Ethanol Exposure and Dendritic Cell Function

by

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ETHANOL EXPOSURE AND DENDRITIC CELL FUNCTION

Audrey H. Lau, Ph.D.

University of Pittsburgh, 2006

The influence of ethanol (EtOH) on multiple dendritic cell (DC) subsets, either in steady state or following mobilization in vivo, has not been characterized. Herein, the generation of mouse bone marrow (BM)-derived DC in fms-like tyrosine kinase 3 ligand was inhibited by physiologically-relevant concentrations of EtOH, with selective suppression of plasmacytoid (p)DC. EtOH reduced surface expression of costimulatory (CD40, CD80, CD86) but not coinhibitory CD274 (B7-H1) molecules on resting or CpG-stimulated DC subsets. IL-12p70 production by activated DC was impaired. Consistent with these findings, EtOH-exposed (E)BMDC exhibited reduced capacity to induce naïve allogeneic T cell proliferation and impaired ability to prime T cells in vivo. Further, T cells from animals primed with EBMDC produced elevated levels of IL-10 following ex vivo challenge with donor alloantigen. DC subsets freshly-isolated from EtOH-fed mice were also examined. Liver DC, inherently immature and resistant to maturation, exhibited little change in low surface cosignaling molecule expression, whereas splenic DC showed reduced expression of cosignaling molecules in response to CpG stimulation. These splenic DC elicited reduced naïve allogeneic T cell proliferation in vitro, while the stimulatory capacity of resting but not CpG-activated liver DC was reduced by EtOH administration. In vivo, hepatic EDC elicited increased capacity to prime T cells compared to control hepatic DC. Conversely, splenic EDC exhibited impaired ability to prime T cells in vivo. This differential capacity of hepatic versus splenic EDC compared to control DC to prime T cells in vivo is likely due to several factors including differential phenotype and migratory capacity. In fact, liver EDC migrate in greater numbers to secondary lymphoid tissue compared to control liver DC. Thus, EtOH impairs cytokine-driven differentiation and function of mDC and pDC in vitro. Hepatic DC from chronic EtOH-fed mice are differentially affected compared to splenic DC. Splenic DC exhibit impaired functional maturation following CpG stimulation while hepatic DC exhibit altered migration to secondary lymphoid tissue. In addition to examining the effects of chronic EtOH exposure on DC, we have

evaluated cell-mediated and humoral responses in EtOH-fed mice. In total, these results indicate potential mechanisms by which alcohol consumption is associated with immunosuppression.

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1.0 INTRODUCTION[1]

Alcoholism is a leading cause of morbidity and mortality. Epidemiological studies have shown that chronic EtOH abuse increases susceptibility to bacterial pneumonia and tuberculosis and exacerbation of the pathologic course of hepatitis C virus (HCV) infection. While some of the effects of alcohol on cells of the immune system are known (i.e. reduced number and altered subsets of lymphocytes as well as impaired cell-mediated immunity), the mechanisms by which EtOH exerts these immunosuppressive effects are still unknown. Based on the known immune compromised status of alcoholics and previous findings of impaired leukocyte function, **we hypothesize that chronic ethanol exposure impairs DC subset development and function, perhaps differentially, and that this impairment results in the compromised ability of alcoholics to mount appropriate responses to TLR ligation.**

There is a clear need to better understand the mechanisms underlying the EtOH-induced immune compromised status of alcohol abusers. Further, a murine model of chronic EtOH consumption should be characterized and evaluated for its potential in evaluating immune function and disease states in relation to human (immune) impairments. In **Chapter 2**, we present our findings regarding the impact of prolonged EtOH exposure on mouse BM-derived mDC and pDC using mainly in vitro approaches. In **Chapter 3**, we expand our observations to DC freshly-isolated from the liver and spleen of EtOH-fed (compared to control) mice. Here, along with ex vivo analyses, we employ adoptive transfer of DC to naïve animals to evaluate their function (ability to migrate and prime T cells). Finally, in **Chapter 4**, we evaluate the effects of chronic EtOH consumption on cell-mediated and humoral immune responses to a model Ag.

[1] Text excerpted from (1, 2), Figure 3 from (1), and Table 2 from (1).

1.1 DENDRITIC CELLS

Although rare, bone marrow (BM)-derived dendritic cells (DC) are the most highly-specialized antigen (Ag)-presenting cells (APC), with ability both to instigate and regulate immune reactivity, and capable of activating T lymphocytes at both low concentrations of Ag and APC/lymphocyte ratios. In addition, DC are well-equipped to migrate from peripheral tissue sites, such as the liver, to regional (secondary) lymphoid organs, where they present Ag to T cells. In the normal steady state, these events may be important in the maintenance of self tolerance. It is now recognized that the microenvironment in which APC develop or are activated influences their function and their effects on T cell populations. Furthermore, different DC subsets have been identified that exhibit distinct functional capabilities.

Once immature DC have taken up residency in peripheral tissue from the blood, they constantly survey their microenvironment through vesicular exchange with other resident cells and the uptake of apoptotic cells and soluble material. Even in the absence of stimulation via pathogen products, other Toll-like receptor (TLR) ligands or alloAg, DC constantly migrate from peripheral tissue sites via the lymphatics to draining lymphoid tissue. These DC may express self-peptide in the context of self major histocompatibility complex (MHC) gene products. While in the normal steady state they are generally immature, expressing low levels of surface MHC and costimulatory molecules, they may also be considered 'semi-mature' if they express moderate levels of T cell costimulatory molecules (in particular, CD80 and CD86). It is thought that, once these DC expressing self-peptide reach secondary lymphoid tissue, they play a role in maintenance of peripheral T cell tolerance via one or more mechanisms that include deletion of Ag-specific T cells via apoptosis, induction of T cell anergy resulting from Ag presentation without appropriate costimulation, and the generation of T regulatory cells (T_{reg}) that actively suppress T cell responses.

When DC are activated or matured by Ag and/or stimuli [i.e. pathogen-associated molecular patterns (PAMPs) or cytokines], they upregulate their Ag processing and presenting ability, co-regulatory molecule expression, and cytokine production, while down-regulating their Ag capture (3-6). These mature DC induce adaptive immune responses through presentation of Ag peptides on MHC class I and class II molecules for presentation to $CD8^+$ and $CD4^+$ T cells, respectively (5). DC can further induce potent T cell immune responses by upregulation of

costimulatory molecules, such as CD40, CD80, and CD86, while further influencing T cells through the secretion of cytokines (3, 4, 6).

Thus, DC can provide (potently) the three signals that naïve T cells require to become activated: (1) T cell receptor (TCR)/MHC ligation; (2) CD28/CD80/CD86 costimulation; and (3) secretion of T helper cell (Th)-inducing cytokines by DC, such as interleukin (IL)-12 and IL-18. In the absence of signal one, naïve and memory T cells fail to become activated. In the absence of signal two, naïve T cells can become anergic or apoptotic. Signal three is important in determining the polarization of the T cell response, such as skewing towards Th1/Th2 or generating T_{reg}. Memory T cells do not need all three signals and therefore can also be activated by non-DC APC, such as macrophages, B cells, and in special cases, some types of endothelial cells (EC) (7-12).

1.1.1 DC Subsets

Various DC subsets have been identified in both human and animal models, each with differential roles in the induction of Ag-specific T cell immune responses (13, 14). The DC subsets that have currently been identified are the myeloid (m) and plasmacytoid (p) subsets. Another commonly studied subset in the mouse is the 'lymphoid-related' DC subset, however, no human counterpart has yet been identified. Murine DC can be identified by expression of CD11c, the integrin-α_x chain.

1.1.1.1 Myeloid and lymphoid-related DC

mDC ($CD11c^+CD8\alpha^-CD11b^+$) and 'lymphoid-related' DC ($CD11c^+CD8\alpha^+CD11b^{-/lo}$) are distinguished by their reciprocal expression of CD8α (homodimer) and CD11b and were thought initially to be of distinct lineage and to exhibit distinct functions (14, 15). Recent evidence has shown that these subsets derive from a common precursor and that rigid lineage affiliations between subsets may not exist (14, 15). In addition to differences in phenotype, mDC and lymphoid-related DC have been shown to differ in tissue localization, and, potentially, in function.

In addition to the high surface expression of CD11b and the absence of CD8α, mDC are also characterized by their low to lack of expression of CD205 (DEC-205). In contrast, 'lymphoid-related' ($CD8\alpha^+$) DC express high levels of CD205. However, when mDC are cultured in vitro or are stimulated with lipopolysaccharide [LPS; Toll-like receptor (TLR)4 ligand], they have been shown to upregulate CD205 (13). Thus, the surface expression of various markers, such as CD205, is not unique to one subset and may be more ubiquitous in expression.

mDC and 'lymphoid-related' DC differ in terms of their localization in secondary lymphoid tissues. Whereas mDC reside in the marginal zones of lymphoid tissue, 'lymphoid-related' DC can be found in the T cell-rich areas of periarteriolar lymphatic sheaths (PALS) (13). However, mDC have been shown to localize in the PALS when stimulated by the proinflammatory TLR4 ligand LPS (13).

Functionally, early experiments with mDC suggested that they were a Th1-inducing DC subset (16, 17), but quickly thereafter, mDC were shown to induce Th2 cells as well (18). The 'lymphoid-related' subset has been suggested to be a more "suppressive" DC subset, reportedly by inducing $CD4^+$ T cell apoptosis (19) or suppressing $CD8^+$ T cell proliferation via suppression of IL-2 production (20). Further, our lab has shown that DC administration of immature or mature $CD8\alpha^+$ DC significantly prolongs mouse heart allograft survival, whereas immature mDC could only delay graft rejection; further, administration of mature mDC accelerated the rejection process (21). More recently, it was shown that populations of mDC containing 3% $CD8\alpha^+$ DC were impaired in their ability to induce an immune response against a tumorigenic self-peptide due to $CD8\alpha^+$ DC secretion of indoleamine 2,3-dioxygenase (IDO), an enzyme of the tryptophan catabolizing pathway which has been shown to inhibit T cell proliferation (22). This suppressive effect of $CD8\alpha^+$ DC on mDC immunogenicity was abrogated by the addition of IL-12 to cultures (22). The effects of additional IL-12 is blocked by further adding IFNγ to these cultures (regain $CD8\alpha^+$ DC-mediated suppression) (23).

In spite of differences in the phenotype, function, and localization of mDC and 'lymphoid-related' DC, it has been suggested that CD8α is a marker of activation and not of lineage/subset demarcation (15). It has been shown that a fraction of $CD8\alpha^-$ DC, after

presentation of parvovirus virus-like particles to $CD8^+$ T cells, upregulate surface expression of CD8α and CD205 (24). Further, Morelli *et al.* (25) showed that splenic marginal zone $CD8\alpha^-$ DC upregulate $CD8\alpha^+$ expression after uptake of intravenous (i.v.)-administered allogeneic apoptotic bodies. Langerhans cells (LC), DC which reside in the skin epidermis, are also interesting in regard to their expression of CD8α, CD11b, and CD205. LC express CD205 and upregulate CD8α expression after CD40 ligation and subsequent migration to lymph nodes (26). Thus, the true role or function of CD8α and associated differences between myeloid and 'lymphoid-related' subsets has yet to be clearly defined.

1.1.1.2 Plasmacytoid DC

pDC were known to exist for over forty years as natural type-1 interferon (IFN)-producing cells, but their hematopoietic origin was not discovered until recently. These cells were recently identified in humans and mice as a subset of DC (27). DC isolated from humans and mice (Figure 1) have similar morphology and function (28-30), showing typical plasmacytoid appearance, with a smooth cell surface, a prominent, lobular nucleus, and clear perinuclear area. Both human and murine pDC produce large amounts of type I IFNs in response to certain types of viruses and microbial products (Reviewed in 31). However, there exist differences in their phenotypical markers. Human pDC express CD123 (IL-3 receptor α chain) but are CD11c negative or low, while murine pDC express CD45R (B220) and CD11c. Recently, a subset of pDC expressing CD19 has been reported with potential potent tolerogenic capability (32, 33).

In spite of the fact that IFNα is a Th1 cytokine (generally considered immunostimulatory), evidence supports pDC as a tolerogenic DC subset (34-37). This theory is supported by studies that show the induction of T_{reg} (27) and production of IDO by pDC (38, 32, 33). However, pDC have also been shown to induce adaptive immunity, with the ability to prime Ag-specific naïve $CD8^+$ T cells and potentiating skewing of Th1 cells (39). Thus, pDC are being studied for their potential as tools for induction of transplant tolerance (40, 41) and autoimmune regulation (39), as well as initiating and amplifying T cell activation (39).

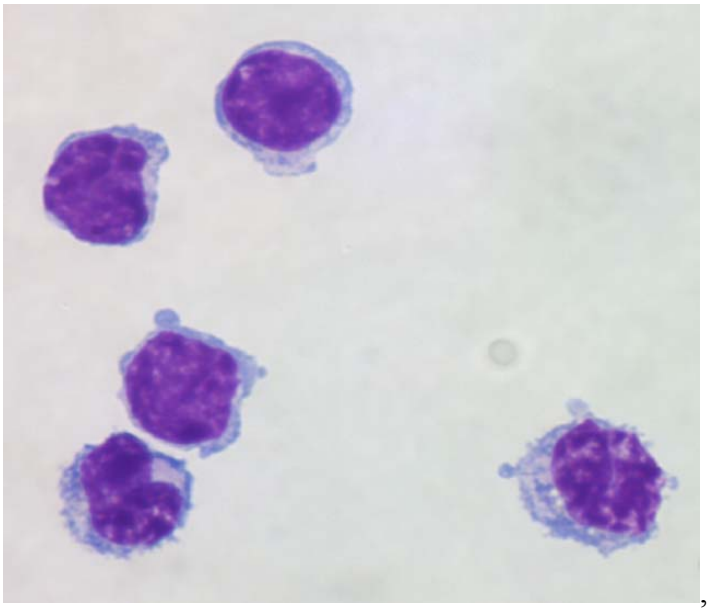 ,

Figure 1. Giemsa stain (x1000) of murine hepatic plasmacytoid (p)DC purified by cell sorting as described in 3.2.8.

1.1.2 Origin and Development of DC

As briefly alluded to above, it was previously believed that murine mDC and 'lymphoid-related' DC arose from common myeloid and lymphoid hematopoietic precursors; thus, the resultant subset monikers (14, 15). However, subsequent studies using mutant mice (either the c^{-}kit-γc^{-} or conditional Notch-1 knockout), which cannot form T cells, showed that 'lymphoid-related' DC were still present, and disproved the theory that 'lymphoid-related' DC were derived from common lymphoid precursors (14, 15). Further, studies have shown that both mDC and 'lymphoid-related' DC can be generated from either progenitor cell type (14, 15). Human pDC were also originally believed to derive from a lymphoid progenitor due to lack of myeloid markers as well as distinct growth requirements (39). However, mouse studies have challenged this hypothesis as a common $CD11c^{+}$ precursor, as well as common lymphoid and myeloid progenitors, can generate both pDC and mDC (15). A DC-specific precursor that only gives rise to DC has yet to be identified.

1.1.3 DC Activation by TLR

TLR are highly conserved major pattern recognition receptors for a variety of pathogenic structures, termed PAMPs. At present, eleven different TLR have been identified that recognize a variety of self, microbial, or synthetic structures, ranging from lipoproteins to double-stranded (ds)RNA to endogenous proteins, such as heat shock proteins (HSP) (42) (Table 1). TLR9 binds bacterial unmethylated deocycytidylate-phosphate-deoxyguanylate oligodeoxynucleotides (CpG) motifs (43, 44) while TLR3 recognizes dsRNA synthesized by viruses (45). Some TLR, such as TLR7 and -8 not only bind viral ssRNA, but they also recognize synthetic anti-viral compounds, such as imidazoquinoline compounds (46) or the guanosine analog Loxoribine (47).

Both leukocyte and DC subsets express different patterns of TLR that differentially affect their ability to respond to the same stimuli. Further, counterpart DC subsets from humans and mice show differential expression of TLR (Table 1) (42, 48). In the mouse, expression of TLR on DC subsets is more ubiquitous than on human DC subsets (Table 1). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of highly-purified, flow-sorted, murine splenic and hepatic pDC performed in our laboratory shows a different and distinct pattern of TLR expression, with most of TLR1-10 variably expressed (De Creus, A *et al.*, unpublished observations; data not shown). Activation of both human and murine pDC via TLR7 and TLR9 leads to high levels of IFN-α production, followed by their development into mature DC (31). The production of IL-12 by human pDC is debated, but IL-12p70 is known to be produced by mouse pDC (31).

TLR are known to signal through two primary pathways, the MyD88 (myeloid differentiation primary-response protein 88) (MyD88-dependent) and TRIF (Toll/Interleukin-1 receptor-domain containing adaptor protein inducing IFN-β) (MyD88-independent) pathways (Figure 2) (42). TLR3 and TLR4 have been shown to signal through the both the MyD88-dependent and -independent pathways, while other pathways appear to rely on the adaptor protein MyD88 for TLR signaling (Figure 2) (42). Further, signaling via all known TLR tested results in an increase in costimulatory molecule expression by DC, which is dependent on the nuclear translocation of nuclear factor-kappa B (NF-κB) (42).

Stimulation of DC via TLR is believed to play a crucial role in regulating innate and adaptive immune responses. Recent studies have indicated a role for TLR (MyD88-dependent

pathways and TLR7) signaling favoring the differentiation of Th1 cells and inhibiting Th2 cells by inducing IL-12 production by APC (49-51). Other studies show that the type of Th response may be dependent upon which TLR is stimulated (52) or upon Ag dose (53). More recently, the effects of simultaneously triggering DC through multiple TLR have been investigated (54). Napolitani *et al.* (54) found that TLR3 and TLR4 acted synergistically with TLR7, TLR8, and TLR9, enhancing and sustaining a Th1-polarizing DC.

One of the most extensively studied TLR is TLR9. In humans, TLR9 expression is restricted to pDC while in mice, TLR9 expression on various DC subsets is ubiquitous (Table 1) (55, 56). Accordingly, pDC are the primary cells that respond to CpG in the human, secreting large amounts of type 1 IFN. In murine systems, however, due to the ubiquitous expression of TLR9, CpGs act directly on pDC as well as other DC subsets (40, 57). Furthermore, and highlighting the inherent differences between murine and human systems, TLR9 in each system preferentially responds to different CpG motifs (58, 59). Thus, although there are differences between TLR expression and possibly function between the human and murine systems, much information can be learned about the role of DC in immune response and more importantly, about manipulation or directing the immune response through DC, by studying TLR signaling in the murine system to examine potential crossover effects or perhaps even therapeutic use in humans

Table 1. Toll-like receptors: ligands and expression on human and murine DC subsets.

Receptor	Ligand*	Expression				
		Human		Mouse		
		mDC†	pDC‡	CD8α+	mDC†	pDC‡
TLR1#, §	Lipoprotein Triacyl lipopeptides	+	+	+	+	+
TLR2	Diacyl lipopeptides Glycopinositolphospholipids Glycolipids HSP70h Lipoarabinomannan Lipopeptides Lipoprotein Lipoteichoic acid LPS¶ (atypical/cylindrical lipid A) Peptidoglycan Phenol-soluble modulin Porins Triacyl lipopeptides Zymosanf	+	-	+	+	+
TLR3	dsRNAv, ** Poly(I:C)s, †† mRNAh	+	-	+	+	-
TLR4	Fibronectinh Fibrinogenh HSP60h HSP70h Hyaluronic acidh Lipoteichoic acid LPS (conical lipid A) MMTV envelope proteinv, ‡‡ RSV fusion proteinv, ## Taxolp	+	-	+	+	+
TLR5	Flagellin	+	-	+/-	+	+
TLR6§	Diacyl lipopeptides Lipoteichoic acid Peptidoglycan Zymosanf	+	+	+	+	+
TLR7	Bropirimines Imidazoquinolines Loxoribines ssRNAv, §§	+	+	-	+	+
TLR8	Imidazoquinolines ssRNAv	+	-	+	+	+
TLR9	Unmethylated CpG¶¶	-	+	+	+	+
TLR10	(Unknown)	?	+	-	-	-
TLR11	Profilin-like molecule from Toxoplasma gondii Uropathogenic bacteria?	ND	ND	+/?	+/?	+/?

*Source of ligand is bacterium unless followed by superscript letter which indicates source of ligand: h, host; f, fungus; p, plant; s, synthetic. †mDC, myeloid DC; ‡pDC, plasmacytoid DC; #TLR, Toll-like receptor; §, requires dimerization with TLR2; ¶LPS, lipopolysaccharide; **dsRNA, double-stranded RNA; ††Poly(I:C), polycytidylic-polyinosinic acid (synthetic compound that mimics dsRNA); ‡‡MMTV, mouse mammary-tumor virus; ##RSV, respiratory syncytial virus; §§ssRNA, single-stranded RNA; ¶¶CpG, deoxycytidylate-phosphate-deoxyguanylate oligonucleotides. Modified from (42, 48) with additional information from (60-62).

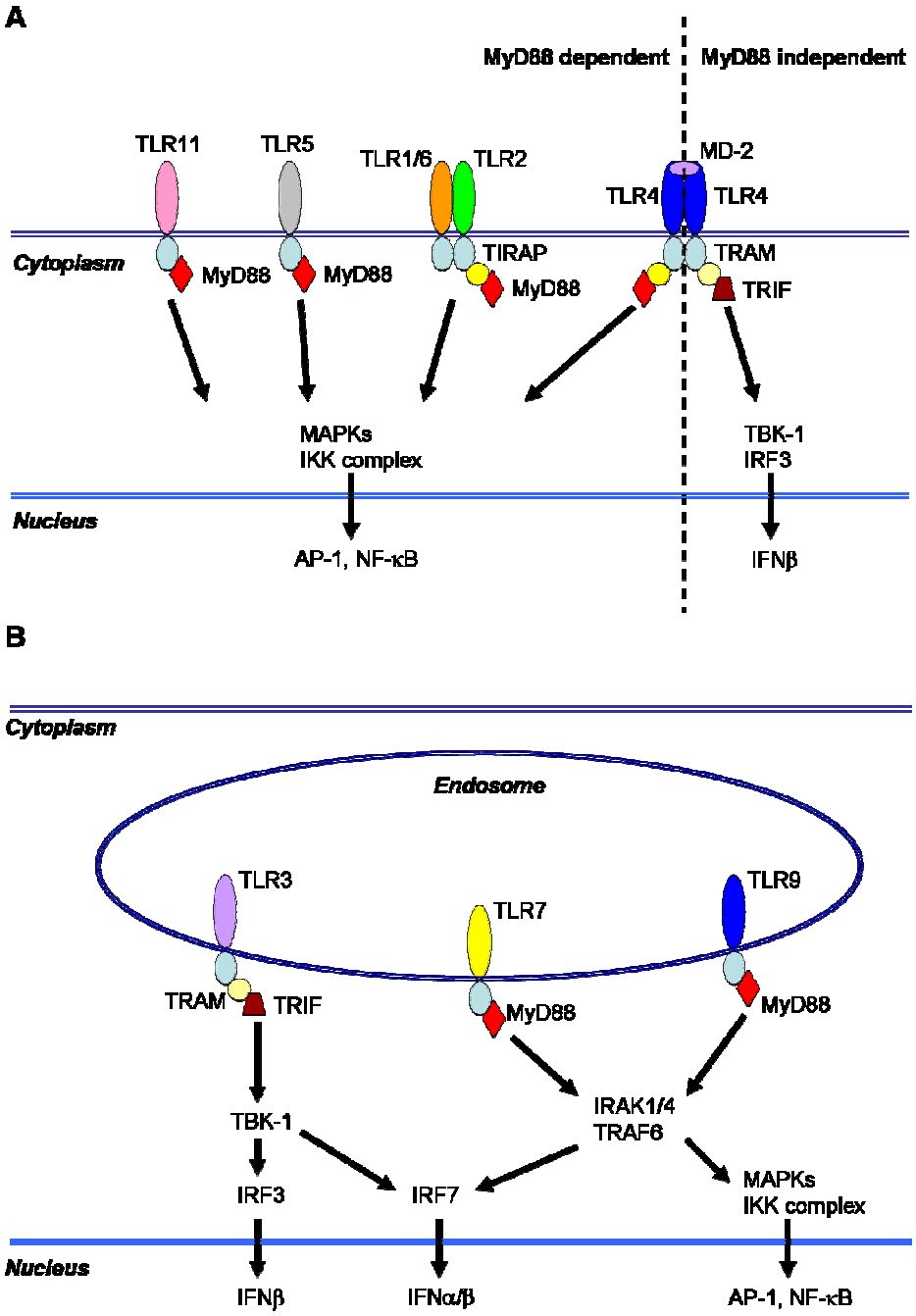

Figure 2. (A) Cell-surface and (B) intracellular TLR signaling pathways.
(A) TLR-1, -2, -4, -5, -6, and -11 are located on the cell surface and, when activated by appropriate ligands, signal through MyD88-dependent or –independent pathways. TLR-1 and -6 cooperate with TLR2. (B) TLR-3, -7, and -9 are localized in intracellular acidic compartments (endosome). TLR3 mediates dsRNA-induced responses via TRAM/TRIF dependent signals while TLR-7 and -9 utilizes the MyD88 signaling pathway. TLR activate via different cell signaling pathways and, ultimately, upregulate the transcription of different inducible genes (i.e. NF-κB, AP-1, IFNs). Abbreviations: AP-1, activating protein-1; IRF, interferon regulatory factor; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary response gene; NF-κB, nuclear factor kappa B; TBK-1, TANK-binding kinase-1; TIRAP, Toll/IL-1R receptor domain-containing adaptor protein; TRAM, TRIF-related adaptor molecule; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β. Modified from (63, 64).

1.1.4 In vivo-derived and in vitro-generated DC

1.1.4.1 Freshly-Isolated DC (Immature DC or DC Progenitors)

$CD11c^+$ DC of phenotypically distinct subsets have been described and isolated from various murine tissues (65-68, 30, 37, 69-71). These include the classic myeloid DC ($CD11b^+$ $CD8\alpha^-$), 'lymphoid-related' DC ($CD11b^-CD8\alpha^+$), and more recently, plasmacytoid DC (pDC; $B220^+$). When $CD11c^+$ DC are freshly-isolated from normal lymphoid or non-myphoid tissues (such as spleen or liver), they are immature in surface phenotype, as characterized by low CD40, CD80, CD86, and MHC II expression. Such immature DC of donor origin, when infused prior to transplantation, prolong organ (72) or pancreatic islet (73) allograft survival, without use of immunosuppressive therapy. The mechanisms by which these freshly-isolated DC promote tolerance when administered in experimental transplant models have not been fully elucidated. Hypotheses to explain this phenomenon include induction of T cell anergy/apoptosis by allogeneic DC expressing low or no levels of classical costimulatory molecules (signal 2), or induction of T_{reg} capable of suppressing alloreactive T cell responses.

Due to the rarity of freshly-isolated tissue-resident DC, mice are typically pre-treated with endogenous hematopoietic growth factors such as granulocyte macrophage colony stimulating factor (GM-CSF) or fms-like tyrosine kinase 3 ligand (Flt3L). Many investigators administer Flt3L intraperitoneally (i.p.) into mice for 10 days before sacrifice, as it expands all three DC subsets, whereas GM-CSF selectively expands the $CD8\alpha^-$ DC population (74, 75). Flt3L dramatically increases DC in the BM, gut-associated lymphatic tissue (GALT), liver, spleen, LN, lung, peritoneal cavity, thymus, and (peripheral) blood, in addition to stem cells and various other leukocyte populations (76). DC from Flt3L-treated mice are otherwise reportedly identical to DC from non-growth factor treated mice.

Freshly-isolated DC may also be innately different in their function depending upon their tissue of origin. For example, the liver microenvironment is high in the immunosuppressive cytokines IL-10 and TGF-β1, that may have direct inhibitory effects on DC development, maturation, and function (77). Freshly-isolated DC from the respiratory tract, intestinal Peyer's patches, and liver have been found to be poor allostimulators of T cells in mixed leukocyte reactions (MLR) (78). Accordingly, these DC are poor synthesizers of bioactive IL-12p70, but

exhibit high levels IL-10 mRNA expression or protein (78). DC isolated from a specific location, such as the liver, may have the potential to promote tolerance because of an inherent tolerogenic capacity, such as in endotoxin tolerance (in response to gut-derived LPS) mediated via TLR4 in liver DC (79).

In addition to potential differences in cytokine production by various tissue-resident DC, these APC may also be the source of potential immunoregulatory proteins. Indeed, there may be preferential or exclusive production of specific immunoregulatory proteins by certain DC subsets. For example, much recent interest has focused on indoleamine-2,3-dioxygenase (IDO) which has been shown to suppress T cell proliferation by catabolizing tryptophan upon which T cell replication is dependent. Several groups have examined the role of IDO in DC function. Mellor *et al.* (80) showed recently that, in mice treated with the B7-CD28 pathway inhibitor CTLA4Ig, splenic DC subsets, including $CD8\alpha^+$ DC, pDC, and bitypic natural killer/DC regulatory cells (81) (NK DC; $DX5^+CD11c^+CD8\alpha^+$), upregulated IDO production. Our laboratory has preliminary data that support the hypothesis that IDO production may be one contributing factor to tolerance induction by freshly-isolated pDC. Freshly-isolated murine splenic pDC strongly express mRNA for IDO, in contrast to myeloid and $CD8\alpha^+$ DC. Similar to other freshly-isolated DC subsets, freshly-isolated (splenic) pDC of donor origin can significantly prolong murine cardiac allograft survival (82).

While it is known that administration of freshly-isolated DC can promote tolerance in the context of experimental transplantation, the specific mechanisms by which different DC subsets achieve this effect are not yet understood. Methods to enhance the capacity of freshly-isolated DC to induce tolerance are under study. In particular, tissue-specific DC are being studied for their differences in maturation and function and thus their ability to promote tolerance. Addition of other therapeutic agents, such as immunosuppressive drugs, other agents that are known to subvert DC maturation, and those that block costimulation, in concert with freshly-isolated or *in vitro*-propagated DC is under investigation.

1.1.4.2 DC generated under specific culture conditions

While freshly-isolated DC have been shown to promote tolerance in experimental models, practical issues concerning their clinical use have arisen. Many studies have used BM-derived DC or DC from other tissues, especially spleen or thymus, which would not normally be

available for human use. Furthermore, the timing of the isolation or propagation of these DC must be considered in relation to when (e.g. in relation to cadaveric organ transplantation or development of autoimmune disease) the DC would be needed for therapeutic administration. A growing area of interest is in culturing DC from BM progenitors or blood-borne precursors under specific conditions which render the DC tolerogenic.

mDC are commonly generated in bulk from BM progenitors by the addition of granulocyte-macrophage colony-stimulating factor (GM-CSF, with or without IL-4) to the culture. mDC generated in this manner are a heterogenous population of immature and semi-mature APC. In the non-obese diabetic (NOD) mouse model of type-1 diabetes, it has been shown that administration of syngeneic BMDC generated with GM-CSF and IL-4 to pre-diabetic mice prevents the onset of autoimmune disease (83). T cells from DC-treated mice were found to have Th2-like properties, such as the production of high levels of Th2 cytokines.

By adding other cytokines or stimuli to DC cultures, the phenotype and function of these cells can be altered. Lutz *et al.* (84) reported that murine DC propagated from BM progenitors in GM-CSF and treated concurrently with a high dose of lipopolysaccharide (LPS) are immature and induce alloAg-specific $CD4^+$ T cell anergy *in vitro*. By altering the culture conditions, Sato *et al.* (85) have generated an 'alternatively-activated' regulatory DC population capable of inducing tolerance in allogeneic BM transplantation. These DC, termed regulatory DC (rDC), are generated with GM-CSF, IL-10 and transforming growth factor (TGF)-β and stimulated with high dose LPS near the end of culture. These rDC express very little CD80 or CD86 but high levels of MHC II. Further, these rDC are very poor stimulators in allogeneic MLR as compared to Vitamin D_3-conditioned DC. In an elegant study using a mouse model of acute graft-versus-host-disease (GVHD), Sato *et al.* (86) found that treatment of recipient mice with host-matched rDC 2 days after transplantation prevented acute lethal GVHD which was Ag-specific and complete. In studying the mechanism by which the rDC exerted their action, $CD4^+$ T cells from rDC-treated recipients were hyporesponsive to stimulation with host-type mature DC, while $CD8^+$ T cells had reduced lytic activity against host-matched target cells. Furthermore, the authors were able to characterize Ag-specific IL-10-producing T_{reg} ($CD4^+$) in protected, rDC-treated mice. More recently, Fujita *et al.* (87) have shown that LPS-induced lethal endotoxemia (sepsis) was prevented with rDC treatment, either as pretreatment or when treatment was given

after onset of disease (2 h post). Further, IL-10-production by these rDC was found to be significantly involved in their inhibitory effects (87).

pDC can also be generated *in vitro* from murine BM cells or human $CD34^+$ stem cells in response to the hematopoietic growth factor fms-like tyrosine 3 kinase ligand (Flt3L) and these cells are an accepted model for the study of pDC activation and function (Reviewed in 31). It has been shown that these cultured pDC exhibit the morphology, known pDC phenotypical markers, and the ability to produce IFN-α as pDC isolated *ex vivo*. As discussed above, in vivo-mobilized freshly-isolated donor splenic pDC can prolong graft acceptance in murine transplant models. Recent evidence suggests that cultured BM-derived pDC, which are also immature in phenotype, can similarly prolong graft survival (88).

It has been shown that BM-derived DC, which can be cultured in large quantities, can be modified by adjusting the culture conditions. Tolerogenic or rDC have been generated that, in experimental animal models, can induce tolerant states. For example, Fugier-Vivier et al. have shown that BMpDC can be used to enchance allogeneic BM cell engraftment and also promote skin graft tolerance (89). Thus, there is considerable potential for the use of this technology in developing DC-based therapy for tolerance induction, e.g. in the context of living-related organ transplantation, which would provide the necessary time for culture and administration of these cells to recipients prior to surgery.

1.1.5 DC Migration

Trafficking of DC is an important component in their ability to regulate immune responses. If there is an inhibition or dysfunction in the migration of DC to either the site of inflammation or subsequently to secondary lymphoid tissues, then DC activation or T cell activation will be impaired. Both adhesion molecules as well as chemokine production/chemokine receptors are important in the migration of DC and will be discussed below. It is also important to note the ubiquitous nature of the expression of adhesion molecules; they are not always limited to sole expression on leukocytes or EC.

1.1.5.1 Adhesion molecules and DC migration

The trafficking of DC in vivo requires interactions between DC and endothelial cells (EC). There are three important stages in the migration of DC – rolling, adhesion/tethering, and transendothelial migration, all which require different classes and families of intercellular adhesion molecules. In both steady-state and inflammatory situations, immature DC exit the bloodstream, via interactions between selectins and integrins on DC with carbohydrate structures and Ig superfamily members on EC for rolling and tethering, respectively, followed by transendothelial extravasation into peripheral tissue. Upon entrance into peripheral tissue, immature DC survey the environment, uptake Ag, begin to mature, and modify their chemokine receptor (CR) expression (90). These maturing DC concurrently downregulate CR for inflammatory chemokines (i.e. CCR5) while upregulating CR for lymphoid chemokines (i.e. CCR7), initiating recruitment of the DC to secondary lymphoid tissues (90).

The initial two steps of migration of DC, leukocyte rolling and tethering, have been studied comprehensively in the human as well as in mouse models. Rolling, the initial step in DC migration, involves weak binding of DC to EC, allowing the DC to survey their environment for chemokine gradients, as well as the expression of adhesion/tethering molecules. This initial step in trafficking requires the expression of selectins and glycosylated carbohydrate structures on DC or EC, such as CD62E/E-selectin (on APC and EC), CD62L/L-selectin (primarily on APC), CD62P/P-selectin (on EC), Sialyl Lewis molecules (SLe, on APC and EC), MAdCAM-1 and GlyCAM-1 [on lymphoid high endothelial venules (HEV)], and CD24 and CD162/P-selectin glycoprotein ligand-1 (on APC) (91, 92).

Once DC have started to roll on EC, stronger cell-to-cell adhesion interactions are mediated by the integrin and IgG superfamily molecules. CD54/intercellular adhesion molecule (ICAM)-1 is important in cell-cell adhesion and is ubiquitously expressed on EC as well as various leukocytes (91-93). Its ligands, CD11a/CD18 [lymphocyte function associated Ag (LFA)-1] and CD11b/CD18 (Mac-1) bind multiple molecules in addition to CD54, but are limited to expression on leukocytes (91). CD11a/CD18 is especially important in mediating cell-cell interactions and subsequent functions with blockade of CD11a/CD18 impairing not only adhesion to EC, but also blocking T cell responses (91). CD102/ICAM-2 and CD50/ICAM-3 are expressed on both EC and leukocytes, although their expression is variable, and are important in mediating adhesion of leukocytes to EC through interaction with ligand CD11a/CD18 (91).

CD106/vascular cell adhesion molecule (VCAM)-1 is located primarily on EC, especially upon activation (93), but is also expressed on multiple non-EC populations, including follicular DC (94, 95). Its ligand, CD49d/VLA-4, is also broadly expressed on leukocytes (91).

Transendothelial migration has recently become the subject of further study. It is well accepted that leukocytes extravasate through paracellular routes, by undergoing diapedesis between EC, with various adhesion molecules, i.e. CD11a/CD18, CD11b/CD18, and CD54, essentially involved (96). However, most transendothelial migration studies have not specifically studied DC extravasation. In paracellular migration, it is believed that leukocytes encounter homophilic interactions of EC junctional molecules, such CD31/platelet endothelial cell adhesion molecule (PECAM)-1, members of the junctional adhesion molecules (JAM) family, and possibly CD99 which, in conjunction with redistribution of vascular endothelial cadherin out of the junction and to the EC cell surface, guide leukocyte migration through the intercellular cleft (97, 98, 96). Another possible mechanism for leukocyte extravasation is via the transcellular pathway. Studies investigating leukocyte movement across the blood brain barrier have shown a transcellular pathway of leukocyte migration through EC, while leaving the EC junctions intact (96). Subsequent studies confirm and further characterize the mechanism of this pathway (99). If these findings hold true, it will be necessary to examine whether this phenomenon is limited to leukocyte migration across the blood-brain barrier and EC lines (i.e. human umbilical vein endothelial cells) or if it represents a more generalized mechanism. Further, it will important to know if this pathway is limited to lymphocytes, as suggested by Niemenen *et al*. (100).

Once DC in the periphery have taken up Ag and begun to mature, they must then undergo reverse transmigration through EC into lymphatic vessels, a process which reportedly requires CD29 (β1-integrin), CD49d/VLA-4, CD49e/VLA-5 (101), tissue factor and multidrug resistance protein 1 (102-104). This process is likely aided in part by CD54 and tissue factor (member of the coagulation cascade) (105, 102), as well as the chemokine gradient of CCL21/secondary lymphoid tissue chemokine (SLC) (106).

Overall, at present, few reports have specifically identified or considered which adhesion molecules may be most important in directing DC migration and extravasation. While most of these studies have been limited to human DC that have differed in the state of maturation and origin (101, 107), our group published the first report examining adhesion molecules involved in

the transendothelial migration of murine DC subsets (108). Elucidation of which adhesion molecules are most important in DC migration would be informative for targeting and optimization of therapies, such as immunotherapy (using DC) or receptor/ligand blockade.

1.1.5.2 Chemokines and DC migration

Chemokines are small, secreted chemoattractant cytokines that are important in inflammatory responses of the host (109, 110). They have the ability to modulate the migration (chemotaxis) of leukocytes, upregulate the expression of adhesion molecules, thus promoting diapedesis and infiltration of cells to sites of inflammation (109, 110), as well as roles in hematopoiesis, organogenesis, tumor metastases, and angiostasis/angiogenesis (111, 112). There are four families of chemokines, C, CC, CXC, and CX3C, named according to the position and separation of the first two amino-terminal cysteine residues of a four-cysteine motif (113). CR are seven-pass-transmembrane G protein-coupled receptors which are differentially expressed on cell subsets (114, 90, 115, 116).

Chemokines are principal in directing DC migration during an immune response. Immature DC express inflammatory CR (113) in anticipation of receiving appropriate signaling/ligation by inflammatory chemokines early in an inflammatory response. Immature DC express variable levels of common inflammatory CR such as CCR1-6 with variable function, especially when comparing BMDC with splenic DC (117-119). These receptors bind the classic chemokines released by inflamed tissues, CCL3/macrophage inflammatory protein (MIP)-1α, CCL4/MIP-1β, and CCL5/Regulated on Activation Normal T cell Expressed and Secreted (RANTES) (116). Recently, Yoneyama *et al*. (120) have also shown that CXCR3 is important in the recruitment of pre-pDC to secondary lymphoid tissues. As DC mature, upregulating MHC II and costimulatory molecules, DC begin to downregulate their inflammatory receptors while upregulating CCR7 and CXCR4 (121, 122). Thus, semi-mature and mature DC migrate to lymphatic vessels, following the CCL21 gradient (115, 123), and, once the DC has departed the lymphatic vessels, are directed to T cell areas of the secondary lymphoid tissue by CCL19/MIP-3β (124, 125) or to B cell areas by CXCL13/B lymphocyte chemoattractant (BLC) (126, 127).

1.2 HEPATIC DC

The unique architecture of the liver allows T cells to interact with resident liver APC, making these latter cells, particularly the uniquely well-equipped DC, ideal candidates for directing immune responses towards immunity or tolerance. We speculate that within the liver microenvironment, production of anti-inflammatory cytokines and other molecules including growth factors, such as IL-10, TGF-β, prostaglandin (PG)E_2 and GM-CSF results in the modulation of APC differentiation, trafficking and function in both health and disease. Much has recently been discussed about the potential of DC to regulate immune responses and to promote tolerance induction. The aforementioned soluble factors may each play a role in 'fashioning' intra-hepatic tolerogenic DC and other APC.

In normal liver, hepatic DC typically reside only around portal triads (Figure 3) (128-130), and, like DC in other peripheral sites, are able to efficiently capture, process and transport Ag to regional lymphoid tissues. All three APC [liver sinusoidal endothelial cells (LSEC), Kupffer cells (KC), DC] internalize Ag by phagocytosis, receptor-mediated endocytosis, or pinocytosis, but their phenotypes differ considerably (9, 10, 131). LSEC and KC express MHC Ags, costimulatory and adhesion molecules and make IL-1 and IFN-γ, suggesting that these cells are at a relatively mature stage (7, 9, 10, 131). Freshly-isolated hepatic DC, on the other hand, are predominantly immature cells, expressing surface MHC but few costimulatory molecules necessary for T cell activation (132, 68, 133). Compared with more mature bone marrow-derived or spleen DC, they stimulate naïve allogeneic T cells only poorly (134, 135, 133).

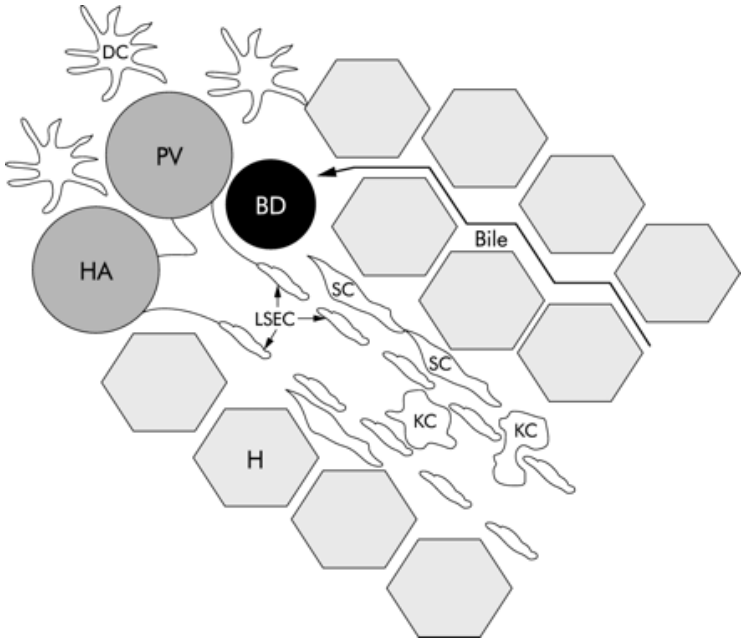

Figure 3. Anatomy of hepatic sinusoids.
The area between the LSEC and hepatocytes, where extracellular matrix and stellate cells reside, is called the space of Disse. Kupffer cells and other immune cells are believed to extravasate through the LSEC fenestrations into the parenchyma. DC normally reside only in the portal areas. Abbreviations: BD, bile duct; DC, dendritic cell; H, hepatocyte; HA, hepatic artery; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell; PV, portal vein; and SC, stellate cell.

1.2.1 The Role of the Liver Microenvironment and Hepatic DC in Tolerogenicity

The immature phenotype of resident hepatic DC, coupled with the inherently unique liver microenvironment, potentially makes these APC different from DC in other tissue sites (i.e. BM, spleen). Although not considered to be an immune privileged site, such as the anterior chamber of the eye or the testis, there are marked similarities between the cytokine milieu of the liver and that of these other sites. KC and LSEC constitutively express the anti-inflammatory cytokines IL-10 and TGF-β, that are upregulated upon stress, while hepatocytes secrete IL-10 in response to autocrine and paracrine TGF-β (136, 137, 9, 131). Lipocytes, another liver-specific cell population that includes Ito and stellate cells, also express increased TGF-β upon activation or stress (136). These cytokines not only affect Th cell differentiation directly (skew to Th2) but also can confer tolerogenicity on DC and other APC by inhibiting their maturation and T cell stimulatory function.

Although mature DC, rich in surface MHC and costimulatory molecules, are potent stimulators of immune (T cell) function, there is now much evidence that DC can be rendered

tolerogenic. Thus exposure of replicating DC progenitors to IL-10 or TGF-β (138) generates DC that are suppressive or tolerogenic. Steinbrink *et al*. (139) showed that culture of immature blood-derived human DC with IL-10 inhibited their maturation. Similar results have been obtained with DC transduced with either IL-10 or TGF-β (140, 141). Lack of adequate costimulatory molecule expression, either due to immaturity or exposure to costimulatory pathway blocking agents, can also result in tolerogenic DC, as shown in both allograft (142) and autoimmune disease (143) models.

1.2.2 Phenotype of Hepatic DC

Many different markers have been used to identify rodent and human DC, including those that are species-specific (Table 2). While none are specific to hepatic DC, variations occur in the level of expression of certain markers compared with others. CD11c is a common but not universal marker for DC detection in the murine system. In addition, other markers, such as CD205, have been used by different groups to identify specific murine DC subsets. The two well-characterized subsets identified in mouse liver as well as in lymphoid tissue, are the 'so-called' myeloid- ($CD8\alpha^{-}CD11b^{+}$) and lymphoid-related ($CD8\alpha^{+}CD11b^{-}$) subsets of DC. pDC have been identified in mouse lymphoid tissues (28-30) and more recently identified and characterized in the murine liver (69-71, 57). These DC are $CD11c^{+}CD11b^{-}CD19^{-}B220^{+}$ and may play crucial roles in anti-viral immunity.

DC have been generated in vitro from mouse liver stem/progenitor cells in response to GM-CSF. These liver-derived DC progenitors (144, 145) are distinct in phenotype from DC freshly-isolated from normal liver and are $CD11c^{lo}CD24^{+}CD44^{+}$. Maturation of DC is associated with upregulation of MHC II, CD80, and CD86 with CD205 being an additional marker used by some groups. Lu *et al*. (146) have also shown that culture of normal murine hepatic non-parenchymal cells (NPC) with IL-3 and CD40L yields a unique population of DC-like cells that are $CD205^{hi}CD11c^{-}B220^{+}CD19^{-}$.

Less diversity has been reported to date for DC markers in the rat and human. OX62, an integrin molecule, is commonly used to detect rat DC (147-149). As in mice, maturity is monitored by surface expression of the CD28/CTLA4 ligands, CD80 and CD86. Two distinct populations of mature rat hepatic DC have been identified: 1) $ED1^{+}ED2^{-}OX6^{+}$ and 2) $ED1^{-}$

ED2$^-$OX6$^+$. In the human, DC are commonly MHC II$^+$ and deficient in the CD28/CTLA4 ligands while in an immature state. Prickett *et al*. (130) found that human liver DC were also CD45$^+$CD11a$^+$CD18$^+$.

Thus it can be seen that there are similarities and disparities amongst hepatic DC populations. Common features to all three species include the lack of or low expression of MHC II and CD28/CTLA4 ligands on immature DC, that are increased upon maturation. CD11c and OX62 are generally considered the definitive markers for mouse and rat, respectively.

1.2.3 Enumeration of Hepatic DC

The normal murine liver has a relatively high total interstitial DC content, about 2-5 fold greater than that of other parenchymal organs, such as the kidney or heart (150). However, when the density of MHC II$^+$ DC between these organs is compared, the liver ranks as the lowest (150).

Specific DC populations, such as the myeloid- and lymphoid-related subsets, studied in other tissues (151, 76, 152) (Table 2), can be found in normal mouse livers. Previous studies have shown that these sub-populations constitute a low percentage of the total tissue-specific DC population. The relative proportions of these two subsets in the liver are similar to those seen in other tissues (151, 76, 152, 68). Each population constitutes ≤1% of the total normal liver NPC population (68).

Liver DC can be isolated from NPC by collagenase digestion followed by metrizamide density centrifugation (153, 134, 68). Although the total number of DC in the liver is greater than that of other parenchymal organs, there are still few cells to work with in comparison to lymphoid tissue. This paucity of cells is especially evident if one wants to work with a specific DC subset. Administration of recombinant human Flt3L, an endogenous hematopoietic growth factor, markedly increases the total number of hepatic DC (68). Furthermore, the yield can be further increased by overnight culture of the isolated DC progenitors with GM-CSF. Under such culture conditions, the percentage of both CD8α^- and CD8α^+ DC can be increased to 10-15% of the total NPC population (68).

Table 2. Phenotype of liver dendritic cells

Species	Maturation Status	Markers	References; Comments
Mouse	Immature	$CD11c^{+}CD40^{lo}CD80^{lo}CD86^{lo}MHCII^{lo}$	(153, 154, 135, 131) 3 subsets:
		1) $CD8\alpha^{-}CD11b^{+}$ 2) $CD8\alpha^{+}CD11b^{-}$ 3) $B220^{+}CD11b^{-}CD19^{-}$	(68, 155) 1) Myeloid-related 2) Lymphoid-related (69-71, 57) 3) Plasmacytoid
		$B220^{-}CD11c^{+}CD205^{-}F4/80^{-}$	(156)
		$CD205^{+}OX2^{+}$	(157, 158, 149)
		$CD11b^{+}CD24^{+}CD44^{+}CD45^{+}CD11c^{lo}$ $CD16/32^{lo}CD40^{lo}CD80^{lo}CD86^{lo}$ $CD205^{lo}F4/80^{lo}$	(144, 145) Generated from liver progenitor cells with GM-CSF; called liver derived DC progenitors.
	Mature	$CD11c^{+}$MHC $II^{hi}CD86^{hi}$	
		$CD11c^{+}CD54^{+}CD205^{+}MHCII^{+}$ $CD11b^{mod}CD86^{mod}$CD11a/ $CD18^{mod}$ $B220^{-}CD3\varepsilon^{-}Gr1^{-}$	(156)
	Other	$CD205^{hi}$ $B220^{+}CD11c^{-}$ $CD19^{-}$	(146) Generated from liver progenitor cells with IL-3 and CD40L. Ig gene rearrangement occurs but no surface expression. Activate then subsequently induce apoptosis of T cells.
Rat	Immature	MHC $II^{+}ANAE^{-}FcR^{-}$	(159) ANAE= α-naphtylacetate esterase = a nonspecific esterase; FcR = Fc Receptor
	Mature	MHC I^{hi}MHC $II^{hi}CD54^{+}OX62^{+}$	(147, 160, 161)
		1) $ED1^{+}ED2^{-}OX6^{+}$ 2) $ED1^{-}ED2^{-}OX6^{+}$	(162) 2 subsets of $OX62^{+}$ cells
		MHC $II^{+}CD54^{+}OX62^{+}CD90^{+}$ CTLA-4 $Counterreceptor^{+}$	(148)
		$CD4^{+}$	(163, 164) Variable expression is common in MHC II^{+} DC and peripheral tissues of rat
Human	Immature	$CD11a^{+}$ $CD45^{+}$MHC II^{+}	(130)
		$CD83^{lo}CD86^{lo}$MHC II^{lo}	(165)
	Mature	$CD200^{+}$	(166)
		$CD83^{+}CD86^{+}$	(167)

The phenotype of the DC obtained from Flt3L-mobilized mice resembles that of DC isolated from normal liver and in situ (153, 134, 144, 168, 157, 68). Drakes *et al*. (157) showed that the administration of Flt3L did not change the phenotype of freshly-isolated hepatic DC, as defined earlier. These Flt3L-treated DC, upon culture with GM-CSF and IL-4 or exposure to a maturation-inducing stimulus, such as extracellular matrix (ECM) protein, increased their surface costimulatory molecule expression and T cell allostimulatory activity (144, 169, 157, 170).

The leukocyte content of the liver and its DC constituency in particular, appear to play an important role in transplant outcome. Thus, when donor hepatic leukocytes either are drastically reduced (171-173) or greatly augmented (174, 175), a switch from tolerance to rejection occurs in murine liver transplantation. In the case of donor leukocyte depletion, transplant tolerance can be restored by replacement of the donor leukocytes (172). Thus, a balance appears to exist between the number of donor hepatic DC and liver tolerogenicity.

1.2.4 APC Functions of Hepatic DC

1.2.4.1 Phagocytosis

Early studies showed that i.v. administration of colloidal carbon (128, 163, 176) or antibody-coated human red blood cells (159) did not result in phagocytosis by DC. It was speculated that liver DC, unlike KC and LSEC (177, 178, 9), did not phagocytose these particles in vivo. However, more recently, elegant studies in the rat by Matsuno *et al*. (160, 179) have shown that carbon-laden DC localize in the celiac nodes within 2 hours of the i.v. administration of carbon particles. Furthermore, it was determined that immature DC were the major population of particle-laden cells that entered the hepatic lymph. It was suggested that these phagocytic DC were recruited from the systemic circulation and were not part of the resident DC population. Interestingly, Iyoda *et al*. (180) have reported that in mice, only the liver-resident $CD8\alpha^+$ DC subset exhibits phagocytic properties in situ.

1.2.4.2 T cell stimulation

Murine liver DC progenitors cultured overnight with or without GM-CSF stimulate naïve allogeneic T cells (134, 174, 135). Abe *et al*. (133) observed that the allostimulatory activity of immature liver-derived DC for memory T cells was not affected by administration of pro-

inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) or IFN-γ. However, addition of Ag (i.e. viral antigen; KLH, keyhole limpet hemocyanin) to immature hepatic DC induced upregulation of MHC II, costimulatory molecules, and T cell allostimulatory activity. Khanna *et al*. (135) found that although cultured immature mouse liver-derived DC were weak stimulators of allogeneic naïve T cells in vitro, their in vivo administration to allogeneic recipients resulted in selectively increased IL-10 production within secondary lymphoid tissue. By contrast, mature bone marrow-derived DC elicited increased IFN-γ but not IL-10 production. Immature hepatic DC therefore resemble freshly-isolated immature respiratory tract DC that poorly stimulate allogeneic T cells and selectively induce Th2 responses (181). These features of liver-derived DC are consistent with hepatic "tolerogenicity" and may play a role in immune response deviation following liver transplantation.

There is as yet little documented information about the T cell stimulatory ability of purified freshly-isolated human liver DC. Based on their immature phenotype in situ (182) (including lack of CD86) and the known properties of circulating peripheral blood DC with an immature phenotype (183), it is likely however that these cells are weak allostimulators.

1.2.4.3 Chemotaxis

Migration of DC to and from peripheral tissue depends on the production of chemokines (CC and CXC) and the expression of specific CR (CCR and CXCR). Because leukocyte migration is a key event in infection and inflammation, chemokine biology is rapidly becoming an important area of study, in relation to elucidation of DC function. Most chemokine receptors are promiscuous and can ligate a variety of different chemokines (184, 113, 185).

In the case of hepatic DC, few studies have been conducted regarding specific chemokine and receptor expression. Drakes *et al*. (186) showed that immature and mature liver-derived DC exhibited similar chemokines and receptors, although with differing levels of expression. Expression was similar to that detected on BM-derived DC. As determined by RNase protection assay (RPA), the chemokine most strongly expressed by both immature and mature liver-derived DC was CCL5. However, CCL3, CXCL1 (MIP-2), and CCL2 (MCP-1; monocyte chemoattractant protein-1) were also expressed by these liver-derived DC. Receptors CCR1 and CCR2 were expressed at comparable levels on these liver DC. CCL5 and CCL3 are among the various chemokines that bind CCR1, while CCL2 binds CCR2. CCL3 expression was greatly

enhanced upon liver DC maturation and stimulation by bacterial LPS or naïve allogeneic T cells also induced chemotaxis of mature liver-derived DC.

Shields *et al*. (187) found that CCR5, for which CCL3 is a ligand, is important in T cell recruitment in both hepatitis C virus (HCV)-infected and normal livers. Goddard *et al*. (188) similarly observed the importance of CCR5 in T lymphocyte recruitment during the inflammatory response in human liver transplantation. The presence of this receptor on T cells, coupled with the production of CCL3 by resident liver cells, implies the existence of DC-T cell interactions within the liver under normal and inflammatory conditions. Further studies are needed to assess the role of chemokines and their receptors in regulation of hepatic DC migration and function.

1.3 ETHANOL AND IMMUNITY

Studies have consistently demonstrated an association between alcohol abuse and increased susceptibility to a variety of infectious diseases. The rates of infection and severity of disease of alcohol abusers with bacterial pneumonia and tuberculosis are higher than nonalcoholics, even with antibiotics (Reviewed in 189). Other studies have shown accelerated histologic and clinical progression of chronic liver disease in patients with chronic HCV and alcohol use (190, 191).

Past analyses on the effects of chronic ethanol (EtOH) exposure on the immune system have shown that serum immunoglobulin levels are increased (192, 193), lymphocytes (including T, B, and NK cells) are reduced in numbers with alteration in subsets, T cells are persistently activated yet there is reduced cell-mediated immunity, and monocytes and neutrophils, while increased in numbers and in activation, have abnormal function (Reviewed in 189). The abnormalities in cell function include altered phagocytosis and migration (194-196) and cytokine expression (Reviewed in 197). In rats and mice, it has been shown that chronic EtOH feeding leads to increased NF-κB which can have numerous consequences on DC function (i.e. increased cytokine and chemokine secretion, maturation) (198, 199). Others have shown in mice and in in vitro cell culture systems that EtOH affects other cell signaling pathways, such as attenuation of activation of STAT1 and STAT3 (Signal Transducer and Activator of Transcription) (200, 201).

It is likely that EtOH has multiple effects on cells which may include changes in phenotype, other signaling pathways, and downstream cellular functions in immune cells, all which may contribute to the mechanism by which EtOH exerts its immunosuppressive effects.

1.3.1 Animal Models of Chronic Ethanol Administration

Several murine models of chronic EtOH administration are currently being used by various investigators. Each model has its advantages and disadvantages; however, each has its role in examining the effects of chronic EtOH exposure on the mouse system (Table 3). It is important to note that many alcohol studies utilize the rat model. Thus, many published studies, although studying chronic EtOH consumption, are not completely correlative in the mouse system as there are differences in the metabolism of EtOH and pathophysiology of EtOH disease between rodent models. Indeed, it has been reported that rat and mice metabolize EtOH with different kinetics. Mice blood alcohol levels (BAL) had a sharper and more rapid rise compared to rats but were followed by a sharp and rapid decline in BAL. In contrast, BAL in rats had more gradual rises and declines (202).

1.3.1.1 Lieber-DeCarli Diet

The Lieber-DeCarli (LD) diet is a totally liquid diet devised over fifty years ago to study alcohol consumption in an animal model while dietary caloric intake was controlled as well as allowing for isocaloric substitutions for pair feeding controls (Table 3) (203). In rats and baboons, this model has proven to be useful in studying early liver injury (204, 205). In these animals, steatosis and oxidative stress changes are found in the liver (204, 205). Even more pronounced effects in the liver, such as fibrosis and inflammation, can be induced in rats when other drugs or agents, such as choline, methionine or Vitamin A, are added to the diet (204).

In the mouse, the EtOH effects of the LD diet on the liver are not equivalent to the changes seen in the rat or baboon. While steatosis occurs, as well as the presence of Mallory bodies - aggregates of intermediate filaments of prekeratin which are present in degenerative hepatocytes and are a classical finding of alcoholic hepatitis (but can be found in other liver disease states) - there are few other pathologic changes in mouse liver (204). Further, mice have been shown not to tolerate the LD diet very well, with most groups either lowering the % EtOH

intake (206) or allowing mice free access to water in addition to the LD diet (207). It has also been reported that the LD diet causes stress effects on mice (207). While this model has its advantages, with no bias from caloric intake between EtOH-treated and control groups, the mortality rate, coupled with the non-physiological method of EtOH intake, have encouraged investigators to continue searching for an alternative mouse model.

1.3.1.2 Tsukamoto-French Model

The Tsukamoto-French murine model (Table 3) essentially administers EtOH to mice intragastrically via permanent gastric cannulation, requiring surgical implantation (208-210). With this model, not only is steatosis in livers observed, but inflammation and pericentral necrosis is found (210). Thus, this model is capable of inducing liver pathology similar to what is seen in human alcoholic liver disease. However, the non-physiologic method of EtOH intake, surgical expertise required, and cost for maintenance of animals following surgery prohibit many groups from utilizing this murine model.

1.3.1.3 Meadows-Cook Model

Meadows first described a chronic alcohol consumption murine model where mice were given 20% w/v EtOH ad libitum as their sole fluid source with free access to diet and water (211). More recently, the Cook group modified the Meadows model by incorporating a one week acclimating period during which the EtOH concentration was increased in two stages to the final 20% w/v EtOH concentration (212). This model has been reported to cause little stress effects, in contrast to the LD diet (207). As seen in mice fed the LD diet, steatosis occurs in the liver, but no other pathologic changes (i.e. inflammation or necrosis) (212). Thus, although lacking many of the late pathologic changes (i.e. inflammation or necrosis) that occur with human alcoholic disease, this model is less stressful to mice and more physiological in EtOH intake compared to the other murine models.

Table 3. Murine models of chronic EtOH administration.

In vivo Model	Administration of EtOH	Lengths of EtOH Administration	Organs studied	Applications of Model	Major Advantages	Major Disadvantages	Seleceted References
Alcohol solution, ad libitum	Mice have free access to EtOH solution and chow.	≥3 w, usually ≥5 w	Spleen, Liver	Functional changes in organ physiology and leukocytes during chronic consumption	Most physiological approach to resemble human behavior. Less stress to mice.	No exact control over ETOH intake. Only low (compared to other models) amounts of EtOH intake are possible. Differences between human and mouse pathology although more studies on model must be completed.	(212, 213, 207, 57)
Lieber-DeCarli diet or similar type of diet	All nutrients provided in a liquid diet that is calorie-adjusted, with EtOH accounting for ≤36% of the caloric intake. Usually no water given ad libitum. Paired controls receive carbohydrates as substitute for calories.	≥10 d, usually no longer than 28 d. Occasional study goes out to 13 w.	Spleen, Liver, Small Intestine, Pancreas	Functional changes in organ physiology and leukocytes during chronic consumption.	No bias in non-alcoholic nutrients as there is equal caloric intake between EtOH and control groups.	Not physiologic. Differences between human and mouse pathology (i.e. liver damage). Mice do not tolerate high EtOH diets well; there tends to be significant mortality rate unless additional water is provided.	(214-217, 201, 207)
Intragastric via gastronomy (Tsukamoto-French model)	EtOH administered via permanent gastric cannulation (surgical implant)	28 d with weekly increase of EtOH content (highest is 34.4% of caloric intake)	Liver	Chronic effect on liver pathology. Likely effects on metabolism and signal transduction.	Ability to generate high blood alcohol levels. Complete control over EtOH intake. Pathology resembles human condition.	Requires surgical expertise. Expensive to maintain. Not physiological. Only for chronic studies.	(218)

Modified from (205).

1.3.2 Ethanol and DC

At present, there have been limited studies investigating EtOH's effects on DC, and all of these studies have been confined to human peripheral blood monocyte-derived DC propagated in vitro (219, 220). Szabo's group have shown that monocyte-derived DC, cultured for 7 days with 25 mM EtOH, have significantly reduced allostimulatory capacity in mixed leukocyte reaction (MLR), reduced expression of CD80 and CD86, as well as altered cytokine production, compared to control monocyte-derived DC (219, 220). NFκB activation, which is upregulated during DC maturation, was not affected in response to LPS stimulation (220). Further, it has been shown that when humans acutely intake EtOH, monocyte-derived DC propagated from their peripheral blood monocytes exhibited reduced allostimulatory capacity (221, 222, 220). Thus, EtOH exposure, both acute and chronic, inhibits the function of human monocyte-derived DC.

Thus, even with the studies by the Szabo group, there is a dearth of knowledge on EtOH's effects of DC differentiation and function. Further, no studies to date have examined the effects of EtOH on murine DC, despite the increased use and role for the mouse model in studying EtOH's effects on immune function.

1.3.3 Ethanol and other leukocytes

Most reports on EtOH's effects on immune cells have focused on monocytes, macrophages, NK cells, and T cells. Furthermore, while a few studies have examined the effects of chronic EtOH exposure, most studies to date have examined the acute effects of EtOH. The predominant theme of these studies is that EtOH exposure, both acute and chronic, has significant inhibitory effects on the function of various leukocyte populations.

Monocytes are precursor cells that already possess migratory, chemotactic, pinocytotic and phagocytic capacity. Depending upon environmental signals, monocytes will differentiate into DC or macrophages (223). Monocytes from human volunteers who consumed acute amounts of alcohol (vodka, 2 ml/kg, in a total volume of 300 ml orange juice, over 30 min) had

significantly reduced allostimulatory capacity compared to control monocytes from the same volunteers (taken before drinking) (221, 222, 220). Further, monocytes, when treated with EtOH and stimulated with LPS in vitro, exhibited inhibited NFκB activation (224, 225, 220) and suppressed TNF-α secretion (226, 227, 225, 228). This finding of suppressed TNFα with acute EtOH exposure is in contrast to findings with chronic EtOH exposure, where there is enhanced TNFα production in response to LPS (229).

TNF-α production in response to acute versus chronic EtOH exposure, similar to that of monocytes, is seen with macrophages (226, 230). Further functional changes in macrophages include decreased phagocytosis (231-233), impaired cytokine production (234-236), and NFκB activation (237, 238). Production of reactive oxygen species is important for macrophage function. It has been shown that acute and chronic EtOH exposure decreases nitric oxide (239), superoxide anion (although differentially depending upon stimuli) (240, 239), and hydrogen peroxide (240) production by alveolar macrophages. However, in the liver, overproduction of oxygen radicals has been suggested as a pathologic mechanism for alcohol-induced liver damage. Accordingly, KC have been shown to be the source of increased production of EtOH-induced superoxide anion with both acute and chronic EtOH exposure (241-244). Thus, depending on the location of the resident macrophage, EtOH has differential effects on the production of superoxide anion.

Alcohol consumption has been associated with increased morbidity and mortality related to malignancies (245, 246). Thus, there is great interest in examining the effects of EtOH on NK cell functions, which are involved in the prevention of tumor development. Current reports on this topic are conflicting - while most studies show that NK cell functions (i.e. cytotoxicity) are inhibited by EtOH (247-251), a few reports have shown no effect (252) or increased (253) NK cell function with EtOH treatment. It should be noted that a seeming tumor-promoting effect of EtOH is transient and within ± 24 hours of EtOH exposure, there was no difference in numbers of tumor metastases between control and EtOH-fed groups (254).

It has consistently been reported that human chronic alcoholics have decreased circulating lymphocytes (255, 256). Similarly, it has been shown that chronic EtOH exposure in mice results in decreased cells numbers in thymus and spleen (257, 258). Further, T cells from human chronic alcoholics exhibit reduced proliferation to stimulation which could not be restored by exogenous IL-2 or IL-1 (259). In rat models, it has been shown that T cells from

chronic EtOH-treated rats fail to proliferate in response to IL-2 and is not due to decreased IL-2 receptor expression. It has also been suggested that the impaired immune responses seen in chronic alcoholics is due to skewing of the Th1/Th2 responses – particularly, a decrease in Th1. Currently, although several reports suggest that chronic EtOH-treatment skews towards Th2 responses, the data are still inconclusive. In support of the Th2 skewing theory, when mice were infected with *Klebsiella pneumoniae*, which is cleared with appropriate Th1 responses, chronic-EtOH fed mice displayed Th2 cytokine skewing (increased IL-10 with decreased IL-12 and IFNγ) with concomitant impaired clearance of infection (260). Further, other reports have shown that EtOH treatment inhibits Th1-associated IL-12 and IFNγ production (261, 262) as well as increased systemic serum IgE (Th2) levels (262). However, in a different study, chronic EtOH-fed mice were infected with *Leishmania major* or *Strongyloides stercoralis* with no difference in clearance of infection between EtOH and control groups (263). Although there was no defect in the Th1 response to clear *L. major*, mice given longer EtOH diets (5 vs. 13 weeks) were associated with increased serum IgG_1 (Th2 Ab) and decreased serum IgG_{2a} (Th1 Ab), indicating a general shift to Th2 responses (263). Thus, length of EtOH exposure is relevant in potential Th1/Th2 skewing although the functional importance of the Th1/Th2 skewing is unknown as EtOH-fed mice were able to clear infection as well as control mice.

Another aspect to consider in the study of T cells in chronic EtOH consumers is the status of their activation. Human alcoholics have been shown to have increased CD69 (264) and CD25 (265, 264) expression, both markers of activation, on T cells. In the mouse model of chronic EtOH consumption, it was shown that CD44, upregulated on T cells upon activation, expression is increased (212). Overall, the mechanism by which EtOH affects T cell proliferation and function is not well understood.

1.3.4 Ethanol and leukocyte migration

It is known that chronic EtOH consumption in humans is associated with increased migration of polymorphonuclear neutrophils (PMN) into the liver, contributing to the pathogenesis of alcoholic liver disease. One theory for the increased leukocyte sequestration and subsequent injury within the liver is that EtOH causes alterations in chemotactic factors and adhesion molecules which contribute to hepatic injury (266, 267). Indeed, it has been shown that chronic

EtOH feeding in rats causes increased levels of endotoxin (267), likely due to increased gut permeability from EtOH consumption, increased serum CXCR2/MIP-2 (high production by KC) (267), and significantly increased expression of CD18 on PMN (268) and CD54 on hepatocytes, LSEC, and KC (267). When an anti-CD18 monoclonal (m)Ab was administered in the rat model, there was attenuation of hepatic injury, indicating a contribution of adhesion molecules in the initiation of the hepatic injury (267). Other studies have imaged hepatic sinusoids and mesenteric venules in the rat model of chronic EtOH consumption and similarly found that LFA-1, CD18 and CD54 play a significant role in EtOH-induced increases in leukocyte adherence and extravasation (269, 266, 270).

In an acute EtOH model, it was shown that EtOH suppressed leukocyte accumulation and EC adhesion molecule expression in a dose-dependent manner (271). Further, it was shown that EtOH inhibited TNF-mediated activation of EC, as determined by suppressed adhesion molecule expression and chemokine production, and inhibited leukocyte adhesion in vitro (271). The reduced adhesion molecule expression and subsequent reduced leukocyte adhesion with acute EtOH exposure is consistent with reports in humans that show similar findings with acute EtOH (272). Similarly, the findings described above using a chronic EtOH model are also in agreement with human studies that have shown significantly higher serum levels of adhesion molecules in chronic alcoholics compared to non-drinkers or moderate drinkers (272). Thus, it is likely that adhesion molecule expression and chemokine production are affected by EtOH exposure, and these alterations may contribute to hepatic injury and inflammatory responses.

1.4 SCOPE OF THE THESIS

The immune compromised status of alcohol abusers may be in part due to impaired function of DC, the most highly specialized APC, in response to TLR ligation. Human alcoholics have been shown to have higher incidence of infection to bacterial pneumonia and tuberculosis (189). Downstream effects of TLR9, which recognizes bacterial DNA and has been shown to induce DC responses, ligation may be affected on DC, contributing to the impaired immune response to bacterial infection. In these studies, we examine the effects of prolonged EtOH exposure on

BMDC, liver and spleen DC subsets in response to TLR9 activation. We have found that Prolonged EtOH exposure: has differential effects on cosignaling molecule expression on BM, liver and spleen DC; reduces the stimulatory capacity of BM and spleen DC in vitro and in vivo; reduces the stimulatory capacity of resting but not stimulated hepatic DC in vitro; increases expression of CD11a on hepatic DC with no effects on expression of adhesion molecules on splenic DC in vivo; inhibits functional CCR7 in hepatic and splenic DC as measured by in vitro chemotaxis to CCL19; and increases the number of hepatic DC migrating to DLN compared to control hepatic DC whereas migration of splenic DC is unaffected by EtOH exposure. Thus, hepatic and splenic DC are shown to be differentially affected by chronic EtOH exposure. In vivo assessment of immune reactivity by specific lytic activity, Ag-induced cytokine production by CD4 and CD8 T cells, as well as serum IgG production reveals no quantifiable differences between control and EtOH-fed mice, which may be affected by length of EtOH exposure in vivo. Thus, the effects of chronic EtOH exposure on DC are complex and differ depending on the subset, location, and activation status. The interrelationship between these effects and immune reactivity will require more extensive analysis.

2.0 EFFECTS OF CHRONIC ETHANOL EXPOSURE ON THE DEVELOPMENT, COSIGNALING MOLECULE EXPRESSION, AND FUNCTION OF PLASMACYTOID AND MYELOID DENDRITIC CELLS GENERATED FROM MURINE BONE MARROW[2]

2.1 INTRODUCTION

The immunobiology of plasmacytoid and myeloid DC has generated much attention as a result of their potential for therapeutic application (273-275, 40). Manipulation and targeting of DC to induce specific immune responses has long been investigated and various strategies are now being tested in the clinical setting (276). With the widespread availability of growth factors and the technology to produce large quantities of DC from BM precursors, the use of various DC subsets will surely be further investigated for their therapeutic potential.

Various drug agents have been used to modify the function of BMDC with varying outcomes in T cell activation. Immunosuppressive drugs such as rapamycin (277) and FK506 (278) have been shown to inhibit the T cell stimulatory capacity of BMDC in distinct manners. Immunosuppressive drugs are used extensively in the treatment of various chronic inflammatory diseases, including allograft rejection. The influence of these drugs on DC development, maturation, and function is under extensive study (279). There is strong evidence that these and other pharmacologic agents may affect DC. Several drugs and vitamins have been studied in detail (i.e. CsA, corticosteroids, 1α,25-Dihydroxyvitamin D_3) while some newer drugs (i.e. the deoxyspergualin derivative LF15-0915) also have inhibitory effects on DC maturation which may have therapeutic implications (279). The influence of these common drugs and vitamins on DC provides potential mechanisms by which DC-T cell interactions can be manipulated to generate T cell unresponsiveness or regulation.

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[2] Data and Text excerpted from (57).

Presently, there have been limited studies investigating EtOH's effects on the development and function of DC. Studies on EtOH administration have revealed numerous inhibitory effects on functions of human blood monocytes (280, 227, 228, 221), monocyte-derived DC (219, 220) and rodent macrophages (226, 234-236). In murine in vivo alcohol intoxication models, studies to date have focused primarily on the deleterious effects of acute and chronic alcohol administration on macrophages (281, 234-236) and of chronic EtOH consumption on splenic T cells (212, 213). Analyses thus far of the influence of prolonged EtOH exposure on DC differentiation and function have been confined to human peripheral blood monocyte-derived DC propagated in vitro (219, 220). No studies to date have examined the effects of chronic EtOH administration on murine DC subsets despite the increased use of the mouse model for alcohol and immune function studies.

We first sought to investigate the effects of prolonged EtOH exposure on the development, phenotype, and function of BM-derived DC subsets. Our studies reveal that while EtOH inhibits both the generation of bone marrow-derived (BM)mDC and BMpDC in vitro, pDC, that are important sources of IFNα (282, 27), appear to be particularly susceptible. EtOH also differentially affects the expression of classic and recently-identified B7 family cosignaling molecules on resting DC subsets and following CpG oligodeoxynucleotide (CpG-ODN) stimulation. Further, EtOH-exposed BMDC have reduced stimulatory capacity for naïve T cells. Interestingly, BMDC exposed to EtOH elicit elevated levels of IL-10 production by allogeneic T cells.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Six- to eight-week old C57BL/10 (B10; $H2K^b$), BALB/c ($H2K^d$), and C3H/HeJ (C3H; $H2K^k$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh Medical Center. Experiments were conducted under an Institutional Animal Care and Use Committee-approved

protocol. The animals were fed a diet of Purina rodent chow (Ralston Purina, St. Louis, MO) and received tap water ad libitum unless specified.

2.2.2 Media, reagents, and Abs

RPMI-1640 was supplemented with 10% or 20% v/v heat-inactivated fetal calf serum (FCS; Atlanta Biologicals, Inc., Norcross, GA), 0.1 mM non-essential amino acids, 2 mM L-glutamine, sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 20 μM 2-β mercaptoethanol (complete medium) (all reagents from Life Technologies, Gaithersburg, MD). Chinese hamster ovary cell-derived human recombinant fms-like tyrosine kinase 3 ligand (Flt3L) was provided by Amgen (Seattle, WA). The TLR9 ligands CpG-DNA class A (CpG-A; ODN2336), CpG-B (ODN1826: TCCATGACGTTCCTGACGTT) and CpG-DNA control (ODN2138) were certified endotoxin-free and obtained from Coley Pharmaceutical (Wellesley, MA). Monoclonal antibodies (mAbs) used for flow cytometry and cell sorting were hamster anti-mouse CD11c [HL3; biotin- or fluorescein isothiocyanate (FITC)-conjugated], hamster IgM anti-CD40 [HM40-3; phycoerythrin (PE)-conjugated], rat anti-CD11b (M1/70; biotin-, FITC- or PE-conjugated), anti-CD45R/B220 (RA3-6B2; biotin-, FITC-, or CyChrome-conjugated) and PE-conjugated anti-CD80 (16-10A1), anti-CD86 (GL1), anti-B7-H1 (MIH5; eBioscience, San Diego, CA), anti-CD49b α_2 integrin (DX5; eBioscience); mouse anti-$H2K^b$ (AF6-88.5) and anti-IA^b β-chain (AF6-120.1) (all mAbs from BD PharMingen, San Diego, CA, unless specified). Isotype-matched control Igs and streptavidin (SA)-CyChrome were from BD PharMingen. Herpes Simplex Virus (HSV)-1 (RE strain of HSV-1) was a kind gift from Dr. R.L. Hendricks (University of Pittsburgh).

2.2.3 Generation and purification of BM-derived DC subsets

Bone marrow (BM)-derived DC were generated as described (40), with minor modifications. Briefly, B10 BM cells were cultured for 8 days in complete medium in 200 ng/ml Flt3L, with or without EtOH. On day 4, 50% of the supernatant was replaced with fresh cytokine-containing medium and fresh EtOH. EtOH-treated cultures were maintained in a chronic EtOH incubator

system (229) to maintain a constant EtOH concentration. EtOH concentrations were measured daily using the nicotinamide adenine dinucleotide (NAD)-alcohol dehydrogenase assay (Pointe Scientific, Inc., Canton, MI) to ensure maintenance of EtOH levels. On day 8, the cells were analyzed by flow cytometry, enriched for $CD11c^+$ cells by incubation with anti-mouse CD11c-coated immunomagnetic beads (10 μl/10^7 cells; Miltenyi Biotec, Auburn, CA) for 15 min at 4°C, then positively selected by passage through a paramagnetic column (MACS; Miltenyi Biotec), yielding a highly-enriched (≥ 90%) $CD11c^+$ population, or flow-sorted as described below.

2.2.4 Flow cytometry and cell sorting

Flow cytometric analyses and cell sorting were performed as described (68, 79) with minor modifications. To avoid non-specific Ab binding, the cells were preincubated with 10% v/v normal goat serum for 20 min at 4°C, then incubated with the mAbs indicated for 45 min at 4°C. Cells incubated with the appropriate isotype-matched control Igs served as negative controls. After washing, biotin-conjugated mAbs were revealed with second step streptavidin-CyChrome. The cells were then analyzed using a Coulter EPICS XL.MCL flow cytometer (Beckman Coulter, Miami, FL). For sorting, CD11c bead-enriched cell suspensions were incubated with anti-CD11c-FITC, anti-CD11b-PE, and anti-CD45R/B220-CyChrome for 45 min at 4°C. $CD11c^{int}B220^+CD11b^-$ (pDC) and $CD11c^+B220^-CD11b^+$ cells (mDC) were then sorted to ≥99% purity using a MoFlo® cell sorter (Cytomation, Fort Collins, CO).

2.2.5 Analysis of apoptosis and necrosis

BMDC exposed to 50 mM EtOH in culture for 1, 4, or 6 days were analyzed for apoptosis by staining of phosphatidylserine translocation with FITC-annexin V in combination with vital dye Propidium Iodide (PI) according to the manufacturer's instructions (BD PharMingen). Cells were costained with CD11c to allow for specific analysis of DC by flow cytometry.

2.2.6 Detection of TLR9 expression by Polymerase Chain Reaction

TRIzol LS Reagent (Life Technologies, Gaithersburg, MD) was added to the bead-purified BMDC suspensions and RNA extracted according to manufacturer's instructions. Before reverse transcription, digestion of DNA was performed with DNase I (Life Technologies). cDNA was synthesized with oligo(dT) as primer using Superscript kit (Life Technologies). Amplication was performed using the following primers; TLR9 (sense) TCCCTGTATAGAATGTG; (anti-sense) TGGAGGCGTGAGAG. HPRT (sense) GTAATGATCAGTCAACGGGGGAC; (anti-sense) CCAGCAAGCTTGCAACCTTAACCA. The samples were amplified for 35 cycles at different annealing temperatures (TLR9, 54°C,: HPRT, 55°C) by a GeneAmp PCR system 2400 (Boston, MA). PCR products were analyzed on agarose gels stained with ethidium bromide and photographed.

2.2.7 Mixed Leukocyte Reaction (MLR)

Spleen cell suspensions from C3H mice were depleted of red blood cells by NH_4Cl treatment, resuspended in warm (37°C) complete medium, then passed over a nylon wool column (37°C for 45 min) to enrich for T cells (purity >85%). Two x 10^5 purified C3H T cells were stimulated with graded numbers of γ-irradiated (20 Gy), bead-purified or flow-sorted DC in complete medium in round-bottom, 96-well plates (Corning, Acton, MA). [^{3}H]TdR (1μCi) was added to each well for the final 16 h of 72 h cultures. Cultures were harvested using a multiple well harvester and [^{3}H]TdR uptake determined using a liquid scintillation counter. Tests were conducted in triplicate and results expressed as mean counts per minute (cpm) ± 1 standard deviation (SD).

2.2.8 Adoptive Cell Transfer

Five x 10^5 bead-purified B10 DC were injected s.c. into one hind footpad of normal allogeneic (BALB/c) mice. Six days later, popliteal lymph node cell suspensions were prepared and the cells cultured in 96-well, round-bottom plates at 2 x 10^5 cells/well in complete medium in the

presence of 2 x 10^5 γ-irradiated (20 Gy) donor or third party splenocytes. After 72 h, supernatants were harvested and cytokine concentrations measured by enzyme linked immunosorbent assay (ELISA). T cell proliferation was quantified by [^{3}H]TdR incorporation, as described above.

2.2.9 Cytokine Quantitation

IFNα (PBL Biomedical Labs, Piscataway, NJ), IFNγ, IL-10 and IL-12p70 (R&D Systems, Inc., Minneapolis, MN) were quantified by ELISA using commercial kits from Biolegend (San Diego, CA) unless otherwise specified, and following the manufacturer's recommended procedures. The detection limits were 12.5 pg/ml for IFNα, 4.0 pg/ml for IFNγ, 30 pg/ml for IL-10, and 7.8 pg/ml for IL-12p70.

2.2.10 Western blot analysis

Cell extracts (10 μg/lane) and positive controls were separated electrophoretically on 10% w/v sodium dodecyl sulfate-polyacrylamide gels, as described (283). Proteins were electroblotted to PolyScreen polyvinylidene fluoride (PVDF) membranes (Perkin Elmer Life Sciences, Inc., Boston, MA) at 200 mA for 1 h. Blots were blocked for 30 min at room temperature with 5% nonfat dry milk in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, Tris-buffered Saline and, 0.05% Tween-20 (TBST). After five 2 min washes with TBST, membranes were incubated overnight at 4ºC with mouse Ab to indoleamine 2,3-dioxygenase (IDO; Chemicon International, Temecula, CA), diluted to 1:1000, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), diluted to 1:1000. After subsequent washes with TBST, the membrane was incubated for 1 h with peroxidase-conjugated goat anti-mouse secondary Ab, diluted 1:10,000 (Jackson Immunoresearch, West Grove, PA). Abs were diluted in TBST. Membranes were developed in Western Lightning Chemiluminescence reagent (Perkin Elmer Lifer Sciences, Inc.) followed by exposure to Kodak Biomax MS Film (Rochester, NY). After the film was developed, Western blots were evaluated by densitometric analysis using Scion Image (Scion Corporation, Frederick, MD).

2.2.11 Statistics

The significances of differences between means were determined using Student's unpaired '*t*' test or Fisher's protected least significant difference test. A value of $p < 0.05$ was considered significant. In certain experiments (Figures 5, 6, 7, 10, 18, 19 and 21), there was variability in the expression of various molecules (percent positive cells, MFI, and/or mRNA or protein levels visualized by PCR or Western blot, respectively) between repeat experiments, and the data shown are thus from one experiment representative of three. In MLR assays, there was variability in the level of proliferation of naïve T cells between the three experiments. However, in each of the three experiments, the same statistical significance was found between experimental and control groups. Thus, the figure shows one experiment representative of three experiments.

2.3 RESULTS

2.3.1 EtOH inhibits the generation of DC subsets from murine bone marrow

The concomitant generation of multiple DC subsets in the presence of EtOH has not been examined. To obtain adequate numbers of DC that included both mDC and pDC, B10 BM cells were cultured with the endogenous DC poietin Flt3L, a cytokine that stimulates the expansion and differentiation of immune cells of myeloid and lymphoid lineages, as described in the Materials and Methods. We tested the influence of prolonged exposure (8 d) to various, physiologically relevant concentrations of EtOH (0-100 mM) on the development of both DC subsets. In control (untreated) cultures, three to four-fold as many mDC as pDC were generated over the 8-day culture period (Figure 4A, B). The yield of both mDC and pDC was reduced significantly and in a dose-related manner at EtOH concentrations >25 mM (Figure 4A, B). However, the two subsets consistently exhibited different sensitivities to EtOH, with pDC being more vulnerable than mDC. pDC were susceptible at concentrations ≥25 mM ($p<0.05$), whereas mDC were reduced significantly in number at ≥50 mM ($p<0.05$). Dotplots of the incidences of

$CD11c^+B220^+$ pDC and $CD11c^+CD11b^+$ mDC in cultures under increasing EtOH concentrations (Figure 4C) confirm the preferential inhibitory effect on the pDC subset. Although the incidence of mDC showed a marked increase between 75 and 100 mM EtOH, the overall numbers of $CD11c^+$ cells decreased substantially. As both subsets were affected significantly at 50 mM, this concentration was used for all subsequent *in vitro* experiments. Flow cytometric analysis of BMDC with Annexin V/PI at various timepoints (Days 0, 1, 4, and 6) revealed no significant difference in the incidence of apoptosis or necrosis between the control and EtOH-treated groups (Table 4). Thus, it is unlikely that the observed inhibition of mDC and pDC generation was due to an effect of EtOH on DC apoptosis.

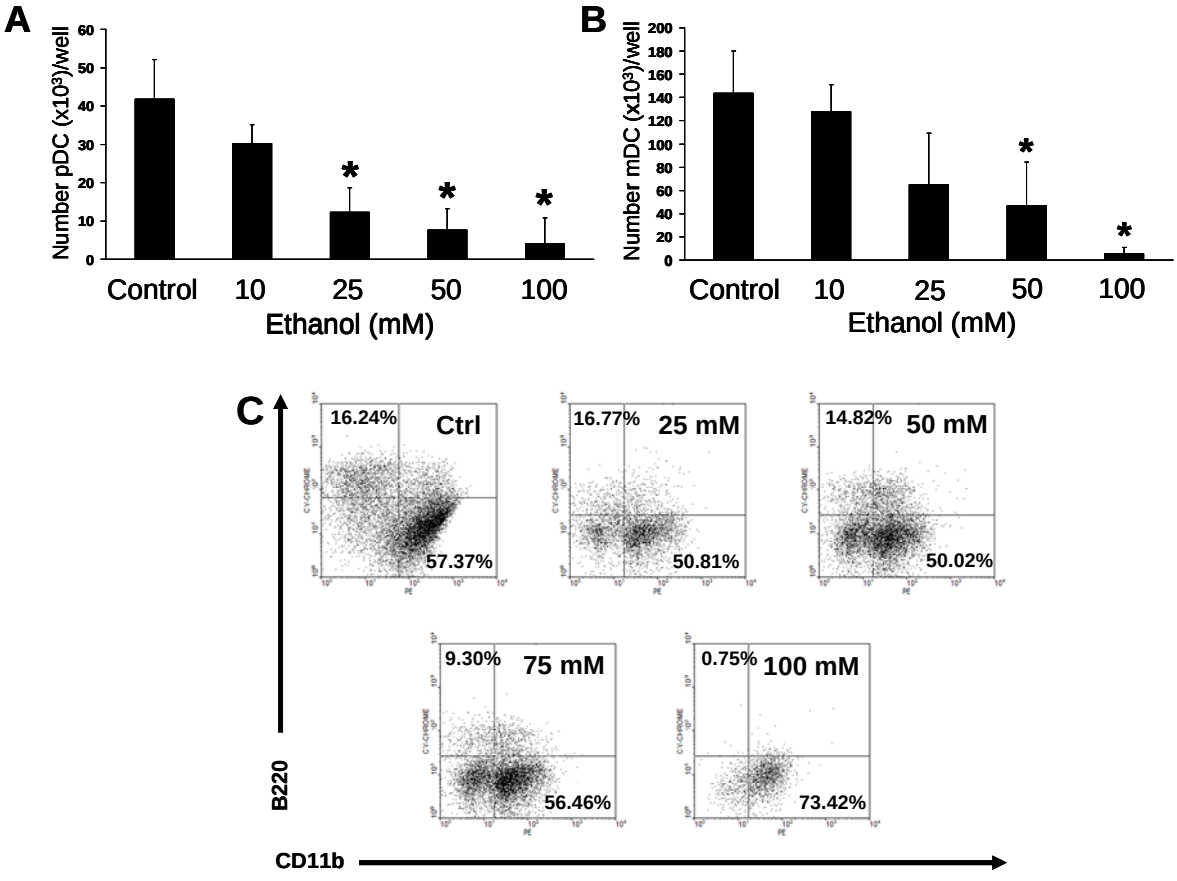


Figure 4. The absolute number of B10 pDC and mDC generated in vitro in response to Flt3L decreases with EtOH concentration in a dose-related manner, with selective depletion of pDC.
Flt3L-stimulated BM cells were exposed to various concentrations of EtOH from the start of culture as described in the Materials and Methods. Day 8 cultures were labeled with anti-CD11c (biotin-conjugated; revealed with SA-CyChrome), anti-CD45R/B220 (FITC-conjugated) and anti-CD11b (PE-conjugated). (A,B), cells were gated on CD11c and analyzed for expression of $B220^+$ (pDC; A) or $CD11b^+$ (mDC; B). Bar charts show the absolute number of cells harvested per well, based on percentage positive cells. (C), day 8 cultures were labeled as above and gated on $CD11c^+$ cells. Dotplots show the distribution of $CD11c^+B220^+$ and $CD11c^+CD11b^+$ subsets. (A,B), data were obtained from 3 experiments. *, $p < 0.05$; in comparison to control. (C), data are from one experiment representative of 3 performed.

Table 4. Prolonged EtOH exposure does not increase apoptosis or necrosis of BMDC.

Length of EtOH Exposure (days)	% Annexin V$^+$		%PI$^+$		% Annexin V$^+$ and PI$^+$	
	Ctrl	EtOH	Ctrl	EtOH	Ctrl	EtOH
1	7.66	6.58	3.25	3.48	10.91	10.06
4	11.93	8.37	1.22	2.53	13.15	10.90
6	1.00	0.93	0.76	0.59	1.76	1.52

Data are from one experiment representative of 3 performed.

2.3.2 Prolonged exposure to EtOH does not affect the expression of TLR9 mRNA

It is currently unknown whether EtOH exposure affects TLR9 expression. Alteration in the expression of this receptor could potentially affect stimulation through the receptor and affect subsequent functional analysis of DC after TLR9 ligation. Thus, TLR9 mRNA expression by PCR was assessed for control and EtOH-exposed (50 mM, 8d) bulk BMDC. PCR analysis revealed no difference in TLR9 mRNA expression between control and EtOH groups (Figure 5).

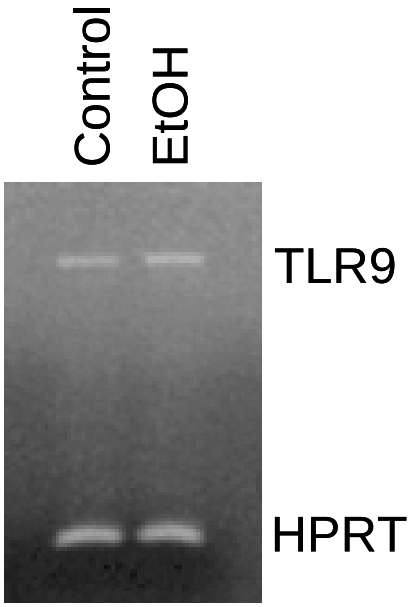


Figure 5. TLR9 mRNA expression by BMDC is not affected by prolonged EtOH exposure.
Flt3L-stimulated BM cells were exposed to 0 (Control) or 50 mM EtOH for 8 days of culture. RNA was extracted from CD11c-bead purified DC and analyzed by PCR for TLR9 and HPRT expression. Data are from one experiment representative of 3 performed.

2.3.3 Prolonged exposure to EtOH selectively modulates B7 family cosignaling molecule expression on unstimulated DC subsets and in response to CpG stimulation

BMDC propagated in the presence of 50 mM EtOH exhibited reduced cell surface expression of CD40 and the classic B7 family costimulatory molecules, CD80 and CD86 (Figure 6A-C). The extent of reduction in costimulatory molecule expression on both mDC and pDC was dependent on the length of exposure to EtOH; longer periods of exposure (≥72h) affected expression more markedly. By contrast, cell surface MHC class I and MHC class II levels were not affected significantly (unpublished results). The strikingly enhanced expression of CD40, CD80 and CD86 induced on both DC subsets by CpG stimulation was also inhibited by exposure to EtOH. CD40 expression appeared to be the most sensitive to the inhibitory effects of EtOH, with reduced expression evident at ≥24h of exposure, whereas CD80 and CD86 levels were reduced on cells exposed to EtOH for ≥48h (Figure 6). Variablity in percent positive cells and MFI was seen between different BM cell cultures; however, the trends reported were consistent. Thus, the data in Figure 6 are from one experiment representative of three.

CD274/B7-H1, is a recently-characterized B7 superfamily coinhibitory molecule (284-288). It is expressed on pDC (289) and mDC (290) and increased upon DC stimulation. Interestingly, and in contrast to the classic costimulatory molecules, basal CD274 expression was not affected by exposure of the BMDC to 50 mM EtOH for up to 8 days (Figure 6D-F). Furthermore, no consistent or significant effect of EtOH was observed on the markedly increased CD274 expression observed on either DC subset exposed to CpG. When the ratio of CD274 to either CD80 or CD86 was examined, a progressive increase was observed with prolonged exposure to EtOH (Table 5).

The reversibility of the effects of EtOH on the expression of costimulatory molecules was also examined. BMDC cultures were set up as described, with or without EtOH treatment (8 days; 50 mM). After 8 days, both groups were maintained in culture under the same conditions, but with no EtOH treatment. The DC were stimulated with CpG at various timepoints (0, 24, 48h) after EtOH removal and mDC and pDC analyzed for costimulatory molecule expression. The results showed that the effects of EtOH on BM-derived mDC and pDC were not readily reversed, as the inhibitory effects of EtOH on BMDC costimulatory molecule expression were still evident up to 48h after removal of EtOH from the cultures (Figure 7).

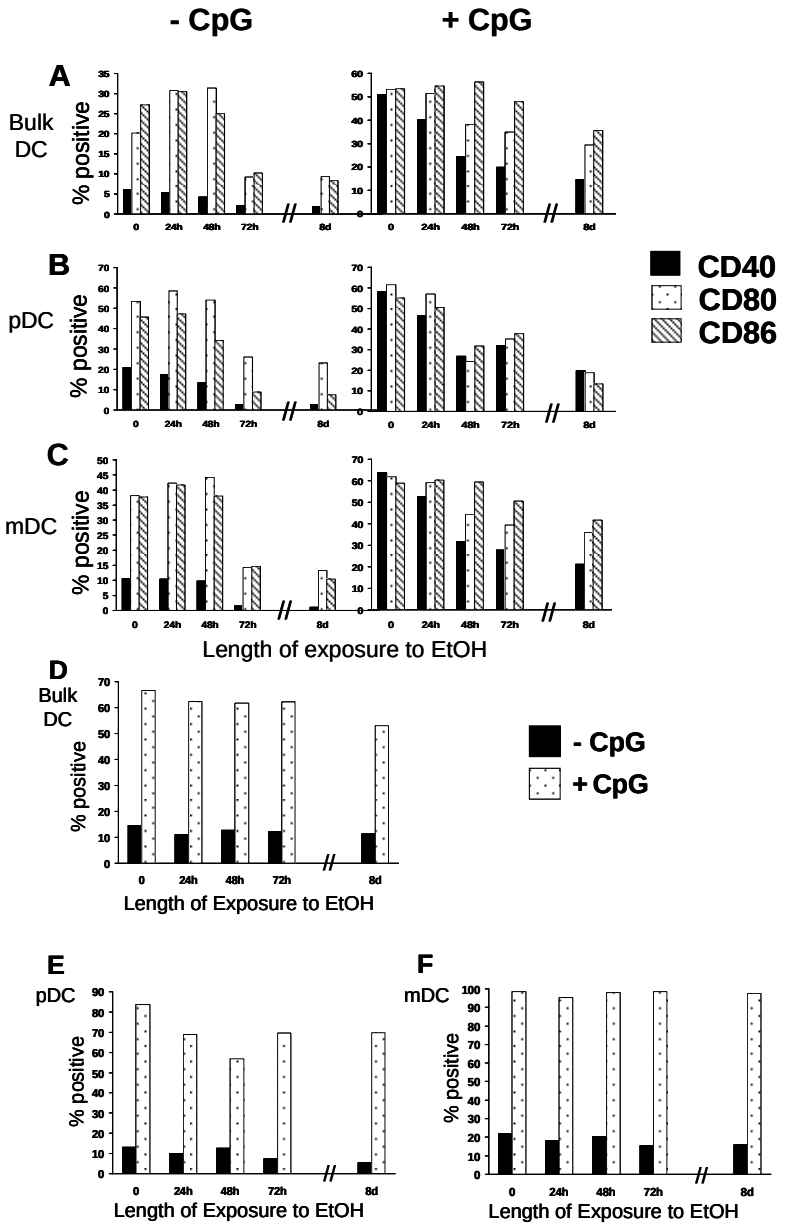


Figure 6. Expression of (A-C) classic costimulatory molecules (CD40, CD80, and CD86) on B10 DC subsets is reduced with prolonged exposure to EtOH, whereas expression of the (D-F) alternative cosignaling molecule CD274/B7-H1/PD-L1 is unaffected, even after prolonged exposure to EtOH.

Flt3L-stimulated BM cell cultures were exposed for various periods to EtOH (50 mM), with or without CpG stimulation before FACS analysis 16 h later. Day 8 cultures were labeled with anti-CD11c (FITC-conjugated) and anti-CD45R/B220 (CyChrome-conjugated) or anti-CD11b (biotin-conjugated and revealed with SA-CyChrome), and either (A-C) anti-CD40, -CD80, -CD86, or (D-F) -B7-H1 (all PE-conjugated). Cells were gated on (A, D), $CD11c^+$ cells, (B, E), $CD11c^+B220^+$ cells (pDC) or (C,F), $CD11c^+B220^-$ cells and expression of (A-C) CD40, CD80, CD86, or (D-F) B7-H1 analyzed. Bar charts show percent positive cells for the molecules indicated. Results are from one experiment representative of three performed.

Table 5. Ratio of CD274/B7-H1/PD-L1 to CD80 or CD86 on BM-derived DC increases with prolonged exposure to EtOH.

Length of Exposure to EtOH	Bulk DC		pDC		mDC	
	B7-H1:CD80	B7-H1:CD86	B7-H1:CD80	B7-H1:CD86	B7-H1:CD80	B7-H1:CD86
0h	0.73	0.54	0.25	0.29	0.58	0.58
0h + CpG	1.25	1.25	1.36	1.52	1.59	1.68
48h	0.39	0.49	0.23	0.37	0.46	0.53
48h + CpG	1.61	1.10	2.35	1.80	2.21	1.65
8d	1.21	1.37	0.24	0.74	1.20	1.54
8d + CpG	1.81	1.49	3.71	5.22	2.72	2.34

Data from Figure 2 (0h, 48h, and 8d of EtOH exposure) are presented as ratios between percent positive cells for CD274 and either CD80 or CD86. Results are from one experiment representative of three performed.

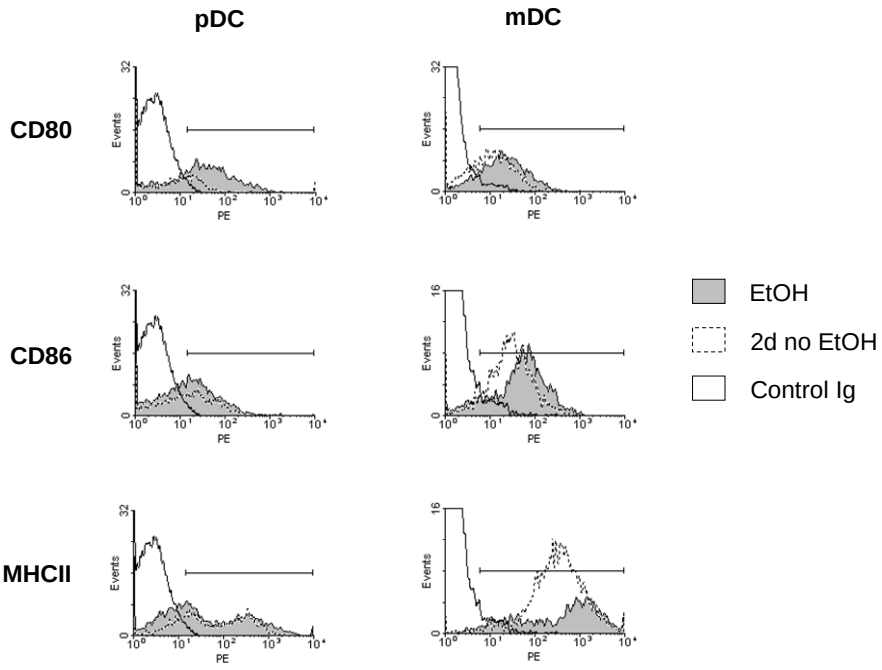


Figure 7. The effects of prolonged EtOH exposure on CD80, CD86 and MHCII expression are not readily reversible.
Flt3L-stimulated BM cell cultures were exposed to EtOH (50 mM) for 8 days after which EtOH was removed from cultures. Cells were stimulated with CpG 16 h prior to FACS analysis. Day 8 and day 10 cultures were labeled with anti-CD11c (FITC-conjugated) and anti-CD45R/B220 (CyChrome-conjugated) or anti-CD11b (biotin-conjugated and revealed with SA-CyChrome), and either anti-CD80, -CD86, or -IA^b (all PE-conjugated). Cells were gated on $CD11c^+B220^+$ cells (pDC) or $CD11c^+CD11b^+$ cells (mDC) and expression of CD80, CD86, or IA^b analyzed. Day 8 EtOH-DC are represented by the shaded histogram and 10 d (last 2 d no EtOH) EtOH-DC by the open dotted outline histogram. Background isotype control staining is represented by the open black outline histogram. Results are from one experiment representative of three performed.

2.3.4 EtOH-treated DC produce less IL-12p70 in response to CpG stimulation and EtOH-treated DC subsets are poor stimulators of naïve T cell proliferation in vitro

The influence of EtOH treatment on the capacity of B10 BMDC to produce IL-12p70, a potent inducer of T cell proliferation, was evaluated. BMDC were bead-purified, stimulated overnight with CpG and culture supernatants assessed for IL-12p70 production, as shown in Figure 8A. EtOH-exposed BMDC produced significantly less IL-12 compared to control DC (Figure 8). Interestingly, production of IFNα in response to CpG-A stimulation was unaffected in BMpDC (Figure 9). To assess the capacity of EtOH-treated control, CpG- or HSV-stimulated B10 DC to stimulate naïve, allogeneic (C3H) T cells, mDC and pDC from control or EtOH-treated BM DC cultures were flow-sorted to >95% purity. CpG was added 16 h before DC sorting, whereas HSV was added to flow-sorted DC for 16 h before testing their function in MLR. As expected, unstimulated pDC were comparatively poor inducers of naïve allogeneic T cell proliferation (Figure 8). By contrast, CpG-stimulated or HSV-infected pDC were much more efficient T cell stimulators. Pre-exposure of the pDC to EtOH (50 mM) strikingly reduced their allostimulatory capacity (Figure 8B, D). mDC were more efficient T cell stimulators than pDC, but EtOH exposure markedly inhibited the allostimulatory activity of control, CpG- or HSV- stimulated mDC (Figure 8C, E).

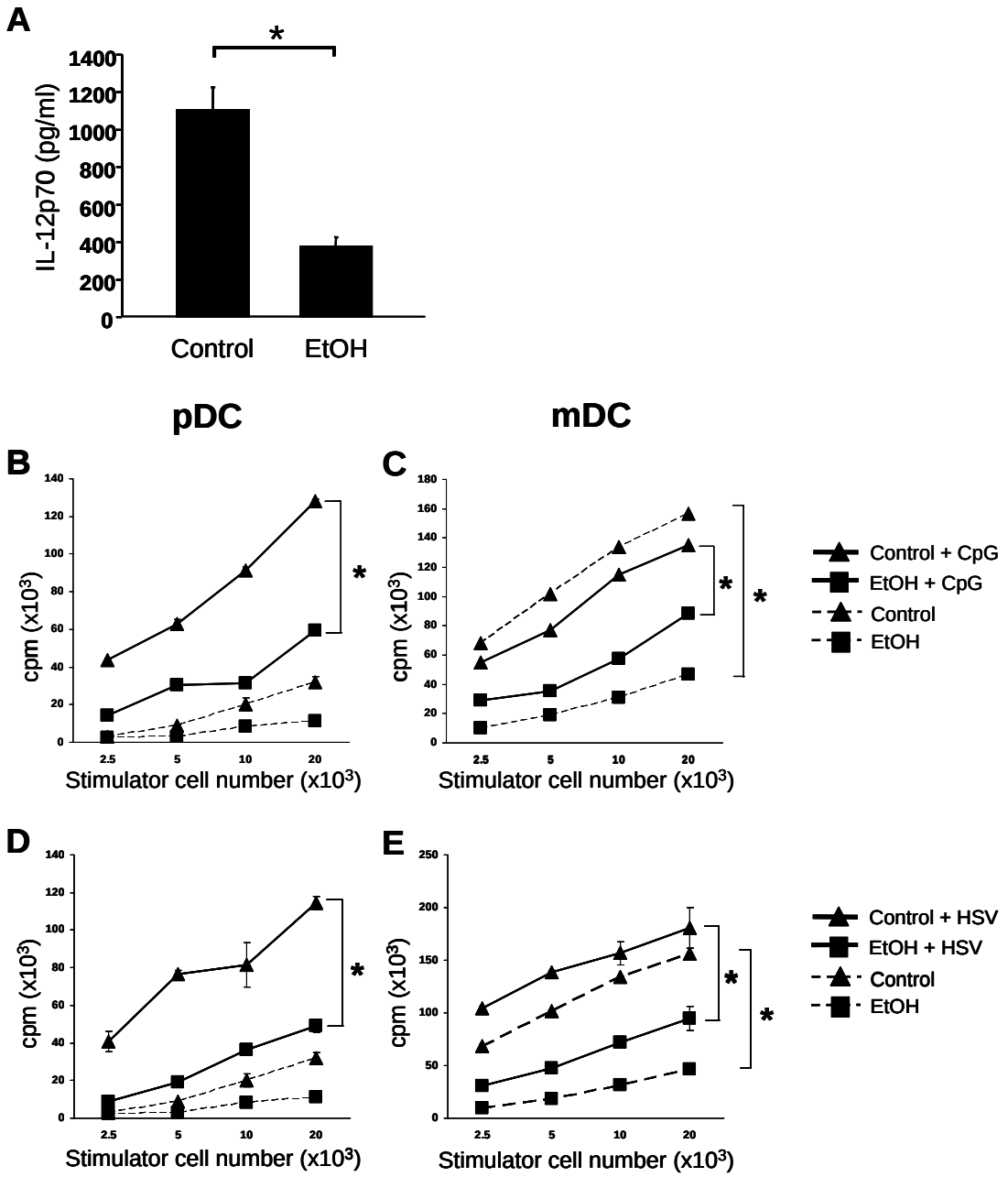


Figure 8. Exposure of B10 BMDC to EtOH significantly reduces their IL-12 production in response to CpG and reduces the stimulatory capacity of pDC and mDC for naïve allogeneic (C3H) T cells.

BM cells were cultured in Flt3L, with or without 50 mM EtOH for 8 days, and with or without CpG (2 μg/ml) or HSV (10 pfu/cell) stimulation. (A), supernatants from 16h CpG-stimulated, bead-purified B10 $CD11c^+$ DC were analyzed for IL-12p70 production. Results are from three experiments. (B,C), cells were stimulated with CpG 16 h prior to sorting. (D,E), alternatively, pDC or mDC were flow-sorted, infected with HSV for 16 h, then used as stimulators in MLR. Flow-sorted pDC ($CD11c^+CD11b^-B220^+$) or mDC ($CD11c^+CD11b^+B220^-$) were irradiated (20 Gy) and graded numbers co-cultured with $2x10^5$ nylon wool-purified allogeneic C3H T cells for 72 h, as described in the Materials and Methods. [^{3}H]TdR was added for the final 16 h of culture. Results are from one experiment representative of three performed and are expressed as means ± 1 SD. *, $p < 0.05$.

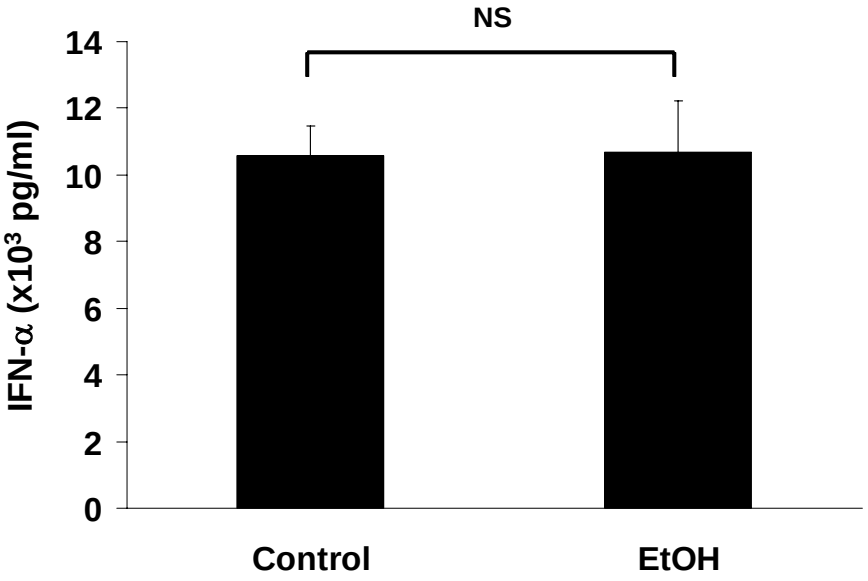


Figure 9. IFN-α production by BMpDC in response to CpG-A stimulation is unaffected by EtOH exposure. BM cells were cultured in Flt3L, with or without 50 mM EtOH for 8 days and flow-sorted for pDC ($CD11c^+CD11b^-B220^+$) as described in Materials and Methods. BMpDC were stimulated for 16 h with CpG-A and supernatants collected for measurement of IFNα production by ELISA. Results are from three experiments.

2.3.5 EtOH-treated DC exhibit unimpaired IDO production

Recently, studies have focused on the role of the tryptophan-catabolizing enzyme indoleamine 2, 3-dioxygenase (IDO) in inhibiting T cell proliferation (291-293), and expression of IDO by DC has been associated with inhibition of T cell function and survival (38, 294). We measured IDO protein levels by western blot and found that EtOH-treated, unstimulated or CpG-stimulated BMDC, spleen DC and liver DC (50 mM) exhibited unimpaired IDO production (Figure 10). Thus, this enzyme is unlikely to play a role in the observed impaired T cell allostimulatory capacity of EtOH-treated BMDC.

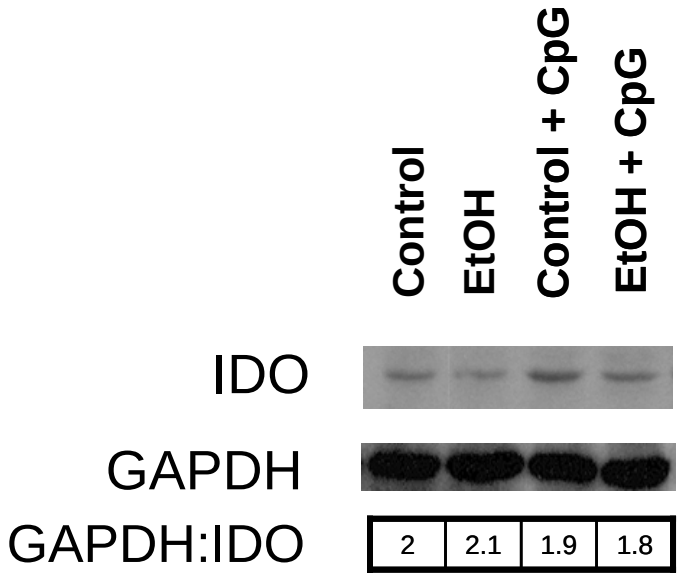


Figure 10. IDO production by BMDC is unchanged by EtOH exposure in vitro.
Protein was isolated from control or CpG-stimulated, bead-purified $CD11c^+$ DC from Flt3L-stimulated B10 cell cultures propagated with or without 50 mM EtOH for 8 days. Western blot analysis was performed for expression of IDO with GAPDH used as the loading control. Densitometric analysis was performed using Scion Image and a ratio of GAPDH to IDO density was computed and presented. Results are from one experiment representative of three performed.

2.3.6 BMDC exposed to EtOH show reduced ability to prime allogeneic T cells in vivo and induce enhanced levels of IL-10 production

Next, we examined the in vivo T cell priming abilities of control and EtOH-treated BMDC (bead-purified to >90% purity, as determined by CD11c staining) by performing adoptive s.c. injection of 5 x 10^5 B10 DC into naïve, allogeneic BALB/c recipients. Six days later, the mice were killed, draining lymph nodes harvested and the total lymph node cells restimulated with donor alloantigen for 72h in MLR. As shown in Figure 11A, EtOH-treated BMDC were significantly less efficient at priming naïve allogeneic T cells in vivo than control BMDC. IL-10 and IFNγ released into the culture supernatants were also measured at 72h. Allogeneic T cells from both groups produced equivalent amounts of IFNγ, but T cells from animals given EtOH-treated DC produced significantly higher levels of IL-10 in comparison to the control group (Figure 11B). Thus, EtOH-treated BMDC were less efficient inducers of naïve allogeneic T cell proliferation in vivo and enhanced IL-10 production by the stimulated T cells.

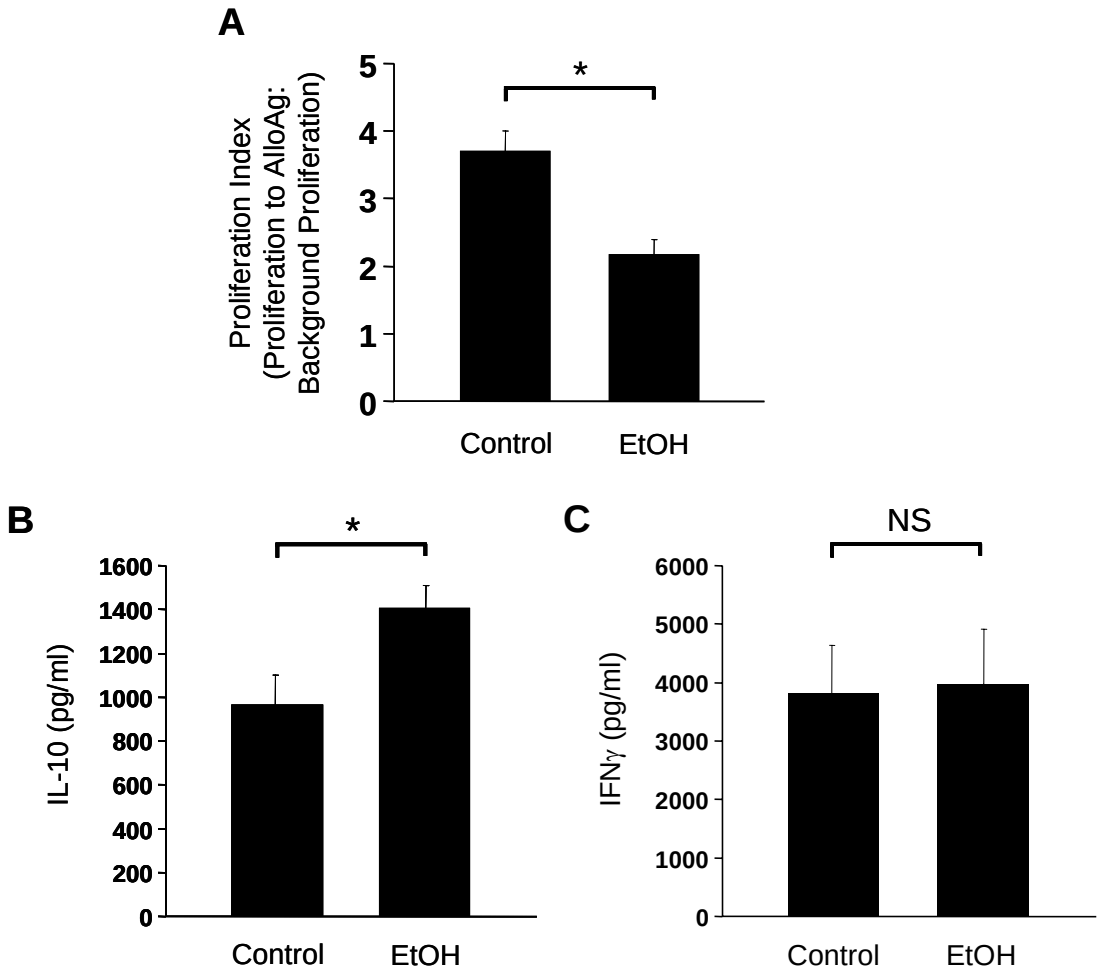


Figure 11. (A), T cell proliferative responses and (B), IL-10 and (C), IFNγ production in ex vivo MLR performed 6 days after s.c. injection of normal BALB/c recipients with bulk, bead-purified B10 CD11c$^+$ DC, propagated with or without 50 mM EtOH for 8 days.

Two x 10^5 T cells were cultured (1:1) with donor cells [bulk, irradiated (20 Gy) B10 splenocytes] for 72 h, as described in the Materials and Methods. (A), [^{3}H]TdR was added for the final 16 h of culture. Results show the ratio of proliferation in response to donor alloantigen to background proliferation of unstimulated LN T cells and are from one experiment representative of five performed. (B), supernatants from the same 72h co-cultures were analyzed for IL-10 and IFNγ by ELISA. Results are from four experiments. *, $p < 0.05$; NS, not significant.

2.4 DISCUSSION

In both humans and animal models, chronic EtOH consumption can adversely affect the immune system and its function (197, 192, 196, 193, 189, 256, 213, 295, 296). With excessive EtOH ingestion, there are reduced T-cell proliferative responses, and impaired delayed–type hypersensitivity reactions (297-299, 261, 300), as well as an increased incidence of infectious diseases, including tuberculosis and hepatitis C viral infection (189-191). Furthermore, prolonged EtOH exposure decreases the Ag-presenting capacity of human monocytes and monocyte-derived DC (222, 220). However, the mechanisms underlying the immune-compromised state of alcoholics have not been fully elucidated. In this study, we have examined the influence of prolonged EtOH exposure on BM-derived mDC and pDC,. We show that prolonged EtOH exposure suppresses the cytokine-induced differentiation of mDC and pDC from normal BM precursors, with a more marked effect on pDC. EtOH also reduces the constitutive and CpG-induced expression of classic B7 costimulatory molecules, while sparing expression of the coregulatory molecule B7-H1 and reducing IL-12p70 production upon DC activation. Consistent with these findings, EtOH impaired the stimulatory capacity of BMDC for naïve allogeneic T cells, both in vitro and in vivo, and enhanced the IL-10-producing capacity of in vivo-primed T cells. Further, chronic EtOH consumption appears to have a more marked inhibitory effect on splenic compared to hepatic DC subsets, as assessed by their phenotypic and functional characteristics.

The complex effects of EtOH on the immune system likely reflect many variables, including the duration (acute vs. chronic) and extent of exposure (EtOH concentration), as well as the influence of local or systemic factors, including cytokines or other immune-modulating agents. Acute alcohol exposure inhibits the production of various cytokines (301, 227, 302, 219, 221, 222, 234, 220, 236), while the generation of human monocyte-derived DC in ≥ 25 mM EtOH reduces the expression of the classic B7 family costimulatory molecules, CD80 and CD86 (220). Our finding that EtOH exposure affects B7 family molecule expression on DC subsets differentially (reduced CD80/CD86, but unaffected B7-H1 expression) confirms and extends these observations. The extent of the impact of EtOH exposure on expression of costimulatory

molecules in response to CpG may reflect the low constitutive level of expression of these molecules by BMDC. Additionally, these effects of EtOH on the expression of key functional cell surface molecules could result from alterations in the cell membrane. Thus, a change in membrane fluidity could affect the formation and stability of lipid rafts, known to contain membrane-associated proteins important in cell signaling. Recent studies on macrophages support this view (234-236, 303). Indeed, there is evidence that EtOH affects signal transduction pathways, such as the mitogen-activated protein kinase (MAPK) pathway in macrophages (234-236). Goral and Kovacs (236) showed recently that acute EtOH exposure inhibited activation of the MAPK pathway when murine macrophages were stimulated with a variety of TLR ligands. Collectively, these data indicate convincingly that EtOH negatively impacts the function of APC.

DC are uniquely well-equipped APC, and their phenotype is important in defining the way that T cells are activated. Szabo *et al.* (221, 222) have shown that EtOH inhibits both the differentiation and full maturation of human mDC, leading to decreased T cell stimulatory capacity. They ascribed this inhibition in part to reduced production of IL-12 (p40/p70) and concomitant, increased production of IL-10 by DC in response to LPS stimulation. Similarly, in the present study, in vitro-generated murine BM-derived mDC and pDC exposed to EtOH showed inhibited differentiation, with a greater effect on pDC development, and reduced IL-12p70 production in response to CpG stimulation. A recent study of recombinant Hep G2 cells exposed to EtOH revealed that the cells were arrested in the G2/M phase, likely due, in part, to impaired Cdc2 activity (304). We propose that in the presence of EtOH, replicating DC progenitors may be affected similarly, explaining the lower total numbers of DC recovered in culture. pDC were affected more markedly, reflecting the poorer survival capacity of these cells in vitro compared with mDC (A.H. Lau and M. Abe, unpublished observations) and their greater sensitivity to potential toxins, such as dexamethasone (305). Alternatively, the cell development signaling pathways for pDC may be specifically affected by EtOH. Hepatic and splenic DC subsets recovered from EtOH-fed, Flt3L-mobilized mice showed no significant reductions compared to Flt3L-treated controls. The apparent discrepancy between the EtOH-mediated reduction in absolute DC numbers in response to Flt3L stimulation in vitro and in vivo may reflect differences in EtOH concentration and metabolism between the different test systems.

EtOH-treated BMDC were poorer stimulators of allogeneic T cells in both in vitro assays and in vivo adoptive transfer experiments. The reduced cell surface expression of CD80 and

CD86, associated with impaired T cell stimulatory capacity, is consistent with the influence of acute alcohol consumption on human monocyte and DC accessory cell function (221). The importance of the CD80/86-CD28 costimulatory pathway in T cell activation is well-recognized (306-308). Further, it has been documented that T cell anergy resulting from the lack of functional CD80/86-CD28 signaling can be overcome by the addition of exogenous IL-2. In this study, we found that the expression of CD80 and CD86, as well as CD40, was reduced on EtOH-treated BMmDC and BMpDC. As expected, stimulation of T cells by these DC was reduced significantly. Addition of exogenous IL-2 did not overcome the anergic state (data not shown), suggesting that alternate mechanisms were responsible for impairment of T cell responsiveness. One possible mechanism whereby EtOH-treated DC may impair T cell responses is signaling via the CD274 (B7-H1)-PD-1 pathway, a recently-identified coregulatory pathway that inhibits T cell activation (309). Unlike the classic costimulatory molecules (CD40, CD80, and CD86), B7-H1 was not reduced significantly on EtOH-treated, CpG-stimulated mDC or pDC. This finding may explain, in part, why the EtOH-treated DC were less stimulatory than control DC for naïve T cells. The high B7-H1 to classical costimulatory molecule (B7-1 and B7-2) ratio that we observed suggests a role for this molecule in the inhibitory effect of EtOH-treated DC on T cell activation (Table 5). Indeed, we have shown elsewhere (40) that blocking of B7-H1 expression on in vitro-generated murine DC enhances their T cell stimulatory ability.

T cells primed in vivo with EtOH-exposed BMDC were found to produce more IL-10 compared to T cells from control BMDC-injected mice, with no differences in IFNγ production. Further, IL-2 production in these cultures did not differ between the EtOH and control groups. It has been shown that autocrine production of IL-10 can lead to T cell unresponsiveness (310). The increased production of IL-10, although not accompanied by changes in IFNγ production, may reflect skewing of T cells to produce more regulatory IL-10 rather than the Th1 cytokine, IFNγ. Mandrekar *et al*. (220) showed that EtOH-treated human monocyte-derived DC exhibited reduced IL-12 and increased IL-10 production, that, when the DC were used as stimulators of naïve $CD4^+$ T cells in vitro, led to reduced IFNγ production. In our experiments, EtOH-treated BMDC were used to prime naïve T cells in vivo, which may account for the differences in cytokine production by T cells between the studies. Further, in our adoptive transfer experiments, responses of $CD8^+$ T cells were not excluded and may have contributed to IFNγ production. The increased IL-10 production by T cells was not reproduced when naïve mice

were primed with hepatic or splenic DC from chronic EtOH-fed mice (Figure 22B). IFNγ (Figure 22B) and IL-2 (data not shown) production by these T cells was also no different between EtOH and control groups. This discrepancy between the influences of in vitro- and in vivo-derived DC may again result from inherent differences in EtOH concentration and metabolism between the different test systems.

In summary, our studies show that the differentiation, phenotypic maturation and T cell stimulatory capacity of EtOH-treated BMDC subsets are impaired. Prolonged exposure to EtOH inhibits BMDC maturation, characterized by reduced expression of surface costimulatory molecules; by contrast, expression of the coregulatory molecule CD274 (B7-H1) is unaffected. Interestingly, EtOH-exposed BMDC, while inducing less Ag-specific T cell proliferation, induce more IL-10 production. Together, these data suggest that chronic EtOH exposure impairs the differentiation and functions of BMDC subsets, and may reflect one mechanism by which alcoholics have compromised immune function.

3.0 EFFECTS OF CHRONIC ETHANOL CONSUMPTION ON HEPATIC AND SPLENIC DENDRITIC CELL PHENOTYPE AND FUNCTION[3]

3.1 INTRODUCTION

As detailed in 2.1, there have been been limited studies investigating EtOH's effects on the development and function of DC. In Chapter 3, we examined the effects of prolonged EtOH exposure on the development and function of mDC and pDC from BM. While these studies are important for determining the possible effects that EtOH may have on the in vivo differentiation and function of DC, there are inherent differences between in vitro-propagated DC and freshly-isolated DC, especially when considering the environment in which DC reside or mature. This functional difference between BMDC or liver or spleen DC has been shown with chemotaxis and migration experiments (119, 75). Further, no studies to date have examined the effects of chronic EtOH administration on murine DC subsets, despite the availability of the mouse model for alcohol and immune function studies. Thus, we sought to utilize the Meadows-Cook murine alcohol model (211, 212) to investigate effects of chronic EtOH on hepatic and splenic DC.

The microenvironment in which DC develop or are activated can markedly influence their function and the outcome of their interactions with T cells. Within the liver, comparatively high levels of interleukin (IL)-10 and transforming growth factor (TGF)-β are produced by various liver cell populations (1). These cytokines can confer tolerogenicity on DC by rendering the cells resistant to maturation and inhibiting their T cell stimulatory function (311). Indeed, liver DC have been implicated in liver transplant tolerance (137). Moreover, compared with splenic DC, murine hepatic DC are refractory to endotoxin stimulation, that appears to reflect comparatively low constitutive levels of TLR4 expression by liver DC (79).

[3] Data and Text excerpted from (57).

Studies have consistently demonstrated an association between alcohol abuse and increased susceptibility to a variety of infectious diseases, including bacterial pneumonia and tuberculosis (Reviewed in 189). Other studies have shown accelerated progression of chronic liver disease in patients with chronic HCV and alcohol use (190, 191). As DC are important mediators of immune function, any inhibitory effects on their function may cause compromised immune function. Of further clinical relevance, DC-based therapies are being investigated in several diseases, including hepatocellular carcinoma, in which chronic alcohol consumption is a major risk factor (312, 313). Thus, evaluating the effects of prolonged EtOH exposure on the phentotype and function of in vivo-derived DC may provide insight into the compromised immune status of alcoholics.

In the present study, we have assessed the influence of chronic EtOH administration on hepatic and splenic DC in vivo using a mouse model. We have found that chronic EtOH administration in vivo exerts a greater inhibitory effect on splenic DC maturation and function (naïve T cell allostimulatory activity) than on maturation-resistant hepatic DC. However, EtOH exposure increases the migratory capacity of hepatic DC in vivo compared to normal control hepatic or splenic DC. Thus, chronic in vivo EtOH exposure exerts differential effects on hepatic and splenic DC.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Six- to eight-week old C57BL/10 (B10; $H2K^b$), C57BL/6 (B6; $H2K^b$), BALB/c ($H2K^d$), and C3H/HeJ (C3H; $H2K^k$) mice were purchased from The Jackson Laboratory and maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh Medical Center. Experiments were conducted under an Institutional Animal Care and Use Committee-approved protocol. The animals were fed a diet of Purina rodent chow and received tap water ad libitum unless specified.

3.2.2 In vivo EtOH administration

B6 mice were separated randomly into control and EtOH-fed groups. Mice in the EtOH group were given 10% w/v EtOH in tap water ad libitum for 2 days, 15% w/v EtOH for 5 days, then 20% w/v EtOH for at least 8 weeks (212). No adverse effects of this EtOH feeding protocol on behavior or weight gain compared with control mice were observed (3.3.2).

3.2.3 Measurement of blood alcohol level

Anti-coagulated blood was collected from mice using heparin and analyzed for blood alcohol levels using a commercial Alcohol Reagent Set kit (Pointe Scientific, Inc., Canton, MI) following the manufacturer's recommended procedures.

3.2.4 Serum alanine aminotransferase and aspartate aminotransferase levels

Blood was collected without heparin and allowed to clot at room temperature for one hour. Blood samples were then centrifuged at 15000 x g at 4°C for 10 min. Serum was collected and then analyzed by UPMC Presbyterian Shadyside Chemistry Labs for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

3.2.5 In vivo CpG administration

Mice received CpG- B (100 μg in 0.1 ml) i.v. via the lateral tail vein, 12 to 16 h before liver and spleen DC isolation.

3.2.6 Histological analysis of liver and spleen

Liver and spleen tissues from control and EtOH-fed mice (8-10 weeks diet) were fixed with formalin then stained with hematoxylin and eosin to compare gross histology.

3.2.7 Immunofluoresence staining of tissue sections

Blocks of liver and spleen were embedded in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN), snap frozen in isopentane/liquid nitrogen, and stored at -80°C. Cryostat sections (8 μm) were fixed in 96% ethanol (10 min), blocked with 10% normal goat serum, and incubated overnight (4°C) with anti-CD11c biotin-mAbs (BD PharMingen). As a second step, slides were incubated with 1:3000 Cy2-streptavidin (Jackson Immunoresearch Lab, West Grove, PA), for 30 min at room temperature. Cell nuclei were stained with DAPI (4,6 diamidino-2-phenylindole; Molecular Probes, Eugene, OR). Slides were fixed in 2% paraformaldehyde, mounted in glycerol/PBS, and examined with a Zeiss Axiovert 135 microscope equipped with appropriate filters and a cooled CCD camera (Photometrics CH250, Tucson, AZ). Signals from different fluorochromes were acquired independently, and montages edited using the Adobe Photoshop software program (Adobe Systems, Mountain View, CA).

3.2.8 Isolation and purification of liver and spleen DC

DC were isolated from livers and spleens of animals given Flt3L [10 μg/day i.p. in phosphate buffered saline (PBS) for 10 consecutive days, as described (68, 79), with minor modifications]. Briefly, a 22 gauge catheter was inserted in the inferior vena cava and blood collected for serum and measurement of blood EtOH levels. Mice were perfused with 20 ml PBS, then livers and spleens removed. Livers were disaggregated and digested for 30-45 min at 37°C in 10 ml type IV collagenase (1 mg/ml; Sigma-Aldrich, St. Louis, MO) supplemented with DNase (100 μg/ml; Roche, Nutley, NJ). Liver non-parenchymal cells were isolated by density centrifugation (428 mg/ml Histodenz; Sigma-Aldrich) at 1200 x *g* for 20 min at 4°C. DC were enriched from bulk splenocytes by density centrifugation (160 mg/ml Histodenz) at 500 x *g* for 20 min at 20°C. Following centrifugation of liver or spleen cell suspensions, the interface cells were collected and washed twice in PBS. $CD11c^+$ cells were then purified using anti-mouse CD11c-coated immunomagnetic beads, as described above.

3.2.9 Antibodies for flow cytometry

Monoclonal antibodies (mAbs) used for flow cytometry and cell sorting were those used in Chapter Three (2.2.2). Additionally, rat anti-mouse PE-CCR7 (4B12; eBioscience), FITC-conjugated or biotinylated-CD11a (LFA-1; 2D7), CD31 (PECAM-1; 390), CD54 (ICAM-1; 1A29), CD62E (E-Selectin; 10E9.6), CD62L (L-Selectin; MEL-14), CD102 (ICAM-2; 3C4), and CD106 (VCAM-1; 429) (all from BD PharMingen).

3.2.10 Media and reagents

Media, Flt3L, TLR9 ligands (CpG) used were the same as in Chapter Three (2.2.2).

3.2.11 Chemotactic agents

Mouse recombinant CC chemokine CCL19/MIP-3β used in chemotaxis assay experiments was obtained from R & D Systems (Minneapolis, MN).

3.2.12 Chemotaxis assay

Assays were performed as described previously (121, 119) with minor modifications. CD11c-bead purified hepatic and splenic DC from control and EtOH-fed mice were stimulated for 16h with 2 μg/ml CpG-B. Stimulated DC (2×10^5) were resuspended in 100 μl 0.5% BSA RPMI 1640 (no FBS, without chemokine) in Transwells® (5 μm pores; Costar, Cambridge, MA) which were placed in 24-well plates with 600 μl of chemokine dilution in 0.5% bovine serum albumin RPMI 1640 per well and incubated for 2 h at 37° C in 5% CO_2 in air. After the incubation period, the Transwells® were removed and migrated DC from the 24-well plates were collected and enumerated using a Coulter Counter (Beckman Coulter). For accurate comparison between experiments, results were expressed as the percentage of migrated DC. Migration assays were performed in duplicate; experiments were repeated at least 3 times.

3.2.13 Migration of DC *in vivo*

Hepatic and splenic CD11c-bead purified DC were labeled cytoplasmically with the green fluorescent dye chloromethylfluorescein diacetate (CMFDA; Molecular Probes, Leiden, The Netherlands) (15 μM; 45 min at 37° C) or carboxyfluoroscein succinimidyl ester (CFSE; Molecular Probes) (5 μM; 15 min 37° C) according to the manufacturer's instructions. After two washes, 3-5x10^6 DC were injected s.c. into one hind footpad of allogeneic recipients (BALB/c). Popliteal DLN were removed 24 h after s.c. injection. Inguinal LN were removed and served as negative controls. LN cells were analyzed by FACS for the percentage of green fluorescent cells. The number of DC migrating to DLN in vivo was determined by the following equation: (% green fluorescent cells in DLN x total number of cells recovered from DLN)/(number of DC injected s.c.).

3.3 RESULTS

3.3.1 Meadows-Cook Murine Model

As discussed, the Meadows-Cook mouse model is one of three possible murine models of chronic ethanol administration that can be used. This model was briefly assessed to ensure that mice had a similar phenotype to reported studies.

3.3.1.1 Blood Alcohol Level

The blood alcohol of mice was measured on the day that they were killed for experiments with blood being drawn 3-4 h after nocturnal cycle (ends at 6 am). Consistent with published reports, the blood alcohol level (BAL) of mice varied, but was significantly elevated compared to control, non-drinking mice (Figure 12), as expected (212).

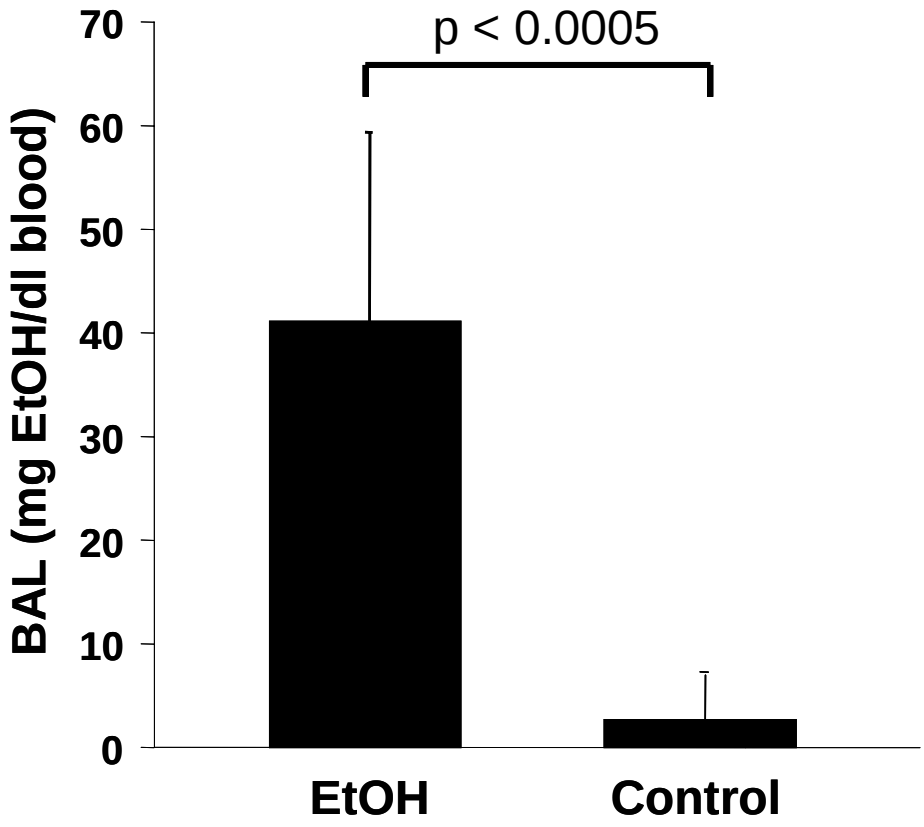


Figure 12. Mice fed EtOH chronically have detectable BAL.
B6 mice were fed EtOH for 8-24 w, as described in the Materials and Methods (3.2.2). Age- and sex--matched control mice received water without EtOH. BAL was quantified as described in Materials and Methods (3.2.3). Results are from 14 experiments and are expressed as mean ± 1 SD.

3.3.1.2 Serum ALT and AST

In alcoholic humans, elevated ALT and AST levels can be diagnostic for liver damage. However, in the Meadows-Cook murine model, it has been reported that there is no significant difference in AST or ALT between control and chronically EtOH-fed (8-24 w) mice (212). Consistent with these reports, there was no significant difference in serum AST or ALT between the two groups (Figure 13).

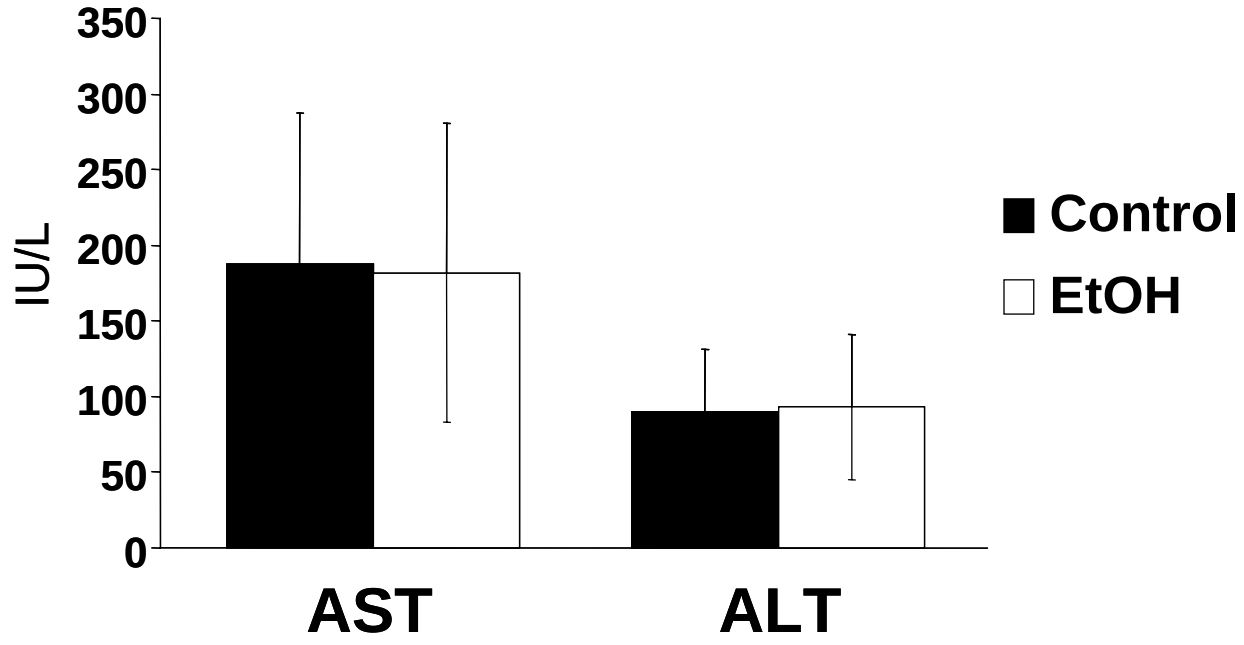


Figure 13. There is no difference in serum AST or ALT between control and EtOH-fed mice.
B6 mice were fed EtOH for 8-24 w, as described in the Materials and Methods (3.2.2). Age-matched control mice received water without EtOH. Serum was collected and AST and ALT quantified as described in Materials and Methods (3.2.4). Results are from nine or eleven experiments and are expressed as mean ± 1 SD.

3.3.1.3 Histologic appearance of livers and spleens

EtOH-fed mice displayed lipid accumulation in the liver compared to control mice in which livers appeared normal (Figure 14A). However, no histologically detectable necrosis, increased inflammatory cells, or other morphologic changes were observed. In contrast, the spleens of EtOH-fed mice showed no gross changes in histology compared to control spleens (Figure 14B).

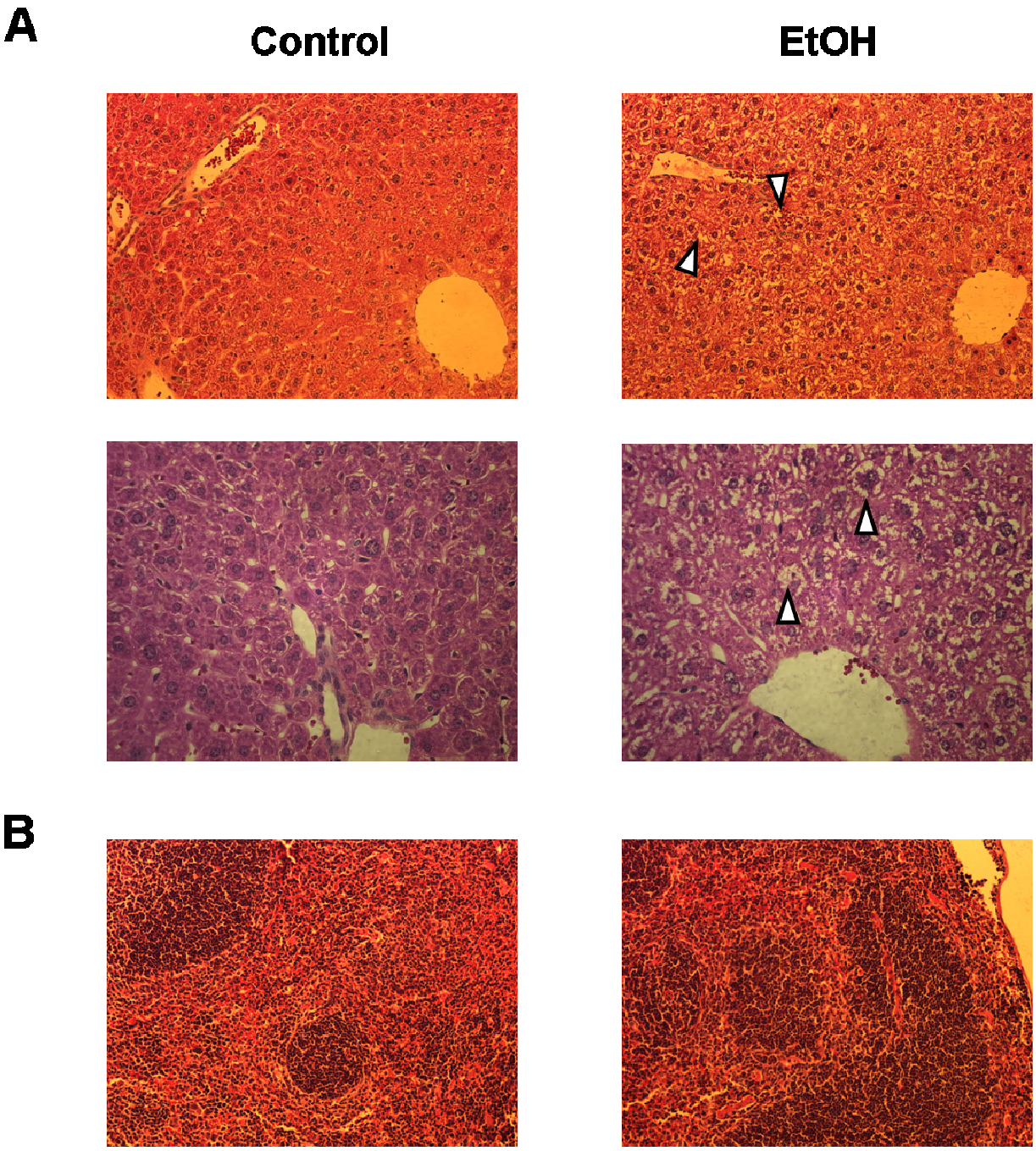


Figure 14. Histological appearance of livers (A) and spleens of (B) control and EtOH-fed mice.
B6 mice were fed EtOH for 8 w, as described in the Materials and Methods (3.2.2). Age-matched control mice received water without EtOH. (A) Control liver sections show normal appearance, whereas the EtOH-fed liver sections show areas of lipid accumulation seen as areas of open space (lack of dye uptake) around hepatocyte nuclei (some representative areas indicated by arrowheads). Top sections: Original magnification, 100x. Bottom sections: Original magnification, 200x; (B) Both control and EtOH-fed spleens exhibited normal gross histology by H & E staining. Original magnification, 10x.

3.3.2 Flt3L treatment of control and EtOH-fed mice

Flt3L has been commonly used in mice to increase the numbers of DC in both livers and spleens (314, 68). However, there have been no reports of Flt3L use in the Meadows-Cook murine model of chronic alcohol intake. Administration of Flt3L can cause changes in the organ weight to body weight ratio as a result of the increased DC and other hematopoietic cell numbers. If prolonged EtOH administration negatively affected DC genesis, it would be expected that there would be a resulting decrease in the organ to body weight ratio, as there was no difference in body weight between the groups after chronic EtOH exposure (Figure 12A) (212). However, there was no difference in the organ to body weight ratio for livers and spleens (Figure 15B), suggesting that there was no difference in DC genesis by Flt3L administration in EtOH-fed mice.

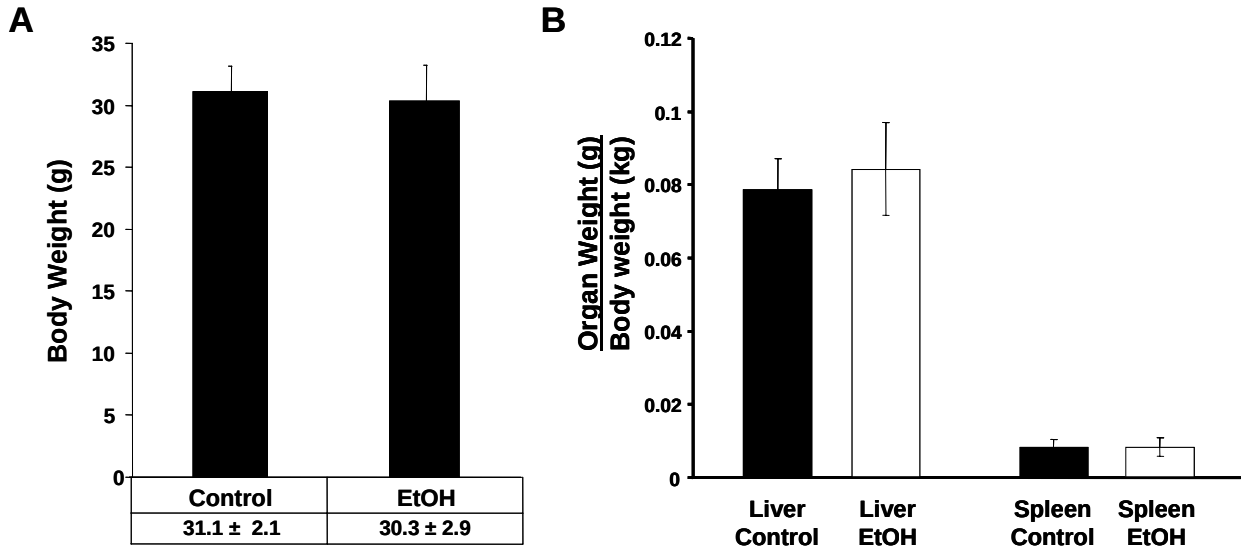


Figure 15. No changes in body weight (A) or organ weight (g)/body weight (kg) (B) between control and EtOH groups.
B6 mice were fed EtOH for 8-24 w, as described in the Materials and Methods (3.2.2). Age-matched control mice received water without EtOH. (A) Mice were weighed before sacrifice; (B) Livers and spleens were weighed after removal and the data expressed relative to body weight. Results are from (A) 14 or (B) 23-25 experiments and are expressed as mean ± 1 SD.

In fact, in vivo chronic EtOH consumption did not lead to significant reductions in absolute DC numbers recovered from Flt3L-mobilized mice (Figure 16). Furthermore, the distribution of DC in the organs was unchanged, with liver DC residing predominantly around portal triads, but also appearing throughout the liver parenchyma, and splenic DC in the marginal zone and red pulp (Figure 17). Thus, prolonged EtOH administration in mice did not affect DC genesis with Flt3L administration in vivo.

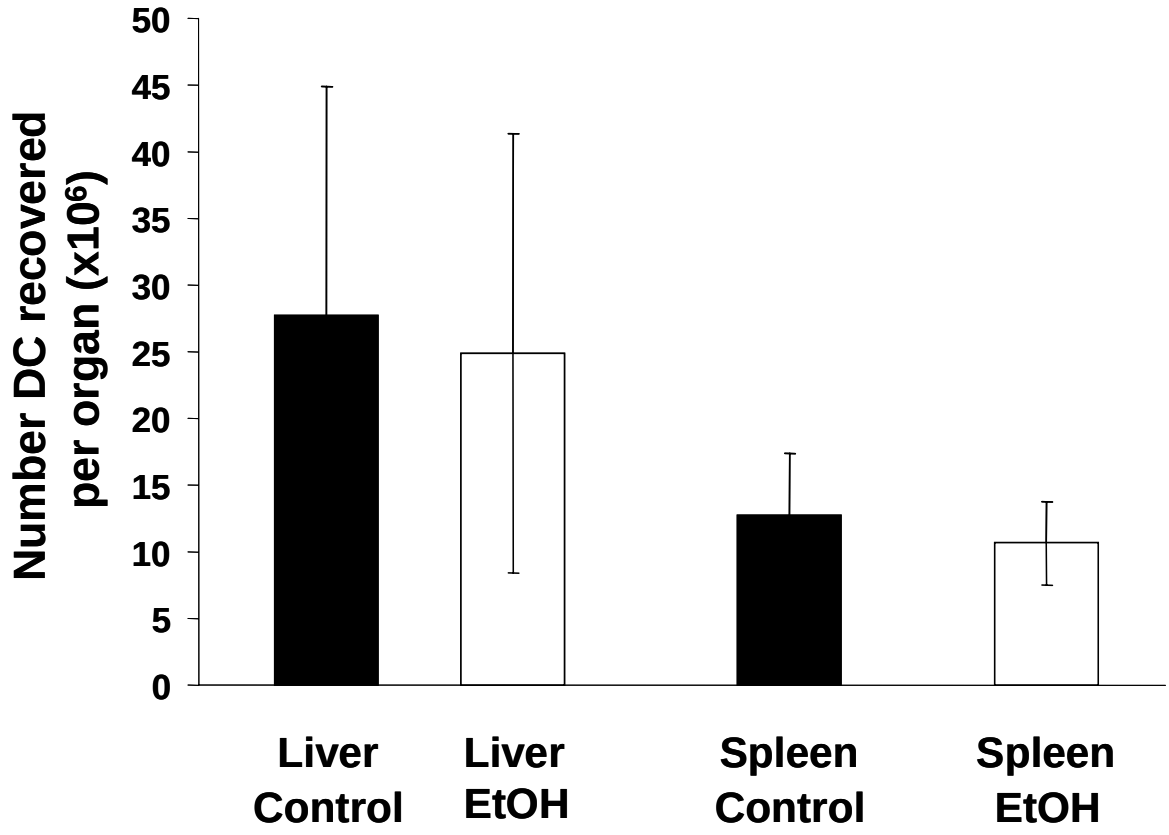


Figure 16. No difference in total number of DC recovered from EtOH-fed mice treated with Flt3L.
B6 mice were fed EtOH for 8-24 w and age-matched control mice received water without EtOH, administered Flt3L and DC recovered from livers and spleens, as described in the Materials and Methods (3.2.2, 3.2.8). Results are from six to seven experiments (representative of many) and are expressed as mean ± 1 SD

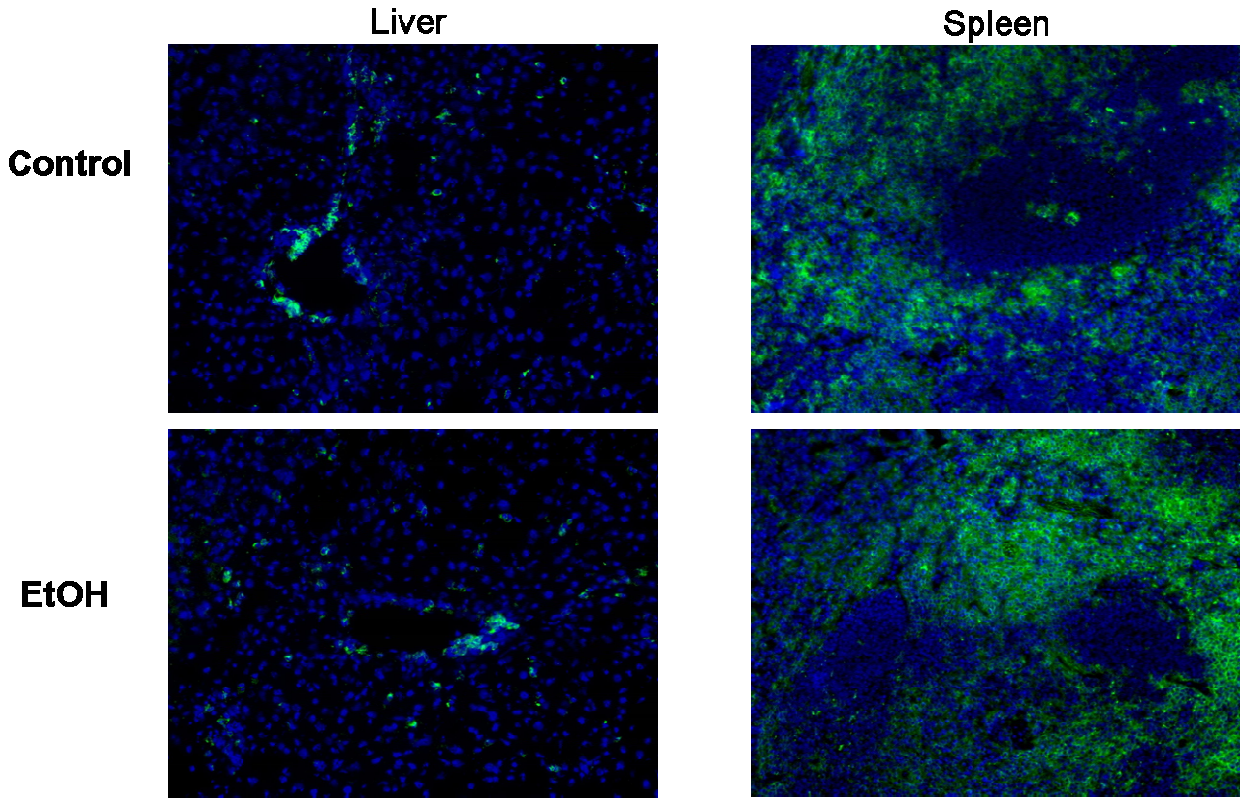


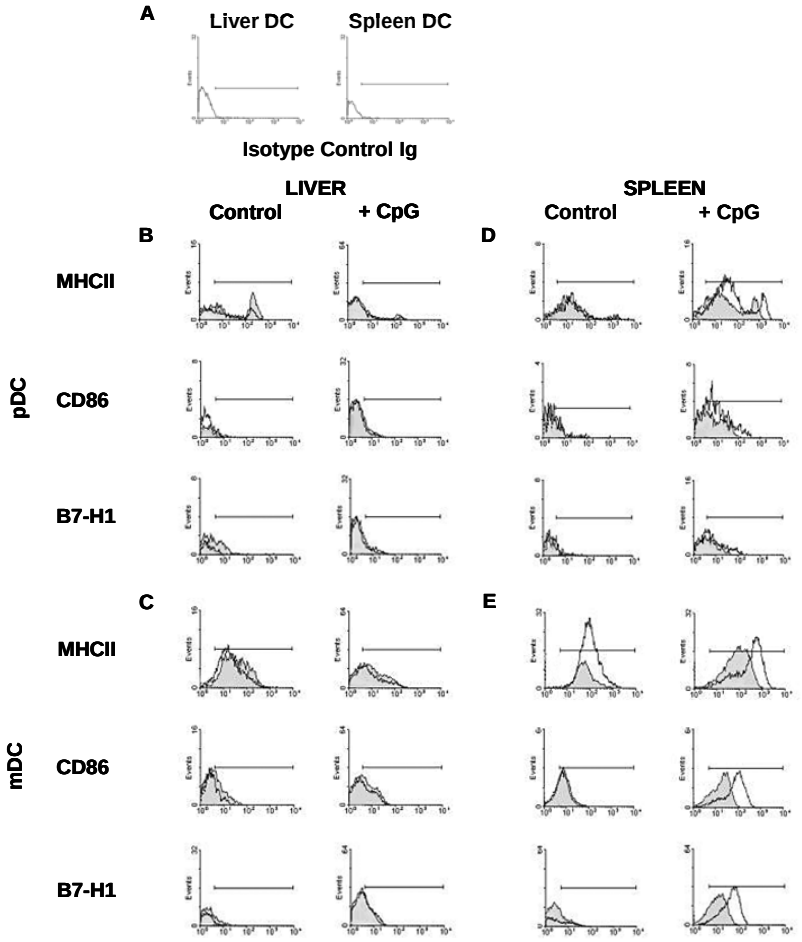
Figure 17. Immunohistochemistry of liver and spleen sections reveals no apparent difference in numbers of DC and localization of DC with Flt3L administration in EtOH-fed mice.
B6 mice were fed EtOH for 8 w, as described in the Materials and Methods (3.2.2). Age-matched control mice received water without EtOH. Liver and spleen sections were stained with biotinylated mAb anti-CD11c, followed by Cy2-streptavidin (green). DAPI (blue) indicates nuclear staining. 200x magnification.

3.3.3 Splenic DC subsets are more susceptible than hepatic DC subsets to in vivo modulatory effects of prolonged EtOH consumption on cosignaling molecule expression

To assess the impact of prolonged EtOH exposure on DC subsets in vivo, we analyzed the maturation capacity of hepatic and splenic DC from mice fed EtOH for 8 weeks. DC were mobilized (with Flt3L) for the final 10 days before harvesting to obtain sufficient numbers of each DC subset for phenotypic and functional analysis. Groups of mice were also given CpG-B (100 μg) i.v. for the final 16 h to determine the influence of chronic EtOH consumption on DC responsiveness to this activating agent in vivo. Freshly-isolated hepatic and splenic DC were purified and analyzed by flow cytometry for the expression of costimulatory molecules (CD40, CD80, and CD86) as well as MHC class I and MHC class II (Figure 18, and unpublished results). As reported previously (68, 79), administration of Flt3L alone did not affect cell surface

expression of these molecules (unpublished results). Freshly-isolated splenic or hepatic mDC and pDC (especially) exhibited very low levels of surface costimulatory molecules. Expression of CD86 was only slightly higher on liver DC subsets in response to in vivo CpG stimulation, whereas more marked upregulation of this molecule was observed on splenic DC (Figure 18). Expression of CD40 and CD80 followed the same pattern as CD86 (data not shown). In mice fed EtOH, the inherently low intensity of CD86 expression on hepatic mDC and pDC differed only modestly in comparison to controls (Figure 18B, C). However, the mean fluorescence intensity (MFI) for this molecule (and CD40 and CD80; data not shown) was lower on spleen DC from EtOH-treatment groups (Figure 18D, E). MHC class II expression was also lower on CpG-stimulated splenic mDC and pDC from EtOH-fed mice, whereas this effect was not evident for hepatic DC subsets that expressed lower constitutive levels of MHC class II.

The expression of CD274 (B7-H1) was also examined on hepatic and splenic DC from chronic EtOH-fed mice (Figure 18). As with the costimulatory molecules, CD274 was expressed at very low levels on freshly-isolated hepatic DC, and was not impaired by long-term (8 w) EtOH administration. Similarly, the higher level of CD274 on unstimulated and (especially) CpG-stimulated splenic DC was not reduced by long-term EtOH consumption. Taken together, these results suggest that the T cell stimulatory function of splenic DC may be more susceptible than that of hepatic DC to the influence of long-term EtOH consumption.



LIVER			Control		+EtOH	
			Control	+CpG	Control	+CpG
MHCII	pDC	%	59.40	28.82	68.79	42.16
		MFI	54.82	28.70	109.05	28.12
	mDC	%	87.23	43.68	95.33	55.73
		MFI	21.68	16.40	33.21	16.74
CD86	pDC	%	4.34	13.77	7.91	20.64
		MFI	8.42	10.84	14.72	9.52
	mDC	%	18.48	28.70	36.88	33.61
		MFI	8.23	12.45	10.35	12.90
B7-H1	pDC	%	10.23	18.95	19.32	11.91
		MFI	10.67	11.86	16.16	8.44
	mDC	%	6.37	20.17	15.62	16.21
		MFI	9.92	10.43	10.20	9.58

SPLEEN			Control		+EtOH	
			Control	+CpG	Control	+CpG
MHCII	pDC	%	88.54	94.06	86.12	86.93
		MFI	22.55	64.41	25.15	42.39
	mDC	%	98.81	97.90	98.08	98.70
		MFI	102.43	240.83	62.12	81.17
CD86	pDC	%	32.60	72.78	33.21	66.34
		MFI	8.25	17.18	7.49	15.21
	mDC	%	67.26	95.52	68.82	85.56
		MFI	10.70	67.90	9.51	20.69
B7-H1	pDC	%	18.79	65.77	25.07	53.00
		MFI	6.76	13.48	6.61	8.76
	mDC	%	40.55	96.75	27.42	81.55
		MFI	13.03	42.40	10.71	14.86

Figure 18. Freshly-isolated hepatic pDC (B) or mDC (C) exhibit little change in surface expression of MHC class II, the classic costimulatory molecule CD86, or the cosignaling molecule CD274/B7-H1/PD-L1 following chronic *in vivo* EtOH feeding, but splenic pDC (D) and mDC (E), that express constitutively higher levels on mDC, exhibit reduced expression of these molecules.

B6 mice were fed EtOH for 8 w, as described in the Materials and Methods. Age-matched control mice received water without EtOH. Mice were treated with Flt3L (10 μg/day, i.p.) for 10 days, then received CpG (10 μg, i.v.) 16 h prior to sacrifice. Bulk, bead-purified $CD11c^+$ hepatic and splenic DC were isolated, labeled with anti-CD11c (biotin-conjugated, revealed with SA-CyChrome) and anti-CD45R/B220 or anti-CD11b (FITC-conjugated) and either anti-IA^b, -CD86, or –B7-H1 (PE-conjugated). Cells were gated on $CD11c^+B220^+$ (pDC) or $CD11c^+CD11b^+$ (mDC) cells and expression of IA^b, CD86, or B7-H1 analyzed. Control DC are represented by the open black outline histogram and EtOH-fed mouse DC by the shaded histogram. Background isotype control staining is shown in (A) and represented by the open black outline histogram. Percent positive cells and MFI for each treatment group are indicated in the tables. Results are from one experiment representative of three performed.

3.3.4 Hepatic and splenic DC from EtOH-treated, control and CpG-stimulated mice are less efficient stimulators of naïve allogeneic T cell proliferation in vitro and in vivo

Freshly-isolated resting and in vivo CpG-B-stimulated hepatic and splenic DC were next analyzed for their ability to stimulate naïve allogeneic T cells. Consistent with the corresponding phenotypic data (Figure 18), splenic DC from mice fed EtOH for 8 w and stimulated in vivo with CpG-B were significantly less efficient inducers of naïve allogeneic T cell proliferation than control CpG-B-stimulated splenic DC (Figure 19). Hepatic DC from control, CpG-B-stimulated mice were poorer stimulators than their splenic counterparts, whereas the stimulatory function of liver DC from CpG-B-stimulated, EtOH-fed mice was similar to that of spleen DC from the same animals (Figure 19).

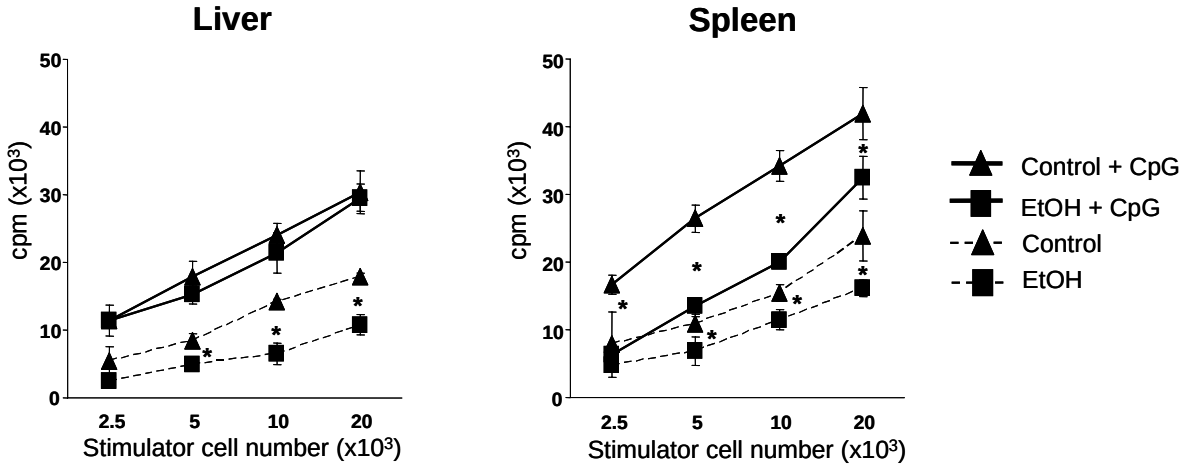


Figure 19. T cell proliferative responses induced by hepatic or splenic DC isolated from EtOH-fed or control B6 mice, with or without CpG stimulation *in vivo*.
Bulk, bead-purified $CD11c^+$ hepatic and splenic DC were isolated from mice given EtOH or water alone for 8 w, Flt3L (10 μg/day, i.p.) for 10 days and CpG (100 μg, i.v.), 16 h prior to sacrifice. Graded numbers of irradiated (20 Gy) DC were co-cultured with $2x10^5$ nylon wool-purified allogeneic C3H T cells for 72 h, as described in the Materials and Methods. $[^3H]$TdR was added for the final 16 h of culture. Results are from one experiment representative of three performed and are expressed as means ± 1 SD. *, $p < 0.05$

The influence of EtOH treatment on the capacity of hepatic and splenic DC to produce IL-12p70, a potent inducer of T cell proliferation, in response to CpG stimulation was evaluated. Hepatic and splenic DC were bead-purified, stimulated overnight with CpG and culture

supernatants assessed for IL-12p70 production, as shown in Figure 20A. Both EtOH-exposed hepatic and splenic DC produced significantly more IL-12p70 compared to control DC (Figure 20A). Interestingly, similar to BMpDC, production of IFNα in response to CpG-A stimulation was unaffected in both hepatic and splenic DC (Figure 20B). It should be noted that bulk hepatic DC, which have a higher percentage of pDC (69-71), produce more IFN-α compared to bulk splenic DC.

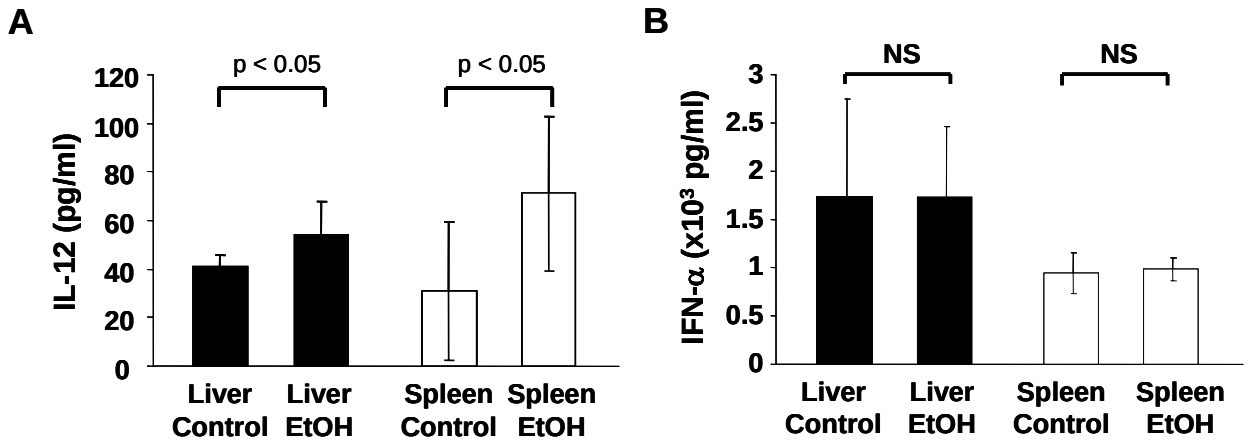


Figure 20. (A) IL-12p70 production by hepatic and splenic DC is increased with prolonged EtOH consumption but (B) IFN-α production is unaffected when DC are stimulated with CpG.
Bulk, bead-purified $CD11c^+$ hepatic and splenic DC were isolated from mice given EtOH or water alone for 8-10 w and Flt3L (10 μg/day, i.p.) for 10 days prior to sacrifice. DC were stimulated for 16 h with (A) CpG-B (2 μg/ml) or (B) CpG-A (2 μg/mL) and supernatants collected for measurement of (A) IL-12 and (B) IFNα production by ELISA. Results are from three experiments and are expressed as means ± 1 SD. *, $p < 0.05$.

We measured IDO protein levels by western blot and found that EtOH-treated, unstimulated or CpG-stimulated spleen DC and liver DC (50 mM) exhibited unimpaired IDO production (Figure 21). Thus, this enzyme is unlikely to play a role in the observed impaired T cell allostimulatory capacity of EtOH-treated BMDC.

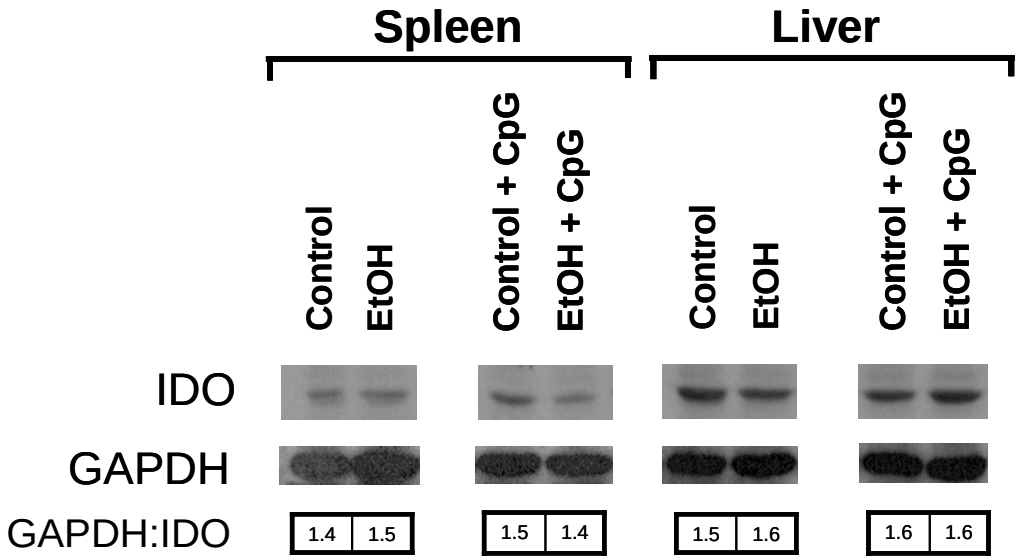


Figure 21. IDO production by hepatic or splenic DC is unchanged by EtOH exposure in vitro or in vivo, respectively.
Bulk, bead-purified $CD11c^+$ hepatic and splenic DC were isolated from mice given EtOH or water alone for 8 w, Flt3L (10 μg/day, i.p.) for 10 days and CpG (100 μg, i.v.), 16 h prior to sacrifice. Western blot analysis was performed for expression of IDO with GAPDH used as the loading control. Densitometric analysis was performed using Scion Image and a ratio of GAPDH to IDO density was computed and presented. Results are from one experiment representative of three performed.

3.3.5 Hepatic, EtOH-exposed DC show increased ability while splenic, EtOH-exposed DC show reduced ability to prime allogeneic T cells in vivo compared to control DC

Next, we examined the in vivo T cell priming abilities of control and EtOH-treated hepatic and splenic DC (bead-purified to >90% purity, as determined by CD11c staining) by performing adoptive s.c. injection of 5 x 10^5 B10 DC into naïve, allogeneic BALB/c recipients. Six days later, the mice were killed, draining lymph nodes harvested and the total lymph node cells restimulated with donor alloantigen for 72h in MLR. As shown in Figure 22A, EtOH-exposed hepatic DC were significantly more efficient at priming naïve allogeneic T cells in vivo than control hepatic DC. In contrast, EtOH-exposed splenic DC were significantly less efficient at priming naïve allogeneic T cells compared to control splenic DC (Figure 22A). IL-10 and IFNγ released into the culture supernatants were also measured at 72h. Allogeneic T cells from both groups produced equivalent amounts of both IL-10 and IFNγ (Figure 22B). Thus, EtOH-treated

hepatic and splenic DC are differentially affected by EtOH-exposure on their capacity to induce naïve allogeneic T cell proliferation in vivo.

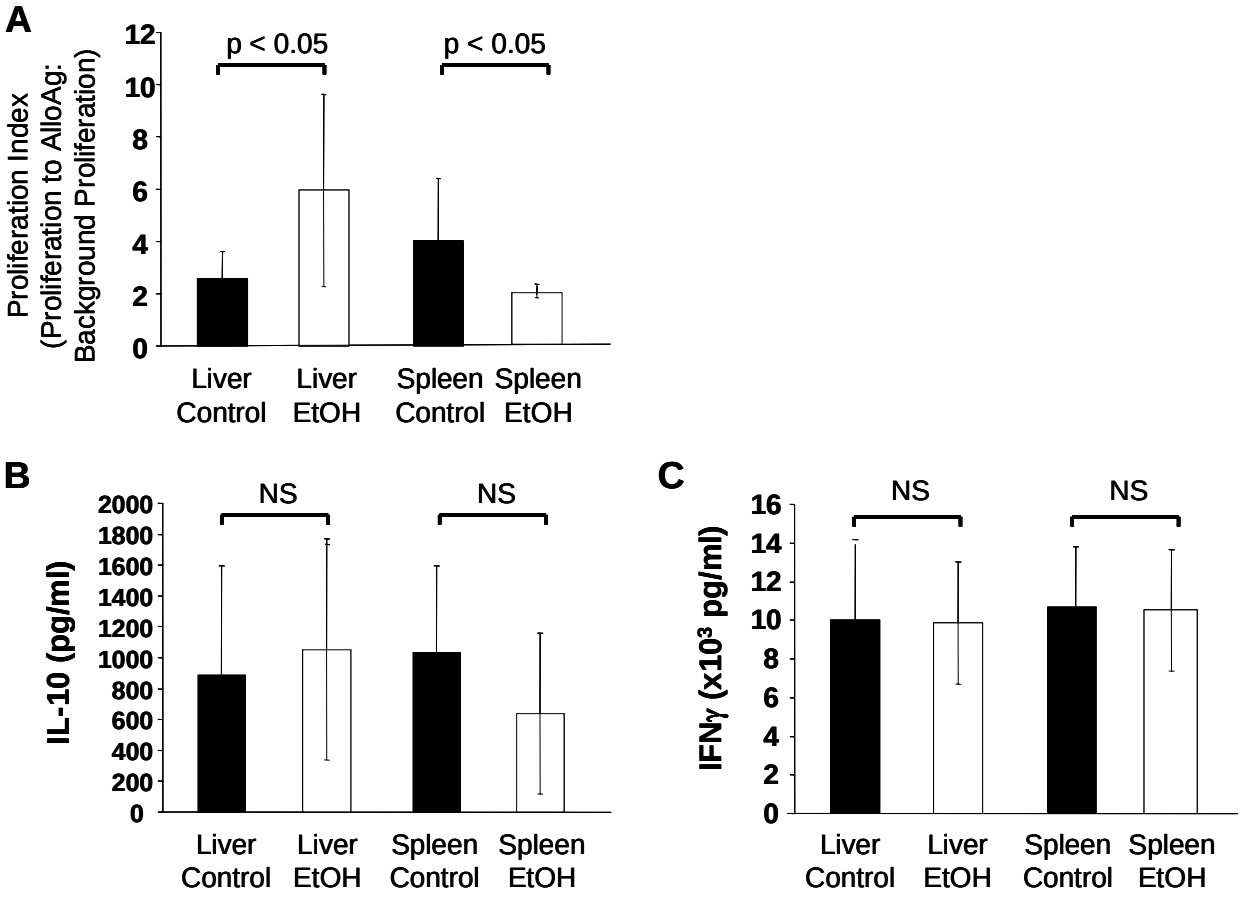


Figure 22. (A), T cell proliferative responses and (B), IL-10 and (C), IFNγ production in ex vivo MLR performed 6 days after s.c. injection of normal BALB/c recipients with bulk, bead-purified B6 hepatic or splenic CD11c$^+$ DC from control or EtOH-fed mice.
Two x 10^5 T cells were cultured (1:1) with donor cells [bulk, irradiated (20 Gy) B6 splenocytes] for 72 h, as described in the Materials and Methods. (A), [^{3}H]TdR was added for the final 16 h of culture. Results show the ratio of proliferation in response to donor alloantigen to background proliferation of unstimulated LN T cells and are from one experiment representative of five performed. (B), supernatants from 72h co-cultures were analyzed for IL-10 and IFNγ by ELISA. Results are from four experiments. *, $p < 0.05$; NS, not significant.

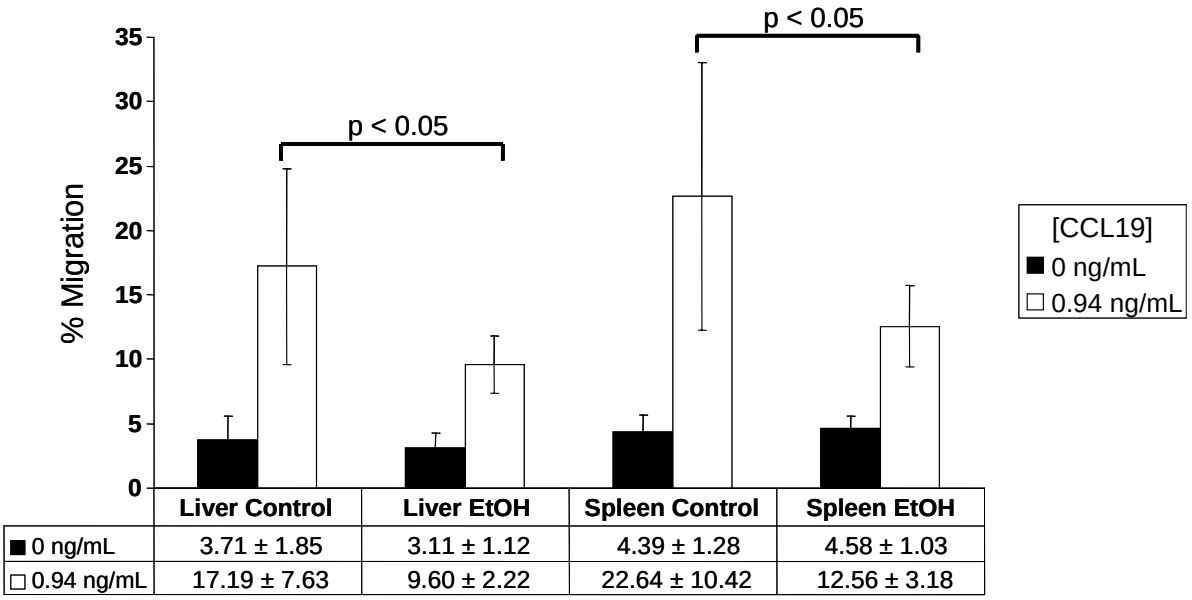
3.3.6 Migration of hepatic and splenic, control and EtOH-exposed DC

3.3.6.1 In vitro migration of CpG-B-stimulated, hepatic and splenic, control and EtOH-exposed DC to MIP3β

Altered chemotactic responses by DC can affect their migration to secondary lymphoid tissue, and subsequently the manner by which T cells are activated. Thus, hepatic and splenic

DC were evaluated for the effects of prolonged EtOH exposure on the ability of cells to migrate to CCL19/MIP-3β, a ligand of CCR7, in vitro.

Previous reports from our lab that show that immature, freshly-isolated, hepatic (75) and splenic (119) DC do not migrate in vitro, precluding the need for in vitro chemotaxis assays to be run for immature DC. Thus, hepatic and splenic DC from control and EtOH-fed mice were matured overnight with CpG-B and then evaluated for their migratory capacity to CCL19 (Figure 23). Previous data from our group have shown that a concentration of 0.94 ng/mL CCL19 is sensitive for detecting differences in migration, while concentrations of 94 μg/mL CCL19 and higher are on the plateau or generate maximal migration responses (119). Thus, two concentrations of CCL19 were assessed - 0.94 ng/mL (Figure 23) and 0.94 μg/mL (as a control to show dose response, data not shown). 2 x 10^5 DC were placed in Transwell® chambers over these two concentrations of CCL19 and after 2 h incubation, the number of DC that migrated from the Transwell® chamber were enumerated. Interestingly, at the lower concentration of CC19, both hepatic and splenic DC from EtOH-fed mice exhibited significantly inhibited migratory response to the chemokine compared to control hepatic and splenic DC (Figure 23). In fact, neither the hepatic nor splenic EtOH-exposed DC displayed chemotaxis to CCL19 (≥ 10% migration over control with no chemokine). However, at 0.94 μg/mL CCL19, this significant reduction was abrogated (data not shown).



	Liver Control	Liver EtOH	Spleen Control	Spleen EtOH
■ 0 ng/mL	3.71 ± 1.85	3.11 ± 1.12	4.39 ± 1.28	4.58 ± 1.03
□ 0.94 ng/mL	17.19 ± 7.63	9.60 ± 2.22	22.64 ± 10.42	12.56 ± 3.18

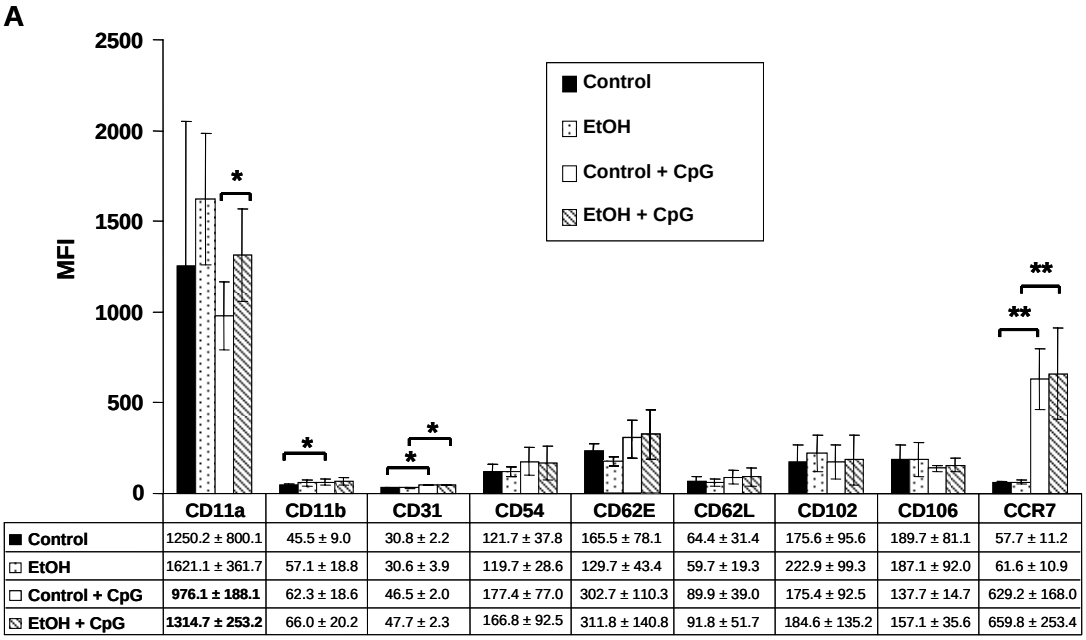
Figure 23. In vitro chemotaxis of hepatic and splenic, control and EtOH-exposed DC to CCL19.
B6 mice were fed EtOH for 8-24 w, as described in the Materials and Methods. Age-matched control mice received water without EtOH. Mice were treated with Flt3L (10 μg/day, i.p.) for 10 days then DC from livers or spleens isolated as described in Materials and Methods then stimulated for 16h with CpG-B (2 μg/mL). DC were placed in the upper wells of Transwell® chambers over CCL19. Positive chemotaxis was considered ≥10% migration over control without chemokines. Bar charts and data table below show % migration to CCL19 expressed as means ± 1 SD. Results are from three experiments; each chemokine dilution was tested in duplicate. *, $p<0.05$.

3.3.6.2 Phenotypic analysis of adhesion molecule and CCR7 expression on freshly-isolated and CpG-B-stimulated, hepatic and splenic, control and EtOH-exposed DC

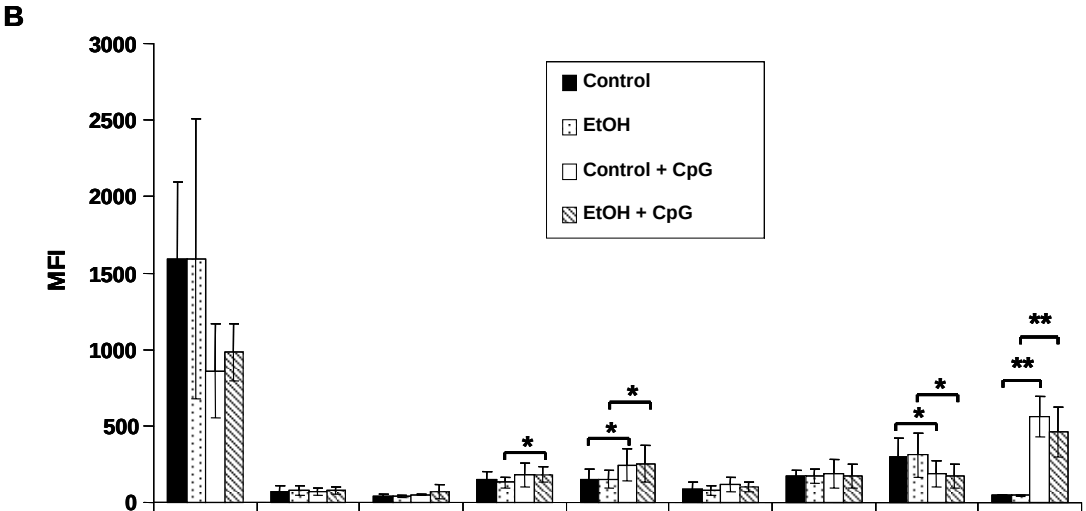
Hepatic and splenic DC were also evaluated for the effects of prolonged EtOH exposure on the expression of various adhesion molecules. Changes in adhesion molecule expression can affect DC migration and subsequently, the ability of DC to prime naïve T cells. Freshly-isolated, immature, hepatic and splenic DC were purified and analyzed by flow cytometry for the expression of surface adhesion molecules (CD11a, CD11b, CD31, CD54, CD62E, CD62L, CD102, and CD106) as well as CCR7 (Figure 24). Freshly-isolated hepatic or splenic DC exhibited low to moderate levels of CD31, CD54, CD62E, CD62L, CD102, and CD106, as expected (Figure 24). Although expression of CD11b (hepatic control only), CD31 (hepatic control and EtOH-exposed), CD54 (splenic EtOH-exposed only), and CD62E (splenic control and EtOH-exposed) were increased with CpG-stimulation (all $p < 0.05$), levels of these adhesion molecules remained low to moderate (Figure 24). Only one adhesion molecule, CD106, was

significantly reduced ($p < 0.05$) upon CpG-stimulation and this was for both control and EtOH-exposed splenic DC.

CD11a was highly expressed on both hepatic and splenic DC that was reduced (not significantly) upon CpG-B stimulation. With CpG-stimulated, hepatic DC, EtOH exposure resulted in significantly higher expression of CD11a compared to control, hepatic DC (Figure 24). Expression of CCR7 was low on freshly-isolated, immature hepatic and splenic DC, and increased significantly with CpG stimulation ($p<0.005$) (Figure 24). However, EtOH-exposure did not affect CCR7 expression. Although there was no difference in the expression of CCR7 on hepatic or splenic DC, as determined by FACS, surface expression was not necessarily reflective of the functional status of receptors. These results suggest that EtOH has limited effects on adhesion molecule expression. In contrast to coregulatory molecules (Figure 18), the effects of EtOH exposure appear to affect CD11a expression on hepatic DC significantly with no significant effects on splenic DC.



	CD11a	CD11b	CD31	CD54	CD62E	CD62L	CD102	CD106	CCR7
Control	1250.2 ± 800.1	45.5 ± 9.0	30.8 ± 2.2	121.7 ± 37.8	165.5 ± 78.1	64.4 ± 31.4	175.6 ± 95.6	189.7 ± 81.1	57.7 ± 11.2
EtOH	1621.1 ± 361.7	57.1 ± 18.8	30.6 ± 3.9	119.7 ± 28.6	129.7 ± 43.4	59.7 ± 19.3	222.9 ± 99.3	187.1 ± 92.0	61.6 ± 10.9
Control + CpG	**976.1 ± 188.1**	62.3 ± 18.6	46.5 ± 2.0	177.4 ± 77.0	302.7 ± 110.3	89.9 ± 39.0	175.4 ± 92.5	137.7 ± 14.7	629.2 ± 168.0
EtOH + CpG	**1314.7 ± 253.2**	66.0 ± 20.2	47.7 ± 2.3	166.8 ± 92.5	311.8 ± 140.8	91.8 ± 51.7	184.6 ± 135.2	157.1 ± 35.6	659.8 ± 253.4



	CD11a	CD11b	CD31	CD54	CD62E	CD62L	CD102	CD106	CCR7
Control	1590.1 ± 503.3	71.5 ± 34.5	40.4 ± 11.7	151.9 ± 49.2	148.9 ± 66.8	87.7 ± 42.6	173.5 ± 40.1	298.7 ± 124.0	46.5 ± 1.3
EtOH	1591.9 ± 914.2	77.1 ± 28.2	42.0 ± 7.6	130.6 ± 36.1	149.5 ± 59.3	79.6 ± 29.2	173.6 ± 45.6	311.1 ± 144.4	45.1 ± 3.0
Control + CpG	859.6 ± 306.0	73.1 ± 23.5	48.9 ± 2.0	179.2 ± 76.9	245.4 ± 103.9	115.9 ± 46.3	184.5 ± 92.7	184.6 ± 84.5	563.1 ± 133.0
EtOH + CpG	983.2 ± 188.5	77.0 ± 20.7	71.1 ± 44.9	182.6 ± 49.8	252.8 ± 120.8	103.6 ± 31.1	170.6 ± 75.5	169.5 ± 78.4	459.3 ± 166.8

Figure 24. (A) Hepatic or (B) splenic DC show no differences in expression of various adhesion molecules or CCR7 when freshly isolated or after CpG stimulation, except for EtOH-exposed, CpG-B-stimulated liver DC (A) that express more CD11a.

B6 mice were fed EtOH for 8-24 w, as described in the Materials and Methods. Age-matched control mice received water without EtOH. Mice were treated with Flt3L (10 μg/day, i.p.) for 10 days, then DC from livers or spleens isolated as described in Materials and Methods. Freshly-isolated DC were either analyzed by FACS or stimulated for 16h with CpG-B (2 μg/mL) for 16 h then analyzed by FACS analysis. DC were labeled with anti-CD11c (FITC- or PE-conjugated) and either anti-CD11a, -CD11b, -CD31, -CD54, CD62E, -CD62L, -CD102, -CD106 (biotin-conjugated and revealed with streptavidin-FITC or FITC-conjugated) or –CCR7 (PE-conjugated). Cells were gated on $CD11c^+$ cells and expression of adhesion molecules or CCR7 analyzed. Bar charts show MFI for the molecules indicated and are expressed as mean ± 1 SD. The table below shows means ± 1 SD. Results are from four-nine experiments. *, $p<0.05$; **, $p<0.005$. In the table below, bolded text is $p<0.05$.

3.3.6.3 In vivo migration of freshly-isolated (immature) and CpG-B-stimulated (mature), hepatic and splenic, control and EtOH-exposed DC

Given the results showing the ability of freshly-isolated, immature, control and EtOH-exposed, hepatic and splenic DC to prime naïve T cells in vivo (Figure 22) as well as the CD11a and CCR7 cell surface expression data (Figure 24), we first examined the migration of these immature hepatic and splenic DC to DLN after s.c. injection by rare-event, flow cytometric analysis. Our group has reported previously (68) that allogeneic mature, liver-derived $CD8\alpha^+$ DC; mature, splenic $CD8\alpha^+$ DC, and immature and mature splenic $CD8\alpha^-$ DC (119) migrate to the DLN of recipients within 24 h of sc injection. Liver and spleen DC from control and EtOH-fed mice were freshly-isolated, labeled with CFSE, then injected s.c. into the hind footpads of allogeneic BALB/c mice. 18-24 h later, popliteal DLN were removed, and total LN cells analyzed rare-event, flow cytometric analysis for percent positive green fluorescent cells. Interestingly, significantly more hepatic DC from EtOH-fed mice migrated to DLN compared to control hepatic DC (Figure 25A). However, these data correlate with adoptive transfer data that shows that hepatic DC from EtOH-exposed mice have increased capacity to induce naïve T cell proliferation in vivo (Figure 22A). If hepatic, EtOH-exposed DC have a greater capacity to migrate to DLN, it may explain the increased T cell activation seen with these DC. In contrast, splenic control or EtOH-exposed DC showed no difference in migration (Figure 25A). Thus, in the case of splenic DC, the reduced capacity of splenic, EtOH-exposed DC to induce naïve T cell proliferation compared to control DC (Figure 22A) likely results from the immature phenotypic status of splenic, EtOH-exposed DC (Figure 18D, E), as seen in in vitro MLR (Figure 19), rather than increased DC numbers reaching T cell areas in the secondary lymphoid tissue.

Next, we examined the in vivo migration of CpG-matured hepatic and splenic DC from control and EtOH-exposed mice to DLN. Freshly-isolated DC from livers and spleens were stimulated with CpG for 16-18 h, labeled with CFSE, then injected and analyzed 24 h later as described above. Again, as with immature DC, significantly more hepatic DC from EtOH-exposed mice migrated to DLN compared to control hepatic DC (Figure 25B). It should be noted that with maturation, more hepatic DC, control and EtOH-exposed, migrated to DLN compared to immature DC (Figure 25), likely due to upregulated CCR7 on mature DC (Figure 24A). Both immature and CpG-matured splenic DC from control and EtOH-exposed mice

showed no difference in migration. In concord with the adhesion molecule expression data (Figure 24), EtOH exposure appears to significantly affect the in vivo migratory capacity of hepatic DC with no significant effects on splenic DC.

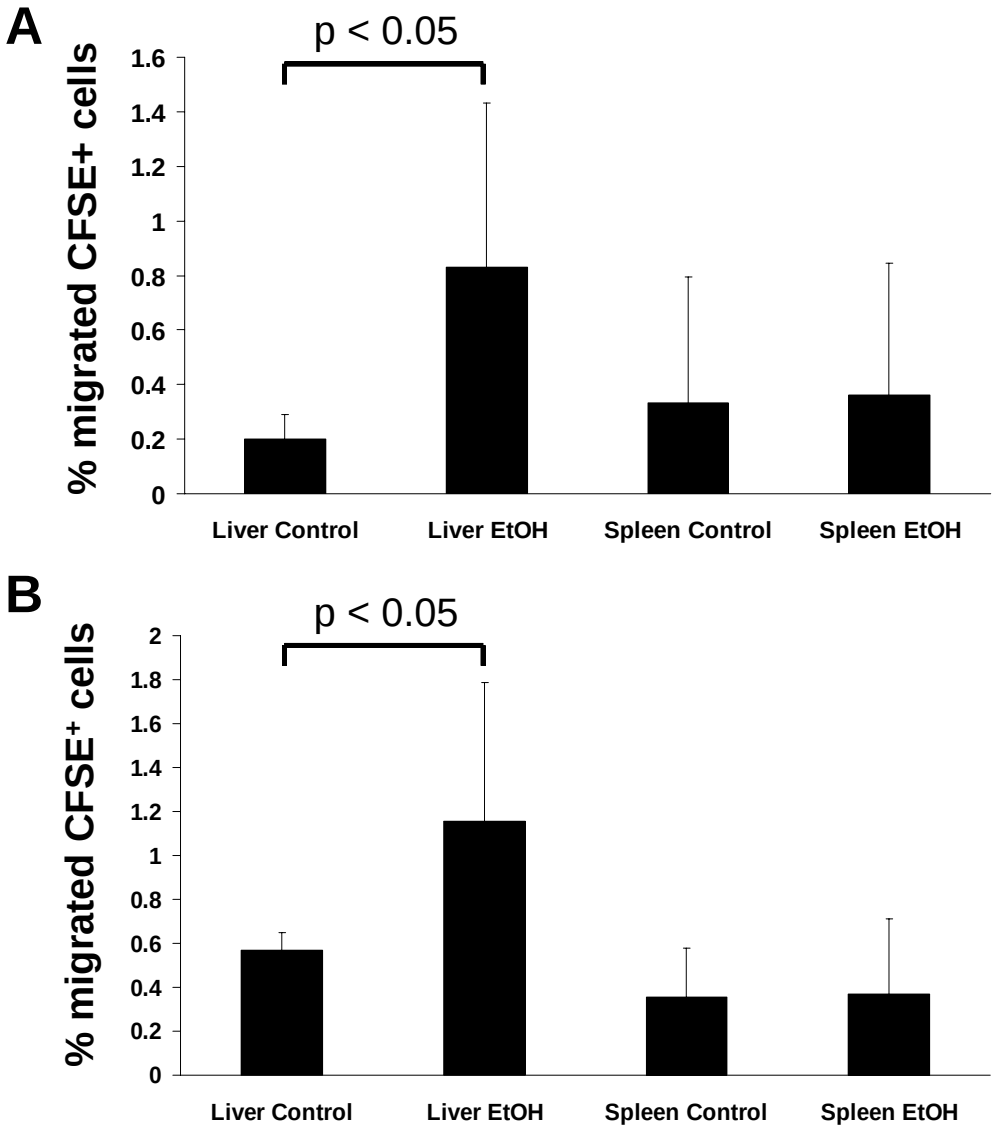


Figure 25. In vivo migration of (A) immature or (B) mature hepatic or splenic DC from control or EtOH-fed mice.

Rare-event FACS analysis was performed on LN cells from DLN isolated 18-24 h after s.c. injection of normal BALB/c recipients with 3-5 x 10^6 bulk, bead-purified, CFSE-labeled B6 $CD11c^+$ DC, isolated from livers or spleens from control or EtOH-fed mice. (A) Immature, freshly-isolated DC; or (B) CpG-stimulated (16 h) DC were analyzed by rare event, FACS analysis for the percentage of green fluorescent cells. The number of DC migrating to DLN in vivo was determined by the following equation: (% green fluorescent cells in DLN x Total number of cells recovered from DLN)/(Number of DC injected s.c.) Results are from three to six experiments. *, $p < 0.05$.

3.4 DISCUSSION

In both humans and animal models, chronic EtOH consumption can adversely affect the immune system and its function (315, 189, 256). With excessive EtOH ingestion, there are reduced T-cell proliferative responses, and impaired delayed–type hypersensitivity reactions (297-299, 261, 300), as well as an increased incidence of infectious diseases, including tuberculosis and hepatitis C viral infection (189-191). Furthermore, prolonged EtOH exposure decreases the Ag-presenting capacity of human monocytes and monocyte-derived DC (222, 220). However, the mechanisms underlying the immune-compromised state of alcoholics have not been fully elucidated. In this study, we have utilized an established murine chronic alcohol consumption model, the Meadows-Cook model, to examine the effects of chronic EtOH administration on the phenotype and function of hepatic and splenic DC. Prolonged EtOH consumption appears to exert a more marked inhibitory effect on splenic compared to hepatic DC subsets, as assessed by their phenotypic and functional characteristics. However, hepatic DC from EtOH-fed mice appear to have increased migratory capacity in vivo, concomitant with their increased CD11a surface expression compared to control hepatic DC.

As discussed in Chapter 1 (1.3) and Chapter 2 (2.4), EtOH exerts complex effects on the immune system that are likely reflect many variables, including the duration (acute vs. chronic) and extent of exposure (EtOH concentration), as well as the influence of local or systemic factors, including cytokines or other immune-modulating agents. Szabo *et al.* (221, 222) have shown that EtOH inhibits the differentiation and full maturation of human mDC, leading to decreased T cell stimulatory capacity. In Chapter 2, we report in vitro-generated murine BM-derived mDC and pDC exposed to EtOH show inhibited differentiation, with a greater effect on pDC, compared to mDC, development (Figure 4). Hepatic and splenic DC subsets recovered from EtOH-fed, Flt3L-mobilized mice showed no significant reductions in DC numbers compared to Flt3L-treated controls (Figure 16). The apparent discrepancy between the EtOH-mediated reduction in absolute DC numbers in response to Flt3L stimulation in vitro and in vivo may reflect differences in EtOH concentration and metabolism between the different test systems.

Currently, there are no reports that define the influence of long-term in vivo EtOH administration on DC phenotype and function. Cook *et al*. (213) have updated a murine model

of chronic EtOH consumption (211) and examined splenic T cells. They found that T cells were more activated, both in phenotype and function, similar to what is found in human chronic alcoholics. The same group also reported (281) that splenic $CD11b^+$ cells recovered from EtOH-fed mice and cultured overnight (a procedure that enhances DC maturation) appeared to be activated, as defined by increased CD80 and CD86 expression. In the present study, we found that freshly-isolated, resting hepatic DC and splenic DC exhibited different phenotypes (liver DC were more immature) and that EtOH consumption reduced splenic DC costimulatory molecule expression, while hepatic DC (that constitutively express lower levels of these molecules) were not noticeably affected. The inhibitory effect of chronic EtOH consumption on splenic DC maturation in response to CpG stimulation was confirmed by in vitro MLR assays and by analysis of host T cell responses following adoptive transfer of EtOH-exposed DC. These data are consistent with those previous reports concerning chronic EtOH consumption and splenic macrophage function. Whereas in the present study we examined the expression of costimulatory molecules on splenic DC activated in vivo, Cook *et al*. (281) cultured splenic $CD11b^+$ cells overnight, in the absence of EtOH, to stimulate upregulation of costimulatory molecules. Conceivably, the manner by which the APC were stimulated may affect the upregulation of costimulatory molecules.

Hepatic DC have been shown to be refractory to stimulation with LPS or pro-inflammatory cytokines (144, 79). This inherent, comparative unresponsiveness may have evolved in response to a need for tolerance to orally-derived Ag and microbial products that are delivered continuously from the gut (1). The comparative inability of liver DC to elicit strong T cell responses may contribute to the persistence of hepatic viral infections and to cancer in the liver (both primary and metastatic) as well as to the tolerogenicity of liver allografts. Further, recent studies of murine liver DC (70, 71) have indicated a higher relative proportion of pDC (versus mDC) in comparison to the spleen. As pDC are being widely investigated for their role in innate and adaptive immunity (282, 27, 39), as well as in immune regulation and tolerance (35, 282), their comparative prominence in the liver may have a significant impact on immunological events within the liver microenvironment. CpG-activated liver DC were not affected significantly in regards to phenotypic maturation and in vitro T cell stimulatory capacity by prolonged EtOH consumption, and we hypothesize that the unique cytokine microenvironment of the liver, the liver's ability to metabolize EtOH, as well as the comparative

resistance of liver DC to maturation, may partially spare these cells from the inhibitory effects of prolonged EtOH exposure.

However, in apparent contrast to the phenotypic maturation and in vitro T cell stimulatory capacity data, when naïve mice were primed with hepatic DC from chronic EtOH-fed mice, these DC were found to have greater Ag-specific T cell proliferation compared to control hepatic DC (Figure 22A). Evaluation of DC migration to DLN showed that hepatic DC from chronic EtOH-fed mice migrated in greater numbers compared to control (Figure 25A). Further, when surface expression of various adhesion molecules were evaluated on DC, CD11a was found to be more highly expressed on EtOH-exposed hepatic DC compared to control (Figure 24A). These data are in agreement with a study that showed that LFA-1 was increased on Jurkat T cells when exposed to acute low concentrations of EtOH and with enhanced adhesion between these EtOH-exposed Jurkat cells and their target cells (Raji cells), even in the absence of specific Ag (316). It can be speculated that the higher expression of CD11a, important for leukocyte adhesion to endothelial cells and thus, subsequently, extravasation, on EtOH-exposed hepatic DC may contribute to its greater migration to secondary lymphoid tissue.

It should be noted, however, that although these in vivo migration studies show increased migration of hepatic DC from EtOH-fed mice, this model of migration is just that – a model. These data may not mimic what actually happens to hepatic DC in vivo in their normal environment. In the liver there are many other factors present, including, but not limited to, the cytokine microenvironment. The increased CD11a in the context of the liver may actually prevent hepatic DC from migrating out of the liver, even when stimulated by TLR9 in vivo. In fact, it has been shown in both mouse and rat studies of chronic EtOH administration that surface expression of CD54 (ICAM-1), the ligand for CD11a/CD18 (LFA-1), is significantly increased on hepatocytes of EtOH-fed animals compared to pair-fed controls (267, 317). Further, when a blocking Ab to CD18 was added to in vitro cultures of polymorphonuclear neutrophils (PMN) and hepatocytes from EtOH-fed rats, AST release was reduced significantly compared to cultures that did not receive the Ab (267). When the blocking Ab was used in chronic EtOH-fed rats in vivo, serum AST levels were significantly reduced compared to rats receiving control Ig Ab (267). Bautista (267) suggests that these observations support the hypothesis that adhesion molecules, in this case CD18 and CD54, are involved in chronic alcohol-induced hepatic injury (increased AST) by enhancing the interaction between leukocytes and hepatocytes. Further, in

vitro data show that hepatic DC from EtOH-fed mice have significantly reduced migration to CCL19 compared to control hepatic DC (Figure 23). It can be speculated that reduced chemotaxis to CCR7 ligands, combined with increased CD54 on hepatocytes and increased LFA-1 on DC, may all contribute to reduced migration of hepatic DC from the liver in chronic EtOH drinkers. This may be one mechanism by which alcoholics are particularly susceptible to infections and viral hepatitis since the ability of the DC to reach draining secondary lymphoid tissue and present Ag to T cells may be impaired.

When splenic DC from control and EtOH-fed mice were used to prime naïve mice, splenic DC from EtOH-fed mice were found to induce reduced Ag-specific T cell proliferation compared to controls (Figure 22A), similar to findings with EtOH-exposed BMDC (Figure 11A). However, the increased IL-10 production by T cells primed in vivo with EtOH-exposed BMDC (Figure 11B) was not reproduced when splenic DC from chronic EtOH-fed mice were used (Figure 22B). IFNγ production by these T cells was also no different between EtOH and control groups. Migration of splenic DC in vivo was also analyzed and showed no difference between control and EtOH-exposed groups (Figure 25). Thus, the reduced ability of splenic DC from EtOH-fed mice to prime naïve T cells in vivo is likely a result of the reduced costimulatory molecule expression and subsequent inhibited stimulatory capacity. The discrepancy between the influences of in vitro- and in vivo-derived DC on T cell priming may result from inherent differences in DC phenotype and function, as well as EtOH concentration and metabolism between the different test systems.

In summary, our studies show that the long-term, in vivo EtOH administration exerts differential effects on liver versus spleen DC subsets. Hepatic DC, that are inherently immature, weakly immunogenic and comparatively resistant to maturation, appear resistant to the inhibitory effects of EtOH on functional maturation and T cell stimulatory capacity. However, the in vivo migratory capacity of hepatic DC from EtOH-fed mice is concomitantly increased with expression of CD11a, in comparison to control hepatic DC. In contrast, secondary lymphoid organ (spleen) DC from mice chronically fed EtOH exhibit impaired functional maturation and function in response to CpG stimulation, with no overall changes in migration or adhesion molecule expression. Together, these data suggest that altered maturation, function, and migration of hepatic and splenic DC, resulting from prolonged EtOH exposure, may be involved in the compromised immune function of alcoholics.

4.0 IMMUNE REACTIVITY IN CHRONIC ETHANOL-CONSUMING MICE

4.1 INTRODUCTION

As discussed in the Introduction (1.3.1), the Lieber-DeCarli (or equivalent) and Meadows-Cook murine models of chronic EtOH consumption, while similar in certain aspects, such as observed pathological changes, have also been shown to exhibit differences, such as mortality rate and overall stress induction. There have been limited studies to date using the Meadows-Cook murine model of chronic EtOH consumption.

There is clinical evidence that chronic EtOH consumption compromises normal host defenses by impairing or altering immune responses (189, 318, 256). Cell-mediated and humoral immunity have been examined in chronic alcoholics. In alcoholic patients, delayed-type hypersensitivity (DTH) responses are impaired (319, 320, 297) and serum Ig (particularly IgG and IgA) levels elevated (321). In mice fed the LD diet for 10-11 days, impaired DTH has been reported (299, 261). Further, in ovalbumin (OVA) T cell receptor (TcR) transgenic (Tg) mice specific for I-A^d-restricted 323-339 OVA peptide (MHC class II restricted; OT-II), it was shown that EtOH significantly inhibited Ag-specific DTH responses (216).

As discussed in 1.3.3, it is theorized that there is Th1/Th2 skewing towards a decrease in Th1 and increase in Th2 responses with chronic EtOH administration. However, the data currently reported are still inconclusive. Further work must be done to fully elucidate the effects of chronic EtOH consumption on cell-mediated immunity. Whereas T cell-dependent responses appear to be inhibited by chronic EtOH feeding (299, 261), other functional effects, i.e. Th1/Th2 skewing, have yet to be determined.

The study of B cell responses and the effects of chronic EtOH has been difficult. Although chronic alcoholics appear to exhibit impaired B cell function, as evidenced by increased circulating serum Ig (321), studies in this patient population have shown that they have

intact T cell-independent antibody (Ab) response to pneumococcal polysaccharide vaccination (322). In vitro studies show different results from those seen in alcoholics, with inhibited Ab production by B cells (323). In the mouse model, it has been shown that alcohol either enhances or does not alter Ab production (262). Thus, the seemingly conflicting data indicate complex effects of chronic EtOH exposure on B cell functions.

A few studies have examined the effects of chronic EtOH consumption on, presumably, the function of CD8 T cells. It has been hypothesized (324) that chronic EtOH sensitizes the liver, by an unknown mechanism, such that activated $CD8^+$ T cells migrating to the liver induce hepatic injury rather than dying by apoptosis, as they normally do (325). Jerrells' group has utilized murine models of *Listeria monocytogenes* and murine cytomegalovirus (MCMV) infection, which can induce liver damage but that can be controlled/cleared in normal mice (324), to study the effects of EtOH consumption on $CD8^+$ T cell function (326, 327). Control of infection to both pathogens requires $CD8^+$ T cells (328, 329). When mice were infected with *L. monocytogenes*, those that consumed EtOH were more susceptible to infection and had greater hepatic injury (326). Further, if immune mice (control mice that had cleared a previous infection) were then fed an EtOH diet and then reinfected, these animals were unable to control the growth of the bacteria (326). Simlarly, when mice were infected with a sub-lethal dose of MCMV to induce hepatitis, EtOH-fed animals were unable to clear the viral infection, unlike the self-limiting hepatitis infection in control mice (327). While neither study has shown definitely that $CD8^+$ T cells mediate the impaired immune responses to either infection, the important role of $CD8^+$ T cells in the clearance of both pathogens suggests an impairing effect of EtOH on $CD8^+$ T cells is likely to be involved at least to some extent.

No reports to date have investigated cell-mediated and humoral responses in the Meadows-Cook model of chronic EtOH consumption. In chapters 3 and 4, we have examined the effects of chronic EtOH exposure on DC function. In this chapter, we evaluate cell-mediated and humoral responses in the Meadows-Cook murine model by examining Ag-specific direct cell killing, CD4 and CD8 T cell responses and serum Ig production.

4.2 MATERIALS AND METHODS

4.2.1 In vivo EtOH administration

B6 mice were separated randomly into control and EtOH-fed groups and fed an EtOH diet as described in Chapter Three (3.2.1, 3.2.2) except that mice were maintained on EtOH for 12-24 weeks (212). No adverse effects of this EtOH feeding protocol on behavior or weight gain compared with control mice were observed.

4.2.2 Immunization of mice with OVA

As described previously (330), Biomag beads (iron oxide; Polysciences, Inc., Warrington, PA) were covalently linked to OVA protein (Sigma-Aldrich) (OVA-Fe) according to the manufacturer's instructions. Naive control and EtOH-fed mice were immunized with OVA-Fe or PBS by bilateral footpad (25 μg) and haunch (75 μg) s.c. injections on day 0 and boosted in the same manner at day 7. In vivo killing assays were run on day 11.

4.2.3 In vivo killing assay

In vivo Ag-specific lytic activity was measured by an in vivo killing assay, as previously described (331, 6, 332). Briefly, mouse splenocytes were collected and pulsed with SIINFEKL (250 ng/ml; Sigma Chemical Co.) and labeled using a high concentration of CFSE (5 μM; $CFSE^{high}$). Mouse splenocytes without SIINFEKL peptide were labeled using a low concentration of CFSE (0.5 μM; $CFSE^{low}$) as an internal control. Ten x 10^6 cells of each population were mixed and injected into mice i.v. via the lateral tail vein. The relative quantity of $CFSE^{high}$ and $CFSE^{low}$ cells in DLN (popliteal and inguinal) was determined by flow cytometry 5 h after injection. Specific lysis was calculated according to the following formula: {1-[(ratio of $CFSE^{low}/CFSE^{high}$ of naive mouse)]/[(ratio of $CFSE^{low}/CFSE^{high}$ of vaccinated mouse)] X 100.

4.2.4 Enzyme-linked immunosorbent spot (ELISPOT)

Spleens from control and OVA-Fe immunized mice were collected at the same time as DLN. $CD4^+$ and $CD8^+$ T cells were enriched by incubation with anti-mouse-CD4- or anti-mouse-CD8-coated immunomagnetic beads (10 μl/10^7 cells; Miltenyi Biotec) for 15 min at 4°C, then positively selected by passage through a MACS column (Miltenyi Biotec), yielding a highly-enriched (>90%) $CD4^+$ and $CD8^+$ T cell population to use as responders in ELISPOT.

Splenic APC (depleted of $Thy1.2^+$ and $CD4^+$ cells) from naive C57BL/6 mice were loaded with OVA (1 mg/mL), SIINFEKL (10 μg/ml), or no Ag for 4 h, washed three times in PBS, and used as target cells for 48-h IFN-γ or IL-5 ELISPOT assays (both plates and Ab sets from BD PharMingen). For the ELISPOT assay, 4×10^4 APC per well and 2×10^5 of either $CD4^+$ or $CD8^+$ T cells (responders) per well were suspended in 200 μL of AIM V® medium (Invitrogen Corp., Carlsbad, CA). OVA-loaded APC were used with $CD4^+$ T cells and SIINFEKL-loaded APC were used with $CD8^+$ T cells. APC with no Ag loaded were used as negative controls for both $CD4^+$ and $CD8^+$ T cells. ELISPOT plates were incubated at 37°C for the indicated time and developed as described in the manufacturer's protocols.

4.2.5 Serum Ig Analysis by ELISA

Serum was collected from each mouse as described in 3.2.4 and analysed by ELISA for Ab titre. Ninety-six-well EIA/RIA [Corning Incorporated Life Sciences (Costar), Acton, MA] flat-bottom plates were coated with 50 μl/well of 100 μg/ml OVA protein in 0.1 M $NaHCO_3$ and incubated overnight at 4°C. Plates were washed twice in PBS supplemented with 0.05% Tween 20 (PBS-Tween), then blocked with 1% bovine serum albumin (w/v) in PBS at room temperature for 2 h. Plates were washed two times with PBS-Tween, then 100 μl of serially diluted serum samples, diluted in PBS supplemented with 10% (v/v) fetal bovine serum (FBS/PBS), were added to the plates. Serial dilutions of reference serum samples of known titer were also assayed on each plate. After samples and reference serum were added, plates were incubated overnight at 4°C. The plates were washed four times with PBS-Tween, and 100 μl of either biotinylated goat anti-mouse IgG_1 mAb (0.4 μg/mL; Caltag Laboratories, Burlingame, CA) or rat anti-mouse $IgG2_a$

mAb (1 μg/ml; BD PharMingen) in FBS/PBS was added to each well and incubated at room temperature for 45 min. Plates were washed five times with PBS-Tween, and 100 μl/well of avidin-conjugated horseradish peroxidase (BD PharMingen) at 1:1000 in FBS/PBS was added to each plate and incubated at room temperature for 30 min. The plates were washed a final five times with PBS-Tween, and 100 μl of substrate, 3,3′,5,5′-Tetramethylbenzidine (TMB) Substrate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) added to each well. Reactions were terminated by addition of 100 μl of TMB Stop solution (Kirkegaard & Perry Laboratories, Inc.) was added to each well. Color development was assessed at 405 nm. Titers of OVA-specific IgG1 and IgG2a were determined by calculating the dilution required to achieve an optical density reading of 0.2, and were expressed as the reciprocal of that dilution.

4.3 RESULTS

4.3.1 In vivo analysis of Ag-specific lytic activity between control and EtOH-fed mice

The function of $CD8^+$ T cells are of great importance in various diseases to which chronic alcoholics appear to be more susceptible, such as viral hepatitis and malignancy. Thus, we compared the in vivo Ag-specific killing capacity in control and chronic EtOH-fed mice. Ag-specific lytic activity was measured in vivo by targeted lysis of Ag-loaded APC in the periphery (Figure 26). Quantification of Ag-specific lysis of target cells demonstrated very similar lytic activity between control and EtOH-fed mice (46.7 ± 10.8% and 48.2 ± 15.0%, respectively) (Figure 26).

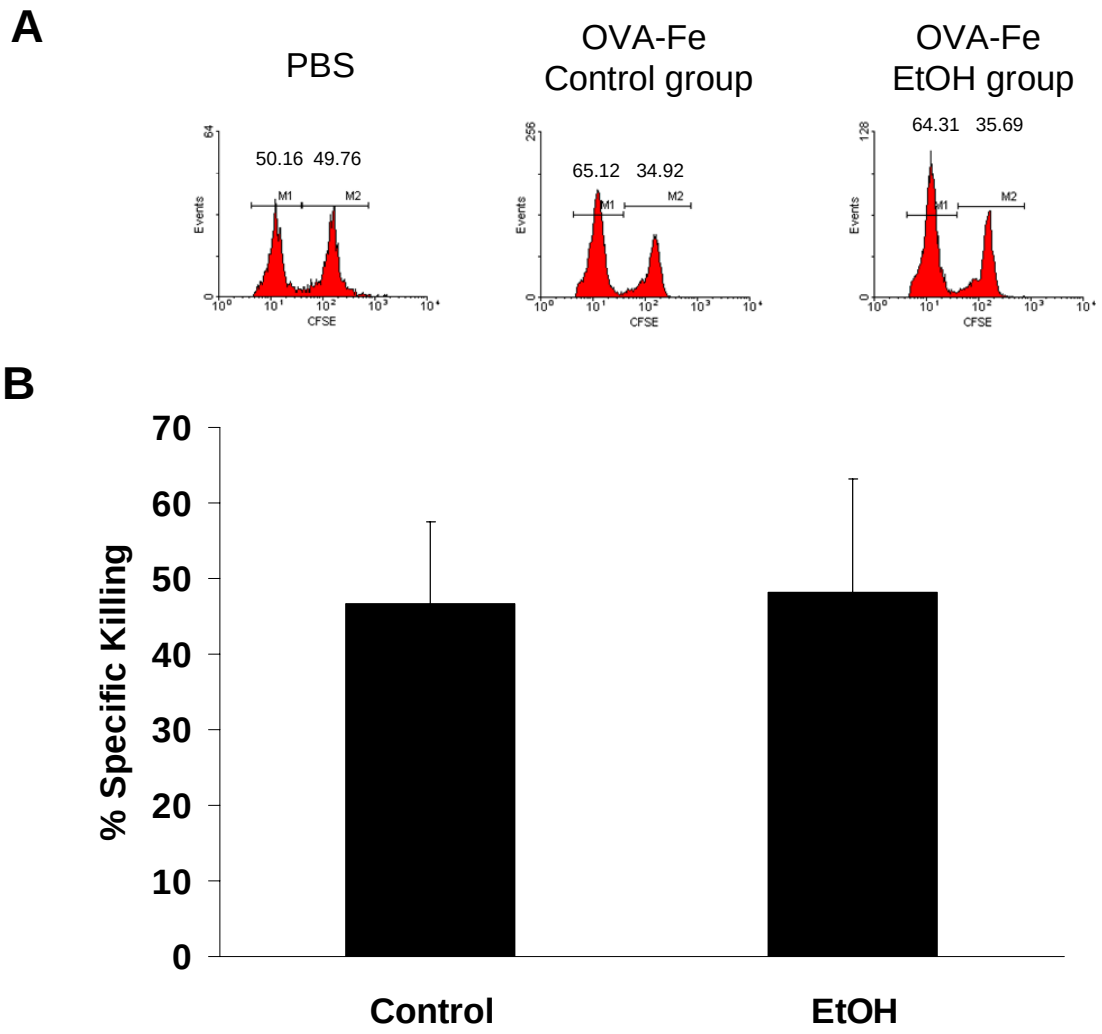


Figure 26. Control and chronic-EtOH fed mice exhibit equivalent in vivo Ag-specific lytic activity.
B6 mice were fed EtOH for 12-24 w, as described in the Materials and Methods. Age-matched control mice received water without EtOH. Mice were immunized with either OVA-Fe or PBS at day 0, boosted at day 7, then injected i.v. with CFSE-labeled, Ag-specific target cells on day 11. 5 h later, popliteal and inguinal LN were removed and assayed by FACS analysis for quantification of Ag-loaded ($CFSE^{high}$) and control (No Ag, $CFSE^{low}$) target cells. (A), Representative histograms of control, PBS (No OVA) immunized group which shows no difference in lysis of SIINFEKL-loaded target cells ($CFSE^{high}$) compared to control target cells ($CFSE^{low}$). In contrast, control-fed or EtOH-fed mice immunized with OVA-Fe display lysis of Ag-loaded target cells ($CFSE^{high}$) compared to control target cells($CFSE^{low}$). Results are from one experiment representative of nine experiments. (B), Average % Ag-specific killing of target cells for control, pair-fed mice and EtOH-fed mice. Results are mean ± 1 SD from nine experiments.

4.3.2 Ag-specfic cytokine production by $CD4^+$ and $CD8^+$ T cells from control and EtOH-fed mice

As discussed above, it has been speculated that chronic EtOH-fed mice exhibit Th1/Th2 skewing. We examined these mice for potential Th1/Th2 polarization by examining Ag-specific cytokine production of IFNγ and IL-5 by ELISPOT and ELISA. $CD4^+$ and $CD8^+$ splenic T cells from the immunized mice were used as responders to Ag-loaded APC in ELISPOT (Figure 27). T cells ($CD4^+$ and $CD8^+$) from control and EtOH-fed mice produced equal amounts of IFNγ in an Ag-specific manner (Figure 27). IL-5 production was extremely low in both groups; in fact, the quantity was too low to quantify (data not shown). Analysis of IFNγ production by ELISA showed that cytokine production was also very low, as expected, considering the ELISPOT data (data not shown). IL-5 production was not detectable by ELISA, also in agreement with ELISPOT data (data not shown). The ELISPOT assay itself was working, as positive control wells, containing T cells stimulated with phytohemagglutinin (PHA), in both IFNγ and IL-5 plates had too many positive cells to count. The lack of difference in IFNγ production between control and EtOH-fed groups indicates a lack of Th1/Th2 skewing in mice fed EtOH for 12-24 weeks.

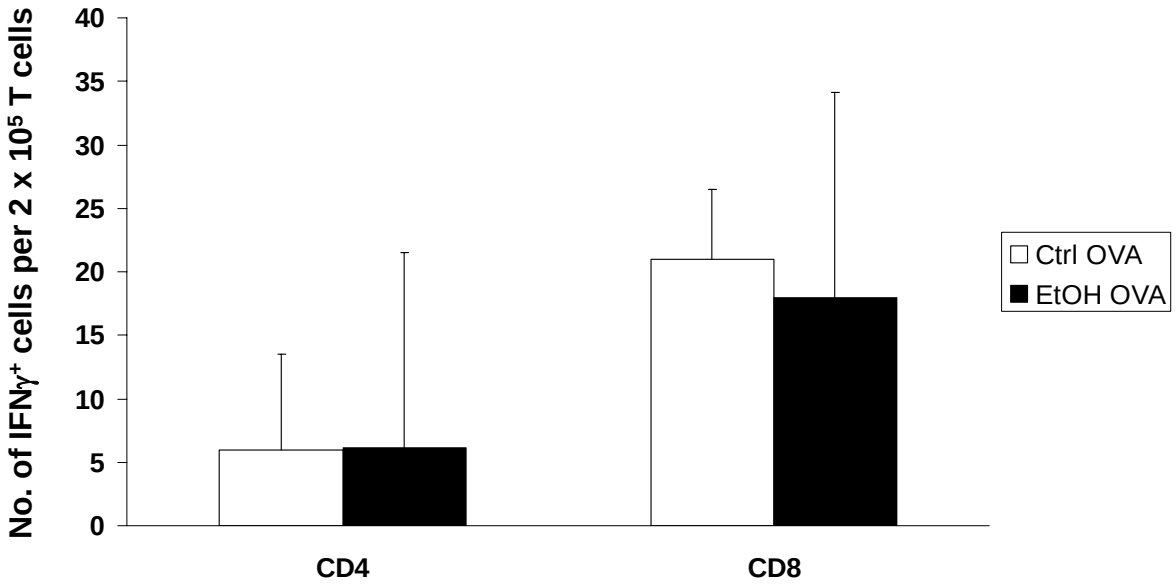


Figure 27. Ag-specific $CD4^+$ and $CD8^+$ T cells from control and EtOH-fed mice produce equivalent amounts of IFNγ.
B6 mice were fed EtOH for 12-24 w, as described in the Materials and Methods. Age-matched control mice received water without EtOH. Mice were immunized with either OVA-Fe or PBS at day 0, boosted at day 7, then tested for in vivo Ag-specific lytic activity on day 11. When mice were killed for DLN for the in vivo killing assay, spleens were removed and $CD4^+$ and $CD8^+$ T cells isolated by magnetic bead isolation as described in the Materials and Methods. Isolated $CD4^+$ and $CD8^+$ T cells were used as responders to Ag-loaded APC in ELISPOT. Results mean ± 1 SD are from nine experiments.

4.3.3 Serum IgG_1 and IgG_{2a} production is equivalent between control and EtOH-fed mice

After two immunizations with OVA-Fe, we observed no difference in humoral immune responses by chronic EtOH consumption (Figure 28). In general, there was considerable mouse to mouse variation in both the control and EtOH-fed groups, with many mice having little to no detectable serum Ig production (Figure 28).

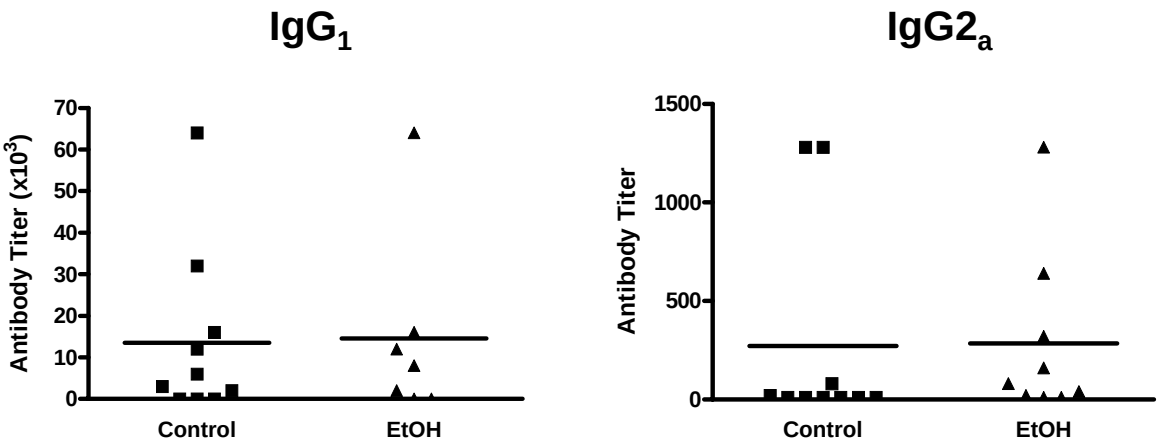


Figure 28. Serum levels of IgG1 and IgG2a are equivalent in control and EtOH-fed mice.
B6 mice were fed EtOH for 12-24 w, as described in the Materials and Methods. Age-matched control mice received water without EtOH. Mice were immunized with either OVA-Fe or PBS at day 0, boosted at day 7, then tested for in vivo Ag-specific lytic activity on day 11. When mice were killed for DLN for the in vivo killing assay, blood was collected without heparin, allowed to clot, then serum collected. Serum was analyzed by ELISA for IgG_1 and IgG_{2a} levels. Horizontal bars indicate mean values. Results are from seven to nine experiments.

4.4 DISCUSSION

As discussed in the Introduction, murine models of chronic EtOH consumption, while in many ways able to mimic the human condition, still exhibit differences. To date, there has been no published report detailing the effects of chronic EtOH consumption on cell-mediated or humoral immunity using the Meadows-Cook model. Studying these effects may provide insight as to the possible immunologic defects that these mice exhibit and, subsequently, the usefulness of this murine model in the study of various immune cells, cell function impairments (i.e. cytokine production by T cells), and viral or bacterial disease models. It should be noted that ultimately, we are examining a murine model which is limited by the length of EtOH exposure, number of immunizations, the animal system (mice do not get cirrhosis), as well as use of a model Ag (OVA) that may not appropriately reflect impaired immune responses in EtOH-fed mice.

The data presented are from mice which have been fed EtOH for 12-24 weeks. The in vivo killing assay shows that there is no difference in Ag-specific lytic activity between control and EtOH-fed mice (Figure 26). In these studies, we used naïve C57BL/6 mice immunized at day 0 and boosted at day 7. It is possible that the length of EtOH-exposure may need to be lengthened in order to see any defects in lytic activity. Furthermore, the killing that was

quantified in Figure 26 could be from either NK cells or $CD8^+$ T cells. The possibilities exist that neither cell population is affected in the Meadows-Cook model, or that one population is compensating for the other population, which is impaired. Considering the existing data on EtOH's effects on NK and $CD8^+$ T cells (326, 247-250, 327, 251), it seems possible that NK cells are impaired by chronic EtOH consumption but that $CD8^+$ T cell activation is enhanced. In order to specifically test the impact of NK cell or $CD8^+$ T cell populations, NK cell deficient mice or OT-II Tg mice would be fed EtOH and Ag-specific lytic activity tested in these mice.

We also found no difference in IFNγ production and a lack of significant IL-5 production between control and EtOH-fed groups (Figure 27). These data suggest no Th1/Th2 polarization in EtOH-fed mice, which is supported by equivalent Ag-specific serum IgG1 and IgG2a levels between control and EtOH-fed mice (Figure 28). At a recent meeting of the Alcohol and Immunology Research Interest Group (Maywood, IL, 2005), it was reported that, in mice immunized with TNP-KLH and adjuvant, CpG for Th1 skewing and alum for Th2 skewing, there was no change in Th2 IgM, IgG_1, or IgE in mice with up to 32 weeks of EtOH consumption on the Meadows-Cook model (333). Th1 responses, as measured by IgG_{2a} and IgG_{2b} production, were decreased in EtOH-fed animals, but only in mice with 32 weeks of EtOH consumption. Overall, it appears that, with >32 weeks EtOH consumption, Th1 responses fail but Th2 responses remain intact. Thus, humoral responses appear to be resistant to the effects of EtOH in the Meadows-Cook model, requiring >32 weeks of EtOH consumption before impairments appear.

Thus, the Meadows-Cook model shows impaired DC function and possible impairments in T cells, B cells, and NK cells. Further evaluation of this model will require mice being fed EtOH for extended periods of time, as well as the possible use of Tg mice, to better evaluate the impact of EtOH on specific cell populations. Overall, it appears that this model will contribute to the evaluation of chronic EtOH consumption on various immune cells and function.

5.0 SUMMARY AND CONCLUDING DISCUSSION

Chronic alcoholics are known to be more susceptible to various diseases, including those caused by bacterial and viral pathogen, such as tuberculosis and viral hepatitis, as well as malignancies (91, 245, 189-191, 246). Various immune cell populations have been evaluated in the past for impairment in function due to EtOH exposure, but the few studies to date have studied the effects of EtOH on DC, the most important APC of the immune system. Further, these studies are limited to human peripheral blood monocyte-derived myeloid DC. With the availability of a mouse model of chronic EtOH consumption (Meadows-Cook model) as well as hematopoietic agents which allow the generation of enough DC to study, the effects of chronic EtOH consumption on DC development and function can now be assessed. Further, by better understanding the cell-mediated and humoral responses in this model, it will be possible to study DC (and other cells, i.e. other leukocytes or endothlelial cells) in the context of various models of infection or disease.

We have determined, herein, that prolonged EtOH exposure affects the development and function of mDC and pDC derived from BM precursors. Both mDC and pDC development were inhibited by EtOH exposure during their generation in vitro in a dose-dependent manner, with pDC being more susceptible to EtOH's effects than mDC (Figure 1). Further, the function of EtOH-treated mDC and pDC, when stimulated with HSV or TLR9 ligand CpG, was inhibited compared to control DC, as determined by phenotypic maturation markers (Figure 6) as well as reduced capacity to stimulate naïve allo-T cell proliferation (Figure 8). More interestingly, when control and EtOH-exposed BMDC were adoptively transferred, EtOH-exposed DC were poorer at priming naïve T cells in vivo (Figure 11A). Further, EtOH-exposed DC primed T cells that produced more IL-10 when restimulated with alloAg ex vivo (Figure 11B). The reduced allostimulatory capacity of EtOH-exposed DC is probably due to their immature phenotype and perhaps also because of their higher CD274/B7-H1 expression (Figure 7). To test if CD274

plays a role in the reduced proliferation of EtOH-exposed BMDC, a blocking Ab to CD274 could be used on the DC in similar in vitro and in vivo experiments. By using a blocking Ab, one could determine the level of involvement of this coregulatory molecule on the impaired function of EtOH-exposed BMDC.

The effects of chronic EtOH exposure on DC freshly-isolated from mice were also assessed. Liver and spleen DC were differentially affected by EtOH exposure, with EtOH seeming to exert a more marked inhibitory effect on spleen DC, as determined by phenotypic (Figure 18) and functional characteristics (Figure 19). Both liver and spleen DC, when freshly-isolated, are immature in phenotype (Figure 18). Thus, they are both poor at stimulating naïve T cell proliferation in comparison to mature DC (Figure 19). Interestingly, although classic phenotypic maturation markers, such as CD40, CD80, and CD86, were all expressed at low levels on both control and EtOH-exposed, freshly-isolated DC, EtOH-exposed liver and spleen DC were both significantly poorer stimulators of naïve allo-T cells in MLR compared to control freshly-isolated liver and spleen DC. These data suggest that there is another mechanism by which EtOH-exposure affects the allostimulatory capacity of immature liver and spleen DC. It is possible that a marker (other coregulatory molecules) not yet tested and/or altered DC cytokine production are involved in the inhibitory function of EtOH-exposed DC. By determining what genes or proteins are affected in liver and spleen DC, such as by microarray, it may be possible to narrow the search for molecules involved in the inhibitory function of EtOH-exposed immature liver and spleen DC.

When liver and spleen DC were stimulated with TLR9 ligand CpG, spleen DC from EtOH-fed mice were inhibited in phenotypic maturation (Figure 18) as well as function (Figure 19) while hepatic DC, which are inherently more resistant to maturation, showed no such inhibitions in response to EtOH exposure (Figure 18 and 18). It is likely that the unique tolerogenic environment of the liver makes resident DC resistant to the effects of EtOH. It is possible that if the liver is exposed to an even more extended diet of EtOH (>12 weeks), liver DC may subsequently lose their resistance to the inhibitory effects of EtOH, perhaps due to altered function in other hepatic cells (i.e. KC, LSEC, hepatocytes).

Interestingly, when immature hepatic and splenic DC were tested for their in vivo capacity to prime naïve allo-T cells, hepatic DC from EtOH-exposed mice had increased ability to prime naïve allo-T cells compared to control hepatic DC (Figure 22A). In contrast, splenic

EtOH-exposed DC had reduced capacity to prime naïve allo-T cells compared to control splenic DC (Figure 22A). The reduced priming ability of EtOH-exposed splenic DC compared to control splenic DC corresponded with in vitro phenotypic maturation and functional data (Figure 18 and 18). However, the increased priming ability of EtOH-exposed hepatic DC compared to control hepatic DC did not correspond with, in fact, seemed to conflict with phenotypic and in vitro functional data (Figure 18 and 18). We then hypothesized that EtOH-exposure might affect the capacity of hepatic (and possibly splenic) DC to migrate to secondary lymphoid tissue. Examination of both immature and mature DC migration to draining lymph nodes in vivo revealed greater migration of EtOH-exposed hepatic DC compared to control hepatic DC and no differences between splenic DC groups (Figure 25). More, adhesion molecule expression data suggest that EtOH-exposure affects expression of CD11a, which is highly expressed on liver DC and is significantly higher in expression on CpG-stimulated, EtOH-exposed hepatic DC (Figure 24A). Altered expression of this adhesion molecule may affect hepatic DC migration which has been shown to be affected by EtOH exposure in vitro and in vivo. Use of a blocking Ab to CD11a on DC would provide insight into its importance in increased hepatic DC migration with EtOH exposure. Similarly, as in vitro migration of hepatic and splenic DC in response to a CCR7 ligand is affected by EtOH exposure (Figure 23), it would be useful to investigate the role of CCR7 on migration. By desensitizing the receptor (by pre-incubation with a CCR7 ligand such as CCL19), we can investigate the importance of CCR7 on DC migration. Further, combining CD11a blockade and CCR7 desensitization in migration experiments would reveal any possible synergistic effects between the adhesion molecule and CCR7 pathways.

Cell-mediated and humoral immune responses of mice fed EtOH on the Meadows-Cook model for 12-24 weeks reveals no differences in comparison to control pair-fed mice (Figure 26-27). A variety of factors could contribute to the apparent absence of effect of chronic alcohol consumption on systemic immune reactivity. As recently presented at the AIRIG 2005 meeting (333), the length of EtOH administration may affect different immune cells at different rates. While we have shown differential deficits in hepatic and splenic DC function from mice consuming EtOH for 8-10 weeks, Waldschmidt *et al.* (333) showed that it was necessary that mice were kept on the diet at least 32 weeks before seeing deficits in humoral immune responses. Another variable may be length of immunization. It is possible that the mice may have needed more immunizations with Ag to show responses or may have needed an adjuvant in order to

generate a strong enough response to detect. In the same study by Waldschmidt *et al.* (333), mice were immunized with Ag and either a Th1 or Th2 adjuvant to promote humoral responses. Further, the timing of immunizations may be important. In our study, we immunized at day 0 and boosted at day 7 then examined sera at day 11. It may be possible that the timings of boosts and sera collection may need to be modified. Waldschmidt *et al.* (333) boosted at day 21 and collected sera at day 28. Thus, many variables may contribute to optimizing the detection of impairments in cell-mediated and humoral immune responses.

The studies discussed herein have provided insight into the field of chronic EtOH exposure and its effects on DC development, phenotypic maturation, and function. These studies can provide a foundation for further studies to investigate the effects of chronic EtOH exposure on immune function and disease processes within a mouse model.

One disease model that could be used to further our work would be to study the effects of EtOH on DC function in the clearance of *Listeria monocytogenes*, a common infectious bacterium that is normally cleared in a healthy individual. We have shown that DC exposed to chronic EtOH treatment are impaired in their responses to stimulation via TLR9, which recognizes bacterial DNA. It has also been shown by Jerrells *et al.* (326) that chronic EtOH consumption by mice prevents their ability to clear infection. By studying DC in mice fed chronic EtOH and infected with *L. monocytogenes*, further insight could be gained into the role of (TLR9 function in) DC in the immune compromised state of chronic alcoholics.

Viral infection is another disease process which is increased in chronic alcoholics. A disease model that could be used to study the effects of EtOH on DC function in the clearance of viral infection would be murine cytomegalovirus (MCMV), a cytopathic β-herpesvirus. In humans, CMV infection of immunosuppressed individuals (i.e. transplant patients, AIDS) is a leading opportunistic infection that can manifest in various clinical symptoms or pathologies, including hepatitis, pneumonitis, and colitis (334). In mice, MCMV causes acute, self-limiting hepatitis depending on both host (i.e. cytokine production) and viral factors (327), with control of viral infection dependent upon $CD8^+$ T cells with contribution from NK cells and $CD4^+$ T cells (335). Importantly, it was recently shown that MyD88 and TLR9 are critically required in the rapid antiviral response (reductions in cytokine production) (336). For DC, MyD88 was required for the upregulation of CD86 expression in response to MCMV (336). It has been reported previously that chronic EtOH in mice infected with MCMV prevents the clearance of

disease (327). In vivo evaluation of EtOH's inhibitory effects on DC (when triggered through TLR9) can be evaluated in the context of a disease state by using this murine viral infection model, which requires TLR9 involvement for the clearance of viral infection.

An alternative murine model of hepatitis caused by viral infection uses lymphocytic choriomeningitis virus (LCMV). The inoculation of adult immunocompetent mice with the Armstrong strain of LCMV leads to a well-characterized acute systemic infection, with the virus replicating in many tissues, including the liver and spleen. LCMV-specific $CD8^+$ CTL responses play a key role in the control of infection within 7-10 days (337). Recently, Belz *et al.* (338) showed that $CD8\alpha^+$ DC are principal in presenting Ag to $CD8^+$ T cells, thus initiating CTL responses. Further, Montoya *et al*. (339) show that initially, $CD8\alpha^+$ DC and mDC are rapidly activated early in LCMV but by day 3 post-infection, the number of pDC, which are type-1 IFN producers, recruited to the spleen was drastically increased. By using type-1 IFN-receptor knockout mice, they show that type-1 IFNs enhance the activation and apoptosis of mDC and $CD8\alpha^+$ DC during early infection, however, type-1 independent pathways for maturation of DC during LCMV infection exist (339). By studying the kinetics of DC activation and recruitment to the spleen, such as Montoya *et al*. (339), we could determine if there is a deficiency in a DC population, either in numbers or function. Our studies of pDC development from BM precursors seemed to show selective developmental inhibition of this subset. It is possible that this same inhibition occurs in the mouse model and that lack of appropriate numbers of pDC to respond to LCMV infection prevents control of infection. Further, EtOH-induced impairments in migration of DC to the spleen or functional defects in ability of DC to initiate appropriate T cell responses may potentially be involved in EtOH's inhibitory effects, and ultimately prevent clearance of LCMV.

Other health consequences of chronic alcohol intake and the immune system could also be examined. For example, it has been well documented that alcohol intake can affect the healing of burn and trauma injury (340), with increased infectious complications. Langerhans cells (LC) or dermal DC could be affected by EtOH and subsequently affect their immune function. Initial studies by Schlueter's group have shown that loss of LC occur within 8 weeks of chronic EtOH feeding and becomes more pronounced with prolonged length of EtOH exposure (341). Further, they found that the migration of DC out of the skin to draining LN was delayed in EtOH-fed mice compared to control mice, although the overall magnitude of LC

migration was equivalent (341). While defective migration of LC may contribute to the immune compromised state in burn and trauma injuries of chronic alcoholics, impaired LC function (i.e. stimulatory capacity or ability to cross-prime) may also be contribute. Thus, it would be worth investigating the effects of chronic EtOH exposure on the function of other cell types, such as the LC.

Our investigations of EtOH's effects on DC development and function are the first in the mouse model and the first to analyze EtOH's effects on liver and spleen DC. The consequences of EtOH exposure on DC in the clinical setting are extensive. Not only are these effects important in host defense and immune response, but also in the combined effects that EtOH may have when used concurrently with other drugs, such as immunosuppressive agents (i.e. Rapamycin, Cyclosporin A, corticosteroids, mycophenolate mofetil). Further, by utilizing our current knowledge of EtOH's effects on DC and by studying the direct effects of chronic EtOH consumption in bacterial and viral infection models, it may be possible to gain further insight into the immune compromised status of alcoholics, especially in the context of liver viral infections (i.e. HBV, HCV).

BIBLIOGRAPHY

1. Lau, AH, and AW Thomson. 2003. Dendritic cells and immune regulation in the liver. *Gut.* 52:307-14.
2. Lau, AH, A de Creus, L Lu, and AW Thomson. 2003. Liver tolerance mediated by antigen presenting cells: fact or fiction? *Gut.* 52:1075-78.
3. Kalinski, P, CM Hilkens, EA Wierenga, and ML Kapsenberg. 1999. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today.* 20:561-67.
4. Lanzavecchia, A, and F Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science.* 290:92-97.
5. Mellman, I, and RM Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell.* 106:255-58.
6. Curtsinger, JM, DC Lins, and MF Mescher. 2003. Signal 3 determines tolerance versus full activation of naive CD8 T cells: dissociating proliferation and development of effector function. *J Exp Med.* 197:1141-51.
7. Lohse, AW, PA Knolle, K Bilo, A Uhrig, C Waldmann, M Ibe, E Schmitt, G Gerken, and KH Meyer Zum Buschenfelde. 1996. Antigen-presenting function and B7 expression of murine sinusoidal endothelial cells and Kupffer cells. *Gastroenterology.* 110:1175-81.
8. Watts, C, and S Powis. 1999. Pathways of antigen processing and presentation. *Rev Immunogenet.* 1:60-74.
9. Knolle, PA, and G Gerken. 2000. Local control of the immune response in the liver. *Immunol. Rev.* 174:21-34.
10. Knolle, PA, and A Limmer. 2001. Neighborhood politics: the immunoregulatory function of organ-resident liver endothelial cells. *Trends Immunol.* 22:432-37.
11. Pober, JS, MS Kluger, and JS Schechner. 2001. Human endothelial cell presentation of antigen and the homing of memory/effector T cells to skin. *Ann N Y Acad Sci.* 941:12-25.
12. Serreze, DV, and PA Silveira. 2003. The role of B lymphocytes as key antigen-presenting cells in the development of T cell-mediated autoimmune type 1 diabetes. *Curr Dir Autoimmun.* 6:212-27.
13. Banchereau, J, F Briere, C Caux, J Davoust, S Lebecque, YJ Liu, B Pulendran, and K Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol.* 18:767-811.
14. Shortman, K, and YJ Liu. 2002. Mouse and human dendritic cell subtypes. *Nat Rev Immunol.* 2:151-61.
15. Ardavin, C. 2003. Origin, precursors and differentiation of mouse dendritic cells. *Nat Rev Immunol.* 3:582-90.

16. Pulendran, B, JL Smith, G Caspary, K Brasel, D Pettit, E Maraskovsky, and CR Maliszewski. 1999. Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc Natl Acad Sci U.S.A.* 96:1036-41.
17. Maldonado-Lopez, R, T De Smedt, P Michel, J Godfroid, B Pajak, C Heirman, K Thielemans, O Leo, J Urbain, and M Moser. 1999. $CD8\alpha^+$ and $CD8\alpha^-$ subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J. Exp. Med.* 189:587-92.
18. Smith, AL, and BF de St Groth. 1999. Antigen-pulsed CD8α+ dendritic cells generate an immune response after subcutaneous injection without homing to the draining lymph node. *J Exp Med.* 189:593-98.
19. Suss, G, and K Shortman. 1996. A subclass of dendritic cells kills CD4+ T cells via Fas/Fas-ligand-induced apoptosis. *J. Exp. Med.* 183:1789-96.
20. Kronin, V, K Winkel, G Suss, A Kelso, W Heath, J Kirberg, H von Boehmer, and K Shortman. 1996. A subclass of dendritic cells regulates the response of naive $CD8^+$ T cells by limiting their IL-2 production. *J. Immunol.* 157:3819-27.
21. O'Connell, PJ, W Li, Z Wang, SM Specht, AJ Logar, and AW Thomson. 2002. Immature and mature $CD8\alpha^+$ dendritic cells prolong the survival of vascularized heart allografts. *J. Immunol.* 168:143-54.
22. Grohmann, U, R Bianchi, ML Belladonna, C Vacca, S Silla, E Ayroldi, MC Fioretti, and P Puccetti. 1999. IL-12 acts selectively on $CD8\alpha^-$ dendritic cells to enhance presentation of a tumor peptide in vivo. *J Immunol.* 163:3100-05.
23. Grohmann, U, R Bianchi, ML Belladonna, S Silla, F Fallarino, MC Fioretti, and P Puccetti. 2000. IFNγ inhibits presentation of a tumor/self peptide by $CD8\alpha^-$ dendritic cells via potentiation of the $CD8\alpha^+$ subset. *J Immunol.* 165.
24. Moron, G, P Rueda, I Casal, and C Leclerc. 2002. $CD8\alpha^-CD11b^+$ dendritic cells present exogenous virus-like particles to $CD8^+$ T cells and subsequently express CD8α and CD205 molecules. *J Exp Med.* 195:1233-45.
25. Morelli, AE, AT Larregina, WJ Shufesky, AF Zahorchak, AJ Logar, GD Papworth, Z Wang, SC Watkins, J Falo, L.D., and AW Thomson. 2003. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood.* 101:611-20.
26. Anjuere, F, G Martinez del Hoyo, P Martin, and C Ardavin. 2000. Langerhans cells acquire a $CD8^+$ dendritic cell phenotype on maturation by CD40 ligation. *J Leukoc Biol.* 67:206-09.
27. Liu, YJ. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu. Rev. Immunol.* 23:275-306.
28. Asselin-Paturel, C, A Boonstra, M Dalod, I Durand, N Yessaad, C Dezutter-Dambuyant, A Vicari, A O'Garra, C Biron, F Briere, and G Trinchieri. 2001. Mouse type 1 IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol.* 2:1144-50.
29. Bjorck, P. 2001. Isolation and characterization of murine plasmacytoid dendritic cells. *Blood.* 98:3520-26.
30. Nakano, H, M Yanagita, and MD Gunn. 2001. $CD11c^+B220^+Gr\text{-}1^+$ cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J. Exp. Med.* 194:1171-78.

31. Hochrein, H, M O'Keefe, and W Hermann. 2002. Human and mouse plasmacytoid dendritic cells. *Hum. Immunol.* 63:1103-10.
32. Munn, DH, MD Sharma, D Hou, B Baban, JR Lee, SJ Antonia, JL Messing, P Chandler, PA Koni, and AL Mellor. 2004. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *J. Clin. Invest.* 114:280-90.
33. Mellor, AL, B Baban, PR Chandler, A Manlapat, DJ Kahler, and DH Munn. 2005. Cutting Edge: CpG oligonucleotides induce splenic $CD19^+$ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN type 1 signaling. *J Immunol.* 175:5601-05.
34. Bjorck, P. 2002. The multifaceted murine plasmacytoid dendritic cell. *Hum. Immunol.* 63:1094-102.
35. Gilliet, M, and YJ Liu. 2002. Human plasmacytoid-derived dendritic cells and the induction of T-regulatory cells. *Hum. Immunol.* 63:1149-55.
36. Coates, PT, SM Barratt-Boyes, L Zhang, VS Donnenberg, PJ O'Connell, AJ Logar, FJ Duncan, M Murphey-Corb, AD Donnenberg, AE Morelli, CR Maliszewski, and AW Thomson. 2003. Dendritic cell subsets in blood and lymphoid tissue of rhesus monkeys and their mobilization with Flt3 ligand. *Blood.* 102:2513-21.
37. Bilsborough, J, TC George, A Norment, and JL Viney. 2003. Mucosal $CD8\alpha^+$ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. *Immunology.* 108:481-92.
38. Fallarino, F, C Vacca, C Orabona, ML Belladonna, R Bianchi, B Marshall, DB Keskin, AL Mellor, MC Fioretti, U Grohmann, and P Puccetti. 2002. Functional expression of indoleamine 2,3-dioxygenase by murine $CD8a^+$ dendritic cells. *Int. Immunol.* 14:65-68.
39. McKenna, K, AS Beignon, and N Bhardwaj. 2005. Plasmacytoid dendritic cells: linking innate and adaptive immunity. *J. Virol.* 79:17-27.
40. Abe, M, Z Wang, A De Creus, and AW Thomson. 2005. Plasmacytoid dendritic cell precursors induce allogeneic T-cell hyporesponsiveness and prolong heart graft survival. *Am. J. Transplant.* 5:1808-19.
41. Huang, Y, M Kucia, F Rezzoug, J Ratajczak, MK Tanner, MZ Ratajczak, CL Schanie, H Xu, I Fugier-Vivier, and ST Ildstad. 2005. FL-mobilized peripheral blood but not FL-expanded bone marrow FC promote establishment of chimerism and tolerance. *Stem Cells.* [Epub ahead of print].
42. Akira, S, and K Takeda. 2004. Toll-like receptor signalling. *Nat Rev Immunol.* 4:499-511.
43. Hemmi, H, O Takeuchi, T Kawai, T Kaisho, S Sato, H Sanjo, M Matsumoto, K Hoshino, H Wagner, K Takeda, and S Akira. 2000. A toll-like receptor recognizes bacterial DNA. *Nature.* 408:740-45.
44. Bauer, M, V Redecke, JW Ellwart, B Scherer, JP Kremer, H Wagner, and GB Lipford. 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *PNAS.* 98:9237-42.
45. Alexopoulou, L, AC Holt, R Medzhitov, and RA Flavell. 2001. Recognition of double-stranded RNA and activation of NF-κB by toll-like receptor 3. *Nature.* 413:732-38.
46. Hemmi, H, T Kaisho, O Takeuchi, S Sato, H Sanjo, K Hoshino, T Horiuchi, H Tomizawa, K Takeda, and S Akira. 2002. Small anti-viral compounds activate immune cells via TLR7-MyD88-dependent signaling pathway. *Nat. Immunol.* 3:196-200.

47. Lee, J, T Chuang, V Redecke, L She, PM Pitha, DA Carson, E Raz, and HB Cottam. 2003. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of toll-like receptor 7. *PNAS.* 100:6646-51.
48. Mazzoni, A, and DM Segal. 2004. Controlling the Toll road to dendritic cell polarization. *J Leukoc Biol.* 75:721-30.
49. Schnare, M, GM Barton, AC Holt, K Takeda, S Akira, and R Medzhitov. 2001. Toll-like receptors control activation of adaptive immune responses. *Nat. Immunol.* 2:947-50.
50. Jankovic, D, MC Kullberg, S Hieny, P Caspar, CM Collazo, and A Sher. 2002. In the absence of IL-12, $CD4^+$ T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an $IL\text{-}10^{-/-}$ setting. *Immunity.* 16:429-39.
51. Ito, T, R Amakawa, T Kaisho, H Hemmi, K Tajima, K Uehira, Y Ozaki, H Tamizawa, S Akira, and S Fukuhara. 2002. Interferon-α and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. *J. Exp. Med.* 195:1507-12.
52. Agrawal, S, A Agrawal, B Doughty, A Gerwitz, J Blenis, T Van Dyke, and B Pulendran. 2003. Cutting edge: Different toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J. Immunol.* 171:4984-89.
53. Boonstra, A, C Asselin-Paturel, M Gilliet, C Crain, G Trinchieri, YJ Liu, and A O'Garra. 2003. Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper Type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. *J. Exp. Med.* 197:101-09.
54. Napolitani, G, A Rinaldi, F Bertoni, F Sallusto, and A Lanzavecchia. 2005. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol.* 6:769-76.
55. Ito, T, R Amakawa, and S Fukuhara. 2002. Roles of toll-like receptors in natural interferon-producing cells as sensors in immune surveillance. *Hum. Immunol.* 63:1120-25.
56. Edwards, AD, SS Diebold, EMC Slack, H Tomizawa, H Hemmi, T Kaisho, S Akira, and C Reis e Sousa. 2003. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by $CD8\alpha^+$ DC correlates with unresponsiveness to imidazoquinolines. *Eur. J. Immunol.* 33:827-33.
57. Lau, AH, MA Abe, and AW Thomson. 2006. Ethanol affects the generation, cosignaling molecule expression, and function of plasmacytoid and myeloid dendritic cell subsets in vitro and in vivo. *J Leukoc Biol.* [Epub ahead of print].
58. Krieg, AM. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol.* 20:709-60.
59. Klinman, DM. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol.* 4:249-58.
60. Kariko, K, H Ni, J Capodici, M Lamphier, and D Weissman. 2004. mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem.* 279:12542-50.
61. Hasan, U, C Chaffois, C Gaillard, V Saulnier, E Merck, S Tancredi, C Guiet, F Briere, J Vlach, S Lebecque, G Trinchieri, and EE Bates. 2005. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol.* 174:2942-50.

62. Yarovinsky, F, D Zhang, JF Andersen, GL Bannenberg, CN Serhan, MS Hayden, S Hieny, FS Sutterwala, RA Flavell, S Ghosh, and A Sher. 2005. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science.* 308:1629-29.
63. Ishii, KJ, and S Akira. 2005. Innate immune recognition of nucleic acids: beyond toll-like receptors. *Int J Cancer.* 117:517-23.
64. Nishiya, T, E Kajita, S Miwa, and AL Defranco. 2005. TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements. *J Biol Chem.* 280:37107-17.
65. Pollard, AM, and MF Lipscomb. 1990. Characterization of murine lung dendritic cells: similarities to Langerhans cells and thymic dendritic cells. *J Exp Med.* 172:159-67.
66. Vremec, D, M Zorbas, R Scollay, DJ Saunders, CF Ardavin, L Wu, and K Shortman. 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med.* 176:47-58.
67. Holt, PG, and PA Stumbles. 2000. Characterization of dendritic cell populations in the respiratory tract. *J Aerosol Med.* 13:361-67.
68. O'Connell, PJ, AE Morelli, AJ Logar, and AW Thomson. 2000. Phenotypic and functional characterization of mouse hepatic CD8α^+ lymphoid-related dendritic cells. *J. Immunol.* 165:795-803.
69. Lian, Z, T Okada, X He, H Kita, YJ Liu, AA Ansari, K Kikuchi, S Ikehara, and ME Gershwin. 2003. Heterogeneity of dendritic cells in the mouse liver: Identification and characterization of four distinct populations. *J. Immunol.* 170:2323-30.
70. Jomantaite, I, N Dikopoulos, A Kroger, F Leithauser, H Hauser, R Schirmbeck, and J Reimann. 2004. Hepatic dendritic cell subsets in the mouse. *Eur. J. Immunol.* 34:355-65.
71. Pillarisetty, VG, AB Shah, G Miller, JI Bleier, and RP DeMatteo. 2004. Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition. *J. Immunol.* 172:1009-17.
72. Fu, F, Y Li, S Qian, L Lu, FG Chambers, TE Starzl, JJ Fung, and AW Thomson. 1996. Costimulatory molecule-deficient dendritic cell progenitors (MHC class II$^+$, CD80^{dim}, CD86$^-$) prolong cardiac allograft survival in nonimmunosuppressed recipients. *Transplantation.* 62:659-65.
73. Rastellini, C, L Lu, C Ricordi, T Starzl, A Rao, and A Thomson. 1995. Granulocyte/macrophage colony-stimulating factor-stimulated hepatic dendritic cell progenitors prolong pancreatic islet allograft survival. *Transplantation.* 60:1366-70.
74. Daro, E, E Butz, J Smith, M Teepe, CR Maliszewski, and HJ McKenna. 2002. Comparison of the functional properties of murine dendritic cells generated in vivo with Flt3 ligand, GM-CSF and Flt3 ligand plus GM-SCF. *Cytokine.* 17:119-30.
75. Abe, MA, AF Zahorchak, BL Colvin, and AW Thomson. 2004. Migratory responses of murine hepatic myeloid, lymphoid-related, and plasmacytoid dendritic cells to CC chemokines. *Transplantation.* 78:762-65.
76. Maraskovsky, E, K Brasel, M Teepe, ER Roux, SD Lyman, K Shortman, and HJ McKenna. 1996. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell sub-populations identified. *J. Exp. Med.* 184:1953.
77. Lau, A, and AW Thomson. 2003. Dendritic cells and immune regulation in the liver. *Gut.* 52:307-14.

78. Morelli, AE, and AW Thomson. 2003. Dendritic cells: regulators of alloimmunity and opportunities for tolerance induction. *Immunol. Rev.* 196:125-46.
79. De Creus, A, M Abe, AH Lau, H Hackstein, G Raimondi, and AW Thomson. 2005. Low TLR4 expression by liver dendritic cells correlates with reduced capacity to activate allogeneic T cells in response to endotoxin. *J. Immunol.* 174:2037-45.
80. Mellor, A, B Baban, P Chandler, B Marshall, K Jhaver, A Hansen, P Koni, M Iwashima, and D Munn. 2003. Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. *J Immunol.* 171:1652-55.
81. Homann, D, A Jahreis, T Wolfe, A Hughes, B Coon, M van Stipdonk, K Prilliman, S Schoenberger, and M von Herrath. 2002. CD40L blockade prevents autoimmune diabetes by induction of bitypic NK/DC regulatory cells. *J Immunol.* 171:1652-55.
82. Bjorck, P, PT Coates, Z Wang, FJ Duncan, and AW Thomson. 2005. Promotion of long-term heart allograft survival by combination of mobilized donor plasmacytoid dendritic cells and anti-CD154 monoclonal antibody. *J Heart Lung Transplant.* 24:1118-20.
83. Feili-Hariri, M, D Falkner, and P Morel. 2002. Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice. *Eur J Immunol.* 32:2021-30.
84. Lutz, MB, RM Suri, M Niimi, AL Ogilvie, NA Kukutsch, S Rossner, G Schuler, and JM Austyn. 2000. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival *in vivo*. *Eur. J. Immunol.* 30.
85. Sato, T, H Yamamoto, C Sasaki, and K Wake. 1998. Maturation of rat dendritic cells during intrahepatic translocation evaluated using monoclonal antibodies and electron microscopy. *Cell & Tissue Research.* 294:503-14.
86. Sato, K, N Yamashita, N Yamashita, M Baba, and T Matsuyama. 2003. Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse. *Immunity.* 18:367-79.
87. Fujita, S, KI Seino, K Sato, Y Sato, K Eizumi, N Yamashita, M Taniguchi, and K Sato. 2006. Regulatory dendritic cells act as regulators of acute lethal systemic inflammatory response. *Blood.* [Epub ahead of print].
88. Abe, M, Z Wang, F Duncan, and A Thomson. 2004. Pre-plasmacytoid dendritic cells propagated from bone marrow induce allogeneic T cell hyporesponsiveness in vivo (Abstract). *Am J Trans.* 4:580.
89. Fugier-Vivier, IJ, F Rezzoug, Y Huang, AJ Graul-Layman, CL Schanie, H Xu, PM Chilton, and ST Ildstad. 2005. Plasmacytoid precursor dendritic cells facilitate allogeneic hematopoietic stem cell engraftment. *J Exo.* 201:373-83.
90. Sallusto, F, CR Mackay, and A Lanzavecchia. 2000. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol.* 18:593-620.
91. http://www.sciencegateway.org/resources/prow/default.htm
92. Carlos, TM, and JM Harlan. 1994. Leukocyte-endothelial adhesion molecules. *Blood.* 84:2068-101.
93. Elangbam, CS, CW Qualls Jr., and RR Dahlgren. 1997. Cell adhesion molecules - update. *Vet Pathol.* 34:61-73.
94. Koopman, G, HK Parmentier, HJ Schuurman, W Newman, CJ Meijer, and ST Pals. 1991. Adhesion of human B cells to follicular dendritic cells involves both the lymphocyte

function-associated antigen 1/intercellular adhesion molecule 1 and very late antigen 4/vascular cell adhesion molecule 1 pathways. *J Exp Med.* 173:1297-304.
95. Lai, AY, SM Lin, and M Kondo. 2005. Heterogeneity of Flt3-expressing multipotent progenitors in mouse bone marrow. *J Immunol.* 175:5016-23.
96. Engelhardt, B, and H Wolburg. 2004. Transendothelial migration of leukocytes: through the front door or around the side of the house? *Eur J Immunol.* 34:2955-63.
97. Muller, WA. 2003. Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol.* 24:326-33.
98. Johnson-Leger, C, and BA Imhof. 2003. Forging the endothelium during inflammation: pushing at a half-open door? *Cell Tissue Res.* 314:93-105.
99. Dejana, E. 2006. The transcellular railway: insights into leukocyte diapedesis. *Nat Cell Biol.* 8:105-07.
100. Nieminen, M, T Henttinen, M Merinen, F Marttila-Ichihara, JE Eriksson, and S Jalkanen. 2006. Vimentin function in lymphocyte adhesion and transcellular migration. *Nat Cell Biol.* 8:156-62.
101. D'Amico, G, G Bianchi, S Bernasconi, L Bersani, L Piemonti, S Sozzani, A Mantovani, and P Allavena. 1998. Adhesion, transendothelial migration, and reverse transmigration of in vitro cultured dendritic cells. *Blood.* 92:207-14.
102. Randolph, GJ, T Luther, S Albrecht, V Magdolen, and WA Muller. 1998. Role of tissue factor in adhesion of mononuclear phagocytes to and trafficking through endothelium in vitro. *Blood.* 92:4167-77.
103. Randolph, GJ, S Beaulieu, M Pope, I Sugawara, L Hoffman, RM Steinman, and WA Muller. 1998. A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. *Proc Natl Acad Sci U.S.A.* 95:6924-29.
104. Muller, WA, and GJ Randolph. 1999. Migration of leukocytes across endothelium and beyond: molecules involved in the transmigration and fate of monocytes. *J Leukoc Biol.* 66:698-704.
105. Randolph, GJ, and MB Furie. 1996. Mononuclear phagocytes egress from an in vitro model of the vascular wall by migrating across endothelium in the basal to apical direction: role of intercellular adhesion molecule 1 and the CD11/CD18 integrins. *J Exp Med.* 183:451-62.
106. Gunn, MD, S Kyuwa, C Tam, T Kakiuchi, A Matsuzawa, LT Williams, and H Nakano. 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med.* 189:451-60.
107. de la Rosa, G, N Longo, JL Rodriguez-Fernandez, A Puig-Kroger, A Pineda, AL Corbi, and P Sanchez-Mateos. 2003. Migration of human blood dendritic cells across endothelial cell monolayers: adhesion molecules and chemokines involved in subset-specific transmigration. *J Leukoc Biol.* 73:639-49.
108. Colvin, BL, AH Lau, AM Schell, and AW Thomson. 2004. Disparate ability of murine $CD8\alpha^-$ and $CD8\alpha^+$ dendritic cell subsets to traverse endothelium is not determined by differential CD11b expression. *Immunology.* 113:328-37.
109. Rossi, D, and A Zlotnik. 2000. The biology of chemokines and their receptors. *Annu Rev Immunol.* 18:217-42.
110. Esche, C, C Stellato, and LA Beck. 2005. Chemokines: key players in innate and adaptive immunity. *J Invest Dermatol.* 125:615-28.

111. Nickel, R, LA Beck, C Stellato, and RP Schleimer. 1999. Chemokines and allergic disease. *J Allergy Clin Immunol.* 104:723-42.
112. Gerard, C, and BJ Rollins. 2001. Chemokines and disease. *Nat Immunol.* 2:108-15.
113. Murphy, PM, M Baggiolini, IF Charo, CA Hebert, R Horuk, K Matsushima, LH Miller, JJ Oppenheim, and CA Power. 2000. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharm. Rev.* 52:145-76.
114. Sallusto, F, B Palermo, D Lenig, M Miettinen, S Matikainen, I Julkunen, R Forster, R Burgstahler, M Lipp, and A Lanzavecchia. 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur J Immunol.* 29:1617-25.
115. Sozzani, S, P Allavena, A Vecchi, and A Mantovani. 2000. Chemokines and dendritic cell traffic. *J Clin Immunol.* 20:151-60.
116. Yoshie, O. 2000. Role of chemokines in trafficking of lymphocytes and dendritic cells. *Int J Hematol.* 72:399-407.
117. Foti, M, F Granucci, D Aggujaro, E Liboi, W Luini, S Minardi, A Mantovani, S Sozzani, and P Ricciardi-Castagnoli. 1999. Upon dendritic cell (DC) activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site. *Int. Immunol.* 11:979-86.
118. Vecchi, A, L Massimiliano, S Ramponi, W Luini, S Bernasconi, R Bonecchi, P Allavena, M Parmentier, A Mantovani, and S Sozzani. 1999. Differential responsiveness to constitutive vs. inducible chemokines of immature and mature mouse dendritic cells. *J Leukoc Biol.* 66:489-94.
119. Colvin, BL, AE Morelli, AJ Logar, AH Lau, and AW Thomson. 2004. Comparative evaluation of CC chemokine-induced migration of murine $CD8\alpha^+$ and $CD8\alpha^-$ dendritic cells and their in vivo trafficking. *J Leukoc Biol.* 75:275-85.
120. Yoneyama, H, K Matsuno, Y Zhang, T Nishiwaki, M Kitabatake, S Ueha, S Narumi, S Morikawa, T Ezaki, B Lu, C Gerard, S Ishikawa, and K Matsushima. 2004. Evidence for recruitment of plasmacytoid dendritic cell precursors to inflamed lymph nodes through high endothelial venules. *Int. Immunol.* 16:915-28.
121. Lin, CL, RM Suri, RA Rahdon, JM Austyn, and JA Roake. 1998. Dendritic cell chemotaxis and transendothelial migration are induced by distinct chemokines and are regulated on maturation. *Eur J Immunol.* 28:4114-22.
122. Penna, G, S Sozzani, and L Adorini. 2001. Cutting edge: selective usage of chemokine receptors by plasmacytoid dendritic cells. *J Immunol.* 167:1862-66.
123. Gunn, MD. 2003. Chemokine mediated control of dendritic cell migration and function. *Semin Immunol.* 15:271-76.
124. Kellerman, SA, S Hudak, ER Oldham, YJ Liu, and LM McEvoy. 1999. The CC-chemokine receptor-7 ligands 6Ckine and macrophage inflammatory protein-3 beta are potent chemoattractants for in vitro- and in vivo- derived dendritic cells. *J. Immunol.* 162:3859-64.
125. Caux, C, S Ait-Yahia, K Chemin, O de Bouteiller, MC Dieu-Nosjean, B Homey, C Massacrier, B Vanbervliet, A Zlotnik, and A Vicari. 2000. Dendritic cell biology and regulation of dendritic cell trafficking by chemokines. *Springer Semin Immunopathol.* 22:345-69.
126. Saeki, H, MT Wu, E Olasz, and ST Hwang. 2000. A migratory population of skin-derived dendritic cells expresses CXCR5, responds to B lymphocyte chemoattractant in

vitro, and co-localizes to B cell zones in lymph nodes in vivo. *Eur J Immunol.* 30:2808-14.
127. Gosling, J, DJ Dairaghi, Y Wang, M Hanley, D Talbot, Z Miao, and TJ Schall. 2000. Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. *J Immunol.* 164:2851-56.
128. Hart, DNJ, and JW Fabre. 1981. Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues but not brain. *J. Exp. Med.* 154:347-61.
129. Daar, AS, SV Fuggle, DNJ Hart, R Dalchau, Z Abdulaziz, JW Fabre, A Ting, and PJ Morris. 1983. Demonstration and phenotypic characterization of HLA-DR positive interstitial dendritic cells widely distributed in human connective tissues. *Transpl. Proc.* 15:311-15.
130. Prickett, TC, JL McKenzie, and DN Hart. 1988. Characterization of interstitial dendritic cells in human liver. *Transplantation.* 46:754-61.
131. Doherty, DG, and C O'Farrelly. 2001. Dendritic cells: regulators of hepatic immunity or tolerance? [letter; comment.]. *J. Hepatol.* 34:156-60.
132. Morelli, AE, PJ O'Connell, A Khanna, AJ Logar, L Lu, and AW Thomson. 2000. Preferential induction of Th1 responses by functionally mature hepatic ($CD8\alpha^-$ and $CD8\alpha^+$) dendritic cells: association with conversion from liver transplant tolerance to acute rejection. *Transplantation.* 69:2647-57.
133. Abe, M, SM Akbar, N Horiike, and M Onji. 2001. Induction of cytokine production and proliferation of memory lymphocytes by murine liver dendritic cell progenitors: role of these progenitors as immunogenic resident antigen-presenting cells in the liver. *J. Hepatol.* 34:61-7.
134. Woo, J, L Lu, AS Rao, Y Li, V Subbotin, TE Starzl, and AW Thomson. 1994. Isolation, phenotype, and allostimulatory activity of mouse liver dendritic cells. *Transplantation.* 58:484-91.
135. Khanna, A, AE Morelli, C Zhong, T Takayama, L Lu, and AW Thomson. 2000. Effects of liver-derived dendritic cell progenitors on Th1- and Th2-like cytokine responses in vitro and in vivo. *J. Immunol.* 164:1346-54.
136. Bissell, DM, S Wang, WR Jarnagin, and FJ Roll. 1995. Cell-specific expression of transforming growth factor-beta in rat liver: evidence for autocrine regulation of hepatocyte proliferation. *J. Clin. Invest.* 96:447-55.
137. Thomson, AW, and L Lu. 1999. Are dendritic cells the key to liver transplant tolerance? *Immunol Today.* 20:27-32.
138. Yamaguchi, Y, H Tsumara, M Miwa, and K Inaba. 1997. Contrasting effects of TGF-β1 and TNF-α on the development of dendritic cells from progenitors in mouse bone marrow. *Stem Cells.* 15:144-53.
139. Steinbrink, K, M Wolfl, H Jonuleit, J Knop, and AH Enk. 1997. Induction of tolerance by IL-10-treated dendritic cells. *J. Immunol.* 159:4772-80.
140. Lee, W, C Zhong, S Qian, Y Wan, J Gauldie, Z Mi, PD Robbins, AW Thomson, and L Lu. 1998. Phenotype, function, and in vivo migration and survival of allogeneic dendritic cell progenitors genetically engineered to express TGF-β. *Transplantation.* 66:1810-17.
141. Takayama, T, Y Nishioka, L Lu, MT Lotze, H Tahara, and AW Thomson. 1998. Retroviral delivery of viral interleukin-10 into myeloid dendritic cells markedly inhibits

their allostimulatory activity and promotes the induction of T-cell hyporesponsiveness. *Transplantation.* 66:1567-74.
142. Thomson, AW, L Lu, N Murase, AJ Demetris, AS Rao, and TE Starzl. 1995. Microchimerism, dendritic cell progenitors and transplantation tolerance. *Stem Cells.* 13:622-39.
143. Khoury, SJ, L Gallon, RR Verburg, A Chandraker, R Peach, P Linsley, LA Turka, WW Hancock, and MH Sayegh. 1996. Ex vivo treatment of antigen presenting cells with CTLA4Ig and encephalitogenic peptide prevents experimental autoimmune encephalomyelitis in the Lewis rat. *J. Immunol.* 157:3700-05.
144. Lu, L, J Woo, AS Rao, Y Li, SC Watkins, S Qian, TE Starzl, AJ Demetris, and AW Thomson. 1994. Propagation of dendritic cell progenitors from normal mouse liver using granulocyte/macrophage colony-stimulating factor and their maturational development in the presence of type-1 collagen. *J. Exp. Med.* 179:1823-34.
145. Drakes, ML, L Lu, VM Subbotin, and AW Thomson. 1997. In vivo administration of flt3 ligand markedly stimulates generation of dendritic cell progenitors from mouse liver. *Journal of Immunology.* 159:4268-78.
146. Lu, L, CA Bonham, X Liang, Z Chen, W Li, L Wang, SC Watkins, MA Nalesnik, MS Schlissel, AJ Demetris, JJ Fung, and S Qian. 2001. Liver-derived $DEC205^{+}B220^{+}CD19^{-}$ dendritic cells regulate T cell responses. *J. Immunol.* 166:7042-52.
147. Brenan, M, and M Puklavec. 1992. The MRC OX-62 antigen: a useful marker in the purification of rat veiled cells with the biochemical properties of an integrin. *J. Exp. Med.* 175:1457-65.
148. Chen-Woan, M, CP Delaney, V Fournier, Y Wakizaka, N Murase, J Fung, TE Starzl, and AJ Demetris. 1996. In vitro characterization of rat bone-marrow derived dendritic cells and their precursors. *J. Leukoc. Biol.* 59:196-207.
149. Gorczynski, L, Z Chen, J Hu, Y Kai, J Lei, V Ramakrishna, and RM Gorczynski. 1999. Evidence that an OX-2-positive cell can inhibit the stimulation of type 1 cytokine production by bone marrow-derived B7-1 (and B7-2)-positive dendritic cells. *J. Immunol.* 162:774-81.
150. Steptoe, RJ, RK Patel, VM Subbotin, and AW Thomson. 2000. Comparative analysis of dendritic cell density and total number in commonly transplanted organs: morphometric estimation in normal mice. *Transpl. Immunol.* 8:49-56.
151. Crowley, MT, K Inaba, MD Witmer-Pack, and RM Steinman. 1989. The cell surface of mouse dendritic cells: FACS analyses of dendritic cells from different tissues including thymus. *Cell. Immunol.* 118:108-25.
152. Wu, L, CL Li, and K Shortman. 1996. Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. *J. Exp. Med.* 184:903-11.
153. Thomson, AW, L Lu, V Subbotin, Y Li, H Noyola, S Qian, AS Rao, AJ Demetris, and TE Starzl. 1994. Propagation of dendritic cell progenitors from mouse liver and their in vivo migration to T-dependent areas of allogeneic lymphoid tissue. *Transpl. Proc.* 26:3484-6.
154. Thomson, AW, ML Drakes, AF Zahorchak, PJ O'Connell, RJ Steptoe, S Qian, and L Lu. 1999. Hepatic dendritic cells: immunobiology and role in liver transplantation. *J. Leukoc. Biol.* 66:322-30.

155. Thomson, AW, PJ O'Connell, RJ Steptoe, and L Lu. 2002. Immunobiology of liver dendritic cells. *Immunol. Cell. Biol.* 80:65-73.
156. Yoneyama, H, K Matsuno, Y Zhang, M Murai, M Itakura, S Ishikawa, G Hasegawa, M Naito, H Asakura, and K Matsushima. 2001. Regulation by chemokines of circulating dendritic cell precursors, and the formation of portal tract-associated lymphoid tissue, in a granulomatous liver disease. *J. Exp. Med.* 193:35-49.
157. Drakes, ML, L Lu, V Subbotin, and AW Thomson. 1997. In vivo administration of flt3 ligand markedly stimulates generation of dendritic cell progenitors from mouse liver. *J. Immunol.* 159:4268-78.
158. Gorczynski, RM, G Levy, and Z Chen. 1999. Hepatic mononuclear cells modulate delivery of immunogenic stimuli by allogeneic dendritic cells. *Transpl. Proc.* 31:856-7.
159. Lautenschlager, I, J Halttunen, and P Hayry. 1988. Characteristics of dendritic cells in rat liver. *Transplantation.* 45:936-9.
160. Matsuno, K, T Ezaki, S Kudo, and Y Uehara. 1996. A life stage of particle-laden rat dendritic cells in vivo: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. *J. Exp. Med.* 183:1865-78.
161. Saiki, T, T Ezaki, M Ogawa, and K Matsuno. 2001. Trafficking of host- and donor-derived dendritic cells in rat cardiac transplantation: allosensitization in the spleen and hepatic nodes. *Transplantation.* 71:1806-15.
162. Sato, T, H Yamamoto, C Sasaki, and K Wake. 1998. Maturation of rat dendritic cells during intrahepatic translocation evaluated using monoclonal antibodies and electron microscopy. *Cell Tiss. Res.* 294:503-14.
163. Steiniger, B, J Klempnauer, and K Wonigeit. 1984. Phenotype and histological distribution of interstitial dendritic cell in the rat pancreas, liver, heart, and kidney. *Transplantation.* 38:169-74.
164. Schon-Hegrad, MA, J Oliver, PG McMenamin, and PG Holt. 1991. Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J. Exp. Med.* 173:1345-56.
165. Ninomiya, T, SMF Akbar, T Masumoto, N Horiike, and M Onji. 1999. Dendritic cells with immature phenotype and defective function in the peripheral blood from patients with hepatocellular carcinoma. *J. Hepatol.* 31:323-31.
166. Goddard, S, N Barclay, and DH Adams. 2001. CD200 (OX2) has limited expression in liver, and is expressed by mature dendritic cells from non-lymphoid tissue. *Scand. J. Immunol.* 54:29 (C7.Tue.2.7/340).
167. Goddard, S, SG Hubscher, P Lane, and DH Adams. 2001. A comparison of dendritic cells migrated from human liver and skin. *Scand. J. Immunol.* 54:29 (C7.Tue.2.7/341).
168. Thomson, AW, L Lu, V Subbotin, Y Li, S Qian, AS Rao, J Fung, and TE Starzl. 1995. In vitro propagation and homing of liver-derived dendritic cell progenitors to lymphoid tissues of allogeneic recipients. *Transplantation.* 59:544-51.
169. Thomson, AW, L Lu, J Woo, AS Rao, TE Starzl, and AJ Demetris. 1995. Exposure to type-I collagen induces maturation of mouse liver dendritic cell progenitors. *Adv. Exp. Med. Biol.* 378:511-8.
170. Drakes, ML, L Lu, HJ McKenna, and AW Thomson. 1997. The influence of collagen, fibronectin, and laminin on the maturation of dendritic cell progenitors propagated from normal or Flt3-ligand-treated mouse liver. *Adv. Exp. Med. Biol.* 417:115-20.

171. Sun, J, GW McCaughan, ND Gallagher, AG Sheil, and GA Bishop. 1995. Deletion of spontaneous rat liver allograft acceptance by donor irradiation. *Transplantation.* 60:233-6.
172. Shimizu, Y, S Goto, R Lord, F Vari, C Edwards-Smith, S Chiba, D Schlect, M Buckley, M Kusano, and N Kamada. 1996. Restoration of tolerance to rat hepatic allografts by spleen-derived passenger leukocytes. *Transpl. Int.* 9:593-95.
173. Bishop, GA, J Sun, DJ DeCruz, KL Rokahr, JD Sedgwick, AG Ross Sheil, ND Gallagher, and GW McCaughan. 1996. Tolerance to rat liver allografts. III. Donor cell migration and tolerance-associated cytokine production in peripheral lymphoid tissues. *J. Immunol.* 156:4925-31.
174. Steptoe, RJ, F Fu, W Li, ML Drakes, L Lu, AJ Demetris, S Qian, HJ McKenna, and AW Thomson. 1997. Augmentation of dendritic cells in murine organ donors by Flt3 ligand alters the balance between transplant tolerance and immunity. *J. Immunol.* 159:5483-91.
175. Steptoe, RJ, W Li, F Fu, PJ O'Connell, and AW Thomson. 1999. Trafficking of APC from liver allografts of Flt3L-treated donors: augmentation of potent allostimulatory cells in recipient lymphoid tissue is associated with a switch from tolerance to rejection. *Transpl. Immunol.* 7:51-7.
176. Witmer-Pack, MD, MT Crowley, K Inaba, and RM Steinman. 1993. Macrophages, but not dendritic cells, accumulate colloidal carbon following administration in situ. *Journal of Cell Science.* 105:965-73.
177. Magnusson, S, and T Berg. 1989. Extremely rapid endocytosis mediated by the mannose receptor of sinusoidal endothelial rat liver cells. *Biochem. J.* 257:651-56.
178. Smedsrod, B, J Melkko, N Araki, H Sano, and S Horiuchi. 1997. Advanced glycation end products are eliminated by scavenger-receptor mediated endocytosis in hepatic sinusoidal Kupffer and endothelial cells. *Biochem. J.* 322:567-73.
179. Matsuno, K, S Kudo, and T Ezaki. 1997. The liver sinusoids as a specialized site for blood-lymph translocation of rat dendritic cells. *Adv. Exp. Med. Biol.* 417:77-81.
180. Iyoda, T, S Shimoyama, K Liu, Y Omatsu, N Akiyama, Y Maeda, K Takahara, RM Steinman, and K Inaba. 2002. The $CD8^+$ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J. Exp. Med.* 195:1289-302.
181. Stumbles, PA, JA Thomas, CL Pimm, PT Lee, TJ Venaille, S Prosksch, and PG Holt. 1998. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. *J. Exp. Med.* 188:2019-31.
182. Kaji, K, K Tsuneyama, Y Nakanuma, K Harada, S Motoko, S Kaneko, and K Kobayashi. 1997. B7-2 positive cells around interlobular bile ducts in primary biliary cirrhosis and chronic hepatitis C. *J. Gastro. Hep.* 12:507-12.
183. Yamamoto, K, SK Akbar, T Masumoto, and M Onji. 1998. Increased nitric oxide(NO) production by antigen-presenting dendritic cells is responsible for low allogeneic mixed leucocyte reaction (MLR) in primary biliary cirrhosis. *Clin. Exp. Immunol.* 114:94-101.
184. Sallusto, F, CR Mackay, and A Lanzavecchia. 2000. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu. Rev. Immunol.* 18:593-620.
185. Nelson, PJ, and AM Krensky. 2001. Chemokines, chemokine receptors, and allograft rejection. *Immunity.* 14:377-86.
186. Drakes, ML, AF Zahorchak, T Takayama, L Lu, and AW Thomson. 2000. Chemokine and chemokine receptor expression by liver-derived dendritic cells: MIP-1alpha

production is induced by bacterial lipopolysaccharide and interaction with allogeneic T cells. *Transpl. Immunol.* 8:17-29.
187. Shields, PL, CM Morland, M Salmon, S Qin, SG Hubscher, and DH Adams. 1999. Chemokine and chemokine receptor interactions provide a mechanism for selective T cell recruitment to specific lver compartments within hepatitis C-infected liver. *J. Immunol.* 163:6236-43.
188. Goddard, S, A Williams, CM Morland, S Qin, R Gladue, SG Hubscher, and DH Adams. 2001. Differential expression of chemokines and chemokine receptors shapes the inflammatory response in rejecting human liver transplants. *Transplantation.* 72:1957-67.
189. Cook, RT. 1998. Alcohol abuse, alcoholism, and damage to the immune system. *Alcohol. Clin. Exp. Res.* 22:1927-42.
190. Corrano, G, and S Arico. 1998. Independent and combined action of hepatitis C virus infection and alcohol consumption on the risk of symptomatic liver cirrhosis. *Hepatology.* 27:914-19.
191. Wiley, TE, M McCarthy, L Breidi, M McCarthy, and TJ Layden. 1998. Impact of alcohol on the histological and clinical progression of hepatitis C infection. *Hepatology.* 28:805-09.
192. Sheron, N. 1994. Alcoholic liver damage - Toxicity, autoimmunity and allergy. *Clin. Exp. Allergy.* 24:503-07.
193. Cook, R, T Waldschmidt, B Cook, D Labrecque, and K McLatchie. 1996. Loss of the $CD5^{+}$ and $CD45RA^{hi}$ B cell subsets in alcoholics. *Clin. Exp. Immunol.* 103:304-10.
194. Cook, RT, JA Keiner, and A Yen. 1990. Ethanol causes acclerated G1 arrest in differentiating HL-60 cells. *Alcohol. Clin. Exp. Res.* 14:695-703.
195. Zuiable, A, E Weiner, and SN Wickramasinghe. 1992. In vitro effects of ethanol on the phagocytic and microbial killing activities of normal human monocytes and monocyte-derived macrophages. *Clin Lab Haematol.* 14:137-47.
196. Silvain, C, C Patry, P Launay, A Lehuen, and RC Monteiro. 1995. Altered expression of monocyte IgA Fc receptors is associated with defective endocytosis in patients with alcoholic cirrhosis. Potential role for IFN-gamma. *J. Immunol.* 155:1606-18.
197. McClain, C, D Hill, J Schmidt, and AM Diehl. 1993. Cytokines and alcoholic liver disease. *Semin. Liver Dis.* 13:170-82.
198. Bautista, AP. 2002. Chronic alcohol intoxication primes Kupffer cells and endothelial cells for enhanced CC-chemokine production and concomitantly suppresses phagocytosis and chemotaxis. *Front. Biosci.* 7:a117-25.
199. Ono, M, B Yu, EG Hardison, MA Mastrangelo, and DJ Tweardy. 2004. Increased susceptibility to liver injury after hemorrhagic shock in rats chronically fed ethanol: role of nuclear factor-kappa B, interleukin-6, and granulocyte colony-stimulating factor. *Shock.* 21:519-25.
200. Osna, NA, DL Clemens, and TM Donohue, Jr. 2003. Interferon gamma enhances proteasome activity in recombinant Hep G2 cells that express cytochrome P4502E1: modulation by ethanol. *Biochem. Pharmacol.* 66:697-710.
201. Jaruga, B, F Hong, WH Kim, R Sun, S Fan, and B Gao. 2004. Chronic alcohol consumption accelerates liver injury in T cell-mediated hepatitis: alcohol disregulation of NF-{kappa}B and STAT3 signaling pathways. *Am. J. Phys. Gastrointest. Liver Phys.* 287:G471-G79.

202. Livy, DJ, SE Parnell, and JR West. 2003. Blood ethanol concentration profiles: a comparison between rats and mice. *Alcohol.* 29:165-71.
203. Lieber, CS, DP Jones, J Mendelson, and LM DeCarli. 1963. Fatty liver, hyperlipidemia and hyperuricemia produced by prolonged alcohol consumption, despite adequate dietary intake. *Trans Assoc Am Physicians.* 76:289-300.
204. de la M Hall, P, CS Lieber, LM DeCarli, SW French, KO Lindros, H Jarvelainen, C Bode, A Parlesak, and JC Bode. 2001. Models of alcoholic liver disease in rodents: a critical evaluation. *Alcohol Clin Exp Res.* 25:254S-61S.
205. Siegmund, S, S Haas, A Schneider, and MV Singer. 2003. Animal models in gastrointestinal alcohol research-a short appraisal of the different models and their results. *Best Pract Res Clin Gastroenterol.* 17:519-42.
206. Mendenhall, CL, F Finkelman, RTJ Means, KE Sherman, VT Nguyen, CE Grossman, SC Morris, S Rouster, and GA Roselle. 1999. Cytokine response to BCG infection in alcohol-fed mice. *Alcohol.* 19:57-63.
207. Sosa, L, D Vidlak, JM Strachota, J Pavlik, and TR Jerrells. 2005. Rescue of in vivo FAS-induced apoptosis of hepatocytes by corticosteroids either associated with alcohol consumption by mice or provided exogenously. *Int Immunopharmacol.* 5:301-14.
208. Tsukamoto, H, RD Reidelberger, SW French, and C Largman. 1984. Long-term cannulation model for blood sampling and intragastric infusion in the rat. *Am J Physiol.* 247:R595-R99.
209. French, SW, K Miyamoto, and H Tsukamoto. 1986. Ethanol-induced hepatic fibrosis in the rat: role of the amount of dietary fat. *Alcohol Clin Exp Res.* 10:13S-19S.
210. Yin, M, MD Wheeler, H Kono, BU Bradford, RM Gallucci, MI Luster, and RG Thurman. 1999. Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice. *Gastroenterology.* 117:942-52.
211. Meadows, GG, SE Blank, and DD Duncan. 1989. Influence of ethanol consumption on natural killer cell activity in mice. *Alcohol Clin Exp Res.* 13:476-79.
212. Song, K, RA Coleman, X Zhu, C Alber, ZK Ballas, TJ Waldschmidt, and RT Cook. 2002. Chronic ethanol consumption by mice results in activated splenic T cells. *J. Leukoc. Biol.* 72:1109-16.
213. Cook, R, X Zhu, R Coleman, Z Ballas, T Waldschmidt, N Ray, D LaBrecque, and B Cook. 2004. T-cell activation after chronic ethanol ingestion in mice. *Alcohol.* 33:175-81.
214. Chen, LH, CY Huang, Y Osio, EA Fitzpatrick, and DA Cohen. 1993. Effects of chronic alcohol feeding and murine AIDS virus infection on liver antioxidant defense systems in mice. *Alcohol Clin Exp Res.* 17:1022-28.
215. Mikszta, JA, C Waltenbaugh, and BS Kim. 1995. Impaired antigen presentation by splenocytes of ethanol-consuming C57BL/6 mice. *Alcohol.* 12:265-71.
216. Schodde, H, S Hurst, M Munroe, T Barrett, and W C. 1996. Ethanol ingestion inhibits cell-mediated immune responses of unprimed T-cell receptor transgenic mice. *Alcohol Clin Exp Res.* 20:890-99.
217. Lopez, MC, B Watzl, LL Colombo, and RR Watson. 1997. Alterations in mouse Peyer's patch lymphocyte phenotype after ethanol consumption. *Alcohol.* 14:107-10.
218. Deaciuc, IV, DE Doherty, R Burikhanov, EY Lee, AJ Stromberg, X Peng, and WJ de Villiers. 2004. Large-scale gene profiling of the liver in a mouse model of chronic, intragastric ethanol infusion. *J Hepatol.* 402:219-27.

219. Dolganiuc, A, K Kodys, A Kopasz, C Marshall, P Mandrekar, and G Szabo. 2003. Additive inhibition of dendritic cell allostimulatory capacity by alcohol and hepatitis C is not restored by DC maturation and involves abnormal IL-10 and IL-2 induction. *Alcohol. Clin. Exp. Res.* 27:1023-31.
220. Mandrekar, P, D Catalano, A Dolganiuc, K Kodys, and G Szabo. 2004. Inhibition of myeloid dendritic cell accessory function and induction of T cell anergy by alcohol correlates with decreased IL-12 production. *J. Immunol.* 173:3398-407.
221. Szabo, G, D Catalano, B White, and P Mandrekar. 2004. Acute alcohol consumption inhibits accessory cell function of monocytes and dendritic cells. *Alcohol. Clin. Exp. Res.* 28:824-28.
222. Szabo, G, A Dolganiuc, P Mandrekar, and B White. 2004. Inhibition of antigen-presenting cell functions by alcohol: implications for hepatitis C virus infection. *Alcohol.* 33:241-49.
223. Delneste, Y, P Charbonnier, N Herbault, G Magistrelli, G Caron, JY Bonnefoy, and P Jeannin. 2003. Interferon-gamma switches monocyte differentiation from dendritic cells to macrophages. *Blood.* 101:143-50.
224. Mandrekar, P, D Catalano, and G Szabo. 1997. Alcohol-induced regulation of nuclear regulatory factor-kappa beta in human monocytes. *Alcohol Clin Exp Res.* 21:988-94.
225. Mandrekar, P, D Catalano, and G Szabo. 1999. Inhibition of lipopolysaccharide-mediated NFκB activation by ethanol in human monocytes. *Int Immunol.* 11:1781-90.
226. Nelson, S, GJ Bagby, G Bainton, and WR Summer. 1989. The effects of acute and chronic alcoholism on tumor necrosis factor and inflammatory response. *J. Infect. Dis.* 160:422-29.
227. Szabo, G, P Mandrekar, L Girouard, and D Catalano. 1996. Regulation of human monocyte functions by acute ethanol treatment: Decreased tumor necrosis factor-alpha, interleukin-1 beta, and elevated interleukin-10 and transforming growth factor-beta production. *Alcohol. Clin. Exp. Res.* 20:900-07.
228. Zhao, X, L Marrero, K Song, P Oliver, SY Chin, H Simon, JR Schurr, Z Zhang, D Thoppil, S Lee, S Nelson, and JK Kolls. 2003. Acute alcohol inhibits TNF-α processing in human monocytes by inhibiting TNF/TNF-α-converting enzyme interactions in the cell membrane. *J. Immunol.* 170:2923-31.
229. Zhang, Z, GJ Bagby, D Stoltz, P Oliver, PO Schwarzenberger, and JK Kolls. 2001. Prolonged ethanol treatment enhances lipopolysaccharide/phorbol myristate accetate-induced tumor necrosis factor-α production in human monocytic cells. *Alcohol. Clin. Exp. Res.* 25:444-49.
230. McClain, CJ, S Barve, I Deaciuc, and DB Hill. 1998. Tumor necrosis factor and alcoholic liver disease. *Alcohol Clin Exp Res.* 22:248S-52S.
231. Bagasra, O, A Howeedy, and A Kajdacsy-Balla. 1988. Macrophage function in chronic experimental alcoholism. I. Modulation of surface receptors and phagocytosis. *Immunology.* 65:405-09.
232. Shiratori, Y, H Jin'nai, H Teraoka, S Matano, K Matsumoto, K Kamii, M Tanaka, and K Okano. 1989. Phagocytic properties of hepatic endothelial cells and splenic macrophages compensating for a decreased phagocytic function of Kupffer cells in the chronically ethanol-fed rats. *Exp Cell Biol.* 57:300-09.
233. Castro, A, DL Lefkowitz, and SS Lefkowitz. 1993. The effects of alcohol on murine macrophage function. *Life Sciences.* 52:1585-93.

234. Goral, J, MA Choudhry, and EJ Kovacs. 2004. Acute ethanol exposure inhibits macrophage IL-6 production: role of p38 and ERK1/2 MAPK. *J. Leukoc. Biol.* 75:553-59.
235. Pruett, SB, C Schwab, Q Zheng, and R Fan. 2004. Suppression of innate immunity by acute ethanol administration: a global perspective and a new mechanism beginning with inhibition of signaling through TLR3. *J. Immunol.* 173:2715-24.
236. Goral, J, and EJ Kovacs. 2005. In vivo ethanol exposure down-regulates TLR2-, TLR4-, and TLR9-mediated macrophage inlammatory response by limiting p38 and ERK1/2 activation. *J. Immunol.* 174:456-63.
237. Fox, ES, CH Cantrell, and KA Leingang. 1996. Inhibition of the Kupffer cell inflammatory response by acute ethanol: NF-κB activation and subsequent cytokine production. *Biochem Biophys Res Commun.* 225:134-40.
238. Spitzer, JA, M Zheng, JK Kolls, C Vande Stouwe, and JJ Spitzer. 2002. Ethanol and LPS modulate NF-κB activation, inducible NO synthase and COX-2 gene expression in rat liver cells in vivo. *Front Biosci.* 7:a99-108.
239. D'Souza, NB, S Nelson, WR Summer, and IV Deaciuc. 1996. Alcohol modulates alveolar macrophage tumor necrosis factor-alpha, superoxide anion, and nitric oxide secretion in the rat. *Alcohol Clin Exp Res.* 20:156-63.
240. Antony, VB, SW Godbey, JW Hott, and SF Queener. 1993. Alcohol-induced inhibition of alveolar macrophage oxidant release in vivo and in vitro. *Alcohol Clin Exp Res.* 17:389-93.
241. Bautista, AP, and JJ Spitzer. 1999. Role of Kupffer cells in the ethanol-induced oxidative stress in the liver. *Front Biosci.* 4:D589-95.
242. Yokoyama, H, M Fukuda, Y Okamura, T Mizukami, H Ohgo, Y Kamegaya, S Kato, and H Ishii. 1999. Superoxide anion release into the hepatic sinusoid after an acute ethanol challenge and its attenuation by Kupffer cell depletion. *Alcohol Clin Exp Res.* 23:71S-75S.
243. Bautista, AP. 2002. Acute ethanol binge followed by withdrawal regulates production of reactive oxygen species and cytokine-induced neutrophil chemoattractant and liver injury during reperfusion after hepatic ischemia. *Antioxid Redox Signal.* 4:721-31.
244. Hasegawa, T, M Kikuyama, K Sakurai, Y Kambayashi, M Adachi, AR Saniabadi, H Kuwano, and M Nakano. 2002. Mechanism of superoxide anion production by hepatic sinusoidal endothelial cells and Kupffer cells during short-term ethanol perfusion in the rat. *Liver.* 22:321-29.
245. Roselle, GA, CL Mendenhall, and CE Grossman. 1993. Effects of alcohol on immunity and cancer. In *Alcohol, immunity, and cancer*. R. Yirmiya, and A. N. Taylor, eds. CRC Press, Boca Raton, p. 3-21.
246. Smith-Warner, SA, D Spiegelman, SS Yaun, PA van den Brandt, AR Folsom, RA Goldbohm, S Graham, L Holmberg, GR Howe, JR Marshall, AB Miller, JD Potter, FE Speizer, WC Willett, A Wolk, and DJ Hunter. 1998. Alcohol and breast cancer in women: a pooled analysis of cohort studies. *JAMA.* 279:535-40.
247. Wu, WJ, RM Wolcott, and SB Pruett. 1994. Ethanol decreases the number and activity of splenic natural killer cells in a mouse model for binge drinking. *J Pharmacol Exp Ther.* 271:722-29.
248. Gallucci, RM, and GG Meadows. 1995. Ethanol consumption reduces the cytolytic activity of lymphokine-activated killer cells. *Alcohol Clin Exp Res.* 19:402-209.

249. Wu, WJ, and SB Pruett. 1999. Ethanol decreases host resistance to pulmonary metastases in a mouse model: role of natural killer cells and the ethanol-induced stress response. *Int J Cancer.* 82:886-92.
250. Collier, SD, and SB Pruett. 2000. Mechanisms of suppression of poly I:C-induced activation of NK cells by ethanol. *Alcohol.* 21:87-95.
251. Zhang, T, CJ Guo, SD Douglas, DS Metzger, CP O'Brien, Y Li, YJ Wang, X Wang, and WZ Ho. 2005. Alcohol suppresses IL-2-induced CC chemokine production by natural killer cells. *Alcohol Clin Exp Res.* 29:1559-67.
252. Meadows, GG, M Wallendal, A Kosugi, J Wunderlich, and DS Singer. 1992. Ethanol induces marked changes in lymphocyte populations and natural killer cell activity in mice. *Alcohol Clin Exp Res.* 16:474-79.
253. Li, F, RT Cook, C Alber, W Rasmussen, JT Stapleton, and ZK Ballas. 1997. Ethanol and natural killer cells. II. Stimulation of human natural killer activity by ethanol in vitro. *Alcohol Clin Exp Res.* 21:981-87.
254. Ben-Eliyahu, S, GG Page, R Yirmiya, and AN Taylor. 1996. Acute alcohol intoxication suppresses natural killer cell activity and promotes tumor metastasis. *Nat. Med.* 2:457-60.
255. Tonnesen, H, JR Andersen, AE Pedersen, and AH Kaiser. 1990. Lymphopenia in heavy drinkers--reversibility and relation to the duration of drinking episodes. *Ann Med.* 22:229-31.
256. Szabo, G. 1999. Consequences of alcohol consumption on host defence. *Alcohol Alcohol.* 34:830-41.
257. Chadha, KC, I Stadler, B Albini, SM Nakeeb, and HR Thacore. 1991. Effect of alcohol on spleen cells and their functions in C57BL/6 mice. *Alcohol.* 8:481-85.
258. Ewald, SJ, and H Shao. 1993. Ethanol increases apoptotic cell death of thymocytes in vitro. *Alcohol Clin Exp Res.* 17:359-65.
259. Spinozzi, F, A Bertotto, F Rondoni, R Gerli, F Scalise, and F Grignani. 1991. T-lymphocyte activation pathways in alcoholic liver disease. *Alcohol.* 73:140-46.
260. Zisman, DA, RM Strieter, SL Kunkel, WC Tsai, JM Wilkowski, KA Bucknell, and TJ Standiford. 1998. Ethanol feeding impairs innate immunity and alters the expression of Th1- and Th2-phenotype cytokines in murine Klebsiella pneumonia. *Alcohol Clin Exp Res.* 22:621-27.
261. Waltenbaugh, C, K Vasquez, and JD Peterson. 1998. Alcohol consumption alters antigen-specific Th1 responses: mechanisms of deficit and repair. *Alcohol. Clin. Exp. Res.* 22:220S-23S.
262. Starkenburg, S, ME Munroe, and C Waltenbaugh. 2001. Early alteration in leukocyte populations and Th1/Th2 function in ethanol-consuming mice. *Alcohol Clin Exp Res.* 25:1221-30.
263. Krolewiecki, AJ, S Leon, PA Scott, TJ Nolan, GA Schad, and D Abraham. 2001. Effect of chronic ethanol consumption on protective T-helper 1 and T-helper 2 immune responses against the parasites Leishmania major and Strongyloides stercoralis in mice. *Alcohol Clin Exp Res.* 25:571-78.
264. Sacanella, E, R Estruch, A Gaya, J Fernandez-Sola, E Antunez, and A Urbano-Marquez. 1998. Activated lymphocytes ($CD25^+$ $CD69^+$ cells) and decreased $CD19^+$ cells in well-nourished chronic alcoholics without ethanol-related diseases. *Alcohol Clin Exp Res.* 22:897-901.

265. Santos-Perez, JL, A Diez-Ruiz, L Luna-Casado, JA Soto-Mas, H Wachter, D Fuchs, and F Gutierrez-Gea. 1996. T-cell activation, expression of adhesion molecules and response to ethanol in alcoholic cirrhosis. *Immunol Lett.* 50:179-83.
266. Takaishi, M, I Kurose, H Higuchi, N Watanabe, T Nakamura, S Zeki, J Nishida, S Kato, S Miura, Y Mizuno, PR Kvietys, DN Granger, and H Ishii. 1996. Ethanol-induced leukocyte adherence and albumin leakage in rat mesenteric venules: role of CD18/intercellular adhesion molecule-1. *Alcohol Clin Exp Res.* 20:347A-49A.
267. Bautista, AP. 1997. Chronic alcohol intoxication induces hepatic injury through enhanced macrophage inflammatory protein-2 production and intercellular adhesion molecule-1 expression in the liver. *Hepatology.* 25:335-42.
268. Bautista, AP. 1995. Chronic alcohol intoxication enhances the expression of CD18 adhesion molecules on rat neutrophils and release of a chemotactic factor by Kupffer cells. *Alcohol Clin Exp Res.* 19:285-90.
269. Ohki, E, S Kato, Y Horie, T Mizukami, H Tamai, H Yokoyama, D Ito, M Fukuda, H Suzuki, I Kurose, and H Ishii. 1996. Chronic ethanol consumption enhances endotoxin induced hepatic sinusoidal leukocyte adhesion. *Alcohol Clin Exp Res.* 20:350A-55A.
270. Ohki, E, S Kato, H Ohgo, T Mizukami, M Fukuda, H Tamai, Y Okamura, M Matsumoto, H Suzuki, H Yokoyama, and H Ishii. 1998. Effect of chronic ethanol feeding on endotoxin-induced hepatic injury: role of adhesion molecules on leukocytes and hepatic sinusoid. *Alcohol Clin Exp Res.* 22:129S-33S.
271. Saeed, RW, S Varma, T Peng, KJ Tracey, B Sherry, and CN Metz. 2004. Ethanol blocks leukocyte recruitment and endothelial cell activation in vivo and in vitro. *J Immunol.* 173:6376-83.
272. Sacanella, E, and R Estruch. 2003. The effect of alcohol consumption on endothelial adhesion molecule expression. *Addict Biol.* 8:371-78.
273. Kuwana, M, J Kaburaki, TM Wright, Y Kawakami, and Y Ikeda. 2001. Induction of antigen-specific human $CD4^+$ T cell anergy by peripheral blood DC2 precursors. *Eur. J. Immunol.* 31:2547-57.
274. Gilliet, M, and YJ Liu. 2002. Generation of human CD8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J. Exp. Med.* 195:695-704.
275. Moseman, EA, X Liang, AJ Dawson, A Panoskaltsis-Mortari, AM Krieg, YJ Liu, BR Blazar, and W Chen. 2004. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol.* 173:4433-42.
276. Ribas, A. 2005. Clinical trials with tumor antigen genetically modified dendritic cells. *Semin Oncol.* 32:556-62.
277. Hackstein, H, T Taner, AF Zahorchak, AE Morelli, AJ Logar, A Gessner, and AW Thomson. 2003. Rapamycin inhibits IL-4-induced dendritic cell maturation *in vitro* and dendritic cell mobilization and function *in vivo*. *Blood.* 101:4457-63.
278. Morelli, AE, MA Antonysamy, T Takayama, H Hackstein, Z Chen, S Qian, NB Zurowski, and AW Thomson. 2000. Microchimerism, donor dendritic cells, and alloimmune reactivity in recipients of Flt3 ligand-mobilized hemopoietic cells: modulation by tacrolimus. *J Immunol.* 165:226-37.
279. Hackstein, H, and AW Thomson. 2004. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat Rev Immunol.* 4:24-34.

280. Verma, BK, M Fogarasi, and G Szabo. 1993. Down-regulation of TNFα activity by acute ethanol treatment in human peripheral blood monocytes. *J Clin. Immunol.* 13:8-13.
281. Zhu, X, RA Coleman, C Alber, ZK Ballas, TJ Waldschmidt, NB Ray, AM Krieg, and RT Cook. 2004. Chronic ethanol ingestion by mice increases expression of CD80 and CD86 by activated macrophages. *Alcohol.* 32:91-100.
282. Colonna, M, G Trinchieri, and YJ Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 5:1219-26.
283. Laemmli, UK. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature.* 227:680-85.
284. Nishimura, H, M Nose, H Hiai, N Minato, and T Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity.* 11:141-51.
285. Freeman, GJ, AJ Long, Y Iwai, K Bourque, T Chernova, H Nishimura, LJ Fitz, N Malenkovich, T Okazaki, MC Byrne, HF Horton, L Fouser, L Carter, V Ling, MR Bowman, BM Carreno, M Collins, CR Wood, and T Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med.* 192:1027-34.
286. Carter, L, LA Fouser, J Jussif, LJ Fitz, B Deng, CR Wood, M Collins, T Honjo, GJ Freeman, and BM Carreno. 2002. PD-1:PD-L inhibitory pathway affects both $CD4^+$ and $CD8^+$ T cells and is overcome by IL-2. *Eur. J. Immunol.* 32:634-43.
287. Selenko-Gebauer, N, O Majdic, A Szekeres, G Hofler, E Guthann, U Korthauer, G Zlabinger, P Steinberger, WF Pickl, H Stockinger, W Knapp, and J Stockl. 2003. B7-H1 (programmed death-1 ligand) on dendritic cells is involved in the induction and maintenance of T cell anergy. *J. Immunol.* 170:3637-44.
288. Zha, Y, C Blank, and TF Gajewski. 2004. Negative regulation of T-cell function by PD-1. *Crit Rev Immunol.* 24:229-37.
289. Abe, M, Z Wang, A de Creus, and AW Thomson. 2005. Plasmacytoid dendritic cell precursors induce allogeneic T-cell hyporesponsiveness and prolong heart graft survival. *Am J Transplant.* 5:1808-19.
290. Yamazaki, T, H Akiba, H Iwai, H Matsuda, M Aoki, Y Tanno, T Shin, H Tsuchiya, DM Pardoll, K Okumura, M Azuma, and H Yagita. 2002. Expression of Programmed Death 1 Ligands by murine T cells and APC. *J. Immunol.* 169:5538-45.
291. Moffett, JR, and MA Namboodiri. 2003. Tryptophan and the immune response. *Immunol. Cell. Biol.* 81:247-65.
292. Mellor, AL, DH Munn, P Chandler, D Keskin, T Johnson, B Marshall, KG Jhaver, and B Baban. 2003. Tryptophan catabolism and T cell responses. *Adv. Exp. Med. Biol.* 527:27-35.
293. Mellor, AL, and DH Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat. Rev. Immunol.* 4:762-74.
294. Terness, P, TM Bauer, L Rose, C Dufter, A Watzlik, H Simon, and G Opelz. 2002. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J. Exp. Med.* 196:447-57.
295. Frank, J, K Witte, W Schrodl, and C Schutt. 2004. Chronic alcoholism causes deleterious conditioning of innate immunity. *Alcohol Alcohol.* 39:386-92.

296. Spies, CD, V von Dossow, V Eggers, G Jetschmann, R El-Hilali, J Egert, M Fischer, T Schroder, C Hoflich, P Sinha, C Paschen, P Mirsalim, R Brunsch, J Hopf, C Marks, KD Wernecke, F Pragst, H Ehrenreich, C Muller, H Tonneson, W Oelkers, W Rohde, C Stein, and WJ Kox. 2004. Altered cell-mediated immunity and increased postoperative infection rate in long-term alcoholic patients. *Anesthesiology.* 100:1088-100.
297. Jayasinghe, R, G Gianutsos, and AK Hubbard. 1992. Ethanol-induced suppression of cell-mediated immunity in the mouse. *Alcohol Clin Exp Res.* 16:331-35.
298. Jerrells, TR, and D Sibley. 1996. Effects of ethanol on T-cell mediated immunity to infectious agents. In *Drugs of Abuse, Immunity and Infections*. H. Friedman, T. Klein, and S. Specter, eds. CRC, Boca Raton, FL, p. 129.
299. Waltenbaugh, C, and JD Peterson. 1997. Ethanol impairs the induction of delayed hypersensitivity in C57BL/6 mice. *Alcohol.* 14:149-53.
300. Nelson, S, and JK Kolls. 2002. Alcohol, host defense and society. *Nat. Rev. Immunol.* 2:205-09.
301. Mandrekar, P, D Catalano, L Girouard, and G Szabo. 1996. Human monocyte IL-10 production is increased by acute ethanol treatment. *Cytokine.* 8:567-77.
302. Szabo, G, L Girouard, P Mandrekar, and D Catalano. 1996. Acute ethanol treatment augments interleukin-12 production in activated human monocytes. *Ann. N. Y. Acad. Sci.* 795:422-25.
303. Dai, Q, J Zhang, and SB Pruett. 2005. Ethanol alters cellular activation and CD14 partitioning in lipid rafts. *Biochem. Biophys. Res. Commun.* 332:37-42.
304. Clemens, DL, LE Calisto, MF Sorrell, and DJ Tuma. 2003. Ethanol metabolism results in a G2/M cell-cycle arrest in recombinant Hep G2 cells. *Hepatology.* 38:385-93.
305. Abe, M, and AW Thomson. 2003. Influence of immunosuppressive drugs on dendritic cells. *Transpl. Immunol.* 11:357-65.
306. Lucas, PJ, I Negishi, K Nakayama, LE Fields, and DY Loh. 1995. Naive CD28-deficient T cells can initiate but not sustain an in vitro antigen-specific immune response. *J. Immunol.* 154:5757-68.
307. Sharpe, AH, and GJ Freeman. 2002. The B7-CD28 superfamily. *Nat. Rev. Immunol.* 2:116-26.
308. Bellou, A, and PW Finn. 2005. Costimulation: critical pathways in the immunologic regulation of asthma. *Curr. Allergy Asthma Rep.* 5:149-54.
309. Chen, L. 2004. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat. Rev. Immunol.* 4:336-47.
310. Becker, JC, C Czerny, and EB Brocker. 1994. Maintenance of clonal anergy by endogenously produced IL-10. *Int. Immunol.* 6:1605-12.
311. Lu, L, W Li, F Fu, FG Chambers, S Qian, JJ Fung, and AW Thomson. 1997. Blockade of the CD40-CD40 ligand pathway potentiates the capacity of donor-derived dendritic cell progenitors to induce long-term cardiac allograft survival. *Transplantation.* 64:1808-15.
312. Tatsumi, T, T Takehara, T Kanto, T Miyagi, N Kuzushita, Y Sugimoto, M Jinushi, A Kasahara, Y Sasaki, M Hori, and N Hayashi. 2001. Administration of interleukin-12 enhances the therapeutic efficacy of dendritic cell-based tumor vaccines in mouse hepatocellular carcinoma. *Cancer Res.* 61:7563-67.
313. Kumagi, T, SM Akbar, N Horiike, K Kurose, M Hirooka, A Hiraoka, Y Hiasa, K Michitaka, and M Onji. 2005. Administration of dendritic cells in cancer nodules in hepatocellular carcinoma. *Oncol. Rep.* 14:969-73.

314. Saunders, D, K Lucas, J Ismaili, L Wu, E Maraskovsky, A Dunn, and K Shortman. 1996. Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor. *J Exp Med.* 184:2185-96.
315. Szabo, G. 1997. Alcohol's contribution to compromised immunity. *Alcohol Health & Res World.* 21:30-41.
316. Ploenie, M, and H Naqvi. 2005. Using teh immunological synapse to monitor activation of Jurkat cells by ethanol. *Alcohol.* 36:127.
317. Pritchard, MT, MR McMullen, Q Wang, JI Cohen, AB Stavitsky, F Lin, ME Medof, and LE Nagy. 2005. Complement contributes to increased hepatic ICAM-1 expression during chronic ethanol-induced early liver injury in mice. *Alcohol.* 36:127.
318. McClain, CJ, S Barve, IV Deaciuc, M Kugelmas, and D Hill. 1999. Cytokines in Alcoholic Liver Disease. *Semin. Liver Dis.* 19:205-19.
319. Gluckman, SJ, VC Dvorak, and RR MacGregor. 1977. Host defenses during prolonged alcohol consumption in a controlled environment. *Arch Intern Med.* 137:1539-43.
320. Tonnesen, H, AH Kaiser, BB Nielsen, and AE Pedersen. 1987. Reversibility of alcohol-induced immune depression. *Br J Addict.* 87:1025-28.
321. Roselle, GA. 1992. Alcohol and the immune system. *Alcohol Health Res World.* 16:16-22.
322. Jerrells, T, AJ Saad, and TE Kruger. 1993. Ethanol-induced suppression of in vivo host defense mechanisms to bacterial infection. In *Drugs of Abuse, Immunity, and AIDS*, Vol. 335. H. Friedman, T. W. Klein, and S. Specter, eds. Plenum Press, New York, p. 153-58.
323. Aldo-Benson, M, L Pratt, and J Hardwick. 1992. Alcohol can inhibit effect of IL-4 on activated murine B cells. *Immunol Res.* 11:117-24.
324. Jerrells, TR. 2002. Role of activated $CD8^+$ T cells in the initiation and continuation of hepatic damage. *Alcohol.* 27:47-52.
325. Park, S, D Murray, B John, and IN Crispe. 2002. Biology and significance of T-cell apoptosis in the liver. *Immunol Cell Biol.* 80:74-83.
326. Saad, AJ, R Domiati-Saad, and TR Jerrells. 1993. Ethanol ingestion increases susceptibility of mice to *Listeria monocytogenes*. *Alcohol Clin Exp Res.* 17:75-85.
327. Jerrells, TR, K Mitchell, J Pavlik, J Jerrells, and D Hoerman. 2002. Influence of ethanol consumption on experimental viral hepatitis. *Alcohol. Clin. Exp. Res.* 26:1734-46.
328. Reddehase, MJ, W Mutter, K Munch, HJ Buhring, and UH Koszinowski. 1987. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. *J Virol.* 61:3102-08.
329. Harty, JT, and MJ Bevan. 1992. $CD8^+$ T cells specific for a single nonamer epitope of Listeria monocytogenes are protective in vivo. *J Exp Med.* 175:1531-38.
330. Kovacsovics-Bankowski, M, K Clark, B Benacerraf, and KL Rock. 1993. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc Natl Acad Sci U.S.A.* 90:4942-26.
331. Mueller, SN, CM Jones, CM Smith, WR Heath, and FR Carbone. 2002. Rapid cytotoxic T lymphocyte activation occurs in the draining lymph nodes after cutaneous herpes simplex virus infection as a result of early antigen presentation and not the presence of virus. *J Exp Med.* 195:651-56.
332. He, Y, J Zhang, Z Mi, P Robbins, and LD Falo, Jr. 2005. Immunization with lentiviral vector-transduced dendritic cells induces strong and long-lasting T cell responses and therapeutic immunity. *J Immunol.* 174:3808-17.

333. Waldschmidt, TJ, LT Tygrett, RA Coleman, and RT Cook. 2005. Long-term ethanol intake in mice results in loss of peripheral B cells and compromised Th cell-driven Ab responses. *Alcohol.* 36:128.
334. Pass, RF. 2001. Cytomegalovirus. In *Fields' Virology*. P. M. H. David, and M. Knife, eds. Lipincott Williams & Wilkins, Philadelphia, p. 2675-706.
335. Lucas, M, U Karrer, A Lucas, and P Klenerman. 2001. Viral escape mechanisms--escapology taught by viruses. *Int J Exp Pathol.* 82:269-86.
336. Delale, T, A Paquin, C Asselin-Paturel, M Dalod, G Brizard, EE Bates, P Kastner, S Chan, S Akira, A Vicari, CA Biron, G Trinchieri, and F Briere. 2005. MyD88-dependent and -independent murine cytomegalovirus sensing for IFN-alpha release and initiation of immune responses in vivo. *J Immunol.* 175:6723-32.
337. Murali-Krishna, K, JD Altman, M Suresh, DJ Sourdive, AJ Zajac, JD Miller, J Slansky, and R Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity.* 8:177-87.
338. Belz, GT, K Shortman, MJ Bevan, and WR Heath. 2005. CD8α^+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo. *J Immunol.* 174:196-200.
339. Montoya, M, MJ Edwards, DM Reid, and P Borrow. 2005. Rapid activation of spleen dendritic cell subsets following lymphocytic choriomeningitis virus infection of mice: analysis of the involvement of type 1 IFN. *J Immunol.* 174:1851-61.
340. Messingham, KA, DE Faunce, and EJ Kovacs. 2002. Alcohol, injury, and cellular immunity. *Alcohol Clin Exp Res.* 28:137-49.
341. Schlueter, AJ, MR Edsen, J Fan, KJ Ness, and JR Marietta. 2005. Alterations in dendritic cell function in a murine model of chronic EtOH exposure. *Alcohol.* 36:128.