kieffer TL, Ruff CT, Buck C, Siliciano RF. Molecular characterization of preintegration latency in human immunodeficiency virus type 1 infection. *Journal of Virology*. 2002;76(17):8518-8531.

137. Kelly J, Beddall MH, Yu D, Iyer SR, Marsh JW, Wu Y. Human macrophages support persistent transcription from unintegrated HIV-1 DNA. *Virology*. 2008;372(2):300-312.

138. Teo I, Veryard C, Barnes H, et al. Circular forms of unintegrated human immunodeficiency virus type 1 DNA and high levels of viral protein expression: Association with dementia and multinucleated giant cells in the brains of patients with AIDS. *J Virol*. 1997;71(4):2928-2933.

139. Pang S, Koyanagi Y, Miles S, Wiley C, Vinters HV, Chen IS. High levels of unintegrated HIV-1 DNA in brain tissue of AIDS dementia patients. *Nature*. 1990;343(6253):85-89.

140. Lahouassa H, Daddacha W, Hofmann H, et al. SAMHD1 restricts the replication of human immunodeficiency virus type 1 by depleting the intracellular pool of deoxynucleoside triphosphates. *Nat Immunol*. 2012;13(3):223-228.

141. Goujon C, Moncorge O, Bauby H, et al. Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. *Nature*. 2013;502(7472):559-562.

142. Van Lint C, Bouchat S, Marcello A. HIV-1 transcription and latency: An update. *Retrovirology*. 2013;10:67-4690-10-67.

143. Barr SD, Ciuffi A, Leipzig J, Shinn P, Ecker JR, Bushman FD. HIV integration site selection: Targeting in macrophages and the effects of different routes of viral entry. *Mol Ther*. 2006;14(2):218-225.

144. Verdin E, Paras P,Jr, Van Lint C. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. *EMBO J*. 1993;12(8):3249-3259.

145. Narayanan A, Kehn-Hall K, Bailey C, Kashanchi F. Analysis of the roles of HIV-derived microRNAs. *Expert Opin Biol Ther*. 2011;11(1):17-29.

146. Triboulet R, Mari B, Lin YL, et al. Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science*. 2007;315(5818):1579-1582.

147. Wang X, Ye L, Hou W, et al. Cellular microRNA expression correlates with susceptibility of monocytes/macrophages to HIV-1 infection. *Blood*. 2009;113(3):671-674.

148. Zhu J, Paul WE. CD4 T cells: Fates, functions, and faults. *Blood*. 2008;112(5):1557-1569.

149. Sodora DL, Allan JS, Apetrei C, et al. Toward an AIDS vaccine: Lessons from natural simian immunodeficiency virus infections of african nonhuman primate hosts. *Nat Med*. 2009;15(8):861-865.

150. Hazenberg MD, Otto SA, van Benthem BH, et al. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS*. 2003;17(13):1881-1888.

151. Lawn SD, Butera ST, Folks TM. Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection. *Clin Microbiol Rev*. 2001;14(4):753-77, table of contents.

152. Giorgi JV, Hultin LE, McKeating JA, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *J Infect Dis*. 1999;179(4):859-870.

153. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med*. 2006;12(12):1365-1371.

154. Carter CA, Ehrlich LS. Cell biology of HIV-1 infection of macrophages. *Annu Rev Microbiol*. 2008;62:425-443.

155. Smith PD, Meng G, Sellers MT, Rogers TS, Shaw GM. Biological parameters of HIV-1 infection in primary intestinal lymphocytes and macrophages. *Journal of Leukocyte Biology*. 2000;68(3):360-365.

156. Honda Y, Rogers L, Nakata K, et al. Type I interferon induces inhibitory 16-kD CCAAT/ enhancer binding protein (C/EBP)ß, repressing the HIV-1 long terminal repeat in macrophages: Pulmonary tuberculosis alters C/EBP expression, enhancing HIV-1 replication. *The Journal of Experimental Medicine*. 1998;188(7):1255-1265.

157. Peng G, Lei KJ, Jin W, Greenwell-Wild T, Wahl SM. Induction of APOBEC3 family proteins, a defensive maneuver underlying interferon-induced anti-HIV-1 activity. *J Exp Med*. 2006;203(1):41-46.

158. Mallardo M, Dragonetti E, Baldassarre F, Ambrosino C, Scala G, Quinto I. An NF-κB site in the 5′-untranslated leader region of the human immunodeficiency virus type 1 enhances the viral expression in response to NF-κB-activating stimuli. *Journal of Biological Chemistry*. 1996;271(34):20820-20827.

159. Rizzi C, Crippa MP, Jeeninga RE, et al. Pertussis toxin B-oligomer suppresses IL-6 induced HIV-1 and chemokine expression in chronically infected U1 cells via inhibition of activator protein 1. *The Journal of Immunology*. 2006;176(2):999-1006.

160. Naif HM, Li S, Ho-Shon M, Mathijs JM, Williamson P, Cunningham AL. The state of maturation of monocytes into macrophages determines the effects of IL-4 and IL-13 on HIV replication. *The Journal of Immunology*. 1997;158(1):501-511.

161. Kedzierska K, Crowe SM, Turville S, Cunningham AL. The influence of cytokines, chemokines and their receptors on HIV-1 replication in monocytes and macrophages. *Rev Med Virol*. 2003;13(1):39-56.

162. Herbein G, Montaner LJ, Gordon S. Tumor necrosis factor alpha inhibits entry of human immunodeficiency virus type 1 into primary human macrophages: A selective role for the 75-kilodalton receptor. *Journal of Virology*. 1996;70(11):7388-7397.

163. Cassol E, Cassetta L, Rizzi C, Alfano M, Poli G. M1 and M2a polarization of human monocyte-derived macrophages inhibits HIV-1 replication by distinct mechanisms. *The Journal of Immunology*. 2009;182(10):6237-6246.

164. Taylor RC, Cullen SP, Martin SJ. Apoptosis: Controlled demolition at the cellular level. *Nat Rev Mol Cell Biol*. 2008;9(3):231-241.

165. Um HD, Orenstein JM, Wahl SM. Fas mediates apoptosis in human monocytes by a reactive oxygen intermediate dependent pathway. *The Journal of Immunology*. 1996;156(9):3469-3477.

166. Kiener PA, Davis PM, Starling GC, et al. Differential induction of apoptosis by Fas–Fas ligand interactions in human monocytes and macrophages. *The Journal of Experimental Medicine*. 1997;185(8):1511-1516.

167. Munn DH, Beall AC, Song D, Wrenn RW, Throckmorton DC. Activation-induced apoptosis in human macrophages: Developmental regulation of a novel cell death pathway by macrophage colony-stimulating factor and interferon gamma. *The Journal of Experimental Medicine*. 1995;181(1):127-136.

168. Perlman H, Pagliari LJ, Georganas C, Mano T, Walsh K, Pope RM. FLICE-inhibitory protein expression during macrophage differentiation confers resistance to fas-mediated apoptosis. *J Exp Med*. 1999;190(11):1679-1688.

169. Mulherin D, Fitzgerald O, Bresnihan B. Synovial tissue macrophage populations and articular damage in rheumatoid arthritis. *Arthritis Rheum*. 1996;39(1):115-124.

170. Perlman H, Pagliari LJ, Liu H, Koch AE, Haines GK,3rd, Pope RM. Rheumatoid arthritis synovial macrophages express the fas-associated death domain-like interleukin-1beta-converting enzyme-inhibitory protein and are refractory to fas-mediated apoptosis. *Arthritis Rheum*. 2001;44(1):21-30.

171. Pagliari LJ, Perlman H, Liu H, Pope RM. Macrophages require constitutive NF-kappaB activation to maintain A1 expression and mitochondrial homeostasis. *Mol Cell Biol*. 2000;20(23):8855-8865.

172. Liu H, Perlman H, Pagliari LJ, Pope RM. Constitutively activated akt-1 is vital for the survival of human monocyte-differentiated macrophages: Role of mcl-1, independent of nuclear factor (nf)-κb, bad, or caspase activation. *The Journal of Experimental Medicine*. 2001;194(2):113-126.

173. El-Deiry WS. Akt takes centre stage in cell-cycle deregulation. *Nat Cell Biol*. 2001;3(3):E71-3.

174. Chugh P, Bradel-Tretheway B, Monteiro-Filho CM, et al. Akt inhibitors as an HIV-1 infected macrophage-specific anti-viral therapy. *Retrovirology*. 2008;5:11-4690-5-11.

175. Osaki M, Oshimura M, Ito H. PI3K-akt pathway: Its functions and alterations in human cancer. *Apoptosis*. 2004;9(6):667-676.

176. Busca A, Saxena M, Kryworuchko M, Kumar A. Anti-apoptotic genes in the survival of monocytic cells during infection. *Curr Genomics*. 2009;10(5):306-317.

177. Crook NE, Clem RJ, Miller LK. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J Virol*. 1993;67(4):2168-2174.

178. Sun C, Cai M, Gunasekera AH, et al. NMR structure and mutagenesis of the inhibitor-of-apoptosis protein XIAP. *Nature*. 1999;401(6755):818-822.

179. Deveraux QL, Leo E, Stennicke HR, Welsh K, Salvesen GS, Reed JC. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO J*. 1999;18(19):5242-5251.

180. Srinivasula SM, Ashwell JD. IAPs: What's in a name? *Mol Cell*. 2008;30(2):123-135.

181. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J*. 1997;16(23):6914-6925.

182. Huang H, Joazeiro CA, Bonfoco E, Kamada S, Leverson JD, Hunter T. The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. *J Biol Chem*. 2000;275(35):26661-26664.

183. Morizane Y, Honda R, Fukami K, Yasuda H. X-linked inhibitor of apoptosis functions as ubiquitin ligase toward mature caspase-9 and cytosolic smac/DIABLO. *J Biochem*. 2005;137(2):125-132.

184. Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P. Toxic proteins released from mitochondria in cell death. *Oncogene*. 2004;23(16):2861-2874.

185. Wu G, Chai J, Suber TL, et al. Structural basis of IAP recognition by smac/DIABLO. *Nature*. 2000;408(6815):1008-1012.

186. Shiozaki EN, Shi Y. Caspases, IAPs and smac/DIABLO: Mechanisms from structural biology. *Trends Biochem Sci*. 2004;29(9):486-494.

187. Sun XM, Bratton SB, Butterworth M, MacFarlane M, Cohen GM. Bcl-2 and bcl-xL inhibit CD95-mediated apoptosis by preventing mitochondrial release of smac/DIABLO and subsequent inactivation of X-linked inhibitor-of-apoptosis protein. *J Biol Chem*. 2002;277(13):11345-11351.

188. Wrzesien-Kus A, Smolewski P, Sobczak-Pluta A, Wierzbowska A, Robak T. The inhibitor of apoptosis protein family and its antagonists in acute leukemias. *Apoptosis*. 2004;9(6):705-715.

189. Sun H, Nikolovska-Coleska Z, Yang CY, et al. Design of small-molecule peptidic and nonpeptidic smac mimetics. *Acc Chem Res*. 2008;41(10):1264-1277.

190. Eschenburg G, Eggert A, Schramm A, Lode HN, Hundsdoerfer P. Smac mimetic LBW242 sensitizes XIAP-overexpressing neuroblastoma cells for TNF-alpha-independent apoptosis. *Cancer Res*. 2012;72(10):2645-2656.

191. Petersen SL, Wang L, Yalcin-Chin A, et al. Autocrine TNFalpha signaling renders human cancer cells susceptible to smac-mimetic-induced apoptosis. *Cancer Cell*. 2007;12(5):445-456.

192. Vince JE, Wong WW, Khan N, et al. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell*. 2007;131(4):682-693.

193. Varfolomeev E, Blankenship JW, Wayson SM, et al. IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell*. 2007;131(4):669-681.

194. Mastrangelo E, Cossu F, Milani M, et al. Targeting the X-linked inhibitor of apoptosis protein through 4-substituted azabicyclo[5.3.0]alkane smac mimetics. structure, activity, and recognition principles. *J Mol Biol*. 2008;384(3):673-689.

195. Raes G, Van den Bergh R, De Baetselier P, Ghassabeh GH. Arginase-1 and Ym1 are markers for murine, but not human, alternatively activated myeloid cells. *The Journal of Immunology*. 2005;174(11):6561-6562.

196. Geissmann F, Gordon S, Hume DA, Mowat AM, Randolph GJ. Unravelling mononuclear phagocyte heterogeneity. *Nat Rev Immunol*. 2010;10(6):453-460.

197. Chambers KA, Parks RJ, Angel JB. Disruption of MAP kinase activation and nuclear factor binding to the IL-12 p40 promoter in HIV-infected myeloid cells. *Clin Exp Immunol*. 2004;137(2):329-340.

198. Jaguin M, Houlbert N, Fardel O, Lecureur V. Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. *Cell Immunol*. 2013;281(1):51-61.

199. Lolmede K, Campana L, Vezzoli M, et al. Inflammatory and alternatively activated human macrophages attract vessel-associated stem cells, relying on separate HMGB1- and MMP-9-dependent pathways. *Journal of Leukocyte Biology*. 2009;85(5):779-787.

200. Zeyda M, Farmer D, Todoric J, et al. Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. *Int J Obes*. 2007;31(9):1420-1428.

201. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annu Rev Immunol*. 1997;15:749-795.

202. Puddu P, Fantuzzi L, Borghi P, et al. IL-12 induces IFN-gamma expression and secretion in mouse peritoneal macrophages. *The Journal of Immunology*. 1997;159(7):3490-3497.

203. Di Marzio P, Puddu P, Conti L, Belardelli F, Gessani S. Interferon gamma upregulates its own gene expression in mouse peritoneal macrophages. *The Journal of Experimental Medicine*. 1994;179(5):1731-1736.

204. Fenton MJ, Vermeulen MW, Kim S, Burdick M, Strieter RM, Kornfeld H. Induction of gamma interferon production in human alveolar macrophages by mycobacterium tuberculosis. *Infection and Immunity*. 1997;65(12):5149-5156.

205. Robinson BW, McLemore TL, Crystal RG. Gamma interferon is spontaneously released by alveolar macrophages and lung T lymphocytes in patients with pulmonary sarcoidosis. *J Clin Invest*. 1985;75(5):1488-1495.

206. Nathan CF, Prendergast TJ, Wiebe ME, et al. Activation of human macrophages. comparison of other cytokines with interferon-gamma. *J Exp Med*. 1984;160(2):600-605.

207. Bosisio, Daniela Polentarutti, Nadia Sironi, Marina Bernasconi, Sergio Miyake, Kensuke Webb, Ginette R. Martin, Michael U. Mantovani,Alberto Muzio, Marta. Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-Î³: A molecular basis for priming and synergism with bacterial lipopolysaccharide. *Blood*. 2002;99(9):3427-3431.

208. Ghalib HW, Whittle JA, Kubin M, et al. IL-12 enhances Th1-type responses in human leishmania donovani infections. *J Immunol*. 1995;154(9):4623-4629.

209. Chowdhury FZ, Ramos HJ, Davis LS, Forman J, Farrar JD. IL-12 selectively programs effector pathways that are stably expressed in human CD8+ effector memory T cells in vivo. *Blood*. 2011;118(14):3890-3900.

210. Micallef MJ, Ohtsuki T, Kohno K, et al. Interferon-gamma-inducing factor enhances T helper 1 cytokine production by stimulated human T cells: Synergism with interleukin-12 for interferon-gamma production. *Eur J Immunol*. 1996;26(7):1647-1651.

211. Robinson D, Shibuya K, Mui A, et al. IGIF does not drive Th1 development but synergizes with IL-12 for interferon-gamma production and activates IRAK and NFkappaB. *Immunity*. 1997;7(4):571-581.

212. Zhang T, Kawakami K, Qureshi MH, Okamura H, Kurimoto M, Saito A. Interleukin-12 (IL-12) and IL-18 synergistically induce the fungicidal activity of murine peritoneal exudate cells against cryptococcus neoformans through production of gamma interferon by natural killer cells. *Infection and Immunity*. 1997;65(9):3594-3599.

213. Busca A, Saxena M, Kumar A. Critical role for antiapoptotic bcl-xL and mcl-1 in human macrophage survival and cellular IAP1/2 (cIAP1/2) in resistance to HIV-vpr-induced apoptosis. *J Biol Chem*. 2012;287(18):15118-15133.

214. Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: Enabling diversity with identity. *Nat Rev Immunol*. 2011;11(11):750-761.

215. Foster-Cuevas M, Wright GJ, Puklavec MJ, Brown MH, Barclay AN. Human herpesvirus 8 K14 protein mimics CD200 in down-regulating macrophage activation through CD200 receptor. *J Virol*. 2004;78(14):7667-7676.

216. Foster-Cuevas M, Westerholt T, Ahmed M, Brown MH, Barclay AN, Voigt S. Cytomegalovirus e127 protein interacts with the inhibitory CD200 receptor. *J Virol*. 2011;85(12):6055-6059.

217. Cameron CM, Barrett JW, Liu L, Lucas AR, McFadden G. Myxoma virus M141R expresses a viral CD200 (vOX-2) that is responsible for down-regulation of macrophage and T-cell activation in vivo. *J Virol*. 2005;79(10):6052-6067.

218. Barclay AN. Membrane proteins with immunoglobulin-like domains - A master superfamily of interaction molecules. *Semin Immunol*. 2003;15(4):215-223.

219. Koning N, van Eijk M, Pouwels W, et al. Expression of the inhibitory CD200 receptor is associated with alternative macrophage activation. *J Innate Immun*. 2010;2(2):195-200.

220. Lolmede K, Campana L, Vezzoli M, et al. Inflammatory and alternatively activated human macrophages attract vessel-associated stem cells, relying on separate HMGB1- and MMP-9-dependent pathways. *Journal of Leukocyte Biology*. 2009;85(5):779-787.

221. Jiang GZ, Kato Y, Sugiyama T, et al. Role of CD86 (B7-2) in triggering of antigen-specific IgE antibody response by lipopolysaccharide. *FEMS Immunol Med Microbiol*. 1998;21(4):303-311.

222. Cooper PJ, Chico ME, Sandoval C, et al. Human infection with ascaris lumbricoides is associated with a polarized cytokine response. *Journal of Infectious Diseases*. 2000;182(4):1207-1213.

223. Thompson CB. Distinct roles for the costimulatory ligands B7-1 and B7-2 in T helper cell differentiation? *Cell*. 1995;81(7):979-982.

224. Natesan M, Razi-Wolf Z, Reiser H. Costimulation of IL-4 production by murine B7-1 and B7-2 molecules. *The Journal of Immunology*. 1996;156(8):2783-2791.

225. Herbert DR, Hölscher C, Mohrs M, et al. Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity*. 2004;20(5):623-635.

226. Kim OY, Monsel A, Bertrand M, Coriat P, Cavaillon JM, Adib-Conquy M. Differential down-regulation of HLA-DR on monocyte subpopulations during systemic inflammation. *Crit Care*. 2010;14(2):R61.

227. Etzerodt A, Moestrup SK. CD163 and inflammation: Biological, diagnostic, and therapeutic aspects. *Antioxid Redox Signal*. 2013;18(17):2352-2363.

228. Moestrup SK, Møller HJ. CD163: A regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. *Ann Med*. 2004;36(5):347-354.

229. Van Gorp H, Delputte PL, Nauwynck HJ. Scavenger receptor CD163, a jack-of-all-trades and potential target for cell-directed therapy. *Mol Immunol*. 2010;47(7-8):1650-1660.

230. Stow JL, Ching Low P, Offenhäuser C, Sangermani D. Cytokine secretion in macrophages and other cells: Pathways and mediators. *Immunobiology*. 2009;214(7):601-612.

231. Fournier ML, Paulson A, Pavelka N, et al. Delayed correlation of mRNA and protein expression in rapamycin-treated cells and a role for Ggc1 in cellular sensitivity to rapamycin. *Mol Cell Proteomics*. 2010;9(2):271-284.

232. Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: New molecules and patterns of gene expression. *The Journal of Immunology*. 2006;177(10):7303-7311.

233. Mosser DM. The many faces of macrophage activation. *Journal of Leukocyte Biology*. 2003;73(2):209-212.

234. Song R, Kim J, Yu D, Park C, Park J. Kinetics of IL-6 and TNF-α changes in a canine model of sepsis induced by endotoxin. *Vet Immunol Immunopathol*. 2012;146(2):143-149.

235. Krausgruber T, Blazek K, Smallie T, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol*. 2011;12(3):231-238.

236. Verreck FAW, de Boer T, Langenberg DML, et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(13):4560-4565.

237. Savan R, Ravichandran S, Collins JR, Sakai M, Young HA. Structural conservation of interferon gamma among vertebrates. *Cytokine Growth Factor Rev*. 2009;20(2):115-124.

238. Wurster AL, Tanaka T, Grusby MJ. The biology of Stat4 and Stat6. *Oncogene*. 2000;19(21):2577-2584.

239. Trinchieri G. Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol*. 1995;13:251-276.

240. Okamura H, Nagata K, Komatsu T, et al. A novel costimulatory factor for gamma interferon induction found in the livers of mice causes endotoxic shock. *Infection and Immunity*. 1995;63(10):3966-3972.

241. Cassol E, Cassetta L, Rizzi C, Alfano M, Poli G. M1 and M2a polarization of human monocyte-derived macrophages inhibits HIV-1 replication by distinct mechanisms. *The Journal of Immunology*. 2009;182(10):6237-6246.

242. Naif H, Ho-Shon M, Chang J, Cunningham AL. Molecular mechanisms of IL-4 effect on HIV expression in promonocytic cell lines and primary human monocytes. *J Leukoc Biol*. 1994;56(3):335-339.

243. Herbein G, Gordon S. 55- and 75-kilodalton tumor necrosis factor receptors mediate distinct actions in regard to human immunodeficiency virus type 1 replication in primary human macrophages. *J Virol*. 1997;71(5):4150-4156.

244. Franchin G, Zybarth G, Dai WW, et al. Lipopolysaccharide inhibits HIV-1 infection of monocyte- derived macrophages through direct and sustained down-regulation of CC chemokine receptor 5. *J Immunol*. 2000;164(5):2592-2601.

245. Verani A, Scarlatti G, Comar M, et al. C-C chemokines released by lipopolysaccharide (LPS)-stimulated human macrophages suppress HIV-1 infection in both macrophages and T cells. *J Exp Med*. 1997;185(5):805-816.

246. Lane BR, Markovitz DM, Woodford NL, Rochford R, Strieter RM, Coffey MJ. TNF-alpha inhibits HIV-1 replication in peripheral blood monocytes and alveolar macrophages by inducing the production of RANTES and decreasing C-C chemokine receptor 5 (CCR5) expression. *J Immunol*. 1999;163(7):3653-3661.

247. Zybarth G, Reiling N, Schmidtmayerova H, Sherry B, Bukrinsky M. Activation-induced resistance of human macrophages to HIV-1 infection in vitro. *J Immunol*. 1999;162(1):400-406.

248. Devadas K, Hewlett IK, Dhawan S. Lipopolysaccharide suppresses HIV-1 replication in human monocytes by protein kinase C-dependent heme oxygenase-1 induction. *Journal of Leukocyte Biology*. 2010;87(5):915-924.

249. Graff JW, Dickson AM, Clay G, McCaffrey AP, Wilson ME. Identifying functional microRNAs in macrophages with polarized phenotypes. *J Biol Chem*. 2012;287(26):21816-21825.

250. Roulston A, Marcellus RC, Branton PE. Viruses and apoptosis. *Annu Rev Microbiol*. 1999;53:577-628.

251. Kiener PA, Davis PM, Starling GC, et al. Differential induction of apoptosis by fas-fas ligand interactions in human monocytes and macrophages. *J Exp Med*. 1997;185(8):1511-1516.

252. Becker S, Warren MK, Haskill S. Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. *J Immunol*. 1987;139(11):3703-3709.

253. Yang J, Liu X, Bhalla K, et al. Prevention of apoptosis by bcl-2: Release of cytochrome c from mitochondria blocked. *Science*. 1997;275(5303):1129-1132.

254. Aronica MA, Goenka S, Boothby M. IL-4-dependent induction of BCL-2 and BCL-X(L)IN activated T lymphocytes through a STAT6- and pi 3-kinase-independent pathway. *Cytokine*. 2000;12(6):578-587.

255. Beug ST, Tang VA, LaCasse EC, et al. Smac mimetics and innate immune stimuli synergize to promote tumor death. *Nat Biotech*. 2014;32(2):182-190.

256. Ramakrishnan V, Painuly U, Kimlinger T, Haug J, Rajkumar SV, Kumar S. Inhibitor of apoptosis proteins as therapeutic targets in multiple myeloma. *Leukemia*. 2014;28(7):1519-1528.

257. Niemand C, Nimmesgern A, Haan S, et al. Activation of STAT3 by IL-6 and IL-10 in primary human macrophages is differentially modulated by suppressor of cytokine signaling 3. *J Immunol*. 2003;170(6):3263-3272.

258. Busca A, Saxena M, Iqbal S, Angel J, Kumar A. PI3K/akt regulates survival during differentiation of human macrophages by maintaining NF-kappaB-dependent expression of antiapoptotic bcl-xL. *J Leukoc Biol*. 2014.

Contribution of Collaborators

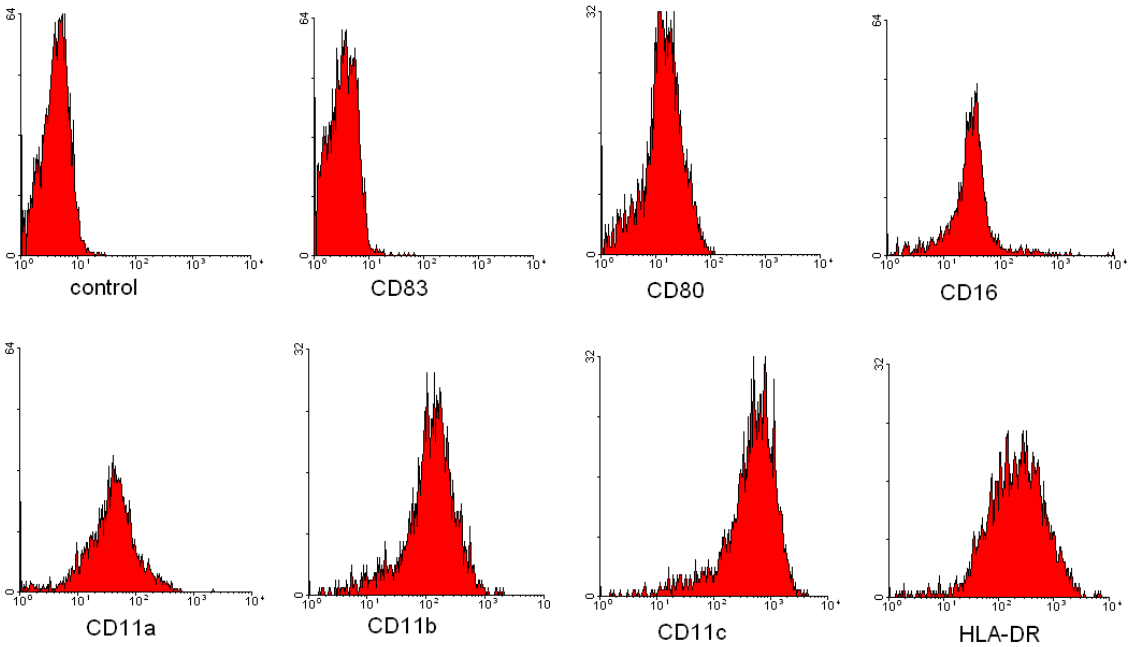
The experiment shown in the Supplementary figure was carried out by Dr. Aurelia Busca

Appendices

Supplementary Figure

Characterization of monocyte-derived macrophages.

MDMs were generated from as mentioned previously with M-CSF over 6 days. Cells were washed and stained with PE-conjugated antibodies against CD83, CD80, CD16, CD11a, CD11b, CD11c and HLA-DR for 15 minutes. Surface expression was assessed by flow cytometry. The result is a representative of 3 independent experiments. Results were generated by Dr. Aurelia Busca.


control
CD83
CD80
CD16
CD11a
CD11b
CD11c
HLA-DR

Authors' Permission

Figure 1. Development and Differentiation of macrophages.

On Tue, Aug 19, 2014 at 1:11 AM,
Subject: Re: request for permission to use figure in thesis
From: Murray, Peter wrote:
No problem

On Behalf Of Salma Iqbal
Sent: Monday, August 18, 2014 11:56 AM
Subject: request for permission to use figure in thesis

Dear Dr Murray and Dr Wynn,

I am writing to request permission to use one of your figures in my Master's thesis. It is Figure 1 from your 2011 review "Protective and pathogenic functions of macrophage subsets". I came across your review while writing the introduction to my thesis which is based on macrophage polarization and was hoping to include your figure in my thesis.

Thank you

Best regards,

Salma Iqbal