

**Inhibition of liver and bone marrow derived dendritic cell maturation and function by
Interleukin-6 activation of Signal Transducer and Activator of Transcription-3**

by

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Dendritic cells (DC) are professional antigen presenting cells bridging the innate and adaptive immune systems by detecting pathogen- and- damage associated molecular pattern (PAMP, DAMP) molecules. This triggers maturation and migration to regional lymph nodes where they stimulate T lymphocytes. In tissues normally exposed to relatively high level of PAMP molecules, such as the liver, DC have a higher threshold to stimulation and therefore maintain an immature phenotype under conditions that would normally stimulate DC maturation. In these studies we tested the hypothesis that interleukin-6(IL-6)/Signal Transducer and Activation of Transcription-3(STAT3) activity increases the activation/maturation threshold of hepatic and bone marrow (BM) DC towards innate immune signals.

Results show that liver nuclear STAT3 activity is significantly higher than other organs and is IL-6-dependent. Hepatic DC in normal wild-type (IL-6^{+/+}) mice are phenotypically and functionally less mature than DC from IL-6-deficient (IL-6^{-/-}) or STAT3 inhibited IL-6^{+/+} mice, as determined by surface marker expression, pro-inflammatory cytokine secretion, and allogenic T-cell stimulation. IL-6^{+/+} liver DC produce IL-6 in response to exposure to PAMPs, but resist maturation compared to IL-6^{-/-} liver DC. Conversely, exogenous IL-6 inhibits LPS-induced IL-6^{-/-} liver DC maturation. Oral antibiotic depletion of commensal gut bacteria in IL-6^{+/+} mice decreased portal blood endotoxin levels, lowered IL-6/STAT3 activity and significantly increased liver DC maturation.

BM derived IL-6^{+/+}DC with elevated STAT3 activity are also significantly less mature than IL-6^{-/-} BMDC. Blocking STAT3 activity increases maturation in IL-6^{+/+} BMDC but not in IL-6^{-/-} BMDC. IL-6^{+/+} BMDC cultured in the presence of elevated IL-6 conditions further decreases maturation and can be reversed by IL-6 neutralization. Compared to IL-6^{-/-} BMDC, IL-6^{+/+} BMDC significantly resisted maturation in response to low concentrations of the PAMP molecules. At higher concentrations of these same ligands stimulation of both IL-6^{+/+} and IL-6^{-/-} BMDC induced maturation.

In conclusion, elevated IL-6/STAT3 activity raises the threshold needed for DC to translate triggers of innate immunity into adaptive immune responses. In the liver, gut-derived bacterial products stimulate hepatic IL-6/STAT3 signaling thereby inhibiting hepatic DC activation/maturation. Manipulating gut bacteria or IL-6/STAT3 activity may therefore be an effective strategy to alter intra-hepatic immune responses.

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PREFACE

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1.0 INTRODUCTION

The ability to translate triggers of innate immunity in peripheral organs and tissues into adaptive immune responses in lymphoid tissues is critical for the protection of organs and organisms from immunological threats. The cells that perform this task are essential for preventing infection and limiting damage, but they also must be tightly regulated as to prevent inappropriate responses to innocuous stimuli that could cause unnecessary, persistent inflammation. Dendritic cells (DC) are antigen-presenting effector cell stimulators that can also induce tolerance. However, the mechanisms controlling DC maturation are unclear. This dissertation will focus on the regulation of DC maturation by signaling through the cytokine IL-6, thereby setting a maturation threshold that must be overcome in order to generate an adaptive immune response.

1.1 DENDRITIC CELLS

Dendritic cells (DC) are the most potent, professional antigen presenting cells (APC) in the body bridging the innate and adaptive immune systems and also inducing tolerance (1-3). DC arise from bone marrow (BM) derived hematopoietic precursor cells (4-8). These DC precursors enter peripheral organs and lymphoid tissue from the circulation where they develop into immature migratory or resident lymphoid DC, respectively (9-11). Additionally, monocytes can function

as DC precursor cells by extravasating into inflamed tissues and differentiating into DC (11-14). Immature DC are remarkable capturers of potential antigenic material in peripheral tissues and, as APC, mature DC can efficiently present antigen to T cells in the lymph nodes. In fact, DC are 10 to 100 times better T cell stimulators than other APC, including monocytes and B cells (15). At the same time, unstimulated, immature DC can induce tolerance in T cells (3, 16).

1.1.1 Dendritic Cell Subsets

DC can be divided into several subtypes (9, 17, 18). The simplest groupings distinguish normal mouse DC in the steady state into pre-cursor DC (pre-DC) or conventional DC (cDC). The steady state refers to the immune system of a normal, specific pathogen-free mouse kept in barrier conditions (9). Pre-DC do not have DC function or morphology, but can develop into DC without cell division. This typically occurs once a progenitor cell has left the bone marrow and entered the blood or peripheral tissues (9). Examples of pre-DC include immature plasmacytoid DC (pDC) and monocytes. cDC are functional DC, acting as sentinels in peripheral tissues and APC in the lymph nodes. In the mouse several different subtypes of cDC exist, with almost all identified by expression of the α_X integrin, CD11c (19). The additional expression of B220 and Gr1 identifies pDC from cDC (20). An exception to this, however, are the liver lymphoid-related DC expressing CD205 and B220 but not CD11c (21).

In humans continually exposed to immunological insults, a steady state akin to that in mice might not be possible, and this might be why identifying human DC subsets to the extent that has been performed in mice has been difficult. Only two human DC subtypes have been identified: myeloid DC expressing $CD11c^+CD14^-CD1a^+$ and pDC with a cell surface profile of $CD11c^-BDCA2^+CD123^+$ (17, 22).

Mouse cDC have been separated based on cell surface expression, location and function (9, 17). Lymphoid tissue cDC can be distinguished based on the expression of CD8 α and CD11b as lymphoid (CD8 α^+ CD11b $^-$) and myeloid (CD8 α^- CD11b $^+$) DC subsets (23-25). CD4 expression has also been used to distinguish subsets of mouse cDC (23). The resident cDC in lymphoid organs typically remain there throughout their lifespan. cDC in peripheral, non-lymphoid, tissues are migratory cells. They exist as immature DC in peripheral tissues and transit to the lymph nodes via the lymphatics upon maturation. The cell surface expression profile of migratory DC in peripheral tissues mimics that of resident lymphoid tissue DC with both CD8 α^+ CD11b $^-$ lymphoid and CD8 α^- CD11b $^+$ myeloid subsets identified (9). However, the function of migratory DC is greatly influenced by their resident tissue microenvironment. For instance, the function of DC from human skin have greater allostimulatory and reduced IL-10 secretion than liver DC (26).

pDC exist as pre-DC in the steady state and, upon activation, acquire the morphology and phenotype of mature DC, including stimulating T-cell proliferation and cytokine production, especially type-I interferons (20, 27). pDC are activated predominantly upon detection of viral particles or bacterial and viral unmethylated cytidine phosphate guanosine (CpG) containing DNA or oligonucleotides via toll-like receptor (TLR) 7 and 9 ligation (28-30). In contrast to mature pDC, immature pDC are poor stimulators of T-cell proliferation, rather they induce anergic T cells secreting the anti-inflammatory cytokine IL-10 (31). Additionally, CpG stimulated pDC have been reported to stimulate the generation of IL-10 secreting CD4 $^+$ CD25 $^+$ regulatory T cells (32).

A final class of cDC arise from monocytes during inflammation and these DC are aptly referred to as inflammatory DC (9, 11-13, 18, 33). These DC can be distinguished by the

expression of CD11b in the absence of CD8 α and CD4 (11). Inflammatory DC are typically not observed in the lymphoid tissues during the steady state, but do migrate to lymph nodes in the course of inflammation (11). However, DC expressing CD11b have been identified in non-lymphoid organs, including the livers of normal mice in the absence of inflammation (34). Lastly, the most common in vitro model of DC using BM cells cultured with GM-CSF and IL-4 generate DC that closely resemble inflammatory DC (35).

1.1.2 Dendritic Cells as Sentinels

In peripheral tissues, immature DC serve the essential roles of sampling the tissue environment and detecting potential innate pathogenic antigen. If “danger” is detected, DC translate this innate message to the cells of the adaptive immune system so an appropriate, specific immune response can be mounted. This process is depicted in Figure 1 and detailed below.

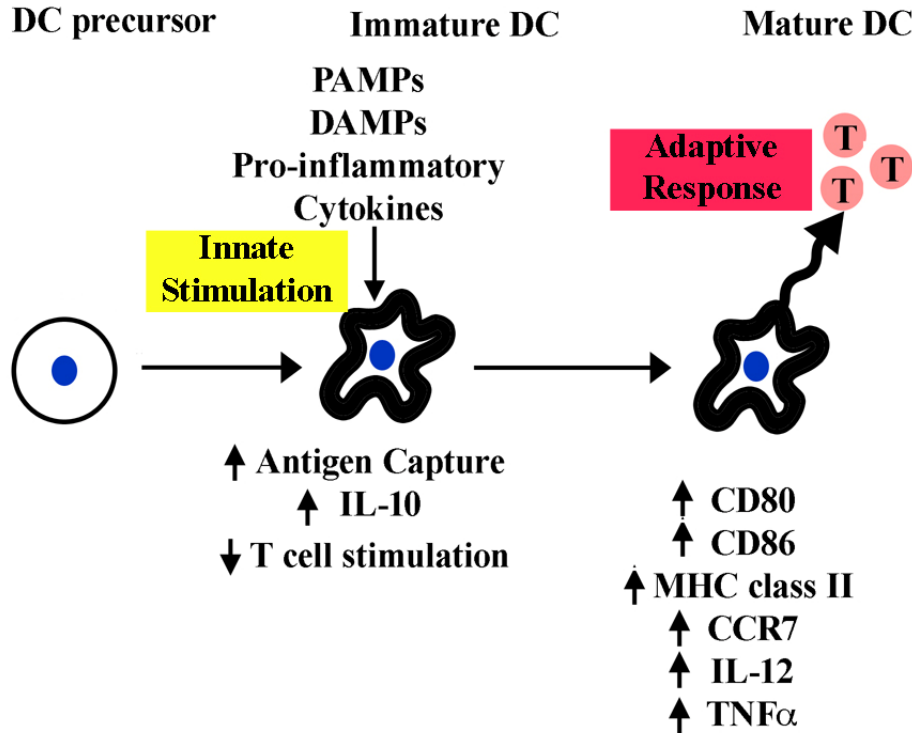


Figure 1. A simplified scheme for DC development and maturation. DC precursors enter peripheral tissues becoming immature DC with elevated antigen capturing ability, anti-inflammatory cytokine (e.g. IL-10), and decreased T cells stimulation capacity. Upon recognition of an innate stimulus, such as PAMPs, DAMPs, or pro-inflammatory cytokines, DC are induced to mature. Following chemokine mediated migration to the lymph nodes, DC can generate an adaptive response stimulating T cells.

1.1.2.1 Antigen Capture

In tissues of peripheral organs, immature migratory DC are the most common DC type and act as sentinels for innate immune triggers. This is accomplished by their ability to capture, engulf, and process endogenous and exogenous antigenic material. Uptake of putative antigen occurs through several mechanisms including phagocytosis, macropinocytosis, and receptor mediated endocytosis using C-type lectin receptors (mannose receptor, DEC-205) and Fc γ receptors (CD64, CD32) (1). The multiple mechanisms possessed by DC for capturing antigen ensures a broad sampling of the tissue microenvironment for potential antigenic material.

1.1.2.2 Detection of Innate Immunity Stimulators

Immature DC are armed with multiple ways to identify and respond to stimulators of innate immunity. These include detecting both exogenous pathogen associated molecular pattern (PAMP) molecules and endogenous damage associated molecular pattern (DAMP) molecules. Multiple types of immunostimulatory PAMP and DAMP molecules have been identified. Examples of PAMP molecules include bacterial and viral products such as bacterial cell wall components (lipopolysaccharide (LPS), peptidoglycans (PGN), pathogenic lipoproteins), Flagellin, fungal zymosan, bacterial and viral CpG containing DNA, and single and double stranded viral RNA (including polyinosinic–polycytidylic acid (poly I:C)).

DAMP molecules are endogenous molecules released from cells following disruption of the plasma membrane typically during necrotic cell damage. They can also be the by-products of degraded extracellular molecules. In normal circumstances, DAMP molecules are intracellular or extracellular molecules with physiological roles. However, the ectopic expression of these molecules can be immunostimulatory. Examples include the extracellular expression of high mobility group box-1 protein (HMGB-1), heat shock proteins, IL-1 α , IL-18, uric acid, ATP and S100 proteins (36-38). Additionally, degraded extracellular matrix including heparin sulfate and hyaluronan act as DAMP molecules (39). Collectively, these endogenous or exogenous triggers of innate immunity and tissue injury have been termed “alarmins” (40).

PAMP and DAMP molecules are detected by pattern recognition receptors (PPR). These proteins are located either on the cell surface, in endosomes and lysosomes, or in the cytosol of DC (40). A summary of PRR molecules and their ligands is summarized in Table 1.

Table 1. Pattern recognition receptors (PRR) and their PAMP and DAMP ligands.

PRR ^a Family	Receptor	Ligands	PRR Family	Receptor	Ligands
TLR ^b			RLR ^g		
	TLR1	Triacyl lipopeptides		RIG-I	dsRNA ssRNA
	TLR2	Lipoprotein/lipopeptides Peptidoglycan Lipoteichoic acid Atypical LPS ^c Zymosan Glycoinositolphospholipids Glycolipids Heat Shock Protein 70 Hyaluronan HMGB-1 ^d		MDA	dsRNA poly I:C
	TLR3	dsRNA poly I:C ^e	NLR ^h		
	TLR4	Lipopolysaccharide Heat shock protein 60 Heat shock protein 70 Lipoteichoic acid Fibronectin Hyaluronan HMGB-1		NOD1 ⁱ	Meso- diaminopimelic acid
	TLR5	Flagellin		NOD2	Muramyl dipeptide
	TLR6	Diacyl lipopeptides Lipoteichoic acid Zymosan		NALP3 ^j	Muramyl dipeptide Bacterial RNA ATP Uric Acid
	TLR7	ssRNA	RAGE ^k		
	TLR8	ssRNA		RAGE	HMGB-1 S100 proteins
	TLR9	Unmethylated CpG ^f DNA Chromatin IgG complex	P2 Receptors		
	TLR10	Unknown		P2X	ATP
	TLR11	Uropathogenic bacteria		P2Y	ATP

^a PRR: pattern recognition receptor; ^b TLR: toll-like receptor; ^c LPS: lipopolysaccharide; ^d HMGB-1: high mobility group box-1; ^e poly I:C: polyinosinic-polycytidylic acid; ^f CpG: cytidine phosphate guanosine; ^g RLR: RIG-I like receptor; ^h NLR: NOD-like receptors; ⁱ NOD: nucleotide oligomerization domain; ^j NALP3: NACHT domain-, leucine-rich-repeat-, and PYD containing protein 3; ^k RAGE: receptor of advanced glycation end products. The material in this table was compiled from the references (38, 40-45).

The number of PRR families that bind PAMP or DAMP molecules has recently been expanded. Historically, PAMP molecules were thought to be principally detected by the toll-like receptors (TLR) (41). These evolutionarily conserved proteins were first identified in *Drosophila* as critical molecules for development and subsequently implicated in anti-fungal responses (46). However, additional PRR molecules were found to participate in the recognition of PAMP and DAMP molecules including nucleotide oligomerization domain (NOD)-like receptors (NLR) (45), retinoid acid inducible protein-I (RIG-I) like receptors (RLR) (43, 44), the receptor of end stage glycation-products (RAGE) (40), and P2 Receptors (47).

Different PRR recognize PAMP and DAMP molecules in specific intracellular compartments or extracellularly. For instance, TLRs recognize PAMP or DAMP molecules either at the cell surface or in the lysosomal or endosomal compartments (42). Alternatively, NLR and RLR proteins detect bacterial and viral pathogens, respectively, in the cytosol (42). Cell surface RAGE can detect a host of DAMP molecules and P2 receptors sense extracellular ATP. These PRR provide an additional measure of detection for PAMP and DAMP molecules and may be responsible for producing an innate response in cells not expressing other PRR molecules. Immature DC also can be activated by endogenously produced cytokines (IFN α/β , TNF α (48)) and signaling molecules (CD40 ligand (49)).

Binding of PAMP and DAMP molecules to PRR initiates signaling through multiple intracellular signaling cascades. Most PRR signaling culminates with the activation of the transcription factor NF κ B, including TLR, NLR, RLR and RAGE. PRR-NF κ B signaling is primarily responsible for transcription of pro-inflammatory genes (40-42, 50). Additionally, the Interferon Response Factor (IRF)-3 and -7 are also activated upon viral RNA detection by TLRs

and RLRs and lead to the transcription of IFN α/β genes along with other cytokines (42). Detection of intracellular bacteria by NLR family members activates Caspase-1, in addition to NF κ B, and this is responsible for the cleavage of pro-IL-1 β into the active, mature IL-1 β (45).

The signal transduction after TLR ligand stimulation has been studied in depth. Ligand binding to most TLR members recruits a complex containing MyD88 and interleukin-1 receptor associated kinase (IRAK)-4 allowing for the phosphorylation of IRAK-1 and the binding of tumor necrosis factor receptor associated factor (TRAF)-6 (41, 50). TRAF-6 and phospho-IRAK-1 can then leave the membrane TLR-MyD88 complex and associate with transforming growth factor β activated kinase (TAK)-1 and TAK1 binding protein-1 (TAB1) and TAB2. This leads to the phosphorylation and subsequent ubiquitination of I κ B allowing NF κ B to translocate to the nucleus to stimulate gene transcription. Additionally, TLR activation of TRAF-6 can also induce nuclear translocation of IRF-5 and the TAK1 stimulation of JNK and p38 MAP Kinase activation leading to pro-inflammatory cytokine production (41, 50).

TLR signaling can be controlled by negative regulation of intracellular signaling and extracellular molecules. Many of these molecules are present only after an initial PAMP stimulation and represent a negative feedback to prevent continued inflammation or induce endotoxin tolerance (51). For instance, elevation of IRAK-M after LPS treatment can inhibit the dissociation of IRAK-1 from IRAK-4 thereby preventing downstream activation of TRAF-6 during subsequent exposure to LPS (52, 53). Additionally, a truncated alternative splice variant of MyD88, MyD88 short (MyD88s) is expressed after LPS exposure in monocytes and cannot bind to IRAK-4 (54). Both ST2 and SIGIRR can bind to intracellular regions of TLRs and interfere with their inability to either recruit intracellular adaptor molecules or facilitate TLR signaling (55, 56). Expression of these factors might be the basis for endotoxin tolerance.

1.1.3 Dendritic Cell Maturation

DC undergo maturation upon stimulation by PAMP or DAMP molecules. This is phenotypically characterized as reduced phagocytosis and increased chemokine mediated migration to lymph nodes where effector cell stimulation can occur. DC migration is typically dependent on the increased expression of the chemokine receptor CCR7 which detects CCL19 and CCL21, two chemokines critical for migration to the lymph nodes (57, 58). CCL21 is constitutively expressed by the lymphatic endothelial cells in peripheral tissues and the high endothelial venules in the lymph nodes, while CCL19 is produced by stromal cells in the T cell area of the lymph nodes (59-61).

Effector cell stimulation by mature DC is facilitated by increased cell surface expression of molecules used in antigen presentation, co-stimulation and cell-cell adhesion. The major histocompatibility complex (MHC) class I and II molecules present antigen engulfed by DC while in the immature state. Upon DC maturation, intracellular MHC class II molecules are rapidly expressed on the cell surface. This allows phagocytosized extracellular antigen to be presented to CD4⁺ T cells. MHC class I molecules primarily present endogenous cytosolic proteins to CD8⁺ T cells. However, DC are among the few cell types able to cross-present exogenous antigen engulfed by the cells on MHC class I molecules (62-65). Antigen in the endosomes or phagosomes can be released into the cytosol for proteosomal processing and subsequent expression on MHC class I complexes. Antigen loading onto MHC class I complexes can occur in the endoplasmic reticulum (ER) or in phagosomes which have fused with the ER and contain the machinery necessary for antigen loading on MHC class I complexes (62-65). Thus, DC can also stimulate CD8⁺ T cells with both endogenous and extracellular antigens.

Mature DC also express adhesion molecules that help maintain interaction with T cells and co-stimulatory molecules that reinforce the MHC-TCR stimulation. The most notable co-stimulatory molecules come from the B-7 family of proteins and include CD80 and CD86 (66-68). These cell surface proteins can interact with CD28 molecules on T cells, providing an activating signal to T cells. However, CD80 and CD86 can also bind to CTLA-4, when expressed on T cells thereby competing with CD28 for B-7 protein binding and, more importantly, providing a negative signal inhibiting T cell activation (69, 70). DC also express CD40 that can produce a reinforcing stimulus to DC upon binding with the CD40 ligand on T cells. DC maturation stimulates pro-inflammatory cytokine secretion, including IL-2, IL-12, IL-4, IFN α , IFN β , IFN γ , TNF α and IL-1 β , which provide growth factors for T cells along with the ability to influence T cell polarization (1, 2).

Mature DC can, therefore, provide three essential signals during the activation of T cells (71). Signal 1 provides the antigen specific MHC-T cell receptor stimulation, while Signal 2 is the co-stimulatory CD80 or CD86 interaction with CD28. Finally, cytokine secretion by DC influences the T cell polarization is termed Signal 3. This enables DC to stimulate antigen specific adaptive immune responses in lymphoid organs.

1.1.4 Tolerogenic Dendritic Cells

Unstimulated DC from peripheral tissues can induce tolerance (3, 16). Innocuous endogenous proteins and apoptotic cells are taken up and presented as potential antigen in lymph nodes by immature DC (72). However, immature DC are poor APC and do not stimulate T cell proliferation, rather they induce T cell anergy and deletion (73, 74). This is consistent with the contribution of DC to the elimination of self-reactive T cells in the thymus during negative

selection process of central tolerance (16, 75). Tolerogenic DC also influence immune responses by secreting anti-inflammatory cytokines, such as IL-10 and TGF β and expressing molecules such as indoleamine 2,3-dioxygenase (IDO) or programmed death ligand 1 (PD-L1) (76). Additionally, DC within the tumor microenvironment are unresponsive and tolerant to the tumor cells (77, 78). Increased STAT3 activity in the tumor or in the DC has been shown to contribute to this immune unresponsiveness (79-82). Tolerogenic DC are also considered potential cellular therapeutics to inhibit immune recognition or inflammation in autoimmunity and transplantation (76).

Maintaining DC tolerance and thereby preventing inappropriate DC maturation in response to PAMP molecules and/or innocuous antigen is an essential role in barrier tissues. Recent evidence suggests that factors secreted by the tissue microenvironment play an important role in preventing APC activation (83). For instance, in the lung, TGF β secreted by alveolar epithelial cells provides an immunosuppressive environment for lung macrophages, even though there is continual exposure to airborne PAMP molecules (84). Similar cytokine suppression of DC occurs at other barrier tissues such as the intestine with IL-10 (85, 86) and the skin with TGF β (87). Both of these cytokines are well known inhibitors of DC maturation (88-90). Thus, by preventing the maturation of DC, cytokines or other factors in the tissue microenvironment might elevate the threshold needed for DC to respond to PAMP molecules.

1.1.5 Liver Dendritic Cells

The phenotype of liver DC differs from that of DC in other peripheral organs, and these differences might allow these cells to contribute to hepatic tolerance. Considering the potential tolerogenic abilities of DC, it is reasonable to speculate that hepatic DC might also contribute to

the overall tolerogenic nature of the liver. The density of DC in liver tissue is lower compared to DC in other peripheral organs (91). However, due to the comparative larger size of the liver, the absolute number of liver DC is greater than other organs (91). Numerically, approximately $1-1.2 \times 10^6$ DC can be isolated from a single normal mouse liver (92, 93) and this represents about 1% of the total liver cells or approximately 20% of the total $CD45^+$ cells (94). In contrast, the percentage of $CD45^+$ cells that are DC in mouse pancreas, heart or kidney is greater than 50% (91). Thus, the total number of DC in the liver is higher compared to other organs, but on a per volume of tissue basis, the density of liver DC is less than other organs (91). The number of hepatic DC can be augmented by hematopoietic growth factor treatment, especially Flt-3 ligand (95) or GM-CSF (96). Intrahepatic DC can be localized to the portal tracts and subcapsular lymphatics, regions that contain the draining lymph ducts, but liver DC are only infrequently observed in the sinusoids (97).

Liver DC have been separated based on expression of $CD8\alpha$, $CD4$, $CD11b$, $NK1.1$ and $B220$ (21, 92, 93, 98, 99). Liver DC expressing $CD11c$ and $B220$ secreted IFN after CpG stimulation and likely represent the plasmacytoid DC (pDC) population (92, 93, 99). The mouse liver pDC population is approximately 5-fold higher than in the spleen (96, 99, 100). Studies have categorized mouse conventional DC into myeloid ($CD8\alpha^-CD11b^+$), lymphoid-related ($CD8\alpha^+CD11b^-$) subtypes, in addition to pDC (101). Additionally, Lu et al have described a unique $B220^+ CD11c^- CD205^+$ DC subtype identified from cultures of hepatic NPC with IL-3 and anti- $CD40$ antibody (21). In human livers, DC have been identified based on $CD11c^+$ (myeloid DC) and $CD123^+$ (plasmacytoid DC) expression, $BDCA1$ (myeloid) and $BDCA2$ (plasmacytoid) expression, as $CD83^+$ expressing cells with dendritic morphology, $CD1a$ expression and MHC class II expression (26, 102-104).

Liver DC are less potent T cell stimulators as compared to splenic DC (99, 105) or skin DC (26) and this may contribute to the overall tolerogenic properties of the liver. Liver DC remain able to respond to PAMP molecules, including LPS, CpG oligonucleotides and polyI:C, by stimulating T cell division and cytokine secretion (IFN α , IFN γ , IL-12, IL-6, TNF α) (92, 100, 106). However, these responses are significantly reduced compared to similarly treated splenic DC. The diminished responses of liver DC to PAMP molecules might be due to a decreased sensitivity to TLR stimuli. Indeed, the expression of the TLR4 gene, whose protein recognizes LPS, is reduced in liver DC compared to splenic derived DC (105). However, no differences in TLR 9 expression were observed between spleen and liver DC (106). Other intracellular regulators of TLR signaling, such as IRAK-M, might contribute to the reduced response of liver DC compared to spleen DC (34). Interestingly, liver and spleen DC respond equally when stimulated with anti-CD40 antibody (105). This suggests that liver DC are capable of maturation in response to some stimuli but not others.

Liver DC also have tolerogenic properties that are apparent, *in vivo*. Reducing the number of hematopoietic cell numbers, including DC in rat liver allografts by irradiation of the graft leads to rejection (107). Moreover, pancreatic islet allograft survival in mice is prolonged when donor liver derived DC are injected into mice 7 days before transplantation (108). Subcutaneous injection of allogeneic liver DC were capable of migrating to regional lymph nodes, but stimulated IL-10 and IL-4 production in mononuclear cells (109). However, when BMDC were injected they induced IFN γ secreting cells (109). This suggests that liver DC might preferentially induce Th₂ cytokines. In contrast to the studies where liver DC prolonged allograft survival, augmenting the number of donor hepatic DC in liver grafts by treating mice with Flt-3 ligand resulted in acute rejection of the liver allografts (110).

Hepatic DC have also been implicated in human liver diseases, including viral hepatitis B and C, primary biliary cirrhosis, and liver allograft rejection (111-115). In non-viral hepatitis diseases, little is known regarding DC function. In HCV and HBV, where DC have been studied in greater detail, peripheral blood DC are less responsive than in patients without disease (111-113). However, a recent study by Lai et al showed that intrahepatic DC from HCV infected patients had greater stimulatory capacity and reduced IL-10 secretion compared to DC from non-infected, inflamed livers (102). Additionally, this study showed a reduced number of pDC in infected livers, consistent with observations of pDC in the blood of HCV patients (102, 116, 117). The differences in myeloid DC maturation between these studies may be a consequence of where the DC are sampled from, with peripheral blood DC being phenotypically different from intrahepatic DC. Lastly, transfecting mouse DC with HBV antigen reduced the allostimulatory capacity of DC (118-120). However, HCV core or NS3 antigen can stimulate immune responses and pro-inflammatory cytokines (121-123).

1.2 THE LIVER

The liver is an organ with diverse functions that include metabolism, digestion, secretion of synthesized proteins, detoxification and excretion of metabolized xenobiotics, and immunological features. Hepatocytes, the main parenchymal cells in the liver are responsible for most of these liver functions. However, other cell populations facilitate hepatocyte functions. For instance, the highly fenestrated liver sinusoidal endothelial cells allow for hepatic blood rich in nutrients, xenobiotics and potential antigenic material contact with the hepatocytes. Biliary epithelial cells provide a barrier duct system isolating bile that contains cholesterol, metabolized

xenobiotics and toxins from the parenchymal cells where it can be damaging. Kupffer cells, the resident macrophage of the liver lining the sinusoids, assist in clearing macromolecules and PAMP molecules. The liver also contains a variety of immune cells that contribute to the immune function in the liver.

The functions of the liver are mainly the consequence of the anatomic location of the liver, being downstream of the gut venous blood flow. Thus, the venous system draining the intestines flows into the liver via the portal vein and comprises over 70% of the blood that perfuses the liver. The remaining blood supply is from the hepatic artery that primarily perfuses the biliary tree and mixes with the portal blood at the entrance to the sinusoids. Gut derived portal blood is rich in intestinal absorbed nutrients, xenobiotics, potential antigens and commensal bacterial PAMP molecules including LPS and CpG oligonucleotides. Therefore, an essential job of the liver is to filter toxic or immunostimulatory molecules from the portal blood prior to entering the general systemic circulation where it could potentially stimulate unwanted immunological responses.

1.2.1 Liver Immunology

The immune liver has distinct properties from other non-lymphoid peripheral organs (124-126). This affords the liver not only protection from potential antigenic material in the portal blood but also confers a state of immune privilege. For instance, liver allografts are spontaneously accepted across complete MHC mismatch in many species (127-130). Additionally, transplantation of non-liver organs are protected from immune attack when preceded by a liver transplant from the same donor (131). Furthermore, human liver allograft recipients require less immunosuppression and have fewer rejection episodes than recipients of other organs (132).

The liver also contributes to the generation of oral tolerance (133-140). Diversion of portal vein blood flow into the vena cava bypassing the liver, eliminates oral tolerance upon secondary challenge (139). Additionally, the direct infusion of antigen into the portal vein also generates systemic tolerance (141-145) while intravenous delivery does not (146, 147). This includes the ability to extend survival of skin, heart, kidney and small intestine grafts when recipients are given intraportal injections of donor cells (148-150). This implicates the liver and the hepatic immune system in generating systemic tolerance to antigens or allografts.

The hepatic tolerance might also have negative effects in that the liver is a permissive site for the perpetuation of pathogens. Infection with hepatotropic viruses such as hepatitis B and C can become chronic in some patients, however the viruses are resolved in others (151, 152). In contrast, hepatitis A viral infection is typically cleared in most patients (153). Liver plasmodium infection initiates an immune response but the infection is not cleared during malaria (154).

1.2.2 Cellular composition of the normal liver

The immunological properties of the liver are the result of a diverse and unique population of hepatic cells, most of which have immunological capacity. The cells of the liver can be divided into two populations, parenchymal and non-parenchymal cells. The liver parenchymal cell, the hepatocyte, accounts for approximately 70% of hepatic cells and is the main liver functional cell. The non-parenchymal cells are a heterogeneous population including liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC), dendritic cells (DC), biliary epithelial cells (BEC), stellate cells and multiple lymphocytes. The percentage of each cell type in the normal liver is shown in Figure 2 and the immunological role of each is discussed in detail below.

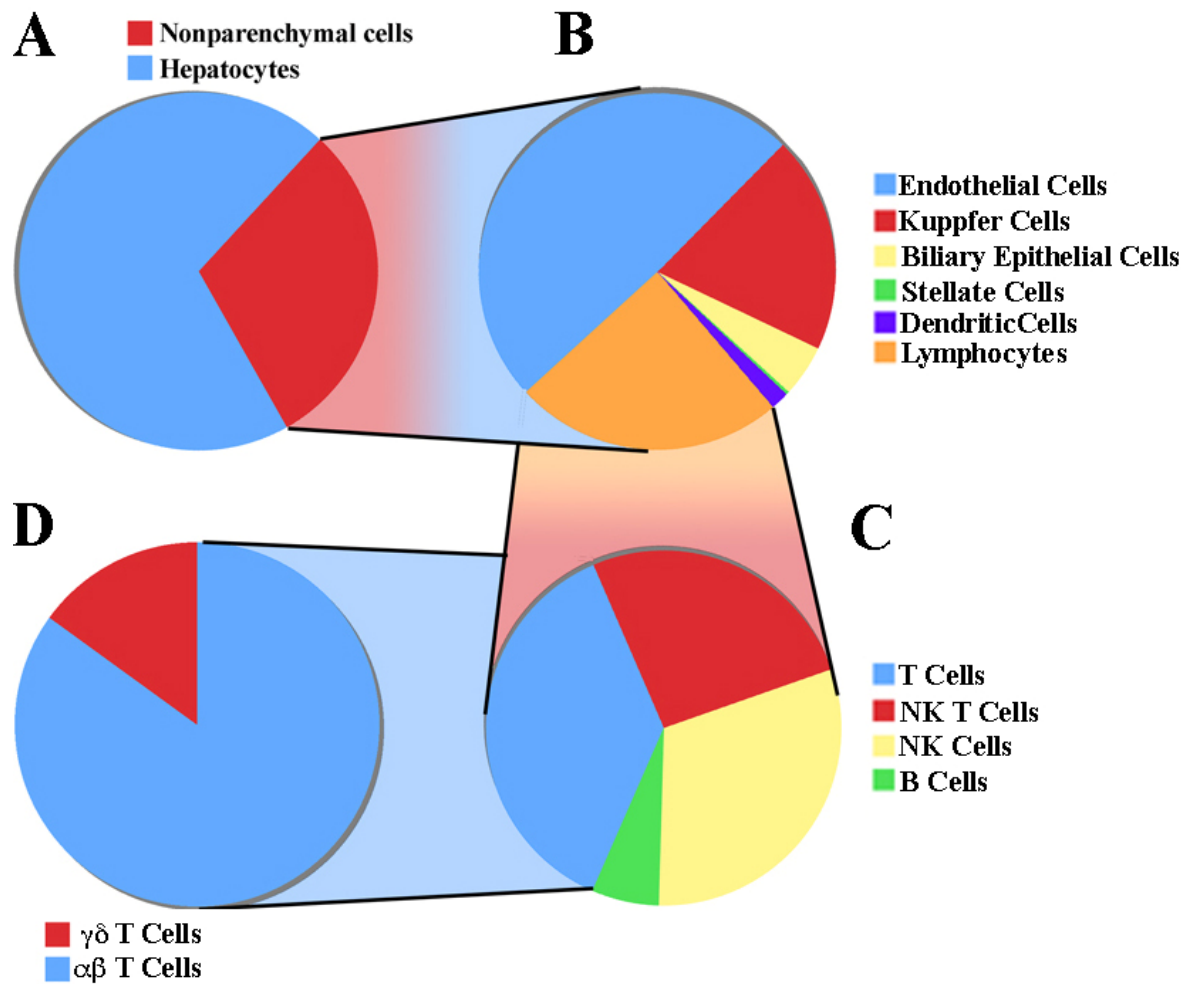


Figure 2. The percentage distribution of cells in the normal liver. **A)** Hepatocytes (70%) and non-parenchymal cells (30%). **B)** The separation of liver non-parenchymal cells. 50% of NPC are endothelial cells, 25% lymphocytes, 20% Kupffer cells, 5% biliary epithelial cells, 1% dendritic cells and less than 1% stellate cells. **C)** The composition of hepatic lymphocytes. 37% are T cells, 31% NK T cells, 26% NK cells and 6% B cells. **D)** The percentage of $\alpha\beta$ T cells (85%) and $\gamma\delta$ T cells (15%) in the liver. Percentages compiled from references (124-126).

1.2.2.1 Hepatocytes

The liver parenchymal cell, the hepatocyte, is the main functional cell in the liver and also has several known immunological characteristics. Mouse hepatocytes can express both MHC class I and II molecules, with the latter induced by IFN γ along with the co-stimulatory molecules, CD80 and CD86 (155-158). This suggests that hepatocytes might be able to act as APC. Indeed, naïve CD8⁺ T-cells can be activated in the liver by hepatocytes, but this activation leads to apoptosis (159-161). The activation and subsequent apoptosis of CD8⁺ T cells by hepatocytes has led to the theory that the liver is a site for elimination of CD8⁺ T cells (162). Hepatocyte microvilli can protrude into the sinusoidal lumen through the LSEC fenestrations and this might allow hepatocytes to interact with lymphocytes in the sinusoids (163).

A key role of hepatocytes in the innate immune response is the synthesis and release of acute phase response proteins. These include the increased secretion of C-reactive protein (CRP), serum amyloid A protein, α_2 -macroglobulin, fibrinogen, haptoglobin, α 1-antitrypsin, α 1-antichymotrypsin, α 1-cysteine proteinase, and complement components C3 and C9, along with decreased albumin secretion (164-167). Hepatocytes secrete acute phase proteins in response to stress-related cytokines such as interleukin (IL)-6 (IL-6), IL-1 and Tumor Necrosis Factor (TNF)- α (168). Thus, hepatocytes contribute to the systemic innate immune reaction against inflammation.

1.2.2.2 Liver sinusoidal endothelial cells

The hepatic sinusoids are lined with unique, highly fenestrated liver sinusoidal endothelial cells (LSEC). These represent the largest fraction (approximately 50%) of non-parenchymal cells in the liver. The fenestrations facilitate the interaction of the blood and hepatocytes along with

permitting the passage of lymphocytes into the space of Disse. Accordingly, LSEC express molecules allowing the adhesion of lymphocytes, including ICAM-1 (CD54), VCAM-1 (CD106) and VAP-1 (169-171). Additionally, the narrow space of the hepatic sinusoids and reduced blood flow help facilitate LSEC-lymphocyte interaction (172).

LSEC also have a great capacity to take up antigen via phagocytosis and receptor mediated endocytosis (173, 174). Additionally, LSEC also have constitutive, albeit low level, expression of MHC class II complexes along with the co-stimulatory molecules CD40, CD80 and CD86 (175, 176). These molecules are upregulated during liver damage and ischemia reperfusion injury (177, 178). Moreover, LSEC MHC class I complexes can also express exogenous antigen through cross-presentation mechanisms (179), but instead of activation, this leads to CD8⁺ T cell tolerance (180). Similar to other liver APC, LSEC MHC class II stimulation of CD4⁺ T cells results in cells being polarized towards a regulatory T cell phenotype that produces IL-10 and IL-4 (176). Thus, LSEC can be considered an intrahepatic resident APC contributing to the tolerogenic immune liver.

1.2.2.3 Kupffer cells

The largest pool of macrophages in the body are the liver Kupffer cells (125). These cells are primarily located in the periportal hepatic sinusoids but are also observed within the space of Disse. Kupffer cells are active phagocytes clearing material from the portal blood, including antigen, apoptotic cells and bacteria (181, 182). In the normal liver, portal blood bacterial endotoxin stimulates Kupffer cells to produce IL-6 (183, 184). In vitro, supraphysiological concentrations of exogenous LPS also stimulates Kupffer cell production of IL-10 and TNF α (185). Depleting Kupffer cells can suppress the generation of systemic tolerance to antigen

injected into the portal vein (186). Kupffer cells, therefore, might contribute to the tolerogenic properties of the liver.

As APC, Kupffer cells stimulate CD4⁺ T cell proliferation, but this response is less than generated by spleen or bone marrow-derived macrophages (185, 187). During liver injury, Kupffer cell production of pro-inflammatory cytokines (IL-12, IL-1 β , TNF α) and reactive oxygen species participates in the activation of innate immune responses and likely contributes to the progression of fibrosis during chronic liver diseases (165, 188).

1.2.2.4 Biliary epithelial cells

The biliary epithelial cells (BEC) create the duct system in the liver transporting bile from the hepatocytes into the intestines. This provides a critical barrier protecting the hepatic parenchyma from the toxins, xenobiotics, digestive components and PAMP molecules in bile. In the normal liver, BEC are immunologically quiescent, but do provide an innate ductal barrier (189). However, similar to the hepatocytes, inflammatory cytokines, such as IFN γ can induce expression of immunologically relevant molecules in BEC. These include the expression of MHC class I and II molecules (190), co-stimulatory molecules including CD40, CD80 and CD86 (191, 192), lymphocyte adhesion molecules such as LFA-3, VLA and ICAM-1 (193, 194), and other molecules such as Fas and Fas Ligand (189, 191, 195) and heat shock proteins (196-198).

BEC are also a frequent immunological target in the liver, as observed in primary biliary cirrhosis (199), acute and chronic allograft rejection (200), biliary atresia (201), and graft versus host disease (202). The susceptibility of BEC is not surprising considering their ability to express inflammatory proteins during inflammation and their close proximity with liver DC (34).

1.2.2.5 Hepatic stellate cells

Hepatic stellate cells, also known as Ito cells, are located within the hepatic space of Disse and are known for their storage of vitamin A and ability to differentiate into extracellular matrix secreting fibroblasts upon activation (203). These cells represent a small percentage of the total liver cells (Figure 2), however others have reported the percentage of stellate cells to be as high as 5-8% of the total liver cells (204). The discrepancies between studies might lie with the ability of these cells to divide in pathological situations upon activation (204).

Conflicting evidence has been reported describing the immune stimulating ability of stellate cells. *In vitro*, activated, but not quiescent stellate cells, can produce cytokines, express cell surface MHC class II complexes and co-stimulatory molecules, and could suppress T cell proliferation via up regulation of PD-L1 and expression of ICAM-1 (205, 206). *In vivo*, islets transplanted with activated stellate cells reduced infiltrating lymphocytes and extended graft survival (207). However, another study demonstrated that stellate cells are functional APC fully capable of stimulating CD4, CD8 and NK T cells by antigen expression on MHC class I and II complexes and CD1 (208). Considering the important contribution of activated stellate cells to the pathogenesis of liver disease, the findings of immunological functions of stellate cells might have significant impact on liver pathobiology. However, further investigation is needed to reconcile the true role of stellate cells in liver immunology.

1.2.2.6 Lymphocytes

The composition of liver lymphocytes differs significantly than the lymphocyte population in the blood. The liver is especially rich in non-traditional lymphocytes, such as natural killer cells (NK cells; CD3⁻CD56⁺), natural killer T cell (NKT cells; CD3⁺CD56⁺) and CD3⁺ T cells expressing the $\gamma\delta$ T cell receptor (TCR) ($\gamma\delta$ T cells). The increased population of NK and NK T

cells provides the liver with a high level of innate protection. The percentage of conventional $\alpha\beta$ TCR CD3⁺ T cells in the liver is lower than in the general circulation (37% versus 75%). Additionally, the CD4:CD8 T cell ratio is shifted toward more CD8⁺ T cells in the liver, while CD4⁺ T cells are more prevalent in the blood (209). There is also an increase in the percentage of T cells not expressing either CD4 or CD8 (double negative) or expressing both CD4 and CD8 (double positive) (209, 210). The percentage of B cells in the liver is similar to the blood.

Most of the CD8⁺ T cells in the liver have an activated phenotype expressing CD25 and CD69 (211). This is likely the result of the ability of the liver to harbor activated or apoptotic CD8⁺ T cells (212, 213). CD8⁺ T cells can be activated in the liver leading to an abbreviated life span and deficient effector function (214). However, CD8⁺ T cells activated in the lymph nodes are fully functional and can respond to antigen on hepatocytes (214). Thus the site of activation, intrahepatic versus the lymph nodes, can influence CD8⁺ T cell activity. Majority of the CD4⁺ T cells in the liver are polarized towards a T-helper 1 (Th₁) phenotype producing IFN γ and TNF α with only about 5% Th₂ CD4⁺ T cells (215).

1.3 INTERLEUKIN-6

1.3.1 Interleukin-6 (IL-6)

IL-6 is a member of a pleiotropic cytokine family also including leukemia inhibitory factor (LIF), oncostatin-M (OSM), cardiotropin-1 (CT-1), ciliary neurotrophic factor (CNTF), cardiotropin-like cytokine (CLC), interleukin-11 (IL-11) and interleukin-27 (IL-27) (216, 217). All of these cytokines signal through a common signal transducing receptor subunit, gp130.

Kishimoto initially identified the protein that would later come to be named IL-6 as a B cell differentiation factor (BCDF). It was given the name B cell stimulatory factor 2 (BSF-2) in accordance with the B cell nomenclature at that time (216, 218, 219). Molecular analysis of genes and proteins with functions similar to IL-6/BSF-2 identified several proteins identical to IL-6/BSF-2. These included 26 kDa protein (220), interferon β_2 (IFN β_2) (221), myeloma/plasmacytoma growth factor (222), hepatocyte stimulating factor (223, 224), macrophage granulocyte inducing factor 2 (225), and cytotoxic T-cell differentiation factor (226). The broad functions implicated by the names of these factors shows the wide range of functions now ascribed to IL-6.

The human IL-6 gene is found on chromosome 7 at location 7p21 and contains 5 exons in both mouse and human. Similarities in the gene sequences between species ranges from 74-59% depending on the exon (227). The IL-6 mRNA encodes an 184 amino acid protein (228) with 42% homology to mouse IL-6 (227). The 5' regulatory region of the IL-6 gene contains several putative sequences controlling IL-6 expression, suggesting that many signaling pathways and transcription factors can influence the IL-6 expression. Motifs identified include a cAMP responsive element (CRE), a c-fos enhancer element, two AP-1 binding sites, a CCAAT box and glucocorticoid responsive elements (227, 229).

1.3.2 IL-6 signaling

IL-6 binds to an exclusive, ligand-specific receptor, IL-6 receptor α (gp80), which forms a heterodimer with gp130 upon ligand binding. gp80 exists in both a membrane bound and soluble form produced by either proteolysis from the cell membrane or by expression of an alternatively spliced mRNA variant of gp80 (230, 231). The presence of soluble gp80 allows for almost all

cells to be sensitive to IL-6 signaling as the gp130 subunit is expressed on nearly all cell types (232, 233).

IL-6-gp80 binding to gp130 rapidly recruits and activates the Janus Kinase (JAK) at the membrane proximal proline rich box 1 and box 2 motifs of gp130 (234, 235). A diagram of IL-6-gp80-gp130 signaling is shown in Figure 3. The recruitment of JAK to the IL-6-gp80-gp130 complex can activate either the Ras-Raf-MEK1-ERK1/2-MAP kinase pathway (236) or the signal transducer and activator of transcription-3 (STAT-3) pathway (237). A negative reciprocal regulation between these pathways usually results in predominance of one signaling pathway over the other (233, 238). Activation of Akt by IL-6 has also been observed in a cell specific manner (233). SHP2 phosphorylation by JAK leads to the formation of a SHP2-Gab-PI3K complex which can activate Akt (239). IL-6 activation of Akt may contribute to the anti-apoptotic properties of this cytokine (240, 241).

IL-6-gp130 stimulation of STAT3 requires phosphorylation of the distal tyrosine residues on gp130 by JAK (233, 242). These tyrosine residues are part of the pYXXQ STAT3 binding motif (242). Alternatively, JAK phosphorylation of the tyrosine residue 759 on gp130 allows docking of the SHP2 and formation of a SHP2-Grb2-SOS complex, which through a GTPase activity, activates Ras and subsequently the Raf-MEK1-ERK1/2-MAP Kinase pathway (242).

The outcome of IL-6-gp130 signaling is highly dependent on the cell type and environmental conditions resulting in mitosis (243-247), migration (248, 249), anti-apoptosis and/or cell survival (250-255), growth arrest (256), acute phase response (253, 257, 258), suppression of innate immunity (79, 259, 260) and differentiation (247, 256, 259, 261-264). Typically, signaling through STAT3 induces the acute phase response, differentiation, anti-apoptosis, survival, migration, suppression of innate immunity and growth arrest. In contrast to

the multiple functions attributed to STAT3 signaling, Ras-Raf-MEK1-ERK1/2 activation typically leads to mitogenesis. As mentioned above, IL-6 activation of Akt also has anti-apoptotic functions (240, 241).

Finally, several mechanisms regulate IL-6-gp130 signaling, most of which act by terminating or preventing STAT3 activation. The protein tyrosine phosphatase SHP2, which is recruited to the gp130 at Tyr759 has an inhibitory effect on JAK/STAT3 signaling (265). This is especially interesting since SHP2 also serves as an adaptor protein facilitating IL-6-gp130- Ras-Raf-MEK1-ERK1/2 and Akt signaling (233, 239) and likely contributes to the negative reciprocal regulation of the two arms of IL-6-gp130 signal transduction (233, 238). The Protein Inhibitor of Activated STAT-3 (PIAS3) can interact with phosphorylated STAT3 and prevent DNA binding to target genes (266). Another inhibitor, the Suppressor of Cytokine Signaling-3 (SOCS-3) binds to gp130 at Try-759, the same binding site as SHP2. This prevents STAT3 activity by inhibiting the phosphorylation of gp130 and STAT3 by JAK, or by inhibiting JAK phosphorylation, itself (267, 268). SOCS-3 functions as a negative feedback loop for IL-6/STAT3 signaling, as STAT3 induces expression of SOCS-3 after IL-6 stimulation (267).

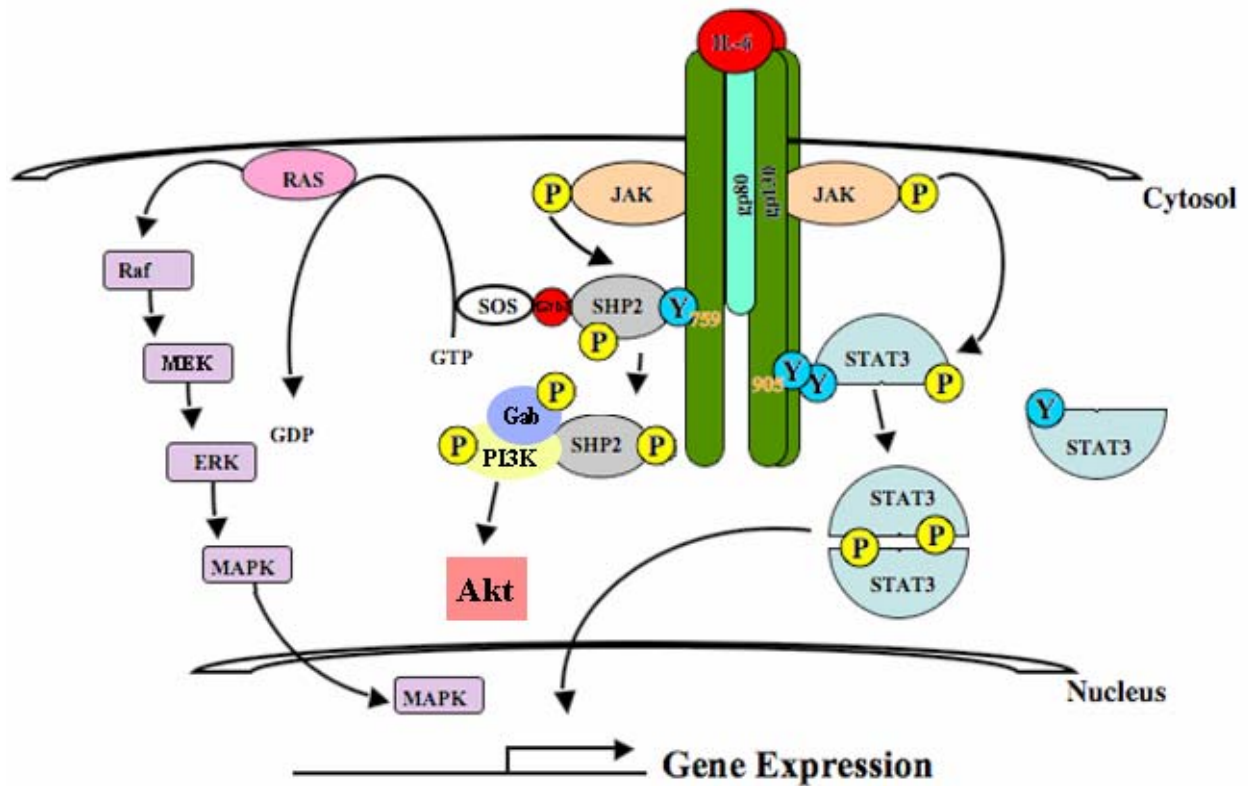


Figure 3. The IL-6/gp80/gp130 signaling cascade. Binding of IL-6 to the gp80 and subsequently gp130 produces a hexameric complex. The Janus Kinase (JAK) is recruited and phosphorylates tyrosine residues on gp130. JAK activity can activate STAT3 activation, SHP2-Ras-Raf-MEK-ERK1/2 or SHP2-PI3K-Akt signaling. Recreated from references (233, 240).

1.3.3 Functions of IL-6

The large number of factors identical to IL-6 reflects this cytokine's wide range of functions, including several roles in the immune system. IL-6 produced by CD4⁺ helper T cells induces B cell differentiation into antibody secreting plasma cells, but does not influence B cell proliferation (228). Several *in vivo* studies also show that IL-6 augments antibody production.

Mice treated with recombinant IL-6 have an elevated antibody response to sheep red blood cells compared to controls not receiving IL-6 (269). Furthermore, the antibody response to vesicular stomatitis virus was almost completely inhibited in IL-6^{-/-} mice (164). Transgenic C57Bl/6J mice over expressing IL-6 displayed polyclonal hypergammaglobulinemia associated with plasmacytosis (270). In the Balb/c mouse strain, transgenic over expression of IL-6 further progressed into monoclonal plasmacytoma with a t(12:15) chromosomal translocation (271). However, *in vitro* proliferation of B cells was unchanged by neutralizing anti-IL-6 treatment (269).

IL-6 also contributes to T cell biology. IL-6 can induce IL-2 secretion (272) and the expression of the IL-2 receptor on T cells (273). Cytotoxic T cell differentiation in mouse and human thymocytes was induced by IL-6 in conjunction with IL-2 and IFN γ (274, 275). CD4⁺ T-cells can be polarized towards T helper (Th)-2 phenotype by IL-6 along with endogenous IL-4 (276). Alternatively, induction of suppressor of cytokine signaling-1 (SOCS-1) by IL-6 inhibits Th1 differentiation (276). Recent evidence has shown that the suppressive ability of regulatory T cells (T_{reg}) is diminished with IL-6 treatment (277). Moreover, the development of T_{reg} cells by TGF β is inhibited in the presence of IL-6 (278). Rather, IL-6 and TGF β drive the generation of IL-17 producing CD4⁺ T-cells (Th-17) (278, 279). These T cells have been implicated in the progression of autoimmunity and microbial infections (280, 281).

IL-6 also contributes to hematopoietic cell differentiation. The formation of multi-potent hematopoietic progenitors is dependent on IL-6 and IL-3 (282, 283) with IL-6 stimulating the entry of hematopoietic stem cells into the cell cycle (284). IL-6 is also able to induce myeloid leukemia cells to differentiate into macrophages (285). Additionally, IL-6 increased the differentiation of bone marrow cells into megakaryocyte in the presence of IL-3 (282). In the

same study, IL-6 had a more broad hematopoietic influence on bone marrow cells from 5-fluoruracil treated mice, with significantly increased granulocyte-macrophage, granulocyte-macrophage-megakaryocyte and megakaryocyte colonies in cultures treated with IL-6 and IL-3 versus IL-3 alone (282). Mice over expressing human IL-6 also generated more megakaryocytes in their bone marrow (270).

IL-6 has several well-known roles in the liver. Most notably, IL-6 (including reports generated when IL-6 was known as hepatocyte stimulating factor) is a potent stimulator of acute phase proteins (APP) by hepatocytes during the acute phase response to inflammation (164, 223, 224). IL-6 produced at the site of injury or infection stimulates hepatocytes production of C-reactive protein (CRP), serum amyloid A protein, α_2 -macroglobulin, fibrinogen, haptoglobin α_1 -antitrypsin, α_1 -antichymotrypsin, α_1 -cysteine proteinase, and complement component C3 and C9, while inhibiting serum albumin levels (164-166, 286).

In the normal liver, IL-6 is chiefly produced by Kupffer cells (183, 184, 287) and is the primary cytokine responsible for the activation of hepatic STAT3 (249, 288). Following insult or injury other hepatic cell types can also produce IL-6, including biliary epithelial cells, dendritic cells, stellate cells and myofibroblasts (289-293). Proliferation of various liver cell types are also stimulated or augmented by IL-6. Hepatocyte proliferation after partial hepatectomy is reduced in IL-6-deficient mice leading to a delay in the restoration of liver mass compared to wild-type mice (294). In vitro, IL-6 is a mitogen (244-246) and motogen (248, 249) for biliary epithelial cells and contributes to the barrier function of the bile ducts, in vivo (248, 249, 288).

Elevated systemic IL-6 is a hallmark of sepsis in addition to other cytokines including TNF α , IFN γ and IL-1 β (295). It is the association with these other cytokines during

inflammation that has caused IL-6 to be categorized a “pro-inflammatory” cytokine. If this is true, then in the absence of IL-6, inflammation should be less. However, evidence shows that IL-6^{-/-} mice have exaggerated inflammatory responses compared to IL-6^{+/+} mice. In mouse models of sepsis IL-6^{-/-} mice have elevated TNF α and IFN γ cytokine production (296), a deficient fever response (297) and increased mortality (298) compared to IL-6^{+/+} mice. This suggests that IL-6 may be acting in an anti-inflammatory role (296, 299). Indeed, IL-6 activation of STAT3 has been shown to inhibit the activation of macrophages and dendritic cells (259, 300-302). Thus, IL-6 likely possesses both pro- and anti-inflammatory properties depending on which arm of the immune system, innate or adaptive, it influences.

IL-6 is elevated in several diseases including autoimmune diseases such as rheumatoid arthritis, systemic juvenile idiopathic arthritis, osteoporosis, psoriasis, primary biliary cirrhosis, and Sjogren’s syndrome (303-306). This is not surprising considering the elevated production of antibodies during autoimmunity and the ability of IL-6 to stimulate B cell differentiation into plasma cells. IL-6 is also a growth factor in tumors, including multiple myeloma (307), renal carcinoma (308) and cholangiocarcinoma (243, 244, 309-311). Other cell types can also use IL-6 as a mitogen, including mesangial cells (312, 313) and biliary epithelial cells (244-246).

2.0 HYPOTHESIS AND AIMS

The underlying hypothesis tested in this dissertation is that IL-6 activation of STAT3 inhibits the maturation and function of dendritic cells. Because of the elevated IL-6/STAT3 signaling in the liver, the inhibition of hepatic DC by IL-6 is expected to be especially prevalent in liver DC. It is predicted that the physiological mechanisms stimulating IL-6 in normal liver, primarily commensal gut derived bacterial PAMP, are essential for the inhibition of liver DC maturation. Moreover, elevated IL-6/STAT3 signaling could raise the threshold needed to stimulate DC maturation to PAMP molecules.

3.0 MATERIALS AND METHODS

3.1 ANIMALS

C57/BL6J IL-6^{-/-}, C57/BL6J and Balb/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed under the guidelines of the University of Pittsburgh IACUC protocol #0701830.

3.1.1 Inhibition of STAT3

STAT3 was inhibited by treating mice for 3 days with 1µg/g body weight JSI-124 (EMD Biosciences, San Diego, CA).

3.1.2 Depletion of gut bacteria

Commensal gut bacteria were depleted by feeding mice for 5 days with 250mg/kg Neomycin Sulfate, 9mg/kg Polymyxin B and 50mg/kg Metronidazole by gavage. Two days after the final antibiotic treatment, animals were sacrificed. Control-treated mice received water only by gavage. Portal blood endotoxin was quantified by Limulus Amebocyte Lysate Endochrome (Charles River, Charleston, SC).

3.2 CELL ISOLATION

3.2.1 Liver NPC isolation and DC selection

Liver DC were isolated from liver non-parenchymal cells. Under isoflurane anesthesia, the abdominal cavity was entered through a mid-line incision. The vena cava was cannulated with a 22-gauge catheter and perfused with 30 ml of ice-cold Hanks buffered saline solution (HBSS) (Invitrogen, Carlsbad, CA) over a 3 min period to remove any blood leukocytes. The portal vein was dissected to allow drainage of the perfusate. Subsequently, livers were flushed with 2 ml of 1mg/ml collagenase B (Roche Applied Science, Indianapolis, IN) in HBSS. Livers were excised and stored on ice until needed.

The liver capsule was removed by gentle scraping with a scissor and the liver was minced in 10 ml of 1mg/ml collagenase solution and incubated at 37°C for 20 min. The digest was then passed through a 100µm nylon mesh and washed with RPMI-5 (RPMI-1640 supplemented with 5% fetal bovine serum, L-glutamine, non-essential amino acids, sodium pyruvate and penicillin-streptomycin-fungizone). Hepatocytes were pelleted by centrifugation at 50 x g and the non-parenchymal cells in the residual supernatant were pelleted at 300 x g. The cell pellet was resuspended in 0.4 ml RPMI-5 for every 0.1 ml cell pellet volume. This suspension was further mixed with 0.7 ml 30% (w/v) Histodenz (Sigma-Aldrich, St Louis, MO) in PBS, the gradient was overlaid with 3 ml ice-cold PBS and centrifuged at 2800 RPM for 20 min. The NPC fraction containing DC was collected from the interface and washed in RPMI-5. After blocking for Fc receptors (CD16/CD32), NPC were incubated with magnetic bead conjugated antibodies against CD11c (Miltenyli, Auburn, CA), washed in MACS buffer (PBS supplemented with

bovine serum albumin and EDTA) and CD11c⁺ DC positively selected on a magnetic column. Three to six livers were pooled for each experiment.

3.2.2 Splenocyte isolation and DC selection

Splenic DC were prepared from spleens injected with a 1 mg / ml collagenase solution, minced, and passed through a 70µm cell strainer. Cells were collected by centrifugation at 1200 RPM. Red blood cells were removed by lysis with 0.14M NH₄Cl in 0.02M Tris-HCl (pH 7.4). CD11c⁺ DC were selected by magnetic bead conjugated CD11c antibodies, similar to the selection of liver DC.

3.2.3 Liver and bone marrow derived DC cultures

Liver derived DC were generated under the influence of GM-CSF as previously reported (314). Liver NPC were isolated by Percoll gradient and cultured in 24 well plates at 2x10⁵ cells per mL in complete RPMI (RPMI supplemented with 5% FBS, L-glutamine, non-essential amino acids, sodium pyruvate and antibiotics (all from Invitrogen, Carlsbad, CA) (cRPMI)) supplemented with 1000U granulocyte-macrophage colony stimulating factor (GM-CSF; Invitrogen) per mL media. The non-adherent DC were greater than 90% CD11c⁺ by flow cytometry (data not shown).

Bone marrow derived DC (BMDC) were prepared from bone marrow isolated from femur and tibia according to the methods of Inaba et al (315). Cells were cultured in cRPMI with 1000U/ml GM-CSF and 10ng/ml IL-4 (Peprotech, Rocky Hill, NJ). Media was replaced

every other day by replacing one-half of the media with fresh media. Non-adherent DC were used on day 6 for all experiments, except where noted.

DC were stimulated to induce maturation by incubation with CpG ODN 1826 (Invivogen, San Diego, CA), lipopolysaccharide (LPS, Sigma-Aldrich) and polyI:C (Sigma). For some experiments cells were treated with 50ng/ml mutein IL-6 (Imclone, New York, NY). In some experiments, day 6 BMDC were collected, washed with cRPMI, and resuspended in media containing one-half fresh media and one-half media collected from day 6 cultures supplemented with GM-CSF and IL-4 (Conditioned Media). Additionally, DC IL-6 was neutralized by the addition of anti-IL-6 antibody (R&D Systems) for 48 hours.

To block STAT3 activity or ERK1/2 activity, DC were treated with JSI-124 or PD98059 (EMD Biosciences, San Diego, CA), respectively, for 48 hours. DC maturation was induced by treatment with LPS (Sigma-Aldrich, St Louis, MO), polyI:C (Sigma) or CpG ODN 1826 (Invivogen, San Diego, CA) for 48 hours.

3.2.4 Mixed lymphocyte culture

Freshly isolated liver DC, spleen DC and BMDC were analyzed for T-cell stimulatory capacity by mixed-lymphocyte reaction (MLR). Nylon wool purified T-cells from Balb/cJ mice were used as allogeneic responders. As a control, syngeneic T-cells from C57Bl/6J mice were used as responders. 5×10^4 T-cells were incubated with γ -irradiated (2000 Rad) liver or spleen DC in triplicate for 3 days. $1 \mu\text{Ci}$ ^3H thymidine was added in the final 18 hours and thymidine incorporation analyzed.

3.3 PROTEIN ANALYSIS

3.3.1 Flow cytometry

Analysis of cell surface proteins was performed by flow cytometry. After isolation, 2×10^5 cells were washed in FACS buffer consisting of PBS supplemented with 1% bovine serum albumin and 0.25% NaN_3 . Fc receptors were then blocked with $1 \mu\text{g}$ of antibody against CD16/CD32 and 1% goat serum in FACS buffer for 10 minutes on ice. Expression of cell surface antigens were assayed using the monoclonal antibodies listed in Table 1. Following three washes in FACS buffer, streptavidin conjugated with phycoerythrin-Cy5 fluorochrome was added for 30 minutes on ice. After an additional three washes, cells were fixed in 1% paraformaldehyde in PBS and stored at 4°C until analysis was performed using either a Coulter XL flow cytometer or BD Biosciences LSR II flow cytometer. Results were analyzed on Coulter EPICS software.

3.3.2 Protein Isolation from Cells and Tissue

Dendritic cell and liver, lung, intestine, heart and spleen protein was isolated using either RIPA buffer for whole cell lysates or by fractionation of nuclear and cytosolic fractions.

RIPA buffer consisted of 1% Triton X-100, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate, 158mM NaCl and 10mM Tris-HCl (pH 7.5) and was supplemented with the following protease inhibitors: 1mM PMSF, $1 \mu\text{M}$ leupeptin, $20 \mu\text{g/ml}$ aprotinin and 1mM sodium orthovanadate.

Nuclear and cytosolic fractions were isolated by treating cells or tissue with a hypotonic solution of 10mM HEPES (pH 7.5), 1.5mM MgCl_2 , 10mM KCl and 0.1% Triton X-100

supplemented with the following protease inhibitors: 1mM PMSF, 1 μ M leupeptin, 20 μ g/ml aprotinin and 1mM sodium orthovanadate on ice for 10 minutes. After centrifugation for 5 minutes at 7000 RPM at 4°C, the supernatant was collected and saved as the cytosolic protein fraction. The residual pellet was resuspended in 20mM HEPES (pH 7.5), 1.5mM MgCl₂, 420mM NaCl, 0.2mM EDTA, 20% glycerol and 0.1% Triton X-100 supplemented with the following protease inhibitors: 1mM PMSF, 1 μ M leupeptin, 20 μ g/ml aprotinin and 1mM sodium orthovanadate and kept on ice for 30 minutes with periodic vortex mixing. After centrifugation at 13,000 RPM for 15 minutes at 4°C, the supernatant was collected and saved as the nuclear protein fraction.

All protein was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL) with BSA standards.

3.3.3 Western Blotting

Fifty-micrograms of nuclear or total protein was separated by electrophoresis on a sodium dodecyl sulfate polyacrylamide gel. Protein was transferred to nitrocellulose membranes in a Tris-Glycine buffer. Verification of transfer was visualized by Ponceau S staining of membranes and Comassie Blue staining of the gel. Membranes were blocked in either 5% non-fat dried milk in tris-buffered saline with 0.05% tween-20 (TBST) for 1 hour and probed overnight at 4°C with specific antibodies diluted 5% non-fat dried milk in TBST listed in Table 2. After washing three times in TBST, membranes were probed with either anti-goat, anti-rabbit, or anti-mouse IgG conjugated with horseradish peroxidase for 1 hour at room temperature. Following three TBST washes, signals were detected by Enhanced Chemiluminescence (Pierce) and exposed to

autoradiography film. Individual bands were quantified using the ImageJ software from the National Institute of Health.

3.3.4 STAT3 DNA Binding Assay

STAT3 activity was measured using a TransFactor STAT3 DNA binding assay (Clontech). Fifty-micrograms of nuclear protein was incubated with STAT3 consensus DNA sequences and subsequently probed with an antibody specific for STAT3. The STAT3-DNA-antibody complex was detected by horseradish peroxidase conjugated secondary antibody.

3.3.5 Cytokine quantification

Cell culture supernatants were analyzed by ProteoPlex Murine Cytokine Array (EMD Biosciences) according to the manufacturer's protocol. Additionally, mouse IL-6 was quantified by ELISA (Invitrogen).

Table 2. Antibodies used in these studies.

Antibody	Conjugate	Source	Application
Anti-CD11c	Biotinylated/ AlexaFluor488	BD Biosciences	FC/IF
Anti-B220	FITC	BD Biosciences	FC
Anti-CD80	PE	BD Biosciences	FC
Anti-CD86	PE/Unconjugated	BD Biosciences	FC
Anti-IA/IE (MHC class II)	PE / AlexaFluor488	BD Biosciences	FC/IF
Anti-CD8 α	Alexa647	eBiosciences	FC
Anti-CD11b	Pacific Blue	eBiosciences	FC
Anti-CCR7	FITC	eBiosciences	FC
Anti-IL-10	Unconjugated	BD Biosciences	WB
Anti- TGF β	Unconjugated	Santa Cruz	WB
Anti-TLR4	Unconjugated	eBiosciences	WB
Anti-TLR9	Unconjugated	eBiosciences	WB
Anti-phosphoSTAT3 (Tyr705)	Unconjugated	Cell Signaling	WB
Anti-STAT3	Unconjugated	Cell Signaling	WB
Anti-IL-6	Unconjugated	R&D Systems	WB
Anti- β -actin	Unconjugated	Sigma-Aldrich	WB
Streptavidin-TRITC		Jackson Immunologicals	IF
Streptavidin-PE-Cy5		BD Biosciences	FC
Streptavidin-PE-Cy7		BD Biosciences	FC

FC: flow cytometry; IF: immunofluorescence; WB: Western blot.

3.3.6 Immunofluorescent Staining

Liver DC were localized by immunofluorescent staining on snap frozen liver tissue embedded in optimal cutting temperature (OCT) solution. Five micron liver sections were fixed for 20 minutes in 96% ethanol at room temperature and air-dried. Endogenous biotin was blocked using an avidin-biotin blocking kit (Vector). Additional blocking was performed with 10% donkey serum for 10 minutes. Tissue was probed with antibodies specific for CD11c, CD86, and MHC class II (IA/IE) overnight. For antibodies conjugated with biotin or unconjugated, tissues were subsequently incubated with streptavidin conjugated FITC or Texas Red, or Texas Red conjugated donkey anti-rat IgG for 1 hour at room temperature. Nuclei were stained with Hoescht dye for 1 minute. Tissues were overlaid with gelvatol anti-fade solution and cover slipped.

Immunofluorescent staining was examined using a Nikon Eclipse Fluorescent microscope. Images were captured using a Spot RT Slider CCD camera and Spot 4.1 software (Diagnostic Instruments, Sterling Heights, MI). Additional modification of images was performed using Adobe Photoshop CS.

3.4 GENE EXPRESSION ANALYSIS

3.4.1 RNA isolation and reverse transcription

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA was quantified by spectrophotometry for absorbance at 260 nm. RNA quality

was determined by the ratio of absorbance at 260nm and 280nm, with a ratio above 1.7 considered acceptable. Additionally, RNA was visualized by agarose gel electrophoresis.

One microgram of total RNA was treated with DNase to remove any contaminating DNA and subsequently subjected to reverse transcription using the Superscript III reverse transcriptase enzyme and random hexamer primers. Complementary DNA (cDNA) was diluted 10-fold and stored at -20°C.

3.4.2 Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Messenger RNA transcripts were quantified by SYBR green real-time RT-PCR. Eight microliters of cDNA was amplified with the Power SYBR Green Master Mix and 250nm forward and reverse gene specific primers in a total volume of 20 μ L. Amplification and detection was performed on an Applied Biosystem 7500Fast Sequence Detection System. Gene expression was analyzed using Applied Biosystems Sequence Detection Software version 1.3.1 using the $\Delta\Delta$ Ct method. Gene expression was expressed as the ratio of the target gene to the housekeeping gene GAPDH.

Table 3. Primers used for quantitative RT-PCR in this study (listed 5'-3').

Gene	Forward	Reverse
IL-10	CCA AGC CTT ATC GGA AAT GA	TTT TCA CAG GGG AGA AAT CG
TGF β	AAA CGG AAG CGC ATC GAA	GGG ACT GGC GAG CCT TAG TT
TLR4	GCT TTC ACC TCT GCC TTC AC	AGC CTT CCT GGA TGA TGT TG
TLR9	ACT TCG TCC ACC TGT CCA AC	TCA TGT GGC AAG AGA AGT GC
IRAK-M	TGA GCA ACG GGA CGC TT	GAT TCG AAC GTG CCA GGA A
GAPDH	TGG CAA AGT GGA GAT TGT TGC C	AAG ATG GTG ATG GGC TTC CCG

3.5 STATISTICAL ANALYSIS

Statistical comparison of groups with a normal distribution was performed by t-test and ANOVA using SPSSv.11 software (SPSS Inc, Chicago, Il). Non-parametric analysis was performed using a Mann-Whitney-U test. A p-value of 0.05 was considered significant.

**4.0 GUT-DERIVED COMMENSAL BACTERIAL PRODUCTS INHIBIT LIVER
DENDRITIC CELL MATURATION BY STIMULATING HEPATIC INTERLEUKIN-6 /
SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION-3 ACTIVITY.**

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4.1 ABSTRACT

Intra-organ dendritic cells (DC) monitor the environment and translate triggers of innate immunity into adaptive immune responses. Liver-based DC are continually exposed, via gut-derived portal venous blood, to potential antigens and bacterial products that can trigger innate immunity. But somehow the liver avoids a state of perpetual inflammation and protects central immune organs from over-stimulation.

In this study we tested the hypothesis that hepatic interleukin-6 (IL-6) / Signal Transducer and Activation of Transcription 3 (STAT3) activity increases the activation/maturation threshold of hepatic DC towards innate immune signals. Results show that liver nuclear STAT3 activity is significantly higher than other organs and IL-6-dependent. Hepatic DC in normal wild-type (IL-6^{+/+}) mice are phenotypically and functionally less mature than DC from IL-6-deficient (IL-6^{-/-}) or STAT3 inhibited IL-6^{+/+} mice, as determined by surface marker expression, pro-inflammatory cytokine secretion, and allogenic T-cell stimulation. IL-6^{+/+} liver DC produce IL-6 in response to exposure to lipopolysaccharide (LPS) and cytidine phosphate guanosine (CpG) oligonucleotides, but are resistant to maturation compared to IL-6^{-/-} liver DC. Conversely, exogenous IL-6 inhibits LPS-induced IL-6^{-/-} liver DC maturation. IL-6/STAT3 signaling influenced liver DC expression of TLR9 and IRAK-M. Depletion of gut commensal bacteria in IL-6^{+/+} mice with oral antibiotics decreased portal blood endotoxin levels, lowered expression of IL-6 and phospho-STAT3 and significantly increased liver DC maturation. **Conclusion:** Gut-derived bacterial products, by stimulating hepatic IL-6/STAT3 signaling, inhibit hepatic DC activation/maturation and thereby elevate the threshold needed for translating triggers of innate immunity into adaptive immune responses. Manipulating gut bacteria may therefore be an effective strategy to alter intra-hepatic immune responses.

4.2 INTRODUCTION

Intra-organ dendritic cells (DC) develop from bone marrow precursors and are potentially the most potent antigen presenting cells. In normal solid organs, resident DC are maintained in a relatively immature state. They sample the microenvironment for pathogen associated molecular pattern (PAMP) danger signals via pattern recognition receptors (PRR) and phagocytotic sampling of potential antigens. Detection of PAMPs induces DC maturation that is marked by reduced phagocytosis, increased co-stimulatory and MHC class II molecule expression, and chemokine-mediated migration to the lymph nodes where they stimulate naïve T-lymphocytes. Through this process DC translate innate immune signals into adaptive immune responses.

Portal blood accounts for seventy-five percent of total hepatic blood flow and it is rich in nutrients, hormones, potential antigens and bacterial products including stimulators of innate immunity (316). The liver filters portal blood of potentially immunogenic materials before it enters the general circulation and thereby protects the central immune organs from over-stimulation. Resident sinusoidal macrophages (Kupffer cells) and specialized liver sinusoidal endothelial cells (LSEC) clear macromolecules (126). Hepatic DC are less responsive to endotoxin exposure and have reduced T-cell stimulatory capacity compared to DC from other organs (105, 314, 317).

Potential mechanisms of liver DC resistance to maturation include lower expression of the LPS-binding TLR4 compared to splenic DC (105). The liver might also influence DC maturation by producing cytokines that inhibit DC maturation. For example, *in vitro*, LSEC and Kupffer cells secrete anti-inflammatory cytokines including TGF β (318) and IL-10 (185). *In vivo*, liver non-parenchymal cells, including Kupffer cells constitutively secrete interleukin-6 (IL-6) (184, 319). But the influence of these cytokines on liver DC has not been directly tested.

IL-6/gp130 signaling is one of the most potent activators of signal transducer and activator of transcription factors-3 (STAT3) (320) and this signaling pathway contributes importantly to hepatic pathophysiology (321). IL-6 can also inhibit DC maturation and function through activation of STAT3 (79, 80, 259, 300, 302) but the effect of gp130/STAT3 signaling on liver DC has not been investigated. The microenvironment of tumors with elevated STAT3 activity suppresses DC responsiveness resulting in immune ignorance (79, 81). This suggests that DC in normal tissues with higher endogenous STAT3 activity, like the liver, might be less responsive to maturational stimuli.

To test this hypothesis, hepatic DC maturation and function were studied in IL-6 wild type (IL-6^{+/+}) and IL-6 deficient (IL-6^{-/-}) and STAT3 inhibited IL-6^{+/+} mice. We show that hepatic STAT3 signaling is dependent significantly on IL-6 and is significantly higher than STAT3 signaling in other organs. Liver DC from IL-6^{-/-} and STAT3 inhibited IL-6^{+/+} are phenotypically and functionally more mature than hepatic DC from IL-6^{+/+} mice. DC maturation in response to bacterial components is also significantly reduced IL-6^{+/+} liver DC. Conversely, IL-6 treatment of IL-6^{-/-} hepatic DC can inhibit their maturation. Antibiotic-treatment reduced portal blood endotoxin and hepatic phospho-STAT3 in IL-6^{+/+} mice resulting in elevated liver DC maturation marker expression. These results implicate gut commensal bacteria and liver IL-6/STAT3 signaling as important mechanisms that raise the threshold for hepatic DC maturation and migration signals.

4.3 RESULTS

4.3.1 Constitutive IL-6/STAT3 signaling is higher in the normal liver than in other organs

Baseline hepatic STAT3 activity is dependent on IL-6 and contributes to liver patho-physiology (321). Since there are no comparisons of IL-6/nuclear STAT3 DNA binding activity among liver and other solid organs, we conducted such an analysis in normal IL-6^{+/+} and IL-6^{-/-} mice. In normal IL-6^{+/+} mice, liver STAT3 activity was significantly greater than STAT3 activity in spleen, lung, intestine and heart (Figure 4).

The IL-6 status also influenced STAT3 activity in all other organs being significantly higher in IL-6^{+/+} than IL-6^{-/-} mouse lung, intestine, and liver. The absolute level and difference, however, in STAT3 activity between IL-6^{+/+} and IL-6^{-/-} mice was most dramatic in the liver.

Treatment of IL-6^{+/+} mice with JSI-124, a chemical inhibitor of STAT3 (80), for 3 days significantly reduced STAT3 activity in liver (Figure 5).

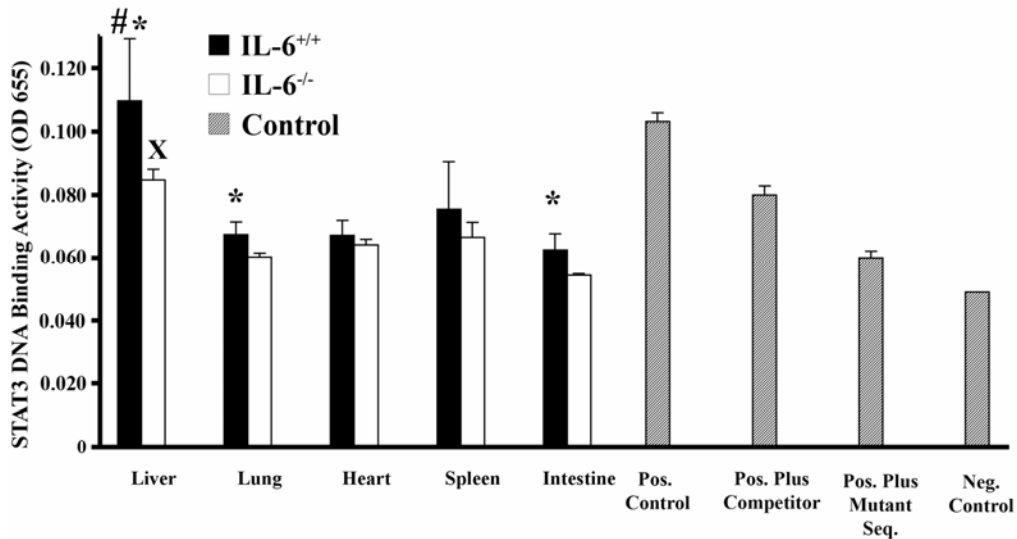


Figure 4. STAT3 DNA binding activity is significantly higher in liver compared to lung, spleen, intestine and heart. Additionally, STAT3 activity is elevated in all organs from IL-6^{+/+} mice compared to IL-6^{-/-} mice, reaching statistical significance in the liver, lung and intestine. The mean STAT3 DNA binding activity \pm standard deviation from 5 individual mice is presented for each organ. HepG2 cells treated with IL-6 are used as a positive control (Pos. Control) and were also hybridized in the presence of competitive oligonucleotide sequences (Pos. Plus Competitor) or mutant STAT3 binding sequences (Pos. Plus Mutant Seq). Lysis buffer alone was used as a negative control (Neg. Control). # P<0.05 versus all other organs tested. X p<0.01 vs all other organs tested except IL-6^{+/+} spleen. * P<0.05 versus IL-6^{-/-}.

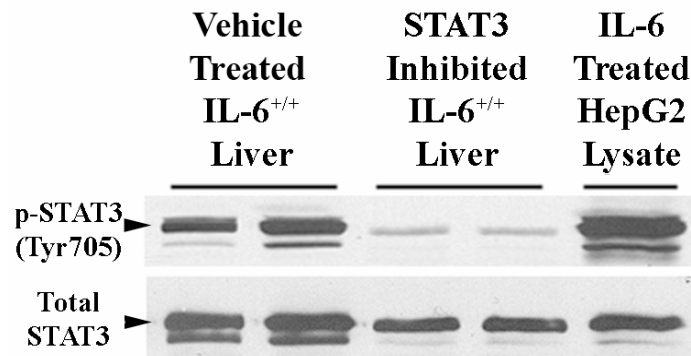


Figure 5. IL-6^{+/+} mice treated with the STAT3 inhibitor JSI-124 have decreased expression of activated phospho-STAT3. Normal IL-6^{+/+} mice were treated for 3 days with 1 μ g/g body weight of JSI-124 (STAT3 Inhibited) or vehicle control and sacrificed 24 hours following the last injection. Liver nuclear lysates were analyzed by Western blot for phospho-STAT3 (p-Tyr705) and total STAT3.

4.3.2 Liver DC are more mature in IL-6^{-/-} than IL-6^{+/+} mice

Elevated IL-6/STAT3 signaling inhibits DC maturation (79, 80, 259, 300, 302), whether the STAT3 activity is in the tissue microenvironment or in the DC themselves. Therefore, we tested the hypothesis that IL-6/STAT3 signaling might be responsible for reduced maturation of hepatic DC observed in normal liver by comparing IL-6^{+/+} and IL-6^{-/-} mice. Maturation was assessed, in situ, by immunofluorescence staining and by flow cytometry of freshly isolated hepatic DC.

As shown before by many groups, dendritic-shaped cells expressing CD11c, CD86, and MHC class II by immunofluorescence localized primarily to the portal tracts (Figure 6A). Many fewer DC were also present around the central veins and in the subcapsular regions (data not shown). In IL-6^{+/+} liver, CD11c⁺ DC co-expressed significantly less CD86 than IL-6^{-/-} or STAT3 inhibited IL-6^{+/+} liver DC (Figure 6). MHC class II co-expression in CD11c⁺ DC was elevated in IL-6^{-/-} and STAT3 inhibited IL-6^{+/+} livers compared to IL-6^{+/+} livers, but did not reach statistical significance (Figure 6).

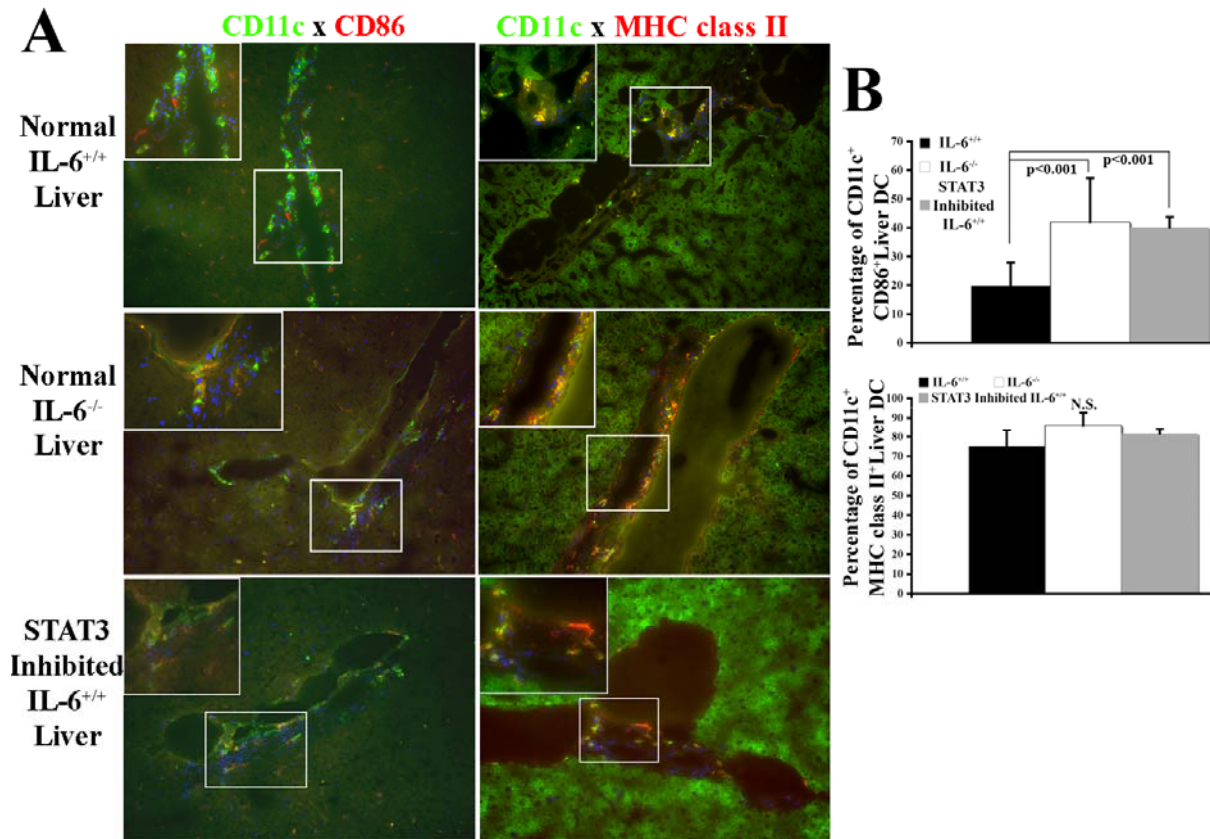


Figure 6. Localization of CD11c⁺ DC and co-expression of CD86 or MHC class II protein by immunofluorescence in IL-6^{+/+}, IL-6^{-/-} or STAT3 inhibited IL-6^{+/+} livers. A) CD11c⁺ liver DC (Green) are predominantly located in the portal tracts with DC also observed around the central veins and in the subcapsular lymphatics (not shown). Liver CD11c⁺ DC from IL-6^{-/-} or STAT3 inhibited IL-6^{+/+} mice co-expressed CD86 (Left Panels, Red) significantly more frequently than IL-6^{+/+} mice. No significant differences were observed in MHC class II (Right Panels, Red) expression. Nuclei were stained with Hoechst dye (Blue). Original magnifications: 200x main image, 1000x inset. B) Quantification of the number of CD11c⁺ DC expressing either CD86 (top) or MHC class II (bottom). Expression of CD86 or MHC class II was examined in DC from at least 10 portal tracts in 4 mice per group. N.S.: Not Significant.

Flow cytometric studies on freshly isolated CD11c⁺ DC from IL-6^{+/+} and IL-6^{-/-} liver confirmed that IL-6^{+/+} liver DC were less mature than IL-6^{-/-} and STAT3 inhibited IL-6^{+/+} hepatic DC. Analysis of co-stimulatory molecule expression in CD11c⁺ DC confirmed that IL-6^{+/+} liver DC had reduced maturation markers expression, CD80 and CD86, and CCR7 than liver DC from

IL-6^{-/-} mice (Figure 7A-B). MHC class II was also elevated in IL-6^{-/-} liver DC, but not significantly so. Reduced expression of CCR7 in IL-6^{+/+} liver DC, the chemokine receptor facilitating CCL19 and CCL21-mediated migration in mature DC (300), suggested that IL-6/STAT3 signaling might inhibit migration of liver DC to the lymph nodes.

Liver CD11c⁺ DC subtypes, based on expression of CD11b and CD8 α (myeloid versus lymphoid) and plasmacytoid (CD11c⁺B220⁺), were also examined in IL-6^{+/+} and IL-6^{-/-} mice. This analysis showed that most liver DC were CD11b⁺ CD8 α ⁻ (myeloid) with minor populations of CD11b⁻ CD8 α ⁺ (lymphoid) and CD11b⁻CD8 α ⁻ (Figure 8A-B). The predominant liver DC subtype (CD11b⁺CD8 α ⁻; myeloid) showed the largest difference in maturation marker expression (CD80, CD86 and MHC class II) between IL-6^{+/+} and IL-6^{-/-} liver DC, but differences were detected across all subtypes examined (Figure 8C-E). Liver DC isolated from IL-6^{-/-} mice also showed a significantly higher percentage of CD11c⁺B220⁺ plasmacytoid DC compared to IL-6^{+/+} liver DC and maturation marker expression was significantly higher in IL-6^{-/-} plasmacytoid liver DC (Figure 9).

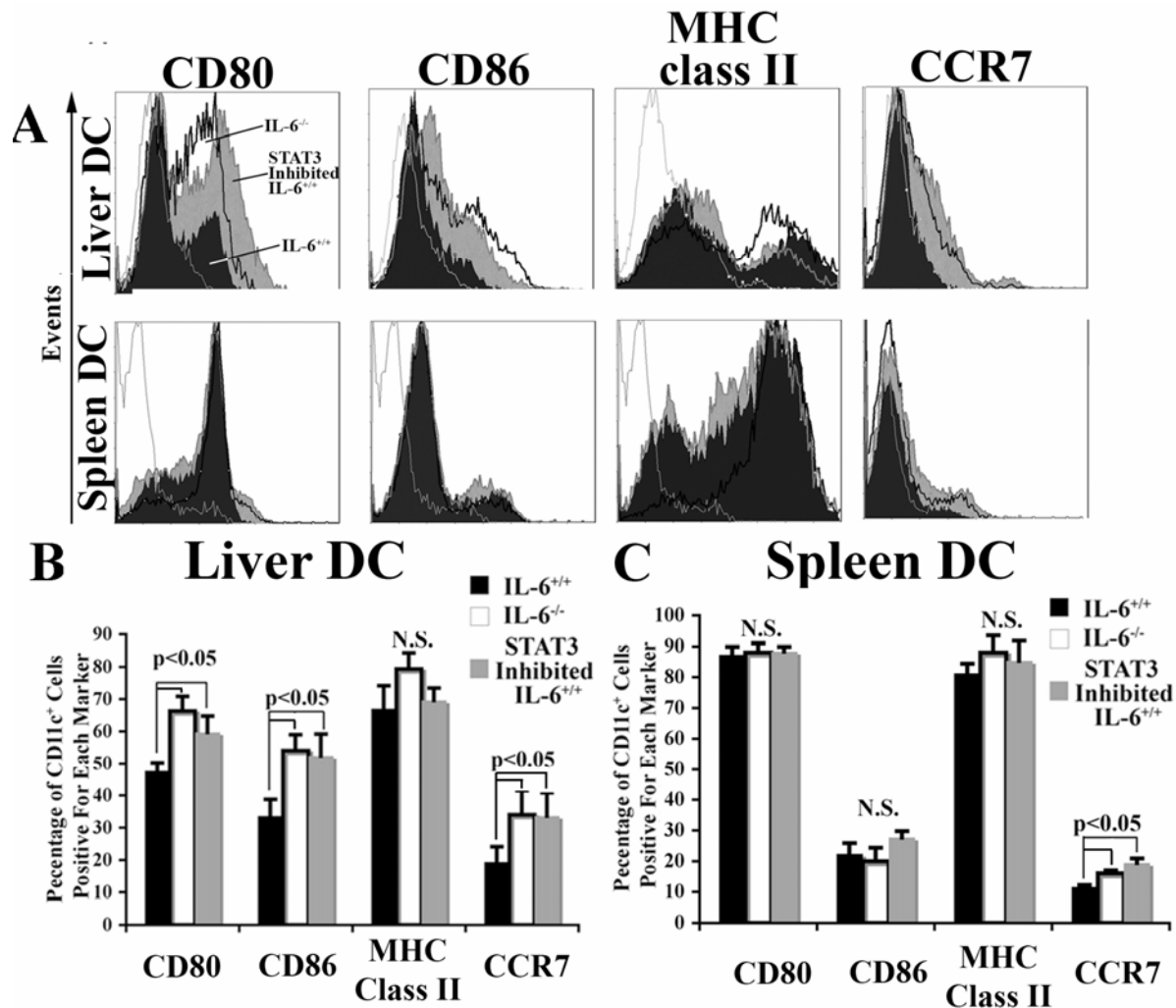


Figure 7. Expression of the maturation markers CD80, CD86, MHC class II and CCR7 in freshly isolated liver and spleen CD11c⁺ DC by flow cytometry. **A)** Histograms are gated on CD11c⁺ populations. DC isolated from IL-6^{+/+} (closed black histogram) liver showed lower expression of all maturation markers compared to IL-6^{-/-} (open black-line histogram) or STAT3 inhibited IL-6^{+/+} (closed gray histogram) liver DC. In comparison, splenic DC isolated from IL-6^{+/+} (closed black histogram), IL-6^{-/-} (open black-line histogram) and STAT3 inhibited IL-6^{+/+} (closed gray histogram) mice showed minimal differences. Isotype controls are shown in the open grey-line histograms. The results are representative of 3 individual experiments. **B)** The percentage of liver and spleen DC expressing CD80, CD86, MHC class II and CCR7. Each bar represents the mean \pm standard deviation of three separate experiments.

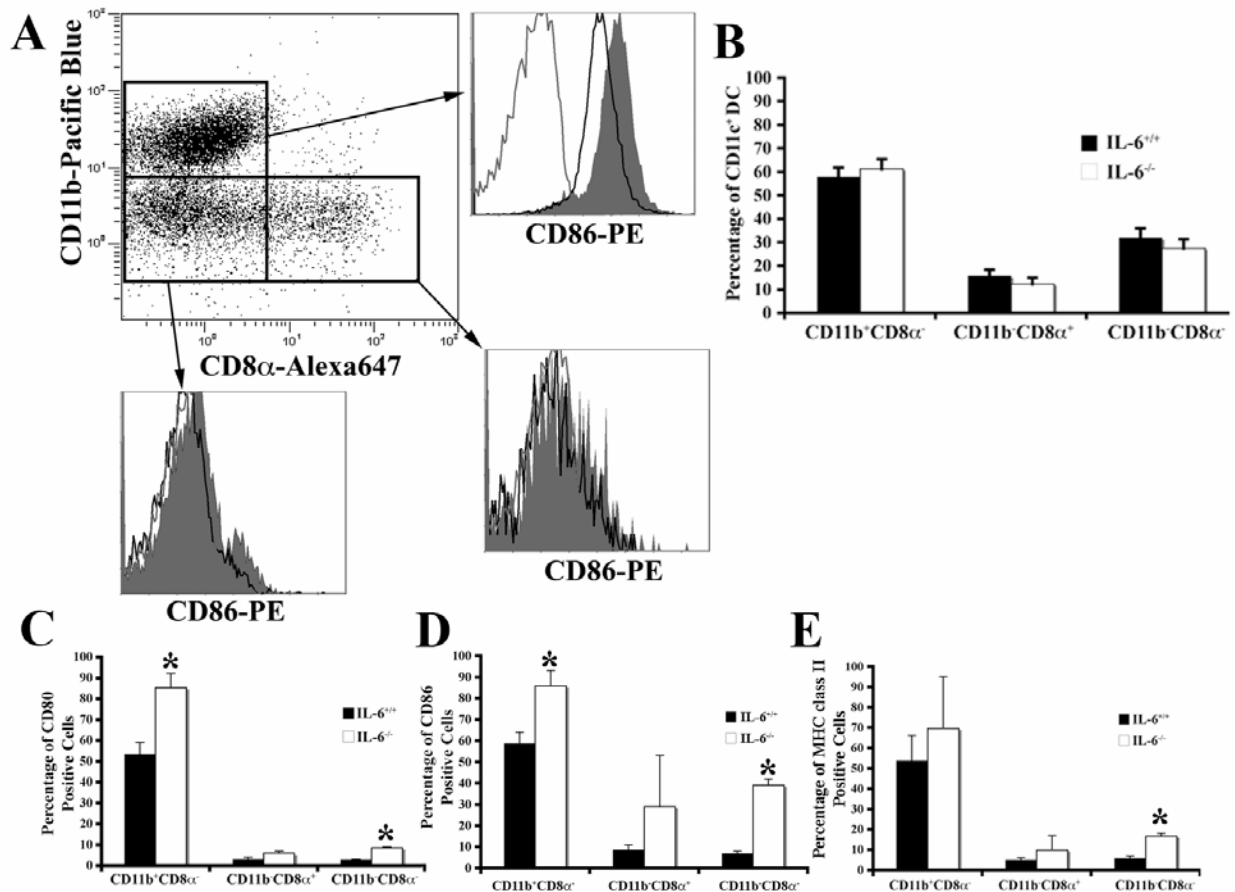


Figure 8. An examination of myeloid and lymphoid subtypes in IL-6^{+/+} and IL-6^{-/-} liver DC. A) A representative dot-plot gated on CD11c showing CD11b and CD8α expression in liver DC. Three populations were observed: CD11b⁺CD8α⁻ (myeloid DC), CD11b⁻CD8α⁺ (lymphoid DC) and a population of CD11b⁻CD8α⁻ liver DC. Representative histograms showing CD86 expression in each subtype is also presented. Open black histogram: IL-6^{+/+} liver DC; Closed gray histogram: IL-6^{-/-} liver DC; Open gray histogram: Isotype control. B) No significant differences were observed between IL-6^{+/+} and IL-6^{-/-} liver DC subtype based on CD11b and CD8α expression. The results are the mean±standard deviation of three independent experiments. (C-E) Quantification of maturation marker expression in each subtype. Expression of CD80 (C), CD86 (D) and MHC class II (E) in liver CD11c⁺ DC subtype populations. Maturation marker expression predominantly occurred in the CD11b⁺CD8α⁻ population. Each bar is the mean±standard deviation of three independent experiments. * P<0.05 versus IL-6^{+/+}.

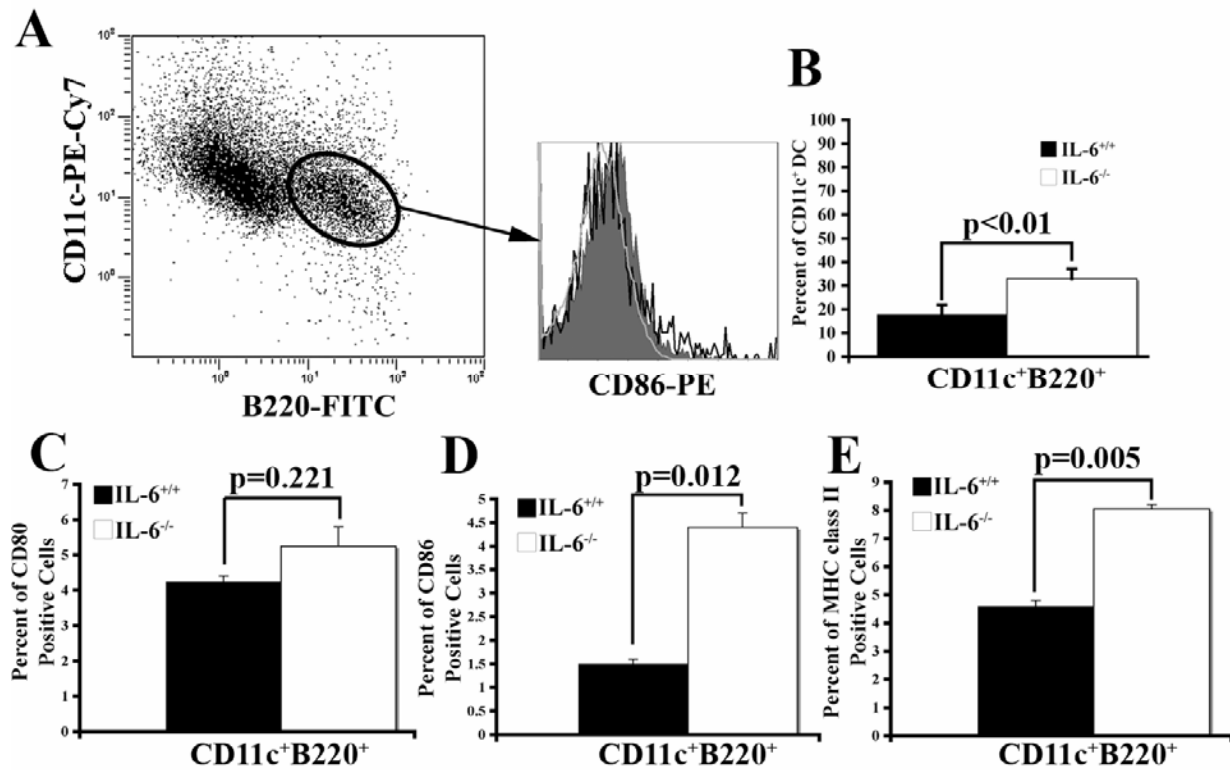


Figure 9. Plasmacytoid liver DC subpopulations in IL-6^{+/+} and IL-6^{-/-} liver DC. A) A representative dot-plot of CD11c and B220 expression in liver DC. The CD11c⁺B220⁺ population circled represents the plasmacytoid liver DC subtype. A representative histogram showing CD86 expression in a population of liver DC gated on CD11c⁺B220⁺. Open black histogram: IL-6^{+/+} liver DC; Closed gray histogram: IL-6^{-/-} liver DC; Open gray histogram: Isotype control. B) IL-6^{+/+} liver DC had significantly less CD11c⁺B220⁺ plasmacytoid DC than IL-6^{-/-} liver DC. The results are the mean±standard deviation of three independent experiments. C-E) Significantly more IL-6^{-/-} plasmacytoid liver DC expressed CD80 (C), CD86 (D) and MHC class II (E) compared to IL-6^{+/+} plasmacytoid liver DC, though under 10% of cells expressed maturation markers.

IL-6/STAT3 signaling had a more pronounced effect on DC maturation marker expression in the liver than in the spleen DC. This is because STAT3 activity is significantly higher in liver than in spleen and the difference in STAT3 activity in the spleen between IL-6^{+/+} and IL-6^{-/-} mice is not significant (Figure 4). Consequently, splenic DC showed minimal, non-significant differences in CD80, CD86 and MHC class II expression between IL-6^{+/+} and IL-6^{-/-}

mice (Figure 7A and C). CCR7 expression was marginally increased in IL-6^{-/-} and STAT3 inhibited IL-6^{+/+} DC compared to IL-6^{+/+} splenic DC.

4.3.3 IL-6/STAT3 signaling impairs liver DC function

DC initiate adaptive immune responses by directly stimulating T-cell activation, in the liver or in regional lymph nodes after migration. DC also indirectly influence T-cell activation through secretion of pro- and anti-inflammatory cytokines. We examined, therefore, liver DC cytokine secretion and T-cell stimulation to gauge their functional characteristics. Cytokine secretion was measured in isolated liver DC cultured in the presence of GM-CSF. IL-6^{-/-} and STAT3 inhibited IL-6^{+/+} liver DC showed significantly more secretion of the pro-inflammatory cytokines IL-1 α and TNF α (Figure 10C and D). Conversely, IL-6^{+/+} liver DC secreted significantly more IL-10 than did IL-6^{-/-} and STAT3 inhibited IL-6^{+/+} liver DC (Figure 10A). Even though STAT3-inhibited IL-6^{+/+} liver DC are more mature than untreated IL-6^{+/+} liver DC (Figure 7), we observed no significant difference in the amount IL-6 secreted (Figure 10B). No detectable IL-4, IL-12 or IFN γ was observed (data not shown), consistent with previous reports of cytokine production from liver DC kept in the presence of GM-CSF (96).

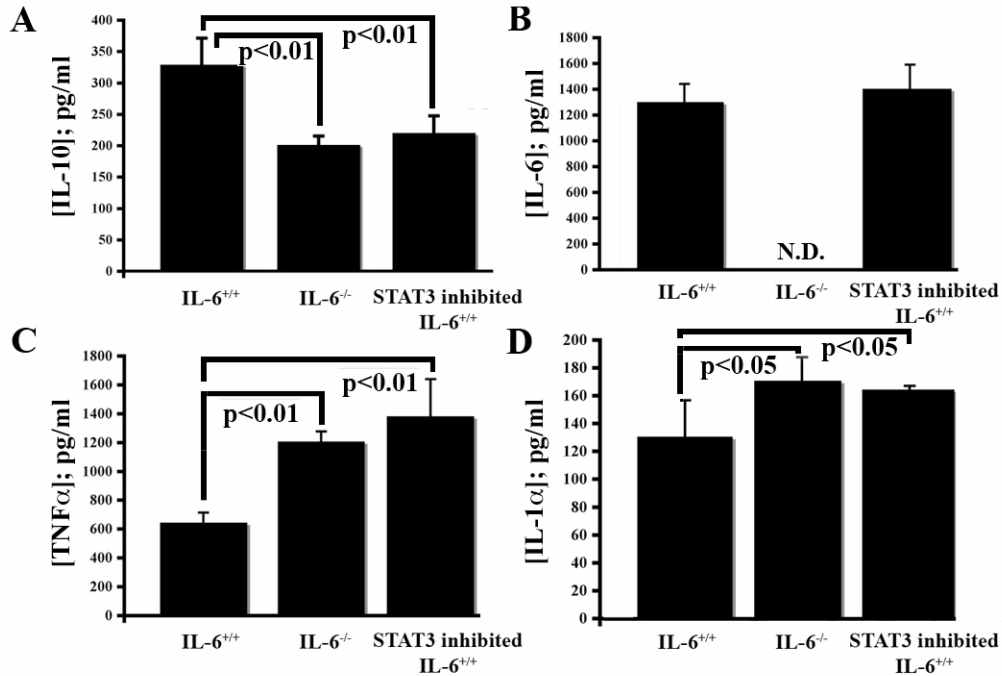


Figure 10. Cytokine secretion by IL-6^{+/+}, IL-6^{-/-} and STAT3 inhibited IL-6^{+/+} isolated liver DC. Production of (A) IL-10, (B) IL-6, (C) TNFα and (D) IL-1α protein was quantified in the media of isolated liver DC cultured for 48 hours in the presence of GM-CSF by the Murine Cytokine ProteoPlex Assay. IL-6^{+/+} liver DC produced significantly greater amounts of the anti-inflammatory IL-10, while IL-6^{-/-} and STAT3 inhibited IL-6^{+/+} liver DC secreted higher amounts of the pro-inflammatory TNFα and IL-1α. IL-6 was equally produced by both IL-6^{+/+} and STAT3 inhibited IL-6^{+/+} liver DC. No IL-6 was detected in IL-6^{-/-} liver DC, as expected. N.D.: Not detectable.

The ability of DC to stimulate T-cell activation was measured in an allogeneic MLR using CD11c⁺ liver DC isolated from IL-6^{+/+}, IL-6^{-/-} or STAT3 inhibited IL-6^{+/+} mice as stimulators and allogeneic splenic T-cells as responders. For all ratios of stimulator to responders tested, IL-6^{+/+} liver DC showed significantly reduced T-cell stimulation compared to IL-6^{-/-} or STAT3 inhibited IL-6^{+/+} liver DC (Figure 11A). IL-6/STAT3 status had little effect when syngeneic T-cells were used as responders (data not shown).

As a comparison, splenic DC were also used as stimulators (Figure 11B) and they were better T-cell stimulators than liver DC, as expected. But IL-6^{+/-} splenic DC remained less potent T-cell stimulators than IL-6^{-/-} or STAT3 inhibited IL-6^{+/-} splenic DC.

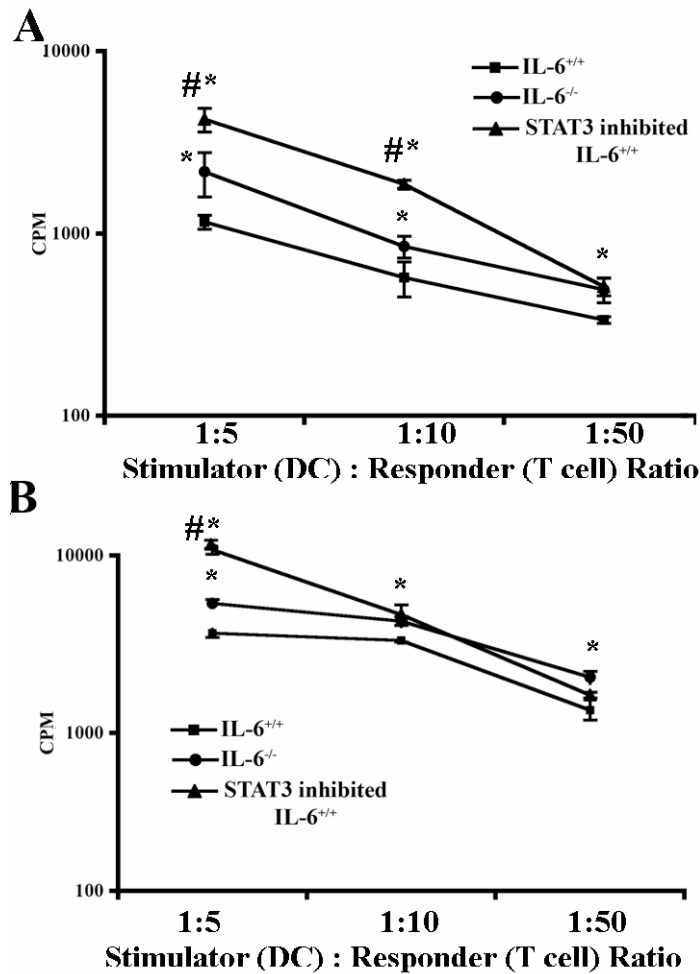


Figure 11. Increased allogenic T-cell stimulation by IL-6^{-/-} and/or STAT3 inhibited IL-6^{+/-} liver DC. Fifty-thousand Balb/cJ nylon wool purified splenic T-cells were stimulated with reducing numbers of γ -irradiated IL-6^{+/-}, IL-6^{-/-} or STAT3 inhibited IL-6^{+/-} liver (A) or splenic (B) DC from C57Bl/6J mice in a mixed lymphocyte reaction (MLR). Overall, splenic DC (B) were more potent stimulators of T-cell proliferation (measured by ³H thymidine incorporation as counts per minute (CPM)) than liver DC (A), as evidenced by differences in the CPM. * P<0.05 versus IL-6^{+/-}. # P<0.05 versus IL-6^{-/-}.

4.3.4 IL-6 status does not influence other anti-inflammatory cytokines

The above data suggest that the IL-6/STAT3 activity-rich milieu of the liver inhibits liver DC maturation. Other cytokines, however, including IL-10 and TGF β , contribute to liver pathophysiology and are known to prevent DC maturation (322). We considered the possibility that the suppressive effect of IL-6 on DC maturation might be mediated through these cytokines and therefore measured their hepatic expression. No significant differences in whole liver IL-10 (Figure 12A-B) or TGF β (Figure 12C-D) mRNA or protein expression were detected between IL-6^{+/+} and IL-6^{-/-} mice. It is likely, therefore, that among these cytokines, IL-6 plays a major role inhibiting hepatic DC maturation in the normal liver.

4.3.5 Increased threshold to TLR stimulation in IL-6^{+/+} liver DC

Because IL-6/STAT3 signaling significantly contributes to the physiological inhibition of hepatic DC maturation, we next determined whether the IL-6 status affects the sensitivity of liver DC maturation to exogenous maturation stimuli. Since the liver is continually bathed in bacterial components, we stimulated liver DC with the bacterial derived TLR agonists LPS and CpG oligonucleotides.

Liver DC were prepared from hepatic non-parenchymal cells cultured with GM-CSF and compared to similarly prepared BMDC (314). Consistent with previous reports (314), unstimulated liver DC expressed less CD86 compared to BMDC (Figure 13A). Both IL-6^{-/-} liver DC and BMDC cultures expressed more CD86 than IL-6^{+/+} liver DC and BMDC cultures. The maturation of liver IL-6^{-/-} DC was less than IL-6^{-/-} BMDC possibly reflecting the elevated basal STAT3 in the IL-6^{-/-} liver (Figure 4).

Following stimulation with 100ng/ml LPS or 1 μ M CpG, liver DC and BMDC CD86 expression increased, but was significantly less in IL-6^{+/+} liver DC (Figure 13A-C). Almost all IL-6^{-/-} liver DC express CD86 after LPS and CpG stimulation, while a significant population of IL-6^{+/+} liver DC resisted maturation. Interestingly, IL-6^{-/-} liver DC were dramatically more sensitive to stimulation with CpG than IL-6^{+/+} liver DC (Figure 13A-B). A similar trend was also apparent between IL-6^{+/+} and IL-6^{-/-} BMDC after CpG treatment though the differences were not as striking as in liver DC (Figure 13A,C).

We speculated that elevated IL-6 secretion might account for the reduced maturation of IL-6^{+/+} liver DC to CpG stimulation. Measurement of IL-6 protein revealed that CpG treated IL-6^{+/+} liver DC secreted nearly 3-fold more IL-6 protein than control treated cells (Figure 13D). In contrast, while LPS treated IL-6^{+/+} liver DC secreted significantly more IL-6 than control treated cells, this response was significantly *less* than CpG treated liver DC. This shows that IL-6^{+/+} liver DC in fact are responsive to CpG treatment as evident by increased IL-6 secretion, and this, in turn, prevents liver DC maturation.

The ability of IL-6 to prevent liver DC maturation was confirmed by treating IL-6^{-/-} liver-derived DC with exogenous IL-6 prior to LPS exposure. IL-6^{-/-} liver DC exposed to IL-6 for 24 hours had a slight effect on CD86 expression compared to untreated cells (Figure 13E). However, IL-6 pre-treatment of IL-6^{-/-} liver DC inhibited LPS induced CD86 expression compared to DC treated with LPS alone (Figure 13E). Collectively, these results show that either endogenous or exogenous IL-6 can prevent DC maturation by TLR stimulation.

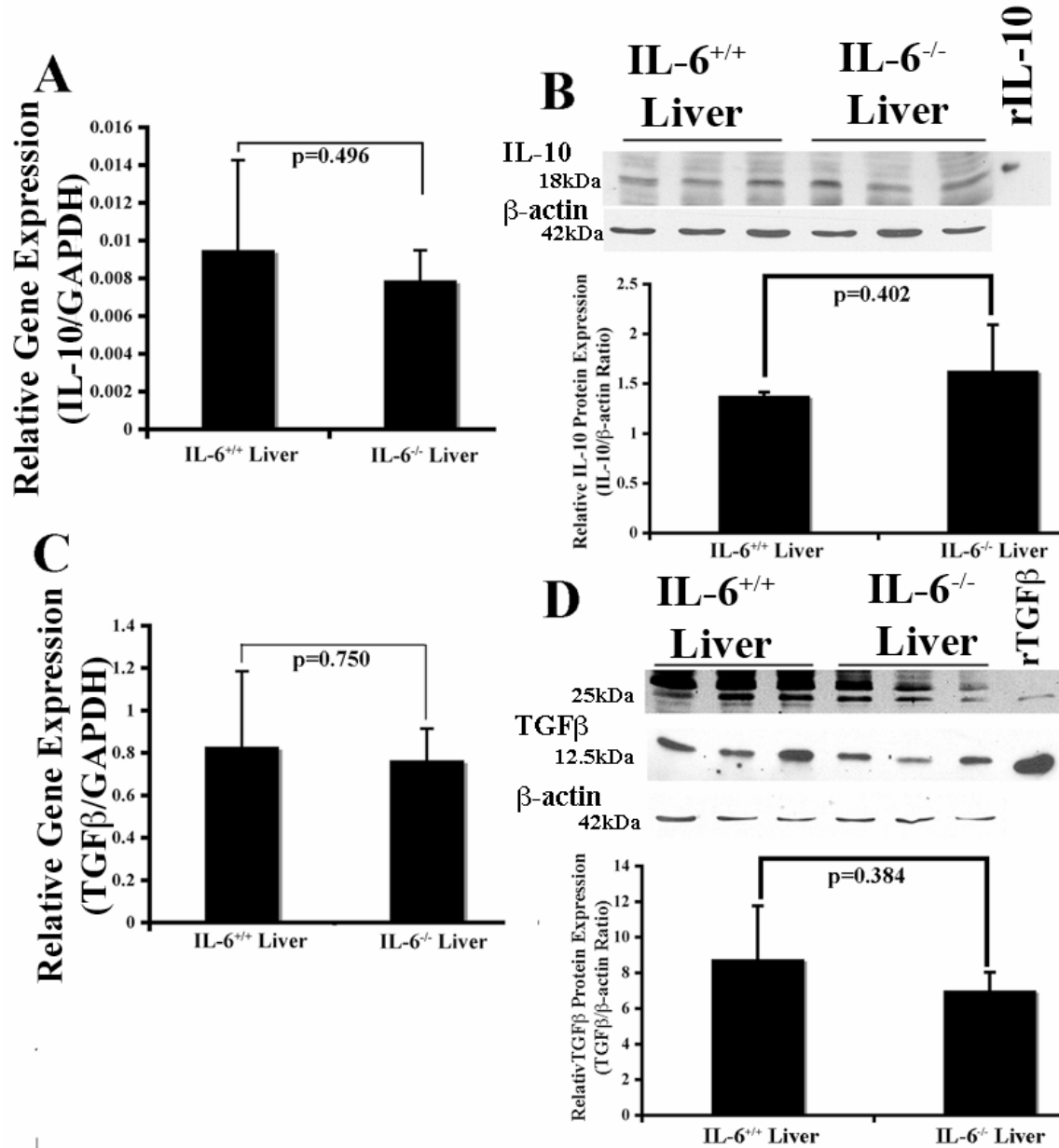


Figure 12. IL-6 status does not influence normal liver IL-10 and TGFβ expression. Livers from IL-6^{+/+} and IL-6^{-/-} mice mRNA and protein were analyzed by quantitative real-time RT-PCR and by western blot, respectively, for IL-10 mRNA (A) and protein (B) and TGFβ mRNA (C) and protein (D). Relative gene expression is reported as the ratio of the target gene to GAPDH. IL-10 protein migrates as an 18kDa band, while TGF-β protein migrates as a 25kDa and 12.5kDa in a denaturing gel. Recombinant IL-10 (rIL-10) and TGF-β (rTGF-β) were used as positive controls. β-actin expression was used as a loading control. Individual protein bands were quantified by densitometry and the relative expression presented graphically as the ratio of the protein to β-actin graphically below each blot.

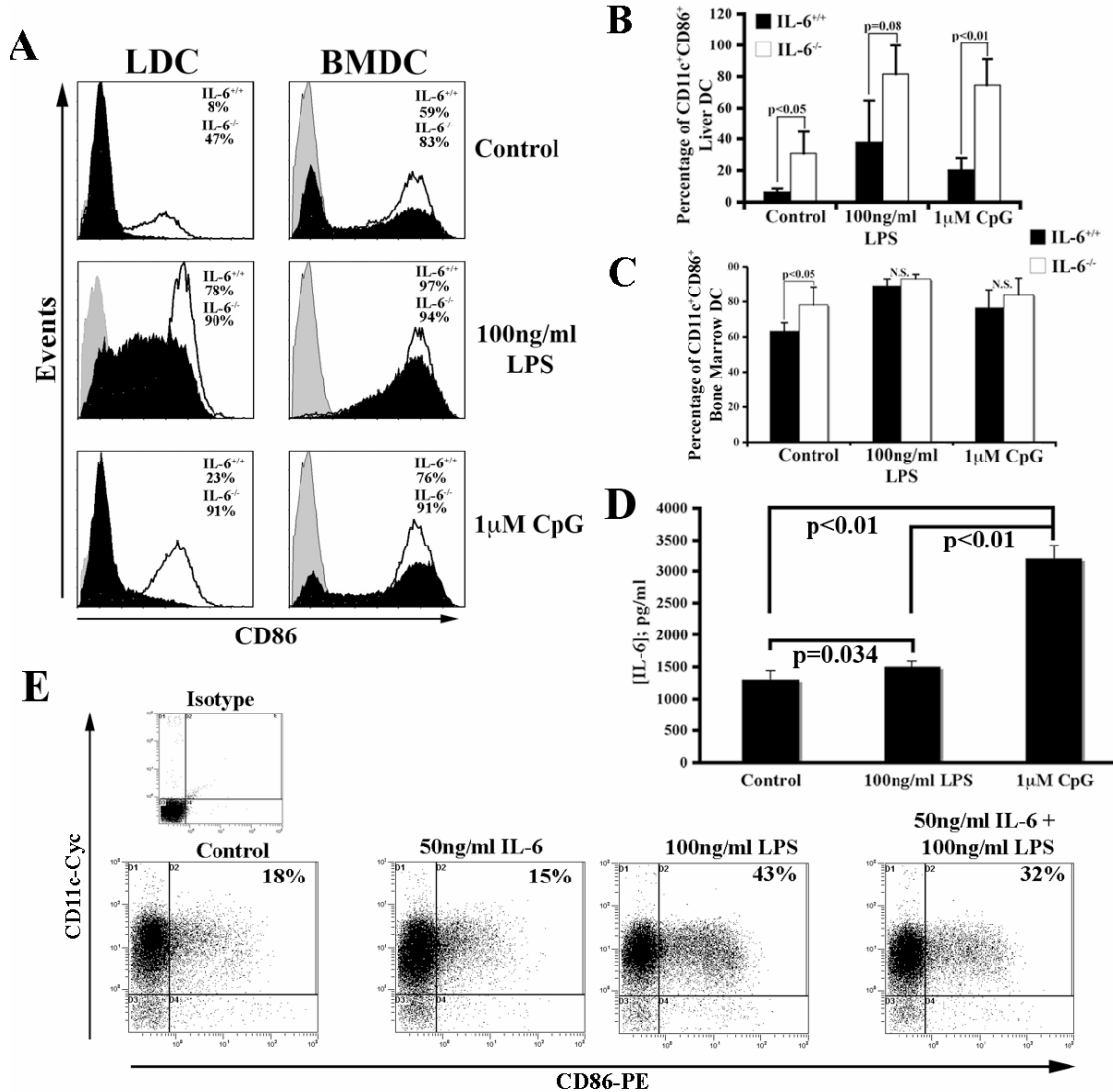


Figure 13. IL-6 reduces the sensitivity of liver DC to stimulation with bacterial TLR agonists. **A)** Liver derived (LDC) and bone marrow derived (BMDC) DC from IL-6^{+/+} (closed black histograms) and IL-6^{-/-} (open black-line histograms) mice were cultured for 48 hours in the presence of the bacterial TLR agonist LPS (100ng/ml) or CpG oligonucleotides (1µM). CD86 expression was analyzed by flow cytometry and gated on CD11c⁺ DC. The percent of CD11c⁺CD86⁺ cells for each strain are listed on each histogram. Overall, liver DC were more resistant to stimulation by TLR agonists than BMDC, but the absence of IL-6 resulted in an increase in CD86 expression. Isotype controls are shown as closed grey histograms. **B-C)** A quantification of CD86 expression in CD11c⁺ liver DC (**B**) or BMDC (**C**). Each bar represents the mean±standard deviation of three independent experiments. N.S.: Not Significant. **D)** IL-6 protein secretion by IL-6^{+/+} liver DC after exposure to 100ng/ml LPS or 1µM CpG oligonucleotides for 48 hours. While IL-6 secretion was significantly higher after TLR stimulation compared to controls, CpG treatment was more potent in inducing IL-6 production. **E)** CD11c⁺ IL-6^{-/-} liver-derived DC were treated for 24 hours in the presence or absence of 50ng/ml IL-6 followed by 48 hours with or without LPS (100ng/ml). DC maturation was assayed by flow cytometry for CD11c and CD86. The percentages of CD11c⁺CD86⁺ DC are reported in each graph. Pre-treatment of IL-6^{-/-} liver DC with IL-6 also reduced the mean fluorescent intensity of CD86 (data not shown).

We also investigated whether the IL-6 status influenced the expression of LPS and CpG recognizing TLR4 and -9. No significant differences were seen in TLR4 and -9 mRNA between IL-6^{+/+} and IL-6^{-/-} liver DC (Figure 14A-B). And while TLR4 protein expression was equal, TLR9 protein expression was significantly lower in IL-6^{+/+} compared to IL-6^{-/-} liver DC (Figure 14C-D). Thus, the reduced TLR9 protein expression in IL-6^{+/+} liver DC may also contribute to the reduced maturation.

Following LPS and/or CpG exposure, TLR signaling is negatively regulated by interleukin-1 receptor associated kinase-M (IRAK-M) (52). Because of the continual hepatic exposure to endotoxin and the blunted maturation response of IL-6^{+/+} liver DC to LPS and CpG, we investigated whether IL-6 influences IRAK-M expression in liver DC. IL-6^{+/+} liver DC (Figure 14E) and whole liver tissue (Figure 14F) expressed significantly more IRAK-M mRNA compared to IL-6^{-/-} liver DC and liver tissue. However, expression of IRAK-M protein revealed an opposite trend: IL-6^{-/-} liver and liver DC had equal or greater IRAK-M expression (data not shown). The discrepancy between mRNA and protein results suggests a complex post-translational regulation of IRAK-M, which we are currently investigating.

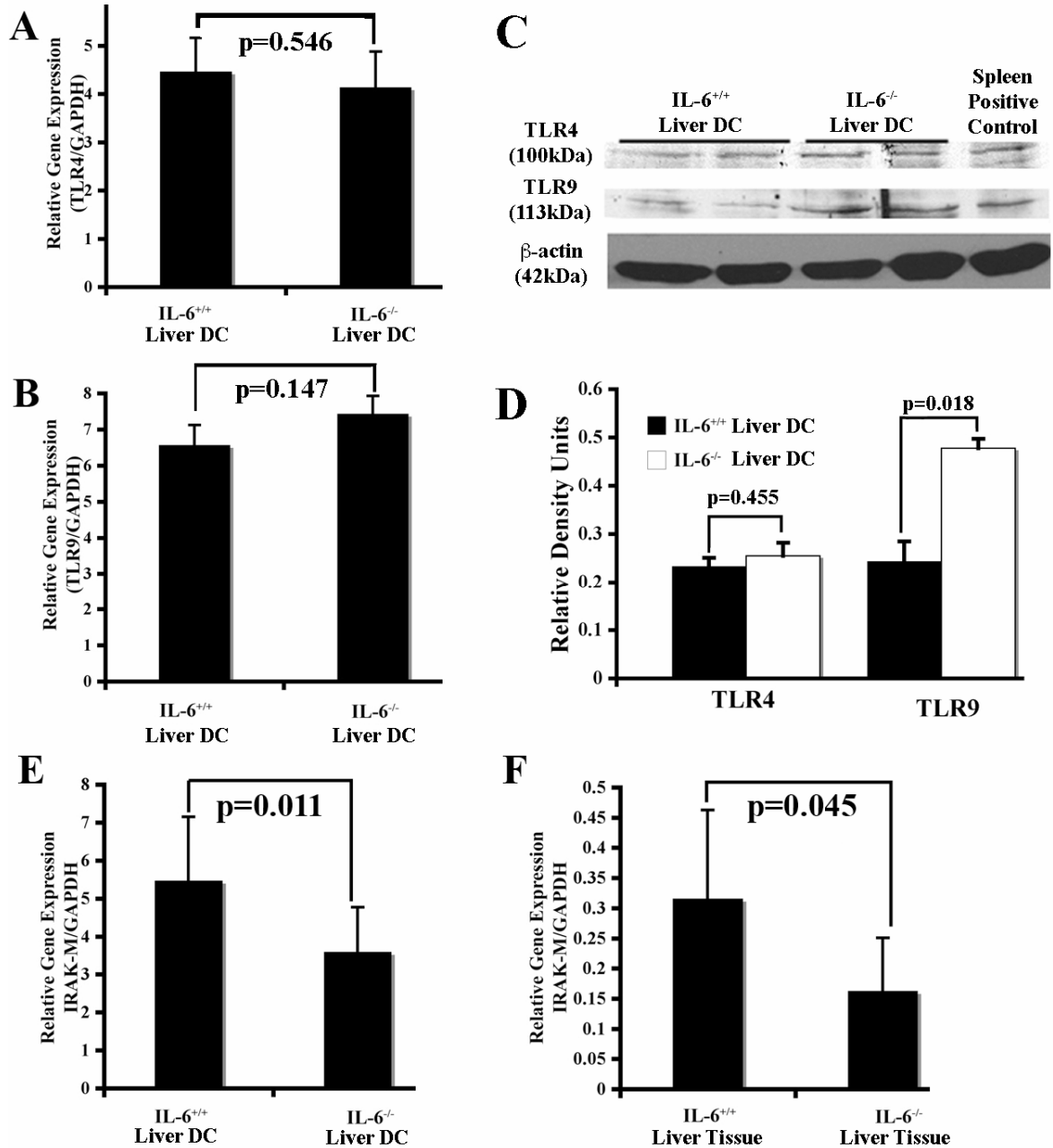


Figure 14. Analysis of TLR4 (A) and TLR9 (B) mRNA and protein (C-D) in freshly isolated IL-6^{+/+} and IL-6^{-/-} liver DC. Gene expression was measured by real-time quantitative RT-PCR and relative gene expression is reported as the ratio of the target gene to GAPDH (A, B). (C) Expression of TLR4 and -9 protein was determined by Western blotting (C) and analyzed by densitometry (D). TLR4 and -9 protein expression were normalized to β-actin expression. Mouse spleen lysate is used as a positive control. (E-F) A negative regulator of TLR signaling, IRAK-M, is elevated in IL-6^{+/+} liver and freshly isolated liver DC compared to IL-6^{-/-}. IRAK-M mRNA was analyzed by quantitative real-time RT-PCR in freshly isolated liver DC (E) and whole liver tissue (F).

4.3.6 Gut-derived commensal bacterial endotoxin inhibits liver DC maturation

If gut-derived bacterial PAMPs are responsible for the IL-6/STAT3 signaling mediated immaturity of liver DC, depletion of commensal bacteria by oral antibiotics should increase DC maturation in IL-6^{+/+} livers. IL-6^{+/+} and IL-6^{-/-} mice were fed antibiotics or control water by gavage for 5 days. Antibiotic treatment significantly reduced the endotoxin levels in the portal blood (Figure 15A) resulting in a significant decrease in liver tissue IL-6 protein (Figure 15B) and nuclear phospho-STAT3 (Figure 15C) expression in IL-6^{+/+} liver tissue compared to control-treated control mice. Control-treated IL-6^{-/-} liver tissue expressed little phosphorylated nuclear STAT3, as expected. Antibiotic-treated IL-6^{-/-} mice, however, showed a significant increase in baseline phosphorylated STAT3 to levels comparable to those seen antibiotic-treated IL-6^{+/+} liver. Preliminary observations suggest that an alternative compensation mechanism may be operative in the IL-6^{-/-} mice, because after antibiotic treatment, they also showed increased glycogen storage, which was absent in all other treatment groups (data not shown).

The decreased portal blood endotoxin, hepatic IL-6 protein, and activated STAT3 in antibiotic-treated IL-6^{+/+} mice resulted in a significant increase in CD80, CD86 and MHC class II expression compared to control-treated IL-6^{+/+} liver DC (Figure 15D). No differences were observed in antibiotic- or control-treated IL-6^{-/-} liver DC, even though STAT3 was elevated in antibiotic-treated IL-6^{-/-} liver. Antibiotic-treatment did not alter the distribution or maturation in the CD11c⁺ liver DC subtype populations (data not shown). These data show gut-derived bacterial products stimulate STAT3 in an IL-6-dependent manner thereby inhibiting liver DC maturation.

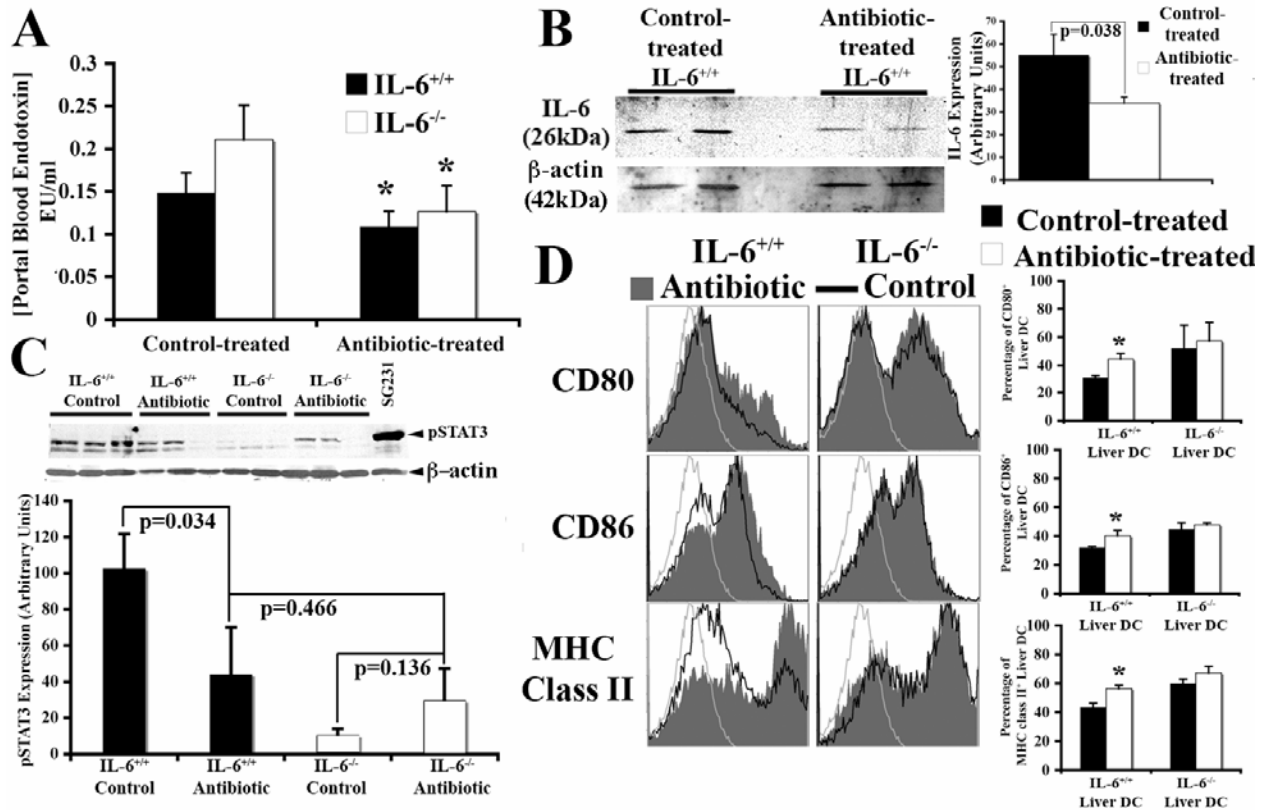


Figure 15. Oral antibiotic-treatment increases IL-6^{+/+} liver DC maturation. A) IL-6^{+/+} and IL-6^{-/-} mice treated with oral antibiotics by gavage had a significant reduction in the concentration of portal blood endotoxin compared to control mice fed water by gavage. * P<0.05 versus control-treated mice. B) Expression of IL-6 protein was measured in liver tissue from control- or antibiotic-treated IL-6^{+/+} mice by Western blot. Livers from antibiotic-treated IL-6^{+/+} mice had significantly lower expression of IL-6 than control-treated mice. C) Antibiotic-treated IL-6^{+/+} liver tissue expressed significantly less phospho-STAT3 than control-treated IL-6^{+/+} livers. IL-6^{-/-} livers from antibiotic-treated mice expressed elevated phospho-STAT3, though this was still lower than antibiotic-treated IL-6^{+/+} liver. Lysate from SG231 cells was used as a positive control. D) The reduced portal blood endotoxin, IL-6 protein and phospho-STAT3 in antibiotic-treated IL-6^{+/+} mice resulted in an increase in IL-6^{+/+} liver DC expression of CD80, CD86 and MHC class II compared to control-treated IL-6^{+/+} mice. Histograms are gated on CD11c⁺ liver DC. No significant differences in liver DC maturation were observed between antibiotic- or control-treated IL-6^{-/-} mice, even though antibiotic-treated IL-6^{-/-} mice had increased phospho-STAT3. Graphs show the mean±standard deviation from 3 separate experiments. * P<0.05 versus control-treated IL-6^{+/+} liver DC.

4.4 DISCUSSION

In this study we show that, under normal physiological circumstances, constitutive hepatic IL-6/STAT3 signaling stimulated by commensal gut bacteria contributes significantly toward maintenance of hepatic DC in a relatively immature state compared to DC from other organs. Since the liver is continually bathed in bacterial components and potential antigens a mechanism preventing liver DC maturation in response to these stimuli would be beneficial. Ironically, these studies show that the bacteria themselves, through endotoxin and CpGs, contribute significantly to hepatic DC hypo-responsiveness. The interdependence between gut bacteria and liver DC raises threshold needed for PAMP molecules to stimulate liver DC activation/maturation and, thereby, translate innate signaling into adaptive immunity.

IL-6 is generally considered to be a pro-inflammatory cytokine because it is elevated along with other pro-inflammatory cytokines, including TNF α , IL-1 and IFN γ , during early stages of inflammatory responses (296). IL-6 also inhibits the suppressive ability of regulatory T-cells (277); stimulates B-cell proliferation and generation of plasma cells (320); and in conjunction with TGF β , drives polarization of CD4⁺ T-cells to a Th₁₇ lineage, which have been linked to autoimmunity (278). Transgenic mice over-expressing IL-6 are also more prone to develop plasmacytosis and exhibit autoimmune characteristics (323).

In contrast, several lines of evidence suggest that IL-6 might also be considered as an anti-inflammatory cytokine. For example, IL-6/STAT3 signaling inhibits macrophage and DC maturation (79-81, 259, 300, 302). And IL-6^{-/-} mice treated with an intraperitoneal injection of *E.coli* and/or LPS have increased mortality (298), a deficient fever response (297), and a

significantly increased TNF α and IFN γ cytokine response (296) compared to IL-6^{+/+} mice. Additionally, granulomas formed after intravenous *Rhodococcus aurantiacus* injection were larger and associated with significant elevations of pro-inflammatory cytokines in IL-6^{-/-} mice compared to IL-6^{+/+} controls (324). Collectively, these studies suggest that IL-6 can both facilitate activation of adaptive cellular immunity while suppressing innate immune responsiveness by acting in a spatio-temporal manner on each arm of the immune response. In this role, IL-6 appears to be involved in setting the threshold for communication between the two arms of the immune system.

IL-6 is a potent activator of hepatic STAT3 activity (325) and widely-recognized as a participant in various aspects of hepatic pathophysiology, including liver regeneration (294), induction of the acute phase response (320), sepsis (295), and biliary tract barrier function and wound repair (321). The current results show that baseline STAT3 activity in the liver is significantly higher than in the spleen, intestine, lung, and heart. Although hepatic STAT3 activity is significantly lower in IL-6^{-/-} compared to IL-6^{+/+} mouse livers, liver STAT3 levels in IL-6^{-/-} mice are still significantly higher than all other organs from both IL-6^{-/-} and IL-6^{+/+} mice. Cytokines responsible for maintenance of STAT3 activity in IL-6^{-/-} liver are currently unknown.

Previous studies showed that liver DC are more immature than DC from other solid organs (317), but aside from reduced TLR4 expression (105), the underlying mechanisms responsible for this immaturity are not fully understood. Diverse subtypes of liver DC, including potentially hyporesponsive populations, may also contribute to the overall liver DC phenotype (92, 96). Our results, however, show that hepatic IL-6 contributes significantly to liver DC immaturity and hyporesponsiveness in all subtypes analyzed.

It has been speculated that anti-inflammatory cytokines such as TGF β and IL-10 are important suppressors of hepatic DC maturation (322). We found, however, no differences in whole liver TGF β and IL-10 mRNA or protein expression, suggesting that the anti-inflammatory effects of IL-6 were not due to elevated expression of these cytokines. This is also consistent with reports showing that IL-10 did not compensate for the absence of IL-6 during LPS mediated inflammation (296). Hepatic IL-6, therefore, is likely an important component of maintaining phenotypic immaturity in liver DC under physiologic conditions.

We show that IL-6 protein is detectable in IL-6^{+/+} liver tissue and is likely produced and utilized within the liver itself. Mouse blood sampled from the portal vein and inferior vena cava had similar IL-6 protein levels (data not shown). IL-6 mRNA is also readily detectable from freshly isolated liver mononuclear cells and micro-dissected Kupffer cells and is significantly higher than in monocytes from the spleen or Peyer's patches (184, 319). Bacterial LPS and CpG induce IL-6 expression in Kupffer cells (326) and liver DC (92). Antibiotic-treated IL-6^{+/+} mice with significantly reduced portal blood endotoxin have decreased hepatic IL-6 expression compared to control-treated mice (Figure 15). This is consistent with data showing lower IL-6 mRNA in livers from gnotobiotic mice compared to specific-pathogen free mice possessing normal gut flora (287).

Lower portal blood endotoxin and hepatic IL-6 in antibiotic-treated IL-6^{+/+} mice resulted in a significant reduction of phospho-STAT3 expression and liver DC that were *more* mature than control-treated IL-6^{+/+} liver DC. Antibiotic-treated IL-6^{-/-} mouse liver DC maturation did not differ from controls. Our results can be melded into a simple, yet elegant, physiological model where elevated portal venous blood commensal gut bacterial products stimulate hepatic IL-6/STAT3 activity, which in turn, inhibits liver DC maturation (Figure 16). Elevated liver IL-

6/STAT3 likely raises the threshold needed by multitude of potential portal venous blood antigens and bacterial products to stimulate systemic adaptive immunity.

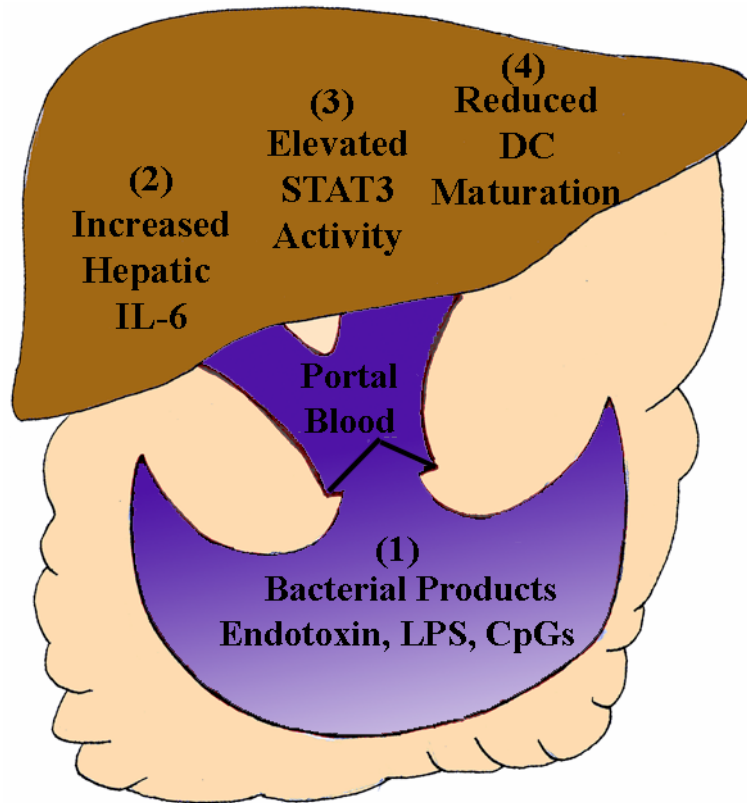


Figure 16. IL-6/STAT3 signaling stimulated by gut-derived bacterial products inhibits liver DC maturation and function. This model provides an explanation for the reduced maturation and function of liver DC compared to DC in other peripheral organs. 1) Bacterial PAMPs from commensal gut bacteria, including endotoxin/LPS and CpG oligonucleotides are elevated in the portal blood. This, in turn, stimulates intrahepatic production of IL-6 (2) and by binding to gp80 and signaling through gp130 activates liver STAT3 (3). As a result of elevated hepatic IL-6/STAT3 signaling liver DC have a higher threshold to maturation inducing stimuli (4).

In vitro, stimulation of IL-6^{+/+} liver DC with either LPS or CpG resulted in dramatically less CD86 expression compared to IL-6^{-/-} liver DC. In contrast, similar treatment of IL-6^{+/+} BMDC resulted in significantly more maturation than hepatic DC. And while IL-6^{+/+} liver DC resisted CpG induced maturation marker expression, CpG-treatment did stimulate DC to produce significantly more IL-6 compared to controls or LPS (Figure 10D). Thus, liver DC do respond to CpG stimulation with elevated IL-6 secretion, possibly through a TLR9-independent pathway, and this maintains DC in a relatively immature state.

It will be important to determine qualitatively or quantitatively whether different molecular mechanisms are needed counteract the inhibitory influence of IL-6/STAT3 signaling on DC maturation. This will help to better understand how immune responses are initiated in the liver or after DC have migrated to regional lymph nodes. IL-6/STAT3 appears to upregulate intracellular negative regulators of TLR signaling, including IRAK-M. After initial TLR agonist stimulation, IRAK-M inhibits TLR signaling by preventing formation of IRAK-1/TRAF6 complexes leading to an endotoxin tolerant state (52). Our results show that IRAK-M mRNA levels were significantly higher in IL-6^{+/+} liver DC and liver tissue compared to IL-6^{-/-} liver tissue. But, IRAK-M protein expression revealed an opposite trend. Since IL-6/STAT3 signaling has not been previously shown to influence the expression of IRAK-M we are currently investigating molecular mechanisms responsible for these observations.

Lastly, the influence of gut bacteria and IL-6/STAT3 signaling on DC allo-stimulation might help explain why long-surviving stable liver allografts recipients can be more readily weaned from immunosuppression (132), and why septic allograft recipients that have elevated serum IL-6 (298) can be withdrawn from all immunosuppression with a low risk of rejection

(327). This study also raises the question whether manipulation of gut bacteria can be exploited therapeutically to either augment or inhibit intra-hepatic immune responses.

4.5 SIGNIFICANCE

It is known that the liver tolerates the continual perfusion with antigen and PAMP rich portal blood. However, the underlying mechanisms maintaining this tolerance are unclear. There has been much focus trying to identify the cellular mechanisms preventing the immune activation of hepatic cells in response to the portal blood PAMPs. Many studies cite Kupffer cell production of IL-10 after LPS stimulation as a cytokine contributor to liver immune quiescence (185). However, the amount of LPS used in this study, 1 μ g/mL, is well above that observed during any pathological event, even bacterial sepsis. On the other hand, IL-6 is physiologically produced by liver Kupffer cells in response to commensal gut bacteria endotoxin and IL-6 potently stimulates liver STAT3 activity. Considering the reports from the tumor immunology field showing that elevated tumor STAT3 activity confers immune unresponsiveness to neoplastic cells, the elevated STAT3 activity in the normal liver might be a similar mechanism to prevent immune activation by gut derived PAMP molecules.

Gut derived PAMP molecules are the principal stimulators of IL-6 in the liver. Using antibiotics to reduce portal blood endotoxin in mice, we show that gut derived PAMP molecule stimulation of IL-6/STAT3 signaling significantly inhibits liver DC function and maturation. It therefore is reasonable to consider commensal gut bacterial PAMP molecules as a kind of immunosuppressive agent for normal liver DC. Studies using functional models of liver immunology should further demonstrate whether manipulation of gut bacteria is a useful

therapeutic tool to alter inflammation in the liver. This could be beneficial in stimulating the immune clearance of chronic hepatitis B and C infections. Additionally, identifying bacterial strains that might further stimulate liver IL-6/STAT3 activity might be used in generating or maintaining liver allograft tolerance.

**5.0 THRESHOLDING EVENTS INVOLVED IN DENDRITIC CELL MATURATION
VIA INTERLEUKIN-6/STAT3 SIGNALING.**

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5.1 ABSTRACT

Professional antigen presenting dendritic cells (DC) bridge the innate and adaptive immune systems by detecting pathogen associated molecular pattern (PAMP) molecules, which trigger DC maturation and migration to regional lymph nodes where they stimulate T lymphocytes. DC in tissues normally exposed to relatively high levels of PAMP molecules have a higher threshold to stimulation than DC at other sites. For example, gut commensal bacteria-dependent constitutive IL-6/STAT3 signaling inhibits DC maturation in normal liver. In this study we tested the hypothesis that IL-6 raises the threshold needed for PAMP molecules to induce DC maturation via upregulation of STAT3 activity. Results show that IL-6 wild-type (IL-6^{+/+}) bone marrow derived DC are significantly less mature than IL-6-deficient (IL-6^{-/-}) DC because of elevated STAT3 activity, which in turn, is significantly influenced via negative reciprocal regulation with MAP Kinase activity. Inhibition of normally high STAT3 activity inhibits DC maturation in IL-6^{+/+} but has no effect in IL-6^{-/-} DC, which normally show little or no constitutive STAT3 activity. A concentration response curve showed that, compared to IL-6^{-/-} DC, IL-6^{+/+} DC significantly resisted maturation in response to low concentrations of LPS, CpG oligonucleotides and polyI:C. Except for CpG oligonucleotides, however, higher concentrations of these same ligands stimulated maturation of both IL-6^{+/+} and IL-6^{-/-} DC. Conditioned media experiments further substantiated that microenvironmental IL-6 levels and STAT3 signaling controlled basal DC maturation, DC response to PAMP molecules, and macrophage proliferation/survival. Thus, environmental IL-6 levels and subsequent gp130/STAT3 signaling significantly influence how DC respond to potential pathogens.

5.2 INTRODUCTION

Dendritic cells (DC) are bone marrow-derived professional antigen presenting cells that bridge innate and adaptive immunity and are involved in tolerance induction/maintenance (1-3). DC maturation and function are influenced by exposure to pathogen associated molecular pattern (PAMP) molecules recognized by pattern recognition receptors (PRR) such as the toll-like receptors (TLR) and other endogenous ligands (328). DC in tissues continually exposed to PAMP molecules, however, are usually resistant to maturational stimuli (83). For example, in the liver we have shown that perfusion with endotoxin-rich portal blood stimulates hepatic interleukin-6 (IL-6) production and constitutive signaling through signal transducer and activator of transcription 3 (STAT3), which in turn, inhibits liver DC maturation (34).

IL-6 is a member of a cytokine family that signals through a common signaling receptor, gp130. Each ligand of the gp130 family activates primarily either the Jak-STAT3 or the Ras-Raf-Erk1/2 MAP Kinase pathways. And negative reciprocal regulation between these pathways usually results in predominance of one over the other (233, 238). IL-6/gp130 signaling is mediated primarily through the STAT3 pathway and IL-6^{-/-} mice phenotype resembles mice with conditional deficiencies of STAT3 (329). Blocking STAT3 activity in wild type mice typically results in stronger signaling through the Ras-Raf-Erk1/2 MAP kinase pathway because of release of reciprocal negative regulation, which is known to exist between these pathways (233, 238).

IL-6 has generally been considered a pro-inflammatory cytokine based largely on its elevation, along with other pro-inflammatory cytokines such as TNF α and IFN γ , during systemic inflammation or injury (296). IL-6 also promotes effector cell activation, B-cell proliferation and differentiation to plasma cells, and inhibition of regulatory T-cell function. In conjunction

with TGF β , IL-6 also drives the differentiation of IL-17 producing CD4⁺ T-cells (277, 278, 330, 331).

Conversely, IL-6 is elevated during sepsis and is associated with a global immunosuppression, including decreased sensitivity of DC to further PAMP stimulation, reduced ability of DC to induce T-cell proliferation, and increased secretion of anti-inflammatory cytokines (295, 332). In general, DC derived from IL-6 deficient (IL-6^{-/-}) mice are also more mature than wild-type (IL-6^{+/+}) DC (259, 300, 302), except for one study that showed somewhat contradictory results (333). IL-6 also prevents the exaggerated expression of pro-inflammatory TNF α and IFN γ in IL-6^{-/-} mice following endotoxin exposure (298). In the liver, IL-6/STAT3 signaling stimulated by commensal gut-bacterial products inhibits hepatic DC maturation and function (34). Collectively, these studies suggest that under some circumstances, IL-6 can exert an anti-inflammatory influence (299).

In this study, we tested the hypothesis that IL-6 signaling activation of STAT3 raises the threshold of maturation for DC in response to PAMPs. Using IL-6^{+/+} and IL-6^{-/-} bone marrow (BM) derived DC we show that predominant IL-6 signaling through STAT3 inhibits DC maturation in response to low concentrations of TLR agonists, but DC maturation does occur when immature DC are exposed to higher concentrations of TLR agonists, except for CpGs. Additionally, DC cultured in the presence of elevated IL-6 resist maturation in response to PAMP stimulation. This suggests that the IL-6/STAT3 signaling by DC elevates the threshold needed for PAMP induced maturation.

5.3 RESULTS

5.3.1 Increased STAT3 activity in IL-6^{+/+} DC inhibits maturation.

IL-6 signaling through gp130 can stimulate both JAK-STAT3 and Ras-Raf-MEK1-ERK1/2 MAP Kinase pathways, although the STAT3 pathway usually predominates (233). The activity, however, of IL-6/gp130: STAT3 or ERK1/2 signaling in DC has not been investigated. Since a negative reciprocal relationship exists between each arm of IL-6-gp130 signaling (238), we analyzed the relative contribution of each pathway and used specific chemical inhibitors in IL-6^{+/+} and IL-6^{-/-} DC to assess the influence of each pathway on DC maturation. DC were examined at day 6 in culture when IL-6 secreted into the culture medium is maximal (Figure 17A). Under baseline culture conditions, STAT3 DNA binding activity and phosphorylated STAT3 expression was significantly higher in IL-6^{+/+} compared to IL-6^{-/-} DC (Figure 17B and F), as expected. Conversely, expression of phosphorylated ERK1/2 was significantly lower in IL-6^{+/+} DC compared to IL-6^{-/-} DC (Figure 17C). These results show that in the basal state IL-6^{+/+} DC shows constitutive STAT3 activity, whereas basal ERK1/2 activity is higher in IL-6^{-/-} DC probably due to an absence of reciprocal negative regulation of STAT3 activity.

The differences in STAT3 and ERK1/2 signaling in IL-6^{+/+} and IL-6^{-/-} DC were associated with altered DC maturation as measured by cell surface expression of CD80, CD86, CCR7, and MHC class II. IL-6^{+/+} DC with elevated STAT3 activity expressed significantly less CD80, CD86, CCR7, and MHC class II indicating a immature phenotype relative to IL-6^{-/-} DC (Figure 17D). In comparison, the predominant gp130-ERK1/2 signaling in IL-6^{-/-} DC resulted in comparatively greater DC maturation (Figure 17D). The influence of gp130 signaling on DC maturation was further substantiated by inhibiting DC STAT3 or MEK1-ERK1/2 with their

respective chemical inhibitors, JSI-124 and PD98059. CD86 expression was used to measure maturation. Blocking the higher STAT3 activity in IL-6^{+/+} DC with JSI-124 significantly increased CD86 expression compared to vehicle treated controls (Figure 17E) while PD98059 inhibition of ERK1/2 had little effect on maturation (Figure 17E). In contrast, JSI-124 treatment did not significantly alter maturation of IL-6^{-/-} DC (Figure 17E), which have significantly lower STAT3 activity than IL-6^{+/+} DC under baseline conditions (Figure 17A). Blocking MEK1-ERK1/2 signaling in IL-6^{-/-} DC, however, resulted in decreased CD86 expression compared to vehicle controls (Figure 17E).

Western blot analysis confirmed the efficacy of the chemical inhibition of STAT3 and MEK1-ERK1/2 signaling. JSI-124 treatment effectively reduced STAT3 phosphorylation in IL-6^{+/+} DC (Figure 17F-G). Similarly, blocking MEK1-ERK1/2 activation in IL-6^{-/-} DC with PD98059 not only reduced phospho-ERK1/2 expression (Figure 17F-G) but also elevated STAT3 activity (Figure 17F and H). These results are consistent with the observations that DC maturation is inhibited in mice with mutations in gp130 resulting in signaling through STAT3 compared to gp130 mutants with predominant Ras-Raf-MEK1-ERK1/2 signaling(259, 302).

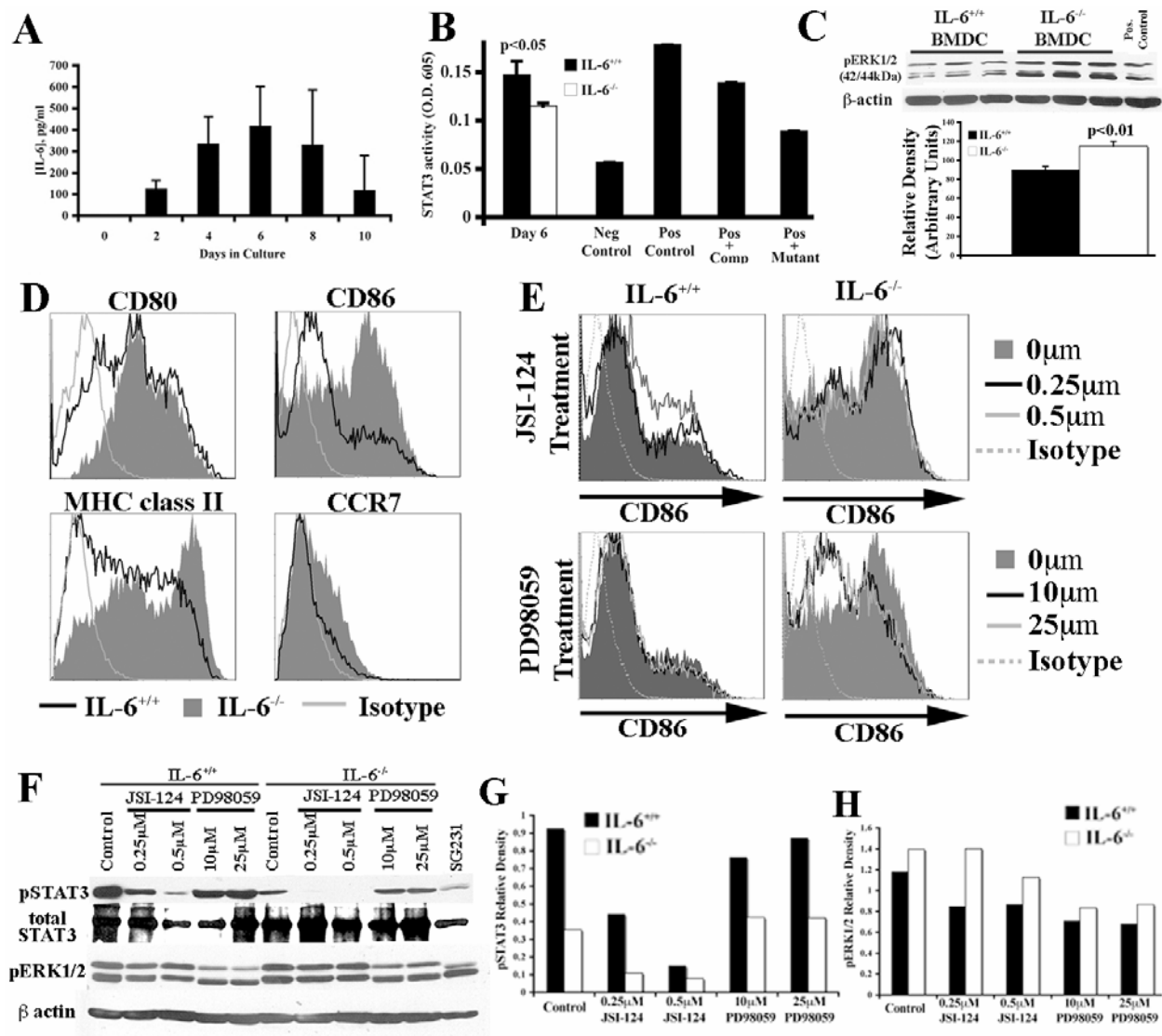


Figure 17. IL-6/STAT3 signaling inhibits DC maturation. A) IL-6 is secreted into the culture medium of bone marrow cells cultured in with GM-CSF and IL-4. IL-6 is detectable by day 2 and peaks at day 6. After day 6, the titers of IL-6 decrease in conjunction with decreased cell viability. B) STAT3 DNA binding activity at day 6 in IL-6^{+/+} DC was significantly higher than in IL-6^{-/-} DC. As controls, HepG2 treated with IL-6 were assayed in the presence or absence of a competitor oligonucleotide sequence or a mutant oligonucleotide sequence. C) Expression of phosphorylated ERK1/2 by Western blot was significantly higher in IL-6^{-/-} DC compared to IL-6^{+/+} DC. Quantification of band intensities by densitometry analysis is shown below the blot. β-actin was used to ensure equal protein loading. D) IL-6^{+/+} DC consistently have reduced expression of the maturation markers CD80, CD86, MHC class II and CCR7 as determined by flow cytometry compared to IL-6^{-/-} DC. Histograms are gated on CD11c⁺ DC populations. E) IL-6^{+/+} and IL-6^{-/-} DC were treated for 48 hours either with the STAT3 inhibitor JSI-124, MEK1-ERK1/2 inhibitor PD98059 or vehicle control. DC were collected and analyzed for CD86 expression by flow cytometry. Histograms are gated on the CD11c⁺ DC population. F) Western blot analysis of phosphorylated STAT3, phosphorylated ERK1/2, total STAT3 and β actin in IL-6^{+/+} and IL-6^{-/-} DC treated with either JSI-124, PD98059 or vehicle controls. Lysates from the SG231 cell line was used as a positive control. G) Relative density quantification of pSTAT3 Western blot. H) Relative density quantification of pERK1/2 Western blot.

5.3.2 IL-6 Alters the Functional Capabilities of Myeloid DC

Compared to functionally mature DC, immature DC show an increased phagocytic activity and reduced ability to stimulate T-cell proliferation (1-3). If IL-6 also influences DC function, in addition to surface marker expression, we would expect these functional endpoints to differ between IL-6^{+/+} and IL-6^{-/-} DC. Particle engulfment, as a measure of DC phagocytosis, was assessed by uptake of phycoerythritan (PE)-labeled 0.04 μ m beads. Receptor-mediated endocytosis, as a measure of DC phagocytosis, was assessed by FITC-Dextran uptake. The percentage and mean fluorescent intensity was measured by flow cytometry in CD11c⁺ DC at time points up to 60 minutes. Consistent with the immature cell surface molecule phenotype, IL-6^{+/+} DC showed significantly greater uptake of FITC-Dextran (Figure 18A, B) and PE-labeled 0.04 μ m beads (Figure 18C, D) compared to more mature IL-6^{-/-} DC. Both strains of DC had minimal uptake of FITC-Dextran when kept at 4°C confirming that the uptake occurring at room temperature was an active process (Figure 18A).

We next compared the ability of IL-6^{+/+} and IL-6^{-/-} DC to stimulate T-cell proliferation in an allogeneic mixed lymphocyte reaction. Decreasing numbers of γ -irradiated C57Bl/6J IL-6^{+/+} and IL-6^{-/-} DC were incubated with 2x10⁵ nylon wool purified splenic Balb/c T-cells for 96 hours. For the last 18 hours, the media was supplemented with 1 μ Ci of [³H]thymidine to measure DNA synthesis. As expected, IL-6^{-/-} DC are significantly more effective stimulators of T-cell proliferation compared to IL-6^{+/+} DC (Figure 18E). No significant T-cell proliferation was observed when syngeneic T-cells were used as responders (data not shown). Collectively, these data show that IL-6 signaling contributes to the functional phenotype of immature DC with increased phagocytosis and reduced T-cell stimulatory ability.

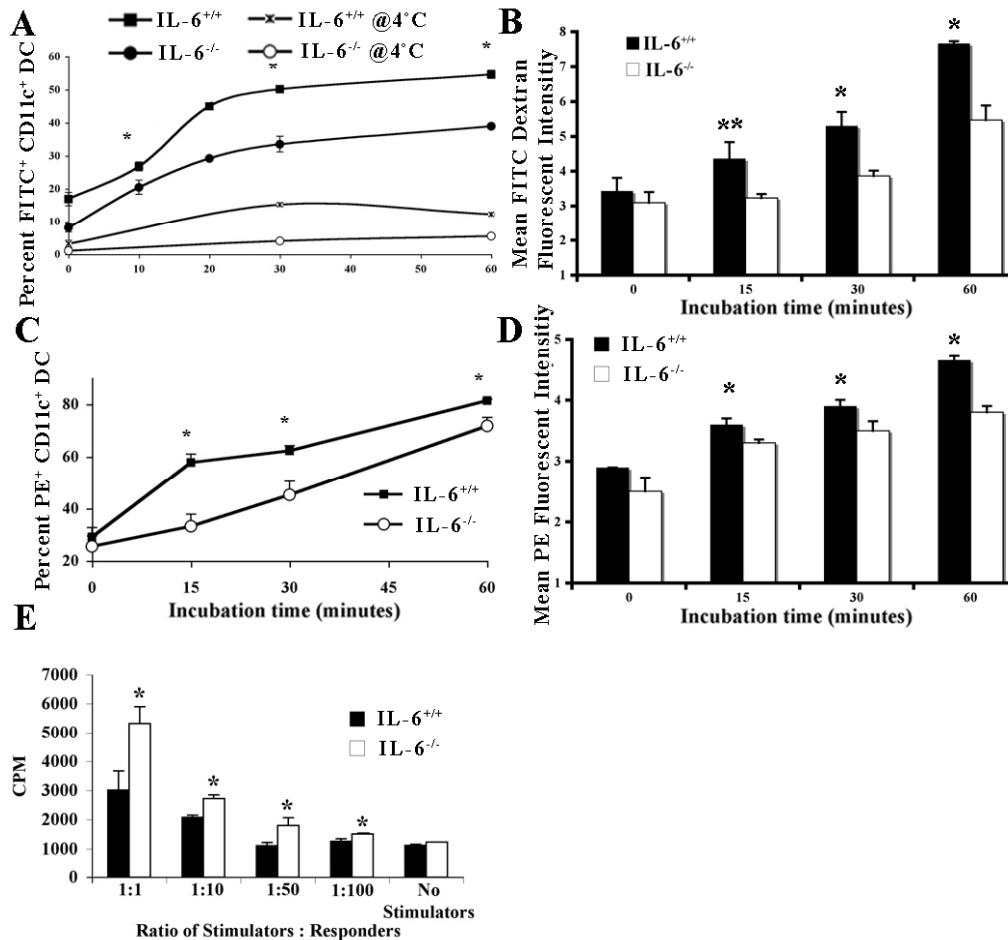


Figure 18. IL-6^{-/-} DC are functionally more mature than IL-6^{+/+} DC. A-B) Phagocytosis was measured by examining the amount of FITC-Dextran (A-B) or 40um PE-microspheres (C-D) engulfed by CD11c⁺ DC over a 60-minute period. The percentage of FITC (A) or PE (C) positive CD11c⁺ DC and the mean FITC (B) and PE (D) fluorescent intensity were quantified by flow cytometry. The more mature IL-6^{-/-} DC had reduced phagocytosis compared to IL-6^{+/+} DC. * P<0.01, ** P<0.05 versus IL-6^{-/-} DC. E) IL-6^{-/-} DC are significantly more potent stimulators of T-cell proliferation than IL-6^{+/+} DC. Decreasing numbers of C57/Bl/6J IL-6^{+/+} and IL-6^{-/-} DC were incubated with nylon wool purified balb/c T-cells in an allogeneic mixed lymphocyte reaction. * P<0.05 versus IL-6^{+/+} DC.

5.3.3 IL-6 signaling decreases the sensitivity to TLR ligands

Our previous analysis of liver DC suggested that IL-6/STAT3 signaling might increase the threshold needed for TLR agonist stimulation to trigger DC maturation (34). We tested, therefore, the response of IL-6^{+/+} and IL-6^{-/-} DC to stimulation with relatively low and high concentration of LPS (TLR4), CpG oligonucleotide (TLR9) and polyI:C (TLR7). DC maturation was assessed by flow cytometric CD86 expression 48 hours after TLR ligand stimulation. Unstimulated IL-6^{+/+} DC expressed significantly less CD86 compared to IL-6^{-/-} DC, as expected (Figure 19). Following stimulation with 0.1ng/ml LPS, 0.1μM CpG oligonucleotides or 1μg/ml polyI:C, almost all IL-6^{-/-} DC expressed CD86 while a significant percentage of IL-6^{+/+} DC resisted maturation. However, when higher concentrations of the TLR agonists were used (100ng/ml LPS, 5μM CpG or 10μg/ml polyI:C) CD86 expression was observed in nearly all IL-6^{+/+} and IL-6^{-/-} DC. A notable exception was that IL-6^{+/+} DC treated with 5μM CpG still showed a significant population of DC that resisted maturation (Figure 19). This response was similar to that of CpG-stimulated IL-6^{+/+} liver DC where the resistance to maturation was associated with significantly higher IL-6 production (34). Also, even though the percentage of IL-6^{+/+} DC expressing CD86 was significantly increased, the mean fluorescent intensity of CD86 expression was significantly lower compared to IL-6^{-/-} DC for all ligands tested. Thus, IL-6 elevates the threshold for DC maturation after TLR stimulation.

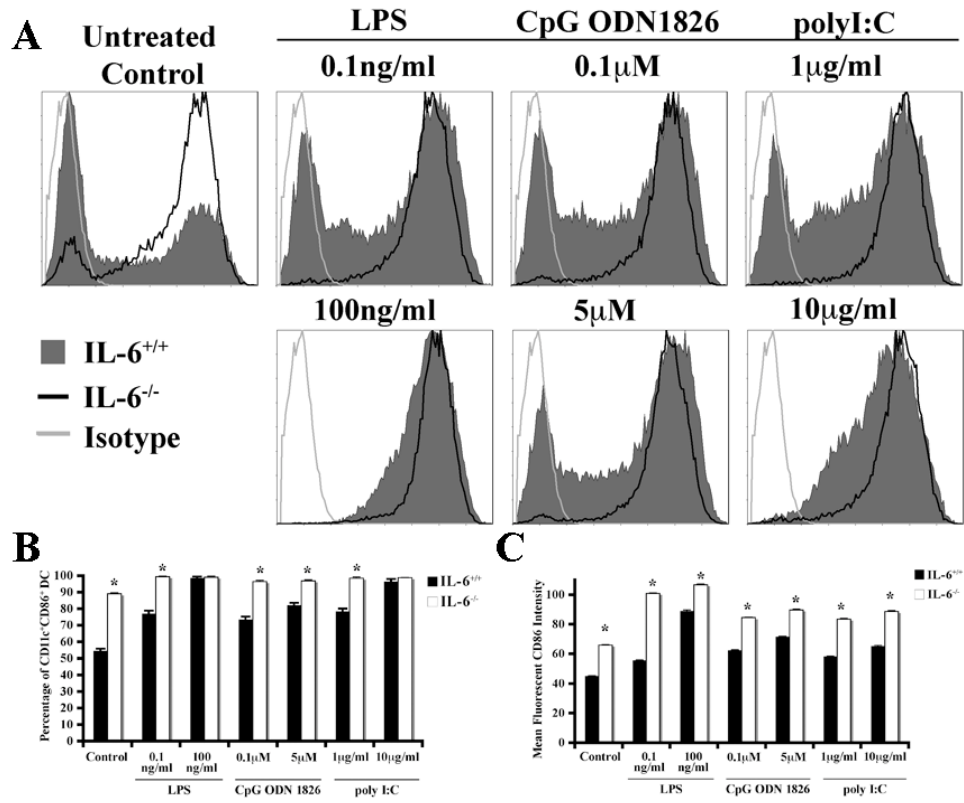


Figure 19. IL-6^{+/+} have an increased threshold toward PAMP molecules than IL-6^{-/-} DC. **A)** Flow cytometry analysis for CD86 in day 6 CD11c⁺ DC stimulated for 48 hours with LPS, CpG or polyI:C. Histograms are gated on the CD11c⁺ population. While nearly all IL-6^{-/-} DC stimulated with lower concentrations of LPS (0.1ng/ml), CpG oligonucleotides (0.1μM) or polyI:C (1μg/ml) expressed CD86, a significant percentage of IL-6^{+/+} DC resisted maturation. At higher concentrations of LPS (100ng/ml), CpG oligonucleotides (5μM) or polyI:C (10μg/ml), IL-6^{+/+} DC responded with an increase in CD86 expression. **B-C)** Quantification of the mean percentage of CD86⁺CD11c⁺ DC (**B**) and the mean CD86 fluorescent intensity (**C**) from 3 individual experiments. * P<0.05 versus IL-6^{+/+} DC.

5.3.4 Autocrine and Paracrine IL-6 signaling decreases the sensitivity to TLR ligands

Since TLR ligands trigger IL-6 production in DC and macrophages (28), we reasoned that secretion of IL-6 into the medium of DC cultures would influence DC maturation. In support of this contention, we observed that when TLR agonists were directly added to DC cultures at day 6, when IL-6 concentrations were highest, DC were more resistant to maturation than if day 6 DC were stimulated in fresh medium (data not shown). To test whether elevated IL-6 at the time of stimulation inhibited DC maturation we cultured IL-6^{+/+} DC in either unconditioned, fresh medium containing minimal IL-6 or in conditioned medium, consisting of one-half IL-6 rich medium from day 6 DC cultures (Figure 17A) and one-half fresh medium. Similar cultures were prepared using DC and conditioned medium from IL-6^{-/-} DC and all cultures were equally supplemented with GM-CSF and IL-4. IL-6^{+/+} DC cultured in conditioned medium for 48 hours had significantly higher concentrations of IL-6 protein compared to IL-6^{+/+} DC cultured in fresh medium (Figure 20A). This resulted in significantly higher STAT3 DNA binding activity in IL-6^{+/+} DC kept in conditioned compared to IL-6^{+/+} cultured in fresh medium (Figure 20B). STAT3 activity in IL-6^{-/-} DC cultured in conditioned medium was significantly higher than IL-6^{-/-} kept in fresh medium, though this was still significantly less than IL-6^{+/+} DC kept in either conditioned or unconditioned media (Figure 20B). Thus, the higher IL-6 levels and STAT3 activity should prevent DC maturation.

Indeed, higher IL-6/STAT3 activity in IL-6^{+/+} DC cultured in conditioned media led to a significantly lower expression of CD86 compared to IL-6^{+/+} DC kept in fresh medium having

lower IL-6/STAT3 activity (Figure 20C-D). No significant differences in CD86 expression were observed when IL-6^{-/-} DC were cultured in either fresh or conditioned medium from day 6 IL-6^{-/-} cultures (Figure 20C-D). Furthermore, treating DC cultures with a neutralizing IL-6 antibody increased CD86 and MHC class II expression in IL-6^{+/+} DC cultured in conditioned medium and to a lesser extent in fresh medium (Figure 20E-F).

Because IL-6^{+/+} DC cultured in the immunosuppressive conditioned media were more immature, we postulated that IL-6^{+/+} DC cultured in conditioned medium would be more resistant to exogenous stimulation by LPS, a representative TLR agonists. IL-6^{+/+} and IL-6^{-/-} DC were cultured in either conditioned or fresh media, prepared as above, in the presence or absence of either 0.1ng/ml or 100ng/ml LPS. DC maturation was examined by flow cytometry 48 hours later. When IL-6^{+/+} DC cultured in conditioned medium were stimulated with 0.1ng/ml LPS, CD86 expression was significantly less compared to IL-6^{+/+} DC cultured in fresh medium. However, the elevated IL-6/STAT3 activity in IL-6^{+/+} DC cultured in conditioned medium was unable to prevent maturation following stimulation with higher concentrations of LPS (Figure 20C-D). IL-6^{-/-} DC kept in either conditioned or fresh media had a strong maturation response to either low or high concentrations of LPS. Thus, when DC are cultured in a microenvironment with elevated IL-6, higher DC STAT3 activity elevates the threshold to stimulation with LPS, though DC are still capable of responding to higher concentrations of PAMPs.

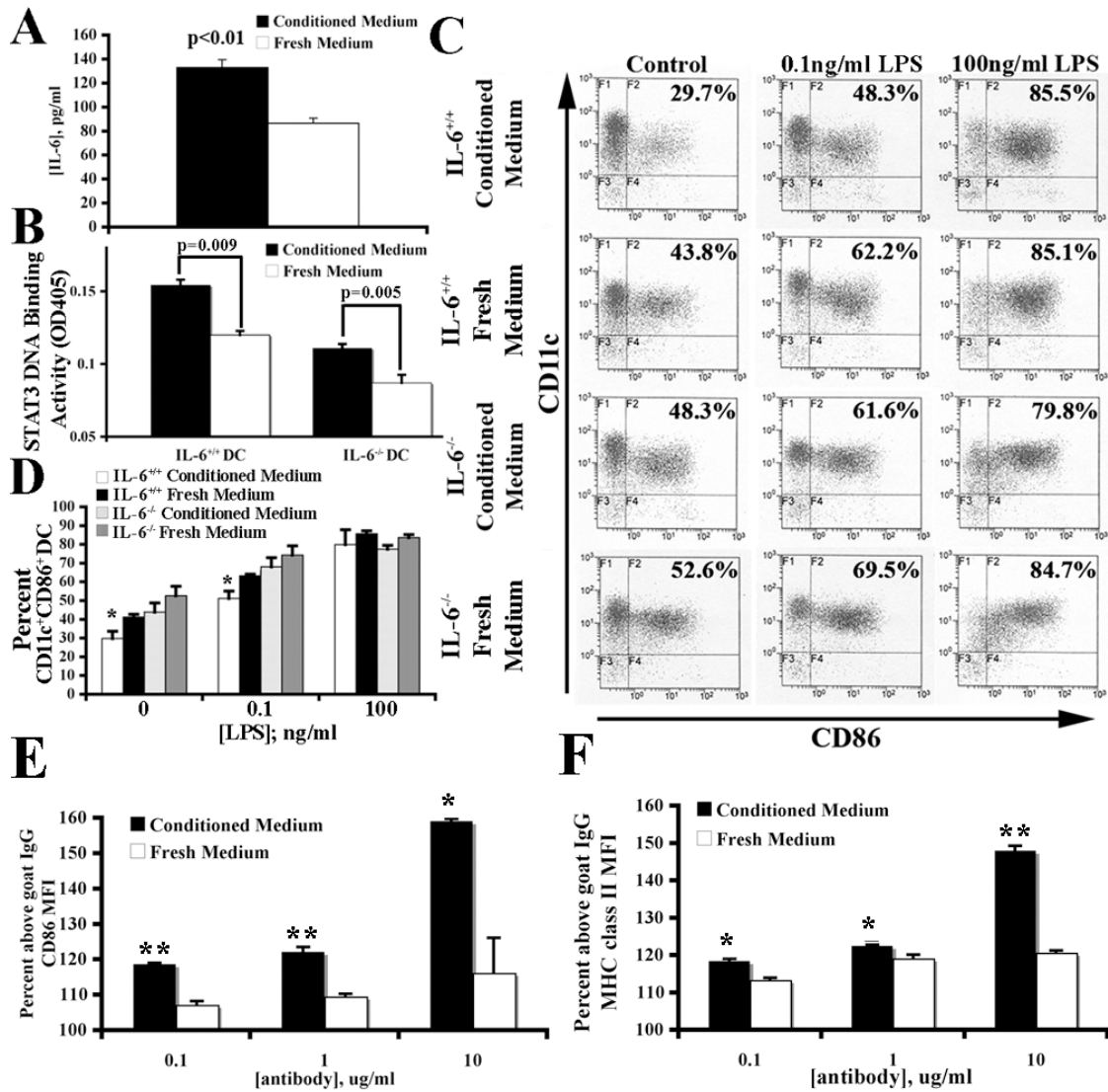


Figure 20. DC cultured in the presence of elevated IL-6 elevates the threshold for stimulation after LPS treatment. A) Culture medium from IL-6^{+/+} DC kept in conditioned medium from day 6 IL-6^{+/+} DC cultures for 48 hours had significantly higher IL-6 protein concentrations compared to IL-6^{+/+} DC cultured in fresh medium. B) IL-6^{+/+} DC kept in conditioned medium for 48 hours also had significantly higher STAT3 DNA binding activity than IL-6^{+/+} DC cultured in fresh medium. A similar trend was seen in IL-6^{-/-} DC, however, STAT3 activity was significantly less than IL-6^{+/+} DC. C) Flow cytometry for CD11c and CD86 in IL-6^{+/+} and IL-6^{-/-} DC cultured in conditioned or unconditioned media supplemented with low (0.1ng/ml) or high (100ng/ml) concentrations of LPS. The results of 3 independent experiments are quantified in (D). Each bar represents the mean \pm standard deviation. * P<0.05 versus all other groups. E-F) Treatment of IL-6^{+/+} DC cultured in either conditioned or fresh medium with neutralizing IL-6 antibody resulted in increased expression of CD86 (E) or MHC class II (F) above that of goat IgG control treated DC. * P<0.05 ** P<0.01 versus fresh media.

5.3.5 IL-6 production in BM cell cultures enhances the number of macrophages but not dendritic cells.

Since paracrine and autocrine IL-6 signaling contributed to the maturational immaturity of myeloid DC we next determined whether it also influenced the generation of DC from bone marrow progenitors. Generation and maturation of myeloid DC derived from IL-6^{+/+} and IL-6^{-/-} bone marrow precursors was studied in the presence of GM-CSF and IL-4 after 6 days in culture when IL-6 secretion into the culture media peaks (Figure 17A). Neither the absolute number (Figure 21A) nor the percentage (Figure 21B) of CD11c⁺ DC in the non-adherent cell fraction was significantly different between IL-6^{+/+} and IL-6^{-/-} BM cultures at day 6 or at any other time-point (data not shown). This is in contrast to a report by Bleier et al (333), who found a significant increase in the number of IL-6^{-/-} DC compared to IL-6^{+/+} BMDC. The discrepancy in results might be due to differences in culture methods between this study (333) and ours. We did, however, observe a significant increase in the number of adherent cells from IL-6^{+/+} BM cultures compared to IL-6^{-/-} BM cultures (Figure 21C and D). Analysis of these cells by flow cytometry showed that over 75% of cells in both strains expressed the macrophage marker F4/80 (Figure 21E).

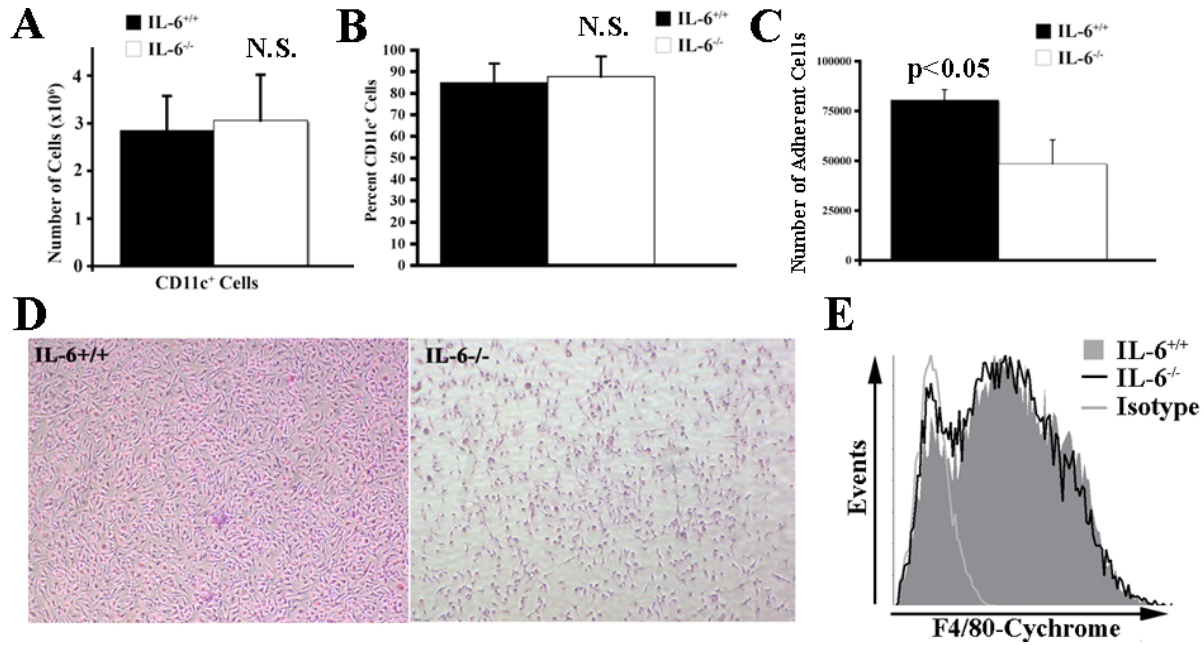


Figure 21. IL-6 influences the number of macrophages, but not the number of non-adherent DC in bone marrow cell cultures. IL-6 in the culture medium did not influence the number (A) or percentage of (B) DC. The number of non-adherent IL-6^{+/+} and IL-6^{-/-} DC were counted at day 6 in culture and analyzed by flow cytometry for CD11c. C) While no difference was observed in the number of non-adherent cells, IL-6^{+/+} BM cultures yield significantly more adherent cells than IL-6^{-/-} cultures. D) Hematoxylin and eosin staining of adherent macrophages at day 6 in culture revealed a dramatic difference between the number of IL-6^{+/+} and IL-6^{-/-} cells. E) Flow cytometric analysis of the adherent cell population for expression of F4/80 showed that over 75% of both IL-6^{+/+} and IL-6^{-/-} adherent cells were macrophages.

5.4 DISCUSSION

IL-6 signaling activates either STAT3 or Ras-Raf-MEK1-ERK1/2 pathway, and negative reciprocal regulation controls signaling through the other pathway so only one arm is dominant (238). Comparing IL-6^{+/+} and IL-6^{-/-} DC revealed that STAT3 activity was significantly higher in IL-6^{+/+} DC while phosphorylated ERK1/2 expression was significantly greater in IL-6^{-/-} DC.

The elevated IL-6/STAT3 signaling in IL-6^{+/+} DC resulted in a reduced cell surface expression of phenotypic maturation markers (CD80, CD86, MHC class II and CCCR7), reduced allogeneic T-cell stimulatory ability and increased phagocytosis compared to IL-6^{-/-} DC. This is in agreement with other studies showing that IL-6 and STAT3 activity can inhibit DC maturation (79, 80, 259, 300, 302). Chemical inhibition of STAT3 activity was able to induce IL-6^{+/+} DC maturation, while inhibitors of MEK1-ERK1/2 had little effect. The opposite effect was observed in IL-6^{-/-} DC; inhibition of STAT3 activity did not significantly alter maturation, but blocking MEK1-ERK1/2 decreased maturation compared to vehicle-treated controls and was associated with an increase in phosphorylated STAT3. Thus, STAT3 appears to be critical in controlling DC maturation.

Our data supports the contention that IL-6 can act as an anti-inflammatory cytokine (299). By elevating the threshold needed for stimuli, such as LPS, IL-6/STAT3 signaling can prevent DC maturation. When DC were stimulated with lower concentrations of PAMP molecules, IL-6^{+/+} significantly resisted maturation while almost the entire IL-6^{-/-} DC population expressed CD86. However, nearly all IL-6^{+/+} and IL-6^{-/-} DC matured after exposure to higher concentrations of TLR agonists, except CpGs. The inability of high concentrations of CpGs to induce maturation in IL-6^{+/+} bone marrow DC is similar to our observation in IL-6^{+/+} liver DC. Rather than inducing maturation, liver DC respond to CpG stimulation by secreting large amounts of IL-6 thereby inhibiting maturation (34).

IL-6 is elevated during sepsis and is associated with decreased functional DC maturation and resistance to further maturation by additional PAMP stimulation (298, 332). This is similar to our observation that IL-6^{+/+} DC cultured in conditioned medium with elevated IL-6 and STAT3 activity have reduced maturation when stimulated with LPS. Elevated IL-6/STAT3

signaling in conditioned medium kept IL-6^{+/+} DC resistant to maturation when stimulated with low concentrations of LPS, whereas IL-6^{+/+} DC cultured in fresh medium had lower IL-6/STAT3 signaling and matured when exposed to low concentrations of LPS. Two lines of evidence show that IL-6 importantly contributes to DC immaturity and resistance to maturational stimuli: 1) IL-6^{-/-} DC cultured in conditioned media from IL-6^{-/-} DC had similar maturation to IL-6^{-/-} DC kept in fresh media; and 2) neutralizing IL-6 antibody added to the IL-6^{+/+} conditioned media restored responsiveness to maturational stimuli. Thus, this culture system, in vitro, effectively mimics a tumor or normal liver microenvironment where elevated STAT3 signaling inhibits DC maturation (79); the same is probably true for sepsis and normal ageing, which are associated with significantly increased circulating IL-6 levels (295, 298, 334).

The thresholding events leading to, or preventing, DC maturation is not unlike those governing neuron activation. The final summation of multiple stimulatory and inhibitory influences results in activation or inhibition of an action potential (335). DC activation is probably regulated in a similar manner. Their large surface area exposes DC to multiple PAMP stimuli and inhibitory mediators. We have shown that high basal STAT3 activity elevates the threshold needed for several PAMPs to trigger DC maturation. Alternatively, as higher concentrations of TLR agonists exceed this threshold, DC maturation can proceed, although the special case for CpGs needs to be investigated further.

The underlying molecular mechanisms preventing DC maturation with elevated IL-6/STAT3 signaling are not well understood. DC maturation in response to TLR agonist stimulation is dependent on NFκB activation (336). The addition of IL-6 to DC treated with LPS has been shown to reduce nuclear translocation of NFκB (300). Activated STAT3 and NFκB can compete for DNA binding when their respective binding sequences overlap or are within

close proximity of each other, as is the case in the $\alpha 2$ macroglobulin promoter (337). It is unknown if NF κ B and STAT3 DNA binding consensus sequences overlap in genes necessary for DC maturation. However, diminished phosphorylated STAT3 in LPS-treated IL-10^{-/-} DC allows for an increased recruitment of the NF κ B c-Rel subunit to the IL-12 promoter, a cytokine typically expressed by mature DC (338). This supports a mechanism where elevated STAT3 DNA binding prevents NF κ B DNA binding and subsequent DC maturation thereby raising the threshold needed for maturation. It could be predicted that once a stoichiometric ratio of NF κ B exceeds activated STAT3, DC maturation would occur.

In addition to the direct role of IL-6 on DC maturation, we also observed a significant increase in the number of F4/80⁺ macrophages generated from IL-6^{+/+} BM compared to IL-6^{-/-} BM cultures. This is consistent with studies showing IL-6 facilitates the generation of macrophages through increasing the M-CSF receptor (301, 339). In contrast with a previous study(333), we observed no difference in the absolute number and percentage of IL-6^{+/+} and IL-6^{-/-} DC in the non-adherent cell fraction. Both macrophages and DC are antigen presenting cells, but DC are generally considered to be more mobile and more potent stimulators of adaptive immune responses than macrophages. Conceivably, an IL-6-mediated increase in the differentiation of myeloid precursors into macrophages rather than DC might influence the overall immune status of the organ.

5.5 SIGNIFICANCE

This study builds upon our work in the liver showing that IL-6/STAT3 signaling inhibits hepatic DC maturation (34). Our results show that if the level of IL-6/STAT3 signaling is increased, DC

have a reduced basal maturation, and more importantly, resist maturation after stimulation with lower concentrations of PAMP molecules. Lowering IL-6/STAT3 by using IL-6^{-/-} DC or reducing IL-6 from the media reduced the threshold towards PAMP stimulation.

Our data can be presented as a simplified model where DC mature in response to lower concentrations of PAMP and DAMP molecules when IL-6/STAT3 signaling is lower (Figure 22). However, when IL-6/STAT3 signaling is elevated, like that normally occurring in the liver, DC have a higher threshold to maturation by PAMPs or DAMPs. This permits DC to resist potentially inappropriate maturation to PAMP or DAMP molecules normally present in the tissue microenvironment, as occurs in the liver, lung, skin and intestines. However, when pathogens or damage exceeds a threshold, DC can mature and stimulate an adaptive immune response. Defining the precise molecular mechanisms of how IL-6/STAT3 signaling inhibits DC maturation or developing methods to inhibit or stimulate IL-6/STAT3 activity could be useful in the modification of immune reactions.

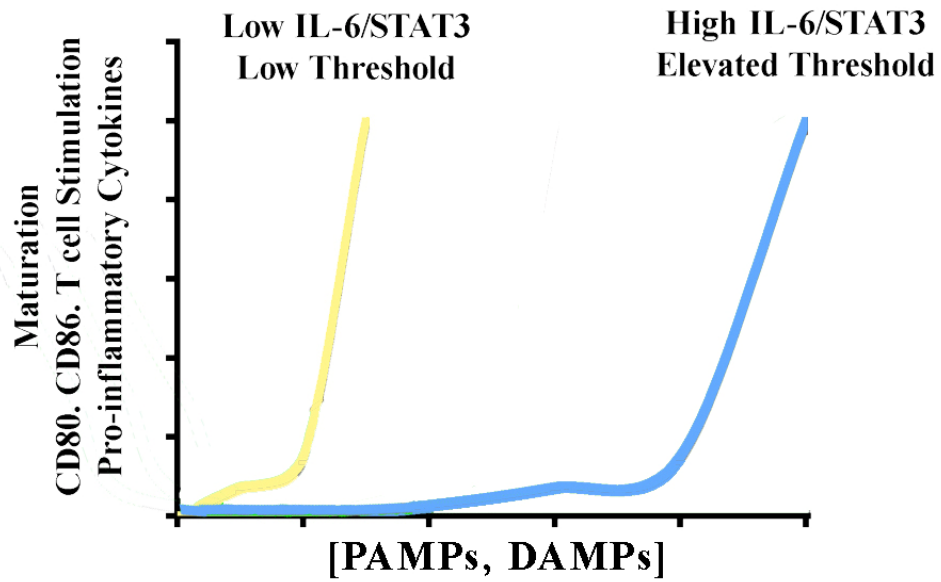


Figure 22. IL-6/STAT3 signaling elevates the threshold toward PAMP and DAMP molecules. During conditions of low IL-6/STAT3 signaling, DC are more sensitive to low concentrations of PAMP or DAMP molecules the threshold for maturation is low. However, when IL-6/STAT3 signaling is high, the threshold to DC maturation is elevated and therefore increased concentrations of PAMP to DAMP molecules are necessary to activate DC.

6.0 FUTURE DIRECTIONS

Our discovery that commensal gut bacteria products stimulate hepatic IL-6/STAT3 activity and thereby inhibits liver DC maturation and function has great implications for controlling hepatic immunity. As the most potent antigen presenting cell, liver DC have the ability to stimulate immune responses. Knowing that IL-6/STAT3 activity prevents liver DC maturation potentially allows altering this signaling pathway to manipulate hepatic immune recognition. For instance, ineffective immune clearance of hepatitis B or C viruses leads to chronic liver disease and cirrhosis. Decreasing hepatic IL-6/STAT3 activity, possibly by decreasing commensal gut bacteria might be effective in increasing hepatic DC maturation and generating intrahepatic immune responses against hepatitis B or C. Alternatively, therapy with neutralizing IL-6 antibody, which is currently in clinical trials for rheumatoid arthritis, might also be effective to lower hepatic IL-6/STAT3 activity and thus increase liver DC maturation and function.

The growth of tumors in the liver, especially metastases of primary tumors from other organs likely also benefits from the tolerant hepatic immune environment. Constitutively active STAT3 in tumors has been shown to be immunosuppressive resulting in a reduced immune recognition of the tumor cells. As IL-6 dependent STAT3 is also elevated in the liver compared to other organs, it is possible, therefore, that by decreasing hepatic IL-6/STAT3 activity, liver DC maturation can be stimulated allowing the immune system to mount an attack against the infection or malignant cells.

While reducing IL-6/STAT3 activity might be an appealing strategy to stimulate hepatic immune activity, elevating hepatic IL-6/STAT3 signaling in liver allograft recipients may be beneficial in preventing rejection. Approximately 15-20% of stable liver allograft recipients can be withdrawn from all immunosuppression without rejection. It is intriguing to speculate that the hepatic STAT3 activity in patients able to be weaned from immunosuppression might be higher compared with those unable to tolerate immunosuppression weaning. This could result in reduced liver DC maturation and the persistence of immature, tolerogenic donor liver DC in the graft. Testing of this hypothesis is currently underway.

Finally, we have shown that reducing gut bacteria can effectively increase DC maturation. While the different bacterial strains populating the gut are known, little information exists about how different bacterial species contribute to stimulating hepatic IL-6/STAT3 activity. This includes differences between gram-negative versus gram-positive strains, the potency of LPS among bacterial strains, and if some bacteria differ in the presence or amount of immunostimulatory CpG containing DNA sequences. Should some bacterial strains be more effective in stimulating hepatic IL-6/STAT3, this might correlate with decreased DC maturation and a more immunosuppressive hepatic microenvironment. Thus, modifying liver immune responses might be as simple as implementing dietary changes or introducing probiotic-containing foods.

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