The role of CD80 and CD86 in macrophage activation and its regulation following LPS stimulation

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Abstract

The binding of CD80/CD86 on the APC to CD28 on the T cell surface provides a second signal for T cell activation. While it was once believed that this interaction represented a one-way signal, resulting in T cell activation, recently, it has been investigated as a bidirectional signaling process. CD80/86 activation produces IL-6 in DCs, but its role in macrophage activation is unknown. Dysregulation of CD80/86 expression has been observed in autoimmune disorders and cancer, and may also influence the development of immune responses including production of cytokines in response to stimulation with TLR-4 ligand, LPS. Therefore, the focus of my project was twofold: 1) to investigate the role of CD80/86 as signaling receptors capable of transmitting extracellular signals, and 2) to determine the TLR-4 activated pathways that regulate CD80/86 expression in human monocyte-derived macrophages (MDMs). Since I demonstrated that activation of CD80/86 alone did not induce expression of the four cytokines investigated, I hypothesized that CD80/86 synergizes with other signaling pathways. I show for the first time that CD80/86 activation synergizes with TLR-4 signaling to produce IL-27 and IL-10 in human MDMs. Since cIAPs play a key role in TLR-4-mediated signaling, I investigated their role in TLR-4- and CD80/86-activated production of IL-10 and IL-27. Degradation of IAPs by SMAC mimetics inhibited LPS-induced IL-10 and IL-27 production in MDMs. However, it did not alter the TLR-4 and CD80/86 synergistic effect on IL-10 and IL-27 production suggesting that IAPs may not play a role in CD80/86 activation of macrophages. Since I have demonstrated this role for IAPs, I extended my studies by examining the involvement of IAPs and other upstream signaling molecules such as SHP-1, RIP1, TRAF2, in modulating the LPS-induced CD80/86 expression. I showed that cIAP2, SHP-1, RIP1, TRAF2 co-localize to form a complex that regulates the LPS-induced CD80 and CD86 expression through AKT-activated p38 MAPK in human macrophages. These findings may lead to the development of novel therapeutic interventions in the treatment of autoimmune diseases.

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List of Abbreviations

α-	anti
Akt	Protein kinase B
AP1	Activating protein-1
APC	antigen presenting cell
APS	ammonium persulfate
ATP	adenosine triphosphate
BCR	B cell receptor
BIR	baculoviral IAP repeat
BMDMs	bone marrow derived macrophage cultures
BMK-1	big MAPK-1
BSA	bovine serum albumin
cIAP	cellular inhibitor of apoptosis
CSF-1	colony stimulating factor 1
CTL	cytotoxic T-lymphocyte
DCs	dendritic cells
DMSO	dimethyl sulphoxide
ECL	enhanced chemiluminescence
ELISA	Enzyme linked immuno-sorbent assay
ERK	extracellular-signal regulated kinase
FLICE	Fas-associated death domain (FADD)-like IL- β converting enzyme
FLIP	FLICE inhibitory protein
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IAP	inhibitor of apoptosis
IFN	interferon
IκB	inhibitor-kappa B
IL-	interleukin
IP	immunoprecipitation
IRAK	IL-1R associated kinases
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
LBP	LPS binding protein
LPS	Lipopolysaccharide
mAb	monoclonal antibody/antibodies
MAPK	mitogen associated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK Kinase kinase
M-CSF	macrophage colony-stimulating factor
MD2	myeloid differentiation protein 2
MDMs	monocyte-derived macrophages
MHC	major histocompatibility complex
mTOR	mammalian target of rapamycin

MyD88	myeloid differentiation factor 88
NIK	NF-κB inducing kinase
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NK cells	Natural killer cells
PAMP	pathogen associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PCR	polymerized chain reaction
PE	phycoerythrin
PerCP	Peridinin chlorophyll protein
PFA	paraformaldehyde
PI	propidium iodide
PI3K	phosphotidyl inositol-3-kinase
PMA	phorbol-12-myristate-13-acetate
PRRs	pattern recognition receptors
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene difluoride
qRT-PCR	semi-quantitative real time polymerase chain reaction
RING	really interesting new gene
RIP1	receptor interacting protein 1
RNS	reactive nitrogen species
ROS	reactive oxygen species
RPM	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate poly acrylamide gel elctrophoresis
SH2	*Src*-homology domains 2
SHP-1	SH2 domain containing phosphatase-1
siRNA	small interfering RNA
SMAC	small mitochondria-derived activators of caspases
TBE	Tris boric acid –EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline and Tween-20
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	transforming growth factor-β
Th cells	T helper cells
Th17	T helper 17 cells
TIR	Toll/IL-1 receptor
TIRAP	Toll-interleukin 1 receptor adaptor protein
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TRAM	TRIF related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
T reg	T regulatory cell
XIAP	x-chromosome linked IAP

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Appendix

1.1 Introduction

1.2 Innate immune system and inflammation

Infectious diseases are a leading cause of morbidity and mortality worldwide. The innate immune system is the first line of defense and the biggest contributor to acute inflammation induced by tissue damage or microbial infection (1). Inflammation is the rapid, coordinated response that leads to the resolution of infection, the repair of damage and the return to the homeostatic state, with minimal damage to the host (2). The survival of the host is largely based on its ability to coordinate a network of cells to recognize and induce the appropriate response for the elimination of the microbes. Monocytes, macrophages, dendritic cells (DCs), neutrophils, eosinophils, mast cells and natural killer (NK) cells represent many of the cells of the innate immune response (3). Neutrophils are among the first cells to be recruited to the site of infection, followed by the recruitment of monocytes, and T and B cells (4). Neutrophils possess highly cytotoxic granules with proteases that are capable of degrading material that has undergone phagocytosis. They also produce reactive nitrogen species (RNS) and reactive oxygen species (ROS) to induce DNA damage, and to further denature proteins and disrupt lipid (5). Chemokines released by neutrophils initiate the continued response by other cells, such as monocytes and macrophages which express pattern recognition receptors (PRRs) on their cell surface (3). These PRRs recognize pathogens associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) on the surface of Gram negative bacteria. For example, the engagement of the toll-like receptor 4 (TLR-4), a transmembrane protein on the surface of macrophages, with its cognate ligand, LPS, will initiate an immune response (3). Other PAMPs include glycolipids, flagellin, lipoproteins, viral RNA and bacterial DNA, and their engagement with PRRs will result in a specific and targeted destruction of the infected cell or organism by phagocytosis or the release of cytotoxic agents (2).

Circulating monocytes constitute between 5-10% of peripheral blood leukocytes (3). They originate from a common myeloid progenitor cell in the bone marrow that is shared with neutrophils and are released into the bloodstream undifferentiated for 1-3 days (3). Upon recruitment into the site of infection/inflammation, they can differentiate into tissue macrophages (Kupffer cells in the liver; microglia in the brain) or myeloid DCs, contributing to host defence and tissue repair (3). To recruit other inflammatory cells to the sites of infections, these cells release cytokines and other proinflammatory mediators to induce changes in the local environment to convert the infected tissue to an inflamed state (2). For example, the release of pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, will initiate leukocyte migration and infiltration, and a flow of plasma to the site of injury (3). Macrophages and DCs are professional antigen presenting cells (APCs) which, upon ingestion of a pathogen, migrate to draining lymph nodes and present pathogen-associated antigens resulting in activation of the adaptive immune response. APCs use HLA/MHC (Human Leukocyte Antigen/Major Histocompatibility Complex) class I and class II molecules to present extracellular peptides to T cells to drive a proper immune response (6). Some viruses prevent expression of the MHC molecule on the surface of APCs to circumvent detection and destruction; however, human APCs that do not express the MHC molecule are removed by NK cells, representing a vital arm of the innate immune system responsible for targeting and lysing cells that do not express MHC molecules (7).

Generated in the thymus, T lymphocytes circulate through the bloodstream and primary lymphoid organs, like the lymph nodes and spleen (3). After encountering APCs, T cells are activated and begin to proliferate and differentiate. This activation requires two signals. The first signal is antigen specific and requires binding of the T cell receptor to the antigenic peptides presented within the context of the MHC molecules (8). This signal is insufficient to elicit an optimal immune reaction and requires the second antigen-independent signal. This second signal is provided by the

binding of CD28 on the T cell surface with one of two costimulatory molecules on the APC: CD80 or CD86 (8). Macrophages represent a large portion of the APCs that express CD80 and CD86 and therefore, play an important role in the activation of the immune response.

1.3 Macrophages

Originating in the bone marrow from $CD34^+$ myeloid progenitor cells, monocytes are released into peripheral blood and enter tissue to replenish tissue macrophage population (9). They can also differentiate into DCs and osteoclasts depending on the local inflammatory milieu (9). The process of monocyte to macrophage differentiation is governed by the binding of the hematopoietic growth factor, the macrophage colony stimulating factor (M-CSF) to its receptor (M-CSFR) on the surface of monocytes. Activation of the M-CSFR results in monocyte changes, beginning with their adherence and development of an elongated, spindle-like appearance (10). Cell cycle genes are activated and the monocyte-differentiated macrophages gain functions such as antigen presentation (10). As key players in the innate immune system, macrophages engulf and digest microorganisms, dead cells, and debris during infection and produce inflammatory mediators, such as cytokines and chemokines, to activate other cell types and kill bacteria (11). By recognizing, processing, and presenting antigen to T cells, macrophages are critical players in bridging the gap between innate and adaptive immunity (11).

Based on the cytokine milieu, macrophages can become polarized and gain specialized functional properties. Similar to the Th1/Th2 nomenclature, polarized macrophages are often characterized as M1 or M2 cells (10). M1 or classically activated macrophages are induced by IFN-γ and TNF, and express higher levels of IL-12 and IL-23 and lower levels of IL-10 (10). They produce antimicrobial effector molecules, such as ROS, and inflammatory cytokines, including IL-1β, TNF and IL-6, and are potent contributors to the Th1 response, specifically mediating resistance to tumors

and intracellular parasites (10). Excessive M1 polarization will inhibit cell proliferation and cause tissue damage (12). In contrast, M2 or alternatively activated macrophages, representing a broad spectrum of cells activated by cytokines such as IL-4 or IL-13, turn off the damaging immune system with anti-inflammatory cytokines and work towards tissue repair (12). Therefore, M2 cells play a critical role in resolving inflammation, promoting cell growth and wound healing. M2 macrophages display an IL-12^{low} and IL-23^{low} phenotype, are poorly microbicidal, but retain an important role as immunomodulators (13). Both subsets of macrophages have important roles in the immune system, and their activation leads to the induction of various branches of adaptive immune responses.

1.4 Pro-inflammatory and Anti-inflammatory Cytokines

Cytokines and chemokines are secreted by immune cells during an inflammatory response as soluble messengers (14). As important cell-cell communicators, these signaling molecules are released, among other innate immune cells, by macrophages to recruit other inflammatory cells and as direct killers (15). Cytokines can be broadly divided into two groups: interferons and interleukins (IL) (14). The IL-12 family of immunoregulatory cytokines play a critical role in bridging the innate and adaptive branches of the immune response. The IL-12 family includes the structurally related, heterodimeric cytokines IL-12, IL-23, IL-27 and IL-35. IL-12 is composed of two subunits, IL-12p40 and IL-12p35. IL-12 is crucial in promoting a Th1 response (16). Upon activation with LPS, macrophages secrete proinflammatory cytokines including TNF and IL-6, followed by the release of other cytokines promoting an inflammatory response, including IL-12 and IL-23 (14). IL-23, through induction of IL-17, promotes a highly pro-inflammatory Th17 response. The role of IL-27 in an immune response has been controversial, as it has been identified as having both a pro- and anti-inflammatory role. However, recently IL-27 has been increasingly recognized as an anti-inflammatory cytokine because of its ability to induce IL-10 and inhibit a Th17 response, mediated

by IL-23 (17). IL-10 is a homodimer that is produced mostly by macrophages, although other immune cells also secrete this cytokine. Upon LPS stimulation, IL-10 inhibits the release of pro-inflammatory cytokines, in particular IL-12 (18), and reduces the presentation of antigen by downregulating the expression of MHC class II molecules (19). Release of anti-inflammatory cytokines such as IL-10, IL-27, TGF-β and IL-4 delimit the inflammatory response resulting in clearance of the infection. While the secretion of IL-12, IL-23, IL-27, and IL-10 are all mediated through different mechanisms in the macrophage, they each carry an important role in mediating a proper immune response.

1.5 B7 receptors

CD80 and CD86 are two important costimulatory molecules part of the B7 family that bridge the gap between the innate and adaptive immune responses. As transmembrane proteins on the surface of APCs, CD80 and CD86 have a large role in exhibiting both the recognition within the innate response and activation of the adaptive response. CD80 and CD86 are members of the immunoglobulin supergene family (IgSF), expressed by hemopoietic cells, including monocytes, macrophages, DCs, and B cells (20). CD80 is a 55 kDa type I hydrophobic transmembrane glycoprotein with a short 19 amino acid cytoplasmic domain (21). On the other hand, CD86 is a 70kDa type 1 membrane glycoprotein, composed of an extended cytoplasmic domain (21). It has been demonstrated that at basal levels, macrophages express low levels of CD80 and constitutively express CD86, but both of these molecules can be induced following LPS or IFN-γ stimulation (22). CD80 and CD86 are expressed as monomers, consisting of a single amino-terminal immunoglobulin variable (IgV)-like, one membrane proximal Ig constant (IgC)-like domain, a transmembrane domain, and a cytoplasmic tail (23). The importance of IgV and IgC domains has been demonstrated. IgV has been shown to modulate B7 protein activity with higher levels of IgV-CD80 transcripts during GVH

disease (24). In the absence of IgC domain, there was over a ten-fold reduction in binding to CD28 and CTLA-4, suggesting that both domains are required for full binding *in vivo* (25).

Two signals are required for the activation of T cells (Figure 1). The first is the antigen-dependent and requires that the MHC molecule present antigen to the T cell receptor. The second is the binding of CD80 or CD86 to CD28 on the surface of T cells (24). After the CD80/CD86-CD28 interaction, T cells are activated, proliferate and differentiate to acquire effector functions (26). They also provide aid to other cells, such as B cells and NK cells, to initiate an adaptive immune response (26). Activated T cells produce cytokines through induction of cytokine genes and mRNA stabilization (27). CD80/CD86-CD28 signals also increase anti-apoptotic activity and T cell survival by upregulating the expression of BCL-X_L (B-cell lymphoma-extra large), a transmembrane molecule that prevents the release of cytochrome c from the mitochondria and subsequent activation of the apoptotic pathway (28). This important interaction is also responsible for lowering the threshold for T cell activation, reducing the number of T cells required for effective cytokine production and immune response (29). In the absence of the secondary signal between CD80/CD86 and CD28, T cells undergo apoptosis or enter a state of anergy, making them unable to produce IL-2 required for their proliferation, even after subsequent stimulation (30).

The careful balance between the positive and negative regulation of T cells is maintained in part by the cytotoxic T-lymphocyte antigen 4 (CTLA-4) expressed on the surface of T cells (26). In addition to interacting with CD28, CD80 and CD86 can also interact with CTLA-4 (also known as CD152), initiating an inhibitory signal, downregulating the proliferation of T cells (8). CTLA-4 is not present on the surface of T lymphocytes during resting state, but instead exists intracellularly. Following T cell activation, the surface expression of CTLA-4 is upregulated making it readily available to bind to CD80 or CD86 followed by induction of an inhibitory signal to prevent an over-activation of the immune response (26). CTLA-4 shares 30% homology with CD28, but has a higher

Figure 1: **Two signals are required to produce a proper immune response.** The first signal constitutes of the presentation of an antigen by the MHC molecule through MHC interaction with the T cell receptor (TCR). The second signal is the binding of CD80 or CD86 to either CD28, resulting in proliferation and differentiation of T cells, or CTLA-4, resulting in cell cycle arrest. If CD80 or CD86 is not present on the surface of the APC, the T cell undergoes apoptosis or anergy.

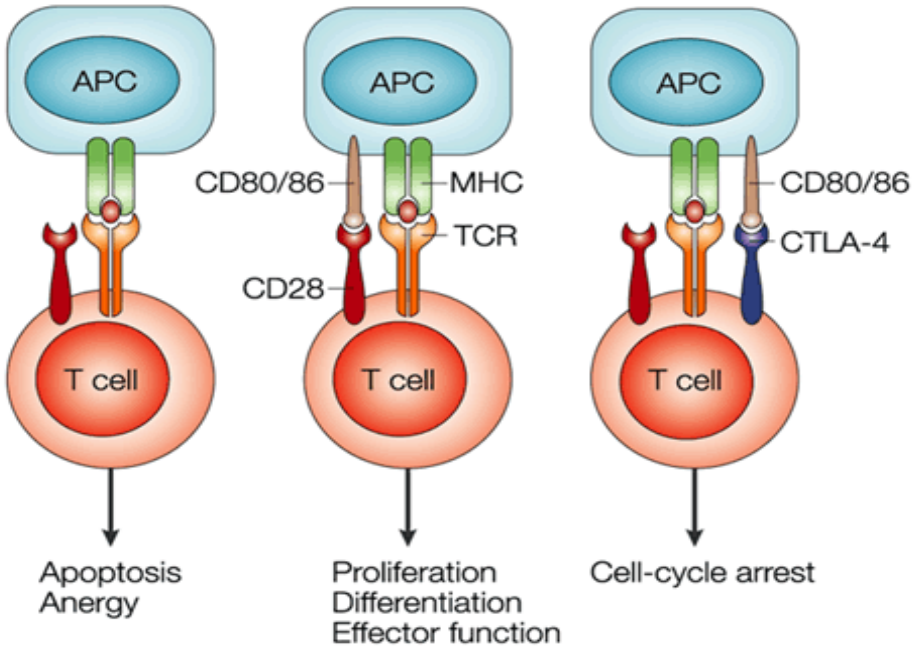


Figure 1 (Alegre *et al*, 2001)

affinity to its ligands, preferentially interacting with CD80/CD86 to terminate an immune response (26). CTLA-4 works by reducing the production of IL-2 and expression of the IL-2 receptor, and by arresting T cells at the G1 phase of the cell cycle (31). Although both CD80 and CD86 bind to CD28 and CTLA-4, recent research has demonstrated that their binding affinities differ; CD80 binds to both receptors with two to three times more affinity, with slower dissociation constants and faster binding kinetics compared to CD86 (22) .

CD80 and CD86 peripheral membrane proteins represent two major members of the B7 family. The B7 family also includes newer members: B7-DC, B7-H1, B7-H2, B7-H3, and B7-H4 expressing their own unique, but often overlapping functions in directing immune functions (32). Preferentially expressed on DCs, B7-DC binds to its receptor PD-1 resulting in T cell proliferation and cytokine production (33). *In vivo* experiments in knockout mice have demonstrated that B7-H1 and B7-H2 have a role in effector responses, particularly in the production of antibodies, and are critical in the tumor immune response (32). B7-H3 has been identified as a major player in cancer research and may play a diverse role in the regulation of growth and differentiation of non-hematopoietic cells (34). B7-H4, highly expressed in human cancers, has been shown to inhibit immune responses, contributing to tumor escape (35). Although interest in the immunological function of these newer members of the B7 family has increased within the past decade, the ongoing research into the elucidation of the mechanisms regulating the expression and function of the first two B7 family members, B7-1 and B7-2, or CD80 and CD86, has expanded tremendously, particularly in their prospects in diagnostics and the development of immunotherapies.

1.6 The role of CD80 and CD86 in Th cell differentiation

CD4+ T helper cells can differentiate into two distinct subtypes of effector cells each with their own set of regulatory cytokines. Th1 cells regulate cell-mediated immune responses through

production of IL-2, IFN-γ, and TNF-β. In contrast, Th2 cells regulate humoral immunity and produce IL-4, IL-5, and IL-10 (36). Both cell types have important implications in the resistance and progression of disease. Following T cell activation, precursor T cells will differentiate preferentially down the Th2 pathway in the presence of IL-4, or down the Th1 pathway in the presence of IL-12. Activation of these cell types by antigens further reinforces their differentiation pattern manifesting by the production of IFN-γ by Th1 cells and IL-4 by Th2 cells (37). More recently, it has been demonstrated that CD80 and CD86 may play a role in the differentiation of these two subtypes of cells. Treatment of murine cells with anti-CD80 antibodies *in vitro* and *in vivo* favored the induction of IL-4 expression and drove the immune response towards a Th2 phenotype, whereas treatment with anti-CD86 antibodies resulted in the production of effector cells of a Th1 phenotype (38). In contrast, another study demonstrated that the presence of CD80 actually inhibited IL-4 production (39). These controversial results warrant further studies aimed at clarifying the role of CD80 and CD86 in the differentiation of Th1 and Th2 cells and in their potential efficacy in the treatment of disease and infection. Particularly, elucidating the molecular mechanisms governing the expression and function of CD80/CD86 in APCs would represent an important step toward the design of new therapeutic strategies for treatment of cancer, immune diseases and infection.

1.7 The role of CD80 and CD86 in disease

CD80 and CD86 have been identified as key regulators of immune activation, tolerance regulation and the skewing of T cell responses in disease models, such as graft-vs-host diseases, cancer, and autoimmune diseases (40).

1.7.1 The involvement of costimulatory molecules in cancer and in the development of therapeutic interventions

There is evidence to suggest that they also have an important role in tumor immunity. The majority of tumor APCs are of low immunogenicity and often lack expression of costimulatory molecules. For example, human gliomas have been shown to suppress immune responses and proliferation of lymphocytes and these effects were attributed to a reduced expression of CD80 and CD86. Topical expression of CD80 and CD86 in murine neuroblastoma cells was able to control tumor growth up to doses greater than one million cells (42). Lack of CD80/CD86 expression in glioma-infiltrating microglia/macrophages (GIMs) also led to the decreased secretion of the cytokines required to initiate an effective innate immune response, such as TNF-α, IL-1, IL-6, and this was in spite of the normal expression levels of TLRs (43). As a result, these GIMs were unable to effectively costimulate and activate T cells (44). Increased T cell activity and cytokine production were restored upon transfection of murine sarcoma cells with CD80/CD86 encoding DNA constructs (45). Furthermore, in experiments where wild type mice were injected with the sarcoma cells transfected with the CD80 expression constructs, half of the mice remained tumor free and exhibited an increase in T cell population and Th1 and Th2 cytokine production, including IFN-γ, which plays an important role in eliciting antigen-specific anti-tumor effects (45). The protective effects of CD86 through the amplification of both local and systemic anti-tumor immunity were also demonstrated in a study where the CD86-transfected mastocytoma P815 tumor cells were injected into mice. More importantly, these mice were also protected against subsequent challenges with the lethal wild-type P815 tumor and this protection was attributed primarily to the enhanced generation of CD8+ T cells and tumor specific cytolytic activity (46). Collectively, these studies demonstrate that induction of CD80/CD86 expression enhances anti-tumor immunity (47). In spite of a growing body of evidence demonstrating a critical role of CD80/CD86 in cancer prevention,

further research is needed to delineate the regulation of CD80/CD86 expression needed to use these molecules as anti-cancer therapeutics.

Evidence emerging from research using human and animal models clearly demonstrates that T cells form an integral part of cancer immune surveillance, in particular, the recognition of tumor associated antigens by CTLs (48). More recently, T cell-associated CTLA-4 molecule, which promotes a negative signal, has been identified as a potential target for the development of anti-cancer therapeutics. CD80/CD86 molecules interact with both CD28 and CTLA-4 expressed on T cells and these interactions modulate the adaptive immune response. For example, the binding of CD80/CD86 to CD28 results in the activation of T cells and the initiation of an adaptive immune response, while the interaction with CTLA-4, which has a higher binding affinity compared to CD28, inhibits excessive T cell activation to prevent host tissue damage and immune over-activation (21). Upon binding to CD80 or CD86, CTLA-4 negatively regulates T cell activation, and as such, plays an important role in T cell homeostasis, through the inhibition of IL-2 production and cell cycle progression (49). Attempts to inhibit CTLA-4 function produced promising results demonstrating enhanced anti-tumor response. For example, the immunopotentiating effects of CTLA-4 blockade by anti-CTLA-4 antibodies in conjunction with CD28-CD80/CD86 stimulation was achieved in a mouse colon cancer model (50). This treatment resulted in a reduced tumor size, particularly in combination with irradiated tumors, and in an increased level of IFN-γ-secreting T cells and tumor-specific CTL activity (50). Similar results were found *in vivo* after administration of anti-CTLA-4 antibodies which led to the rejection of pre-existing tumors (51). The long term effects of this treatment have also been studied in mice treated with anti-CTLA-4 antibodies, in combination with irradiated cancer cells engineered to produce granulocyte and macrophage colony stimulating factor (GM-CSF) (52). These mice rejected previously established tumors and were found to be refractory to secondary tumor challenge, suggesting the development of immunological memory

(52). Clinically, anti-CTLA-4 antibodies, such as ipilimumab and temozolomide have shown promising results and have been approved for treatment for patients with metastatic melanoma, particularly since they show low toxicity and can penetrate the blood-brain barrier, making them ideal for treatment against brain cancers (53, 54).

1.8 CD80/CD86 and transplant rejection

The importance of these two costimulatory molecules was recognized in transplantation immunology experiments. It was first thought that only antigen presentation was required to activate lymphocytes, but upon the discovery that tissue cells of non-hemapoietic origin were not readily rejected during transplantation, it was believed that a second signal provided by APCs was required for lymphocyte activation and graft rejection (55).

Transplant rejection is an immune response by the recipient's immune system that results in T cell mediated rejection and destruction of the transplanted tissue. Graft-versus-host (GVH) disease remains the principal risk involved in organ and bone marrow transplantation (56), and remains the leading cause of morbidity and mortality after lung and heart transplantations (57). Rejection of transplanted allografts is dependent on T cell activation, which requires its engagement with the APC. CD80 and CD86 play pivotal roles in this T-cell dependent process, and attempts have been made to modulate the CD80/CD86-CD28 signaling pathway to stop this rejection (56). Because CTLA-4 has been identified as providing an inhibitory signal to T cells, it has been suggested as a potential therapeutic target for graft-vs-host disease. The development of a membrane bound anti-CTLA-4 antibody expressed on B cells *in vivo* demonstrated a profound inhibited T cell proliferation and cytokine production *in vitro* and *in vivo* (58). The antibody prevented the rejection of allogeneic tumor cells by antigen-specific CD8+ T cells *in vivo* (56) and protected NOD (non-obese diabetic) mice from developing spontaneous autoimmune diabetes (59). In mice and rats, CTLA-4Ig treatment

prolonged the acceptance and survival of cardiac allografts (60, 61). Mice treated with CTLA-4Ig in a xenogeneic islet transplant model prevented human pancreatic islet cell rejection (62). Taken together, CTLA-4 and the CD80/CD86-CD28 pathway play a critical role in controlling T-cell responses to foreign antigen and present novel targets for future research.

1.10 CD80 and CD86 as signaling molecules

The role of macrophages and the importance of the costimulatory markers CD80 and CD86 in T cell activation have been well characterized. Particularly, the studies investigating the role of CD80 and CD86 in autoimmune diseases and cancer clearly demonstrated lack of T cell activation and depletion of T cell numbers in the absence of CD80 or CD86 surface expression (63). While the biological significance of CD28 ligation has been well described, more recently, CD28 has emerged as a potential agonist ligand of B7 receptor molecules. Until now however, little is known about the role of CD80 and CD86 as signaling molecules. Both molecules are comprised of two highly glycosylated extracellular Ig-like domains linked to a transmembrane domain and a cytoplasmic tail (64). The importance of the cytoplasmic tail has been highlighted in studies where interaction of tailless CD80 molecules with CD28 was examined. These tailless molecules could not promote antibody-induced cytoskeleton-dependent redistribution and capping in epithelial and lymphoid cells (65). These results suggested that the cytoplasmic region of CD80 is involved in the localization and redistribution of CD80 molecules on cell surfaces and is therefore important for effective costimulation. Further studies by the same group identified a small, 30-kilodalton (kDa) phosphoprotein that associated with the cytoplasmic tail of CD80 in activated cells (66), however identification of this protein and its function have not been investigated further.

More recently, the role of CD80 and CD86 as signaling molecules has been investigated in DCs where it has been demonstrated that CD80/CD86 transduce signals to the DC. In particular,

following engagement of CD80 or CD86 with CD28, DCs have been shown to produce IL-6, a proinflammatory cytokine that is necessary for full T cell activation (67). To elucidate signal transduction pathways activated by engagement of CD80 or CD86, Koorella *et al* demonstrated that treatment of DCs with anti-CD80 and anti-CD86 antibodies, or with a recombinant fusion protein CD28-Ig led to induction of IL-6 expression through activation of the PI3K/AKT pathway and transcription factor, NF-κB (67). Moreover, employing murine DCs, it was also shown that anti-CD28-Ig induced both IL-6 and IFN-γ production, and blockade of p38 MAPK activity resulted in complete suppression of IL-6 production (68). This bidirectional signaling presents new potential avenues for the development of therapeutic interventions targeting these costimulatory molecules. As a result, deciphering the CD80/CD86 signaling pathway and the molecules that govern their expression is essential. Since toll like receptors (TLRs) serve as a model for cell activation pathways, it was of interest to study them in the context of macrophage activation.

1.11 Toll like receptor signalling

Toll like receptors (TLRs) are cognate pattern recognition receptors (PRRs) that act as guards against invading organism that bear pathogen-associated molecular patterns (PAMPs), such as Gram-negative bacterial LPS, and damage-associated molecular pattern molecules (DAMPs), such as heat shock proteins (69). By recognizing specific molecular patterns that are found in a broad range of microbial pathogens, an inflammatory response can be triggered for eradication of the pathogen (69). These type I transmembrane glycoproteins function as homo- or heterodimers, and are structurally characterized by extracellular leucine-rich repeats and toll/IL-1 receptor (TIR) signaling domains (70). The TLRs are broadly divided into two classes depending on cellular component where they engage their ligands. TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6 are expressed on the cell surface, while TLR-3, TLR-7, TLR-8, and TLR-9 bind to their cognate ligand in the endosome (71). TLR

signaling leads to multiple outcomes depending on the cell type responding to the stimuli; for example, cell differentiation and proliferation, induction of inflammatory/regulatory genes, antibody class-switching production (71). All TLRs except TLR-3 utilize MyD88 for signal transduction, whereas TLR-3 signals through TRIF (71). Myeloid differentiation factor 88 (MyD88), TIRAP (Toll-interleukin-1 receptor domain containing adaptor protein), TRIF (toll receptor-associated activator of interferon), and TRAM (toll receptor associated molecule) are examples of TIR-domain containing adaptors that are activated upon ligation of TLRs (72). Upon binding of LPS to TLR-4, the TRIF-dependent, also known as the MyD88 independent, and MyD88 dependent pathways are activated. MyD88 was first characterized as an essential component for activating the innate immune system through the activation of nuclear-factor KB (NF-κB) and mitogen-activated protein kinases (MAPKs) (72). For example, after infection, LPS or bacterial endotoxin will interact with LPS-binding protein (LBP), which facilitates its subsequent interaction with CD14 found on the surface of monocytes/macrophages. CD14 recruits an accessory protein MD2, which in turn binds to TLR-4 expressed on APCs (73). Activation of the TLR-4 signaling pathway is initiated following recruitment of TIRAP to the TIR domain of TLR and MyD88; this results in the translocation of TRIF to the complex, which also requires the recruitment of TRAM (Figure 2) (69). Such formed adaptor protein complex activated a cascade of signalling molecules, eventually leading to the activation of the transcription factors NF-κB and interferon regulatory factors (IRFs), which in turn induce the expression of various inflammatory cytokines, type 1 interferons, and chemokines. In addition to the TRIF mediated protein complexes, TLR-associated adaptor protein MyD88 also interacts with signaling proteins forming a MyD88-dependent signaling complex. In response to stimuli, MyD88 recruits members of the interleukin-1 receptor associated kinase (IRAK) family, which are sequentially phosphorylated and dissociated from MyD88, resulting in the activation of tumor necrosis factor receptor-associated factor 6 (TRAF-6). This in turn activates transforming growth

Figure 2: **Schematic representation of Toll Like Receptor (TLR) Family**. Numerous pathways are induced upon engagement of TLRs. In particular, binding of LPS to its cognate receptor TLR-4 induces NF-κB is the central regulator of immune responses.

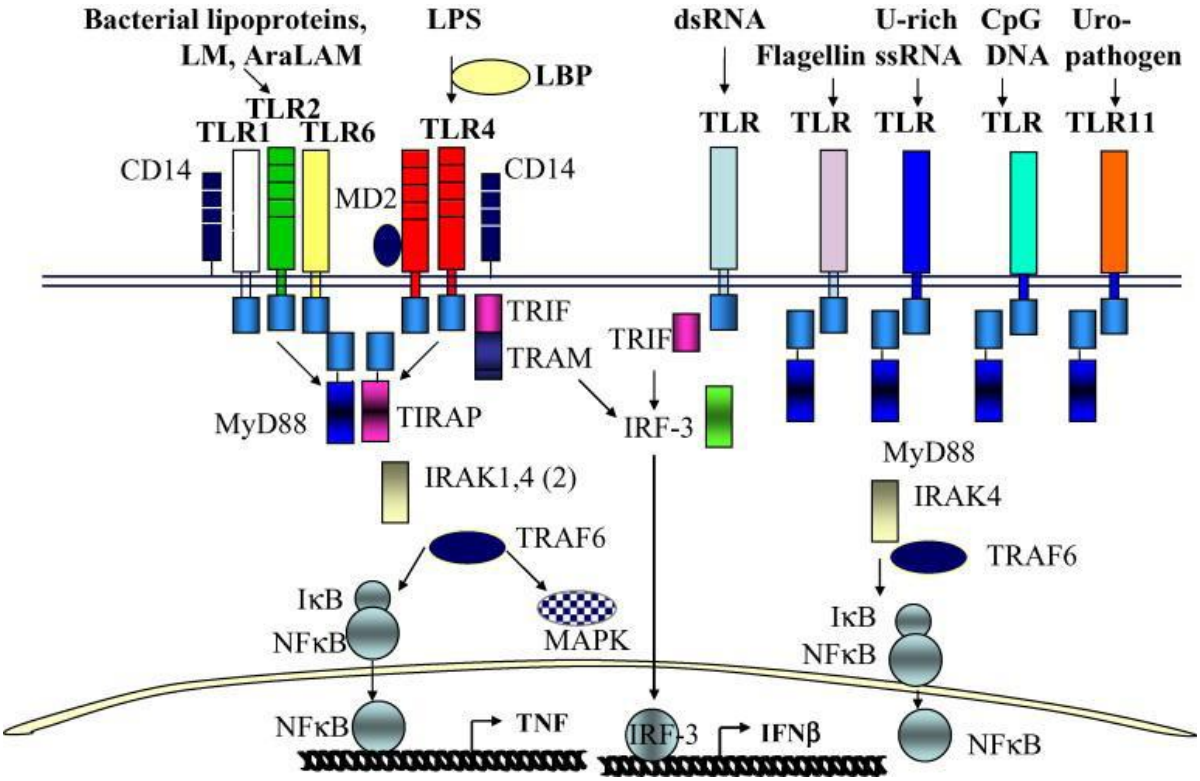


Figure 2 (Quesniaux *et al*, 2004)

factor-β-activated protein kinase 1 (TAK1), which is a member of the MAP3K family, in a ubiquitin-dependent fashion. TAK1 then activates the IKK complex which leads to the activation of NF-KB (Figure 2), while simultaneously activating other members of the MAP kinase family (72). NF-κB is the central regulator of immune responses that are involved in the proliferation and survival of cells and is responsible for inducing the expression of various cytokines and chemokines, including IL-12 (70). NF-κB is a heterodimer composed of the p65 and p50 subunits, residing in the cytoplasm in an inactive form by interacting with inhibitor NF-κB proteins (IκB). Through the stimulation by the TLR-4 ligand, LPS, IκB proteins are phosphorylated by an IKK complex, releasing NF-κB into the nucleus. Activating protein-1 (AP-1) family proteins control various cellular processes including differentiation, proliferation and apoptosis and are activated by the TLR-4 pathway. Among the AP-1 family proteins, c-Jun plays a central role in the inflammatory response, and the activation of AP-1 is mediated by MAP kinases, such as c-Jun N-terminal kinase (JNK), p38, and extracellular signal regulated kinase (ERK) (72). Dysregulation of TLR-4 signaling has been implicated in the development of autoimmune diseases, such as multiple sclerosis.

1.12 TLR-4 and the activation of the MAPKs and PI3K pathway

In addition to the canonical MyD88-dependent and a MyD88-independent TRIF-mediated pathway, engagement of TLR-4 also results in the activation of the MAPK and the phosphoinositide-3-kinase pathways (71). The MAPKs are key players in cellular responses, such as cell growth and survival, proliferation, differentiation, neuronal response, and apoptosis (74). This family of serine/threonine protein kinases is highly conserved among eukaryotes and the dysregulation of MAPK signaling has been implicated in cancer, and immune and neuronal diseases (75, 76). In mammals, the triple kinase module consisting of a MAPK kinase kinase (MKKK) phosphorylates and activates a MAPK kinase (MKK) that activate the terminal MAPK by dual phosphorylation of the

threonine and tyrosine residues (77). Six distinct groups of MAPKs have been characterized in mammals: extracellular signal-regulated kinases (ERK1/2), jun NH_2 terminal kinases (JNK1/2/3), p38 (p38 α, β, δ), ERK7/8, ERK3/4 and ERK5. To date, ERK1/2, JNKs, and p38 kinases are the best characterized (77). Once they are activated, MAPKs can phosphorylate the target substrates, such as transcription factors (77). In particular, activation of ERK1/2 and p38 has been implicated in the regulation of cell proliferation and differentiation, whereas phosphorylation of JNK has been specifically implicated in the production of cytokines, such as TNF-α (78-80). Crosstalk between signaling cascades also contributes to the continuing complexity of the MAPK signal transduction pathway. For example, MAPKK activates both JNK and p38α (77).

The PI3K pathway is activated by many different signaling molecules, and it is crucial for cell growth and cell survival. PI3Ks are a family of lipid kinases consisting of three classes (class I, class II, and class III) (71). Class IA PI3Ks, composed of p110α, p110β, and p110δ subunits, are critical in immune cell function (81). The subunits are regulated by p85α or p85β regulatory subunits; the p85 subunit engages to phosphorylated tyrosines to release the inhibitory pressure p85 places upon the catalytic p110 PI3K subunit (82). The PI3K complex is recruited with phosphorylated tyrosines, bringing PI3K to the lipid membrane, in close proximity with its lipid substrate (71). The activation of the PI3K pathway culminates in the phosphorylation and activation of AKT, a serine/threonine-specific protein kinase that has an important role in various cellular processes, such as apoptosis, cell proliferation and migration, and transcription (83). While it is known that LPS induces all three types of MAPKs and the PI3K pathway, little is known about their role in the regulation of LPS-induced CD80 and CD86 expression in macrophages.

1.13 Inhibitor of Apoptosis Proteins and SMAC mimetics

One of the main anti-apoptotic families of caspase inactivating proteins are the inhibitor of apoptosis proteins (IAPs) (84). The activity of IAPs is antagonized by the release of pro-apoptotic proteins known as a second mitochondrial activator of caspases (SMAC) from the mitochondria (84). Since the inappropriate regulation of apoptosis is one of the hallmarks of many diseases, including cancer, understanding the role of IAPs is an attractive strategy for developing new classes of drug therapy.

Described over 20 years ago, the *iap* baculovirus gene was originally found to inhibit apoptosis in virally infected *Spodoptera frugiperda* insect cells (85). They are characterized by the presence of a variable number of highly conserved Baculoviral IAP repeat (BIR) motifs, a sequence of ~70 amino acids with three anti-parallel beta sheets and four alpha helices, allowing BIRs to mediate protein-protein interactions (86). They can also contain additional functional regions, such as a RING domain or caspase-associated recruitment domain (CARD), important for protein interactions and ubiquitin ligase activity (87). Eight mammalian IAPs have been identified: cellular IAP1 (cIAP1), cIAP2, X-chromosome linked IAP (XIAP), neuronal apoptosis inhibitory protein (NAIP), livin, survivin, IAP-like protein 2 (ILP2) and baculovirus inhibitor of apoptosis repeat containing ubiquitin-conjugating enzyme (BRUCE) (87). In mammals, cIAP1, cIAP2 and XIAP and their role in apoptosis have been studied extensively. The BIR2 and BIR3 in these molecules have been identified as being the important motifs in the binding of caspases and apoptosis-regulatory molecules (87). For example, the common anchoring motif on BIR3 found on cIAP1 and cIAP2 selectively targets caspase 9, preventing its activation and the activation of caspase 3 and 7, whereas BIR2 prevents the activation of caspase 3 and 7 specifically (88, 89). Recent studies have identified BIR1 as being critical for the interaction of cIAPs and with tumor necrosis factor (TNF) associated factors (TRAFs), in particular, TRAF2 (90). Through the association of their BIR and TRAF-N domains, the cIAP1- and

cIAP2-TRAF2 interaction regulates receptor-mediated apoptosis through TNF receptor 1- and 2-associated complexes (90). The degree of cell death is tightly controlled by IAPs and their importance can be highlighted in neurodegenerative disorders and cancer. For example, patients with spinal muscular atrophy were found to have partly deleted NAIP (86) and pancreatic cancer has been associated with an overexpression of cIAP2, survivin, livin and XIAP (91).

In response to apoptotic stimuli, SMAC, also known as Direct IAP Binding protein with Low pI (DIABLO), undergoes proteolytic processing and is subsequently released from the mitochondria into the cytosol to inactivate several IAPs, including cIAP1, cIAP2, and XIAP (92). The first four amino acids (Ala-Val-Pro-Ile) on the N terminal of this proapoptotic molecule (the AVPI tetrapeptide binding motif) binds to a surface groove on the BIR3 motif of IAPs, directly competing with the ATPF tetrapeptide of caspase 9, thereby interfering with the interaction between caspase 9 and the IAP (93, 94). Since they block apoptosis at the downstream effector phase, IAPs represent a particularly interesting target for drug therapy. SMAC mimetics were designed to overcome the apoptosis resistant tumor cells by preventing the interaction between caspases and IAPs (95). This small molecule induces cell death without the mitochondrial release of apoptotic factors by mimicking the AVPI tetrapeptide and binding to the BIR3 domain (94), leading to the rapid ubiquitination and proteasomal degradation of cIAP1 and cIAP2, but not XIAP, although the method by which auto-ubiquitination is induced is not entirely clear (90). Increasing the abundance of pro-apoptotic proteins such as SMAC is a more attractive therapeutic strategy for tumors because it could limit the toxicities associated with other therapies, such as recombinant cytokine therapy (96).

1.14 IAPs and NF-κB signaling

While the use and testing of SMAC mimetics in cancer therapeutics has demonstrated their ability to reveal the apoptosis-opposing properties of IAPs, accumulating evidence has suggested

that cIAP1 and cIAP2 may also be involved in signal transduction pathways, in particular, TNF-α-mediated NF-κB activation (87). Upon binding to its receptor, TNF-R1, TNF-α exerts its effect by rapidly recruiting the TNFR-associated death domain (TRADD) protein and the receptor-interacting protein 1 (RIP1), which consequently recruits TRAF2 to form a large complex (97). It is believed that during this recruitment, cIAP1 and cIAP2 interact with TRAF2 in a TNF-dependent manner and can bind to TNF receptors (98). TRAF2, cIAP1 and cIAP2 facilitate the ubiquitination of RIP1, a crucial event for the propagation of the signal (99). RIP1 serves as a docking site for inhibitor of κB (IκB) kinase (IKK) α/β/γ heterocomplex, which, when phosphorylated, will phosphorylate IκBα, an inhibitory protein. IκBα signals for its ubiquitination and proteasomal degradation, allowing NF-κB to translocating into the nucleus. Here, it regulates target genes, in particular, prosurvival genes such as FLICE inhibitory protein and cIAP2, inhibiting the caspase-8-mediated apoptotic pathway that is concomitantly engaged by TNF-α (97). In the non-canonical pathway, receptors such as CD40 activate NF-κB inducing kinase (NIK) which also results in the translocation of NF-κB to the nucleus (87). Blocking NF-κB activation resulted in decreased TNF production and protected the cells from SMAC mimetic-induced apoptosis (100). Therefore, TNF-α-mediated cell survival is dependent on the proper NF-κB induction.

1.15 IAPs and Pattern Recognition Receptor (PRR) signaling: cIAPs and TLR-4 signaling

IAP proteins are critical regulators of the NF-κB signaling and the expression of genes that control innate and adaptive immunity, inflammation, and cell survival and migration. The canonical pathway for the activation of NF-κB is essential for the innate immune response (101), while the non-canonical pathway is vital for the adaptive immune response and the development and maintenance of lymphoid organs (102). After recognition of PAMPs by their respective PRRs, the inflammatory response is activated and mediated by the NF-κB pathway and the MAP kinases

(MAPK), which increase the transcription of genes that encode cytokines, chemokines, adhesion molecules, and antimicrobial peptides to recruit inflammatory and phagocytic cells to the infection site (103). After stimulation with LPS, the MyD88 dependent pathway is activated resulting in the recruitment of TRAF3 and TRAF6 to form the MyD88-assembled signaling complex (104). In this complex, TRAF3 undergoes TRAF6-, cIAP1-, and cIAP2-dependent degradative ubiquitination promoting cytosolic translocation of the entire signaling complex, activating MAPKs, and inducing in inflammatory genes (105). Depletion of cIAP1 and cIAP2 with SMAC mimetics blocked p38 and JNK MAPK activation via TLR-4 (105). Furthermore, depletion resulted in a reduction in LPS-induced cytokine production, with no effect on interferon responses, indicating an absence of a role for cIAPs in the MyD88-independent/TRIF-mediated pathway (105).

IAPs have also been identified as regulators of the cytoplasmic signaling pathway initiated by the nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 receptors, as well as the retinoic acid-inducible gene (RIG)-1 receptor. NOD1 and NOD2 are PRRs that require cIAP1, cIAP2 (106) and XIAP (107) to elicit a proper immune signal. Upon recognition of bacterial peptidoglycan, NOD1 and NOD2 self-oligomerize and recruit RIP2, cIAP1, cIAP2, and XIAP (106). This complex induces the ubiquitination of RIP2 by cIAP1 and cIAP2, resulting in the downstream activation of MAPK p38 and JNK (108). The importance of IAPs was highlighted in cIAP1-, cIAP2-, and XIAP-deficient mice that were treated with the NOD1 and NOD2 ligands. They failed to activate NF-κB and MAPK resulting in a reduction in immune signaling and cytokine expression, and this was attributed to their ubiquitination of RIP2, similar to their role in TLR signaling (106, 107). Similarly, IAPs have also been associated in the type I interferon response mediated through RIG-1, the cytoplasmic PRR that recognizes viral double-stranded RNA through their helicase domain (109). Upon recognition, RIG-1 is recruited to the mitochondrial antiviral signaling (MAVS) protein, which interacts with

TRAF3 to induce a type I interferon response (110). cIAPs ubiquitinate TRAF3 in order to enhance interferon regulatory factor 3 (IRF-3) and NF-κB to induce an antiviral immune response (111).

In summary, there exists compelling evidence to suggest that IAPs do not simply have an anti-apoptotic role. These proteins have important roles in NF-κB activation and MAPK signaling, and the use of SMAC mimetics has been identified as significant antagonists that can be potentially used in cancer therapies.

1.16 The role of tyrosine phosphorylation the LPS/TLR-4 pathway

Phosphorylation is a fundamental mechanism that provides a quick and reversible change known to alter the function of proteins; it is important in the regulation of normal cellular processes, such as cell growth and differentiation (112). Aberrant phosphorylation of tyrosine residues can result in excessive cell proliferation and disease (113). Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) control the tyrosine phosphorylation of signalling molecules (113). While PTKs catalyze the phosphorylation of tyrosine residues, PTPs dephosphorylate the phosphotyrosine residues, maintaining equilibrium in biological systems (113).

More recently, the role of tyrosine phosphorylation in the LPS/TLR-4 pathway has been investigated. Binding of LPS to TLR-4 initiated the phosphorylation of signaling molecules and the activation of protein tyrosine kinases (PTKs) (114). The observation that a broad spectrum of tyrosine kinase inhibitors prevented LPS-induced cytokine production and protected against septic shock attracted considerable attention in research (115). This protection was correlated with the ability of the inhibitors to block LPS-induced tyrosine phosphorylation of ERK2 MAPK proteins and the subsequent release of TNF-α in murine macrophages (115). The induction of other cytokines, such as IL-1β and IL-6, has also been shown to require the activation of PTKs (116). The SRC family of tyrosine kinases make up the largest group of non-receptor tyrosine kinases. The 9 members of this

group are: Src, Hck, Fyn, Lyn, Fgr, Lck, Blk, Yes, and Yrk, each containing an N-terminal unique domain SH3, SH2 and tyrosine kinase domain (114). They are differentially expressed in various cell types, with members of the innate immune system being Src, Fyn, Fgr, Lyn and Hck (117). Activation of TLR-3, TLR-4, and TLR-9 in macrophages was shown to upregulate PTP expression, and triggered an inhibited TNF-α, IL-6 and IFN-β production, impairing NF-κB activation (118).

PTPs have been described as both positive and negative regulators of signaling processes. For example, the SRC homology region 2 (SH2) domain-containing tyrosine phosphatase-1 (SHP-1) negatively regulates the cellular responses in hematopoietic cells initiated by colony stimulating factor (CSF-1), IL-3, c-Kit, erythropoietin, and IFN-α/β, but positively regulates epidermal growth factor and IFN-γ in astrocytes (116). SHP-1 has been strongly associated with the LPS/TLR-4 pathway; it has been shown that SHP-1 regulates the activation of ERK1/2 MAPK and NF-κB in bone marrow derived macrophages (116). The importance of SHP-1 has been highlighted in SHP-1-deficient mice; these mice manifest autoimmune disorders and a phenotype that correlates with autoantibody production, a consequence of the altered production of inflammatory cytokines, such as IL-6 (119). A proximal function of SHP-1 in conjunction with PTKs has been suggested; similar to underexpression of SHP-1, treatment with the PTK-specific inhibitor, Herbimycin A, also inhibited LPS-induced IL-6 production (116).

1.17 The regulation of CD80 and CD86 expression

Upon binding of LPS to its cognate receptor CD14/TLR-4, a cascade of signaling events is induced resulting in gene activation and expression of proteins. In particular, signaling molecules and transcription factors that regulate the expression of CD80 and CD86 have been elucidated (121). CD80 expression can be induced by a number of stimuli other than LPS, including IL-4, anti-B cell receptor (BCR), antibody or anti-CD40 antibody, and stimulation of monocytic cells with LPS and

IFN-γ (20, 22, 120). CD80 and CD86 are crucial for T cell differentiation and activation of an immune response. It is therefore likely that immunoregulatory cytokines and mitogens, such as LPS, would modulate CD80/CD86-mediated signals to enhance the activation of T cells (22). In LPS-stimulated human monocytes, CD80 expression has been shown to be regulated by the (interferon regulatory factor) IRF-7 transcription factor through the activation of JNK (121). Further studies identified a distinct CD80-responsive element corresponding to the IRF-7 binding site, located between 84 and 72 base pair region upstream of the transcription start site of the CD80 gene (121). In LPS-stimulated human monocytes, p38 and JNK MAPK have been shown to regulate CD86 expression, through an IL-10-dependent and IL-10-independent pathways (8). LPS stimulation of normal human monocytes results in the downregulation of CD86 expression, mediated via endogenously produced IL-10 involving p38 MAPK (8). However, THP-1 cells are noncompliant to the inhibitory effects of IL-10, and CD86 expression was upregulated in an IL-10-independent manner following LPS stimulation in these cells (8). The regulation of CD80 and CD86 in DCs has also been investigated. One group demonstrated the role of JNK MAPK in the LPS-induced CD80 and CD86 expression in human monocyte-derived DCs (122). Another group identified a critical role for p38 MAPK in the LPS- and TNF-α-induced CD80/CD86 expression human monocyte-derived DCs (123).

There are conflicting studies on the functional differences between CD80 and CD86. While some reports have demonstrated that they provide similar costimulatory signals for T cell activation and cytokine production, others have shown contrasting effects (64). Two DNA vaccination studies have demonstrated that CD86, but not CD80, supported an HIV peptide-specific cytotoxic T lymphocyte response (124, 125). More recently, CD80 has been identified as a more potent ligand for CTLA-4 based on its higher affinity and enhanced avidity, whereas CD86 is a more effective CD28 ligand (126). On the other hand, mice that were CD80 or CD86 deficient demonstrated the overlapping nature of their functions (127). Although the reason for these conflicting studies is not

clear, it could be due to differences in their experimental approach, such as the ligands used, or due to the differences in their expression kinetics. Further studies are required to elucidate the critical, and potentially different, role for these two molecules.

Modulation of CD80 and CD86 expression on APCs may alter the development of immune responses. While the role of MAPK in their regulation has been studied in some cell types, there has been limited work looking into the regulation of CD80 and CD86 in macrophages. To better understand their role as a target for therapeutics, the signaling molecules involved in the regulation of CD80 and CD86 in macrophages must be studied extensively.

1.18 Rationale

The activation of T cells is a crucial requirement for the initiation of effective immune responses, and is dependent on the engagement of T cells with CD80/CD86 on the APC. The role of CD80/CD86 in the activation of T cells has been well characterized, but more recently these B7 molecules have been investigated as signaling receptors in DCs. It has been shown that stimulation of DCs with anti-CD80/CD86 antibodies induced IL-6 expression through the activation of the PI3K pathway (67). Furthermore, Jain *et al* demonstrated that CD86 stimulation enhanced the effect of TLR-2 stimulation on resting B cells (41). However, the role of CD80 and CD86 in macrophage activation remains unknown. Macrophages are a potent source of cytokines particularly anti-inflammatory IL-10 andthe IL-12 family of cytokines including IL-12, IL-23 and IL-27. Hence stimulation of macrophages via CD80 and/or CD86 activation alone or in concert with LPS may have a profound effect on the production of IL-10, IL-12, IL-23 and IL-27 and consequent development of immune responses.

Dysregulation of CD80 and CD86 expression on macrophages has been observed following HIV infection, in autoimmune disorders, and in cancer. Therefore, dysregulated expression of CD80 and/or CD86 may also influence the production of IL-10, IL-12, IL-23 and IL-27 by macrophages in response to stimulation with TLR-4 ligand, LPS and consequent development of immune response. Determining signaling molecules and the signaling pathways that govern their regulation may provide novel strategies to control diseases such as autoimmune disorders and cancer. Therefore, the focus of this project was twofold: 1) to investigate the role of CD80 and CD86 as signaling receptors capable of transmitting extracellular signals either alone or in synergy with LPS leading to the production of anti-inflammatory IL-10 and the IL-12 family of cytokines including IL-12, IL-23 and IL-27, and 2) to determine the TLR-4 activated pathways that regulate CD80/CD86 expression in human macrophages.

Since cIAPs play a key role in TLR-4-mediated signaling (128), it is reasonable to hypothesize that cIAPs may play a role in CD80 and/or CD86 mediated activation either alone or in concert with LPS activation of MDMs. cIAPs have also been shown to interact with TRAFs in the TLR-4 mediated signaling pathways (129). Therefore, it is possible that cIAPs either alone or in association with TRAFs and other interacting signaling molecules such as RIP1 or SHP-1 may regulate the expression of CD80 and CD86 in MDMs stimulated by the TLR-4 pathway.

1.19 Hypothesis

Activation of CD80 and CD86 receptors either alone or in synergy with TLR-4 enhances expression of cytokines such as IL-10 and IL-12 family of cytokines including IL-12, IL-23 and IL-27. In addition, IAPs which play a key role in TLR-4-mediated signaling, either alone or in association with TRAFs and other interacting signaling molecules such as RIP-1 or SHP-1 may regulate the expression of CD80 and CD86 in MDMs stimulated by the TLR-4 pathway.

1.20 Objectives

1. To determine whether activation of CD80 and CD86 receptors alone, or in synergy with TLR-4 altered cytokine expression of IL-10 and IL-12 family of cytokines.
2. To determine the role of TLR-4 activated signaling molecules, such as cIAP, SHP-1, RIP1 and TRAF2, and signaling pathways, such as MAPKs and PI3K, in LPS-induced expression of CD80 and CD86 in human MDMs.
3. To determine the role of TLR4-activated signalling molecules including MAPKs and PI3K/AKT in the expression of CD80 and CD86 in human MDMs, and their association with the upstream signalling molecules.

2.1 Materials and Methods

2.2 Reagents

Chemical inhibitors SB203580 (pp38 inhibitor), SP600125 (pJNK inhibitor), PD98059 (pERK inhibitor), LY294002 (pAKT inhibitor), sodium stibogluconate (SHP-1 inhibitor), and SU6656 (pSrc inhibitor) were purchased from Calbiochem (La Jolla, California). Lipopolysaccharide (LPS) was obtained from *Escherichia* coli 0111:B4 (Sigma-Aldrich, St. Louis, Missouri). Second mitochondria-derived activator of caspases (SMAC) mimetics, LN730, was a generous gift of Dr. R. Korneluk at the Children's Hospital of Eastern Ontario, Apoptosis Research Centre.

2.3 Cell culture

2.3.1 Human Monocyte-derived Macrophages (MDMs)

Human blood was obtained from healthy volunteers with written consent, according to a protocol approved by the Ethics Review Committee of The Ottawa General Hospital. Peripheral blood mononuclear cells (PBMCs) were extracted using Ficoll-Paque (GE Healthcare, Buckinghamshire, UK) to create a density gradient following centrifugation of whole blood at 1600 RPM for 45 mins at 4° C in Allegra X-12R centrifuge (Beckman Coulter, Brea, California). The cell layer containing mainly PBMCs was collected and counted. To generate MDMs, monocytes were isolated via the adherence method. PBMCs were resuspended at 4 x 10^6 cells/ml in Iscove's Modified DMEM 1X media (Sigma-Aldrich). The cells were seeded at 1 ml/well in 12 well polystyrene plates (Thermoscientific, Rochester, New York) and monocytes were allowed to adhere to the plate for 3 hrs in a humidified environment at 37° C, 6% CO_2/air mixture. Non-adherent cells were washed off and adherent cells were cultured for six days with complete media containing and 100 units/ml penicillin and 100 µg/ml gentamicin (Sigma-Aldrich) and 10% v/v fetal bovine serum (GE Healthcare) and 10 ng/ml recombinant human macrophage colony stimulating factor (M-CSF)

(R&D Systems, Minneapolis, Minnesota). SnCells were washed and media with M-CSF was replenished every two days for six days. Macrophages were characterized by Flow Cytometry, using phycoerythrin (PE)-conjugated anti-CD11a, -CD11b, -CD11c, -CD80, -CD83, -CD16 and HLA-DR antibodies (BD Biosciences, Franklin Lakes, New Jersey). Windows Multiple Document Interface (WinMDI) version 2.8 software (J. Trotter, Scripps Research Institute, San Diego, California) was used to create histograms.

2.4 MAP kinase and PI3K inhibition

To determine the effect of p38, JNK, ERK1/2, PI3K, SHP-1, and SRC on the LPS-induced expression of CD80 and CD86, MDMs were treated for 2 hrs with their pharmacological inhibitors SB203580, SP600125, PD98059, LY294002, sodium stibogluconate, and SU6656 (Calbiochem), respectively, for 2 hrs, followed by LPS stimulation (1 μg/mL) for 15 mins, 4 hrs, or 24 hrs, for Western Blot, RT-PCR, and ELISA/Flow Cytometry, respectively. MDMs were treated with 50nM of SMAC mimetics for 24 hrs, before LPS stimulation. After various treatments, the viability of cells was >95% viable, as tested via Flow Cytometry.

2.5 Transfection of MDMs with small interfering RNA (siRNA)

All control and specific siRNAs were purchased from Santa Cruz Biotechnology, Santa Cruz, California. MDMs (1 x 10^6 cells/well) were seeded in 250 μL of serum-free media. 4 μM of the Transfection Reagent (Santa Cruz Biotechnology) and 4 μM of siRNA specific to p38, AKT, SHP-1, SRC, RIP1, TRAF2, cIAP1 and 2, and a nonspecific control pool (siRNA control) were incubated for 30 mins, before adding this mixture to the cells at a final ratio of 40:4 of transfection reagent to siRNA. After 24 hrs of incubation at 37° C, 200μL complete media was added to stop the transfection and

cells rested overnight at 37° C. The following day, cells were treated with 1 µg/ml LPS for another 15 mins or 24 hrs and then were collected for Western blotting and Flow Cytometry.

2.6 Western blot analysis

MDMs (1 x 10^6) lysates were prepared and total protein concentration of the cell lysates was determined using Bradford protein assay kit (Bio-Rad Laboratories, Hercules, California). Equal amounts of proteins (30 µg) were subjected to SDS-PAGE before being transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories) using a wet transfer blotting cell apparatus (Bio-Rad Laboratories). Membranes were then probed overnight at 4° C with 1:1000 concentration of antibodies against SHP-1 (BD Biosciences), cellular IAP1 (cIAP1), cIAP2, TRAF2, RIP1, pAKT, AKT, pp38, p38, pJNK, JNK, pERK, ERK (all from Cell Signaling, Danvers, Massachusetts). The membranes were then washed and blocked with 5% skim milk in TBS-T, followed by 1:1000 goat anti-rabbit or goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories). To ensure equal loading, the membranes were stripped of the primary antibodies and reprobed with antibodies specific for their unphosphorylated forms, or with a loading control such as anti-GAP-DH (Cell Signaling). Immunoblots were visualized using the Amersham enhanced chemiluminescence (ECL) Western Blotting detection system using the Chemigenius Bio-imaging system and the GeneSnap software (Syngene, Cambridge, England).

2.7 Flow Cytometry

CD80 and CD86 expression on MDMs was determined by flow cytometric analysis. For FACS staining, 0.5 x 10^6 MDMs in a polystyrene tube (Falcon, Lincoln Park, New Jersey) were washed with PBS and stained with 2.5 µL of PE-conjugated CD80 and CD86 antibodies (BD Biosciences) for 30 mins at 4° C. After staining, cells were washed with PBS and centrifuged at 2200 RPM for 5 mins to

remove excess antibodies. 10,000 events were collected using FACSCanto Flow Cytometer (BD Biosciences) and analyzed using the WinMDI version 2.9 software (J Trotter, Scripps Institute, San Diego, California).

2.8 RNA isolation and semi-quantitative RT-PCR analysis for CD80 and CD86

MDMs were stimulated with 1 μg/mL of LPS for 4 hrs before collecting and washing cell pellets. Total RNA was extracted from cells using RNeasy Plus Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Using a spectrophotometer Nanodrop 2000C (Nanodrop Products, Wilmington, Delaware), the concentration of purified RNA was measured and 50 ng/μL of purified RNA was added to the master mix. To prepare the RT-PCR mastermix, the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, California) was used: 7.5 μL 10X reverse transcriptase buffer, 3 μL dNTPs, 7.5 μL random primers, 3.75 μL reverse transcriptase and 53.25 μL ddH_2O. RT-PCR was carried out using GeneAmp PCR system 2700 amplifier (Applied Biosystems). Following this, real-time PCR reactions were prepared using 12.5 μL Taqman DNA polymerase, 1.25 μL primer pairs and 8.75 μL ddH_2O using 7500 Real Time PCR System. The transcript expression levels were shown relative to the control, β-actin by calculation of cycle threshold (Ct) values in amplification plots (Applied Biosystems). Primers were as follows:

CD80 sense (5'-AGT ACA AGA ACC GGA CCA TC-3'); CD80 antisense(5'-GGC GTA CAC TTT CCC TTC TC-3'); CD86 sense(5'-AGG ACA AGG GCT TGT ATC AA-3'); CD86 antisense(5'-ATT GCT CGT AAC ATC AGG GA-3'); β-actin sense(5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'); β-actin antisense(5'-CTA GAA GCA TTT GCG GTG GAG GAT GGA GGG-3').

2.9 Treatment with Anti-CD80 and Anti-CD86 antibodies

Using 500 ng/mL antibodies that neutralize CD80/CD86-induced IL-2 secretion in T cells and a monoclonal mouse IgG_1 isotype control (all from R&D systems), MDMs were treated for 24 hrs in combination with low levels of LPS (1-10 ng) for 24 hrs before IL-12p40, IL-23, IL-27 and IL-10 expression was measured by ELISA.

2.10 Cytokine measurement by ELISA (Enzyme-linked Immunosorbent assay)

MDM supernatants were collected in order to measure IL-12p40 (Invitrogen, Burlington, Canada), IL-23 (R&D systems), IL-27 (R&D systems), and IL-10 (BD Biosciences) cytokine production. 96 well plates (Corning Incorporated, Corning, New York) were coated overnight at 4 °C with ELISA coating buffer (4.24 g of Na_2CO_3 and 5.04 g $NaHCO_3$ in 1L ddH_2O, pH 8.2) and IL-12p40, IL-23, IL-27, or IL-10 capture antibody as per the manufacturer's instructions. The plates were washed three times with PBS containing 0.05% Tween-20 (Bio-Rad Laboratories), and blocked for 2 hrs with PBS containing 1% BSA or 10% FBS, according to the manufacturer's protocol. After blocking, the plates were washed again, and standards and test supernatants were added and incubated overnight. On the third day, the plates were washed and biotinylated secondary antibodies for anti-IL-12p40, IL-23, IL-27 and IL-10 were added, followed by the addition of streptavidin horse radish peroxidase. The color reaction was carried out using 3,3', 5,5'-tetramethylbenzibidine (TMB) substrate solution and 450 nm liquid stop solution for TMB microwell substrates (BioFX Laboratories, Owings Mills, Maryland). Cytokine concentrations were measured by absorbance using Bio-Rad iMark microplate reader and data were analyzed using Microplate Manager 6 software (Bio-Rad Laboratories).

2.11 Immunofluorescence

MDMs were cultured and differentiated in 12 well plates containing round glass coverslips (Electron Microscopy Sciences, Hatfield, Pennsylvania). Cells were fixed in 4 % paraformaldehyde (Sigma-Aldrich) for 30 mins at 37° C, washed three times with PBS, before incubating with 50 mM NH_4Cl (Sigma-Aldrich) in PBS for 10 mins to quench the cells. Cells were washed again and permeabilized with 0.1% Triton-X in PBS for 10 mins. The coverslips were then incubated overnight at 37° C with two primary antibodies SHP-1 (BD Biosciences), RIP1 (Cell Signaling), TRAF2 (Cell Signaling), or cIAP2 (Cell Signaling) at 1:300 ratio of 5% FBS. The next day, coverslips were washed three times with PBS and incubated for 1 hr in AlexaFluor goat anti-rabbit (red) or anti-mouse antibody (green) (Invitrogen). The coverslips were then washed again three times with PBS and immersed in mounting media (Dako, Glostrup, Denmark) with DAPI (Molecular Probes, Eugene, Oregon), before being visualized by confocal microscopy.

2.12 Statistical Analysis

Data were plotted and analysis was done using GraphPad Prism 5. Means were compared by the Student's t test or ANOVA and results are expressed as a mean ± standard deviation of at least three experiments.

2.13 Ethics Statement

Informed written consent was provided by all healthy participants based on the study protocol approved by the Research Ethics Boards of the Research Institute, Children's Hospital of Eastern Ontario and the Ottawa Hospital Research Institute, University of Ottawa, Ottawa, Canada.

3.1. Results

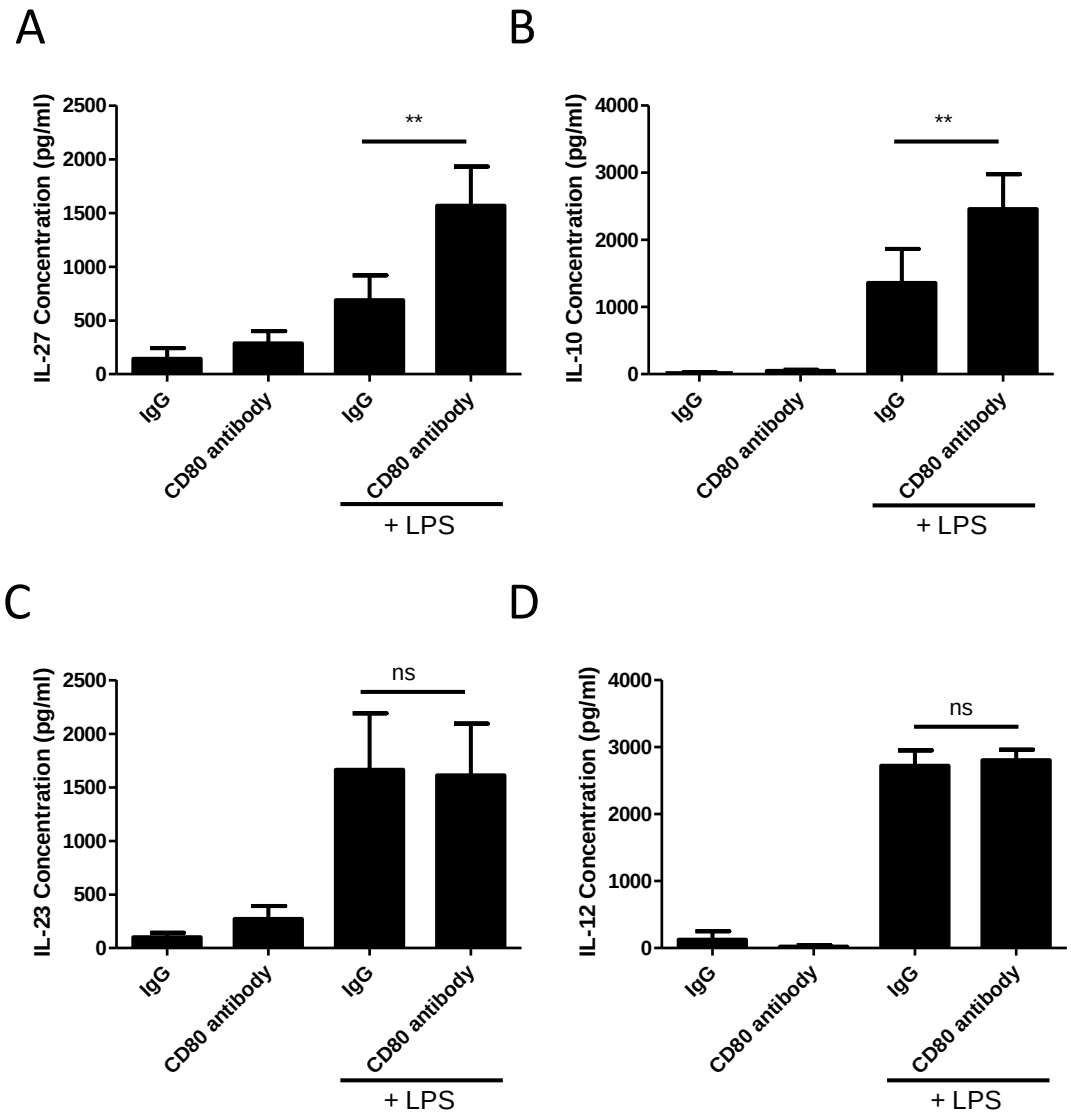
3.2. CD80 and CD86 activation synergizes with TLR-4 signaling pathway to regulate IL-10 and IL-27 production in human MDMs

After encountering an APC, T cells are activated by two important signals. The first antigen-specific signal requires the TCR to bind to the MHC molecule and the second signal is provided by the binding of CD28 with one of two costimulatory molecules on the surface of the APC (8). Macrophages represent a large portion of the APCs that express these molecules. While the role of CD80 and CD86 as a second signal for T cell activation is well characterized, it is not well understood if this interaction with T cells results in macrophage activation. More recently, the role of CD80 and CD86 as a signaling molecule has been investigated in dendritic cells. Stimulation of DCs with anti-CD80/CD86 antibodies has been shown to induce IL-6 expression through the activation of PI3K/AKT pathway (67). Anti-CD80 and anti-CD86 antibodies have also been shown to neutralize CD80/CD86-induced IL-2 secretion in T cells by binding to their binding pockets and were therefore used to represent the interaction between CD28/CTLA-4 on the surface of the T cell with CD80 and CD86. However, little is known about this bidirectional signaling in macrophages. To explore this, human MDMs were treated with either anti-CD80/anti-CD86 antibodies or with a control antibody IgG for 24 hours (Figure 3 and Figure 4). Antibody treatment alone resulted in no changes in the production of these cytokines.

Innate immune cells, such as macrophages, respond to various bacterial components by providing regulatory signals to other immune cells through the secretion of cytokines (14). Recent evidence has demonstrated that the downstream effects of Toll like receptor activation can combine with the effects seen after stimulation of costimulatory molecules to bolster downstream functions (41). Jain *et al* demonstrated that CD86 costimulation enhanced the effect of TLR-2 stimulation on resting B cells, resulting in substantial increases in the activation, differentiation,

Figure 3: **CD80 activation by anti-CD80 antibodies synergize with TLR-4 signaling in human MDMs to produce IL-27 and IL-10, but not IL-12 and IL-23.**

MDMs ($1x10^6$ cells/ml) were treated with 500 ng/mL of the anti-CD80 antibody or isotype matched control IgG_1 control antibodies, in the presence or absence of 10 ng/mL of LPS for 24 hours. Production of A) IL-27 (n=12), B) IL-10 (n=8), C) IL-23 (n=10), and D) IL-12p40 (n=6) protein was measured by ELISA. Values in bar graph are means of at least three independent experiments ±SEM; nonsignificant (ns); *p≤0.05; **p≤0.005; ***p≤0.0005.

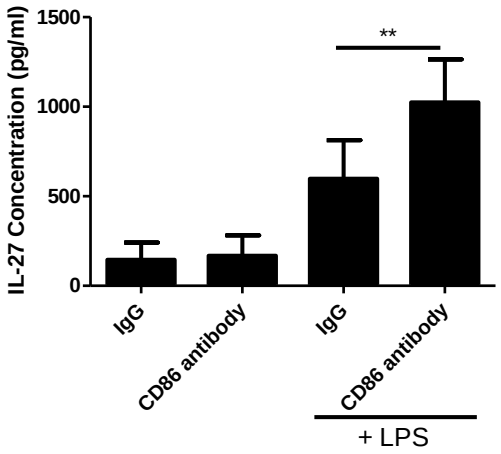

A
B
C
D
IL-27 Concentration (pg/ml)
IL-10 Concentration (pg/ml)
IL-23 Concentration (pg/ml)
IL-12 Concentration (pg/ml)
**
ns
IgG
CD80 antibody
+ LPS

survival, and antigen uptake capacity of these cells (41). In view of these findings, I wanted to explore whether CD80/CD86 molecules also promote signaling via with TLR-4 pathway in MDMs. To explore this process, human MDMs were treated simultaneously with either anti-CD80 or anti-CD86 antibody or with a control antibody, IgG, and with LPS (10 ng/mL) for 24 hours (Figure 3 and Figure 4). Various concentrations of LPS (1 ng/mL-1 ug/mL) were tested and a low dose was selected for these experiments (10 ng/mL) (data not shown). Supernatants were collected and assessed for IL-27, IL-10, IL-23, and IL-12p40 secretion by ELISA. The addition of LPS alone, or LPS with IgG control antibody, induced production of all four cytokines. In contrast, treatment with LPS and anti-CD80 antibody together showed a significantly enhanced production of IL-27 and IL-10 protein (Figure 3A and 3B). Similarly, treatment with LPS and anti-CD86 antibody together demonstrated increased IL-27 and IL-10 production (Figure 4A and 4B). This effect, however, was not seen with IL-12p40 and IL-23 after similar treatment with either anti-CD80 or anti-CD86 antibodies, along with LPS. There was no significant difference in production of IL-23 or IL-12p40 in MDMs stimulated with LPS alone versus with LPS and anti-CD80 antibody together (Figure 3C and 3D). Similarly, there was no significant difference in IL-23 or IL-12p40 secretion following stimulation with anti-CD86 antibody and LPS (Figure 4C and 4D). Taken together, these results suggest that engagement of CD80/CD86 promotes signals which synergize with the TLR-4 pathway to induce IL-10 and IL-27 production in human MDMs, but not IL-12p40 and IL-23.

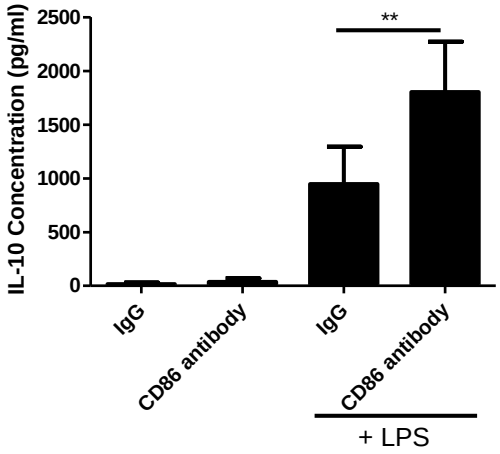
Figure 4: **CD86 activation by anti-CD86 antibodies synergize with TLR-4 signaling in human MDMs to produce IL-27 and IL-10, but not IL-12 and IL-23.**

MDMs ($1x10^6$ cells/ml) were treated with 500 ng/mL of the anti-CD86 antibody or isotype matched control IgG_1 control antibodies, in the presence or absence of 10 ng/mL of LPS for 24 hours. Production of A) IL-27 (n=10), B) IL-10 (n=8), C) IL-23 (n=10), and D) IL-12p40 (n=6) protein was measured by ELISA. Values in bar graph are means of at least three independent experiments ±SEM; nonsignificant (ns); *p≤0.05; **p≤0.005; ***p≤0.0005.

A


IL-27 Concentration (pg/ml)
1500
1000
500
0
**
IgG
CD86 antibody
IgG
CD86 antibody
+ LPS

B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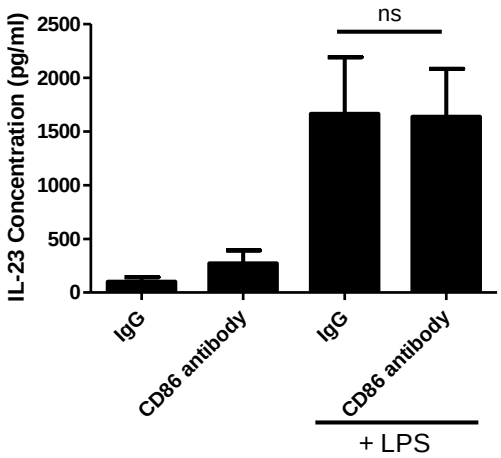
2000
1500
1000
500
0
**
IgG
CD86 antibody
IgG
CD86 antibody
+ LPS

C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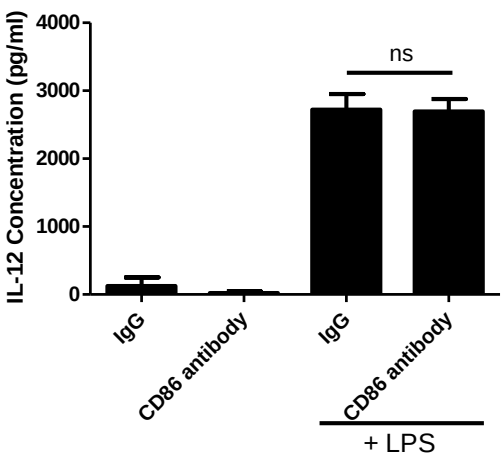
2000
1500
1000
500
0
ns
IgG
CD86 antibody
IgG
CD86 antibody
+ LPS

D


IL-12 Concentration (pg/ml)
4000
3000
2000
1000
0
ns
IgG
CD86 antibody
IgG
CD86 antibody
+ LPS

3.2.1 Inhibitor of apoptosis proteins regulate LPS-induced IL-10 and IL-27 expression, but are not involved in the CD80/CD86-induced IL-10 and IL-27 production

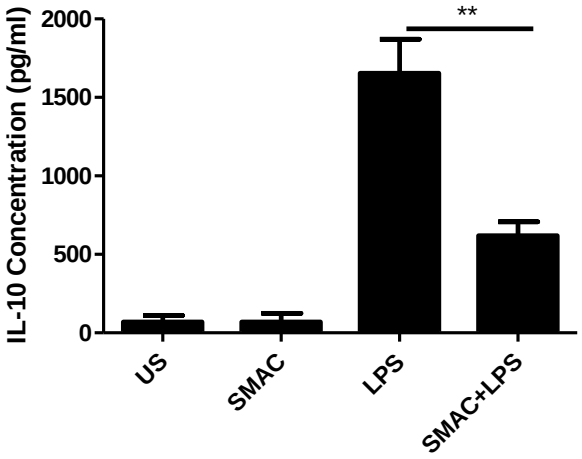
IAPs have emerged as important regulators of innate immune signaling downstream of TLR-4 (128). In particular, they have been identified as having a crucial role in the activation of downstream NF-κB, which are induced by the binding of LPS to TLR-4. Furthermore, depletion of cIAPs was shown to specifically block LPS-induced p38 and JNK MAPK activation and the induction of cytokine gene transcription in murine bone-marrow derived macrophages (105). Tseng *et al* demonstrated that cIAPs were present in the MyD88-assembled complex and were essential for the induction of inflammatory cytokine genes, such as TNF, IL-6 and IL-12β, in response to LPS (105). To investigate the role of IAPs in the LPS-induced production of IL-10 and IL-27, MDMs were treated with 50 nM/mL SMAC mimetics for 24 hours, followed by LPS stimulation for another 24 hours. Biological activity of SMAC mimetics is demonstrated in Figure 5C. ELISA was used to measure the protein expression levels. Treatment with SMAC mimetics alone had no effect on IL-10 and IL-27 production, but LPS-induced IL-10 and IL-27 expression was abrogated after SMAC mimetic treatment (Figure 5A and 5B, respectively). Therefore, IAPs regulate the LPS/TLR-4-induced IL-10 and IL-27 production in human MDMs.

Since IL-10 and IL-27 expression was shown to at least regulated at least in part through the CD80/CD86 synergistic relationship with TLR-4, I wanted to investigate whether synergism of CD80/CD86 and TLR-4 is subject to regulation by IAPs. I pretreated the cells with 50 nM/mL SMAC mimetics as aboveand then treated the MDMs with anti-CD80 or anti-CD86 antibody in concert with a low dose of LPS (10 ng/mL). Consistent with the previous results, the LPS-induced IL-10 and IL-27 expression was enhanced upon addition of anti-CD80/CD86 antibody, but these preliminary results suggested that this synergistic relationship may not be subject to regulation by IAPs (Figure 6).

Figure 5: **IAPs regulate LPS-induced IL-10 and IL-27 expression.**

MDMs ($1x10^6$ cells/mL) were pretreated with SMAC mimetics (50 nM/mL) for 24 hours, following with stimulation in the presence or absence of LPS for 24 hours. A) IL-10 and B) IL-27 production was measured by ELISA. SMAC mimetics specifically block cIAP2 expression (C). Values in bar graph are means of at least three independent experiments ±SEM; *p≤0.05; **p≤0.005; ***p≤0.0005 with at least n=3.

A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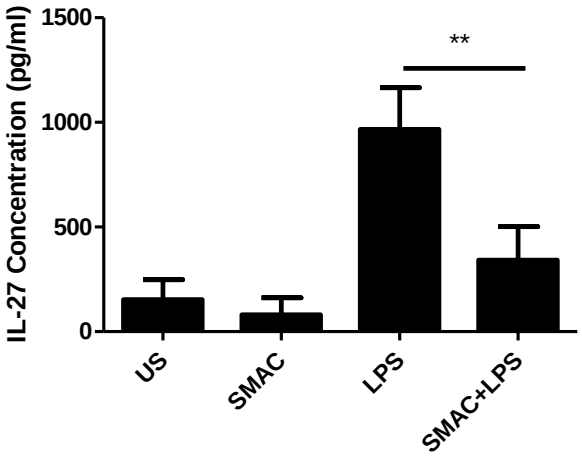
2000
1500
1000
500
0
**
US
SMAC
LPS
SMAC+LPS

B


IL-27 Concentration (pg/ml)
1500
1000
500
0
**
US
SMAC
LPS
SMAC+LPS

C

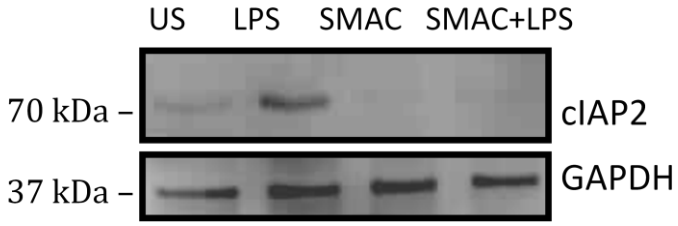
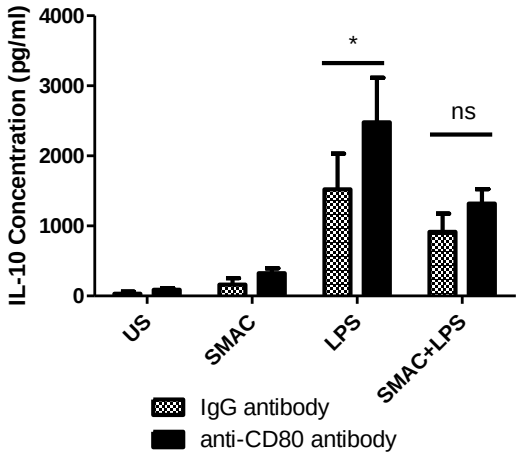

US
LPS
SMAC
SMAC+LPS
70 kDa –
cIAP2
37 kDa –
GAPDH

Figure 6: **Blocking of IAPs by SMAC mimetics inhibits TLR-4 signaling to produce IL-10 and IL-27, but do not appear to affect CD80 and CD86 signaling synergistic effect.**

MDMs ($1x10^6$ cells/mL) were pretreated with SMAC mimetics (50 nM/mL) for 24 hours, following with cells were stimulated with 500 ng/mL of either anti-CD80 or anti-CD86 or isotype matched IgG_1 control antibodies, in the presence or absence of 10 ng/mL LPS for 24 hours. IL-10 (A and B) and IL-27 (C and D) protein secretion was measured by ELISA. Values in bar graph are means of at least three independent experiments ±SEM; nonsignificant (ns); *p≤0.05; **p≤0.005; ***p≤0.0005 with at least n=3.

A


IL-10 Concentration (pg/ml)
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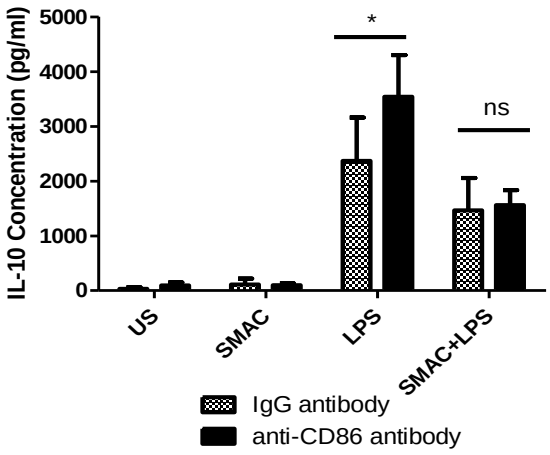
3000
2000
1000
0
*
ns
US
SMAC
LPS
SMAC+LPS
IgG antibody
anti-CD80 antibody

B


IL-10 Concentration (pg/ml)
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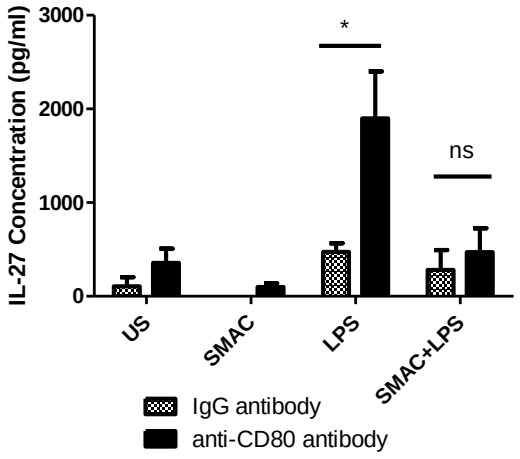
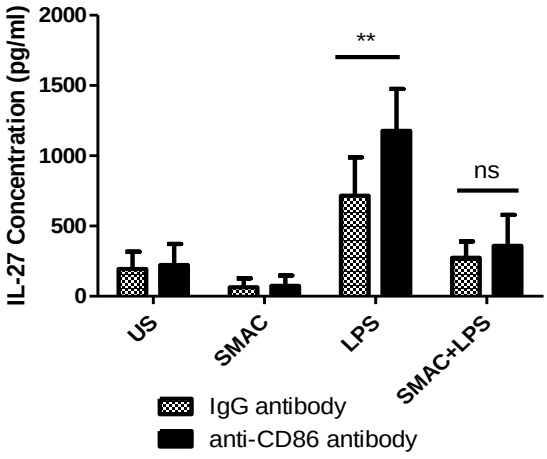
4000
3000
2000
1000
0
*
ns
US
SMAC
LPS
SMAC+LPS
IgG antibody
anti-CD86 antibody

C


IL-27 Concentration (pg/ml)
3000
2000
1000
0
*
ns
US
SMAC
LPS
SMAC+LPS
IgG antibody
anti-CD80 antibody

D


IL-27 Concentration (pg/ml)
2000
1500
1000
500
0
**
ns
US
SMAC
LPS
SMAC+LPS
IgG antibody
anti-CD86 antibody

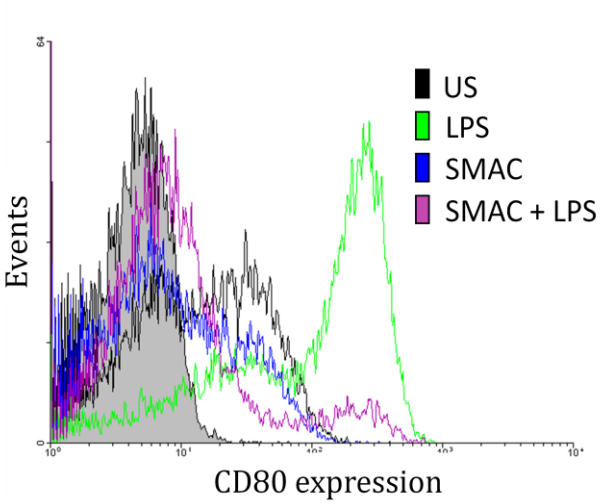
3.3. Regulation of CD80 in LPS-induced monocyte-derived macrophages

3.3.1 Inhibitor of Apoptosis Proteins (IAPs) regulate LPS-induced CD80 and CD86 surface protein expression

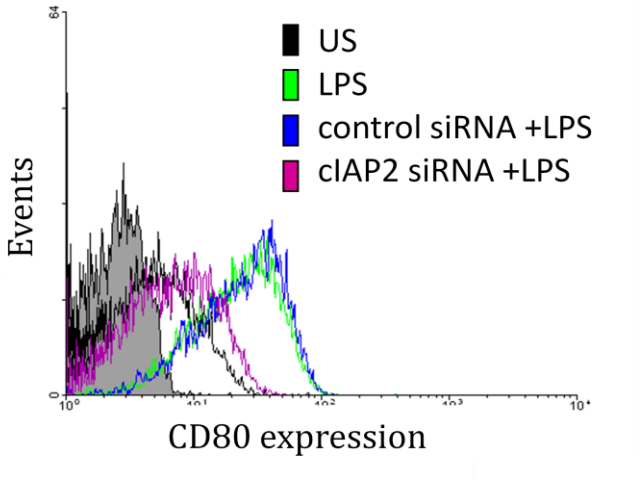
In light of the influence of CD80 expression on the activation of T cells, and the importance of this activation on immune responsiveness and disease progression, understanding the regulation of CD80 and CD86 expression could facilitate the development of immune therapies. The regulation of these two costimulatory molecules has been characterized in monocytes and dendritic cells. In particular, the LPS/TLR-4-activated MAPKs and PI3K molecules have been identified as important regulators of CD80 and CD86 expression in these cells (8, 22, 122). Recently, accumulating evidence has suggested that IAPs may be involved in signal transduction pathways, in particular, TNF-α-mediated NF-κB activation (87). Therefore, I investigated the role of IAPs in the regulation of CD80 and CD86 upregulation in response to LPS. Human MDMs were isolated from peripheral blood mononuclear cells (PBMCs) and treated for 24 hours with 50 nM/mL of SMAC mimetics and LPS for another 24 hours, and CD80 and CD86 surface protein expression was measured by Flow Cytometry. Inhibition of cIAPs by SMAC mimetics prevented the LPS-induced CD80 protein expression (Figure 7A), suggesting that cIAPs are positive regulators of CD80 surface expression. I also investigated the role of cIAP2 in the regulation of CD86 expression in human MDMs and obtained similar results. CD86 protein expression was upregulated following LPS treatment, and this upregulation was positively regulated by IAPs, as CD86 expression returned to basal levels upon treatment with SMAC mimetics (Figure 8).To confirm the role of IAPs in the regulation of LPS-induced CD80 and CD86 surface protein expression, MDMs were transfected with cIAP2 specific siRNA for 24 hours, followed by LPS stimulation for 24 hours. CD80 (Figure 7B) and CD86 (Figure 8B) surface expression was measured by Flow Cytometry. As compared to the cells transfected with control siRNA, MDMs with effective cIAP2 knockdown demonstrated a decreased CD80 and CD86 expression. Silencing of cIAP2 was confirmed via Western Immunoblotting (Figure 7C).

Figure 7: **IAPs positively regulate the LPS-induced CD80 surface expression in MDMs.**
A: SMAC mimetics inhibit LPS-induced CD80 surface expression.
B and C: cIAP2 specific siRNA inhibits LPS-induced CD80 surface expression.
MDMs ($1x10^6$ cells/mL) were treated with 50 nM/mL of SMAC mimetics for 24 hours followed by LPS stimulation for 24 hours. CD80 surface expression was measured by Flow Cytometry (A). These results were confirmed with siRNA (B), where MDMs ($1x10^6$ cells/mL) were transfected with cIAP2 or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours. Confirmation of cIAP2 silencing was demonstrated by Western Immunoblotting (C).

A


US
LPS
SMAC
SMAC + LPS
Events
CD80 expression

B


US
LPS
control siRNA +LPS
cIAP2 siRNA +LPS
Events
CD80 expression

C

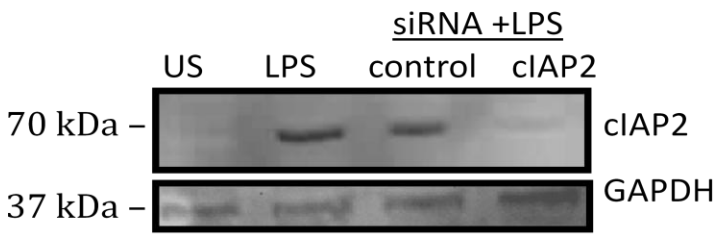
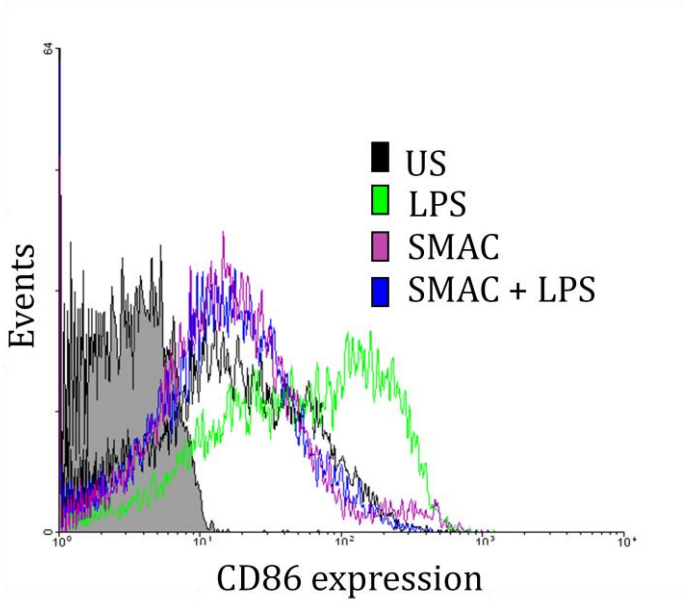
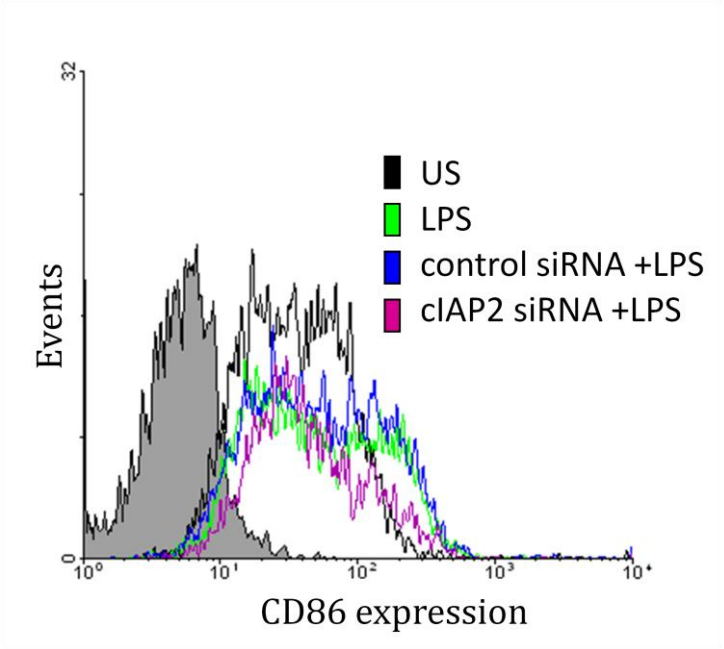

siRNA +LPS
US
LPS
control
cIAP2
70 kDa –
cIAP2
37 kDa –
GAPDH

Figure 8: **IAPs positively regulate the LPS-induced CD86 surface expression in MDMs.**
A: SMAC mimetics inhibit LPS-induced CD86 surface expression
B: cIAP2 specific siRNA inhibits LPS-induced CD86 surface expression.
MDMs ($1x10^6$ cells/mL) were treated with 50 nM/mL of SMAC mimetics for 24 hours followed by LPS stimulation for 24 hours. CD86 surface expression was measured by Flow Cytometry (A). These results were confirmed with siRNA (B), where MDMs ($1x10^6$ cells/mL) were transfected with cIAP2 or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours.

A


64
0
Events
US
LPS
SMAC
SMAC + LPS
10⁰
10¹
10²
10³
10⁴
CD86 expression

B

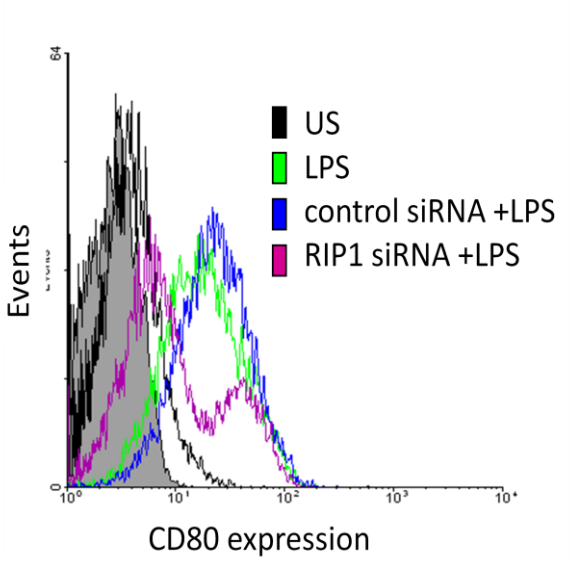

32
0
Events
US
LPS
control siRNA +LPS
cIAP2 siRNA +LPS
10⁰
10¹
10²
10³
10⁴
CD86 expression

3.3.2 RIP1 and TRAF2 regulate the LPS-induced CD80 surface protein expression

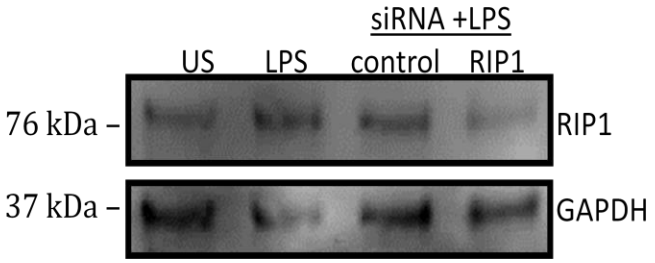
Upon engagement of LPS to its cognate receptor TLR-4, assembly of the TLR4–associated protein complex is initiated. The association of a group of proteins called complex I have been studied downstream of TNF-α receptor. Binding of TNF to the TNF receptor results in the recruitment of molecules that make up complex I, including RIP1 and TRAF2, and TNF-α-induced activation of NF-κB (129). TRAF1 and TRAF2 signaling molecules in turn recruit cIAP1 and cIAP2. By maintaining RIP1 in an ubiquitinated state, this complex prevents the formation of complex II (129). Because of their close association with IAPs and their role in the regulation of downstream transcription factor NF-κB, I investigated the role of RIP1 and TRAF2 in the CD80 and CD86 expression in response to LPS. MDMs were treated with RIP1 or TRAF2 specific siRNA for 24 hours followed by LPS stimulation for 24 hours and the CD80 and CD86 protein expression was examined by Flow Cytometry. MDMs treated with control siRNA together with LPS resulted in an upregulation of CD80 and CD86 surface expression (Figure 9 and Figure 10). Treatment with siRNA specific to RIP1 or TRAF2 together with LPS reduced CD80 and CD86 cell surface protein expression (Figure 9 and Figure 10), suggesting that LPS-induced CD80 and CD86 protein expression are regulated by RIP1 and TRAF2.

Figure 9: **RIP1 and TRAF2 mediate LPS-induced CD80 expression in MDMs.**
MDMs ($1x10^6$ cells/mL) were transfected with RIP1 (A) or TRAF2 (C) siRNA, or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours. CD80 surface expression was measured by Flow Cytometry (left panel). Confirmation of RIP-1 (B) and TRAF2 (D) silencing was demonstrated by Western Immunoblotting (right panel).

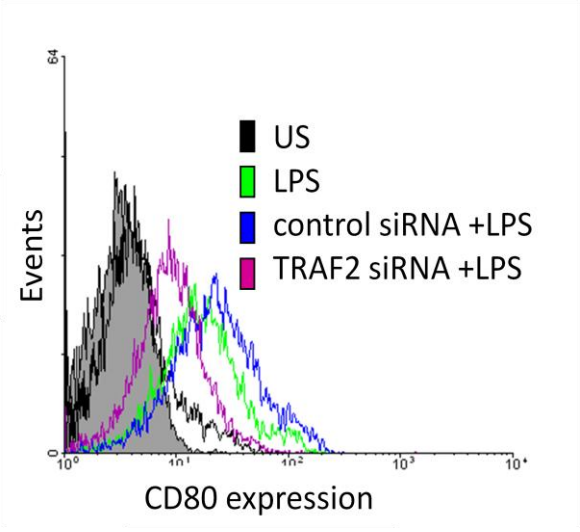
A


US
LPS
control siRNA +LPS
RIP1 siRNA +LPS
Events
CD80 expression

B


siRNA +LPS
US
LPS
control
RIP1
76 kDa –
RIP1
37 kDa –
GAPDH

C


US
LPS
control siRNA +LPS
TRAF2 siRNA +LPS
Events
CD80 expression

D

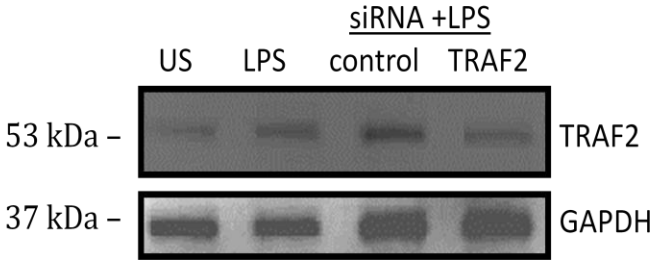
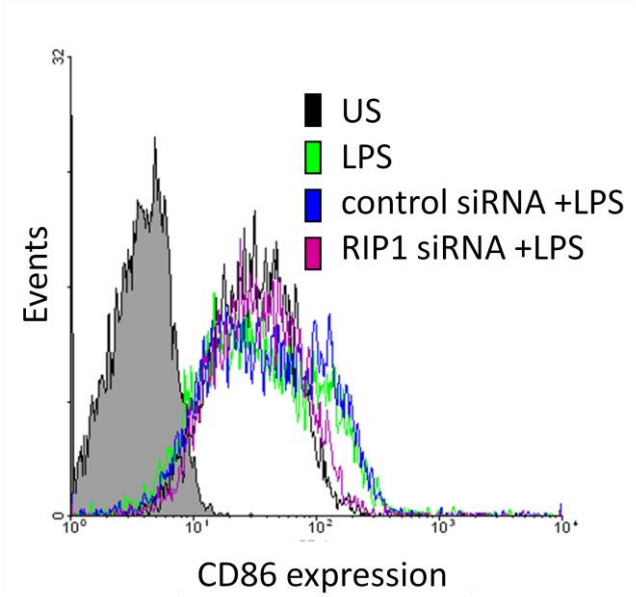
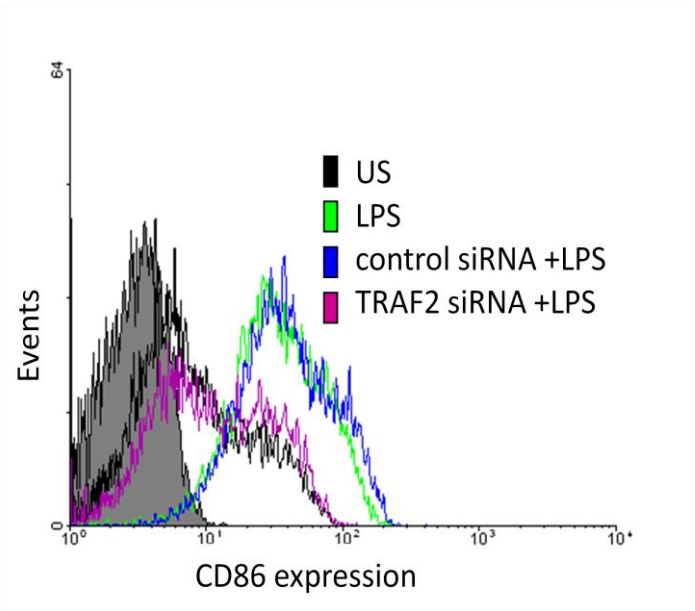

siRNA +LPS
US
LPS
control
TRAF2
53 kDa –
TRAF2
37 kDa –
GAPDH

Figure 10: **RIP1 and TRAF2 mediate LPS-induced CD86 expression in MDMs.**
MDMs ($1x10^6$ cells/mL) were transfected with A) RIP1 or B) TRAF2 siRNA, or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours. CD86 surface expression was measured by Flow Cytometry.

A


US
LPS
control siRNA +LPS
RIP1 siRNA +LPS
Events
CD86 expression

B


US
LPS
control siRNA +LPS
TRAF2 siRNA +LPS
Events
CD86 expression

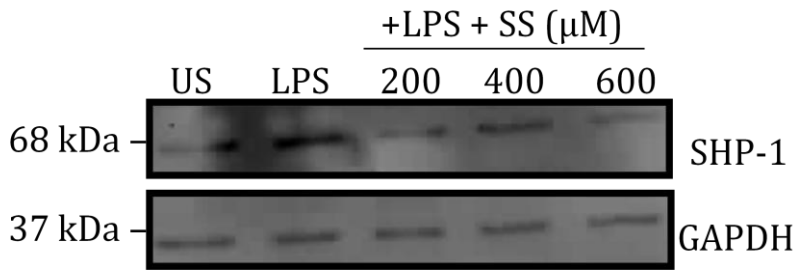
3.3.3 SHP-1 positively regulates CD80 and CD86 protein in human MDMs

Protein tyrosine phosphatase (PTP) SHP-1 has been shown to regulate numerous signaling pathways in hematopoietic cells. In particular, disruption of SHP-1 functions has been implicated autoimmunity and in lymphomas (131). For example, SHP-1 negatively regulates the signaling molecules activated by the T and B cell antigen receptors (132). SHP-1 has been primarily recognized as a negative regulator of signal transduction pathways in hematopoietic cells. More recently, however it has been demonstrated that SHP-1 functions as a positive regulator of cytokine production downstream of TLR-4 receptor (132). However, the role of SHP-1 in the LPS-induced regulation of CD80 expression remains unknown. To examine the involvement of SHP-1 in the regulation of LPS-induced CD80/86 expression, human MDMs were treated for 24 hours with various concentrations of the specific SHP-1 inhibitor, sodium stibogluconate (SS), followed by stimulation with 1 μg/mL of LPS for 24 hours. The biological activity of sodium stibogluconate was confirmed with Western Immunoblotting (Figure 11A). As expected from previous experiments, LPS stimulation induced higher levels of CD80 (Figure 11B) and CD86 surface expression (Figure 12A), and this was inhibited by SS in a dose-dependent manner, suggesting that SHP-1 is a positive regulator of TLR4-induced CD80 and CD86 expression.

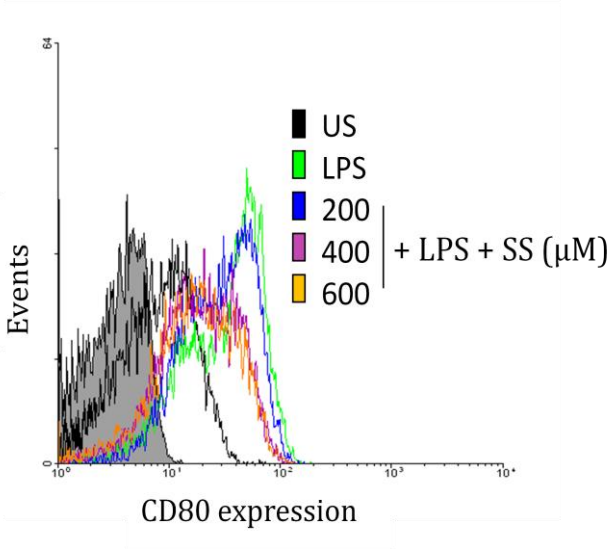
The role of SHP-1 in the regulation of LPS-induced CD80 and CD86 protein expression was confirmed using siRNA transfection. MDMs were transfected with SHP-1 specific siRNA for 24 hours, followed by LPS stimulation for another 24 hours for protein measurement by Flow Cytometry. Western Immunoblotting was used to demonstrate the knockdown of SHP-1 expression, as compared to control siRNA (Figure 11D). SHP-1 specific siRNA significantly reduced the CD80 (Figure 11C) and CD86 (Figure 12B) surface protein expression relative to the expression in cells transfected with control siRNA. This suggests that SHP-1 plays a key role as a positive regulator of LPS-induced CD80 and CD86 surface expression in human macrophages.

Figure 11: **SHP-1 positively regulates the LPS-induced CD80 surface expression in MDMs.**
A: Sodium stibogluconate inhibits SHP-1 expression.
B: Inhibition of SHP-1 reduced CD80 surface expression.
C and D: SHP-1 specific siRNA inhibits LPS-induced CD80 surface expression.
MDMs ($1x10^6$ cells/mL) were treated with indicated concentrations of sodium stibogluconate (SS) for 24 hours, followed by LPS stimulation for 24 hours. A) SS inhibitor decreased SHP-1 expression as measured by Western Immunoblotting. The effect of SS on CD80 surface expression was determined by Flow Cytometry. These results were confirmed with siRNA, where MDMs ($1x10^6$ cells/mL) were transfected with siRNA specific to SHP-1 or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours. CD80 surface expression was measured by Flow Cytometry (D). Confirmation of SHP-1 silencing was demonstrated by Western Immunoblotting (E).

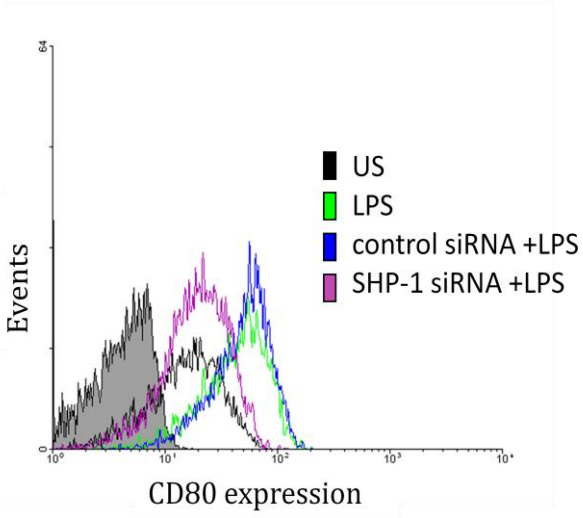
A


+LPS + SS (μM)
US
LPS
200
400
600
68 kDa
SHP-1
37 kDa
GAPDH

B


US
LPS
200
400
600
+ LPS + SS (μM)
Events
CD80 expression

C


US
LPS
control siRNA +LPS
SHP-1 siRNA +LPS
Events
CD80 expression

D

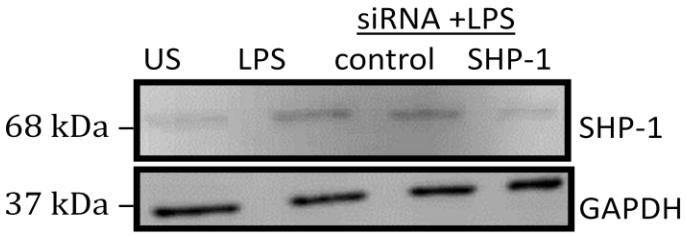
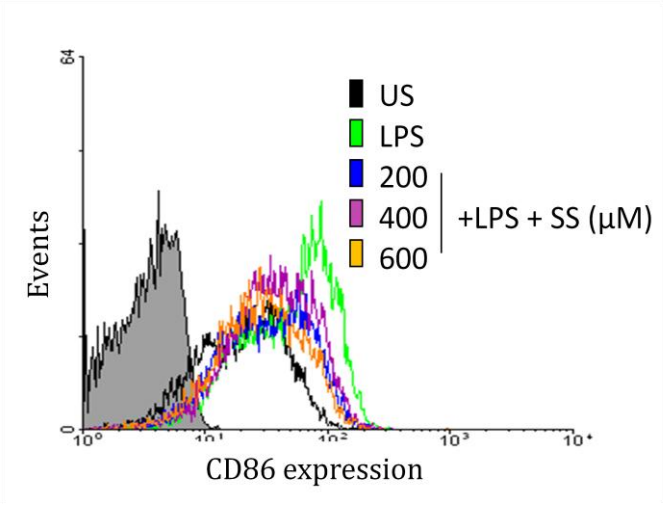
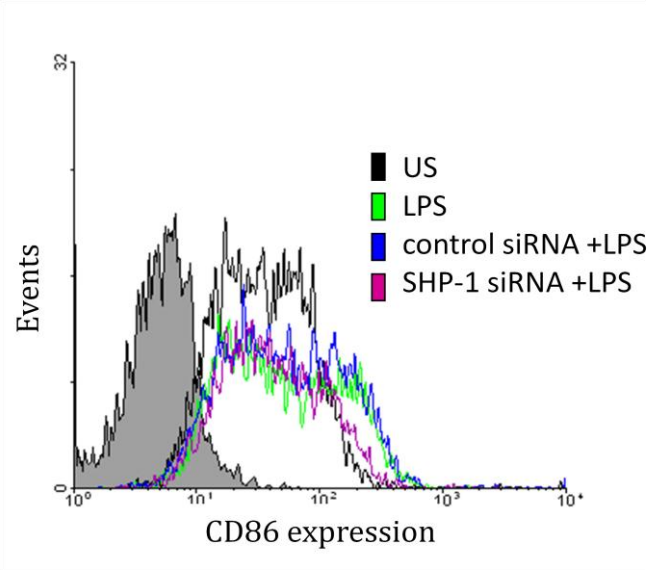

siRNA +LPS
US
LPS
control
SHP-1
68 kDa
SHP-1
37 kDa
GAPDH

Figure 12: **SHP-1 positively regulates the LPS-induced CD86 surface expression in MDMs.**
A: Inhibition of SHP-1 reduced CD86 surface expression.
B: SHP-1 specific siRNA inhibits LPS-induced CD86 surface expression.
MDMs ($1x10^6$ cells/mL) were treated with indicated concentrations of sodium stibogluconate (SS) for 24 hours, followed by LPS stimulation for 24 hours. CD86 surface expression was measured by Flow Cytometry (A). These results were confirmed with siRNA (B), where MDMs ($1x10^6$ cells/mL) were transfected with SHP-1 or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours. CD86 cell surface expression after transfection was measured by Flow Cytometry (B).

A


64
0
Events
US
LPS
200
400
600
+LPS + SS (μM)
10⁰
10³
10⁴
CD86 expression

B


32
0
Events
US
LPS
control siRNA +LPS
SHP-1 siRNA +LPS
10⁰
10³
10⁴
CD86 expression

3.3.4 SHP-1, cIAP2, RIP1 and TRAF2 co-localize in human MDMs

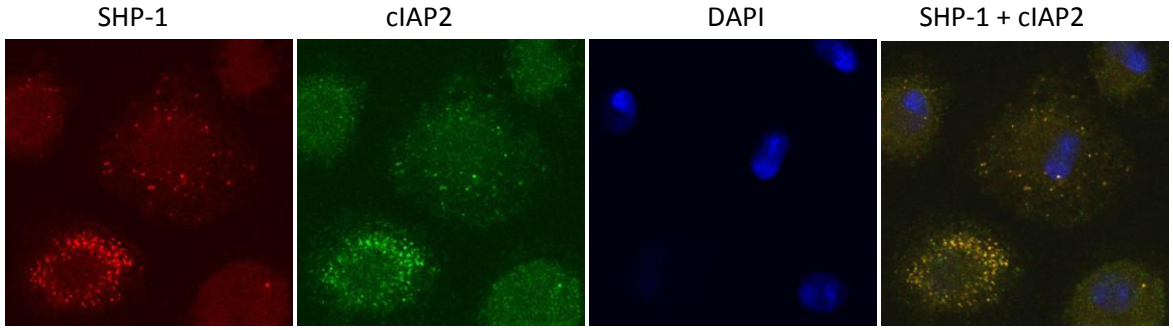
Since it is known that RIP1 and TRAF2 interact to form a complex with cIAP2 (106) (confirmed in Figure 14A and Figure 14B), I wanted to investigate whether SHP-1 also co-localized with cIAP2. For this, MDMs on slides were fixed and permeabilized before being incubated overnight with two primary antibodies, SHP-1 (anti-mouse), RIP1 (anti-rabbit), TRAF2 (anti-rabbit) or cIAP2 (anti-rabbit/anti-mouse). These proteins were visualized by confocal microscopy. Figure 13A demonstrates that these two proteins co-immunofluoresce, suggesting their co-localization within the macrophage. Furthermore, I demonstrated that SHP-1 co-localized with RIP1 (Figure 13B) and TRAF2 (Figure 13C). Taken together, these results suggest that SHP-1, cIAP2, RIP1 and TRAF2 co-localize in human macrophages.

3.3.5 PI3K pathway regulates the LPS-induced CD80 and CD86 protein expression

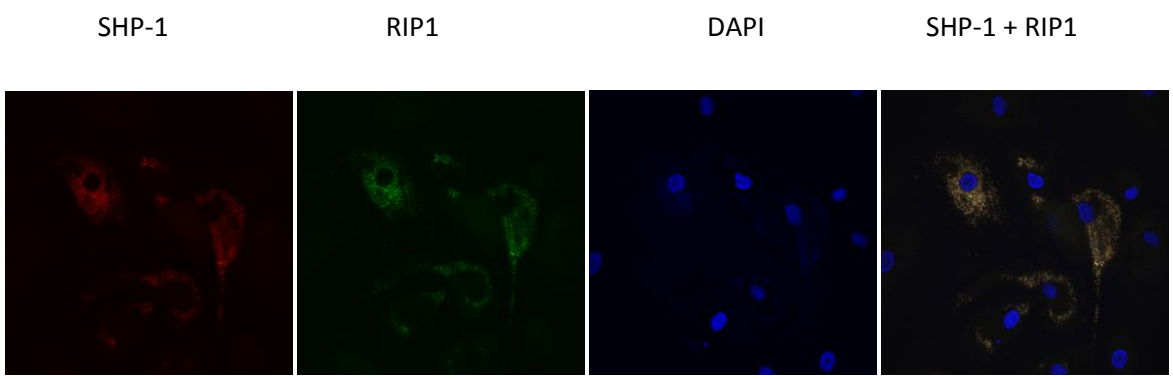
Engagement of LPS to its receptor TLR-4 induces a host of cellular responses, including the activation of the PI3K pathway (133). The PI3K pathway is crucial for cellular growth and cell survival and more recently, its role in the regulation of LPS-induced CD80 and CD86 expression in B cells has been investigated. Attridge *et al* have demonstrated that a potent and sustained CD86 upregulation in murine B cells triggered by the addition of IL-21 is mediated through a PI3K-dependent mechanism (134). Hence, I investigated whether PI3K modulated CD80 and CD86 expression in human macrophages. Since the activation of the PI3K pathway culminates in the phosphorylation of AKT, LY294002 a highly specific inhibitor that abolishes PI3K-dependent AKT phosphorylation, but does not inhibit other lipid and protein kinases, such as MAPKs, was used (135). MDMs were treated for 2 hours with LY294002 and then stimulated for 24 hours with LPS to measure CD80 and CD86 surface protein expression. Figure 15B demonstrates the dose-dependent decrease in LPS-induced CD80 protein expression. CD80 mRNA expression was measured after treatment with LY294002

Figure 13: **SHP-1 co-localizes with cIAP2, RIP1, and TRAF2 in human macrophages.**
A: SHP-1 and cIAP2 co-localize
B: SHP-1 and RIP1 co-localize
C: SHP-1 and TRAF2 co-localize
MDMs ($1x10^6$ cells/mL) were stained with anti-mouse SHP-1 antibody, anti-rabbit cIAP2 antibody, anti-rabbit RIP1, or anti-rabbit TRAF2, and then analyzed using immunofluorescence.

A


SHP-1
cIAP2
DAPI
SHP-1 + cIAP2

B


SHP-1
RIP1
DAPI
SHP-1 + RIP1

C

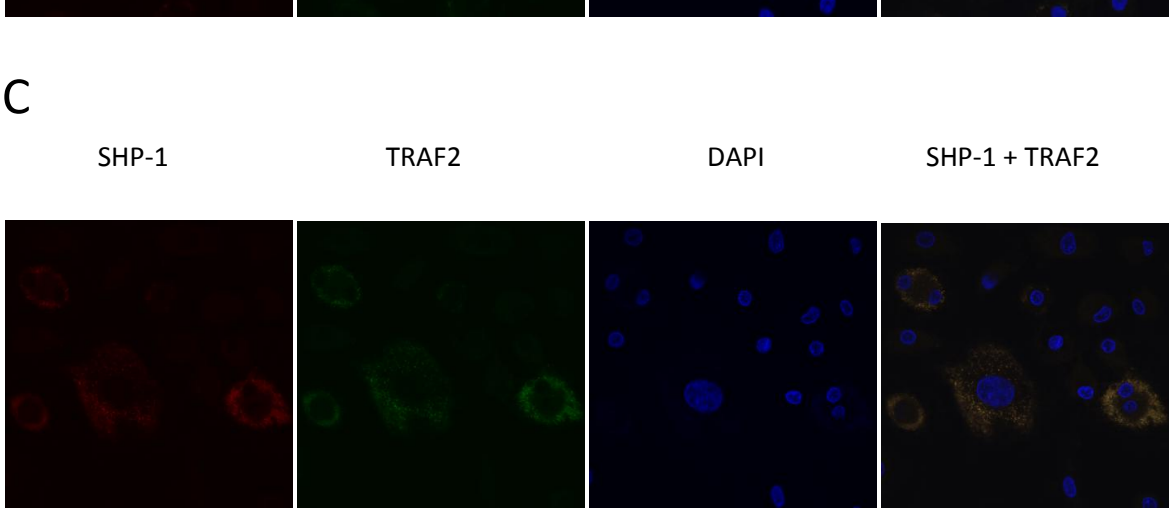
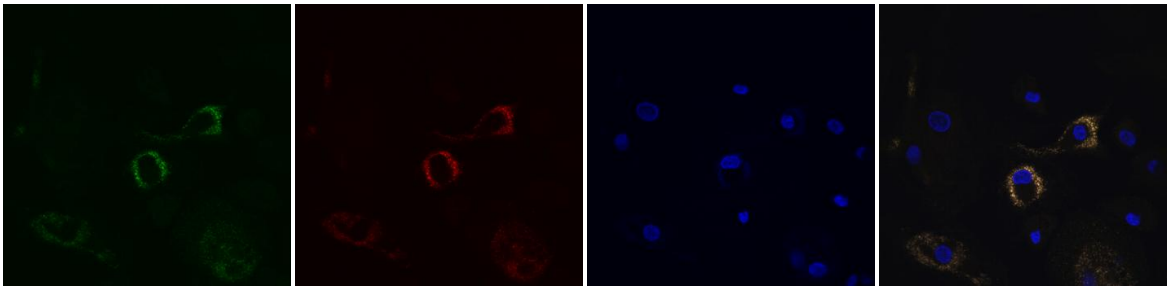

SHP-1
TRAF2
DAPI
SHP-1 + TRAF2

Figure 14: **cIAP2 co-localizes with RIP1 and TRAF2 in human macrophages.**
A: cIAP2 and RIP1 co-localize
B: cIAP2 and TRAF2 co-localize
MDMs ($1x10^6$ cells/mL) were stained with anti-mouse cIAP2 antibody, anti-rabbit RIP1, or anti-rabbit TRAF2, and then analyzed using immunofluorescence.

A

cIAP2 RIP1 DAPI cIAP2 + RIP1



B

cIAP2 TRAF2 DAPI cIAP2 + TRAF2

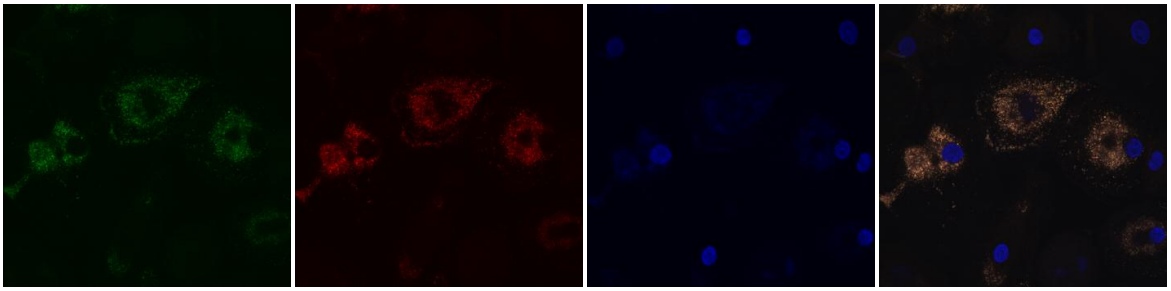
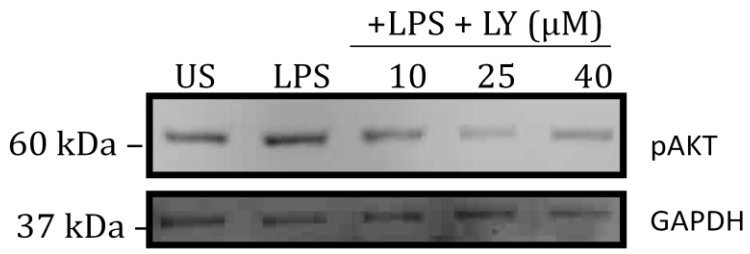
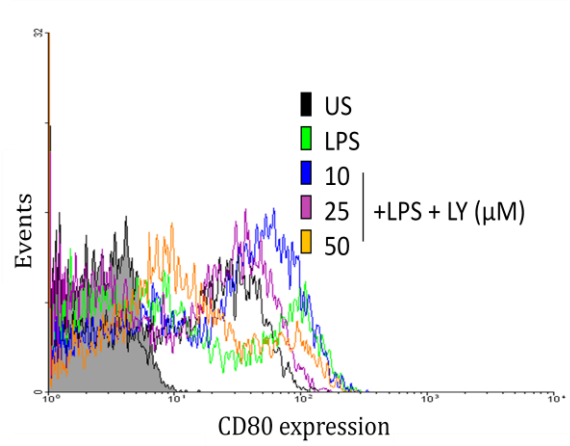


Figure 15: **PI3K positively regulates the LPS-induced CD80 surface expression in MDMs.**
A: LY294002 inhibits phospho-AKT expression.
B and C: Inhibition of pAKT reduced CD80 surface and mRNA expression.
D and E: AKT specific siRNA inhibits LPS-induced CD80 surface expression.
MDMs ($1x10^6$ cells/mL) were treated with indicated concentrations of LY294002 for 2 hours, followed by LPS stimulation for A) 15 mins B) 24 hours and C) 4 hours. A) Biological activity assay of LY inhibitor measured by Western Immunoblotting. CD80 surface and mRNA expression was measured by Flow Cytometry (B) and qRT-PCR (C), respectively. These results were confirmed with siRNA, where MDMs ($1x10^6$ cells/mL) were transfected with AKT or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours. CD80 cell surface expression after transfection was measured by Flow Cytometry (D). Confirmation of AKT silencing was demonstrated by Western Immunoblotting (E). Values in bar graph are means of at least three independent experiments ±SEM; *p≤0.05; **p≤0.005; ***p≤0.0005 as measured by One-Way Anova with at least n=3.

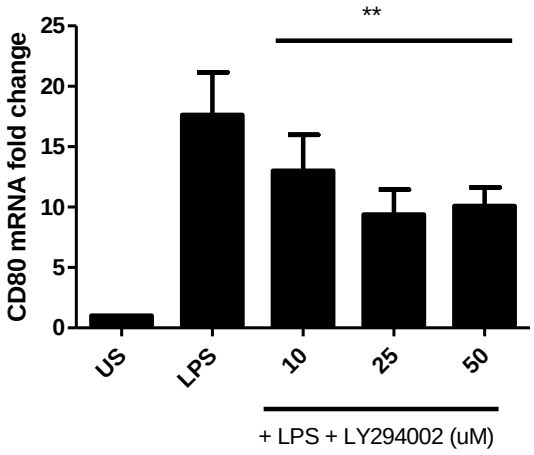
A


+LPS + LY (μM)
US
LPS
10
25
40
60 kDa –
pAKT
37 kDa –
GAPDH

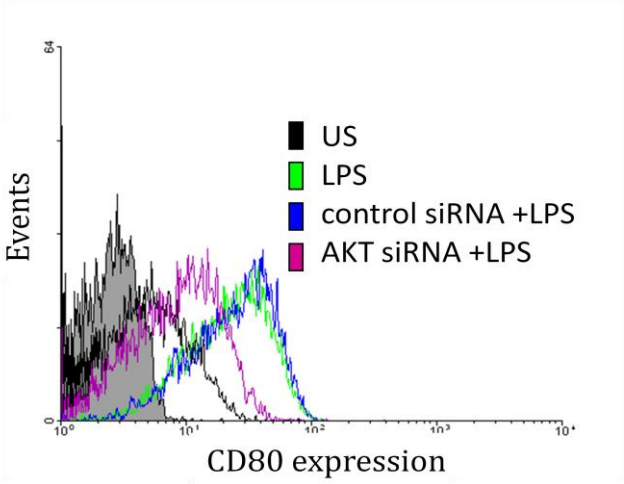
B


US
LPS
10
25
50
+LPS + LY (μM)
Events
CD80 expression

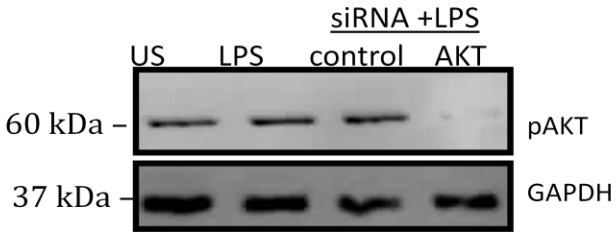
C


**
CD80 mRNA fold change
25
20
15
10
5
0
US
LPS
10
25
50
+ LPS + LY294002 (uM)

D


US
LPS
control siRNA +LPS
AKT siRNA +LPS
Events
CD80 expression

E

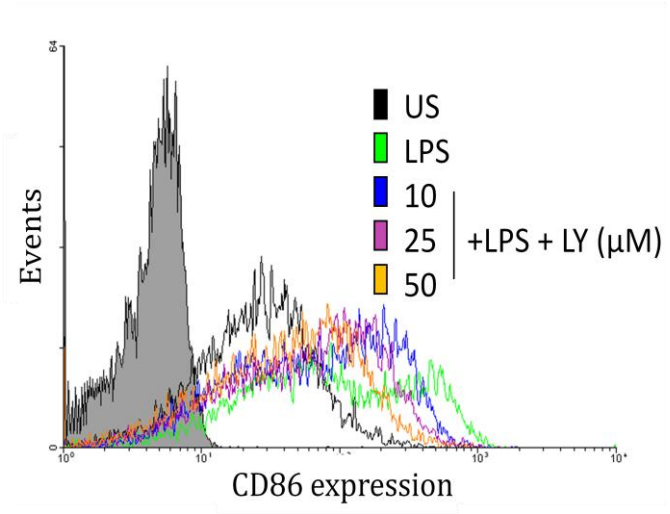

siRNA +LPS
US
LPS
control
AKT
60 kDa –
pAKT
37 kDa –
GAPDH

inhibitor for 2 hours and LPS stimulation for 4 hours. A dose dependent decrease was also observed in LPS-induced CD80 mRNA expression (Figure 15C). The biological activity of the inhibitor was demonstrated by Western Immunoblotting (Figure 15A). This effect was confirmed with AKT specific siRNA that effectively knocked down the expression of AKT in the macrophages (Figure 15E). As compared to the control siRNA, the AKT specific siRNA demonstrated a reduced LPS-induced CD80 expression in human MDMs (Figure 15D), confirming the results obtained with the biological inhibitor.

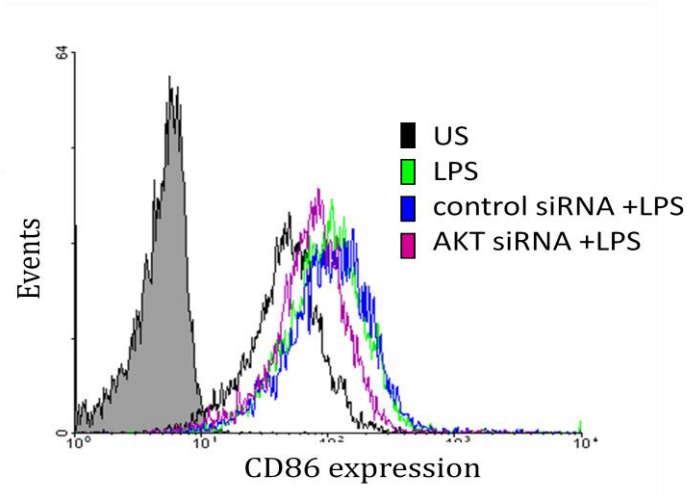
With respect to the role of PI3K in CD86 expression, Figure 16A demonstrates the dose-dependent decrease in CD86 surface protein expression after treatment with the biological inhibitor, LY294002. This result was confirmed after MDMs were treated with AKT specific siRNA (Figure 16B). As compared to the cells treated with control siRNA, MDMs showed a significantly reduced CD86 protein expression after transfection with the AKT specific siRNA. Taken together, these results suggest that the PI3K pathway is a positive regulator of the LPS-induced CD80 protein and mRNA expression, as well as the CD86 protein expression.

Figure 16: **PI3K positively regulates the LPS-induced CD86 surface expression in MDMs.**
A: Inhibition of pAKT reduced CD86 surface expression.
B: AKT specific siRNA inhibits LPS-induced CD86 surface expression.
MDMs ($1x10^6$ cells/mL) were treated with indicated concentrations of LY294002 for 2 hours, followed by LPS stimulation for 24 hours. CD86 surface expression was measured by Flow Cytometry (A). These results were confirmed with siRNA, where MDMs ($1x10^6$ cells/mL) were transfected with AKT specific or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours. CD86 cell surface expression after transfection was measured by Flow Cytometry (B).

A


Events
64
0
US
LPS
10
25
50
+LPS + LY (μM)
CD86 expression

B


Events
64
0
US
LPS
control siRNA +LPS
AKT siRNA +LPS
CD86 expression

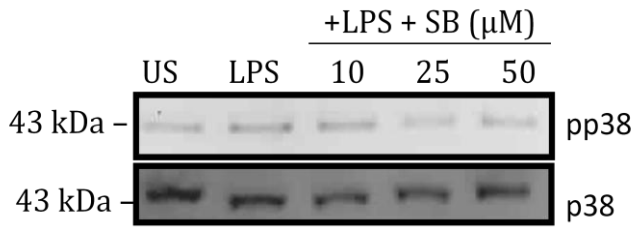
3.3.6 P38, but not JNK or ERK MAPK regulate the LPS-induced CD80 and CD86 expression

In recent years, significant progress has been made in elucidating members of the LPS signaling pathways that are involved in the regulation of CD80 and CD86 expression (8, 22, 122, 123). In particular, Mitogen-activated protein kinases (MAPKs) have been shown to be key players in regulating cellular responses such as proliferation and differentiation in human monocytes (8, 22). Three main families of MAPKs have been shown to be activated by LPS: extracellular signal-regulated protein kinases (ERK1 and ERK2), c-Jun N-terminal kinases (JNKs) and the p38 MAPK/stress-activated protein kinases (8). In human monocytes, the LPS-induced CD86 expression has been shown to be regulated by p38 and JNK MAPK (8). Furthermore, regulation of CD80 expression in monocytic cells was shown to be mediated through the activation of JNK MAPK (121). The role of MAPKs in the regulation of CD80 and CD86 expression has also been explored in DCs. Nakahara *et al* and Arrighi *et al* have demonstrated that JNK MAPK (122) and p38 MAPK (123) are involved in the LPS-induced CD80 and CD86 expression in human DCs (122). The modulation of CD80 and CD86 expression by MAPKs has also been highlighted in other cell types. Therefore, it was of interest to investigate the role of MAPKs in the expression of CD80 and CD86 in human macrophages.

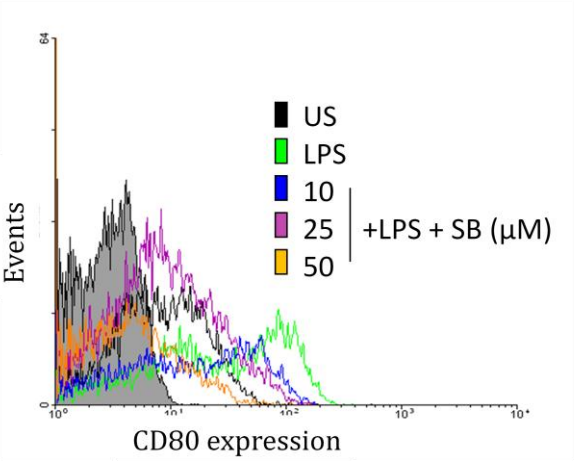
To investigate the role of p38 MAPK in the LPS-induced CD80 and CD86 expression, MDMs were treated with SB203580, a specific inhibitor for p38 MAPK, for 2 hours and then stimulated with LPS for 24 hours. Treatment with LPS resulted in a significant upregulation in CD80 protein expression, and this was reduced with increasing doses of the p38 inhibitor, suggesting that p38 MAPK is a positive regulator of the LPS-induced CD80 surface protein expression (Figure 17B). These data were confirmed using p38 specific siRNA (Figure 17D). As compared to the control siRNA, silencing p38 resulted in a decreased CD80 surface protein expression. The effect of p38 inhibition on CD80 mRNA expression was examined by RT-PCR. Figure 17C demonstrates the dose dependent

Figure 17: **p38 MAPK positively regulates the LPS-induced CD80 surface expression in MDMs.**
A: SB203580 inhibits phospho-p38 expression.
B and C: Inhibition of pp38 reduced CD80 surface and mRNA expression.
D and E: p38 specific siRNA inhibits LPS-induced CD80 surface expression.
MDMs ($1x10^6$ cells/mL) were treated with indicated concentrations of SB203580 for 2 hours, followed by LPS stimulation for A) 15 mins B) 24 hours and C) 4 hours. A) Biological activity assay of SB inhibitor measured by Western Immunoblotting. CD80 surface and mRNA expression was measured by Flow Cytometry (B) and qRT-PCR (C), respectively. These results were confirmed with siRNA, where MDMs ($1x10^6$ cells/mL) were transfected with p38 or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours. CD80 cell surface expression after transfection was measured by Flow Cytometry (D). Confirmation of p38 silencing was demonstrated with Western Immunoblotting (E). Values in bar graph are means of at least three independent experiments ±SEM; *p≤0.05; **p≤0.005; ***p≤0.0005 as measured by One-Way Anova with at least n=3.

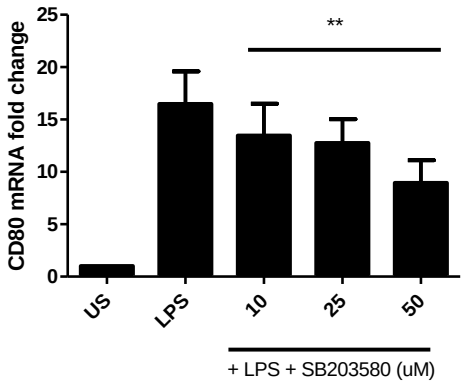
A


+LPS + SB (μM)
US
LPS
10
25
50
43 kDa –
pp38
43 kDa –
p38

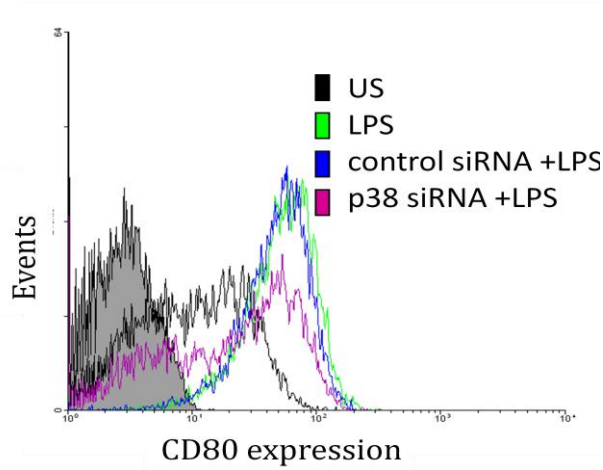
B


US
LPS
10
25
50
+LPS + SB (μM)
Events
CD80 expression

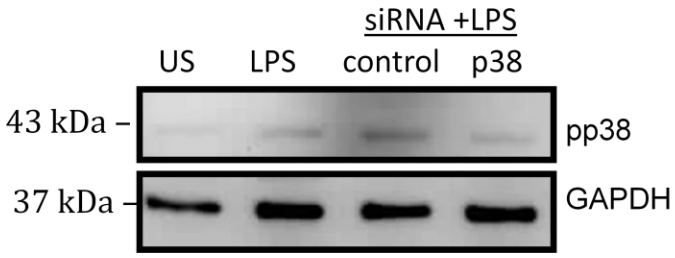
C


CD80 mRNA fold change
25
20
15
10
5
0
**
US
LPS
10
25
50
+ LPS + SB203580 (uM)

D


US
LPS
control siRNA +LPS
p38 siRNA +LPS
Events
CD80 expression

E


siRNA +LPS
US
LPS
control
p38
43 kDa –
pp38
37 kDa –
GAPDH

decrease of CD80 mRNA transcripts after treatment with the p38 inhibitor for 2 hours and LPS stimulation for 4 hours. Collectively, this data demonstrates that CD80 surface protein and mRNA are positively regulated by p38 MAPKinase. Similarly, I investigated the role of p38 MAPK in the regulation of CD86 protein expression. P38 MAPK expression was required for LPS-induced CD86 expression, as the inhibition of this molecule resulted in a dose dependent decrease in CD86 protein expression as measured by Flow Cytometry (Figure 18A). After treatment with p38 specific siRNA, there was a marked decrease in CD86 surface protein expression (Figure 18B). Taken together, this data demonstrates that p38 MAPK is a crucial regulator of the CD86 protein.

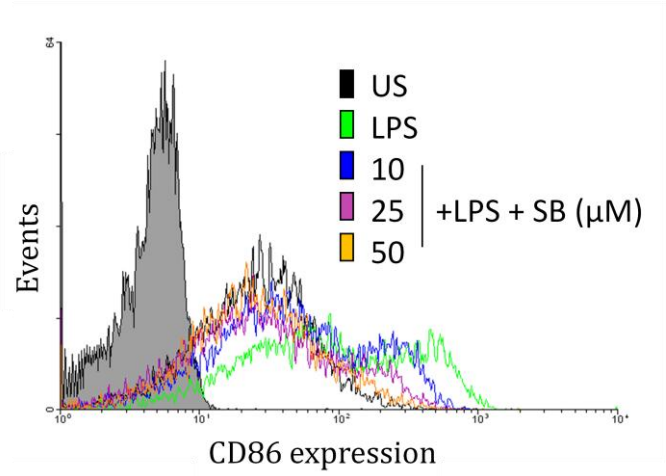
In parallel, I also investigated the role of JNK and ERK MAPK in the regulation of LPS-induced CD80 and CD86 expression. Using various doses of SP600125, the specific inhibitor for JNK MAPK, I showed that JNK MAPK did not play a role in the LPS-induced CD80 or CD86 surface protein expression (Figure 19). To investigate the role of ERK MAPK in the regulation of LPS-induced CD80 and CD86 expression, I employed the specific inhibitor PD98059 in increasing doses and measured its effect on CD80/CD86 surface protein and mRNA expression. Using three concentrations of PD98059, I demonstrated that ERK MAPK is not involved in the LPS-induced CD80 surface protein (Figure 20B) and mRNA expression (Figure 20C). Similarly, ERK MAPK was found to not be involved in the LPS-induced CD86 surface protein (Figure 20D).

3.3.7 SHP-1 and IAPs regulate activation of downstream PI3K/p38 signaling axis

Since I identified that IAPs, SHP-1 signaling molecule, and PI3K and p38 MAPK pathways positively regulate CD80 and CD86 expression, I wanted to elucidate the mechanism by which this occurs. For this, I inhibited SHP-1 using sodium stibogluconate (SS) and measured the phosphorylation of AKT and p38 molecules. I demonstrated that SHP-1 positively regulated the activation of both PI3K signaling (Figure 21A) and p38 MAPK (Figure 21B). In addition, using SMAC

Figure 18: **p38 MAPK positively regulates the LPS-induced CD86 surface expression in MDMs.**
A: Inhibition of pp38 reduced CD86 surface expression.
B: p38 specific siRNA inhibits LPS-induced CD86 surface expression.
MDMs ($1x10^6$ cells/mL) were treated with indicated concentrations of SB203580 for 2 hours, followed by LPS stimulation for 24 hours. CD86 surface expression was measured by Flow Cytometry (A). These results were confirmed with siRNA, where MDMs ($1x10^6$ cells/mL) were transfected with p38 or control siRNA (CSI) for 24 hours, followed by LPS stimulation for 24 hours. CD86 cell surface expression was measured after transfection by Flow Cytometry (C). Values in bar graph are means of at least three independent experiments ±SEM; *p≤0.05; **p≤0.005; ***p≤0.0005 as measured by One-Way Anova with at least n=3.

A


US
LPS
10
25
50
+LPS + SB (µM)
Events
CD86 expression

B

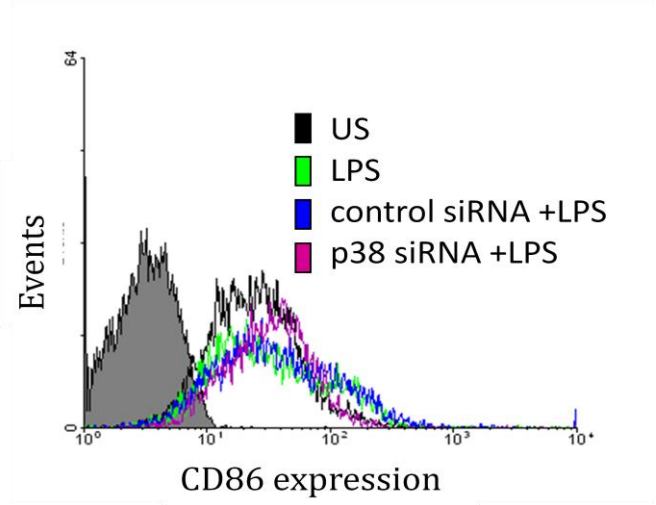

US
LPS
control siRNA +LPS
p38 siRNA +LPS
Events
CD86 expression

Figure 19: **JNK MAPK does not regulate the LPS-induced CD80 or CD86 surface expression in MDMs.**

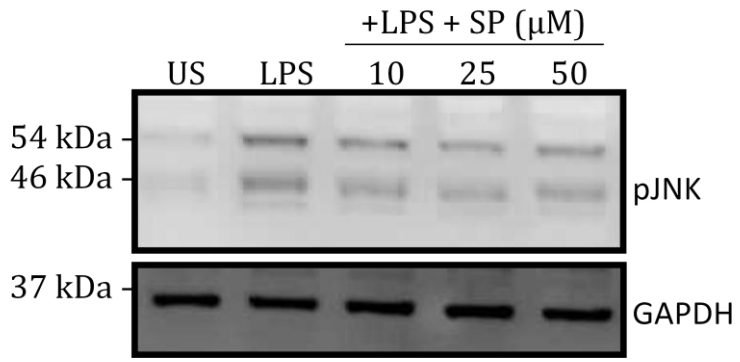
A: SP600125 inhibits phospho-JNK expression.

B and C: Inhibition of pJNK reduced CD80 surface and mRNA expression.

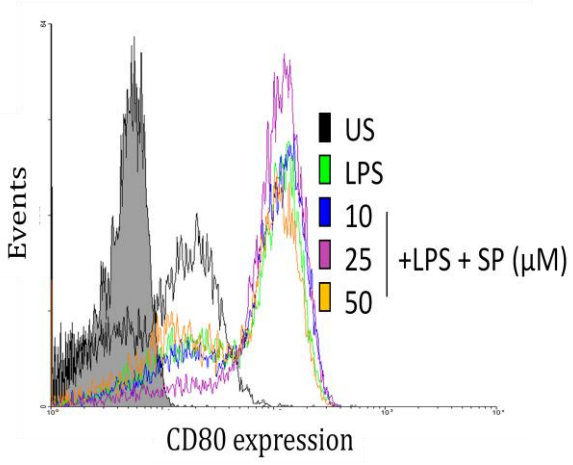
D: Inhibition of pJNK reduced CD86 surface expression.

MDMs ($1x10^6$ cells/mL) were treated with indicated concentrations of SP600125 for 2 hours, followed by LPS stimulation for A) 15 mins, B) and D) 24 hours, and C) 4 hours. A) Biological activity assay of SP inhibitor measured by Western Immunoblotting. CD80 surface and mRNA expression was measured by Flow Cytometry (B) and qRT-PCR (C), respectively. Similarly, CD86 surface expression was also measured by Flow Cytometry (D). Values in bar graph are means of at least three independent experiments ±SEM; *p≤0.05; **p≤0.005; ***p≤0.0005 as measured by One-Way Anova with at least n=3.

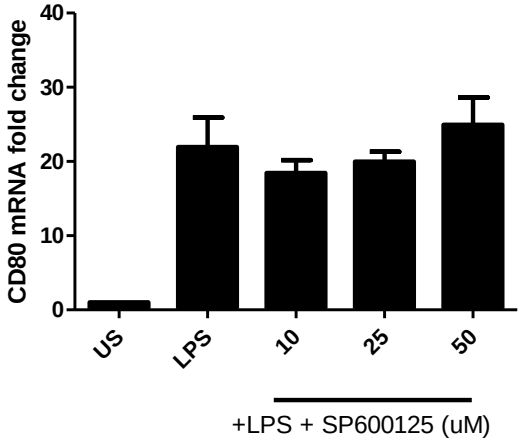
A


+LPS + SP (μM)
US
LPS
10
25
50
54 kDa
46 kDa
pJNK
37 kDa
GAPDH

B


Events
US
LPS
10
25
50
+LPS + SP (μM)
CD80 expression

C


CD80 mRNA fold change
40
30
20
10
0
US
LPS
10
25
50
+LPS + SP600125 (uM)

D

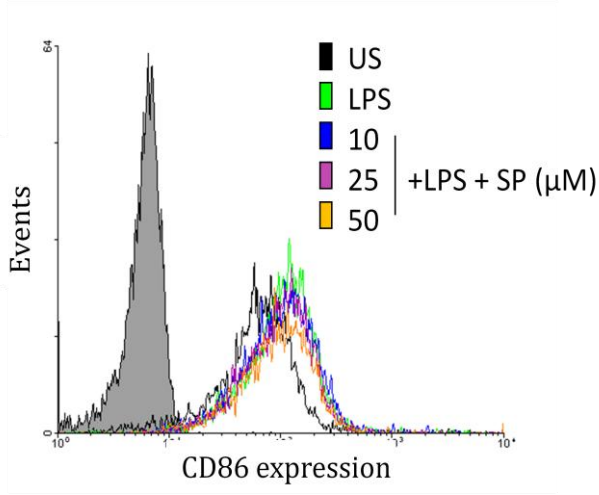
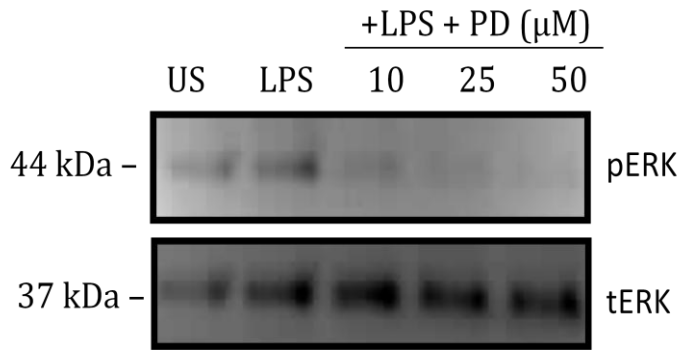
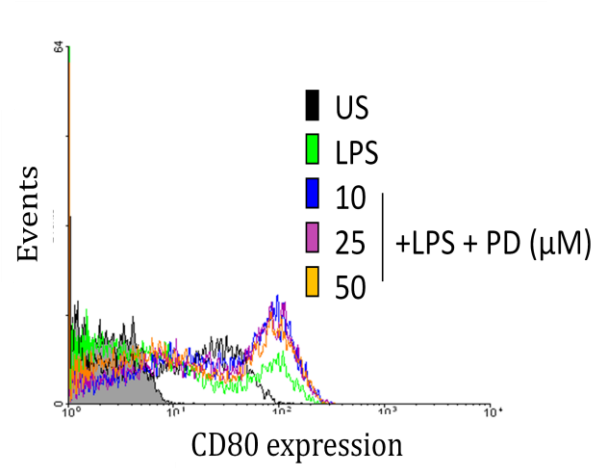

Events
US
LPS
10
25
50
+LPS + SP (μM)
CD86 expression

Figure 20: **ERK MAPK does not regulate the LPS-induced CD80 and CD86 surface expression.**
A: PD98059 inhibits phospho-ERK expression.
B and C: Inhibition of pERK reduced CD80 surface and mRNA expression.
D: Inhibition of pERK reduced CD86 surface expression.
MDMs ($1x10^6$ cells/mL) were treated with indicated concentrations of PD98059 for 2 hours, followed by LPS stimulation for A) 15 mins, B) and D) 24 hours and C) 4 hours. A) Biological activity assay of PD inhibitor measured by Western Immunoblotting. CD80 surface and mRNA expression was measured by Flow Cytometry (B) and qRT-PCR (C), respectively. Similarly, CD86 surface and mRNA expression was also measured by Flow Cytometry (D). Values in bar graph are means of at least three independent experiments ±SEM; *p≤0.05; **p≤0.005; ***p≤0.0005 as measured by One-Way Anova with at least n=3.

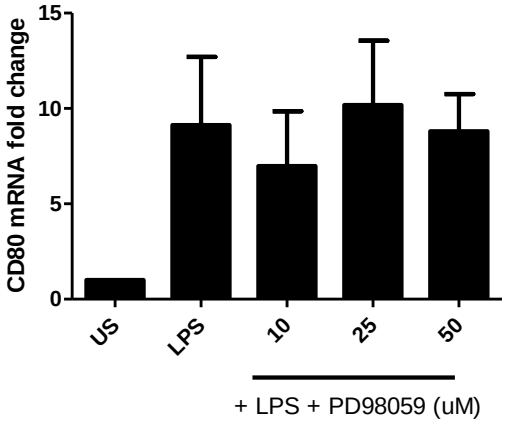
A


+LPS + PD (μM)
US
LPS
10
25
50
44 kDa –
pERK
37 kDa –
tERK

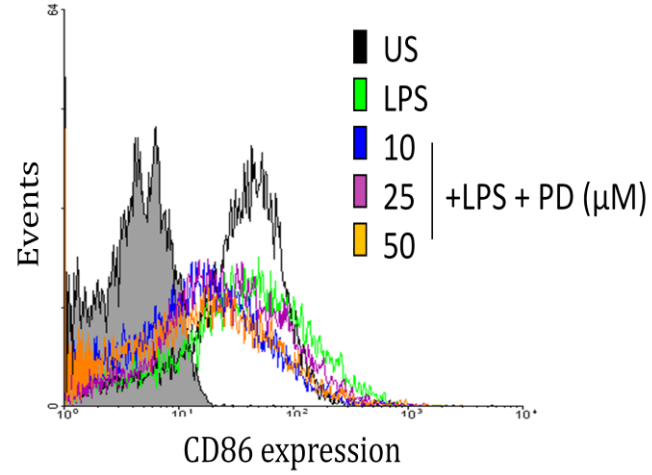
B


Events
US
LPS
10
25
50
+LPS + PD (μM)
CD80 expression

C


CD80 mRNA fold change
15
10
5
0
US
LPS
10
25
50
+ LPS + PD98059 (uM)

D

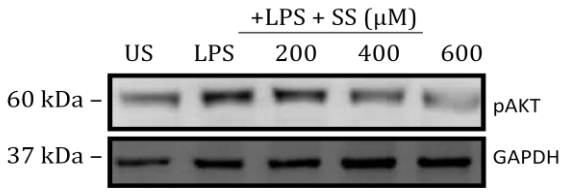

Events
US
LPS
10
25
50
+LPS + PD (μM)
CD86 expression

mimetics to block IAPs, I demonstrated that IAPs regulate PI3K (Figure 21C) and p38 MAPK activation (Figure 21D). Furthermore, since PI3K has been shown to regulate p38 phosphorylation in a murine model (133), I investigated whether inhibition of PI3K would alter p38 activation. For this, I inhibited PI3K using LY294002 and measured p38 phosphorylation by Western Immunoblotting, demonstrating that PI3K positively regulates p38 activation (Figure 21E). Taken together, these results suggest that upon LPS stimulation, activation of SHP-1 and IAP molecules activates PI3K pathway which further activates p38 MAPK, and together these molecules regulate CD80 and CD86 expression.

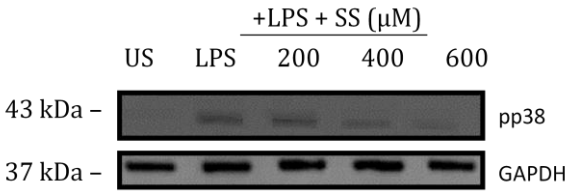
Figure 21: **SHP-1 and IAP activity regulate the activation of AKT/p38 signalling axis in LPS-stimulated MDMs.**

MDMs ($1x10^6$ cells/mL) were treated with indicated concentrations of sodium stibogluconate (SS), SMAC mimetics, and LY294002 followed by LPS stimulation for 15 min. Phosphorylation of AKT (A) and p38 MAPK (B) molecules after treatment with SS, and after treatment with SMAC mimetics (C and D) were measured by Western Immunoblotting. P38 MAPK phosphorylation after LY treatment (E) was also measured by Western Immunoblotting.

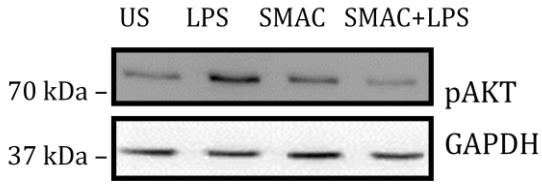
A


+LPS + SS (μM)
US
LPS
200
400
600
60 kDa –
pAKT
37 kDa –
GAPDH

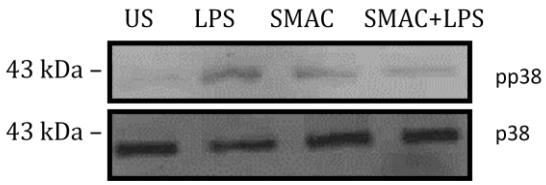
B


+LPS + SS (μM)
US
LPS
200
400
600
43 kDa –
pp38
37 kDa –
GAPDH

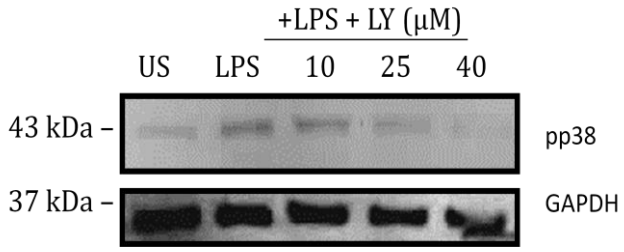
C


US
LPS
SMAC
SMAC+LPS
70 kDa –
pAKT
37 kDa –
GAPDH

D


US
LPS
SMAC
SMAC+LPS
43 kDa –
pp38
43 kDa –
p38

E


+LPS + LY (μM)
US
LPS
10
25
40
43 kDa –
pp38
37 kDa –
GAPDH

4.1 Discussion

CD80 and CD86 play a key role in the activation of effective immune responses, and as such in the modulation of diseases (136). Expressed on the surface of APCs including macrophages, these costimulatory molecules bind to their cognate receptors, CD28 and CTLA-4, resulting in the activation, proliferation, and differentiation of T cells. In the absence of CD80 or CD86 engagement, T cells are unable to produce IL-2 or proliferate in response to antigen, and instead enter a state of anergy or undergo apoptosis (30). While it was once believed that the binding of B7 molecules to CD28/CTLA-4 represented a one way signal, resulting in T cell activation, more recently, this interaction has been investigated as a bidirectional signaling process. CD80 and CD86 were shown to transduce signals to DCs, suggesting that these proteins act as signaling receptors (67, 68). Hence, CD80 and CD86 induction on macrophages may impact the development of an immune response. LPS, a TLR-4 ligand is a potent inducer of cytokines, including IL-10 and the IL-12 family of cytokines, and a variety of surface molecules including CD80 and CD86 on macrophages. Therefore, understanding the regulation of CD80 and CD86 expression, and decoding their potential as signaling receptors within macrophages may provide new avenues for therapeutic treatment of autoimmune disorders.

The focus of this project was twofold: 1) to investigate the role of CD80 and CD86 as signaling receptors capable of transmitting extracellular signals, and 2) to determine the TLR-4 activated pathways that regulate CD80/CD86 expression in human macrophages. I show for the first time that CD80/CD86 activation synergizes with TLR-4 signaling to produce IL-27 and IL-10 in human MDMs. I have also demonstrated that cIAPs play a key role in TLR-4-mediated signaling, as inhibition of IAPs with SMAC mimetics inhibited LPS-induced IL-10 and IL-27 production in MDMs, but these preliminary results suggested that this synergistic relationship may not be subject to regulation by

IAPs. Furthermore, I have shown that LPS-induced CD80 and CD86 expression was regulated by cIAP2-SHP-1-RIP1-TRAF2 complex through AKT-activated p38 MAPK in human macrophages.

To investigate the role of CD80 and CD86 as signaling receptors, I employed anti-CD80 and anti-CD86 antibodies and measured the production of IL-10 and IL-12 family of cytokines. Anti-CD80/anti-CD86 antibodies have been shown to neutralize CD80/CD86-induced IL-2 secretion in T cells by binding to the CD80/CD86 binding pocket. These antibodies represent an experimental model for the engagement of CD28/CTLA-4 by these B7 molecules. My results demonstrate that activation of CD80/CD86 alone did not induce expression of any of the four cytokines investigated. This is in contrast to DCs where stimulation with anti-CD80/CD86 antibodies induced IL-6 expression through the activation of the PI3K pathway (67). This difference could be attributed to possibly higher surface expression of these molecules exhibiting stronger activation. This could also suggest significant differences between the responses and signaling pathways in macrophages versus DCs. Further studies are warranted to look at other cytokines, including IL-6. Jain *et al* demonstrated that CD86 stimulation enhanced the effect of TLR-2 stimulation on resting B cells (41). I, therefore, hypothesized that CD80/CD86 synergizes with other signaling pathways activated by other ligands. In particular, I investigated the role of LPS/TLR-4 pathway, because of its ability to activate several signaling cascades. Since LPS is a potent mitogen, I employed suboptimal concentrations of LPS (10 ng/mL) to investigate the crosstalk between the CD80/CD86 and the TLR-4 signaling pathways. Surprisingly, treatment of cells with anti-CD80/CD86 antibodies and LPS induced significantly higher levels of IL-10 and IL-27 production, as compared to LPS-stimulated cells alone, without affecting the production of IL-12 and IL-23. My results clearly suggest that although CD80/CD86 activation on its own did not activate macrophages to induce IL-10 and IL-12 family of cytokines, CD80/CD86 activation synergizes with TLR-4 pathway to induce production of IL-10 and IL-27. TLR-dependent synergistic activity has been recognized in murine macrophages and DCs previously. Makela *et al*

showed that stimulation of TLR-8 together with TLR-3 or TLR-4 in monocyte-derived DCs led to synergistic IL-10, IL-12, IL-6 and TNF-α mRNA expression and cytokine production (137). Synergistic TNF- α expression has also been seen in murine macrophages in response to TLR-9 and TLR-4 stimulation (138).

While IAPs were originally identified as having a primary role in the prevention of cell death, IAPs have now been identified as key players in the TLR-4 pathway. cIAP1 and cIAP2 are involved in signal transduction pathways, in particular TNF-α-mediated NF-κB activation (87). To analyze the role of IAPs in innate immune signaling, I used SMAC mimetic chemical compounds. SMAC mimetics were designed as a means of counteracting the activity of IAPs and inducing cell death, particularly because of the high level of IAPs found in certain forms of malignancies, such as leukemia (139). I have demonstrated that 50 nM/mL of SMAC mimetics is sufficient to exhibit degradation of IAPs in human macrophages, without inducing cell death. In this study, I demonstrated for the first time that IAPs mediate the IL-10 and IL-27 secretion in human MDMs. In murine macrophages, IAPs have been shown to regulate the LPS induced production of proinflammatory cytokines such as IL-6 and TNF-α (140). However, inhibition of IAPs did not alter IL-10 production in murine macrophage (105). In contrast, my results show that IAPs positively regulate LPS-induced IL-10 expression in human MDMs, suggesting that the role of IAPs may differ across species. The role of IAPs has also been investigated in signaling through another class of PRRs, NOD-like receptor activated signaling pathways (128). Downstream of NOD is RIP2, and similar to their role in TLR signaling, cIAPs are required for ubiquitination of RIP2, which in turn is required for the activation of NF-κB. As result, depletion of IAPs was shown to result in reduced cytokine and chemokine secretion in response to stimulation of NOD receptors in murine cells (141).

Due to their role in TLR/NOD signaling, I investigated whether IAPs were involved in the CD80/CD86-TLR-4 synergistic regulation of IL-10 and IL-27 production. Treatment with SMAC

mimetics alone did not affect the CD80/CD86 signaling pathway. Furthermore, these preliminary results suggest that SMAC mimetics had no significant effect on the CD80/CD86-TLR-4 synergistic pathway. Taken together, these results suggest that IAPs inhibit LPS-induced IL-10/IL-27 expression without any impact on CD80/CD86-mediated activation of human MDMs. Further studies are required to confirm this role for IAPs. In particular, my results could be substantiated by IAP-specific siRNAs and by exploring the far-reaching effects of the CD80/CD86 pathway through the measurements of other cytokines related to inflammation, such as IL-6 and TNF-α. While these results demonstrate for the first time the existence of a CD80/CD86 signaling pathway in macrophages, further studies are required to fully elucidate this pathway and the molecules involved specifically with the use of Fc chimeras of CTLA-4 and CD28. This would present a more specific model for the interaction between CD80/CD86 and CD28/CTLA-4.

Activation of the TLR-4 pathway has also been implicated in the upregulation of CD80 and CD86 expression on the surface of monocytes and dendritic cells (8, 22, 123). Initiation of an inflammatory response following association of LPS with TLR-4 results in the induction of cellular responses to Gram-negative bacterial infections. In monocytic cells, LPS stimulation modulates the expression of costimulatory molecules CD80 and CD86 (21, 121). Since these molecules play a crucial role in the activation of T cells, the LPS-enhanced CD80 and CD86 expression may provide a mechanism for the amplification of T cell activation, and the augmentation of the immune response. The molecular mechanism by which the expression of CD80 and CD86 is controlled is poorly understood in human macrophages. In particular, the role of signaling molecules, such as cIAPs, SHP-1, RIP1 and TRAF2, and signaling pathways, such as MAPK and PI3K, have not been investigated.

Since I have demonstrated the role of IAPs in the induction of IL-10 and IL-27 via the LPS/TLR-4 signaling, I extended my studies by examining the involvement of IAPs in modulating the

LPS-induced CD80 and CD86 expression. My results showed that blocking IAP expression by either SMAC mimetics or cIAP2-specific siRNA led to a reduced expression of CD80 and CD86 after LPS stimulation. These results suggest that IAPs regulate LPS-induced CD80 and CD86 expression.

Since IAPs form a complex with RIP1 and TRAF2 and regulate downstream transcription factor NF-κB (129), it was of interest to determine if these molecules also played a role in the regulation of CD80 and CD86 expression. Using RIP1-specific and TRAF2 –specific siRNA, I demonstrated that RIP1 and TRAF2 knockdowns demonstrated a significantly lower surface expression of CD80 and CD86 in MDMs, as compared to MDMs transfected with control siRNAs. Taken together, my results suggest that IAPs, RIP1, and TRAF2 regulate LPS-induced CD80 and CD86 surface expression.

It has been shown that binding of LPS to TLR-4 induces tyrosine phosphorylation of signaling molecules and activates protein tyrosine kinases (PTKs) (113). The competing activities of protein tyrosine phosphatases (PTPs) and PTKs regulate the phosphorylation of key signaling molecules (113). My results demonstrate that PTP SHP-1, through the activation of the PI3K pathway and the p38 MAPK pathway, is a required positive regulator of the LPS-induced expression of CD80 and CD86 in MDMs. The involvement of SHP-1 was demonstrated by employing pharmacological inhibitors, such as sodium stibogluconate and SHP-1 specific siRNAs. It should be noted however that the relatively low transfection efficiency of siRNA shown in Western Immunoblots may explain the incomplete abrogation of LPS-induced CD80 and CD86 expression.

Since it has been shown that cIAPs, RIP1, and TRAF2 form a complex in MDMs, I investigated the inclusion of SHP-1 in this complex. Using confocal microscopy, I demonstrated that SHP-1 co-localizes, as judged by co-immunofluorescence, with cIAP2, RIP1 and TRAF2. Moreover, my results further show that cIAP2, RIP1 and TRAF2 co-immunofluoresce. These results suggest that cIAP2-RIP1-TRAF2-SHP-1 together co-localize and may form a complex within the macrophage to regulate

the LPS-induced CD80 and CD86 expression. These results need to be further confirmed by co-immunoprecipitation studies.

It is well established that LPS stimulation results in the activation of the MAPK and PI3K pathways in most cell types including monocytes and macrophages (133). Recently, MAPKs were identified as key modulators involved in the regulation of CD80 and CD86 expression in monocytes and dendritic cells. For example, Kim *et al* demonstrated the mandatory role of p38 MAPK in the upregulation of CD80 and CD86 expression in Vitamin C treated DCs (142). Similar results were obtained after stimulation with LPS and TNF-α, where inhibition of p38, but not ERK MAPK was shown to inhibit maturation of human blood-derived DCs, preventing upregulation of both CD80 and CD86 (123). In support of these results, our laboratory has also demonstrated the role of LPS-induced MAPKs in the regulation of CD80 and CD86 expression in human monocytic cells (8, 22). Lim *et al* have identified the roles for p38 MAPK in the regulation of CD86 expression in human monocytes(121). Furthermore, JNK MAPK was shown to be involved in the LPS-induced CD80 and CD86 expression in human promonocytic THP-1 cells (8, 121). PI3K has also been identified as a key modulator in LPS-induced CD80/CD86 expression. In particular, PI3K was shown to upregulate CD86 expression in Epstein Barr Virus-transformed B cells after ligation with CD40/CD40 homodimer (143). More recently, IL-21-induced CD86 expression has also been recognized as a PI3K-dependent mechanism in B cells (144), further demonstrating the crucial role that this pathway plays. In light of these observations, I investigated the role of the MAPK and PI3K pathways in the regulation of CD80 and CD86 expression in human macrophages in the context of upstream cIAP2-RIP1-TRAF2-SHP-1 complex. Inhibition of PI3K and p38 MAPK by LY294002 and SB203580, and by AKT-specific and p38-specific siRNAs, respectively, significantly decreased surface expression of both CD80 and CD86 in LPS-stimulated MDMs. Similar studies were employed to investigate the role of other MAPKs, JNK and ERK, but inhibition of these proteins with specific inhibitors showed no effect on the CD80 or

CD86 surface or protein expression. These results were also confirmed using JNK1/2-specific and ERK-specific siRNAs (data not shown), suggesting the specific role of p38 MAPK in the regulation of CD80/CD86 surface expression. Taken together, these results suggest that the PI3K pathway and p38 MAPK are positive regulators of the LPS-induced CD80 and CD86 surface protein expression.

PTP SHP-1 and IAPs are critical proteins for the activation of signaling molecules downstream of the TLR-4. For example, SHP-1 has been identified as a key regulator in the activation of ERK1/2 MAPK and NF-κB in bone marrow derived macrophages (116). Furthermore, IAPs regulated LPS-induced p38 MAPK expression (128). I have demonstrated that in human MDMs, IAPs, SHP-1, p38 MAPK and PI3K pathways are crucial for the LPS-induced upregulation of CD80 and CD86 expression. To elucidate whether IAPs and SHP-1 regulate CD80/CD86 expression through p38 MAPK and PI3K activation, I inhibited SHP-1 and IAP expression using sodium stibogluconate and SMAC mimetics respectively, and examined the level of phosphorylation of p38 MAPK and AKT by Western Immunoblotting. I demonstrated that SHP-1 and IAPs are necessary for the LPS-induced activation of the p38 MAPK and PI3K pathways.

The PI3K pathway has been shown to enhance LPS-induced MAPK expression in monocytes and murine macrophages (133, 145). To determine if PI3K and p38 MAPk constitute two distinct pathway or a single pathway to induce CD80/CD86 expression, I inhibited the PI3K pathway and showed that p38 MAPK activation is inhibited. This suggests that the LPS-induced CD80 and CD86 activation in human MDMs occurs through a complex formed between cIAP2-RIP1-TRAF2-SHP-1, which activates the PI3K pathway, and subsequently p38 MAPK to upregulate CD80 and CD86 surface expression.

While I recognize that the drawback of using inhibitors to demonstrate their role is their non-specific nature and toxicity at higher concentrations, I have examined the viability of my cells treated with various concentrations of these inhibitors by staining apoptotic cells with propidium

iodide (PI). I have also used low concentrations of each inhibitor and ensured that effects shown were dose-dependent. In addition, viable cells observed using FACS analysis were gated accordingly to eliminate apoptotic cells. I have also confirmed all of my findings with the use of specific siRNAs. For the experiments involving SMAC mimetics, I used a very low dose (50 nM/mL) to ensure IAP degradation, without the induction of cell death. As a result, I can conclude that none of the inhibitors used in my studies caused apoptosis at any concentration and did not exert nonspecific effects at the concentration used.

4.1.1 Regulation of CD80 and CD86 at the transcriptional level

Understanding the regulation of gene expression at the transcriptional level is crucial requirement to completely elucidate the pathway by which CD80/CD86 expression is regulated. To confirm the results obtained at the transcriptional level, I treated MDMs for 2 hours with their respective inhibitors or 24 hours with SMAC mimetics, followed by LPS stimulation for 4 hours, the required time for the measurement of transcripts (146-148). RNA was isolated using RNeasy Plus Mini Kit and RT-PCR was used to measure transcript levels. The mRNA results obtained were not consistent with the results obtained at the protein level. As suggested by my committee members, I am incorporating these results in the appendix along with its discussion in this chapter.

4.1.2 CD80 Transcription

Treatment with LPS resulted in an increase in CD80 mRNA transcription, similar to CD80 protein levels (Figure 24A). However, IAPs and SHP-1 did not have a significant effect on LPS-induced CD80 mRNA expression (Figure 25A and Figure 25C, respectively). On the other hand, inhibition of PI3K and p38 MAPK in LPS-stimulated MDMs resulted in a reduced CD80 protein and mRNA expression (Figure 15C and Figure 17C, respectively).

4.1.3 CD86 Transcription

Unlike the upregulation of CD86 protein expression after LPS treatment, CD86 mRNA expression was surprisingly decreased after treatment with LPS (Figure 24B). IAPs and SHP-1 appeared to have no significant role in the CD86 mRNA expression (Figure 25B and Figure 25D, respectively). Furthermore, using LY294002, SB203580 and PD98059, the inhibitors specific for PI3K, p38 MAPK and ERK MAPK, respectively, were not involved in the CD86 mRNA expression (Figure 26A-D). These results were inconsistent with protein results that demonstrated that IAPs, SHP-1, PI3K and p38 MAPK were required for CD86 surface expression (Figure 8A, Figure 12A, Figure 16A, and Figure 18A, respectively). It is also inconsistent with previous results that demonstrated that LPS stimulation resulted in increased CD86 surface expression. Investigating the role of JNK MAPK in the regulation of CD86 expression demonstrated that, surprisingly, JNK MAPK negatively regulated CD86 mRNA transcription (Figure 26C). Inhibition of JNK MAPK with SP600125 resulted in a dose dependent increase of CD86 mRNA, but there were no significant changes in CD86 protein expression after inhibition of JNK (Figure 19D).

The differential regulation of gene expression at the post-transcriptional or post-translational level is widespread in eukaryotes (149). It is a fundamental process for the synthesis, assembly and localization of the macromolecular structures in cells, and is achieved through a highly interconnected program regulated at diverse levels (150). For example, translational repressors, such as eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs) have been shown to govern the generation of induced pluripotent stem cells (iPSCs) (151) through the addition of a 5' guanine cap which significantly increased mRNA stability and translational efficiency (151). The protein half-life is a major post-translational factor that can vary from a few seconds to a few days and is dependent on the intrinsic protein stability, the post-translational modifications, such as phosphorylation and ubiquitination (152).

More recently, the advancement in technologies related to mRNA/protein analysis has demonstrated that the correlation between expression of mRNA and protein is relatively poor, attributed to the differential regulation of mRNA stability, expression, and degradation (153). For example, Fournier *et al* assessed the expression of proteins following rapamycin treatment (153) and demonstrated that of the 56 proteins that exhibited increased expression, 26 proteins did not show correlation with an increase in corresponding mRNAs, some even demonstrating a significant decrease in mRNA expression (153). They concluded that some proteins were more stable in the presence of rapamycin, rather than in its absence and as a result, the increased protein expression was not attributed to an increase in mRNA. Further studies are needed to investigate this discordance between mRNA and protein expression.

These studies can be expanded by examining the stability of the CD80 and CD86 mRNA and protein and by determining their half-lives using actinomycin D and cycloheximide to inhibit transcription and protein biosynthesis, respectively. The reduced CD86 mRNA expression and increased protein expression after LPS treatment could be explained by a long half life for the CD86 protein. The lower mRNA transcription could address the moderate increase in surface protein expression.

One of the drawbacks of using qRT-PCR is the normalization and reference genes used. In these experiments, β-actin was used as the housekeeping gene as it remains constant despite treatments, and all transcript levels were quantified relative to β-actin. Using expression microarrays could present another method of assessing CD80/CD86 mRNA as it provides an absolute transcript concentration (152). Further studies are required to better understand this differential regulation at the protein and transcriptional level.

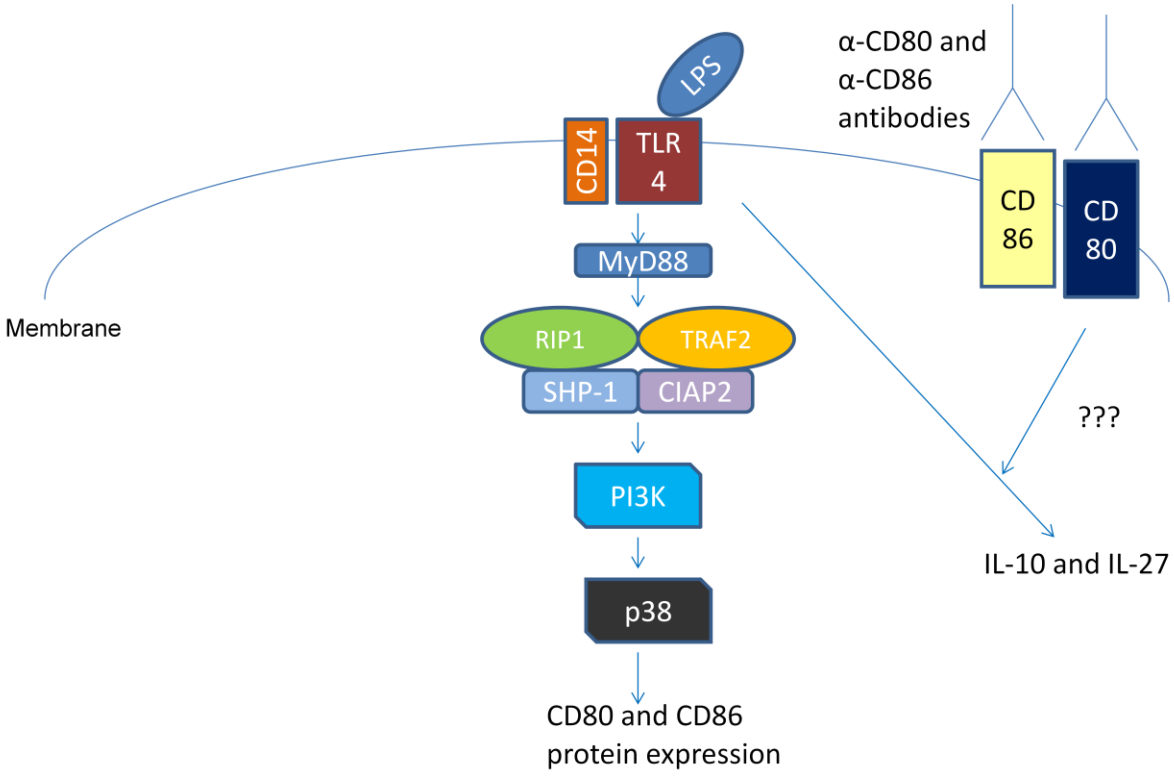
In summary, the focus of this project was to investigate the role of CD80 and CD86 as a signaling molecule and to determine the proteins that regulate their expression in human

macrophages. I have shown for the first time that activation of the CD80 or CD86 molecules synergizes with TLR-4 signaling to produce IL-27 and IL-10 in human macrophages. I have also demonstrated that inhibition of IAPs by SMAC mimetics diminished LPS-induced IL-10 and IL-27 expression. Finally, I have shown that cIAP2-SHP-1-RIP1-TRAF2 complex regulate the activation of AKT/p38 MAPK signaling, and this pathway regulates the expression of CD80 and CD86 in human macrophages.

Concluding Remarks, Future Directions and Significance

The activation of T cells requires the costimulatory signals provided by CD80 and CD86 molecules on the surface of APCs (127). CD80 and CD86 have a critical role in the activation of an effective immune response and the modulation of diseases (136). This study has demonstrated for the first time that activation of the CD80/CD86 pathway synergizes with the TLR-4 pathway to induce IL-10 and IL-27 production in human macrophages, but not IL-12 and IL-23 (Figure 22). I have also shown for the first time that while IAPs regulate the TLR-4-induced IL-10 and IL-27 production, they are not involved in the CD80/CD86-TLR-4 synergistic regulation of IL-10 and IL-27 in macrophages. These preliminary results call for further studies employing the Fc chimeras for CTLA-4 and CD28, which would provide a more representative model for the interaction between CD80/CD86 and their receptors CD28/CTLA-4. Furthermore, the investigation into the role of IAPs in the TLR-4 and CD80/CD86 pathways could be confirmed using siRNAs. The synergy between CD80/CD86 and TLR-4 pathway did not appear to regulate IL-12 and IL-23 expression, which could suggest that the pathway that regulates the IL-12 and IL-23 expression is independent of this CD80/CD86-TLR-4 pathway that regulates IL-10 and IL-27 expression, but further studies would be required to better understand how these cytokines are modulated. To explore this, studies could be conducted looking at the role of this synergistic pathway in the modulation of other cytokines, such as IL-6 which has been shown to be modulated by CD80 and CD86 signaling pathways in DCs (67). Furthermore, future studies could focus on the synergistic relationship of CD80/CD86 with other pathways. For example, TLR-4 and TLR-3 are partially analogous as both receptors trigger TRIF-mediated signaling, resulting in Type I IFN production (154). Studies could investigate whether activation of CD80/CD86 could synergize with TLR-3 or other TLRs to induce other cytokines. Furthermore, it would be of interest to explore the pathways activated by CD80/CD86 molecules, such as JAK/STAT, PI3K or MAPKs.

Figure 22: **The working model for the CD80/CD86 activation, and the pathway that governs their activation.** Upon LPS stimulation of TLR-4, cIAP2-SHP-1-RIP1-TRAF2 form a complex and induce AKT-activated p38 MAPK to regulate CD80 and CD86 expression. Activation of CD80 or CD86 pathway synergizes with the TLR-4 pathway to produce IL-10 and IL-27 in human macrophages.


α-CD80 and
α-CD86
antibodies
LPS
CD14
TLR
4
CD
86
CD
80
MyD88
Membrane
RIP1
TRAF2
SHP-1
CIAP2
???
PI3K
IL-10 and IL-27
p38
CD80 and CD86
protein expression

In light of this crucial role for CD80 and CD86 in the regulation of downstream cytokine expression, I investigated the molecules that govern their regulation in response to LPS. Through the use of specific inhibitors and siRNAs, I have determined that cIAP2, SHP-1, RIP1, and TRAF2 molecules are positive regulators of the CD80 and CD86 surface protein expression. Furthermore, I have demonstrated that the p38 MAPK and PI3K pathway positively regulate the CD80 and CD86 expression in macrophages. My results have also demonstrated that JNK and ERK MAPK are not involved in the regulation of CD80 or CD86 surface protein expression. Taken together, I have demonstrated that macrophages employ the cIAP2-SHP-1-RIP1-TRAF2 complex to regulate activation of the AKT/p38 signaling axis downstream of TLR-4 after LPS stimulation, and together, this pathway positively regulates the CD80 and CD86 surface protein expression (Figure 22).

At the transcriptional level, I have demonstrated that treatment with LPS resulted in an increase in CD80 mRNA, but IAPs and SHP-1 did not have a significant effect on LPS-induced CD80 expression. This is in contrast to CD80 protein expression, which was shown to be positively regulated by both IAP and SHP-1. Inhibition of the PI3K and p38 MAPK pathways showed consistent mRNA and protein expression. I have also shown that LPS stimulation resulted in increased CD86 protein levels, but surprisingly, decreased CD86 mRNA expression. I have also shown that while JNK MAPK does not appear to regulate the expression of these costimulatory molecules at the protein level, it does appear to negatively regulate CD86 mRNA expression. This could suggest a separate pathway by which the expression of CD80 and CD86 is reduced. The RNA/protein discordance suggests that further studies are required to better understand this differential regulation at the protein and transcriptional level. It would also be of interest to identify the transcription factors involved in the context of the pathways described.

The differential regulation of gene expression at the post-transcriptional or post-translational level is widespread in eukaryotes (149). The protein half-life is a major post-

translational factor that can vary from a few seconds to a few days (152). My results can be expanded by examining the stability of the CD80 and CD86 mRNA and protein by determining their half-lives. Furthermore with the advancement in technologies related to mRNA/protein analysis, expression microarrays could be used to assess CD80/CD86 mRNA as it provides an absolute transcript concentration (152). Further studies are required to better understand this differential regulation at the protein and transcriptional level.

In conclusion, my studies have attempted to identify the pathways that govern the CD80 and CD86 expression and the bidirectional signaling in macrophages. My results demonstrate that activation of CD80 or CD86 pathways synergize with the LPS/TLR-4 pathway to activate IL-10 and IL-27 expression. Furthermore, CD80 and CD86 protein expression is regulated by a cIAP2-SHP-1-RIP1-TRAF2 complex through AKT-activated p38 signaling axis. Understanding the regulation of CD80/CD86 molecules, and the bidirectional signaling in macrophages, could provide insight into possible therapeutic interventions for the treatment of cancer and autoimmune diseases.

Appendix

Figure 23: **Characterization of monocyte-derived macrophages**. PBMCs (4 x 10^6 cells/mL) were plated and supplemented with 10 ng/mL of macrophage colony stimulating factor (M-CSF) for 6 days. Cells were washed and stained with PE-conjugated antibodies for 15 minutes. Flow Cytometry was used to assess surface expression of CD83, CD80, CD16, CD11a, CD11b, CD11c, and HLA-DR. Results were generated by Dr. Aurelia Busca.

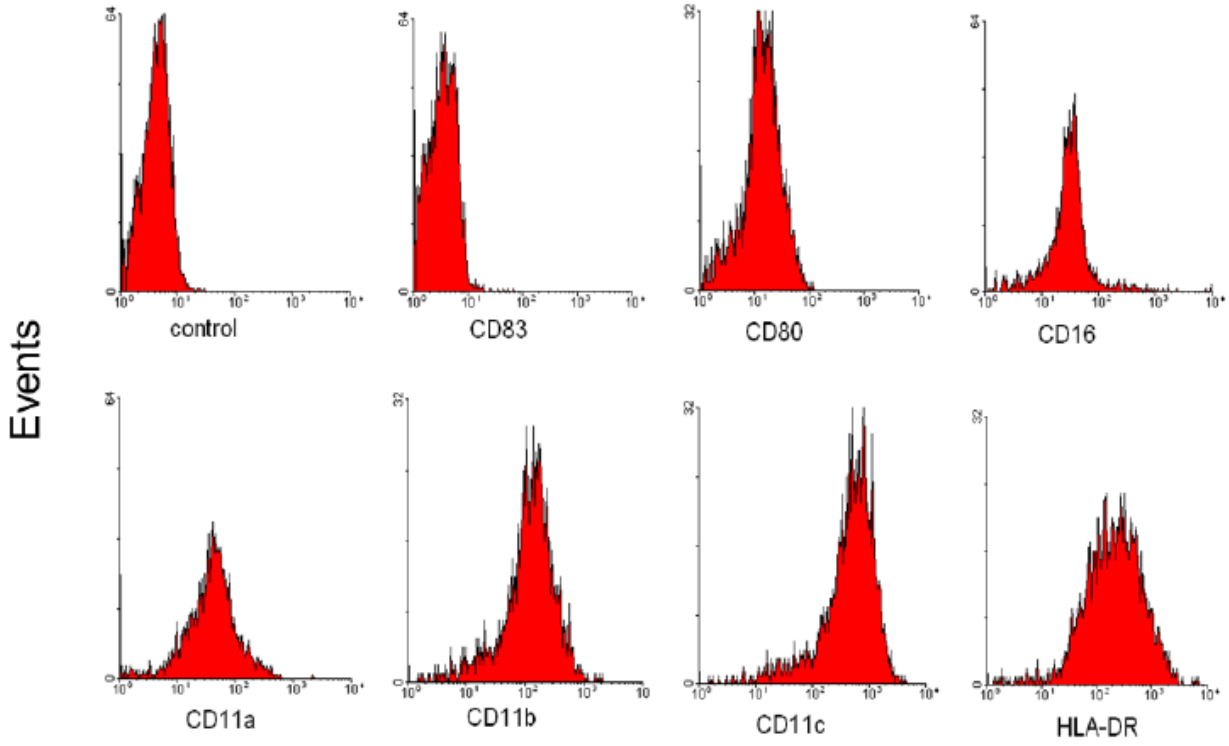
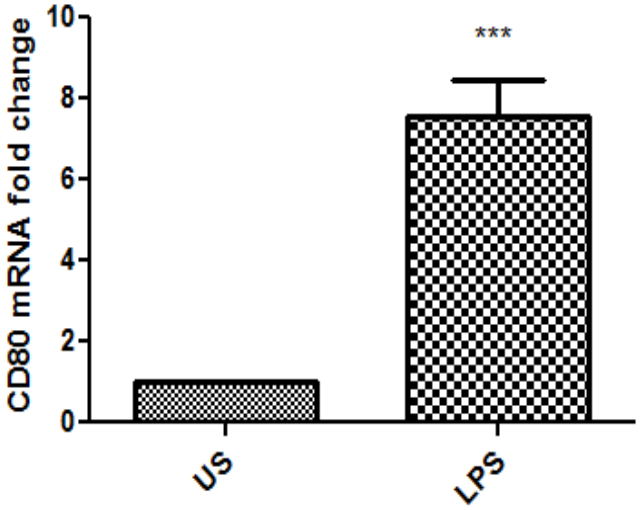

Events
control
CD83
CD80
CD16
CD11a
CD11b
CD11c
HLA-DR

Figure 24: **CD80 and CD86 mRNA expression after treatment with LPS**. MDMs were treated with LPS for 4 hrs. qRT-PCR was used to measure CD80 (A) or CD86 (B) mRNA expression. Values in bar graph are means of at least three independent experiments ±SEM.

A


CD80 mRNA fold change
10
8
6
4
2
0
***
US
LPS

B

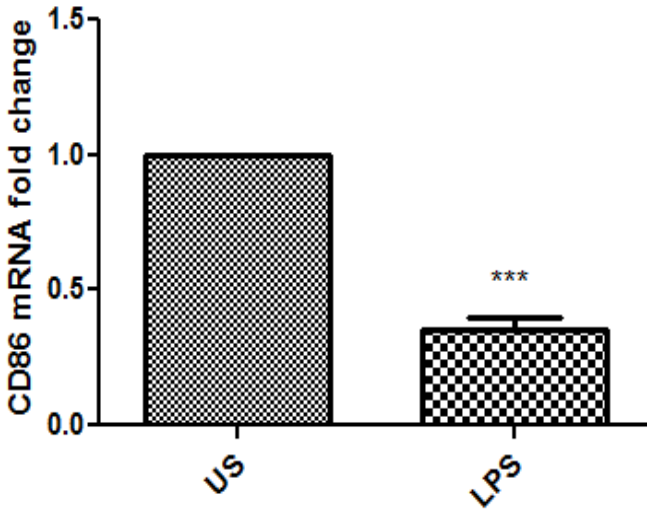

CD86 mRNA fold change
1.5
1.0
0.5
0.0
***
US
LPS

Figure 25: **CD80/CD86 mRNA expression after treatment with SMAC mimetics or sodium stibogluconate**. MDMs were treated with their respective inhibitors for 24 hrs, followed by LPS stimulation for 4 hrs. qRT-PCR was used to measure CD80 (left side) or CD86 (right side) mRNA expression. Values in bar graph are means of at least three independent experiments ±SEM.

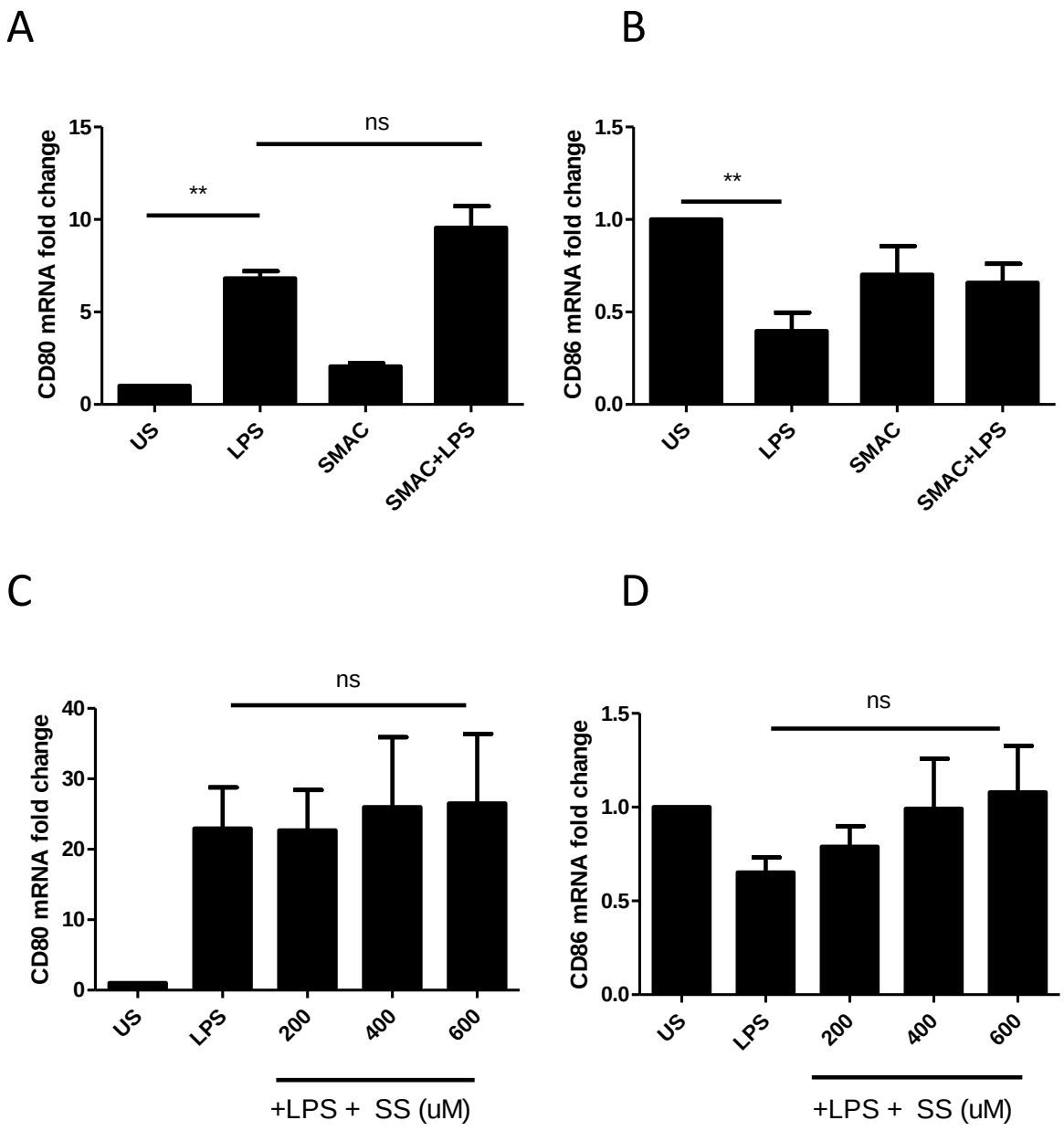
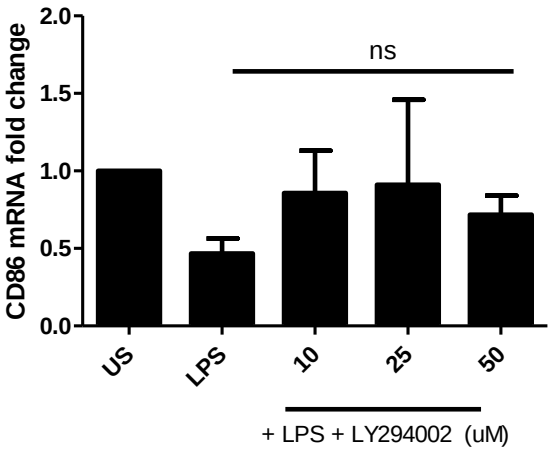
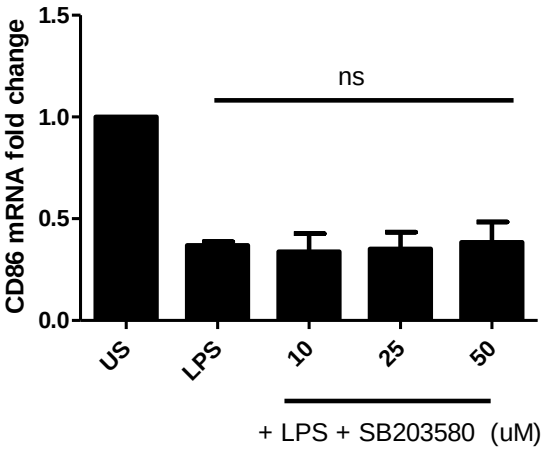

A
B
C
D
CD80 mRNA fold change
CD86 mRNA fold change
ns
**
US
LPS
SMAC
SMAC+LPS
200
400
600
+LPS + SS (uM)

Figure 26: **CD86 mRNA expression after treatment with PI3K and MAPK inhibitors.** MDMs were treated with their respective inhibitors for 2 hrs, followed by LPS stimulation for 4 hrs. qRT-PCR was used to measure CD86 mRNA expression. Values in bar graph are means of at least three independent experiments ±SEM.

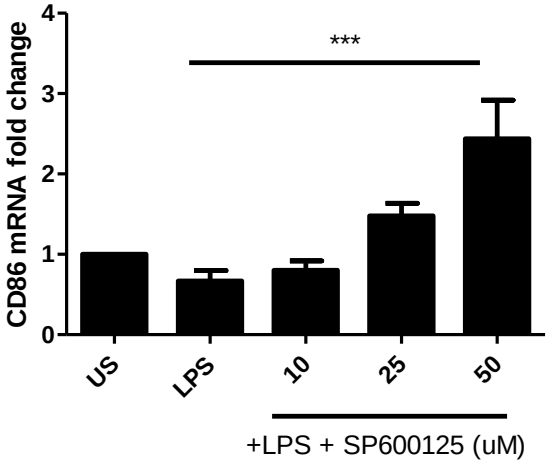
A


CD86 mRNA fold change
2.0
1.5
1.0
0.5
0.0
ns
US
LPS
10
25
50
+ LPS + LY294002 (uM)

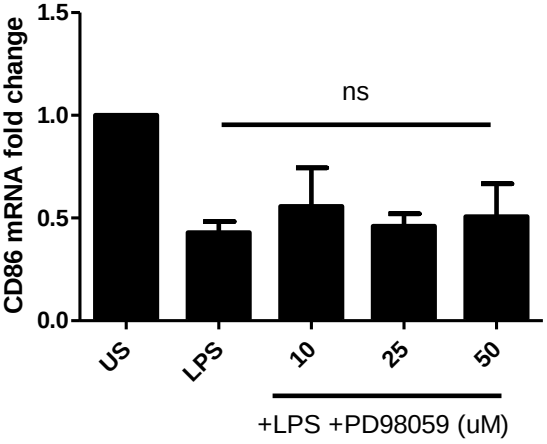
B


CD86 mRNA fold change
1.5
1.0
0.5
0.0
ns
US
LPS
10
25
50
+ LPS + SB203580 (uM)

C


CD86 mRNA fold change
4
3
2
1
0
***
US
LPS
10
25
50
+LPS + SP600125 (uM)

D


CD86 mRNA fold change
1.5
1.0
0.5
0.0
ns
US
LPS
10
25
50
+LPS +PD98059 (uM)

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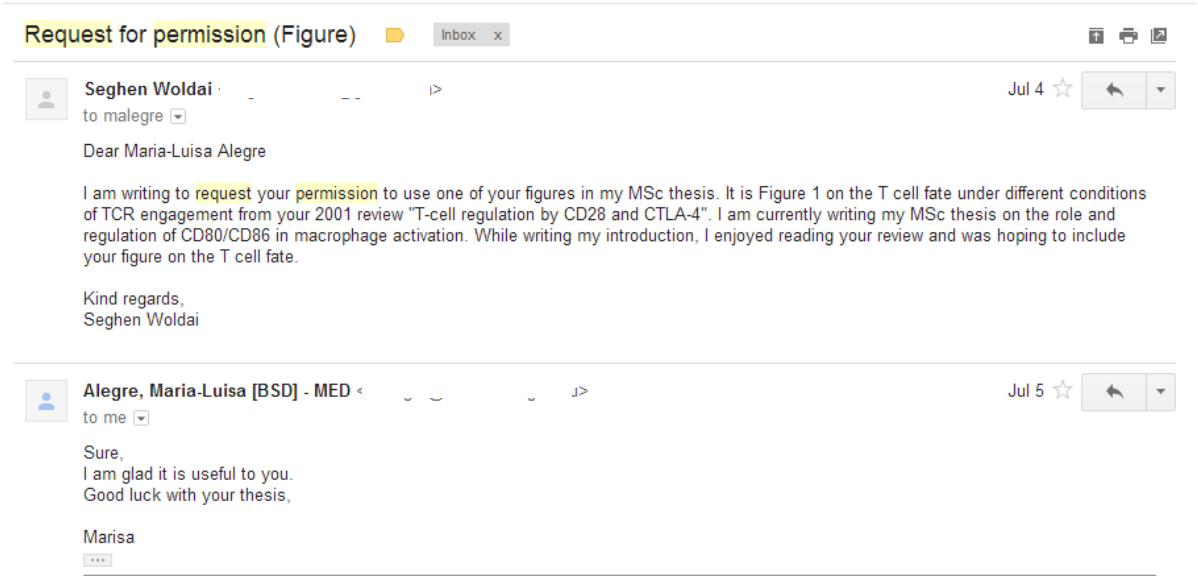

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Seghen Woldai · Jul 4

to malegre

Dear Maria-Luisa Alegre

I am writing to request your permission to use one of your figures in my MSc thesis. It is Figure 1 on the T cell fate under different conditions of TCR engagement from your 2001 review "T-cell regulation by CD28 and CTLA-4". I am currently writing my MSc thesis on the role and regulation of CD80/CD86 in macrophage activation. While writing my introduction, I enjoyed reading your review and was hoping to include your figure on the T cell fate.

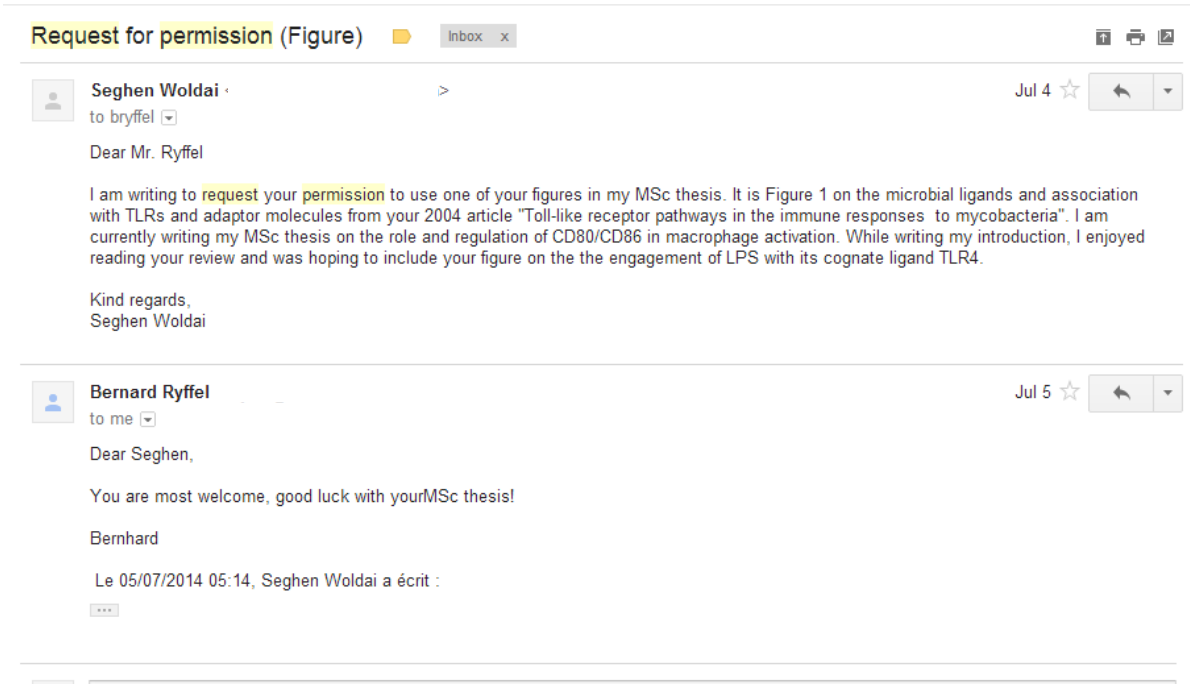
Kind regards,
Seghen Woldai

Alegre, Maria-Luisa [BSD] - MED · Jul 5

to me

Sure,
I am glad it is useful to you.
Good luck with your thesis,

Marisa



Request for permission (Figure) Inbox x

Seghen Woldai Jul 4

to bryffel

Dear Mr. Ryffel

I am writing to request your permission to use one of your figures in my MSc thesis. It is Figure 1 on the microbial ligands and association with TLRs and adaptor molecules from your 2004 article "Toll-like receptor pathways in the immune responses to mycobacteria". I am currently writing my MSc thesis on the role and regulation of CD80/CD86 in macrophage activation. While writing my introduction, I enjoyed reading your review and was hoping to include your figure on the the engagement of LPS with its cognate ligand TLR4.

Kind regards,
Seghen Woldai

Bernard Ryffel Jul 5

to me

Dear Seghen,

You are most welcome, good luck with yourMSc thesis!

Bernhard

Le 05/07/2014 05:14, Seghen Woldai a écrit :